Spectroscopic and biochemical insight into an electron-bifurcating [FeFe] hydrogenase

Nipa Chongdar\textsuperscript{1} · Krzysztof Pawlak\textsuperscript{1} · Olaf Rüdiger\textsuperscript{1} · Edward J. Reijerse\textsuperscript{1} · Patricia Rodríguez-Maciá\textsuperscript{1} · Wolfgang Lubitz\textsuperscript{1} · James A. Birrell\textsuperscript{1} · Hideaki Ogata\textsuperscript{1,2}

Received: 12 October 2019 / Accepted: 21 November 2019 / Published online: 10 December 2019
© The Author(s) 2019

Abstract
The heterotrimeric electron-bifurcating [FeFe] hydrogenase (HydABC) from \textit{Thermotoga maritima} (\textit{Tm}) couples the endergonic reduction of protons (H\textsuperscript{+}) by dihydronicotinamide adenine dinucleotide (NADH) (\(\Delta G^{\circ} \approx 18 \text{ kJ mol}^{-1}\)) to the exergonic reduction of H\textsuperscript{+} by reduced ferredoxin (Fd\textsubscript{red}) (\(\Delta G^{\circ} \approx -16 \text{ kJ mol}^{-1}\)). The specific mechanism by which HydABC functions is not understood. In the current study, we describe the biochemical and spectroscopic characterization of \textit{Tm}HydABC recombinantly produced in \textit{Escherichia coli} and artificially maturated with a synthetic diiron cofactor. We found that \textit{Tm}HydABC catalyzed the hydrogen (H\textsubscript{2})-dependent reduction of nicotinamide adenine dinucleotide (NAD\textsuperscript{+}) in the presence of oxidized ferredoxin (Fd\textsubscript{ox}) at a rate of \(\approx 17 \text{ µmol NADH min}^{-1} \text{ mg}^{-1}\). Our data suggest that only one flavin is present in the enzyme and is not likely to be the site of electron bifurcation. FTIR and EPR spectroscopy, as well as FTIR spectroelectrochemistry, demonstrated that the active site for H\textsubscript{2} conversion, the H-cluster, in \textit{Tm}HydABC behaves essentially the same as in prototypical [FeFe] hydrogenases, and is most likely also not the site of electron bifurcation. The implications of these results are discussed with respect to the current hypotheses on the electron bifurcation mechanism of [FeFe] hydrogenases. Overall, the results provide insight into the electron-bifurcating mechanism and present a well-defined system for further investigations of this fascinating class of [FeFe] hydrogenases.

Graphic abstract

---

Nipa Chongdar and Krzysztof Pawlak have contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00775-019-01747-1) contains supplementary material, which is available to authorized users.

Extended author information available on the last page of the article
Keywords  [FeFe] hydrogenase · Electron bifurcation · Spectroscopy · Electrochemistry · Ferredoxin

Abbreviations

NADH  Dihydronicotinamide adenine dinucleotide
NAD⁺  Nicotinamide adenine dinucleotide
Fdox  Oxidized ferredoxin
Fdred  Reduced ferredoxin
ADT  2-azapropane 1,3-dithiolate
FMN  Flavin mononucleotide
FTIR  Fourier transform infrared
EPR  Electron paramagnetic resonance
FAD  Flavin adenine dinucleotide
CW  Continuous wave
OCP  Open-circuit potential
PCET  Proton-coupled electron transfer
FBEB  Flavin-based electron bifurcation

Introduction

[FeFe] hydrogenases catalyze the reversible interconversion of protons (H⁺) and electrons to hydrogen (H₂) at very high rates, with negligible energy waste [1–3]. Their active center, the H-cluster, consists of a unique [2Fe] cluster ([2Fe]H) tethered covalently by a cysteine thiolate to a standard [4Fe–4S] cluster ([4Fe–4S]H) (Fig. 1a) [4, 5]. The Fe ions in the [2Fe]H subsite are coordinated by CO and CN⁻ ligands and also by a bidentate 2-azapropane 1,3-dithiolate (ADT) ligand that bridges the two irons of [2Fe]H (Fig. 1a). The H-cluster is buried inside a highly optimized protein scaffold, which tunes its catalytic efficiency and provides pathways for the transport of protons, electrons, and gases (the substrate H₂ as well as inhibitors such as CO and O₂) [6].

During H₂ conversion, the H-cluster passes through several redox states [1]. In the active oxidized state (Hox), the [2Fe]H subcluster is in a mixed valent [Fe(I)Fe(II)] state, and the [4Fe–4S]H subcluster is oxidized (2 +). One-electron reduction of the [4Fe–4S]H subsite forms the Hred state [7]. Protonation of the ADT in the Hred state is coupled to electron transfer from the reduced [4Fe–4S]H to the [2Fe] cluster, yielding the HredH⁺ state [8]. A second electron reduction forms the most reduced state, HsredH⁺ ([4Fe–4S]1+–[Fe(I)Fe(I)]) [9]. Rearrangement of the HsredH⁺ state gives the Hhyd state, where a terminal hydride is bound to the [2Fe] subsite with an Fe(II)Fe(II) configuration and [4Fe–4S]H remains reduced [10, 11]. Protonation of Hhyd leads to H₂ production and regeneration of the Hox state.

Based on amino acid sequence phylogeny, the [FeFe] hydrogenases have been classified into three major groups: (I) prototypical and electron-bifurcating; (II) ancestral; and (III) sensory [12]. The physiological roles of the prototypical [FeFe] hydrogenases are far better understood than the other classes. While the prototypical [FeFe] hydrogenases use ferredoxin or cytochrome as their sole redox partner, the electron-bifurcating [FeFe] hydrogenases use both ferredoxin (Fd) and dihydronicotinamide adenine dinucleotide (NADH) simultaneously during H₂ evolution [13–16].

Electron bifurcation is a process, whereby an endergonic redox reaction and an exergonic redox reaction are directly coupled, and is an alternative energy conservation mechanism to the well-known chemiosmotic coupling mechanism [16, 17]. First described by Peter Mitchell in the Q-cycle of mitochondrial complex III [18], it was only about a decade ago that the involvement of electron bifurcation in the metabolism of anaerobic microorganisms was discovered [19]. Subsequent investigations showed that in a variety of reactions in anaerobic metabolism, electron bifurcation is involved [20], among them: coupling of the endergonic oxidation of NADH (Eₐ₀′ = −320 mV) to the exergonic oxidation of reduced ferredoxin (Fdred) (Eₐ₀′ ≈ −450 mV) to reduce
H\(^+\) to H\(_2\) \((E'_\text{H} = -420\ \text{mV})\). The overall reaction is the recycling of NADH, generated by the oxidation of sugars, driven by Fd\(_\text{red}\) oxidation and can be described in full as \([14, 16, 21–23]\):

\[
\text{NADH} + 2\text{Fd}_{\text{red}} + 3\text{H}^+ = \text{NAD}^+ + 2\text{Fd}_{\text{ox}} + 2\text{H}_2 \quad (1)
\]

This reaction is essential in many organisms as various metabolic steps only yield enough energy to reduce nicotinamide adenine dinucleotide (NAD\(^+\)), but NADH is not capable of reducing protons. Thus, by coupling NADH oxidation to ferrodoxin oxidation, protons can be used as a terminal electron acceptor \([20, 21]\).

Electron-bifurcating [FeFe] hydrogenases are widely found in the genomes of anaerobic bacteria belonging to the phyla of Bacteroidetes, Firmicutes, Spirochaetes, Thermotogae, and Fusobacteria \([12]\). Bacteria belonging to Bacteroidetes and Firmicutes phyla are commonly found in mammalian guts, and as H\(_2\) is an abundant metabolite in the gastrointestinal tract, hydrogenases found in the above-mentioned organisms play an important role in colonic H\(_2\) metabolism \([24]\). Interestingly, in a recent bioinformatics study, it has been shown that a wide variety of hydrogenases found in the bacteria of the human gut belong to the electron-bifurcating class \([25]\). Therefore, understanding the metabolism of these organisms may also be important for medical applications.

Electron-bifurcating [FeFe] hydrogenases have been characterized from a number of organisms; however, the main focus of all these studies were their biochemical properties \([14, 21, 27–29]\). Even though the electron-bifurcating [FeFe] hydrogenase from Thermotoga maritima (Tm) has been spectroscopically characterized \([26, 30]\), ambiguities in the understanding of its biophysical properties still persist. In this work, based on the previous efforts of recombinant expression and artificial maturation of [FeFe] hydrogenases, we have developed a recombinant system to overexpress TmHydABC in E. coli followed by its artificial maturation into the bifurcation mechanism and even more importantly provide the basis for further investigation of this unresolved phenomenon.

Materials and methods

Activity assays

Hydrogen oxidation activity of TmHydABC and TmHydA was measured by following the reduction of 1 mM benzyl viologen (at 600 nm, \(\varepsilon_{600} = 7.8 \ \text{mM}^{-1} \ \text{cm}^{-1}\)) in 200 mM H\(_2\)-saturated potassium phosphate buffer, pH 8. The reactions were performed at various temperatures (30–80 °C) by the addition of 25–50 ng protein to 1 mL of the above-mentioned reaction buffer in 1.5 mL plastic cuvettes and the change in absorbance was measured using an Ocean Optics DH-mini UV–Vis–NIR light source and a USB2000 + XR1-ES detector, operated by the SpectraSuite™ software. The desired reaction temperatures were achieved using a temperature controlled cuvette holder (CUV-QPOD-2E-ABSKit, Ocean Optics). All values are the average of three measurements after subtracting the value of the blank measurement. The other details are described in the figure captions.

The NAD\(^+\) and ferrodoxin-dependent H\(_2\) oxidation assay of TmHydABC was performed at 70 °C by addition of 300–600 ng of the protein to 1 mL of H\(_2\) saturated 200 mM potassium phosphate buffer, pH 8. The formation of NADH (monitored at 340 nm, \(\varepsilon = 6.2 \ \text{mM}^{-1} \ \text{cm}^{-1}\)) and reduction of ferrodoxin (monitored at 430 nm, \(\varepsilon = 12 \ \text{mM}^{-1} \ \text{cm}^{-1}\)) were determined from the change in absorbance measured using a spectrophotometer.

To determine the methyl viologen dependent hydrogen production, 250–500 ng of protein was added to 10 mM methyl viologen reduced with 100 mM sodium dithionite.
(NaDT) in 200 mM potassium phosphate buffer pH 8 in 2.5 mL plastic tubes with rubber stoppers. The reaction mixture was purged with argon for 5 min and incubated at the desired reaction temperature for 10 min before 0.5 mL of the headspace gas was extracted for analysis. The head space gas was then analyzed by gas chromatography. Hydrogen content was quantified by comparison with a 100% H₂ standard. All values are the average of three measurements after subtracting the value of a blank measurement.

**Electrochemistry**

Protein film electrochemistry experiments were performed using a gas-tight three electrode setup inside an anaerobic glovebox (MBraun) filled with N₂. The cell temperature was controlled by a water-jacket system. A pyrolytic graphite edge disk (0.03 cm², Momentive Materials) was used as a controlled by a water-jacket system. A pyrolytic graphite ard. All values are the average of three measurements after subtracting the value of a blank measurement.

**FTIR spectroscopy**

FTIR spectroscopy was performed using home-built water-cooled sample holders accommodated in a Bruker Vertex v80 spectrometer. Samples (10 µL) were placed between two CaF₂ (Korth Kristalle, Altenholz) windows, separated by a 50 µm Teflon spacer, and sealed with rubber rings. The temperature of the sample holder was maintained using a water circulator system (Huber). The electrochemical cell was filled with a buffer mixture of MES, HEPES, TAPS, CHES, and sodium acetate adjusted to pH 5, 6, 7, 8, 9, and 10. Cyclic voltammetry was performed under 100% H₂ with a 2000 rpm rotation rate (Princeton Applied Research model 636A). The potentials were converted to the standard hydrogen electrode (SHE) using a conversion of + 241 mV from SCE.

**EPR spectroscopy**

X-band EPR spectra were recorded on a Bruker ELEXSYS E500 CW X-band EPR spectrometer. The temperature of the samples was controlled using an Oxford Instruments ESR900 helium flow cryostat connected to an ITC503 temperature controller. The measurement parameters were: microwave frequency 9.64 GHz, time constant 81.92 ms, conversion time 81.92 ms, and modulation frequency 100 kHz. The microwave power and temperature were varied between measurements and are indicated in the figure legends.

The EPR samples (200 µL) were transferred anaerobically to 4 mm (o.d.) quartz EPR tubes and frozen in liquid nitrogen. All spectra were analyzed with home-written scripts in MATLAB. Spectral simulations were performed using the EasySpin package [37]. Spin quantification was achieved by comparison with a 1 mM CuSO₄, 10 mM EDTA standard.

**Results**

**Characterization of apo-TmHydABC and apo-TmHydA**

The apo-TmHydABC and apo-TmHydA were produced recombinantly in *E. coli* (details of construct preparation, heterologous expression, and purification are provided in the Supplementary Information). Iron quantification of apo-TmHydABC and apo-TmHydA indicated the presence of 35 ± 2 and 19 ± 1 moles of iron per mole of protein, respectively, in good agreement with the expected values (36 for apo-TmHydABC and 20 for apo-TmHydA) based on the number of iron–sulfur (FeS) clusters, and on the previously reported data on TmHydABC and TmHydA isolated from the native organism [26]. Flavin quantification gave 0.2 ± 0.05 moles of FMN per mole of protein. Inclusion of
riboflavin during protein overexpression did not enhance the FMN content. Thus, it is likely that the majority of the FMN is lost during purification, as has been demonstrated also for native \( T_{m} \text{HydABC}[26]. \)

Apo-\( T_{m} \text{HydABC} \) and apo-\( T_{m} \text{HydA} \) were analyzed using UV–Vis and continuous wave (CW) electron paramagnetic resonance (EPR) spectroscopy (Figure S5). In both cases, the UV–Vis spectrum showed broad bands in the range from 300 to 600 nm (Figure S5A) typical for [2Fe–2S] and [4Fe–4S] clusters. EPR spectra from apo-\( T_{m} \text{HydABC} \) and apo-\( T_{m} \text{HydA} \) reduced with sodium dithionite are shown in Figure S5B. The EPR spectrum of apo-\( T_{m} \text{HydABC} \) at 40 K is similar to that observed with the reduced native protein and has \( g \) values and relaxation properties consistent with [2Fe–2S] clusters (Figure S5B, upper panel) [26]. The EPR spectrum of apo-\( T_{m} \text{HydABC} \) at 10 K appears to be more complex than that at 40 K, with the broadening of the feature at \( g \approx 2.02 \) and appearance of a new feature at \( g \approx 1.88 \) (Figure S5B, upper panel). This phenomenon was also observed in the native protein and was attributed to the weak dipolar interactions between the clusters [26]. The EPR spectrum of apo-\( T_{m} \text{HydA} \) is very similar to the apo-\( T_{m} \text{HydABC} \) EPR spectrum at 40 K (Figure S5B, lower panel). Interestingly, at 10 K the EPR spectrum of \( T_{m} \text{HydA} \) does not show such a large contribution from the broad feature as apo-\( T_{m} \text{HydABC} \). Nevertheless, it can be concluded that apo-\( T_{m} \text{HydABC} \) and apo-\( T_{m} \text{HydA} \) contain both [2Fe–2S] and [4Fe–4S] clusters essentially identical to that of the proteins purified from the native organism.

### Activity assays with artificial partners

The apo-\( T_{m} \text{HydABC} \) and apo-\( T_{m} \text{HydA} \) were artificially maturated using the previously published in vitro method (see Supplementary Text 3 for details) to generate the holoenzymes [34, 35]. Activity assays with holo-\( T_{m} \text{HydABC} \) and holo-\( T_{m} \text{HydA} \) in solution were performed to obtain the rates of \( \text{H}_2 \) oxidation coupled to benzyl viologen reduction and \( \text{H}_2 \) production coupled to methyl viologen oxidation. At 30 °C, \( T_{m} \text{HydABC} \) and \( T_{m} \text{HydA} \) oxidized \( \text{H}_2 \) at a rate of \( \approx 500 \text{ U mg}^{-1} \) and \( \approx 800 \text{ U mg}^{-1} \), respectively (1 U = 1 µmol min\(^{-1}\)) (Fig. 2a). The \( \text{H}_2 \) production activities of \( T_{m} \text{HydABC} \) and \( T_{m} \text{HydA} \) at 37 °C were found to be \( \approx 270 \text{ U mg}^{-1} \) and \( \approx 150 \text{ U mg}^{-1} \), respectively (Fig. 2b). While both activities increased with temperature, a stronger effect was found for \( \text{H}_2 \) oxidation (Fig. 2). Compared to \( T_{m} \text{HydABC} \) isolated from the native organism the artificially maturated protein showed significantly higher activity [38]. This could be related to a higher conversion of the CO-inhibited state \( \text{Hox–CO} \) to the active states in the artificially maturated protein, while in the native organism, a larger fraction of the isolated protein is in the inactive \( \text{Hox–CO} \) state.

### Catalytic activity using protein film electrochemistry

The catalytic activity of \( T_{m} \text{HydABC} \) and \( T_{m} \text{HydA} \) was also measured electrochemically by adsorbing the enzymes on pyrolytic graphite electrodes (Fig. 3). Due to protein

---

**Fig. 2** Activity assays of artificially maturated \( T_{m} \text{HydABC} \) and \( T_{m} \text{HydA} \). a Temperature dependence of \( \text{H}_2 \) oxidation of both proteins was measured in the range from 30 to 80 °C with benzyl viologen as the electron acceptor. b \( \text{H}_2 \) production activity of \( T_{m} \text{HydABC} \) and \( T_{m} \text{HydA} \) was measured at 37 °C and 70 °C with reduced methyl viologen as electron donor.
desorption at increased temperatures, the maximum temperature that could be used was 35 °C. Under these conditions reasonably stable films were formed such that individual voltammograms could be measured over a range of different pH values. From these measurements, it was clear that higher current densities are achieved with TmHydA (up to −0.9 mA cm⁻² for H⁺ reduction) (Fig. 3b) than TmHydABC (up to −0.045 mA cm⁻² for H⁺ reduction) (Fig. 3a), but otherwise, the two enzymes behaved in a similar fashion showing a bias towards H⁺ reduction at neutral to low pH. The difference in current densities between TmHydA and TmHydABC could be related to the larger size of TmHydABC (160 kDa) compared to TmHydA (72 kDa), which may lead to higher electroactive coverage (surface concentration of the electroactive enzyme) for TmHydA, as more enzymes can fit into the same surface area. The oligomeric nature of the enzyme can also influence the electroactive coverage: TmHydABC forms a tetramer of trimers in solution (around 700 kDa), while TmHydA behaves as a monomer or dimer (Figures S2 and S3). Protein film electrochemistry reveals that for both TmHydABC and TmHydA, the H⁺ reduction current is strongly pH dependent, dominating over H₂ oxidation at pH values below 8, while the H₂ oxidation current is relatively pH independent (Fig. 3). Both enzymes show reversible electrochemical behavior with essentially no over-potential requirement in either direction (Fig. 3). The behavior of TmHydA is reminiscent of that from Clostridium pasteurianum and Clostridium acetobutylicum [FeFe] hydrogenases both of which show a bias towards H⁺ reduction at neutral pH [9, 39].

Catalytic activity using physiological partners

The TmHydABC enzyme isolated from the native organism was unable to produce H₂ with reduced ferredoxin as the sole electron donor [14]. However, when reduced ferredoxin and NADH were added together as electron donors, TmHydABC could evolve H₂ [14]. Thus, TmHydABC acts as an electron-confering (reverse of bifurcation) hydrogenase, which converges electrons from two different sources to reduce protons to H₂ [14, 23]. To check if the recombinantly produced and artificially maturated TmHydABC is able to perform electron bifurcation, we followed the reverse reaction, i.e., we monitored H₂ oxidation coupled to reduction of NAD⁺ and ferredoxin. The ferredoxin (TmFd1) used for this assay is a single [4Fe–4S] cluster containing ferredoxin from T. maritima (details of its purification and characterization are provided in the Supplementary Text 4 and Figure S8). TmHydABC was not able to reduce TmFd1 in an H₂-saturated assay mixture containing FMN but lacking NAD⁺ (indicated by the unchanged absorbance intensity at 430 nm) (Fig. 4a). As soon as NAD⁺ was added to the assay, a decrease in the absorbance at 430 nm was observed, indicating reduction of TmFd1 (Fig. 4a). Concomitantly, NADH production was detected by an increase of the absorbance at 340 nm (Fig. 4a). The changes in the absorbance intensities at 340 nm and 430 nm were not observed in the absence of TmHydABC, indicating that the H₂-dependent reduction of NAD⁺ and TmFd1 is indeed catalyzed by TmHydABC.

The above observation was also confirmed by EPR spectroscopy (Fig. 4b). The reaction mixture containing NAD⁺ and TmFd1, but lacking TmHydABC, does not show EPR signals corresponding to the reduced [4Fe–4S] cluster of TmFd1 (Fig. 4b, trace i). Similarly, no [4Fe–4S] cluster EPR signal was observed when the reaction was performed without NAD⁺ (Fig. 4b, trace ii). The typical rhombic feature of the reduced [4Fe–4S] cluster originating from TmFd1 was observed only when the reaction mixture contained both NAD⁺ and catalytic amounts of TmHydABC (Fig. 4b, trace iii). These results indicate that our holo-TmHydABC is
The specific activity of NADH production by *Tm* HydABC in the presence of *Tm* Fd1 at 70 °C was found to be \( \approx 16.6 \, \text{U mg}^{-1} \) (Table S1), which is well within the range that was obtained for other electron-bifurcating [FeFe] hydrogenases [21, 27–29, 40]. The stoichiometry of NADH produced vs *Tm* Fd1 reduced was found to be \( \approx 2.01 \) (± 0.1), indicating that the reaction proceeds as expected according to Eq. (1) (Figure S9). Like the electron-bifurcating [FeFe] hydrogenase from *Desulfovibrio fructosovorans* (Hnd) [29], *Tm* HydABC was also able to reduce NADH in the absence of *Tm* Fd1, however, at \( \approx 35 \)-fold lower rate (Table S1). The specific activity of NADH production was found to be \( \approx 5 \)-fold lower (3.4 ± 0.5 \, \text{U mg}^{-1}) than when flavin adenine dinucleotide (FAD) was added to the assay buffer instead of FMN (Table S1). A similar observation was also made in the case of natively isolated *Tm* HydABC that showed approximately 50% activity when FAD was added instead of FMN [14].

It is interesting to note that even in the absence of any FMN or FAD artificially maturated *Tm* HydABC could form NADH at \( \approx 4 \)-fold lower rate (4.7 ± 0.6 \, \text{U mg}^{-1}) than when excess FMN was added to the assay mixture (Table S1, Figure S10). This residual activity appears to arise from the fraction of *Tm* HydABC molecules that retained the FMN cofactor (0.2 ± 0.05 moles of FMN per mole of *Tm* HydABC). From this observation, it can be concluded that the flavin cofactor required for the optimal bifurcation activity of *Tm* HydABC is FMN and not FAD. Furthermore, this observation supports the idea that only one FMN per HydABC trimer is required for bifurcation and that a second flavin is highly unlikely to be present. Since the addition of excess FMN leads to an \( \approx 4 \)-fold increase in rate, this indicates that the FMN content has likewise increased \( \approx 4 \)-fold (from \( \approx 0.2 \) FMN per HydABC to \( \approx 0.8 \)). If there were two sites, excess FMN would be expected to increase the FMN content \( \approx 10 \)-fold, leading to a greater than tenfold increase in the rate. This finding has important implications for the bifurcation mechanism, which are discussed below.

**Spectroscopic characterization of the holo-protein**

The CO and CN⁻ ligands coordinated to the iron atoms in the H-cluster have characteristic stretching vibrations in the range 2150–1750 cm⁻¹, where the characteristic amide vibrations from the protein backbone are absent. Since the stretching vibrations of these ligands are sensitive to changes in oxidation state of the metal center, Fourier transform infrared (FTIR) spectroscopy is an effective tool to study the redox intermediates of the H-cluster. The characteristic FTIR bands for the various redox states of some well-characterized [FeFe] hydrogenases are presented in Table S2.

EPR spectroscopy is an additional tool providing information about the paramagnetic species present in the various redox states of the hydrogenase. Characteristic g values for the redox states of some well-characterized hydrogenases are presented in Table S3.
Characterization of the oxidized state

After artificial maturation, both *Tm*HydABC and *Tm*HydA were obtained in a mixture of oxidation states (Figure S6). Various redox agents were used to enrich the individual oxidation states. Figure 5 shows the FTIR and EPR spectra of *Tm*HydABC and *Tm*HydA after oxidizing the proteins with NAD⁺ and thionine, respectively. In both cases, the FTIR spectra (Fig. 5a) are characterized by five major FTIR bands (2090, 2076, 1964, 1939, and 1802 cm⁻¹). The positions of these bands are comparable to those reported for the Hox state of *Tm*HydABC isolated from the native organism [30] and also to those reported for other [FeFe] hydrogenases (Table S2). It is, therefore, reasonable to assume that the H-cluster of both *Tm*HydABC and *Tm*HydA are mostly in the Hox state under the applied oxidizing conditions.

In the Hox state, both irons in the [2Fe]H subsite are in a low spin configuration leading to an $S = 1/2$ ground state for Hox. In this electronic configuration, the H-cluster shows a rhombic EPR spectrum with two $g$ values above 2.0. Figure 5b shows the CW X-band EPR spectra of oxidized *Tm*HydABC and *Tm*HydA samples at 20 K, 0.1 mW microwave power. The samples were prepared in the same way as for the FTIR measurements. The experimental spectra are overlaid with spectral simulations (dotted magenta lines) and the component spectra are shown underneath. The red trace (Component 1) corresponds to the Hox state, the blue trace (Component 2) corresponds to the Hox–CO state and the gray trace (Component 3) corresponds to one of the reduced F-clusters.

**Fig. 5** FTIR and EPR spectra of artificially maturated *Tm*HydABC and *Tm*HydA under oxidizing conditions. **a** Top panel shows the FTIR spectrum of 400 µM *Tm*HydABC oxidized with 20 mM NAD⁺ and the lower panel shows the FTIR spectrum of 400 µM *Tm*HydA oxidized with 1 mM thionine. Both samples were in 0.1 M Tris–HCl pH 8 buffer, 0.15 M NaCl and the spectra were measured at room temperature. The peaks belonging to the Hox state are shaded in red and those belonging to the Hox–CO state are shaded in blue. **b** CW X-band EPR spectra of the oxidized *Tm*HydABC and *Tm*HydA samples at 20 K, 0.1 mW microwave power. The samples were prepared in the same way as for the FTIR measurements. The experimental spectra are overlaid with spectral simulations (dotted magenta lines) and the component spectra are shown underneath. The red trace (Component 1) corresponds to the Hox state, the blue trace (Component 2) corresponds to the Hox–CO state and the gray trace (Component 3) corresponds to one of the reduced F-clusters.

For both *Tm*HydABC and *Tm*HydA, Component 1 is a rhombic EPR spectrum with almost identical $g$ values; 2.102, 2.044, and 1.998 for *Tm*HydABC and 2.103, 2.045, and 1.999 for *Tm*HydA (Fig. 5b). These $g$ values are in good agreement with the $g$ values of the Hox state of other [FeFe] hydrogenases (Table S3). Thus, the Hox state is also observed for *Tm*HydABC and *Tm*HydA by EPR after oxidative treatment. The additional Component 2 can be assigned to the Hox–CO state, and indications of its presence were also observed in the FTIR spectra (Fig. 5a, peaks shaded in blue). The third component with $g$ values of [2.013, 1.950, 1.917] for *Tm*HydABC and [2.005, 1.949, 1.918] for *Tm*HydA (Table S4) can be assigned to reduced FeS clusters based on the similarity to the spectra of reduced apo-*Tm*HydABC and apo-*Tm*HydA (Figure S5B). When the EPR spectrum of the NAD⁺ oxidized *Tm*HydABC sample was measured at higher temperature (40 K), signals corresponding to Component 3 could still be observed, which indicated that this signal is originating from a slowly relaxing [2Fe–2S] cluster (Figure S11). EPR spin quantitation on the oxidized holo-*Tm*HydABC sample gave a spin content of ≈ 0.6 spins/molecule of protein for the Hox component.
and \( \approx 0.1 \) spins/molecule of protein for the \( \text{Hox} - \text{CO} \) component. Therefore, the total spin content of the H-cluster is approximately 0.7 spins/molecule of protein. In the case of native \( \text{TmHydABC} \) the spin content originating from the H-cluster was found to be \( \approx 0.1 \) spin/molecule of protein [26], indicating a larger proportion of intact H-clusters in the \( \text{TmHydABC} \) sample prepared by our method, which could also explain the higher activity found in solution compared with that reported for the native enzyme [26].

**Characterization of the CO-inhibited state**

CO is a well-known competitive inhibitor for [FeFe] hydrogenases by occupying the open coordination site of the H-cluster to form the \( \text{Hox} - \text{CO} \) state, a well-defined state showing characteristic EPR and FTIR signals [1, 41, 42]. FTIR and EPR spectra of the \( \text{Hox} - \text{CO} \) state of \( \text{TmHydABC} \) and \( \text{TmHydA} \) are shown in Fig. 6.

FTIR spectra of CO-inhibited \( \text{TmHydABC} \) and \( \text{TmHydA} \) are virtually identical and are characterized by the same IR bands at 2092 and 2085 cm\(^{-1}\) for the \( \text{CN}^- \) ligands; 2008, 1970 and 1961 cm\(^{-1}\) for the terminal CO ligands and 1807 cm\(^{-1}\) for the bridging CO ligand. The FTIR bands of the CO ligands are in very good agreement with those recently published for native \( \text{TmHydABC} \) [30]. Both the \( \text{CN}^- \) and CO IR-stretching frequencies are very similar to those reported for the \( \text{Hox} - \text{CO} \) state of other [FeFe] hydrogenases (Table S2).

The experimental EPR spectra of CO-inhibited \( \text{TmHydABC} \) and \( \text{TmHydA} \) indicate the presence of two paramagnetic species. The almost axial EPR spectra with \( g \) values [2.064, 2.008, 2.005] for \( \text{TmHydABC} \) and [2.062, 2.011, 2.006] for \( \text{TmHydA} \) are similar to those from the \( \text{Hox} - \text{CO} \) state of other [FeFe] hydrogenases (Table S3) and arise from the low spin iron centers in the mixed valence [Fe(I) Fe(II)]\(_{\text{H}}\), analogous to the \( \text{Hox} \) state. The oxidized sample of \( \text{TmHydABC} \) isolated from the native organism showed features at \( g = 2.070, 2.024, \) and 2.002 [26]. The EPR lines at 2.070 and 2.002 are similar to the \( \text{Hox} - \text{CO} \) EPR signature observed here for artificially maturated \( \text{TmHydABC} \) or for other [FeFe] hydrogenases. The second paramagnetic species observed in the EPR spectra of the \( \text{Hox} - \text{CO} \) state can be assigned to a reduced [2Fe–2S] cluster, as it has \( g \) values and line shapes similar to those observed for the third component in the EPR spectra of the \( \text{Hox} \) samples.

![Fig. 6](image-url)  
*Fig. 6 FTIR and EPR spectra of \( \text{TmHydABC} \) and \( \text{TmHydA} \) after CO inhibition. a FTIR spectra of CO-inhibited (\( \text{Hox} - \text{CO} \)) artificially maturated \( \text{TmHydABC} \) and \( \text{TmHydA} \). The ‘as-isolated’ protein samples (\( \approx 400 \) µM) were purged for 20 min with 100% CO, followed by incubation for an additional 60 min. Spectra were measured at room temperature. The peak marked with an asterisk belongs to an unidentified species. b CW X-band EPR spectra of CO-inhibited (\( \text{Hox} - \text{CO} \)) \( \text{TmHydABC} \) and \( \text{TmHydA} \) measured at 20 K and 0.1 mW microwave power. Approximately, 150 µM of each protein in 0.1 M Tris–HCl buffer pH 8, 0.15 M NaCl, 20% glycerol, were purged for 20 min with 100% CO, before measuring the spectra. The experimental spectra are overlaid with the spectral simulations (dotted magenta line) and the components are shown underneath.*
Characterization of the reduced state

Reduction of the Fe-centers in the H-cluster causes red-shifts of the FTIR peaks (with respect to $H_{ox}$) of the CO and CN$^-$ ligands due to increases of electron density in anti-bonding ligand orbitals, which lengthens the CO and CN$^-$ bonds [43]. This effect is largest when reduction takes place at the [2Fe]H subcluster (20–50 cm$^{-1}$); however, small red-shifts ($\approx$10 cm$^{-1}$) are also observable when the [4Fe–4S]H subcluster is reduced [8]. When reduced with sodium dithionite, FTIR spectra of $Tm$HydABC and $Tm$HydA are identical and showed five major IR bands at 2075, 2037, 1956, 1919, and 1887 cm$^{-1}$ (Fig. 7a). All these bands are significantly red-shifted (by 20–50 cm$^{-1}$) as compared to the $H_{ox}$ state indicating reduction of the [2Fe]H subsite. They resemble closely those for the $H_{red}H^+$ state identified in other [FeFe] hydrogenases (see Table S2). It is to be noted here that in the $H_{red}H^+$ state, the bridging CO vibration is absent in both $Tm$HydABC and $Tm$HydA; however, an additional peak is observed at 1956 cm$^{-1}$ (Fig. 7a), which may indicate that in the $H_{red}H^+$ state the bridging CO becomes terminal, as was proposed for the $H_{red}H^+$ state in the [FeFe] hydrogenases from *Chlamydomonas reinhardtii* (*CrHydA1*) and *Desulfovibrio desulfuricans* (*DdHydAB*) [8, 44, 45]. However, in a recent study, it was suggested that the bridging CO can be retained in the $H_{red}H^+$ state under certain conditions [46].

Due to the antiferromagnetic spin coupling in the [Fe(I)Fe(I)] unit, the H-cluster in the $H_{red}H^+$ state is EPR silent, but the reduced F-clusters give rise to a characteristic EPR spectrum consisting of multiple contributions from [4Fe–4S]$^+$ and [2Fe–2S]$^+$ clusters (Fig. 7b) very similar to the EPR spectrum observed for the reduced apo-enzyme (Figure S5B) and that of the reduced native enzyme [26, 38, 47]. Interestingly, the dominant contribution with $g$ values ([2.004, 1.950, 1.920] for $Tm$HydABC and [2.004, 1.955, 1.923] for $Tm$HydA) is also present in the $H_{ox}$ and $H_{ox–CO}$ states (Figs. 5b, 6b) and was tentatively assigned to one of the two [2Fe–2S]$^+$ clusters in the HydA subunit.

**Spectroelectrochemical characterization of $Tm$HydABC**

Spectroelectrochemical FTIR was used previously in studies of several [FeFe] hydrogenases to investigate their equilibrium redox properties and to calculate midpoint potentials [7, 44]. Here, we apply this method to characterize the potential dependence of the observed redox states in $Tm$HydABC.
The reductive titration of \( \text{TmHydABC} \) was initiated at an open-circuit potential (OCP) of \( \approx -230 \) mV vs SHE. The FTIR spectrum recorded at this potential suggested that the protein is mainly in the \( \text{Hox} \) state (Fig. 8a). As the potential of the cell was decreased, the peaks corresponding to the \( \text{Hox} \) state decreased in intensity and were replaced by the peaks that were previously observed in the dithionite reduced enzyme (Fig. 8a). However, when the potential was decreased lower than \( \approx -500 \) mV, the intensity of these peaks decreased but no new peaks appeared, indicating irreversible degradation of the H-cluster (Fig. 8a, b).

The reductive titration curve obtained by plotting the intensities of the bands at 1939 \( \text{cm}^{-1} \) (\( \text{Hox} \)) and 1887 \( \text{cm}^{-1} \) (\( \text{HredH}^+ \)) vs. the applied potential could be fitted using the Nernst equation corresponding to a one-electron reduction (Fig. 8b, c). This fitting revealed that the state characterized by bands at 2075, 2037, 1956, 1919, and 1886 \( \text{cm}^{-1} \) is indeed one electron reduced with respect to the \( \text{Hox} \) state, and further supporting that this state is \( \text{HredH}^+ \). The redox potential associated with the \( \text{Hox/}\text{HredH}^+ \) transition for \( \text{TmHydABC} \) at pH 8 (Fig. 8b) was found to be \( -420 \pm 5 \) mV, which is similar to that reported for \( \text{DdHydAB} \) [44, 45].

This difference is explained by the influence of the proximal F-cluster on the redox potential of the \([4\text{Fe}–4\text{S}]_{\text{H}}\) subcluster. Redox anti-cooperativity (redox interaction between the F- and H-clusters that decreases the reduction potential of the H-cluster when the proximal F-cluster is reduced and vice versa), disfavors reduction of the \([4\text{Fe}–4\text{S}]_{\text{H}}\) subcluster, thus promoting direct PCET from the proximal F-cluster to the \([2\text{Fe}]_{\text{H}}\) subcluster upon reduction of the H-cluster. This mechanism increases the apparent pKa of the ADT amine moiety [45]. During the spectroelectrochemical titration of \( \text{TmHydABC} \) at pH 8, we did not observe any \( \text{Hred} \) state (Fig. 8). The \( \text{Hred} \) state was also not observed when the FTIR spectrum of dithionite reduced \( \text{TmHydABC} \) was measured at pH 10 (Figure S12). The exceptionally high apparent pKa of the ADT amine in \( \text{TmHydABC} \) could be the result of structural modifications in the H-cluster binding pocket with respect to \( \text{DdHydAB} \) and \( \text{CrHydA1} \) that reinforce the effect of redox anti-cooperativity with the proximal F-cluster(s).

Artificial maturation with the unprotonatable \([2\text{Fe}]_{\text{ADT}}\)-anologue \([2\text{Fe}]_{\text{PDT}}\) [48, 49] confirmed that an unprotonated reduced state of \( \text{TmHydABC} \) is indeed accessible. The \([2\text{Fe}]_{\text{PDT}}\)-maturated \( \text{TmHydABC} \) exhibits an \( \text{Hred} \) state with very low signal intensity and broadened peaks (Figure S13). The reason for this is unclear, but may be related to some conformational freedom of the \([2\text{Fe}]\) site in this enzyme. Furthermore, we found no evidence for the formation of a two electron reduced state (the \( \text{HredH}^+ \) state) in our spectroelectrochemistry data (Fig. 8). Formation of
the H$_{\text{red}}$H$^+$ state was proposed to be essential for CrHydA1 during H$_2$ production [9]. For DdHydAB, an [FeFe] hydrogenase containing F-clusters, it was proposed that it can avoid the formation of the H$_{\text{red}}$H$^+$ state, as the nearby F-clusters can accept the second electron and can tunnel it quickly to the H-cluster during catalysis [45]. A similar phenomenon is also likely to be operative in TmHydABC leading to the absence of H$_{\text{red}}$H$^+$, although the protein is catalytically highly active. It should be noted that in a recent kinetics study on native TmHydABC, the bands assigned to H$_{\text{red}}$H$^+$ in our study were attributed to the doubly reduced state H$_{\text{red}}$H$^+$ with some contribution from H$_{\text{red}}$H$^+$ (referred to as “H$_{\text{red}}^+$” and “H$_{\text{red}}^+$”, respectively) [30]. We believe that as the positions of the FTIR bands of the H$_{\text{red}}$H$^+$ and H$_{\text{red}}$H$^+$ states are very similar, correct assignment of bands to these two states is difficult without electrochemical titrations. The main conclusions of this kinetics study, however, are not affected by this misassignment [30]. The protonated reduced state did occur under proton reduction conditions and was identified as a catalytically active state [30].

**Discussion**

In this study, the heterotrimeric electron-bifurcating [FeFe] hydrogenase from *T. maritima*, TmHydABC, and its catalytic subunit TmHydA was produced by recombinant expression and artificial maturation. With artificial redox partners, semisynthetically produced TmHydABC and TmHydA showed significantly higher H$_2$ production (550 ± 50 U mg$^{-1}$ and 475 ± 60 U mg$^{-1}$) and H$_2$ oxidation activities (1300 ± 140 and 2000 ± 200 U mg$^{-1}$) at 70 °C compared to the enzyme isolated from the native organism [26]. Native TmHydABC was previously found to use both reduced ferredoxin and NADH as electron donors for H$_2$ production, thereby acting as an electron-conveying hydrogenase [14]. Here, we show that the semisynthetically produced holo-TmHydABC could catalyze the reverse reaction, i.e., H$_2$-dependent reduction of ferredoxin only in the presence of NAD$^+$, demonstrating that the enzyme is also capable of the electron bifurcation reaction.

We have used FTIR and EPR spectroscopy to analyze the salient features of the H-cluster in TmHydABC and TmHydA under various conditions. Under oxidizing conditions, both TmHydABC and TmHydA showed spectral properties (positions of the FTIR peaks and EPR g values) similar to the H$_{\text{ox}}$ state of prototypical [FeFe] hydrogenases (non-electron bifurcating) (Fig. 5, Tables S1 and S2). Furthermore, when we treated TmHydABC and TmHydA with CO, FTIR, and EPR spectroscopic features of the H$_{\text{ox}}$-CO state could be identified. This observation implies that like prototypical [FeFe] hydrogenases, the H-cluster of electron-bifurcating hydrogenases is also inhibited by CO. All these results suggest that properties of the H-cluster of TmHydABC are similar to those of the prototypical [FeFe] hydrogenases. This was expected as the amino acid sequences surrounding the H-cluster pocket are well conserved in electron-bifurcating and non-electron-bifurcating [FeFe] hydrogenases [50]. However, the previously published EPR spectroscopic analysis of native TmHydABC did not identify the characteristic EPR signal of the H$_{\text{ox}}$ state [26], which led to the speculation that the H-cluster of electron-bifurcating enzymes is different from prototypical enzymes [51].

Electron bifurcation refers to the process of splitting the electrons from a single electron donor to two different electron acceptors, one with higher redox potential and the other with lower redox potential than that of the electron donor [16, 17]. Electron-bifurcating enzymes usually contain at least one flavin along with several other redox centers and it is presumed that the bifurcation reaction happens at this flavin as this cofactor is capable of both one and two electron transfer reactions (flavin-based electron bifurcation, FBE) [16, 51]. In general, the flavin centers responsible for FBE show special redox properties [52]. These flavins display ‘crossed’ redox potentials: they undergo 2e$^-$ reduction from the flavoquinone state to the flavohydroquinone state and, then, upon 1e$^-$ oxidation, form a highly reducing flavosemiquinone state that can transfer electrons to low potential electron acceptors.

FBE has been suggested to operate in TmHydABC and other electron-bifurcating [FeFe] hydrogenases [23]. Protein sequence analysis of TmHydABC and other electron-bifurcating [FeFe] hydrogenases indicates the presence of one FMN-binding site in HydB. Based on the sequence similarity between HydB and the NuoF (Nqo1) subunit of respiratory complex I [23], whose FMN does not engage in electron bifurcation, it seems unlikely that the FMN in HydB would be the site of electron bifurcation. Thus, the involvement of a second flavin for electron bifurcation was proposed in TmHydABC [23]. Here, we showed that TmHydABC containing 0.2 FMN per heterotrimer catalyzed the electron bifurcation reaction with a rate of 28% of that in the presence of added excess FMN. This fits with the idea that only one FMN per trimer is necessary for electron bifurcation. If two FMN sites were present then the 0.2 FMN would be split between the two sites, with 0.1 FMN per site (or 10% occupancy of each site) assuming equal affinity for both sites. By the law of probabilities then, only 1% of HydABC would have both sites occupied. If both FMN sites were essential for the catalytic mechanism (one for NADH oxidation and one for electron bifurcation), then, in this scenario, the addition of excess FMN should fill up all empty FMN sites and increase the activity accordingly by about 100-fold. Therefore, the modest fourfold increase in activity, contradicts the proposition of a second flavin being the bifurcation center in TmHydABC.
More recently, an alternative model of the bifurcation mechanism in [FeFe] hydrogenases has been postulated by Peters et al. where the H-cluster was hypothesized to be the electron bifurcation site [51]. The rationale behind this hypothesis was that the H-cluster fulfills one of the key requirements of being an electron-bifurcation center, as it is capable of reducing reactions and can exist in different oxidation states. Like electron-bifurcating flavins, the intermediate redox state of the H-cluster should be strongly reducing in order for it to be an electron bifurcation center [52]. Our spectroelectrochemical analysis of \(Tm\)HydABC shows that the H-cluster forms a stable reduced state (\(H_{\text{red}}\)). The \(2e^-\)-reduced state (\(H_{\text{red}}^+\)) was not observed under our experimental conditions; possibly due to a very low redox potential of the \(H_{\text{red}}\) transition imposed by redox anti-cooperativity between the H-cluster and the reduced F-clusters [45]. This observation implies that the H-cluster does not show ‘crossed-over’ redox behavior similar to electron-bifurcating flavins. Although according to Zhang et al. redox centers with uncrossed redox potentials can also theoretically act as bifurcating centers under certain conditions [55].

A possible mechanism would be that \(H_2\) oxidation at the H-cluster produced a highly reducing \(H_{\text{red}}^+\) species, which quickly transfers an electron to a nearby iron–sulfur cluster with a very negative redox potential. This would then be followed by downhill electron transfer to the ferredoxin-binding site. The \(H_{\text{red}}^+\) state would then transfer an electron to a different iron–sulfur cluster with a more positive redox potential. We think that this is rather unlikely to be the case in \(Tm\)HydABC, due to the high sequence similarity between \(Tm\)HydA and the structurally characterized [FeFe] hydrogenase from \(C.\) \(\text{pasteurianum}\) [4, 54]. \(Cp\)HydA contains four accessory iron–sulfur clusters, but only one of them is within electron transfer distance of the H-cluster. Thus, a second proximal iron–sulfur cluster in \(Tm\)HydA seems unlikely.

We hypothesize, instead, that a complicated arrangement of iron–sulfur clusters, and interactions between them may facilitate a novel elegant electron-bifurcating mechanism. The arrangement of cofactors and subunits in \(Tm\)HydABC remains unknown. However, the strong homology between \(Tm\)HydA, \(Tm\)HydB, and \(Tm\)HydC, and the complex I subunits \(Tt\)Nqo1, \(Tt\)Nqo2, and \(Tt\)Nqo3 from \(T.\) \(\text{thermophilus}\) [53], may indicate a similar arrangement of subunits.

Fig. 9 Proposed arrangement of subunits and cofactors in \(Tm\)HydABC. The \(Tm\)HydABC subunits are homologous to the Nqo1, Nqo2, and Nqo3 subunits of the structurally characterized complex I from \(T.\) \(\text{thermophilus}\). Based on this homology, the arrangements of the conserved cofactors can be predicted. The figure shows the protein subunits Nqo1 (HydB, green), Nqo2 (HydC, blue), and Nqo3 (HydA, pink) in the cartoon representation (PDB: 4HEA [53]), with the cofactors from Nqo1 and Nqo2 from complex I, and the cofactors from the [FeFe] hydrogenase \(Cp\)HydA (PDB: 4XDC [54]), overlaid. \(Cp\)HydA was aligned to Nqo3 in Pymol giving almost perfect alignment of the homologous clusters. HydA contains an additional [2Fe–2S] cluster, for which \(Cp\)HydA does not contain a homologous cluster, and HydB contains an additional two [4Fe–4S] clusters and one [2Fe–2S] cluster, for which complex I does not contain homologous clusters.
and cofactors (Fig. 9). In this arrangement, HydC would be located on one side of HydB, positioning the [2Fe–2S] cluster of HydC close to the FMN cofactor of HydB. Meanwhile, HydA would be located on the opposite side of HydB with the surface exposed [2Fe–2S] cluster of HydA in electrical contact with the surface exposed [4Fe–4S] cluster of HydB. This arrangement would not be compatible with the previously proposed Fd-binding site being HydC. Instead, Fd would interact with the His-ligated [4Fe–4S] cluster of HydA, as has been proposed for the CpHydA [56]. With the H-cluster in one direction, the FMN in another direction and the Fd-binding site in a third direction, this arrangement temptingly implicates the trinity of iron–sulfur clusters in HydA as a potential bifurcation site. How, such an arrangement could operate to regulate electron transfer from the H-cluster in one direction or another is unclear, but we speculate that the His-ligated [4Fe–4S] cluster in HydA could play an important role. Further investigations are underway to investigate this possibility. Crucially, our recombinant method for producing TmHydABC represents the perfect system to perform such in-depth mechanistic studies of the electron-bifurcating mechanism, since it provides an efficient way to produce very high yields of pure protein, as well as making it easy to produce site directed mutations to directly test these ideas.

**Conclusion**

In this study, we have developed a method of producing the heterotrimeric electron-bifurcating [FeFe] hydrogenase from *T. maritima* using recombinant expression and artificial maturation. The time efficiency of the recombinant expression method prevented protein damage and led to high catalytic activity for both TmHydABC and TmHydA, outperforming enzymes isolated from the native organism. Our preparation was competent in the electron bifurcation system to perform such in-depth mechanistic studies of the electron-bifurcating mechanism, since it provides an efficient way to produce very high yields of pure protein, as well as making it easy to produce site directed mutations to directly test these ideas.

**Acknowledgements**

Open access funding provided by Max Planck Society. We thank Yvonne Brandenburger for helping with sample preparation and biochemical characterization and Tabea Mufeldt for synthesis of the complexes. We also thank Norbert Dickmann (MALDI TOF–MS), Dr. Philip Schulze and Sylvia Ruthe, MPI-Kohlenforschung (gas chromatography), and Ingeborg Heise (synthesis) for their assistance. The work was supported by the Max Planck Society and in part by JSPS KAKENHI Grant number 16K21748 (H. O.). J. B. acknowledges the Deutsche Forschungsgemeinschaft Priority Programme “Iron–Sulfur for Life: Cooperative Function of Iron–Sulfur Centers in Assembly, Biosynthesis, Catalysis and Disease” (SPP 1927) Project BI 2198/1-1.

**Compliance with ethical standards**

**Conflict of interest**

The authors declare that they have no conflict of interest.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

**References**

1. Lubitz W, Ogata H, Rüdiger O, Reijerse E (2014) Chem Rev 114:4081–4148
2. Peters JW, Schut GJ, Boyd ES, Mulder DW, Shepard EM, Broderick JB, King PW, Adams MW (2015) Biochim Biophys Acta 1853:1350–1369
3. Glick BR, Martin WG, Martin SM (1980) Can J Microbiol 26:1214–1223
4. Peters JW, Lanzilotta WN, Lemon BJ, Seefeldt LC (1998) Science 282:1853–1858
5. Nicolet Y, Piras C, Legrand P, Hatchikian CE, Fontecilla-Camps JC (1999) Structure 7:13–23
6. Winkler M, Esselborn J, Happe T (2013) Biochim Biophys Acta 1827:974–985
7. Silakov A, Kamp C, Reijerse E, Happe T, Lubitz W (2009) Biochemistry 48:7780–7786
8. Sommer C, Adamska-Venkatesh A, Pawlak K, Birrell JA, Rüdiger O, Reijerse EJ, Lubitz W (2017) J Am Chem Soc 139:1440–1443
9. Adamska A, Silakov A, Lambertz C, Rüdiger O, Happe T, Reijerse E, Lubitz W (2012) Angew Chem Int Ed Engl 51:11458–11462
10. Mulder DW, Guo Y, Ratzloff MW, King PW (2017) J Am Chem Soc 139:83–86
11. Reijerse EJ, Pham CC, Pellenschikov V, Gilbert-Wilson R, Adamska-Venkatesh A, Siebel JF, Gee LB, Yoda Y, Tamasaku
K, Lubitz W, Rauchfuss TB, Cramer SP (2017) J Am Chem Soc 139:4306–4309
12. Greening C, Biswas A, Carere CR, Jackson CJ, Taylor MC, Stott MB, Cook GM, Morales SE (2016) ISME J 10:761–777
13. Sondergaard D, Pedersen CN, Greening C (2016) Sci Rep 6:34212
14. Schut GJ, Adams MW (2009) J Bacteriol 191:4451–4457
15. Huang H, Wang S, Moll J, Thauer RK (2012) J Bacteriol 194:3689–3699
16. Buckel W, Thauer RK (2018) Chem Rev 118:3862–3886
17. Peters JW, Miller AF, Jones AK, King PW, Adams MW (2016) Curr Opin Chem Biol 31:146–152
18. Mitchell P (1975) FEBS Lett 59:137–139
19. Li F, Hinderberger J, Seedorf H, Zhang J, Buckel W, Thauer RK (2008) J Bacteriol 190:843–850
20. Muller V, Chowdhury NP, Basen M (2018) Annu Rev Microbiol 72:331–353
21. Zheng YN, Kahnt J, Kwon IH, Mackie RI, Thauer RK (2014) J Bacteriol 196:3840–3852
22. Buckel W, Thauer RK (2018) Front Microbiol 9:208–216
23. Buckel W, Thauer RK (2013) Biochem Biophys Acta 1827:94–113
24. Nakamura N, Lin HC, McSweeney CS, Mackie RI, Gaskins HR (2010) Annu Rev Food Sci Technol 1:363–395
25. Wolf PG, Biswas A, Morales SE, Greening C, Gaskins HR (2016) Gut Microbes 7:235–245
26. Verhagen MF, O’Rourke T, Adams MW (1999) Biochim Biophys Acta 1412:212–229
27. Schuchmann K, Muller V (2012) J Biol Chem 287:31165–31171
28. Wang S, Huang H, Kahnt J, Thauer RK (2013) J Bacteriol 195:1267–1275
29. Kpebe A, Benvenuti M, Guendon C, Rebai A, Fernandez V, Le Laz S, Etienne E, Guigliarelli B, Garcia-Molina G, de Lacey AL, Baffert C, Brugna M (2018) Biochim Biophys Acta Bioenerg 1859:1302–1312
30. Greene BL, Schut GJ, Adams MWW, Dyer RB (2017) ACS Catal 7:2145–2150
31. Pan G, Menon AL, Adams MW (2003) J Biol Inorg Chem 8:469–474
32. Verhagen MF, O’Rourke TW, Menon AL, Adams MW (2001) Biochem Biophys Acta 1505:209–219
33. Birrell JA, Laurich C, Reijerse EJ, Ogata H, Lubitz W (2016) Biochemistry 55:4344–4355
34. Esselborn J, Lambertz C, Adamska-Venkatesh A, Simmons T, Berggren G, Noth J, Siebel J, Hemschemeier A, Arteiro V, Reijerse E, Fontecave M, Lubitz W, Happe T (2013) Nat Chem Biol 9:607–609
35. Birrell JA, Rüdiger O, Reijerse EJ, Lubitz W (2017) Joule 1:61–76
36. Moss D, Nabedyk E, Breton J, Mantele W (1990) Eur J Biochem 187:565–572
37. Stoll S, Schweiger A (2006) J Magn Reson 178:42–55
38. Juszczak A, Aono S, Adams MW (1991) J Biol Chem 266:3834–3841
39. Kertess L, Wittkamp F, Sommer C, Esselborn J, Rüdiger O, Reijerse EJ, Hofmann E, Lubitz W, Winkler M, Happe T, Apfel UP (2017) Dalton Trans 46:16947–16958
40. Wang S, Huang H, Kahnt J, Mueller AP, Kopek M, Thauer RK (2013) J Bacteriol 195:4373–4386
41. Lemon BJ, Peters JW (1999) Biochemistry 38:12969–12973
42. Pierik AJ, Hulstein M, Hagen WR, Albracht SP (1998) Eur J Biochem 258:572–578
43. Buckel W, Reijerse E, van Gestel M (2007) Chem Rev 107:4331–4365
44. Roseboom W, De Lacey AL, Fernandez VM, Hatchikian EC, Albracht SP (2006) J Biol Inorg Chem 11:102–118
45. Rodriguez-Macíà P, Pawlak K, Rüdiger OM, Reijerse EJ, Lubitz W, Birrell JA (2017) J Am Chem Soc 139:15122–15134
46. Ratzloff MW, Artz JH, Mulder DW, Collins RT, Furtak TE, King PW (2018) J Am Chem Soc 140:7623–7628
47. Smith ET, Adams MW (1994) Biochim Biophys Acta 1206:105–112
48. Siebel JF, Adamska-Venkatesh A, Weber K, Rumpel S, Reijerse E, Lubitz W (2015) Biochemistry 54:1474–1483
49. Adamska-Venkatesh A, Krawietz D, Siebel J, Weber K, Happe T, Reijerse E, Lubitz W (2014) J Am Chem Soc 136:11339–11346
50. Poudel S, Tokmina-Lukaszewska M, Colman DR, Refai M, Schut GJ, King PW, Maness PC, Adams MW, Peters JW, Bothisher N, Boyd ES (2016) Biochim Biophys Acta 1860:1910–1921
51. Peters JW, Beratan DN, Schut GJ, Adams MWW (2018) Chem Commun 54:4091–4099
52. Nitschke W, Russell MJ (2012) BioEssays 34:106–109
53. Baradaran R, Berrisford JM, Minhas GS, Sanazan JA (2013) Nature 494:443–448
54. Esselborn J, Muraki N, Klein K, Engelbrecht V, Metzler-Nolte N, Apfel UP, Hofmann E, Kurisu G, Happe T (2016) Chem Sci 7:959–968
55. Zhang P, Yuly JL, Lubner CE, Mulder DW, King PW, Peters JW, Beratan DN (2017) Acc Chem Res 50:2410–2417
56. Artz JH, Mulder DW, Ratzloff MW, Lubner CE, Zadkorya OA, LeVan AX, Williams SG, Adams MW, Jones AK, King PW, Peters JW (2017) J Am Chem Soc 139:9544–9550

**Affiliations**

Nipa Chongdar¹ · Krzysztof Pawlak¹ · Olaf Rüdiger¹ · Edward J. Reijerse¹ · Patricia Rodríguez-Macíà¹ · Wolfgang Lubitz¹ · James A. Birrell¹ · Hideaki Ogata¹,²

¹ Max Planck Institute for Chemical Energy Conversion, Stiftstrasse 34-36, 45470 Mülheim an der Ruhr, Germany
² Institute of Low Temperature Science, Hokkaido University, Kita-19, Nishi-8, Kita-ku, Sapporo 060-0819, Japan

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.