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## Introduction

Hydrogenases catalyze the reversible cleavage of dihydrogen, thereby making use of earth–abundant transition metals. Among them, O<sub>2</sub>-tolerant [NiFe]-hydrogenases are particularly attractive in view of a remarkable catalytic performance under usually inhibiting oxic conditions.<sup>1,2</sup> Buried deeply in the large

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## Hydroxy-bridged resting states of a [NiFe]hydrogenase unraveled by cryogenic vibrational spectroscopy and DFT computations<sup>†</sup>

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The catalytic mechanism of [NiFe]-hydrogenases is a subject of extensive research. Apart from at least four reaction intermediates of  $H_2/H^+$  cycling, there are also a number of resting states, which are formed under oxidizing conditions. Although not directly involved in the catalytic cycle, the knowledge of their molecular structures and reactivity is important, because these states usually accumulate in the course of hydrogenase purification and may also play a role in vivo during hydrogenase maturation. Here, we applied lowtemperature infrared (cryo-IR) and nuclear resonance vibrational spectroscopy (NRVS) to the isolated catalytic subunit (HoxC) of the heterodimeric regulatory [NiFe]-hydrogenase (RH) from Ralstonia eutropha. Cryo-IR spectroscopy revealed that the HoxC protein can be enriched in almost pure resting redox states suitable for NRVS investigation. NRVS analysis of the hydrogenase catalytic center is usually hampered by strong spectral contributions of the FeS clusters of the small, electron-transferring subunit. Therefore, our approach to investigate the FeS cluster-free, <sup>57</sup>Fe-labeled HoxC provided an unprecedented insight into the [NiFe] site modes, revealing their contributions in a spectral range otherwise superimposed by FeS cluster-derived bands. Rationalized by density functional theory (DFT) calculations, our data provide structural descriptions of the previously uncharacterized hydroxy- and water-containing resting states. Our work highlights the relevance of cryogenic vibrational spectroscopy and DFT to elucidate the structure of barely defined redox states of the [NiFe]-hydrogenase active site.

> subunit of the heterodimeric functional unit, the [NiFe] active site contains a Ni and an Fe ion bridged by two cysteine (Cys) residues; two additional terminal Cys bind exclusively to the Ni, while two CN<sup>-</sup> and one CO ligands participate in the coordination of the Fe (Fig. 1).  $H_2$  splitting releases two protons that are transferred to nearby proteinaceous H<sup>+</sup> acceptors. The concomitantly released electrons are channeled through an FeS cluster chain located in the small subunit to the hydrogenase's redox partner.<sup>3-5</sup> Although [NiFe]-hydrogenases have been the subject of intensive research for decades, many questions remained open regarding the nature of some resting states as well as catalytically relevant intermediates. Infrared (IR) spectroscopy represents a valuable technique to monitor the redoxsensitive CO and CN<sup>-</sup> ligands of the active site, and electron paramagnetic resonance (EPR) spectroscopy provides important electronic and structural information on the active site redox states.6-8 During catalysis, nickel serves as a redox-active metal center, while iron retains an Fe<sup>II</sup> low-spin configuration throughout the entire catalytic cycle (Fig. 1, left panel).<sup>1,9</sup> However, some redox states are EPR-silent, and direct IR spectroscopic detection of possible hydride H<sup>-</sup> and hydroxy OH<sup>-</sup>

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<sup>†</sup> Electronic supplementary information (ESI) available: Materials and methods and supplementary results including Fig. S1–S20 (PDF). Selected DFT normal mode animations (GIF format) relevant to NRVS bands (ZIP archive). Optimized structures (XYZ format) for all DFT-computed models (ZIP archive). See DOI: 10.1039/d0sc05022a

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**Fig. 1** (Left) Schematic representation of the subunit composition of the RH and the proposed catalytic cycle of [NiFe]-hydrogenases. The states involved in the H<sub>2</sub> transformation include the Ni<sub>a</sub>–S intermediate that presumably binds H<sub>2</sub> in the bridging position between the nickel and the iron ions. H<sub>2</sub> splitting results in a two-electron reduced Ni<sub>a</sub>–SR state characterized by a bridging hydride. Release of one electron and one proton results in a hydride-carrying Ni<sub>a</sub>–C species, which is a tautomeric form of the Ni<sub>a</sub>–L state. Release of another electron and a further proton restores the Ni<sub>a</sub>–S intermediate.<sup>14</sup> Protonation of a terminal cysteine as well as the bridging hydride are highlighted in red. The Ni and its oxidation state are depicted in green. (Right) Schematic representation of the [NiFe]-hydrogenase large subunit HoxC and proposed structures of its resting states. The as-isolated HoxC protein occurs in a mixture of the Ni<sub>r</sub>–S<sub>1</sub> and Ni<sub>r</sub>–S<sub>1</sub> and Ni<sub>r</sub>–S<sub>1</sub> and Ni<sub>r</sub>–L intermediates (left panel), and the OH<sup>-</sup>/H<sub>2</sub>O active site ligands assigned in this work are depicted in bold case letters.

bridging ligands at the active site is hindered by strong spectral contributions of the protein and/or the solvent. Furthermore, resonance Raman (RR) spectroscopy has also been applied for the analysis of metal-ligand vibrations of [NiFe]-hydrogenase intermediate states characterized by a vacant bridging position between Ni and Fe (*i.e.* Ni<sub>a</sub>–S and Ni<sub>a</sub>–L, Fig. 1).<sup>10,11</sup>

Nuclear resonance vibrational spectroscopy (NRVS) is a synchrotron-based technique that allows selective observation of vibrational modes of Mössbauer-active nuclei. Using the <sup>57</sup>Fe nuclear resonance at 14.4 keV, NRVS has provided valuable structural information about [NiFe]-,12-14 [FeFe]-,15,16 and [Fe]hydrogenases.<sup>17</sup> A typical NRV spectrum of [NiFe]-hydrogenase comprises dominant bands of Fe-S(-Fe) stretching and bending modes in the region between 100 and 420 cm<sup>-1</sup>, which are mostly related to the FeS clusters of the electron transport chain, as well as Fe-CO/CN stretching and bending modes of the [NiFe] active site in the 400–650 cm<sup>-1</sup> spectral region. Most of the iron atoms in the enzyme are located in the FeS clusters; therefore their spectral contributions surpass the signals of the single active site iron. This results often in poorly resolved signals of the Fe-CO/CN modes, which have a relatively low intensity. Furthermore, certain bands related to the FeS clusters and the [NiFe] active site are superimposed, hindering their unambiguous assignment.12,14,18 Nevertheless, a combined approach of NRVS and density functional theory (DFT) has

provided significant structural details of the [NiFe] active site in catalytic intermediates, revealing the existence of a hydride-containing  $Ni_a$ -SR intermediate.<sup>13</sup>

Apart from H<sub>2</sub>-cycling intermediates, several resting states are formed under non-turnover conditions.<sup>1</sup> Although these states are not directly involved in the catalysis, the knowledge of their molecular structure is important, because they accumulate in the course of hydrogenase purification and often require extensive reactivation procedures. Additionally, they may also play a role *in vivo* during hydrogenase maturation protecting the active site from redox inactivation.<sup>19</sup>

Recently, we isolated the catalytic subunit HoxC from the O<sub>2</sub>tolerant regulatory [NiFe]-hydrogenase (RH) of *Ralstonia eutropha*. HoxC hosts exclusively the [NiFe] active site and exhibits little activity due to the predominance of inhibited resting states.<sup>19</sup> Therefore, the HoxC protein is an ideal target for a selective and detailed spectroscopic investigation of the catalytic center in its resting states (Fig. 1) without interference from the auxiliary FeS clusters. In this work, we applied lowtemperature IR and NRVS techniques together with DFT calculations on the <sup>57</sup>Fe-labeled HoxC protein to gain structural information of these so-far poorly characterized [NiFe]hydrogenase states. We uncovered the vibrational bands of the active site in a broad spectral range, previously superimposed by the Fe–S vibrations. This enabled us to define new

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structural determinants of the catalytic center, namely a bridging hydroxy-ligand and a protonated Ni-bound cysteine residue, as key elements of the two resting states. These new finding may represent benchmarks for future NRVS studies on site-selectively labeled hydrogenases as well as biomimetic [NiFe] compounds.

### **Results and discussion**

# IR detection of CO and CN<sup>-</sup> ligands of the [NiFe] active site at ambient and cryogenic temperatures

The as-isolated HoxC protein  $(HoxC_{ai})$  contains a stoichiometric amount of the [NiFe] active site, which resided in the



Fig. 2 IR spectra of HoxC<sub>ai</sub>, HoxC<sub>ox</sub> and RH (top to bottom) taken at 283 K and 85 K. (a) IR spectral region characteristic of CO and CN stretching modes recorded at 283 K. The spectrum of HoxC<sub>ai</sub> exhibits spectral contributions of the Ni<sub>r</sub>-S<sub>I</sub> (red) and Ni<sub>r</sub>-S<sub>II</sub> (dark blue) resting states, respectively. The spectrum of RH is dominated by signals attributed to the Ni<sub>a</sub>-S state (labeled in black). The IR data of HoxC<sub>ox</sub> comprise contributions from two paramagnetic oxidized Ni<sub>r</sub>-B' (minor species) and Ni<sub>r</sub>-B" states labeled in purple and blue, respectively. (b) IR spectral region characteristic of CO and CN stretching modes recorded at 85 K for the same samples. Contributions from the corresponding CN absorptions of Fe(CN)<sub>6</sub><sup>3-</sup> and Fe(CN)<sub>6</sub><sup>2-</sup> present in the chemically oxidized HoxC<sub>ox</sub> were subtracted for the sake of clarity.<sup>19</sup>

diamagnetic and isoelectronic Nir-SI and Nir-SII states at ambient temperature (Fig. 1 and 2a).1,19 A previous study showed that these states can be partially interconverted by shifting the pH of the buffer.19 This has been interpreted with the presence of a protonatable bridging ligand at the active site, attributed to a hydroxy (OH<sup>-</sup>) group (Fig. 1).<sup>20-22</sup> Native RH, for comparison, resided preferentially in the Ni<sub>a</sub>-S state characterized by a vacant bridging position and a CO absorption in the IR spectrum at 1943 cm<sup>-1</sup>, which remains stable over a broad pH range (Fig. S1<sup>†</sup>).<sup>19,23</sup> As NRVS is conducted under cryogenic conditions (estimated sample temperature of 40-80 K), it is advisable to record the corresponding IR spectra of the HoxC and RH proteins at similarly low temperatures to allow a direct comparison of the two spectroscopic techniques. In fact, the mixture of the Nir-SI and Nir-SI states, observed for HoxCai at 283 K, converted into an almost pure Nir-SI species at 85 K characterized by a CO absorption at 1955  $\text{cm}^{-1}$  (Fig. 2b). According to our previous interpretation,<sup>19</sup> the Nir-SI and Nir-SI species just differ in the protonation state of the bridging ligand  $(OH^{-} vs. H_2O)$ . Thus, cryogenic temperatures seem to stabilize the hydroxy ligand (Fig. 1), which is presumably related to the temperature-dependent changes of the active site surrounding. The enrichment of the Ni<sub>r</sub>-S<sub>I</sub> state turns out to be reversible. Indeed, setting the temperature of the HoxC sample to 298 K (RT) restored the original Nir-SI/Nir-SII ratio (Fig. S2<sup>+</sup>). Native RH, by contrast, retained a single CO absorption at 1946 cm<sup>-1</sup> in the IR spectrum, *i.e.* it maintained the Ni<sub>a</sub>-S state at 85 K (Fig. 2b). Thus, HoxC and RH exhibit different IR spectra both at room temperature and under cryogenic conditions, reflecting at least slightly divergent active site structures.

In our previous work, we showed that  $HoxC_{ai}$  can be oxidized by ferricyanide, revealing a species denoted as  $HoxC_{ox}$ .<sup>19</sup> According to X-ray, EPR and DFT studies, standard [NiFe]hydrogenases form paramagnetic Ni<sup>III</sup> species such as the Ni<sub>r</sub>-B state upon oxidation (Fig. 1).<sup>1,24–26</sup> The IR spectrum of  $HoxC_{ox}$ , recorded at room temperature, displayed bands assigned to Ni<sub>r</sub>-B-like species termed Ni<sub>r</sub>-B' and Ni<sub>r</sub>-B'', respectively.<sup>19</sup> Lowtemperature IR measurements performed on  $HoxC_{ox}$  revealed the Ni<sub>r</sub>-B'' state, characterized by a CO absorption at 1979 cm<sup>-1</sup>, as the predominant species at 85 K (Fig. 2b). The oxidation of the nickel site was confirmed by EPR measurements (Fig. S3†).<sup>19</sup> The fact that almost pure Ni<sub>r</sub>-S<sub>I</sub> and Ni<sub>r</sub>-B'' states were observed in  $HoxC_{ai}$  and  $HoxC_{ox}$ , respectively, allowed for establishing DFT models, which were subsequently used to interpret the lowtemperature IR and NRV spectra.

# DFT modeling of the HoxC active site in the $Ni_r$ -S- and $Ni_r$ -B-like states

DFT calculations were used to model the structural details and vibrational spectra of the [NiFe] active site in the HoxC<sub>ai</sub>/HoxC<sub>ox</sub> proteins. In the absence of experimentally resolved crystallographic structures of either HoxC or native RH, the DFT modeling was based on the corresponding X-ray structure of the membrane-bound hydrogenase (MBH) from the same organism.<sup>27</sup> The model setup is described in detail in the ESI<sup>†</sup> Materials and methods. In comparison to the large subunit of

MBH, our HoxC homology model revealed a larger active site pocket with a lower number of contacts between the protein side chains and the metal ligands (Fig. S4<sup>†</sup>). The DFT calculations were performed for the reduced, diamagnetic Ni<sub>r</sub>–S-like [Ni<sup>II</sup>Fe<sup>II</sup>] species with an overall spin of zero and for the oxidized, paramagnetic Ni<sub>r</sub>–B-like [Ni<sup>III</sup>Fe<sup>II</sup>] state with a spin of 1/2. According to previous structural predictions for the reversibly inhibited ready-states of [NiFe]-hydrogenases,<sup>1,28,29</sup> our models contained a bridging hydroxy ( $\mu$ OH subscript in the model notations) ligand at the active site (Fig. S5–S7<sup>†</sup>). For the Ni<sub>r</sub>–S species, local energy minima were also obtained with a water molecule serving as either a metal-bridging ( $\mu$ H<sub>2</sub>O), a Niterminal (Ni–H<sub>2</sub>O),<sup>28,29</sup> or an Fe-terminal (Fe–H<sub>2</sub>O) ligand (Fig. S8–S10<sup>†</sup>). All models revealed a hydrogen bonding between the guanidinium group of the conserved arginine residue and

Cys60 Cys63 а Ni<sub>r</sub>-S<sub>µOH</sub>SH Cys482 Fell Nill Cys479 Arg411 Asp102 Cys60 Cys63 b S<sub>Ni-H2C</sub> Cys482 Nil Fell Cys479 Arg41 Asp102 Cys60 Cys63 С Ni<sub>r</sub>-B<sub>µOH</sub><sup>SH</sup> Cys482 Fell Nill Cys479 Arg411 Asp102

Fig. 3 DFT models: (a) Ni<sub>r</sub>-S^{SH}\_{\mu OH} of the reduced Ni<sub>r</sub>-S<sub>1</sub> state, (b) Ni<sub>r</sub>-S^{S-}\_{Ni-H\_2O} of the reduced Ni<sub>r</sub>-S<sub>1</sub> state, and (c) Ni<sub>r</sub>-B^{SH}\_{\mu OH} of the oxidized Ni<sub>r</sub>-B" state, displaying the [NiFe] cofactor metal ligands and their contacts with the nearby side chains in HoxC. For alternative models and the entire HoxC homology model employed, see Fig. S4–S10.†

the alternative metal-bound  $\mu$ OH/H<sub>2</sub>O species (Fig. S6–S10†). The DFT model set was further extended by considering the Ni-bound terminal cysteine Cys479 with either a protonated thiol (SH superscript in the model notations), as, *e.g.*, in the Ni<sub>r</sub>–S<sup>SH</sup><sub>µOH</sub> model shown in Fig. 3a, or a deprotonated thiolate (S<sup>-</sup>, as shown in Fig. 3b and S6–S10†). This is in line with the proposed protonation of the corresponding cysteine residue in the catalytically relevant Ni<sub>a</sub>–SR and Ni<sub>a</sub>–L states of [NiFe]-hydrogenases.<sup>30–34</sup> We also examined the second Ni-bound terminal cysteine Cys60 as a possible protonation site. The corresponding Ni<sub>r</sub>–S<sup>Cys60–SH</sup> model is shown in Fig. S6.†

Notably, the structural optimization of the Ni<sub>r</sub>-S<sub>uOH</sub> models revealed an over-elongated bond between the Ni<sup>II</sup> ion and the bridging sulfur ligand of Cys482,19,28,35 whose length depended on the protonation state of the Ni-terminal cysteines. The Ni<sub>r</sub>- $S_{\mu OH}^{SH}$ , Ni<sub>r</sub>- $S_{\mu OH}^{Cys60-SH}$  and Ni<sub>r</sub>- $S_{\mu OH}^{S-}$  models yielded Ni<sup>II</sup>...S(Cys482) distances of 2.7, 2.8 and 3.0 Å, respectively, consistent with an essentially lost coordination. All other Ni/Fe–S (Cys  $\neq$  Cys482) distances remained within a narrow bonding range of  $\sim$ 2.2–2.4 Å across the entire model set. No bond elongation or lost coordination between the Ni<sup>III</sup> ion and the bridging sulfur ligand of Cys482 was found for the Ni<sub>r</sub>-B<sub>µOH</sub> models, as, *e.g.*, in the  $Ni_r$ - $B_{\mu OH}^{SH}$  model (Fig. 3c), in line with available crystallographic data.24 To account for the flexibility of the Ni coordination sphere, the present DFT models included explicitly the two short CXXC protein sequence spacers spanning the two cysteine pairs ligating the nickel (Fig. S4<sup>†</sup>).

# DFT reproduces the experimental IR spectra of the $\rm Ni_r\text{-}S_I$ and $\rm Ni_r\text{-}B''$ states of HoxC

DFT calculations based on the  $\rm Ni_r\text{-}S^{SH}_{\mu OH}$  and  $\rm Ni_r\text{-}B^{SH}_{\mu OH}$  models (Fig. 3a and c, or S6 and S7<sup>†</sup>) predict CO and CN stretching frequencies as well as relative IR intensities close to those detected in the low-temperature IR measurements (Fig. 2b, S11a and b<sup>+</sup>). While the absolute positions of the DFT-simulated CO and CN bands in proteins commonly rely on a linear fit procedure (described in the ESI† Materials and methods), the corresponding direction of band shifts is independent of the fitting procedure and reflects a polarization of the Fe-bound CO/CN ligands. Thus, a charge increase of 1+ per active site unit results in a predicted blue-shift of the CO stretching frequency of  $\sim$ 20-30 cm<sup>-1</sup>. This is reflected by the simulated IR bands from a series of models at three levels of charge, *i.e.* (i)  $Ni_r-S^{S-}_{\mu OH}$ , (ii)  $Ni_r-B^{S-}_{\mu OH}/Ni_r-S^{SH}_{\mu OH}$  and (iii)  $Ni_r-B^{SH}_{\mu OH}$  (Fig. S11†). The blue shift of  $\sim$ 20–30 cm<sup>-1</sup> is in a good agreement with the experimentally monitored spectral shift of 24 cm<sup>-1</sup> observed for the Ni<sub>r</sub>-S<sub>I</sub> (HoxC\_{ai}) and Ni\_r-B^{\prime\prime} (HoxC\_ox) states (Fig. S11a and b†). This is consistent with the oxidation of the nickel ion from Ni<sup>II</sup> to Ni<sup>III</sup> for the Ni<sub>r</sub>-S<sub>I</sub> to Ni<sub>r</sub>-B'' conversion (Fig. S3<sup>†</sup>),<sup>22,36,37</sup> assuming that the protonation status of Cys479 was unchanged. Altogether, the experimental and theoretical data are consistent with a total charge increase of 1+ at the [NiFe] active site when moving from HoxCai to HoxCox. Thus, the comparison of the experimental and DFT-computed IR spectra does not allow a conclusive statement whether or not Cys479 is protonated.

The low-temperature IR spectrum of HoxCai also revealed traces of the Nir-SII species, identified through the CO band at 1943 cm<sup>-1</sup>, which is shifted just by 12 cm<sup>-1</sup> compared to the CO band at 1955 cm<sup>-1</sup> of Ni<sub>r</sub>-S<sub>I</sub> (Fig. 2b). Therefore, the Ni<sub>r</sub>-S<sub>I</sub> and Nir-SII states carry rather an identical charge. Among the different Nir-S models explored, a red-shifted CO band with the same active site charge was computed assuming a terminal H<sub>2</sub>O ligand at the Ni ion and a deprotonated Cys479 (Ni<sub>r</sub>– $S_{Ni-H_2O}^{S-}$ , Fig. 3b, S12† and 1). The relative energy difference between the  $Ni_r\!-\!S^{S-}_{Ni-H_2O}$  and  $Ni_r\!-\!S^{SH}_{\mu OH}$  models is just 2.0 kcal mol<sup>-1</sup>. Thus, the corresponding Ni<sub>r</sub>-S<sub>II</sub> and Ni<sub>r</sub>-S<sub>I</sub> species may coexist in a temperature-dependent dynamic equilibrium with a slight preference for Ni<sub>r</sub>-S<sub>I</sub> (Fig. S2<sup>†</sup>). Yet, an alternative rationalization of the minor red-shifted Nir-SII is that it represents a species singly deprotonated with regard to the Ni<sub>r</sub>-S<sub>I</sub> state, still carrying the bridging hydroxy ligand (Ni<sub>r</sub>- $S^{S-}_{\mu OH}$  vs.  $Ni_r\text{-}S^{SH}_{\mu OH},$  Fig. S11a†). A recent computational study supported the  $Ni_r\text{-}S^{SH}_{\mu OH}$  and  $Ni_r\text{-}S^{S-}_{Ni-H_2O}$  active site assignment for the Ni<sub>r</sub>-S<sub>I/II</sub> states.35

# Experimental and DFT-computed NRV spectra of the active site in $HoxC_{ai}$ and $HoxC_{ox}$ proteins

The NRV spectra of <sup>57</sup>Fe-enriched HoxC<sub>ai</sub> and RH are shown in Fig. 4. As native RH has three [4Fe4S] clusters, bands originating from the FeS clusters dominate the corresponding spectrum. The spectral contributions of the [NiFe] site are relatively weak and visible only in the high-frequency region of 420–600 cm<sup>-1</sup> (Fig. 4).<sup>12</sup> Further details about the NRVS characterization of RH are provided in the ESI Supplementary results and Fig. S13 and S14.<sup>†</sup> The most intense active site bands in RH are detected at 553 cm<sup>-1</sup> and 596 cm<sup>-1</sup> and are attributed mainly to Fe–CO stretching and bending modes.<sup>12</sup> Bands below 530 cm<sup>-1</sup>, which are due to Fe–CN and mixed Fe–CO/CN modes, could not be properly resolved due to their relatively low intensity.

NRV spectra for the HoxCai and HoxCox samples were obtained under conditions identical to those applied for the cryogenic IR measurements. The NRV spectrum of the HoxCai sample, which mostly resided in the Nir-SI state (Fig. 2b), comprises main bands at 554, 600 and 612  $\text{cm}^{-1}$  in addition to features at 507, 474, 445 and 427 cm<sup>-1</sup> (Fig. 4). The corresponding spectrum of HoxCox, which mostly resided in the Nir-B" state (Fig. 2b), is shown in Fig. S15.† Due to the absence of the FeS clusters, the observed bands could be attributed exclusively to the [NiFe] center. This also led to a notable increase of the intensity of the characteristic Fe-CO/CN vibrations in the  $\sim$ 400–600 cm<sup>-1</sup> region compared to the RH spectrum (Fig. 4, red shaded rectangle). Moreover, we also observed the spectral contributions of the active site in the low-frequency region with a main band at  $180 \text{ cm}^{-1}$ . This spectral region of the HoxC NRV spectrum (Fig. 4, 5 and S15<sup>†</sup>) is shaped by the (i) torsional/'breathing' modes of the cofactor ( $\sim 150-220$  cm<sup>-1</sup>), (ii) [NiFe] core displacements relative to the protein framework  $(\sim 50-150 \text{ cm}^{-1})$ , and (iii) displacements of the cofactor in phase with the nearby side chains or 'acoustic' modes (below  $\sim$ 50 cm<sup>-1</sup>). The intermediate-frequency region ( $\sim$ 250 to 400 cm<sup>-1</sup>), containing high-intensity FeS cluster bands in the



Fig. 4 NRVS partial vibrational density of states (PVDOS) of <sup>57</sup>Felabeled RH (bottom) and its large subunit HoxC (top), in their isolated forms, normalized to an integrated PVDOS of 3. Different spectral regions are indicated with arrows using the following color code: red, bands related to Fe-CO/CN of the [NiFe] active site; orange, bands related to [NiFe] site/protein modes; blue, bands related to Fe- $\mu$ S modes involving bridging cysteines; olive, bands related to the Fe-S modes of the [4Fe4S]-clusters. Representative bands in the RH and HoxC spectra are labeled with numbers in black. The corresponding NRVS data including the error bars are presented in Fig. S20.†

uniformly <sup>57</sup>Fe-labeled RH spectrum, is largely free of distinct bands of HoxC. The only vibrations of a similar type in the active site are Fe– $\mu$ S(Cys) stretching modes of the two bridging cysteines, detectable in the region of 270–380 cm<sup>-1</sup> (Fig. S19g†). These contributions cannot be detected in the presence of (NRV-active) FeS clusters (Fig. 4). Consistent with lowtemperature IR spectroscopic measurements (Fig. 2b), the spectral differences of the NRVS data indicate that the catalytic centers of RH (Ni<sub>a</sub>–S) and HoxC<sub>ai</sub> (Ni<sub>r</sub>–S<sub>I</sub>) reside in different states (Fig. 1).

The DFT models described above (Fig. S6–S10<sup>†</sup>) were used to generate the <sup>57</sup>Fe-PVDOS profiles of the HoxC<sub>ai</sub> and HoxC<sub>ox</sub> proteins in the Ni<sub>r</sub>–S<sub>I</sub> and Ni<sub>r</sub>–B" states, respectively (Fig. S16 and S17<sup>†</sup>). Fig. 5 shows a comparison of the HoxC<sub>ai</sub> experimental spectrum (0–700 cm<sup>-1</sup> region) with the DFT-calculated spectra based on the Ni<sub>r</sub>–S models. The computed data based on the Ni<sub>r</sub>–S<sup>S–</sup><sub>µOH</sub> and Ni<sub>r</sub>–S<sup>SH</sup><sub>µOH</sub> models – both carrying a bridging hydroxy group – reproduce the experimental spectrum very well (Fig. 5a, S16 and S18<sup>†</sup>).

Yet, an alternative hydroxy-bridged Ni<sub>r</sub>–S model in which Cys60 is protonated (Ni<sub>r</sub>–S<sup>Cys60-SH</sup><sub>µOH</sub>) revealed a distinct ~20 cm<sup>-1</sup> splitting of the high-intensity ~550 cm<sup>-1</sup> band and is therefore rather incompatible with the experimental spectrum (Fig. 5b). All the other models, carrying either bridging H<sub>2</sub>O (Ni<sub>r</sub>–S<sup>S+</sup><sub>µH<sub>2</sub>O</sub> and Ni<sub>r</sub>–S<sup>S+</sup><sub>µH<sub>2</sub>O</sub>, Fig. 5c), a terminal, Fe-bound water molecule (Ni<sub>r</sub>–S<sup>S+</sup><sub>Fe-H<sub>2</sub>O</sub> and Ni<sub>r</sub>–S<sup>S+</sup><sub>Fe-H<sub>2</sub>O</sub>, Fig. 5d) or a terminal, Ni-bound water molecule (Ni<sub>r</sub>–S<sup>S+</sup><sub>Fe-H<sub>2</sub>O</sub> and Ni<sub>r</sub>–S<sup>S+</sup><sub>Ni-H<sub>2</sub>O</sub>, Fig. 5d) or a terminal, Ni-bound water molecule (Ni<sub>r</sub>–S<sup>S-</sup><sub>Ni-H<sub>2</sub>O</sub> and Ni<sub>r</sub>–S<sup>S+</sup><sub>Ni-H<sub>2</sub>O</sub>, Fig. 5e), led to <sup>57</sup>Fe-PVDOS signatures with an inferior match to the experimental spectrum of HoxC<sub>ai</sub>. A closer examination of the low-frequency region (0–200 cm<sup>-1</sup>) revealed that the Ni<sub>r</sub>–

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Fig. 5 Comparison of the experimental NRV spectrum of the HoxC<sub>ai</sub> sample (Ni<sub>r</sub>-S<sub>1</sub>, grey trace reiterated in (a–e)) overlaid with the corresponding DFT-calculated <sup>57</sup>Fe-PVDOS bands using alternative [Ni<sup>II</sup>Fe<sup>II</sup>] models, schematically shown on the right side of the spectra. The DFT spectra derived using either protonated (solid lines) or deprotonated (broken lines) Cys479 are shown for the following models: (a) best-fit Ni<sub>r</sub>-S<sup>SH</sup><sub>DOH</sub> and Ni<sub>r</sub>-S<sup>SH</sup><sub>DOH</sub> and Ni<sub>r</sub>-S<sup>SH</sup><sub>DOH</sub> and Ni<sub>r</sub>-S<sup>SH</sup><sub>DOH</sub> (Fig. S6†); (c) Ni<sub>r</sub>-S<sup>SH</sup><sub>DH2O</sub> and Ni<sub>r</sub>-S<sup>SH</sup><sub>DE-H2O</sub> (Fig. S8†); (d) Ni<sub>r</sub>-S<sup>SH</sup><sub>E-H2O</sub> and Ni<sub>r</sub>-S<sup>SH</sup><sub>DOH</sub> (Fig. S9†); (e) Ni<sub>r</sub>-S<sup>SH</sup><sub>NI-H2O</sub> and Ni<sub>r</sub>-S<sup>SH</sup><sub>DOH</sub> and Ni<sub>r</sub>-S<sup>SH</sup><sub>DOH2O</sub> (Fig. S10†). The DFT spectrum derived from the alternative hydroxy model with protonated Cys60, Ni<sub>r</sub>-S<sup>Cys60-SH</sup>, is highlighted in (b). Both  $\mu$ OH<sup>-</sup> models in (a) reproduce the experimental data in the Fe-CO/CN region above 400 cm<sup>-1</sup>. Minor differences in the low-frequency spectral region around 180 cm<sup>-1</sup> (Fig. S16†) strengthen the Ni<sub>r</sub>-S<sup>SH</sup><sub>DOH</sub> model, in line with the better prediction of the experimental IR absorptions for the CO and CN stretching modes (Fig. S11a†).

 $S^{\rm SH}_{\mu OH}$  model, which carries a protonated Cys479, provides a more accurate fit (solid line in Fig. 5a) to the experimental spectrum than the unprotonated form (Ni<sub>r</sub>–S^S–<sub>\muOH</sub>, dotted line in Fig. 5a, see also Fig. S16†). In this respect, the shape and position of the high-intensity band at  $\sim 180~{\rm cm}^{-1}$  in the calculated HoxC spectrum were found remarkably sensitive to the specific  $\mu OH^-/(\mu/Ni-/Fe-)H_2O$  ligand coordination as well as to the protonation of the Ni-bound terminal cysteine(s) (Fig. 5 and S16†).

Analogously to low-energy NRVS maxima in the spectral region characteristic of FeS clusters, preferentially attributed to S–Fe–S bending vibrations (Fig. S13 and S14a<sup>†</sup>),<sup>14,38</sup> the active site normal modes forming the ~180 cm<sup>-1</sup> band comprise

'delocalized' Cys(S)–Ni/Fe–(S)Cys bending modes involving entire side chain motions of the four cysteines. Additional details on the effects influencing the <sup>57</sup>Fe-PVDOS profiles are provided in the ESI† Supplementary results. The experimental spectrum of HoxC<sub>ox</sub> was best reproduced by DFT-computed data based on the Ni<sub>r</sub>–B<sup>SH</sup><sub>POH</sub> model, which contains a protonated Cys479 cysteine (Fig. S17†). Thus, the Ni<sub>r</sub>–S<sub>I</sub> and Ni<sub>r</sub>–B" states differ only in the oxidation level of the Ni ion, and the Ni<sub>r</sub>–S<sub>I</sub> to Ni<sub>r</sub>–B" transition represents a one-electron oxidation of Ni<sup>II</sup> to Ni<sup>III</sup>.<sup>20,22,36</sup> This is fully consistent with our DFT-supported IR analysis (Fig. 2b and S11†) and the EPR measurements (Fig. S3†). Accordingly, the NRVS data of the HoxC<sub>ox</sub> sample revealed red-shifts of 8–15 cm<sup>-1</sup> of bands in the region above



Fig. 6 Comparison of the experimentally observed and DFT-calculated <sup>57</sup>Fe-PVDOS bands in the 400–650 cm<sup>-1</sup> spectral region for HoxC<sub>ai</sub> and HoxC<sub>ox</sub>. Experimental spectra (top: red trace, HoxC<sub>ai</sub>; blue trace, HoxC<sub>ox</sub>) are compared to their DFT-calculated counterparts based on the corresponding models that contain either protonated (middle: Ni<sub>r</sub>-S<sup>SH</sup><sub>µOH</sub> and Ni<sub>r</sub>-B<sup>SH</sup><sub>µOH</sub> states) or deprotonated Ni-bound terminal cysteine (bottom: Ni<sub>r</sub>-S<sup>SH</sup><sub>µOH</sub> and Ni<sub>r</sub>-B<sup>SH</sup><sub>µOH</sub> models are in better agreement with the NRVS data, including their shift magnitudes marked in the figure. The corresponding NRVS data including the error bars are presented in Fig. S20.†

500 cm<sup>-1</sup> dominated by the Fe–CO stretching vibrations compared to the HoxC<sub>ai</sub> sample (Fig. 6), thereby reflecting the oxidation of the active site.

The red-shifts of this magnitude are predicted by the corresponding DFT models with protonated Cys479 (Ni<sub>r</sub>-S<sup>SH</sup><sub>uOH</sub>  $\rightarrow$  $Ni_r-B_{\mu OH}^{SH}$ ). The related models without terminal cysteine protonation  $(Ni_r-S^{S-}_{\mu OH} \rightarrow Ni_r-B^{S-}_{\mu OH})$  revealed smaller band shifts, which do not reflect the experimental data (Fig. 6). Notably, while the oxidation of the active site led to a red-shift of the Fe-CO/CN vibrations in NRVS (Fig. 6), a blue-shift was observed for the IR stretching frequencies of the active site CO/CN<sup>-</sup> ligands (Fig. 2b). This can be rationalized by charge polarization effects at the Fe site. Stronger bonds within the diatomic ligands, reflected by the blue-shifted IR bands related to the CO and CN stretching vibrations, imply, in turn, weaker Fe-CO/CN bonds, resulting in a red-shift of the corresponding NRVS bands. The accurate prediction of this redox-dependent vibrational shift relies on the inclusion of explicit interactions between the HoxC protein environment and the [NiFe]-cofactor, as confirmed by our computational test using an alternative minimal model omitting all the residues except the four cysteines directly coordinating the two metals (ESI Supplementary results and Fig. S18c<sup>†</sup>).

Taking advantage of the good agreement between the experimental and computed vibrational spectra, we were able to unveil the most prominent bridging hydroxy  $\mu$ OH<sup>-</sup> displacements in the active site of HoxC (Fig. S19†). The largest  $\mu$ OH<sup>-</sup> displacements were correlated with the  $\mu$ O(H)-PVDOS profile (Fig. S19b†). In the corresponding Ni<sub>r</sub>-S<sup>SH</sup><sub>µOH</sub>/Ni<sub>r</sub>-B<sup>SH</sup><sub>µOH</sub> models, we deduced Ni- $\mu$ OH stretches at 506/494 cm<sup>-1</sup> (Fig. S19c†), Fe- $\mu$ OH stretches at 424/409 cm<sup>-1</sup> (Fig. S19d†), and Ni- $\mu$ OH-Fe wagging modes at 284/268 cm<sup>-1</sup>, in which the  $\mu$ OH<sup>-</sup> ligand moves perpendicularly to the Ni-Fe axis of the active site. The most relevant Fe/Ni- $\mu$ OH modes are depicted in Fig. 7. In spite of their inherently low <sup>57</sup>Fe-PVDOS intensities, the Ni- $\mu$ OH and Fe- $\mu$ OH stretches gain intensity *via* vibrational coupling to the Fe-CO/CN coordinates. They are associated with the



Fig. 7 Arrow-style representation of important Fe/Ni- $\mu$ OH vibrational modes from representative DFT models for the Ni<sub>r</sub>-S<sub>I</sub> (model Ni<sub>r</sub>-S<sup>H</sup><sub>D</sub><sub>D</sub>, top) and Ni<sub>r</sub>-B" (model Ni<sub>r</sub>-B<sup>H</sup><sub>D</sub><sub>D</sub>, bottom) states. The corresponding vibrational energies (cm<sup>-1</sup>) are indicated in the top left of each panel. Left to right: Ni- $\mu$ OH-Fe wagging, Fe- $\mu$ OH stretching, and Ni- $\mu$ OH stretching modes. The stick-style mode intensities relevant to the interpretation of the <sup>57</sup>Fe-PVDOS spectra are marked in Fig. S18b and S19b-d.† Animations of these and other normal modes are available as part of the ESI.†

experimental NRVS features at 507/502 (Ni– $\mu$ OH) and 427/420 cm<sup>-1</sup> (Fe– $\mu$ OH) of Ni<sub>r</sub>–S<sub>I</sub>/Ni<sub>r</sub>–B", respectively (Fig. S16a/S17a†). Because of their relatively low intensities, no specific bands in the experimental NRV spectra could be confidently associated with the essentially pure Ni– $\mu$ OH–Fe wagging modes.

### Conclusions

With the present study, we provide insight into the active site structure of two interconvertible redox states of a [NiFe]hydrogenase active site by means of vibrational spectroscopy and concomitant DFT calculations. Specific 57Fe enrichment of the catalytic subunit HoxC of the regulatory [NiFe]-hydrogenase from R. eutropha resulted in a strong enhancement of the active site spectral features, as revealed by NRVS analysis. The absence of FeS clusters enabled the observation of low-intensity Fe-CO/ CN modes as well as active site bands usually superimposed by the strong spectral contribution of FeS cluster signals. By combining NRVS with cryo-IR spectroscopy, we targeted the two hydroxy-bridged  $Ni_r$ -S<sub>I</sub> and  $Ni_r$ -B" states of the [NiFe] active site, which turned out to differ just in the oxidation state of the Ni ion. In fact, the two DFT-based homology models of the catalytic center  $Ni_r-S^{SH}_{\mu OH}$  and  $Ni_r-B^{SH}_{\mu OH}$ , comprising a bridging OH<sup>-</sup> ligand and a protonated terminal cysteine, reproduced accurately the active site bands obtained by NRV and cryo-IR spectroscopy.

Previous studies suggested a protonatable bridging hydroxy ligand between Ni and Fe ions in the Ni<sub>r</sub>–S state(s),<sup>21,22,39,40</sup> whose removal produces a catalytically competent Ni<sub>a</sub>–S intermediate.<sup>30,41</sup> Our results provide structural details for these states, including a protonated terminal cysteine, which is consistent with previous studies supporting a conserved protonation state in the Ni<sub>r</sub>–B and Ni<sub>r</sub>–S<sub>I</sub> resting states.<sup>22,36,37,42</sup> Additionally, the presence of an acid-base couple (OH<sup>-</sup> and H<sup>+</sup>) at a close distance in the HoxC active site suggests that water removal might be a crucial step of hydrogenase maturation. This step might be mediated by the attachment of the FeScarrying small subunit, enabling the formation of H<sub>2</sub>-metabolizing catalytic intermediates. Recent results on the *in vitro* assembly of large and small subunits of RH from *R. eutropha* seem to corroborate this mechanism.<sup>43</sup>

Another important outcome of the present work is that temperature has a strong impact on the equilibrium distribution of the enzymes' redox states. Therefore, samples measured at ambient temperature by, *e.g.*, IR spectroscopy, should be used with care as a reference for experiments performed at cryogenic temperatures. This conclusion proved to be valid also for [FeFe]-hydrogenases.<sup>44,45</sup> It can probably be extended to the structural characterization of other biological and synthetic catalysts, where spectroscopic measurements at room temperature, *e.g.* IR, UV-visible, are used to interpret data collected using cryogenic techniques, including EPR and synchrotronrelated X-ray crystallography, NRVS and XAS.

In summary, our study demonstrated the power of advanced vibrational spectroscopic techniques at cryogenic temperatures in combination with DFT calculations to elucidate the molecular active site structure of metalloenzymes. Here, we enriched and studied two hydroxy-containing redox states of [NiFe]-hydrogenases. In case catalytic intermediates can be trapped in a similar manner, this strategy will be useful to unravel their corresponding active site architectures.

### Conflicts of interest

There are no conflicts to declare.

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### References

- 1 W. Lubitz, H. Ogata, O. Rüdiger and E. Reijerse, *Chem. Rev.*, 2014, **114**, 4081–4148.
- 2 H. S. Shafaat, O. Rüdiger, H. Ogata and W. Lubitz, *Biochim. Biophys. Acta Bioenerg.*, 2013, **1827**, 986–1002.
- 3 D. Tombolelli and M. A. Mroginski, *J. Phys. Chem. B*, 2019, **123**, 3409–3420.
- 4 P. A. Ash, R. Hidalgo and K. A. Vincent, *ACS Catal.*, 2017, 7, 2471–2485.
- 5 R. M. Evans, E. J. Brooke, S. A. M. Wehlin, E. Nomerotskaia, F. Sargent, S. B. Carr, S. E. V. Phillips and F. A. Armstrong, *Nat. Chem. Biol.*, 2016, **12**, 46–50.
- 6 W. Lubitz, E. Reijerse and M. van Gastel, *Chem. Rev.*, 2007, **107**, 4331–4365.
- 7 M.-E. Pandelia, H. Ogata and W. Lubitz, *ChemPhysChem*, 2010, **11**, 1127–1140.
- 8 R. P. Happe, W. Roseboom, A. J. Pierik, S. P. J. Albracht and K. A. Bagley, *Nature*, 1997, **385**, 126.
- 9 J. E. Huyett, M. Carepo, A. Pamplona, R. Franco, I. Moura,
  J. J. G. Moura and B. M. Hoffman, *J. Am. Chem. Soc.*, 1997,
  119, 9291–9292.
- 10 M. Horch, J. Schoknecht, M. A. Mroginski, O. Lenz, P. Hildebrandt and I. Zebger, J. Am. Chem. Soc., 2014, 136, 9870–9873.
- 11 E. Siebert, M. Horch, Y. Rippers, J. Fritsch, S. Frielingsdorf, O. Lenz, F. Velazquez Escobar, F. Siebert, L. Paasche, U. Kuhlmann, F. Lendzian, M.-A. Mroginski, I. Zebger and P. Hildebrandt, *Angew. Chem., Int. Ed.*, 2013, **52**, 5162–5165.
- S. Kamali, H. Wang, D. Mitra, H. Ogata, W. Lubitz,
   B. C. Manor, T. B. Rauchfuss, D. Byrne, V. Bonnefoy,
   F. E. Jenney, M. W. W. Adams, Y. Yoda, E. Alp, J. Zhao and
   S. P. Cramer, *Angew. Chem., Int. Ed.*, 2013, 52, 724–728.

- 13 H. Ogata, т. Krämer, H. Wang, D. Schilter. V. Pelmenschikov, M. van Gastel, F. Neese. T. B. Rauchfuss, L. B. Gee, A. D. Scott, Y. Yoda, Y. Tanaka, W. Lubitz and S. P. Cramer, Nat. Commun., 2015, 6(1), 7890.
- 14 L. Lauterbach, H. Wang, M. Horch, L. B. Gee, Y. Yoda, Y. Tanaka, I. Zebger, O. Lenz and S. P. Cramer, *Chem. Sci.*, 2015, 6, 1055–1060.
- 15 R. Gilbert-Wilson, J. F. Siebel, A. Adamska-Venkatesh, C. C. Pham, E. Reijerse, H. Wang, S. P. Cramer, W. Lubitz and T. B. Rauchfuss, *J. Am. Chem. Soc.*, 2015, 137, 8998–9005.
- 16 E. J. Reijerse, C. C. Pham, V. Pelmenschikov, R. Gilbert-Wilson, A. Adamska-Venkatesh, J. F. Siebel, L. B. Gee, Y. Yoda, K. Tamasaku, W. Lubitz, T. B. Rauchfuss and S. P. Cramer, *J. Am. Chem. Soc.*, 2017, 139, 4306–4309.
- Y. Guo, H. Wang, Y. Xiao, S. Vogt, R. K. Thauer, S. Shima,
  P. I. Volkers, T. B. Rauchfuss, V. Pelmenschikov,
  D. A. Case, E. E. Alp, W. Sturhahn, Y. Yoda and
  S. P. Cramer, *Inorg. Chem.*, 2008, 47, 3969–3977.
- 18 J. Preissler, S. Wahlefeld, C. Lorent, C. Teutloff, M. Horch, L. Lauterbach, S. P. Cramer, I. Zebger and O. Lenz, *Biochim. Biophys. Acta Bioenerg.*, 2018, **1859**, 8–18.
- G. Caserta, C. Lorent, A. Ciaccafava, M. Keck, R. Breglia, C. Greco, C. Limberg, P. Hildebrandt, S. P. Cramer, I. Zebger and O. Lenz, *Chem. Sci.*, 2020, 11, 5453–5465.
- 20 H. Tai, Y. Higuchi and S. Hirota, *Dalton Trans.*, 2018, 47, 4408-4423.
- 21 S. Kurkin, S. J. George, R. N. F. Thorneley and S. P. J. Albracht, *Biochemistry*, 2004, **43**, 6820–6831.
- B. Bleijlevens, F. A. van Broekhuizen, A. L. De Lacey, W. Roseboom, V. M. Fernandez and S. P. J. Albracht, *J. Biol. Inorg Chem.*, 2004, 9, 743–752.
- 23 F. Roncaroli, E. Bill, B. Friedrich, O. Lenz, W. Lubitz and M.-E. Pandelia, *Chem. Sci.*, 2015, **6**, 4495–4507.
- 24 S. Frielingsdorf, J. Fritsch, A. Schmidt, M. Hammer, J. Löwenstein, E. Siebert, V. Pelmenschikov, T. Jaenicke, J. Kalms, Y. Rippers, F. Lendzian, I. Zebger, C. Teutloff, M. Kaupp, R. Bittl, P. Hildebrandt, B. Friedrich, O. Lenz and P. Scheerer, *Nat. Chem. Biol.*, 2014, **10**, 378–385.
- 25 M. van Gastel, M. Stein, M. Brecht, O. Schröder, F. Lendzian,
  R. Bittl, H. Ogata, Y. Higuchi and W. Lubitz, *J. Biol. Inorg Chem.*, 2006, 11, 41–51.
- 26 Ch. Geßner, O. Trofanchuk, K. Kawagoe, Y. Higuchi, N. Yasuoka and W. Lubitz, *Chem. Phys. Lett.*, 1996, 256, 518–524.

- 27 J. Fritsch, P. Scheerer, S. Frielingsdorf, S. Kroschinsky,B. Friedrich, O. Lenz and C. M. T. Spahn, *Nature*, 2011, 479, 249–252.
- 28 T. Krämer, M. Kampa, W. Lubitz, M. van Gastel and F. Neese, *ChemBioChem*, 2013, **14**, 1898–1905.
- 29 P. E. M. Siegbahn, J. W. Tye and M. B. Hall, *Chem. Rev.*, 2007, 107, 4414–4435.
- 30 H. Tai, K. Nishikawa, Y. Higuchi, Z. Mao and S. Hirota, *Angew. Chem., Int. Ed.*, 2019, **58**, 13285–13290.
- 31 H. Ogata, K. Nishikawa and W. Lubitz, *Nature*, 2015, **520**, 571–574.
- 32 A. M. Escorcia and M. Stein, Front. Chem., 2018, 6, 164.
- 33 G. Dong and U. Ryde, J. Biol. Inorg Chem., 2016, 21, 383-394.
- 34 P. E. M. Siegbahn and R.-Z. Liao, *ACS Catal.*, 2020, **10**, 5603–5613.
- 35 R. Breglia, C. Greco, P. Fantucci, L. De Gioia and M. Bruschi, *Inorg. Chem.*, 2019, **58**, 279–293.
- 36 A. L. De Lacey, A. Pardo, V. M. Fernández, S. Dementin, G. Adryanczyk-Perrier, E. C. Hatchikian and M. Rousset, *J. Biol. Inorg Chem.*, 2004, 9, 636–642.
- 37 C. Fichtner, C. Laurich, E. Bothe and W. Lubitz, *Biochemistry*, 2006, **45**, 9706–9716.
- 38 L. Lauterbach, L. B. Gee, V. Pelmenschikov, F. E. Jenney, S. Kamali, Y. Yoda, M. W. W. Adams and S. P. Cramer, *Dalton Trans.*, 2016, 45, 7215–7219.
- 39 H. Tai, L. Xu, S. Inoue, K. Nishikawa, Y. Higuchi and S. Hirota, *Phys. Chem. Chem. Phys.*, 2016, **18**, 22025–22030.
- 40 H. Tai, L. Xu, K. Nishikawa, Y. Higuchi and S. Hirota, *Chem. Commun.*, 2017, **53**, 10444–10447.
- 41 Y. Ilina, C. Lorent, S. Katz, J. Jeoung, S. Shima, M. Horch, I. Zebger and H. Dobbek, *Angew. Chem., Int. Ed.*, 2019, 58, 18710–18714.
- 42 C. Stadler, A. L. de Lacey, Y. Montet, A. Volbeda, J. C. Fontecilla-Camps, J. C. Conesa and V. M. Fernández, *Inorg. Chem.*, 2002, **41**, 4424–4434.
- 43 G. Caserta, C. Lorent, V. Pelmenschikov, J. Schoknecht, Y. Yoda, P. Hildebrandt, S. P. Cramer, I. Zebger and O. Lenz, ACS Catal., 2020, 10, 13890–13894.
- 44 J. A. Birrell, V. Pelmenschikov, N. Mishra, H. Wang, Y. Yoda,
  K. Tamasaku, T. B. Rauchfuss, S. P. Cramer, W. Lubitz and
  S. DeBeer, *J. Am. Chem. Soc.*, 2020, 142, 222–232.
- 45 C. Lorent, S. Katz, J. Duan, C. J. Kulka, G. Caserta, C. Teutloff,
  S. Yadav, U.-P. Apfel, M. Winkler, T. Happe, M. Horch and
  I. Zebger, *J. Am. Chem. Soc.*, 2020, 142, 5493–5497.