Asymmetry in the Ligand Coordination Sphere of the [FeFe] Hydrogenase Active Site Is Reflected in the Magnetic Spin Interactions of the Aza-propanedithiolate Ligand

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ABSTRACT: [FeFe] hydrogenases are very active enzymes that catalyze the reversible conversion of molecular hydrogen into protons and electrons. Their active site, the H-cluster, contains a unique binuclear iron complex, [2Fe]H, with CN− and CO ligands as well as an aza-propane-dithiolate (ADT) moiety featuring a central amine functionality that mediates proton transfer during catalysis. We present a pulsed 13C-ENDOR investigation of the H-cluster in which the two methylene carbons of ADT are isotope labeled with 13C. We observed that the corresponding two 13C hyperfine interactions are of opposite sign and corroborated this finding using density functional theory calculations. The spin polarization in the ADT ligand is shown to be linked to the asymmetric coordination of the distal iron site with its terminal CN− and CO ligands. We propose that this asymmetry is relevant for the enzyme reactivity and is related to the (optimal) stabilization of the iron-hydride intermediate in the catalytic cycle.

The active site of [FeFe] hydrogenases, called the “H-cluster”, features a two-iron subcluster that is coordinated by CN− and CO ligands as well as a unique 2-aza-propane-1,3-dithiolate (ADT) moiety featuring a central amine functionality that mediates proton transfer during catalysis. We present a pulsed 13C-ENDOR investigation of the H-cluster in which the two methylene carbons of ADT are isotope labeled with 13C. We observed that the corresponding two 13C hyperfine interactions are of opposite sign and corroborated this finding using density functional theory calculations. The spin polarization in the ADT ligand is shown to be linked to the asymmetric coordination of the distal iron site with its terminal CN− and CO ligands. We propose that this asymmetry is relevant for the enzyme reactivity and is related to the (optimal) stabilization of the iron-hydride intermediate in the catalytic cycle.

Isotope Labeling. For the 13C-ENDOR experiments described herein, we took advantage of a triply labeled (2 × 13C) Hox iron-hydride intermediate in the [FeFe] hydrogenase active site. This labeling is achieved by introducing 13C atoms into the methylene carbons of the ADT ligand through isotope exchange with 13C-labeled methanol (MeOH-13C) to replace the endogenous 12C of the active site by 13C at an iron site (Fe4) distal to the [2Fe]H subcluster. The catalytically active oxidized state (Hox) is characterized by a mixed valence redox configuration [Fe(II)Fe(I)] in the [2Fe]H subcluster. This state features a so-called “frustrated Lewis pair” (FLP) with the electrophilic Fe3 center adjacent to the Bronsted basic amine of the ADT. The FLP splits Hox heterolytically placing a proton on the amine and a hydride on Fe3. This Fe3−H species is immediately oxidized through the electron transport chain connected to the iron core of the H-cluster. This oxidation converts the hydridic Fe3−H to an acidic Fe3−H species. The subsequent stages in the catalytic cycle (Figure 1) feature several protonation, reduction, and proton coupled electron transfer (PCET) steps. This multistep sequence results in a very flat energy landscape, which explains the very high catalytic rates observed in [FeFe] hydrogenases (up to 10,000 s−1).
$^{57}$Fe, 2 $\times$ $^{13}$C, 4 $\times$ $^{2}$H) synthetic precursor of [2Fe]$_{3}$H$_{3}$, initially prepared for ongoing nuclear resonance vibrational spectroscopy (NRVS) experiments. The new synthetic route for this compound is described in the Supporting Information (SI) section A and Scheme S1. The [FeFe] hydrogenase from Chlamydomonas reinhardtii (CrHydA1) was produced using artificial maturation$^{12,13}$ of the apoenzyme expressed in E. coli with the triply labeled [2Fe]H$_{3}$ precursor (see Figure 2). The enzyme was allowed to oxidize under an N$_{2}$ atmosphere (so-called "auto-oxidation") to produce the H$_{ox}$ state. This is characterized by a mixed valence Fe(II)Fe(I) binuclear subcluster showing a characteristic rhombic $S = 1/2$ EPR spectrum with $g$-parameters (2.1008, 2.0398, 1.9966) (see Figures 2 and S2).$^{1,14}$ The EPR and FTIR spectra (Figure S1) are consistent with those obtained previously for the native H$_{ox}$ state.$^{1}$

**Mims-ENDOR.** Orientation-selective $^{13}$C Mims ENDOR (see Experimental Section in the SI and Scheme 1) was recorded at Q-band (34 GHz) at field positions corresponding to the range from $g = 2.0$ to $g = 2.1$ (Figure 3). In order to facilitate spectral fitting, the ENDOR spectra were normalized to the same amplitude and symmetrized with respect to the Larmor frequency (indicated by the arrows in Figure 3).

At the $g = 2.0$ and $g = 2.1$ positions (magnetic field values of 1219 and 1158.7 mT, respectively), only molecules with one of their $g$-axes ($x$ or $z$, respectively) oriented along the magnetic field contribute to the ENDOR spectrum. These positions are called "pseudo single crystal positions". In these spectra, clearly two doublets centered at the $^{13}$C nuclear Larmor frequency (12.85 MHz at 1200 mT) are observed corresponding to the nuclear spin transitions of the two $^{13}$C nuclei in the ADT ligand. The different splitting and line-width of the two doublets indicate that the two $^{13}$C nuclei have significantly different hyperfine interaction (HFI) tensors differing in magnitudes and orientations. ENDOR spectra recorded at intermediate field positions contain contributions from molecules with multiple orientations giving rise to an EPR transition at the effective $g$-value corresponding to the field position. These multiple orientations lead to a broadening of the ENDOR spectra showing "powder-type" features. The line-shapes at, e.g., 1165.8 and 1205.4 G are reminiscent of "near axial hyperfine anisotropy" for the $^{13}$C nuclei. Therefore, in order to reduce the number of free variables, spectral fitting was started assuming axial HFI tensors for both $^{13}$C nuclei. First order perturbation theory was employed to calculate the ENDOR frequencies. The $(1 - \cos(2\pi A))$ dependence of the ENDOR amplitude was taken into account. This procedure provided a fair but nonoptimal fit to the experimental ENDOR spectra. Subsequently, the rhombicity parameter was relaxed, and individual scaling of the two ENDOR components was applied. The final fitted parameters are listed in Table 1 and Figure S4. Given the local mirror symmetry of the ADT ligand (Figure 2), it is surprising that the two $^{13}$C HFI-tensors have

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Figure 1. Structure of the H-cluster in [FeFe] hydrogenase from Chlamydomonas reinhardtii (CrHydA1) and the proposed catalytic cycle.

Figure 2. Left: Schematic structure of the H-cluster in its H$_{ox}$ state, triply isotope labeled with $^{57}$Fe, as well as $^{13}$C and $^{2}$H in the aza-propane-dithiolate (ADT) ligand. Right: Q-band EPR spectrum (pseudo modulated FID detected) of our H$_{ox}$ preparation. Fitted $g$-values: (2.1008, 2.0398, 1.9966) (see Figures 2 and S2).$^{1,14}$ A small contribution (<5%) from the H$_{ox}$-CO state is evident from the feature at $g = 2.006$ and is marked by an asterisk. Full analysis of the EPR spectrum is presented in Figure S2.
significantly different magnitudes (1.9 vs 1.2 MHz for the isotropic component).

Mims TRIPLE. In order to determine the relative signs of the two fitted $^{13}$C HFIs, a triple resonance experiment was conducted at a field position corresponding to $g = 2.02$ (magnetic field position near 1218 mT, see Figure 3) where the two ENDOR doublet features are nicely separated and still relatively sharp (Figure 4 and Scheme 2). Adjusting the radiofrequency pumping pulse (RF1) to one of the ENDOR transitions inverts the spin state populations corresponding to this transition. This also affects (reduces) the population difference of the other nuclear spin transition in the same $m_s$.

Table 1. Experimental and DFT-Predicted $^{13}$C HFI Anisotropic and Isotropic (Fermi Contact) Spin Couplings (MHz) for the Two Methylene Carbons A1 and A2 in the ADT Ligand in the Hox State

| A1 (MHz) | A2 (MHz) |
|----------|----------|
| $X$ | $Y$ | $Z$ | iso | $X$ | $Y$ | $Z$ | iso |
| exptl | | | | | | | |
| $A_{native}$ | 1.20 | 1.46 | 3.08 | 1.91 | | | | |
| $A_{cis}$ | 1.23 | 1.50 | 3.20 | 1.97 | | | | |
| $A_{cis}$ | 1.41 | 1.33 | 0.50 | 0.94 | | | | |
| $A_{crot}$ | 0.84 | 1.06 | 2.74 | 1.55 | | | | |
| $A_{crot}$ | 1.44 | 1.08 | 0.17 | 0.78 | | | | |
| $A_{tet}$ | 0.46 | 0.30 | 0.01 | 0.13 | | | | |

The experimental (exptl) and representative DFT ($A_{native}$) values are in bold. Other values are from alternative isomeric DFT models shown in Figure S7.
Pumping the high frequency line of the sharp $^{13}$C doublet leads to a reduction in amplitude of the low frequency line of the broad $^{13}$C doublet (upper spectra in Figure 4). Likewise, pumping the low frequency line of the sharp doublet causes a reduction in amplitude of the high frequency line of the broad doublet. We must, therefore, conclude that the signs of the two $^{13}$C hyperfine interactions are opposite (Tables 1 and S1). This implies that at one of the ADT carbon nuclei a negative ($\beta$) spin density is localized.

**DFT Calculations.** In order to obtain more insight into the underlying mechanism of this spin polarization phenomenon, we conducted DFT calculations using a [2Fe]$_{H}$ subcluster model in which the (oxidized) [4Fe$-4S$]$_{H}$ subcluster ($S = 0$) is modeled by a protonated thiol (ethyl-SH) group (Figure S5). The representative model is called “A” due to the “axial” conformation of the ADT$-\text{NH}^-$ moiety, with its proton pointing toward Fe$_{o}$. Using this modeling level, previously referred to as “S (small)”$^{17,18}$ the magnitude and sign of the experimental $^{13}$C-ADT HFIs for both carbon nuclei could be best reproduced. In Figure S6 the spin-density distribution of the (i) corresponding (see the DFT methods described in the SI) singly occupied molecular orbital (SOMO), (ii) formally doubly occupied MOs (Total-SOMO), as well as (iii) that of all MOs (Total) of the binuclear subsite are displayed with two contour values. A summary of these DFT results is presented in Figure 5.

**Figure 4.** Mims TRIPLE resonance experiment (see pulse sequence in Scheme 2) at $B = 1218$ mT. The black traces represent the (unsymmetrized) $^{13}$C TRIPLE experiment with the pumping frequency RF1 off resonance at the $^{13}$C Larmor frequency, i.e., equivalent to the $^{13}$C ENDOR spectrum at $B = 1218$ mT (see Figure 3). The red traces represent the $^{13}$C Mims TRIPLE experiment with the RF1 pumping frequency tuned to one of the sharp ENDOR transitions. The “TRIPLE effect” is indicated by the red asterisks.

**Scheme 2. Pulse Sequence of the $^{13}$C Mims Triple Resonance Experiment**

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| mw | mw | T | mw | echo |
|----|----|---|----|------|
|   |    |   |    |      |
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“See Scheme S2 for extra details.

**Figure 5.** Top (a): Isosurfaces of total spin density at the $5 \times 10^{-4}$ a.u. cutoff (corresponding to the Fermi contact HFI term $A_{iso}(^{13}C) = 0.6$ MHz) for the representative $H_{nm}$ $S = 1/2$ DFT model $A_{native}$, showing positive (blue) density on the “right” $^{13}$C and negative (green) spin density on the “left” $^{13}$C nuclei. The model itself is shown in thin wire. Bottom (b): View of DFT model $A_{native}$ along the $F_{e_{p}}-F_{e_{d}}$ axis displaying the orientation of the calculated methylene $^{13}$C hyperfine tensors (Table S1). For extended information, see Figures S6 and S7.
Notably, the corresponding SOMO has 74% contribution from the Fe$_2$ 3d shell. It turns out that the “left” methylene carbon, i.e., the carbon on the same side as the distal terminal CO ligand, has negative spin density due to spin polarization since the SOMO has only a vanishing contribution on this atom. The “right” carbon nucleus (on the same side as the distal CN$^-$ ligand) has positive spin density due to delocalization of the SOMO onto this center. No other MOs noticeably contribute to the spin densities at the two $^{13}$C nuclei. Although very small in their magnitude, the calculated atomic spin populations at the two ADT carbon nuclei (Table S3) are nevertheless consistent with the observed $^{13}$C HFI values. It is, therefore, tempting to assume that this asymmetric spin distribution is associated with the asymmetric ligand configuration at the distal iron center Fe$_d$. To verify this hypothesis, two isomeric DFT models were investigated in which the positions of the CN$^-$ and CO ligands were swapped at either Fe$_p$ (‘A$^{p\text{-}(CN)2}$’ or Fe$_q$ (‘A$^{q\text{-}(CN)2}$’), see Figure S7. Obviously, swapping CN$^-$ ligands at both Fe sites creates a pseudomirror image of the native [2Fe]$_4$H isomer (‘A$^{p\text{-}(CN)2}$’). A full mirror image can only be obtained if the protonation position of the ethyl-SH ligand is also swapped. Exchanging the Fed changes the spin-polarization in the ADT ligand (Table 1). The behavior described is rationalized by the [Fe(II)pFe(I)d] electronic structure of the [2Fe$_4$]$_4$H subcluster in the H$_{\text{ox}}$ state with its S = 1/2 spin localization at the Fe(I)d distal iron site, while the Fe(II)$_p$ proximal site remains low-spin.19,20 Other investigators DFT models are $E_{\text{native}}$, having the –NH– proton in its alternative “equatorial” position pointing toward Fe$_p$ and “A$^{d\text{-}rot}$” with CN$^-$ at Fe$_d$ rotated to the position trans to the bridging CO ligand;22 these two models represent [2Fe$_4$]$_4$H subcluster structural alternatives often discussed in the literature. While the two alternatives $E_{\text{native}}$ and A$^{d\text{-}rot}$ predict the same orientation of the $^{13}$C-ADT HFI tensors as seen in $A_{\text{native}}$, they produce an inferior match to the experimental HFI values (Figure S7, Tables 1 and S1). Interestingly, all the above-described ‘A’ (axial) isomeric models are found essentially equi-energetic within 1 kcal/mol, while the ‘E’ (equatorial) isomer is calculated +5 kcal/mol higher in its energy. Two additional isomeric ligand swap isomers have both CN$^-$ ligands at either Fe$_p$ (‘A$^{p\text{-}(CN)2}$’ or Fe$_d$ (‘A$^{d\text{-}(CN)2}$’); these two structures are predicted to have significantly higher (+123/129 kcal/mol) relative energies. Notably, placing two negative CN$^-$ ligands at Fe$_d$ in the A$^{d\text{-}(CN)2}$ isomer produces an inverted [Fe(II)$_p$Fe(II)$_d$] oxidation pattern for the iron sites, having Fe$_p$ as the spin center (Table S2).

The local CN$^-$/CO ligand asymmetry at the distal iron site, which features the open coordination in the H$_{\text{ox}}$ state, seems to be the key to inducing the spin-polarization in the ADT ligand. A further result of this asymmetry is the spin polarization of the methylene protons as was observed in our paramagnetic NMR studies.11 Since in our [2Fe$_4$]$_4$H precursor the methylene protons have been labeled with $^2$H, we also performed $^2$H Mims ENDOR experiments at the three canonical field positions (g$_x$, g$_y$, g$_z$) (see Figure S8). Signals of up to three $^2$H nuclei could be observed and show hyperfine couplings up to 0.6 MHz. The signals are, however, much weaker than those of the $^{13}$C nuclei, which, currently, precludes the application of a triple $^2$H ENDOR experiment.

To address the question of the relevance of the asymmetry in coordination of the distal iron atom for the catalytic cycle, we investigated how it influences the stabilization of the hydride-bound H$_{\text{hyd}}$ state, which is formed from the 2e$^-$-reduced (vs H$_{\text{ox}}$), protonated H$_{\text{red}}$H$^+$ state (see Figure 1). We calculated the energetics for this process for three isomers: the native configuration ‘A’ with one terminal CN$^-$ on each [2Fe$_4$]$_4$H iron, ‘p-(CN)2’ with both CN$^-$ ligands coordinated at the proximal iron, and ‘d-(CN)2’ with both CN$^-$ ligands coordinating the distal iron (see Figures S7 and S9). Clearly, the non-native isomeric configurations are of academic interest only since they do not occur in [FeFe] hydrogenase and cannot be synthesized. Moreover, the relative energies of the non-native isomers are around 130 kcal/mol higher than that of the native configuration (Figure S9). Since in our truncated model the [4Fe–4$^2$S]$_4$H subcluster is absent, H$_{\text{red}}$H$^+$ is indistinguishable from H$_{\text{red}}$H$^+$. This also means that the models are diamagnetic, and no magnetic effects can be modeled. Nevertheless, it can be assumed that the thermodynamics of the Fe$_d$–H$^+$ hydroxide formation (i.e., the H$_{\text{red}}$H$^+$-to-H$_{\text{red}}$H$^+$ intramolecular proton transfer from the ADT–NH$_3^+$–bridgehead to the distal iron) is well represented since the [4Fe–4$^2$S]$_4$H subcluster is unlikely to have a major impact on this process. It should be noted that the hydroxide formation can involve a transition from terminal CO in the H$_{(\text{ox})}$ state to bridging CO in H$_{(\text{ox})}$ (see Figures 1 and S9). It is not clear if this ligand rearrangement also occurs to its full extent in the enzyme on the time scale of catalysis. For the ‘A’ native and p-(CN)2 isomers, the H$_{\text{red}}$H$^+$ hydroxide formation is predicted exothermic: $\Delta$H$_{\text{native}}$ = −3.4 kcal/mol, $\Delta$H$_{(p\text{-}(CN)2)}$ = −7.8 kcal/mol. For the d-(CN)2 isomer, the hydroxide formation is endothermic, $\Delta$H$_{(d\text{-}(CN)2)}$ = +16.8 kcal/mol. For the p-(CN)2 model, the distal iron Fe$_d$ has a lack of charge density (in the absence of CN$^-$ coordination), providing extra stabilization of the hydroxide. However, model d-(CN)2 has excess charge density (with two CN$^-$ coordinating) at the distal iron, which prevents stabilization of the hydroxide at Fe$_d$. The charge density is more equally distributed over the two-iron core of [2Fe$_4$]$_4$H in the native configuration and seems to provide the perfect compromise to sufficiently stabilize the hydroxide state for fast catalysis yet not overstabilizing it, in order to avoid formation of a thermodynamic sink, which would strongly slow down the catalysis. We, therefore, conclude that the asymmetry of the ligand coordination at Fe$_d$ ensures a flat energy landscape for the hydroxide formation and transfer during the reversible catalytic cycle of [FeFe] hydrogenase. This finding significantly advances the design strategies for artificial hydrogen conversion catalysts: Apart from the ligand geometry, also the charge balance at the catalytic iron center needs to be carefully controlled.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcllett.9b02354.

New synthetic route for the $^5$Fe labeled ADT precursor, experimental and computational procedures, supplementary figures and tables (PDF)

Cartesian coordinates of structurally optimized DFT models (ZIP)
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Notes
The authors declare no competing financial interest.

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REFERENCES
(1) Lubitz, W.; Ogata, H.; Rüdiger, O.; Reijerse, E. Hydrogenases. *Chem. Rev.* 2014, 114, 4081–4148.
(2) Stephan, D. W.; Erker, G. Frustrated Lewis Pair Chemistry: Development and Perspectives. *Angew. Chem., Int. Ed.* 2015, 54, 6400–6441.
(3) Birrell, J. A.; Rüdiger, O.; Reijerse, E. J.; Lubitz, W. Semisynthetic Hydrogenases Propel Biological Energy Research into a New Era. *Joule* 2017, 1, 61–76.
(4) Sommer, C.; Adamska-Venkatesh, A.; Pawlak, K.; Birrell, J. A.; Rüdiger, O.; Reijerse, E. J.; Lubitz, W. Proton Coupled Electronic Rearrangement within the H-cluster as an Essential Step in the Catalytic Cycle of FeFe Hydrogenases. *J. Am. Chem. Soc.* 2017, 139, 1440–1443.
(5) Rodríguez-Maciá, P.; Pawlak, K.; Rüdiger, O.; Reijerse, E. J.; Lubitz, W.; Birrell, J. A. Inter-cluster Redox Coupling Influences Protonation at the H-cluster in [FeFe] Hydrogenases. *J. Am. Chem. Soc.* 2017, 139, 15122–15134.
(6) Glick, B. R.; Martin, W. G.; Martin, S. M. Purification and Properties of the Periplasmic Hydrogenase from *Desulfovibrio desulfuricans*. *Can. J. Microbiol.* 1980, 26, 1214–1223.
(7) Hatchikian, E. C.; Forget, N.; Fernández, V. M.; Williams, R.; Cammak, R. Further Characterization of the [Fe]-Hydrogenase from *Desulfovibrio desulfuricans* ATCC 7757. *Eur. J. Biochem.* 1992, 209, 357–365.
(8) Silakov, A.; Reijerse, E. J.; Albracht, S. P. J.; Hatchikian, E. C.; Lubitz, W. The Electronic Structure of the H-cluster in the [FeFe]-hydrogenase from *Desulfovibrio desulfuricans*: A Q-band 57Fe ENDOR and HYSCORE Study. *J. Am. Chem. Soc.* 2007, 129, 11447–11458.
(9) Silakov, A.; Reijerse, E. J.; Lubitz, W. Unraveling the Electronic Properties of the Photoinduced States of the H-Cluster in the [FeFe] Hydrogenase from *D. desulfuricans*. *Eur. J. Inorg. Chem.* 2011, 2011, 1056–1066.
(10) Silakov, A.; Wenk, B.; Reijerse, E.; Albracht, S. P. J.; Lubitz, W. Spin Distribution of the H-cluster in the H4-CO state of the [FeFe] Hydrogenase from *Desulfovibrio desulfuricans*: HYSCORE and ENDOR Study of 14N and 13C Nuclear Interactions. *J. Biol. Inorg. Chem.* 2009, 14, 301–313.
(11) Rumpel, S.; Ravaer, E.; Sommer, C.; Reijerse, E.; Fares, C.; Luchinat, C.; Lubitz, W. 1H NMR Spectroscopy of [FeFe] Hydrogenase: Insight into the Electronic Structure of the Active Site. *J. Am. Chem. Soc.* 2018, 140, 131–134.
(12) Berggren, G.; Adamska, A.; Lambertz, C.; Simmons, T. R.; Esselborn, J.; Atta, M.; Gambarelli, S.; Mouesca, J. M.; Reijerse, E. J.; Lubitz, W.; Happe, T.; Artero, V.; Fontecave, M. Biomimetic Assembly and Activation of (FeFe)-Hydrogenases. *Nature* 2013, 499, 66–69.
(13) Esselborn, J.; Lambertz, C.; Adamska-Venkatesh, A.; Simmons, T.; Berggren, G.; Noth, J.; Siebel, J. F.; Hemschemeier, A.; Artero, V.; Reijerse, E. J.; Fontecave, M.; Lubitz, W.; Happe, T. Spontaneous Activation of [FeFe]-Hydrogenases by an Inorganic [2Fe] Active Site Mimic. *Nat. Chem. Biol.* 2013, 9, 607–609.
(14) Lubitz, W.; Reijerse, E.; van Gastel, M. [NiFe] and [FeFe] Hydrogenases Studied by Advanced Magnetic Resonance Techniques. *Chem. Rev.* 2007, 107, 4331–4365.
(15) Schweiger, A.; Jeschke, G. Principles of Pulse Electron Paramagnetic Resonance; Oxford University Press: Oxford, 2001.
(16) Harmer, J. R. Hyperfine Spectroscopy - ENDOR. *Eur. J. Med. Chem.* 2016, 5, 1493–1514.
(17) Pham, C. C.; Mulder, D. W.; Pelmenschikov, V.; King, P. W.; Ratzloff, M. W.; Wang, H.; Mishra, N.; Alg, E. E.; Zhao, J.; Hu, M. Y.; Tamasaku, K.; Yoda, Y.; Cramer, S. P. Terminal Hydride Species in [FeFe]-Hydrogenases Are Vibrationally Coupled to the Active Site Environment. *Angew. Chem., Int. Ed.* 2018, 57, 10605–10609.
(18) Reijerse, E. J.; Pham, C. C.; Pelmenschikov, V.; Gilbert-Wilson, R.; Adamska-Venkatesh, A.; Siebel, J. F.; Gee, L. B.; Yoda, Y.; Tamasaku, K.; Lubitz, W.; Rauchfuss, T. B.; Cramer, S. P. Direct Observation of an Iron-Bound Terminal Hydride in [FeFe]-Hydrogenase by Nuclear Resonance Vibrational Spectroscopy. *J. Am. Chem. Soc.* 2017, 139, 4306–4309.
(19) Fiedler, A. T.; Brunold, T. C. Computational Studies of the H-Cluster of Fe-Only Hydrogenases: Geometric, Electronic, and Magnetic Properties and Their Dependence on the [Fe2S4 ] Cubane. *Inorg. Chem.* 2005, 44, 9322–9334.
(20) Greco, C.; Silakov, A.; Bruschi, M.; Ryde, U.; De Gioia, L.; Lubitz, W. Magnetic Properties of [FeFe]-Hydrogenases: A Theoretical Investigation Based on Extended QM and QM/MM Models of the H-Cluster and Its Surroundings. *Eur. J. Inorg. Chem.* 2011, 2011, 1043–1049.
(21) Pelmenschikov, V.; Birrell, J. A.; Pham, C. C.; Mishra, N.; Wang, H.; Sommer, C.; Reijerse, E.; Richers, C. P.; Tamasaku, K.; Yoda, Y.; Rauchfuss, T. B.; Lubitz, W.; Cramer, S. P. Reaction Coordinate Leading to H2 Production in [FeFe]-Hydrogenase Identified by Nuclear Resonance Vibrational Spectroscopy and Density Functional Theory. *J. Am. Chem. Soc.* 2017, 139, 16894–16902.
(22) Singleton, M. L.; Bhuvanesh, N.; Reibenspies, J. H.; Darenbourg, M. Y. Synthetic Support of De Novo Design: Sterically Bulky [FeFe]-Hydrogenase Models. *Angew. Chem., Int. Ed.* 2008, 47, 9492–9495.