

Thermodynamic landscape of the redox-centres in the electron-confurcating [FeFe]-Hydrogenase (*TmHydABC*) of *Thermotoga maritima*

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ABSTRACT

The heterotrimeric [FeFe]-hydrogenase (*TmHydABC*) from *Thermotoga maritima* synergistically uses electrons from ferredoxin and NADH to reduce protons to hydrogen enabling the anaerobic metabolism of carbohydrates and other molecules. The precise mechanism by which this flavin-based electron confurcation is achieved is still unknown. Here, the redox properties of the cofactors in *TmHydABC* were characterized via chemical and electrochemical redox titrations monitored by EPR- and UV/Vis-spectroscopy. Deconvolution of nine out of eleven iron-sulfur clusters harboured by the apo-enzyme was achieved by (a) exploiting the distinct relaxation properties (and hence temperature-dependencies) of the EPR-signals, (b) making use of spectral idiosyncrasies of [4Fe-4S] versus thioredoxin-like [2Fe-2S] clusters in UV/Vis-spectroscopy and (c) using the individual HydA, HydB and HydC subunits and C-terminal domains of HydA and HydB. Furthermore, electrostatic interactions between certain neighbouring clusters and a paramagnetic interaction between the flavin and one of the iron-sulfur clusters were revealed. The electrochemical parameters of the flavin were obtained both via UV/Vis-spectroscopy and EPR-analysis of its semi-reduced state in isolated HydB. This semi-reduced state was found to correspond to a neutral flavosemiquinone and a pK_a (attributed to the N5-proton) was extracted from the pH-dependence of its redox midpoint potential. The electrochemical properties of the flavin in the entire enzyme appear to differ significantly from those in the isolated HydB subunit. The flavin and iron-sulfur cluster redox properties indicate a dynamic electrochemical landscape, potentially contributing to its mechanism.

1. Introduction

Electron bifurcation/confurcation is the coupled redox reaction of a two-electron redox compound with two individual one-electron redox centres of differing nature and electrochemical potential, one of them usually featuring a more negative redox midpoint potential than the two-electron compound while the second one has a more positive midpoint potential [1–3]. The phenomenon of electron bifurcation was first observed in mitochondrial Complex III (also known as the cytochrome bc_1 -complex) [4] and later interpreted within an electrochemical framework by Mitchell [5,6]. In the bc_1 complex, a quinol

reduces, in a coupled manner, a lower potential heme b_L and a higher potential Rieske iron-sulfur cluster. A more in-depth thermodynamic functional model for this process was presented by Crofts et al. (2013) [1] and more recently shown to be applicable to the entire family of Rieske/cytb complexes [7].

In 2008, an analogous reaction scheme was proposed to explain the endergonic reduction of ferredoxin by the electron transferring flavo-protein (Etf) [8,9]. The role of the quinone as a pivotal two-electron redox compound in electron bifurcation/confurcation may, thus, also be played by the flavin cofactor [10].

Flavin-based electron bifurcation (FBEB) and confurcation (FBEC)

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occur in a variety of different enzymes [11–13]. The majority of these flavoenzymes seem to perform electron bifurcation/confurcation reactions resembling those described for Rieske/cytb complexes, that is, electron transfer between two one-electron redox partners of respectively high and low redox potentials and one two-electron redox partner of intermediate redox potential. A subgroup of FBEB/EC-enzymes, however, stands out by virtue of the fact that the direct high potential electron acceptor of the bifurcating reaction is an obligatory two-electron redox compound, i.e. NAD(H) or NADP(H), hampering straightforward transposition of the functional models previously developed to rationalize these EB processes.

This subgroup of “NAD(P)-dependent” FBEB-enzymes has recently been recognized to constitute a large NAD(P)H dehydrogenase family comprising [FeFe]- and [NiFe]-hydrogenases, formate dehydrogenases as well as respiratory Complex I [3,14,15]. Some of them, however, are not thought to be electron bi/confurcating in the above-described manner, i.e., coupled to the concomitant redox transition of ferredoxin.

The unifying features of all representatives in this group are the presence of a structurally well-conserved flavin-containing module catalyzing the redox conversion of NAD(P) and of an iron-sulfur-centre-rich module mediating charge transfer between the NAD(P)-binding module and the respective catalytic centres in the diverse enzymes. The fact that electrons are bifurcated/confurcated towards/from one-electron redox substrates (mostly ferredoxins) in one branch and the obligatorily two-electron compound NAD(P) in the other branch substantially complicates the elaboration of functional models. A very promising approach to rationalize this process initially involved the presence of two rather than one flavin cofactor in these enzymes [2,3]. Crystallographic and cryo-EM structure models of proteins from this family of enzymes resolved during recent years did not confirm this hypothesis [16–20], sending the respective community back to the drawing board.

In order to develop mechanistic models for the electron bifurcating/confurcating process in this group of enzymes, two crucial parameters need to be known: (a) the structural arrangement of the involved cofactors including an assessment of potential domain movements/conformational changes modulating this arrangement and (b) the electrochemical parameters (i.e., redox midpoint potentials) of the redox centres participating in the bifurcating/confurcating reaction together with an assessment of potential variations thereof in response to conformational changes.

Over the last years, multiple 3D-structures of four major representatives of the NAD(P)-dependent bifurcating/confurcating enzymes belonging to this NAD(P)H dehydrogenase family have been determined and our knowledge of the architecture of these complexes has improved substantially [17,19–22], although a number of structural/conformational details are still controversial.

By contrast, to the exception of Complex I, the electrochemical characterization of these enzymes remains rudimentary at best. Although in Complex I the initial electron transfer from the flavin follows the electron bifurcation scheme [3,14,23], the enzyme has evolved to carry out significantly differing catalytic processes shuttling all electrons to quinone and coupling this electron transfer to transmembrane proton pumping [24–26]. Transposability of results obtained on Complex I towards the remainder of the enzyme superfamily therefore remains doubtful.

To contribute to a better understanding of the Fd-dependent subgroup of this family, we have in this work tried to obtain for the first time a comprehensive catalogue of the redox properties of the relevant cofactors in the electron confurcating, NADH-dependent [FeFe]-hydrogenase, HydABC, from the hyperthermophilic bacterium *Thermotoga maritima* (TmHydABC).

Recent structural efforts using cryo-EM have elucidated that the trimeric TmHydABC functionally forms a dimer of heterotrimers [19]. The detailed molecular model features 10 iron-sulfur clusters per heterotrimer, four of the binuclear [2Fe-2S]- and six of the cubane,

tetranuclear [4Fe-4S]-type (Fig. 1) in addition to the catalytically active H-cluster, consisting of a [4Fe-4S]_H cluster linked to a [2Fe]_H cluster.

Half of these iron-sulfur centres (called B2, A1, A2, A3 and A4 in Furlan et al. (2022) [19]) are found in the well-resolved central core of TmHydABC and form a chain connecting the two active sites, i.e. (a) the essential flavin-molecule, FMN, as the binding site of NADH and proposed electron bifurcating site in the HydB subunit and (b) the proton-reducing H-cluster in the HydA subunit.

The other five iron-sulfur clusters (termed A5, B1, B3, B4 and C1) in TmHydABC are arranged outside of this chain and some of them are harboured by conformationally flexible domains (Fig. 1).

Three of these, one in each subunit (A5, B1 & C1) are cysteinyl-coordinated [2Fe-2S]-clusters, situated within domains featuring the thioredoxin-like protein fold (in the following called Trx-like [2Fe-2S]-clusters as introduced by Meyer, Andrade, Einsle (2011) [27]). Members of this family of thioredoxin-like ferredoxins display well-defined UV/Vis and EPR-signals [27].

The two remaining iron-sulfur clusters B3 and B4, peripheral to the “main wire”, are [4Fe-4S]-centres contained within a 2×[4Fe-4S]- (so-called bacterial-type) ferredoxin domain. This domain represents a C-terminal extension of the flavin-containing HydB subunit and was found to be conformationally mobile [19].

Mutagenesis studies suggest functional indispensability of the Trx-like [2Fe-2S]-clusters B1 and C1 as well as of the [4Fe-4S]-clusters of the bacterial-type ferredoxin domain, B3 and B4 [20,21]. The Trx-like [2Fe-2S]-cluster A5, however, is not conserved in all members of this enzyme family [14] and two recently published cryo-EM structures of the homologous NfnABC showed a Zn-ion in place of cluster B1 in these enzymes [28,29].

We here present the assignment of UV/Vis and EPR-spectra, redox potentials and electrostatic as well as magnetic interactions of almost all iron-sulfur clusters in TmHydABC. These parameters have been obtained using electron paramagnetic resonance (EPR) and UV/Vis-spectroscopy on the entire HydABC complex, as well as its separate subunits, and domains thereof.

2. Materials and methods

2.1. Protein expression and purification

Thermotoga maritima's electron confurcating hydrogenase “apo”-HydABC, the “apo”-HydA subunit and the HydB subunit were expressed and purified as described in Chongdar et al. (2020) [30] with minor modifications: to ensure maximal FMN occupation, 50 µg/ml riboflavin was added to the culture medium of *E. coli* and 10-fold molar excess FMN were added to the sample prior to the gel-filtration column. It is noteworthy that the label “apo” refers only to an incompletely matured (and hence inactive) H-cluster, thus lacking the [2Fe]_H subcluster [31]. All other iron-sulfur clusters are present in the expressed enzyme. The isolated HydC subunit was recovered during HydABC purification.

The genes encoding the separate C-terminal 8Fe-ferredoxin domain of HydB (TmHydB_{CTD}) and the C-terminal thioredoxin domain of HydA (TmHydA_{CTD}) were generated by PCR on the pASK-IBA17+/tmhydabc expression plasmid using the following primers: TmHydB_{CTD} 5'-AAGAAATACGTCATCAATCCAG -3' (forward) and TmHydB_{CTD} 5'-GCCCTGAAAATAAAGATTCTCAC -3' (reverse) and HydA-CT 5'-AAGATGTGGAAATACTGCCC -3' (forward) and HydA-CT 5'-GCCCTGAAAATAAAGATTCTCAC -3' (reverse) and expressed and purified following the same protocol as apo-HydA.

The Strep-tag remained uncleaved in all samples. Concentrated proteins were stored, frozen and shipped in 100 mM Tris-HCl, 150 mM NaCl, pH 8. Protein concentration was determined by absorbance at 280 nm using an experimentally determined (protein quantitation with the Biorad DC protein quantitation assay) extinction coefficient of 250 mM⁻¹ cm⁻¹ for HydABC, 108 mM⁻¹ cm⁻¹ for HydA, 20 mM⁻¹ cm⁻¹ for HydC and 112 mM⁻¹ cm⁻¹ for HydB. FMN concentration in HydB

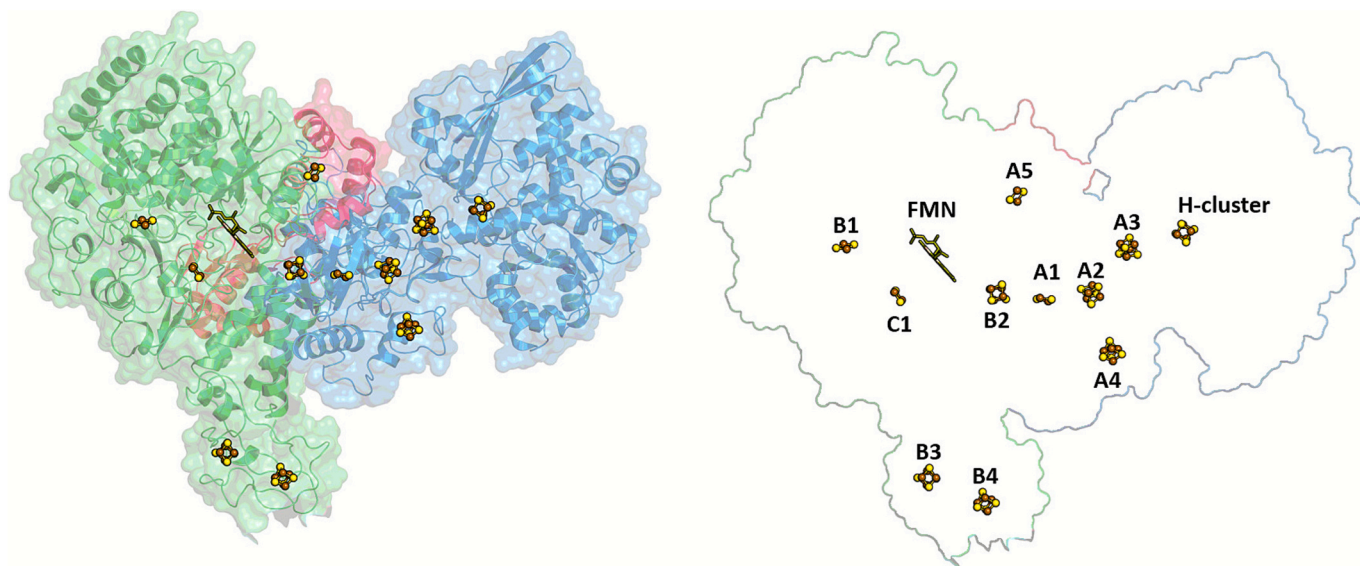


Fig. 1. Schematic representation of the redox cofactors in the cryo-EM structure model of the [FeFe]-Hydrogenase apo-*TmHydABC* complex of *T.maritima* without matured H-cluster (PDB ID: 7P92). Nomenclature of redox cofactors according to Furlan et al. (2022) [19].

samples was determined by absorbance at 450 nm ($\epsilon_{\text{FMN}} = 12.2 \text{ mM}^{-1} \text{ cm}^{-1}$, [32]) of the supernatant after protein denaturation in 2.25 M KCl, in 50 mM NaPO_4 buffer, pH 7 at 100°C for 30 min and centrifugation at 1400g for 30 min. Protein concentration was corrected for the contribution of the FMN at 280 nm and the protein:FMN ratio determined to be close to 1 for HydB samples. Flavin content of apo-HydABC was determined to be at about 0.6 FMN/protein.

2.2. Optical spectroscopy

UV/Vis spectra were recorded on a Cary 5E or Cary 4000 spectrophotometer. Samples were prepared anaerobically in a glovebox and sealed in stoppered Quartz cuvettes before being transferred to the spectrometer for analysis.

Spectra of redox mediators were recorded on a diode array spectrometer (Ocean Optics, DH-2000-BAL light source, USB2000 + UV-Vis-ES detector) in a glove box (MBraun LabMaster SP) constantly flushed with N_2 . $177.5 \mu\text{M}$ anthraquinone-2-sulfonate (AQ1S, $E_m^{0\cdot} = -228 \text{ mV}$, ThermoFisher, CAS: 131-08-8) and $2.75 \mu\text{M}$ benzyl-viologen (BV, $E_m^{0\cdot} = -350/540 \text{ mV}$, Merck, CAS: 1102-19-8) were prepared in buffer mix A with 80 mM KCl and 50 mM sodium acetate (Merck, CAS: 127-09-3, $\text{pK}_a = 4.54$), MES (ROTH, CAS: 4432-31-9, $\text{pK}_a = 6.15$), HEPES (ROTH, CAS: 7365-45-9, $\text{pK}_a = 7.5$), Tris (ROTH, CAS: 77-86-1, $\text{pK}_a = 8.07$) and glycine (ROTH, CAS: 56-40-6, $\text{pK}_a = 9.6$), adjusted to pH 7. Optical spectra of the mediator mix ($10 \mu\text{M}$ AQ1S, $1 \mu\text{M}$ BV and $16 \mu\text{M}$ methyl viologen (MV, $E_m^{0\cdot} = -440/-772 \text{ mV}$, Acros Organics, CAS: 1910-42-5) in buffer mix A as used for redox titrations were also recorded.

2.3. Electrochemical redox titrations followed by optical spectroscopy

Sample preparations and measurements were carried out in the glove box (MBraun LabMaster SP). $150 \mu\text{l}$ protein sample (containing 15 to $30 \mu\text{M}$ HydB or 8.5 to $15 \mu\text{M}$ HydABC) in buffer mix A adjusted to pH values in the range of 5.2 to 9.2 and containing the mediator mix were injected into the electrochemical setup [33], SI). The setup consists of a capillary non-modified gold working electrode (WE), an Ag/AgCl_2 reference electrode (RE) in 3 M KCl (WPI, DRIREF-2SH) and a platinum counter electrode (CE). The setup was calibrated before and after each measurement by cyclic voltammetry with ferrocene methanol ($E = 233 \text{ mV}$ vs Ag/AgCl_2 in 3 M KCl, 97 %, Sigma-Aldrich, CAS: 1273-86-5) in 0.5 M KCl [34]. The experimental protocol involved reduction of the sample to

-600 mV for HydB or -700 mV for HydABC and subsequent incremental oxidation in steps of 20 mV to -100 mV followed by reduction back to the fully reduced state in similar steps. Two complete oxidation/reduction cycles were performed, and ambient potential was measured and adjusted using a Metrohm potentiostat (Autolab/PGSTAT101). After each potential step and equilibration for 10 min, spectra were recorded on a diode array spectrometer (Ocean Optics, DH-2000-BAL light source, USB2000 + UV-Vis-ES detector), connected to the electrochemical cell via optical fibers (QP-600-2-SR) and a 320 nm cutoff filter to avoid photo damage of FMN. The potentiostat together with the spectrometer were controlled by a LABVIEW script (Version 2021).

2.4. Chemical redox titrations followed by EPR spectroscopy

Redox titrations were performed in a Jacomex glove box under nitrogen atmosphere in a conical vessel with an Ag/AgCl reference electrode, calibrated before and after each experiment against a saturated quinhydrone solution ($E_m^{0\cdot} = +280 \text{ mV}$ vs SHE, Sigma Aldrich, CAS: 106-34-3) in 1 M MOPS buffer, pH 7, at RT. Titrations were performed in buffer mix B: MES (Sigma, CAS: 4432-31-9, $\text{pK}_a = 6.15$), HEPES (ROTH, CAS: 7365-45-9, $\text{pK}_a = 7.5$), Tricine (ROTH, CAS: 5704-04-1, $\text{pK}_a = 8.15$), AMPSO (Sigma, CAS: 68399-79-1, $\text{pK}_a = 8.9$) and KCl at 50 mM each, adjusted to pH values between 6 and 9. 3 ml of $10\text{--}30 \mu\text{M}$ protein in buffer mix B was stirred for 1 h with mediators (depending on experiments, 10 or $100 \mu\text{M}$ each AQ1S, BV, MV and anthraquinone-1,5-disulfonate (AQ2S, $E_m^{0\cdot} = -184 \text{ mV}$, TCI Chemicals, CAS: 853-35-0; the lower mediator concentration was used for detection of the semi-reduced FMN radical to minimize spectral contamination by radicals due to mediators). $1 \mu\text{l}$ -aliquots of 200 mM sodium dithionite and 10 mM potassium hexacyanoferrate(III) stock solutions were used to adjust ambient potentials. After equilibration, $200 \mu\text{l}$ of sample were transferred into an EPR tube and frozen inside the glove box in a cold-finger in ethanol cooled to -50°C to -60°C .

2.5. Evaluation of data from optical and EPR redox titrations

Redox potentials were determined by fitting the signal amplitude changes of the EPR signals of A1 and A5 recorded on HydABC, B1 recorded on HydB as well as the optical signal of B1 to a Nernst curve (Fig. 2) and the g_x signal at 1.91 recorded on HydABC to a sum of three Nernst curves (Fig. 3).

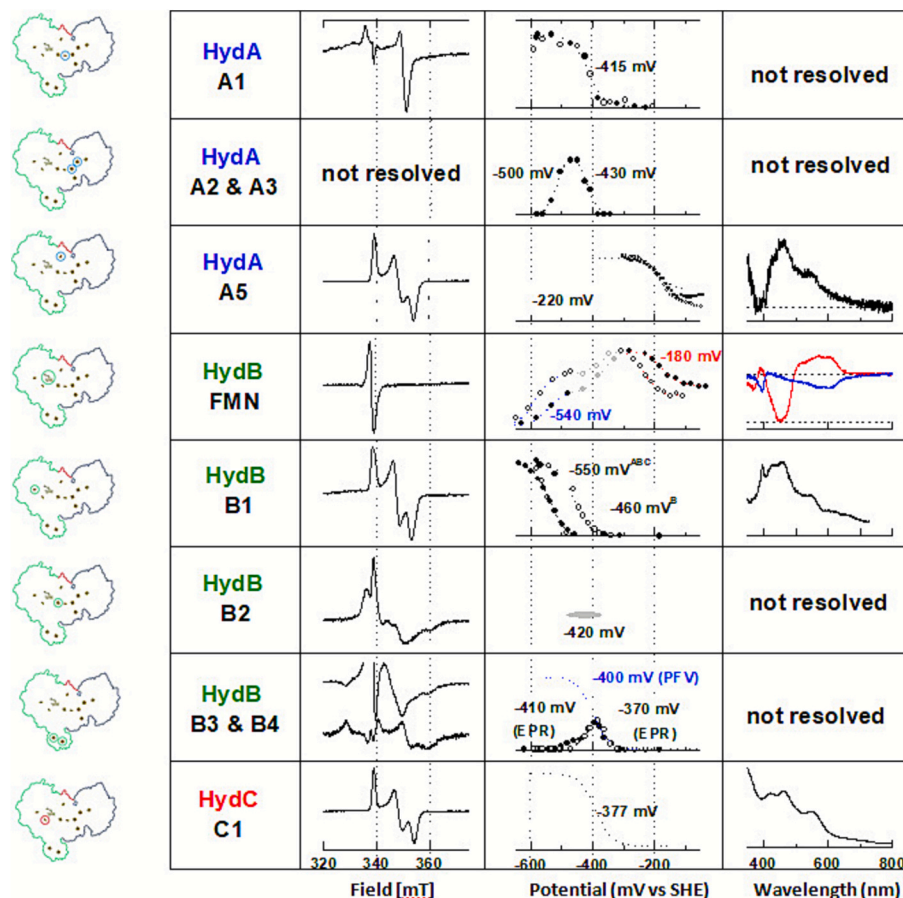


Fig. 2. Overview of spectral and redox properties of the cofactors of *TmHydABC*. Columns show from left to right: pictogram of the enzyme with the respective cofactor circled, nomenclature of the centres (according to Furlan et al. 2022 [19]), EPR-spectra, redox behaviour, UV/Vis spectra.

A1: EPR spectrum: HydABC, -424 mV spectrum from a redox titration at pH 9, 50 K, 6.32 mW from which the spectrum of centre C1 was subtracted. Redox behaviour: amplitude of the $g_z = 2.018$ signal as a function of potential at pH 9, 50 K, 6.32 mW. UV/Vis spectrum could not be deconvoluted.

A2/A3: Redox behaviour: HydABC: amplitude changes of the $g_z = 2.045$ peak at 15 K, 6.32 mV, pH 9. EPR and UV/Vis spectra could not be resolved.

A5: EPR spectrum: isolated Trx-like domain (HydA-CT) reduced by dithionite at 50 K, 6.32 mW, pH 8.5. Redox behaviour: amplitude changes at 450 nm (oxidative titration: open circles, reductive titration: closed circles), pH 8. At higher potentials absorbance changes of the flavin overlap; data points attributed to A5 were fitted by a Nernst curve with $E_m = -220$ mV (dotted line). UV/Vis spectrum: difference spectrum between spectra at -194 mV and -274 mV at pH 8 corrected for contributions of BV.

FMN: EPR spectrum: HydB at -303 mV and pH 8.5, 50 K, 6.32 mW. Redox behaviour: amplitude changes at 604 nm at pH 6.5 (dotted line) and pH 8.3 (dash-dotted line) fitted to a sum of three $n = 1$ -Nernst curves (red: $FMN_{NSR/OX}$, blue: $FMN_{RED/NSR}$, gray: iron-sulfur clusters at 370 mV). UV/Vis spectra: difference spectra at pH 8.34, -268 mV minus -188 mV (red, NSR/OX) and -649 mV minus -528 mV (blue, NSR/RED). Global fit analysis [57] of data recorded at pH 6.5 resulted in identical spectra.

B1: EPR spectrum: HydB at pH 9 and -540 mV, 50 K, 6.32 mW. Redox behaviour: amplitude of the g_z signal of HydB at pH 9 and absorbance changes at 400 nm at pH 7 for HydABC. UV/Vis spectrum: difference spectrum (-700 mV and -430 mV) of HydABC, pH 7.

B2: EPR spectrum: difference spectrum (-481 mV minus -445 mV) at pH 9, 15 K, 6.32 mW after subtraction of the signals from centre B1. Redox behaviour: the gray ellipsoid indicates the potential range where the centre undergoes its redox transition (see text and Fig. 5 for further explanations).

B3/B4: EPR spectra: difference spectra of HydB, pH 7 (-340 mV minus -270 mV (lower spectrum, high potential cluster); -435 mV minus -367 mV (upper spectrum, interaction spectrum with both clusters reduced)), 15 K, 6.32 mW. Redox behaviour: amplitude changes of the $g_z = 2.06$ peak on HydB (closed circles) and HydABC (open circles), dotted line represents an $n = 1$ Nernst curve with an E_m of -400 mV as found by PFV on the isolated C-terminal domain of HydB.

C1: EPR spectrum: dithionite reduced HydC at 50 K, 6.32 mW, pH 8.5. Redox behaviour: simulation of a Nernst curve with an E_m of -377 mV [30]. UV/Vis spectrum: dithionite reduced HydC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The redox behaviour of the g_z signal from the A2/A3 and B3/B4 2 [4Fe-4S] pairs was fitted to a sum of two interdependent Nernst curves according to:

$$A = c \cdot \left(\frac{1}{1 + \left(\frac{10 \exp((E_1 - E_2)/2.303 R^* T / F / 2)}{10 \exp((E_2 - E_1)/2.303 R^* T / F / 2)} \right)^n} \right) + \left(\frac{10 \exp((E_2 - E_1)/2.303 R^* T / F / 2)}{10 \exp((E_1 - E_2)/2.303 R^* T / F / 2)} \right)^n + \text{off}$$

with A: measured signal amplitude, c: full amplitude of the 1-electron reduced high potential cluster, E1 and E2: redox midpoint potentials of the two redox transitions, R: universal gas constant, F: Faraday con-

stant, T: Temperature in K, off: offset (adapted from [35]).

The optical signals of the semi-reduced flavin, recorded on HydB, were fitted to a sum of two Nernst curves.

2.6. Electron paramagnetic resonance (EPR) spectroscopy

EPR spectra were recorded on a Bruker Elexsys E-500 X-band EPR spectrometer equipped with an ER4012ST rectangular cavity and an ESR900 helium flow cryostat (Oxford Instruments). Experimental parameters are given in the Figure legends.

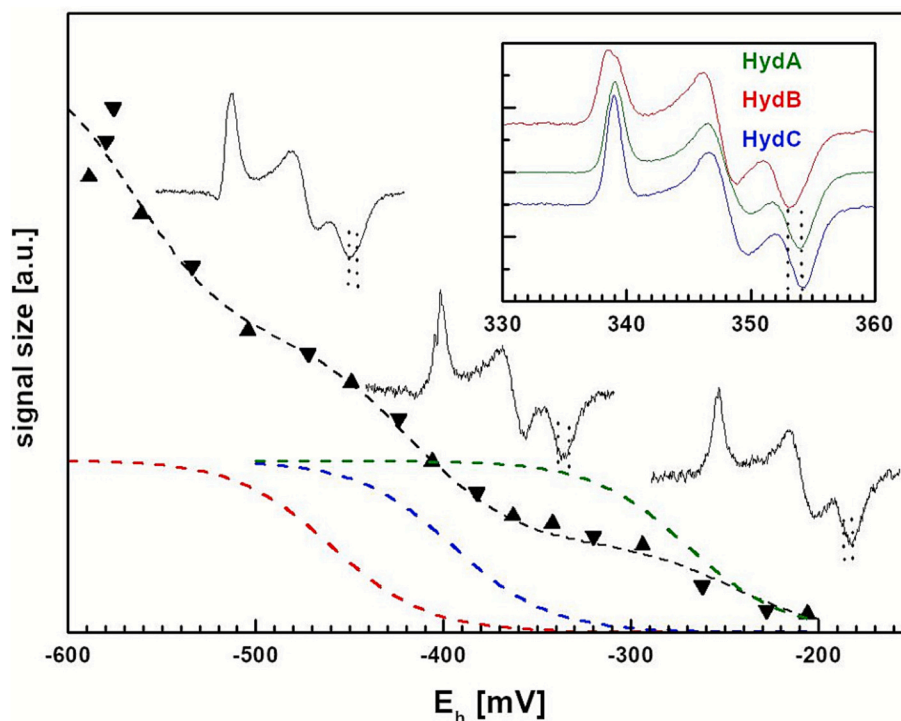


Fig. 3. Thioredoxin (Trx)-like [2Fe-2S]-clusters in apo-*TmHydABC*. Amplitude at $g = 1.9116$ (g -value with equal amplitude for each Trx-like centre) of *TmHydABC* (pH 9) as a function of the potential (up triangle: reductive titration, down triangle: oxidative titration), fitted to a sum of three $n = 1$ Nernst curves (black dashed line, $E_m = -270$ mV, -420 mV, -550 mV). Colored dashed lines: redox behaviour of HydA (blue, $E_m = -415$ mV), HydC (red, $E_m = -377$ mV) and HydB (green, $E_m = -460$ mV) Trx-like [2Fe-2S]-cluster as determined on the isolated subunits. Spectra obtained from apo-*TmHydABC* are shown in black, the inset shows the spectra as obtained on the isolated subunits. Parameters: T: 120 K, MWP: 6.4 mW, MWF: 9.48 GHz, MA: 5 G, MF: 100 kHz modulation frequency, RG 50 dB, time constant: 82 ms. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.7. Protein film voltammetry (PFV)

1 μ l protein solution in 100 mM TRIS/HCl, 150 mM NaCl, pH 8 was applied to a glassy carbon electrode in a glove box under N_2 atmosphere (Belle Technologies). A saturated calomel electrode was used as reference and platinum wire as a counter electrode. Cyclic voltammetry was conducted in 100 mM TRIS/HCl, 500 mM NaCl, pH 8 at room temperature without rotation at a scan rate of 50 mV/s using a PalmSens EmStat3 [30]. The analysis of the non-catalytic voltammogram in Suppl. Fig. S2 was performed with the program QSoas (open source available at qsoas.org) [37]. A spline baseline was subtracted to remove the contribution of the capacitive current, and a sum of two one-electron Nernstian peaks (eq. 1 in Léger and Bertrand (2008) [38]) of equal intensities was fitted to the resulting Faradaic current. The procedure was repeated to analyze the forward and backward sweeps.

3. Results

The high number of iron-sulfur clusters in *TmHydABC* substantially hampers identification and characterization of individual redox centres due to spectral superposition both in optical and EPR spectroscopy. We have therefore assisted the examination of the native enzyme complex by a parallel study of the individually expressed protein subunits HydA, HydB and HydC as well as of selected cofactor-carrying domains thereof.

While at cryogenic temperatures below 20 K, all types of iron-sulfur clusters are generally easily detectable, meanwhile the cubane [4Fe-4S]-clusters rapidly broaden to beyond detection at temperatures above around 20 K, and the plant-type [2Fe-2S]-cluster broadens above around 60 K, leaving the only EPR spectra visible those of the thioredoxin-type [2Fe-2S] clusters and the FMN radical. We, therefore, collected EPR spectra at 15 K, 50 K and 120 K.

As shown below, in *TmHydABC* as well as in all studied subunits and

fragments of the enzyme, this procedure allowed us to tease apart the contributions arising from [2Fe-2S]-clusters which were selectively visible at $T \geq 50$ K, devoid of appreciable spectral overlap from the cubane clusters. Due to the very low redox midpoint potentials of some redox cofactors in the *TmHydABC* enzyme, in chemical redox titrations for EPR experiments high pH values were required to attain near-full reduction of the cofactors. Electrochemically induced UV/Vis redox titrations were not hampered by the limited reducing power of dithionite and could thus be performed down to -700 mV (vs SHE). However, optical spectra of iron-sulfur clusters are broad and are therefore difficult to deconvolute.

We will, in the following, first present the results gathered on these individual subunits and fragments of the whole enzyme and then try to integrate the obtained spectral and redox parameters into the dataset measured on the entire enzyme. The deconvolution of the contributions of individual centres to the data recorded on the subunits and the entire enzyme is detailed in the Result section. The g -values of all EPR species deduced from this analysis are summarized in Table 1. The optical and EPR-spectra as well as redox titration curves are shown in Fig. 2, which presents an overview of the here gathered results. In the Discussion section we will try and interpret interactions between cofactors and differences observed for individual cofactors when comparing their behaviour in individual subunits and in the entire enzyme. Finally, in the conclusion section the main characteristics of the thermodynamic landscape of *TmHyd* are synthesised and put in the light of the yet to be discovered reaction mechanism of this class of enzymes.

3.1. HydA and its C-terminal [2Fe-2S]-domain

As discussed previously, the HydA-subunit of *TmHydABC* corresponds to a fusion of a *Chlamydomonas*-type [FeFe]-hydrogenase H-domain [31] with an (N-terminal) “Y-junction module” [14], and a (C-

Table 1EPR and redox characteristics of the iron-sulfur clusters in HydABC of *T. maritima*.

Subunit	Cluster	Nature	g_1 / g_z	g_2 / g_y	g_3 / g_x	E_m (mV, pH 7)
A	A1	Plant-type [2Fe-2S]	2.018	1.937	1.937	-415 ^{ABC}
	HP (A2/A3)	8Fe -Fd [4Fe-4S]	~2.045	?	?	-430 ^{ABC}
	LP (A2/A3)	8Fe -Fd [4Fe-4S]	—	—	—	-500 ^{ABC}
	A4	His-[4Fe-4S]	—	—	—	—
	A5	Trx-like [2Fe-2S]	1.998	1.945	1.913	~ -250 ^{ABC}
B	B1	Trx-like [2Fe-2S]	1.999	1.949	1.917	-460 ^B -550 ^{ABC}
	B2	[4Fe-4S]	~2.02 [*]	~1.94	1.87	-420 ^B
	HP (B3/B4)	8Fe -Fd [4Fe-4S]	2.06	1.93	1.89	-370 ^B
	LP [*] (B3/B4)	8Fe -Fd [4Fe-4S]	2.04 > 2.00 [*]	1.96	~1.94	-410 ^B
	C1	Trx-like [2Fe-2S]	1.998	1.945	1.913	-377 ^C -420 ^{ABC}

^{ABC} Redox potential obtained in the entire HydABC complex.^B Redox potential obtained in the isolated HydB subunit only.^C Redox potential obtained in the isolated HydC subunit only [30].^{*} g-value close to the radical $g = 2$ signal during redox titration could not be attributed with certainty.^{*} The g-values appearing with the redox transition of the LP cluster describe the interaction spectrum between the B3/B4 clusters.

terminal) Trx-like [2Fe-2S] domain. The catalytic H-cluster was not matured in the samples studied in this work, meaning that the enzyme was inactive for proton reduction and hydrogen oxidation, permitting equilibrium redox titrations to be performed even at ambient potentials conducive to redox conversion between protons and H₂ in a matured enzyme.

An initial spectroscopic characterization of the isolated HydA protein was reported by Chongdar et al. (2020) [30]. However, individual redox potentials of the ensemble of its iron-sulfur centres have not yet been determined. A stepwise reduction of the HydA-subunit showed that in the spectra measured at 50 K, the Trx-like [2Fe-2S]-cluster A5, recognizable by its rhombic spectrum below $g = 2$ [27], became observable at less reducing potentials than the plant-type [2Fe-2S]-cluster A1 with its characteristic axial signal. Furthermore, the A5 cluster also appeared earlier than the bulk of the [4Fe-4S]-clusters A2, A3 and A4, identifying cluster A5 as the highest potential iron-sulfur centre of the HydA subunit. For the sake of assigning individual Trx-like clusters in the entire enzyme (see below), we recorded the EPR-spectrum of cluster A5 in the heterologously, separately expressed C-terminal domain which holds exclusively this cluster (Fig. 3). Further characterization of the iron-sulfur centres was achieved on the entire HydABC enzyme.

3.2. The HydC-subunit

HydC has been examined previously and both its spectral and redox parameters have been reported [33]. Here, we recorded again the spectra of the single Trx-like cluster C1 present in this subunit (Fig. 2, row 8) to attribute each of the individual Trx-like clusters present in HydA, HydB and HydC to the electrochemical species observed in the entire *TmHydABC* enzyme (Fig. 3). The redox midpoint potential of cluster C1 of -377 mV at pH 8 was adopted from Birrell et al. (2016) [33] and is represented in Fig. 2 (8th row, 4th column).

3.3. The HydB-subunit and its C-terminal 2×[4Fe-4S]-domain

HydB harbors the FMN moiety together with four iron-sulfur centres, that is, the Trx-like cluster B1, the cubane [4Fe-4S]-cluster B2 (in close

vicinity of the flavin) as well as the bacterial ferredoxin-like protein carrying clusters B3 and B4. We performed redox titrations on the HydB subunit monitored by EPR-spectroscopy at pH values in the range of 6.5 to 9 as well as by UV/Vis-spectroscopy at pH 5.2 to 9.2. To avoid spectral contributions from mediators in the EPR spectra, samples were also reduced step-by-step via addition of sodium dithionite, in the absence of mediators and a measuring electrode.

In EPR redox titrations at 50 K and higher, a single iron-sulfur cluster was observed to become reduced at -460 mV with the spectral characteristics of a Trx-like [2Fe-2S]-centre [27]. Since there is only one Trx-like-cluster in isolated HydB (Fig. 1), this EPR species was attributed to cluster B1 (Fig. 2, 5th row, 3rd column).

The C-terminal domain of the HydB protein structurally corresponds to a bacterial-type ferredoxin and was expressed heterologously to be investigated by PFV. It yielded a single wave centered around -400 mV (see Suppl. Fig. S2), which could be equally well interpreted as arising from two independent redox species with midpoint potentials of -370 mV and -410 mV, or from a single species with a potential of -390 mV. EPR spectra recorded on this domain partially or fully reduced by dithionite were different (see below, Suppl. Fig. S3), arguing for the presence of two iron-sulfur clusters. The amount of the sample of the C-terminal HydB domain was not sufficient for a redox titration monitored by EPR at 15 K but this experiment was carried out on the entire subunit. The titration (Suppl. Fig. S1) showed the appearance of signals due to the reduction of an iron-sulfur centre below -300 mV with g-values of 2.06/1.93/1.89. These spectral features weren't visible at 50 K and above, identifying this spectrum as originating from a single [4Fe-4S]-cluster. On progressive reduction, the spectrum was distorted: an increase in signal amplitude below $g = 2$ was accompanied by a decrease of intensity of the g_z -peak of the [4Fe-4S]-cluster spectrum at $g = 2.06$ (Suppl. Fig. S1). Evaluating the amplitude of the $g = 2.06$ signal as a function of potential (Fig. 2 7th row, 4th column) resulted in a bell-shaped titration curve that can be rationalized by two models: the two clusters involved could have identical redox potentials and spectra with the spin-coupled two-electron reduced population building up at lower potential than the one-electron reduced state [39]. Otherwise, both clusters may feature distinct redox potentials and the EPR spectrum observed at higher redox potentials reflects the reduction of one of the two clusters, whereas reduction of the second cluster at lower redox potentials induces the distortion of the spectrum due to spin-coupling between the two reduced clusters. Such a behaviour is characteristic for bacterial-type 2[4Fe-4S]-cluster-ferredoxins where it reflects paramagnetic interactions between the two neighbouring cubane clusters in their reduced state [40–42]. A fit of the data to a sum of two interdependent Nernst curves (see method section) resulted in redox potentials of the two clusters close to -400 mV, with a redox midpoint potential difference of less than 50 mV, in agreement with the PFV data above (Suppl. Fig. S2).

The EPR spectrum of the higher potential component (Fig. 2, 7th row, 3rd column lower spectrum) can be observed in relatively pure form at less reducing potentials (-340 mV), while the spectrum at lower potentials (-435 mV minus -367 mV) represents the interaction spectrum of the two clusters (7th row, 3rd column, upper spectrum). To obtain the pure interaction spectrum shown in Fig. 2 we subtracted the contribution of the higher potential component from the spectral changes occurring between -367 mV and -435 mV, i.e. before further clusters contributed to the spectra. The g_x signal at 1.89 from the high potential cluster was thereby minimized. Spectra obtained on the isolated domain by stepwise addition of sodium dithionite were altered (broadened g_y line and shifted g_z peak) as compared to those measured on the HydB subunit (see Suppl. Fig. S3, as black dashed line), in line with observations on other isolated bacterial-ferredoxin-like proteins or domains which frequently show higher g-strains than their native, enzyme-bound, counterparts (e.g. Psac [43]). Obviously, the question of which of the two spectral/redox species belongs to structural cluster B3 and which one to B4 remains open.

At potentials below -450 mV, the reduction of a further [4Fe-4S]-cluster was suggested by the appearance of a well-defined g_z -peak at $g = 2.02$ together with an additional contribution to the g_x -region at 1.87. Calculating difference spectra and subtracting the contribution of the [2Fe-2S]-cluster B1 yielded the shape of this additional spectral species, which, for lack of other candidates in the structure, was attributed to centre B2. This attribution is corroborated by very similar EPR parameters of the structurally equivalent cluster N3 in the homologous complex I [44]. We tried to analyze its redox properties by tracing the potential-dependent intensity of the mentioned g_z -peak since the spectral convolution of the remaining EPR spectral features hampered evaluation of the g_y - and g_x -lines. This g_z peak, however, was increasingly obscured at very low potentials by signals of mediators and in particular of the viologens. Despite these limitations, we unambiguously saw the g_z peak appearing and increasing below -450 mV. Surprisingly, however, no redox difference spectral features attributable to a [4Fe-4S]-cluster were detected in UV/Vis titrations on HydB in this region of ambient potentials as will be discussed in more detail below.

None of the above discussed iron-sulfur centres in the HydB subunit displayed any significant pH-dependence between pH 6 and pH 9.

At ambient potentials of -500 mV and lower, the field positions of basically all observed lines started to slightly shift in different directions, resulting in derivative signals over the whole spectral range in the redox difference spectra at 15 K as well as at 50 K. This phenomenon was observed in redox titrations on HydB at all pH-values and we will provide a tentative rationalization below.

In EPR redox titrations, the presence of mediator signals around $g = 2$ strongly hampers the observation of enzyme-associated radical species. To be able to assess the potential presence of flavin-derived radicals, we have gradually reduced HydB samples via addition of increasing amounts of sodium dithionite in the absence of mediators and therefore

a measuring electrode. After the disappearance of spurious amounts of a [3Fe-4S]-cluster spectrum (visible in all redox titrations followed by EPR and becoming reduced at around -100 mV) which we interpret to arise from a small fraction of degraded [4Fe-4S]-clusters), an isotropic, radical-type signal became observable at $g = 2$. This signal was observed at temperatures from 8 K to 160 K and down to the highest reduction states achieved with dithionite. An EPR spectrum of this radical species is shown in Fig. 2 (4th row, 3rd column and Suppl. Fig. S4). The width of the isotropic $g = 2$ feature was 1.9 mT, which corresponds well to that of a neutral semi-reduced flavin radical [45].

An in-depth characterization of the electrochemical parameters of this flavin radical was achieved via electrochemically-controlled redox titrations monitored by UV/Vis-spectroscopy which are less strongly affected by the absorbance changes of mediators. Indeed, redox-difference spectra characteristic for a transition from the oxidized flavin to its neutral semi-reduced form were observed in the range of -100 mV to -250 mV. The corresponding difference spectra (Fig. 2, 4th row, 5th column, red line) showed a decrease of the broad 450 nm-peak arising from the oxidized (OX) form of FMN with the concomitant appearance of multiple absorbance bands at higher wavelengths (500–625 nm region) characteristic for a neutral ("blue") semi-reduced (NSR) species [45]. Fig. 2 (4th row, 5th column, blue line) depicts the dependence of this spectral form on ambient potentials at pH 6.5 and 8.3. The full pH-dependence of the OX-NSR transition of the FMN is shown in Fig. 4. As in the mediator-free EPR experiments described above, the reduction of the flavin to its semi-reduced form occurred at higher potentials than the reduction of the iron-sulfur clusters and the spectral transformation of the flavin upon its first reduction could be observed without contributions from those clusters. Further reduction of the semi-reduced flavin to its 2-electron reduced state was observed below -500 mV, well below the redox transitions of the iron-sulfur

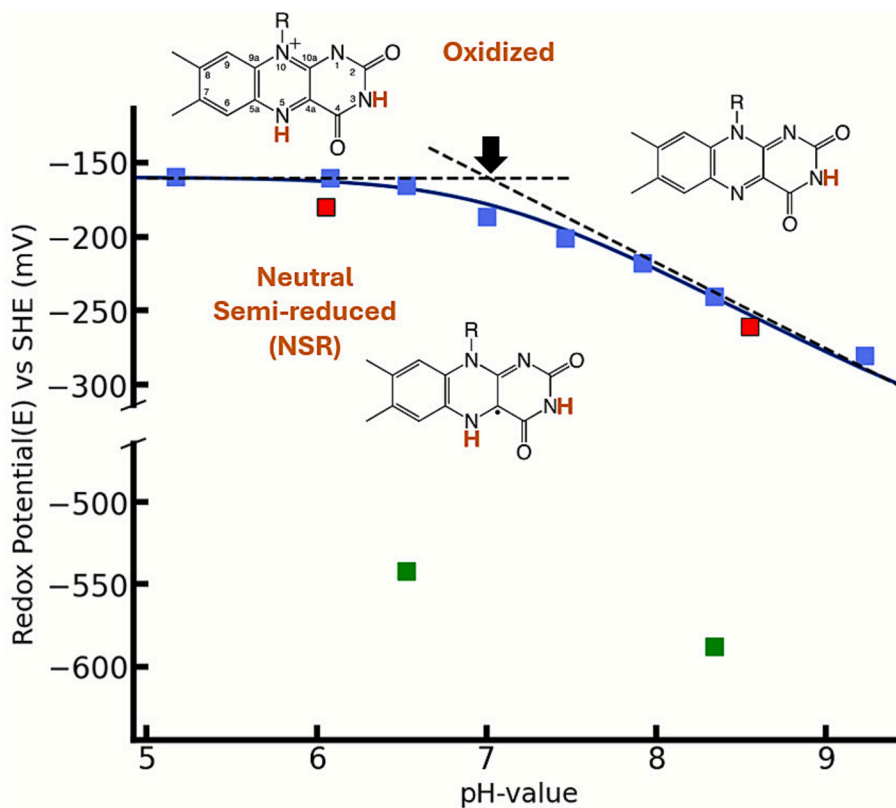


Fig. 4. Dependence of FMN $E_{\text{OX-NSR}}$ and $E_{\text{NSR-RED}}$ redox potential in HydB on pH.

One-electron redox potentials obtained by spectro-electrochemical redox titrations for FMN in HydB: blue squares: $E_{\text{OX-NSR}}$, green squares: $E_{\text{NSR-RED}}$. Red squares $E_{\text{OX-NSR}}$ determined by EPR-spectroscopy. Dashed line: 0 mV/pH and -60 mV/pH, solid line: fit of the data points to the Henderson Hasselbach equation, with a pK_a of 7 (black arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clusters. These signals, however, were distorted by viologen absorbance changes and could only be evaluated at two pH-values (Fig. 2, 4th row, 5th column, blue line and Fig. 4). The blue dashed line in Fig. 5 retraces the redox dependence of the flavin NSR-radical at pH 8.3 (without the contributions of the iron-sulfur clusters at this wavelength) for the sake of comparison with the results obtained by EPR as described in the following.

To further corroborate that the radical feature in EPR and the flavin-type UV/Vis difference spectra belong to the same redox species, we have performed an EPR redox titration on HydB using significantly higher protein concentrations and lowering the amount of mediators present. Under these conditions, the radical species could be directly observed in EPR. Due to sample limitations, we performed these titrations only at two representative pH-values (pH 6 and pH 8.5) and will in the following describe in more detail the phenomena observed at pH 8.5.

While the appearance of the radical signal between -100 mV and -300 mV (Fig. 5, orange data points) closely followed the optical titration curve (blue dashed line) of the neutral semi-reduced flavin signal at a comparable pH-value (pH 8.3), a further increase in amplitude of the 1.9 mT-wide EPR line between -350 and -450 mV was observed. No counterpart to this increase in intensity was found in the UV/Vis-titrations. Below -500 mV, the radical signal diminished substantially, in accordance with a reduction of the semi-reduced to the fully reduced form just as observed in the UV/Vis-monitored titration (blue dashed line in Fig. 5). A qualitatively similar behaviour was observed at pH 6 (not shown).

While overall in the HydB subunit (just as in all other subunits and in the entire enzyme), UV/Vis- and EPR-monitored redox titrations yielded closely corresponding results (whenever similar parameters could be assayed), we are here left with two major discrepancies.

- (a) Cluster B2: The appearance well below -400 mV of the $g_z = 2.02$ peak arising from the $[4\text{Fe-4S}]$ -cluster B2 while no cubane cluster was observed to get reduced in this potential range during UV/Vis-monitored titrations.

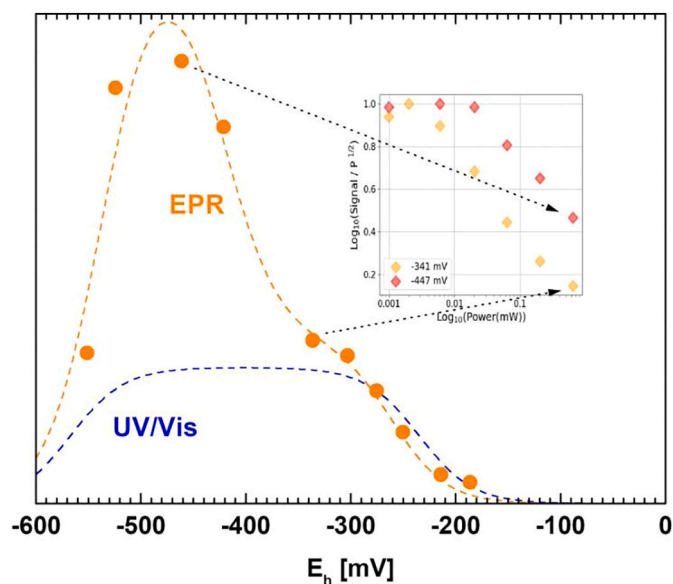


Fig. 5. Amplitude of the signal of the semi-reduced flavin in optical (blue, without contributions of iron-sulfur clusters, pH 8.3) and EPR (orange) redox titrations. Inset: 50 K Saturation behaviour of the FMN NSR in HydB at pH 8.5 at -341 mV (iron-sulfur centres mainly oxidized) and -447 mV (iron-sulfur centres mainly reduced). The dashed orange curve is a sum of three Nernst curves with $n = 1$ and midpoint potentials of -270 mV, -421 mV and -530 mV. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

- (b) The FMN: the EPR titration of the radical signal attributed to the flavin's semi-reduced state features an additional increase not observed in UV/Vis-monitored titrations. This apparent increase in signal size of the radical cannot be straightforwardly explained as a redox phenomenon.

A common rationalization of both discrepancies (a) and (b) is suggested by the structural features of the *TmHydABC*-enzyme as determined by cryo-EM [19]. Cluster B2 and the flavin are by far the most closely spaced redox centres of the entire enzyme with an edge-to-edge distance of only 6.8 Å. Such short spacings allow for both electrostatic and paramagnetic interactions to occur. Centre B2 in its paramagnetic (i. e. reduced) state represents (by virtue of its nature as a $[4\text{Fe-4S}]$ -cluster and as evidenced by its temperature-dependence in EPR) a fast-relaxing spin system while the semi-reduced flavin shows the slowly relaxing, easily saturating properties typical for organic radicals. Due to the close proximity between the two centres, saturation transfer from the FMN to cluster B2 is very likely to occur. Since our EPR redox titrations of the radical were performed under partially saturating conditions (to obtain sufficient signal amplitudes), the reduction of cluster B2 may partially desaturate the flavin signal via an opening-up of additional relaxation pathways and thus increase the size of the signal.

To assess this possibility, we have measured saturation curves for the radical signal at potentials before (i. e. at higher potentials than) the onset of the additional increase in signal size (-336 mV) and roughly at its maximum (-461 mV) (see inset to Fig. 5). A substantially faster relaxation is indeed observed at -461 mV yielding a significant desaturation of the radical signal under the experimental conditions (indicated by the dotted arrows in the inset) used to record the spectra of this EPR-titration. The redox midpoint potentials determined by UV/Vis for both the OX/NSR and NSR/RED transitions as well as the desaturation factor obtained from the inset of Fig. 5 were used to fit the EPR-titration data for the flavin by assuming that the increasing desaturation follows a Nernst-behaviour due to reduction of cluster B2. The midpoint potential of this cluster therefore was the only free fitting variable and an E_m of -421 mV for cluster B2 was obtained. The dashed orange line in Fig. 5 represents this best fit to the data points.

The appearance of cluster B2's g_z -peak at 2.02 at potentials well below -400 mV can equivalently be rationalized by assuming that this peak is at different g -values (and likely obscured by the strong radical signals due to mediators) while B2 magnetically interacts with the spin on the semi-reduced flavin. Only reduction towards the (diamagnetic) fully reduced flavin renders the $g_z = 2.02$ peak observable. Unfortunately, the quality of the data points in the titration of this g_z -peak was insufficient for performing fitting procedures as described above for the flavin radical.

Spectral shifts of EPR signals appear at low redox potentials where no redox transition of iron-sulfur clusters are observed in UV/Visible experiments. These signal shifts cannot be attributed to a redox transition of iron-sulfur centres and possible interpretations will be proposed in the discussion section.

3.4. Correlation between redox potentials and sequence idiosyncrasies in the three Trx-like $[2\text{Fe-2S}]$ -clusters

The three Trx-like clusters A5, B1 and C1 were found to exhibit substantially differing redox midpoint potentials spanning the range from about -250 mV to around -450 mV in the respective isolated subunits (Fig. 2, Fig. 3, Table I). While the overall structures of all three Trx-like domains are similar, the polypeptide stretches surrounding the $[2\text{Fe-2S}]$ -clusters feature a number of amino acid differences. Previously, the isolated HydB subunit was mutated at the Val131-position to mimic the sequence specificities of the other two Trx-like domains present in the enzyme (harbouring clusters A5 and B1) [33]. Position 131 was predicted to be instrumental in differentiating the three Trx-like ferredoxins based on multiple alignments and structure-comparisons of

members of this structural family of iron-sulfur proteins. The Val-to-Asn mutant (corresponding to the sequence pattern surrounding the A5-cluster) was found to display a shift of about 80–90 mV towards more positive values while the Val-to-Leu variant (mimicking the B1-cluster) titrated 30–40 mV lower than the HydC wild type [33]. The values determined in our work closely mirror the potential shifts observed in the HydC-variants mutated at the sequence position corresponding to Val131 in the wild type HydC protein and thus corroborate the previous pinpointing of this sequence residue as crucially influencing the electrochemical properties of the cluster in Trx-like domains.

3.5. The entire *TmHydABC* enzyme

In the same way as HydB, the apo-enzyme *TmHydABC* (without matured H-cluster, see material and methods) was subjected to redox titrations in the range of pH 6.5 to 9 monitored by EPR (at 8 K, 15 K, 50 K and 120 K) and over a range of pH 5.2 to 9.2 by UV/Vis-spectroscopy. In EPR at 50 K and 120 K, only [2Fe-2S]-centres were detected and contributions from the cubane clusters were negligible. Due to the different relaxation properties of the Trx-like and the plant-type [2Fe-2S]-centres, we selectively observed the former at 120 K where the signals of the plant-type [2Fe-2S]-centres are broadened. Fig. 3 shows the dependence of the amplitude of the Trx-like [2Fe-2S]-centres' g_x -signal (at 354 mT) on ambient potential. The dotted Nernst-curves in the bottom of the Figure represent the midpoint potentials as determined on the isolated subunits. Redox difference spectra for the three distinguishable redox waves are indicated next to these individual transitions, compared to the spectral characteristics of the Trx-like centres observed on the individual subunits and shown in the inset. The three centres (also depicted in Fig. 2, second column) differ slightly by the position of their g_x and g_y signals. Similar observations were recently published for the hydrogenase from a cyanobacterium [46]. The Trx-like [2Fe-2S]-centres observed in the isolated HydA (cluster A5) and HydC (cluster C1) feature virtually identical g -tensors (Table 1) while that seen in HydB (cluster B1) has its g_x - and g_y signal shifted to higher g -values (indicated by dotted vertical lines in the inset to Fig. 3). The respective positions are marked by vertical lines on the spectra of the individual redox waves observed for the entire complex. The position of the g_x signal showed that the spectrum obtained for the highest potential wave corresponds to either HydA or HydC. Since the determined redox dependence in the *TmHydABC* enzyme for the highest potential wave corresponds well to that of the A5 Trx-like [2Fe-2S]-centre in the HydA protein (green dashed Nernst curve in Fig. 3), we consider that this redox wave corresponds to the titration of HydA's Trx-like cluster. The second Trx-like redox wave in HydABC lies at slightly lower midpoint potentials (−415 mV) than that of the centre in the HydC protein (−377 mV [33], blue dashed Nernst curve) while the redox difference spectrum of this redox component is found halfway between those of HydA/C and of HydB. Since the redox transition occurs in the same range as that of the plant-type [2Fe-2S]-cluster A1, the strongly axial $g_{//}$ -line of A1 (see Fig. 2, 1st row, 3rd column) may overlay and shift the position of the Trx-like g_x signal to higher g -values. For this reason and for lack of an alternative interpretation (see below), we consider it very likely that the intermediate redox wave observed in *TmHydABC* at higher temperatures, that is, 50 K and 120 K, arises from the HydC cluster C1. The redox difference spectrum corresponding to the lowest potential wave closely matches that recorded in the HydB subunit and should therefore arise from the Trx-like centre B1. In contrast to the two other Trx-like [2Fe-2S]-clusters, this centre's redox midpoint potential was strongly shifted towards lower potentials, to about −550 mV, in the entire HydABC enzyme as compared to the isolated HydB subunit (red dashed Nernst curve).

Since all [4Fe-4S]-centres (observed at 15 K and 8 K) became reduced below −300 mV, the A5 Trx-like [2Fe-2S]-centre in the C-terminal domain of HydA represents the highest potential iron-sulfur cluster of the entire *TmHydABC* enzyme. In line with these EPR results, the highest potential redox species observed in UV/Vis-spectroscopy of

TmHydABC showed the typical spectral features of a Trx-like [2Fe-2S]-centre with bands around 333, 420, 460 and 550 nm [27]. Its redox potential was found between −220 mV and −270 mV in different experiments (Fig. 2, 3rd row, 4th column). This variability in redox behaviour is most probably due to the small signal amplitude and the overlap with absorbance changes of the flavin. The amplitude of this signal was indeed three times smaller than the Trx-like spectrum of B1 resolved at lower redox potentials. This is in line with the observation made on *TmHydABC* in EPR, where the high potential wave was systematically of smaller amplitude (by a factor of 2 to 3) than the signals of C1 and B1 (Fig. 3). We attribute this to a sub-stoichiometric presence of A5 in the heterologously expressed complex, possibly due to partial proteolytic digest of the mobile Trx-like domain from HydA.

The A1- (plant-type [2Fe-2S]-) centre's axial EPR-signature overlapped the EPR spectra of the Trx-like clusters. Its g_z -peak (at $g = 2.018$, Fig. 2, 1st row, 3rd column), however, is positioned outside the magnetic field range of the Trx-like clusters and therefore served to determine the redox midpoint potential of A1 to −415 mV (Fig. 2, 1st row, 4th column). The isolated HydA subunit could not be redox-titrated due to sample limitations and we therefore cannot assess whether the E_m -value of this centre may be influenced by the assembly of the individual subunits into the entire enzyme as observed for B1. At variance with B1, however, A1 is surface-exposed in the cryo-EM structure of the entire enzyme and no obvious changes in the protein environment between the isolated subunit and the entire complex are expected.

The properties of the seven [4Fe-4S]-centres in *TmHydABC* were more difficult to deconvolute, both in EPR and in UV/Vis-spectroscopy. The spectral features attributed to the C-terminal ferredoxin domain of HydB, including the bell-shaped titration curve of the g_z -peak at 2.06 of the higher potential centre (see above) were observed at the same potentials in the HydABC complex as in HydB and represent the highest potential pair of [4Fe-4S]-clusters. The dependence of the g_z -peak on ambient potential is depicted in Fig. 2 (7th row, 4th column, open symbols) and corresponds well to that observed in the isolated HydB protein (closed symbols). A similar phenomenon, i.e., an appearing and then disappearing g_z -peak (at $g = 2.045$) is also observed below −420 mV in titrations on HydABC but not on HydB. The paramagnetic centres responsible for this effect therefore need to reside in the HydA protein and, more specifically, its Y-junction module. As discussed in Zuchan et al. (2021) [14], the Y-junction module is composed of three distinct domains, that is, a plant-type [2Fe-2S]-ferredoxin, a single [4Fe-4S]-cluster domain and a bacterial-type $2 \times [4Fe-4S]$ -ferredoxin. This latter domain is structurally related to the C-terminal ferredoxin domain of HydB and it therefore seems very likely to us that the observed bell-shaped titration behaviour of the $g_z = 2.045$ peak at potentials between −400 mV and −500 mV arises from paramagnetic interactions of the two [4Fe-4S]-clusters in the bacterial ferredoxin domain of the Y-junction module. We therefore ascribe the redox potentials deduced from the titration curve shown in Fig. 2 (2nd row, 4th column) to clusters A2 and A3. By contrast, cluster A4 of the Y-junction module, that is, the cluster in the mono-cubane domain and standing out from all other cubane clusters of the enzyme by featuring a histidine ligand to the cluster (see Zuchan et al. (2021) [14]) and the [4Fe-4S]_H moiety from the H-cluster, could not be deconvoluted in the HydA protein nor in the entire HydABC enzyme from the bulk of low potential centres. A4 may have very fast relaxation properties and a mixed spin state as reported for the homologous N5 of complex I [47] and thus escaped our attention or appeared below −340 mV with the bulk of the other FeS signals. The latter explanation probably also holds true for the [4Fe-4S]_H moiety from the H-cluster. Furthermore, we were unable to deconvolute cluster B2 from the bulk of overlapping centres at low potential in *TmHydABC* but readily determined its properties in the isolated HydB subunit (see above).

In contrast to what was observed for HydB, no radical signal attributable to semi-reduced forms of the FMN moiety was detected in *TmHydABC* in EPR, even under the specific conditions used to monitor

the neutral semi-reduced flavin in HydB (as described above). Our EPR data therefore indicates that the flavin features crossed-over potentials for the individual redox transitions in the entire enzyme and therefore behaves as an $n = 2$ component in equilibrium redox titrations. Apparently in line with that, the optical redox-difference spectra across all tested pH values do not show the typical features of any semi-reduced flavin [48] (to the exception of signals of very small amplitude below pH 7 for the neutral form and at pH 9 for the anionic form, Suppl. Fig. S5A). This supports a largely direct transition from the fully oxidized to the fully reduced state, consistent with the EPR data. However, even in absence of signs for a semi-reduced state, the potential-dependent changes in the flavin absorption band at 450 nm generally followed a one-electron ($n = 1$) Nernst behaviour across the pH range of 5.2 to 9.2. A notable exception was observed at pH 8, where the data followed a two-electron ($n = 2$) Nernst curve, with a redox potential of -160 mV (Fig. S5B). The data with respect to the formation of a semi-reduced flavin radical in *TmHydABC* are therefore inconsistent between the EPR- and the UV/Vis-monitored redox titrations but even more so within the optical experiments themselves. At the time being, we therefore refrain from drawing firm conclusions as to whether the flavin features crossed-over or uncrossed potentials for the individual redox transitions in the entire *TmHydABC* enzyme.

4. Discussion

In this work, we have exploited specific EPR and optical signatures of redox cofactors in the confurcating [FeFe]-hydrogenase *TmHydABC* from *Thermotoga maritima* and determined a first, almost comprehensive, data set for their thermodynamic characteristics. These results may assist investigations of the entire enzyme family [14] and allow detecting the influence of mutants on specific clusters such as for example the integrity of the B3 cluster after deletion of B4 [29].

In this enzyme a H^+/H_2 ($E' = -414$ mV, [49]) converting catalytic centre on one side of the protein complex is connected to an $NAD^+/NADH$ ($E' = -320$ mV, [49]) and Fd_{ox}/Fd_{red} ($E' = -420$ mV, [50]) converting unit on the other side via a chain of iron-sulfur clusters (i.e., centres A1, A2, A3 and B2) all featuring redox midpoint potentials around and below -400 mV. Since the enzyme, in its natural cellular host, must be expected to operate at dissolved H_2 -concentrations well below those corresponding to 1 atm H_2 in the gas phase [51], the effective redox midpoint potential of the H_2/H^+ couple will be substantially more oxidizing than -400 mV. The thermodynamic bias of the electron transfer chain between the flavin module and the hydrogenase centre therefore should favour electron transfer in the direction of proton reduction to H_2 . While we cannot attribute a precise E_m -value to centre A4, that is, the histidine-ligand-containing cubane cluster of the Y-junction module, its redox midpoint potential is likely within those of the bulk of clusters titrating below -400 mV, since otherwise the centre would have been detectable at 8 K and 15 K in the higher potential range during our redox titrations, unless it escaped our attention due to fast relaxation properties. Incidentally, the cryo-EM structure shows that this cluster is not part of the direct path of electron transfer from the flavin module to the hydrogenase centre but resides at the interface of the dimeric enzyme [19]. Although the distance of 8.8 Å as measured in the structure [19] would be in line with inter-monomer electron transfer mediated by this cluster, its deletion by site-directed mutagenesis in HydABC of *Thermoanaerobacter kivui* does not influence electron bi/confurcating activity [20] as measured in vitro.

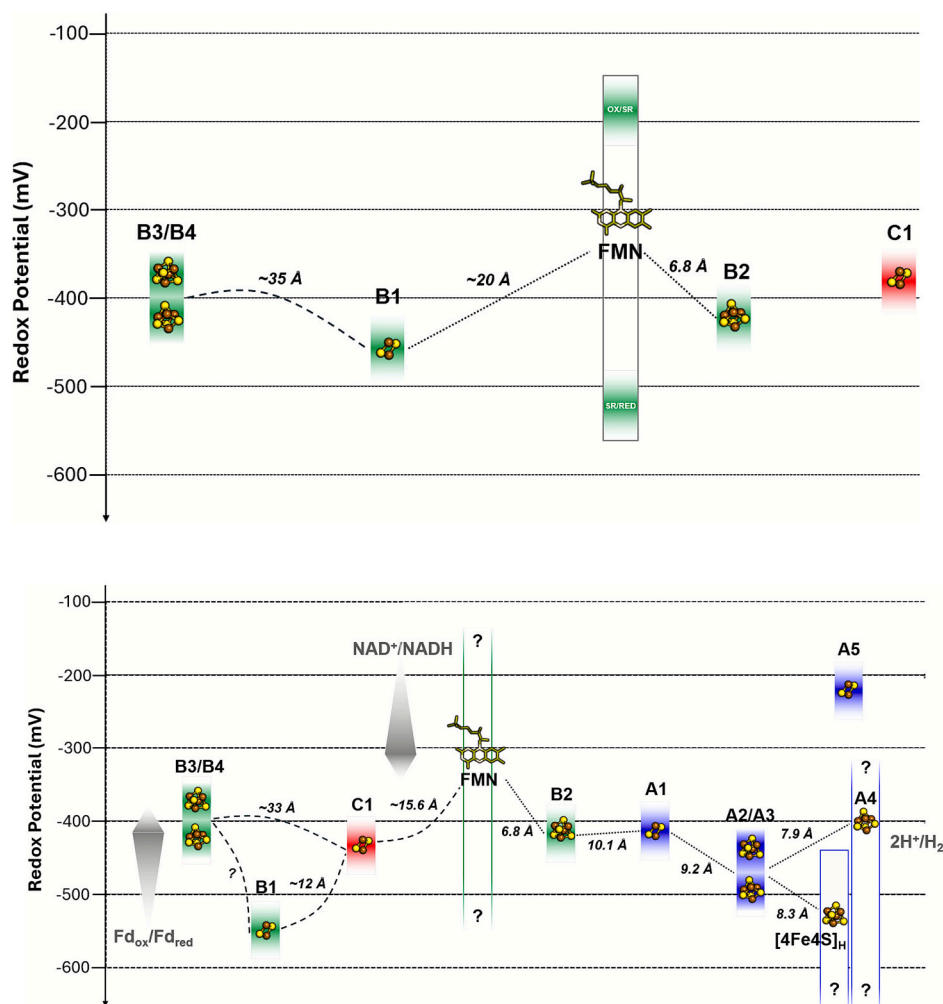
The redox centres surrounding the flavin moiety show more electrochemical heterogeneity than those in the above-mentioned electron wire. Four of the centres (C1, B2, B3 and B4) were found to titrate at potentials higher than -425 mV while centre B1 was found to be substantially more reducing.

We characterized the FMN's redox properties on the isolated HydB subunit. In this sample, the flavin was consistently (that is, via all experimental approaches) found to feature strongly uncrossed potentials

for its two individual redox transitions. The transition from the oxidized to the semi-reduced form was determined to occur between -160 and -260 mV (depending on pH, Fig. 4), which makes it the least reducing redox centre of the entire enzyme. Both optical and EPR properties provide evidence that the semi-reduced form is in the neutral, i.e., N(5) protonated state [52]. The second transition from this neutral semi-reduced state to the two-electron, fully-reduced FMN was observed at around -550 mV. Therefore, both the overall redox midpoint potential of the two-electron transition of the flavin and the stability constant of the semi-reduced species are drastically shifted with respect to FMN in aqueous solution [53].

The here observed one-electron redox transition of the flavin in HydB, ($E_{ox/nsr}$) is puzzling since it is well above the redox midpoint potentials of all iron-sulfur centres. Only the A5 Trx-like [2Fe-2S] cluster at 37 Å distance from the flavin and therefore beyond reach for fast electron transfer comes close in redox potential. It is notable that cluster A5 lies in a thioredoxin fold that is attached to the C-terminal end of HydA by a long linker sequence that may allow it to explore other positions in the protein and potentially approach the flavin. No potential oxidant for the semi-reduced flavin inside the enzyme is obvious from our data and a continued turnover of the flavin site is difficult to envisage under these conditions. We have in the past (for other reasons) discussed the possibility that the flavin moiety in this enzyme (and related ones) may transition between two (or more) different types of redox behaviour [14], which might help to address some of the mechanistic problems outlined in the introduction section. The pH-dependences of the FMN's individual redox potentials as observed in the isolated HydB subunit strongly resemble those reported for flavodoxins [54,55], where also a neutral semi-reduced form is observed, albeit without an observable pK_a in the examined pH-range. Since UV/Vis and EPR spectral parameters of this neutral semi-reduced redox-species are indicative for protonation of the N₅-nitrogen atom of the isoalloxazine moiety [52], our observed pH-dependence is most straightforwardly rationalized as shown in Fig. 4. Similar to flavodoxins, the recorded pH-dependence would result from an upshift (as compared to flavin in aqueous solution) of the $pK_{a,ox}$ value of the N₅-proton by about 6–7 units to roughly pH 7. While in the structures of flavodoxins, there is clear evidence for a stabilizing effect on N₅-protonation by the protein environment of the flavin [55,56], unfortunately there is no structure model of the isolated HydB subunit available. Meanwhile, in the structure of the entire *TmHydABC* enzyme, no obvious candidate for such a stabilization of the N₅-proton is seen, which would predict a redox behaviour of the flavin more closely resembling that of the moiety in aqueous solution, that is, compatible with our results obtained by EPR but not by UV/Vis-spectroscopy on the *TmHydABC*-enzyme.

The redox transition of centre B1 was observed to occur at about a hundred millivolts more negative ambient potentials in the entire enzyme complex than in the isolated HydB subunit (Fig. 2, Row 5, 3rd column). This effect can be attributed to electrostatic interactions between B1 and C1 of HydC in *TmHydABC*. Centre B1 is exposed to the surface of the HydB subunit in the cryo-EM structure of *TmHydABC* and buried by HydC in the entire complex with B1 and C1 12 Å apart [19]. Intriguingly, the Trx-like [2Fe-2S]-cluster domain of the HydC subunit was seen mobile and not always occupying its binding site at the HydB subunit positioning C1 in electron-transferring distance to the flavin. Similar observations were made with the homologous [NiFe]-hydrogenase [17] (without published PDB ID), while preliminary low-resolution data confirm such a structural state also for *TmHydABC*. Our data together with these structural findings suggest the possibility of conformation-dependent substantial alterations of cluster B1's redox parameters, a property potentially important for the catalytic mechanism of the flavin module. This may, however, not be a general property of this enzyme family since the recent structure resolution of NfNABC from *Thermococcus sibiricus* and *Caldicellulosiruptor saccharolyticus* revealed the absence of cluster B1 in these homologous enzymes and its replacement by a non-redox active Zn-ion [28,29], an observation that is



Scheme 1. Representation of the redox potentials (this work) and respective position [19] of the cofactors in the isolated HydB and HydC subunits (A) and the HydABC complex (B). For A4 and [4Fe-4S]_H the redox midpoint potentials could not be resolved but an upper limit can be given for [4Fe4S]_H. For FMN in HydABC no value was determined so far. Straight lines indicate distances between cofactors as observed from the structure. Bent dashed lines indicate possible conformational changes of the co-factor carrying domains resulting in variable distances between cofactors. Shortest distances observed in a structure so far are given for indication. Colour code as in Fig. 1. Redox ranges of the substrates are given for orientation. The redox potential of the H⁺/H₂ largely depends on environmental conditions and is not further specified here.

in contrast to the work of Katsyv et al. (2023, [20]) who showed by mutagenesis studies the importance of this cluster for catalysis.

Further EPR spectral changes were observed over the entire spectral range of iron-sulfur clusters at very low redox potentials i.e. below -500 mV in HydB and in *TmHydABC*, in a redox range where only B1 undergoes redox transition in HydABC. The difference spectra rather indicate spectral shifts (resulting in derivative shaped features) rather than typical iron-sulfur cluster spectra. As the only plausible variable that could explain the spectral shifts of several iron-sulfur signals at low potential we see the reduction of the semi-reduced flavin. Since other iron-sulfur clusters than B2 are further away from the flavin and direct couplings are less probable, conformational changes linked to the redox transitions of the flavin could be at the origin of these observations

5. Conclusion

In this work we set out to determine the redox properties of the cofactors of the confurcating [FeFe]-hydrogenase from *Thermotoga maritima* (Scheme 1). The redox midpoint potentials of the iron-sulfur clusters all fall in a narrow range between -370 mV and -500 mV. An exception is the Trx-type [2Fe-2S] cluster A5, which is not conserved across all enzymes in this family and located in the C-terminal region of

HydA, and exhibits a significantly higher potential of -250 mV. Cluster B1 in the vicinity (~ 20 Å away) of the catalytically important flavin revealed two redox potentials, depending on the presence or absence of the close-by cluster C1. Cluster C1 itself also exhibits a redox potential shift in the complex when compared to the isolated domain. Taking into account structural work that suggest the domain carrying the cluster C1 is mobile [17,19–21], these potential shifts may reflect functional importance. The flavin radical itself could be characterized in the isolated HydB subunit, but the lack of similar redox behaviour in the full enzyme points to the redox flexibility of this cofactor as well. Furthermore, substrate (NAD⁺/NADH)-binding could also influence the redox properties of the flavin. Large scale changes in the redox properties of the flavin and its surrounding cofactors may thus be the missing piece that will allow us to unravel the puzzle of this not yet understood electron confurcation mechanism.

CRediT authorship contribution statement

Kilian Zuchan: Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Data curation. **Nina Breuer:** Resources. **Christoph Laurich:** Resources, Methodology. **Wolfgang Nitschke:** Writing – review & editing, Writing – original draft,

Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Frauke Baymann:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **James A. Birrell:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbabo.2025.149577>.

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