High-Resolution ENDOR Spectroscopy Combined with Quantum Chemical Calculations Reveals the Structure of Nitrogenase Janus Intermediate E₄(4H)

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INTRODUCTION

The Mo-dependent nitrogenase enzyme system comprises two component proteins. The Fe protein delivers electrons one at a time to the MoFe protein, where they are utilized to reduce the substrate at the active-site iron–molybdenum cofactor ([7Fe-9S-Mo-C-R-homocitrate]; FeMo-co, Figure 1). We have established the proposed Lowe–Thorneley (LT) kinetic model for the nitrogenase function, developed in the 1970s and 1980s, which describes the kinetics of transformation among catalytic intermediates, denoted Eₙ, where n is the number of electrons delivered from the nitrogenase Fe protein to the catalytic FeMo-co of the MoFe protein (Figure 2). A defining feature of this scheme is the accumulation of 4[e⁻/H⁺] before N₂ is reduced and the obligatory formation and release of one mole of H₂ per mole of N₂ reduced, with a limiting stoichiometry for enzyme-catalyzed nitrogen fixation given by eq 1,

\[ \text{N}_2 + 8\text{e}^- + 16\text{ATP} + 8\text{H}^+ \rightarrow 2\text{NH}_3 + \text{H}_2 + 16\text{ADP} + 16\text{Pi} \]  

which implies a stoichiometric enzymatic requirement for 8[e⁻/HH⁺] per N₂ reduced rather than the chemically required 6[e⁻/HH⁺], a central feature that was both baffling and disputed and eventually even doubted by those who originally proposed it.

The key state in this scheme is E₄(4H), the “Janus” intermediate, which has accumulated four of the eight reducing equivalents required by eq 1: E₄(4H) sits at a transition in the
The bridging mode likewise lowers the reactivity of these equivalents stored as two [Fe\(\alpha\) hydrides, a [Fe\(\alpha\) bridges have very similar electronic properties. It further showed that they are differently oriented relative to the g-tensor of FeMo-co, but the analysis was unable to provide a satisfactory picture of the relative spatial relationships of the two bridging moieties. Our DFT study of the re/oa equilibrium yielded as the lowest-energy form of E4(4H) the structure E4(4H)\(^{(o)}\) shown in Figure 3 with parallel Fe–H–Fe planes bridging two pairs of “anchor” Fe on the Fe2,3,6,7 face of FeMo-co. Slightly higher in energy was structure E4(4H)\(^{(f)}\), with one bridging and one terminal hydride, and still higher was E4(4H)\(^{(g)}\), in which a single pair of anchor Fe supports two bridging hydrides, a “dihydride” dibridge. However, the differences in energy between these states were not beyond the uncertainties of the calculation, and a structure of the VFe protein obtained by Einsle and co-workers indeed led them to propose a dibridge structure analogous to E4(4H)\(^{(f)}\). Finally, a quite different “open” structure, E4(3H;CH), with one [Fe–H–Fe] bridging hydride and a protonated interstitial carbide rather than a second hydride,
denoted as E₄(3H;CH), was found at quite high energy. Other DFT studies proposed this structure to play a critical role in nitrogenase activity.²⁸⁻³¹ Siegbahn further suggested that E₄(3H;CH) is the precursor of an Fe-bound methyl intermediate where FeMoco has accumulated 7[e⁻/H⁺], denoted here as P-7 (referred to as E₅₀ in ref 29), which was proposed to be the central catalytic species that undergoes reduction of H₂.²₈,²₉

To resolve the nature of hydride binding to the Janus intermediate, we have carried out extensive high-resolution ¹H ENDOR measurements using the resolution-enhancing "stochastic"³² CW-ENDOR protocol performed on instrumentation greatly improved since our original study.⁹ These are complemented by the use of a recently developed pulsed-ENDOR procedure to obtain absolute signs for hyperfine couplings³³ and by Mims pulsed ²H ENDOR measurements. These measurements are coupled to the structural DFT models through the use of the analytical point-dipole Hamiltonian for the electron–nuclear dipolar interaction of the hydride to its "anchoring" Fe ions, a powerful approach that overcomes limitations inherent in both experimental interpretation and computational chemistry accuracy. The result of this protocol is the identification of the lowest-energy structure of the Janus intermediate: E₄(4H)¹⁴ (Figure 3).

### MATERIALS AND METHODS

#### Materials and Protein Purification.
All reagents were obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ) and were used without further purification. Argon, N₂, and acetylene gases were purchased from Air Liquide America Specialty Gases LLC (Plumsteadville, PA).

The E₄(4H) intermediate has been characterized and behaves similarly in three Azotobacter vinelandii MoFe variants: wild type,³⁴ ³¹ singly substituted α-70Ile, and doubly substituted α-195His→Gln MoFe protein.⁹ Because use of the variants permits the freeze trapping of MoFe with higher populations of E₄(4H),⁹ experiments were carried out both with the singly and doubly substituted MoFe protein. The enzymes were purified and samples were prepared as has been described.³⁵

#### ENDOR Spectroscopy.
Q-band CW ENDOR spectra were collected at 2 K on a spectrometer³⁶ that had been upgraded with a low-noise microwave amplifier; temperature 2 K was achieved with a helium immersion Dewar. Relaxation effects in the original "Feher-style" CW ENDOR protocol,³⁷,³⁸ (100 kHz field modulation; phase-sensitive detection of the dispersion, rapid-passage signal; 2 K) were overcome here by the use of Stochastic ENDOR, as first reported by Brueggeman and Niklas,³⁹ which is a hybrid method that employs field-modulated CW-EPR but pulsed rf.³² Further improving signal-to-noise ENDOR spectra collection employed broadening of the rf to 100 kHz.³⁰ Q-band pulsed Mims and Davies ENDOR spectra⁴¹ were collected at 2 K on a spectrometer with a helium immersion Dewar as previously reported.⁴² The Mims ENDOR intensities exhibit nulls (suppression holes) when \( Aτ = n (n = 0, 1, 2, ...) \), where \( A \) is the...
hyperfine coupling \( (A) \) and \( r \) is the time between the second and third microwave pulses in the three-pulse sequence.

For a single molecular orientation and for nuclei with nuclear spin \( I = 1/2 \) \((^1H)\), the ENDOR transitions for the \( m_g = \pm 1/2 \) electron manifolds are observed, to first order, at frequencies \( v_e = n \nu_e \pm A/2 \), where \( \nu_e \) is the nuclear Larmor frequency and \( A \) is the orientation-dependent hyperfine coupling. At the low- and high-field edges of the EPR spectrum \( (g_1 \) and \( g_2 \), respectively), ENDOR interrogates a single molecular orientation \("single-crystal-like" position). The complete hyperfine tensor principal values and their orientations relative to \( g \) are obtained by analyzing a 2D field-frequency \("orientation-selective"\) pattern of ENDOR spectra taken at numerous fields across the EPR envelope, as described \(^{41-45}\) ENDOR simulations were performed in MATLAB with the easyspin \((v \text{ 5.0)}\) toolbox.\(^{46}\)

Signs of the hyperfine couplings (more specifically, the sign of \( \Delta \nu_\text{mic} \)) were obtained by the PESTRE protocol,\(^{45}\) a pulsed-ENDOR multisequence comprising multiple Davies ENDOR sequences, carried out in three distinct experimental phases: (I) an EPR saturation phase \((r/f \text{ off})\) of 100 Davies sequences whose spin–echo intensities quickly converge to the steady-state \("baseline" \((\text{BSL});\)) (II) 24 sequences, each of which contains a fixed rf frequency; and (III) an EPR recovery phase \((r/f \text{ off})\) of 388 sequences during which the spin echo intensity of the spin system polarized by the ENDOR transitions induced in II, the dynamic reference level \((\text{DRL})\), relaxes to BSL. When \( A_{\text{mic}} > 0 \) \((\text{and} \Delta g_{\text{mic}} > 0)\), if \( \nu_e/\nu_\text{mic} \) is being interrogated, the DRL relaxes to the BSL from below/above. When \( A_{\text{mic}} < 0 \), the opposite behavior is observed.

**Density Functional Theory Calculations.** The structure of the \( E_\text{4} \) isomers investigated in the present study \((\text{Figure 3})\) was taken from previous BS-DFT calculations.\(^{14}\) All structures were obtained for an electron count for \( E_\text{4} \) in the \([\text{Mo}^{3+}\text{Fe}^{2+}\text{Fe}^{4+}]\) oxidation state.\(^{5,19}\) The adopted structural model of the active site comprises 198 atoms and includes FeMo-co with truncated modifications of the \( \alpha-27S^{\text{III}}, \alpha-442^{\text{III}} \) ligands \((\text{Azotobacter vinelandii numbering}), \text{R-homocitrate, and all of the MoFe protein residues and water molecules that engage hydrogen-bonding interactions with the FeMo-co or are known to be important in the catalytic activity \((\nu-70^\text{Val} \text{ and } \nu-96^\text{Glu})\).**

**RESULTS**

**ENDOR Spectroscopy of the Hydrides in \( E_\text{4}(4H) \).** The implementation of the stochastic ENDOR protocol, coupled with the improved spectrometer sensitivity, yields spectra of remarkable resolution with strong orientation selection, as illustrated in Figure 4. A full 2D field-frequency \(^1H\) stochastic CW ENDOR pattern has been collected across the EPR envelope for \( E_\text{4}(4H) \) in \( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \) buffers, and the extent of the improvement is seen by comparison with the 2D pattern reported previously \((\text{Figure S1})\). However, before presenting the new 2D pattern, we first navigate a "decision tree" that leads to the assignment of the features within the spectra to one of the two hydrides, \( H_1 \) and \( H_2 \). This in turn enables the analysis of the 2D pattern in terms of the magnitudes and relative signs of the two hyperfine coupling tensors. We then describe the determination of the absolute signs of these tensors and, central to this report, the relative orientations of the principal axes of the two tensors. Finally, we employ the analytical anchor model for the anisotropic hyperfine coupling tensor of a bridging hydride, which allows us to use the symmetries of the \([\text{Fe}^{-H-\text{Fe}}]\) hyperfine tensors and their relative orientations for \( H_1 \) and \( H_2 \) to distinguish among the proposed structures for the \( E_\text{4}(4H) \) intermediate \((\text{Figure 3})\).

**Alternative Models for \(^1H\) ENDOR Patterns.** As shown in Figure 5A, the single-crystal-like \(^1H\) ENDOR spectra of \( E_\text{4}(4H) \) collected at \( g_1 \) and \( g_3 \) show hyperfine-split doublets from two strongly coupled protons; the peaks disappear in samples prepared in \( \text{D}_2\text{O} \) \((\text{not shown})\), showing that these hydrides are exchangeable. The resolution of two signals immediately shows that the two protons are magnetically inequivalent, with different hyperfine-tensor orientations and/or principal values. However, as shown below, at intermediate fields the responses from the two protons spread and overlap strongly. As a first "branch-point" in a decision "tree" for determining the hyperfine tensors, one must correlate the signals denoted as \( H_1 \) and \( H_2 \) at \( g_1 \) with the two signals at \( g_5 \) \((\text{Figure 5A})\). In one alternative, \( A(H_1) > A(H_2) \) \((\text{both} g_1 \text{and} g_3 \text{signals}) \), whereas a bridging hydride is expected to give a hyperfine tensor whose anisotropic component is nearly rhombic. There are four alternative models for assigning the binding mode to the two distinct hydridic species bound to FeMo-co \((\text{Figure 5B})\): Both hydrides may be terminal \((\text{denoted} \ T/T)\) or bridging \((B/B)\); one hydride may be terminal and the other bridging, which itself comprises two cases differing in the assignment to \( H_1 \) and \( H_2 \) \((T/B \text{ or} B/T)\). In addition, for each of these four models, the hydride signals can evolve as the magnetic field is moved across the EPR envelope according to either a crossing or noncrossing scenario.

As described in the SI, a painstaking process of simulation/analysis has established that the hydride \(^1H\) spectra obey the crossing \(B/B\) model. Overlaid on the 2D pattern of ENDOR spectra in Figure 6 are sums of optimized simulations that employ the parameters for this model that are listed in Table 1. Figure S5 displays the 2D pattern and simulations of Figure 6 as well as the 2D pattern overlaid with the individual simulations for the two hydrides. These provide the basis for determining the listed signs of the hyperfine interactions, which are established in the next subsection.

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**Figure 4.** \(^1H\) stochastic-field modulation \((35 \text{ GHz})\) detected \((\text{stochastic CW})\) ENDOR spectra of \( E_\text{4}(4H) \) acquired at \( g_1 = 1.991 \) and 1.964 \((\text{black})\) and simulations generated as described below \((\text{red})\). The signal with \( \nu_e(1H) \approx 3 \text{ MHz} \), with both positive and negative features, represents transient responses from weakly coupled, more distant protons.\(^1\) Experimental conditions: microwave frequency, 35.045 GHz; modulation amplitude, 2.7 G; microwave power, 1 \( \mu \text{W}\); rf power, 10 W; temperature, 2 K; stochastic sequence \((\text{sample/delay/rf})\), 1/3/3 ms; the bandwidth of the rf excitation was broadened to 100 kHz.
The two hyperfine tensors have nearly equal isotropic components, and both anisotropic (traceless) tensor contributions not only are completely rhombic, namely, with one null component and the remaining two components having equal magnitude and opposite signs, but also have equal-magnitude nonzero components for the two hydrides. Of central importance to this study is the relative orientation of these two tensors, to which we return below. As can be seen in Figure 6, the simulations generated with the hyperfine tensors listed in Table 1 provide a remarkably precise correspondence with the extremely high resolution experimental stochastic CW spectra across the 2D pattern plus the scaled 2H spectra. For completeness and for use below, Figure S5 shows both the 2D pattern with summed simulations and the same pattern showing the individual simulations for H1 and H2.

**Absolute Signs of Hydride Hyperfine Couplings.** Earlier, it was impossible to obtain absolute signs of the H1 and H2 hyperfine couplings, and thus of \( a_{\text{anis}} \) or the relative orientations of the positive/negative components of the two

**Table 1. Experimentally Determined [Fe-\(^1^H-Fe\)] \(^1^H\) Hyperfine Coupling Parameters for E\(_4\)(4H)**

<table>
<thead>
<tr>
<th></th>
<th>H1</th>
<th>H2</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A )</td>
<td>+[11.6, 24.8, 38.0]</td>
<td>+[35.6, 9.2, 22.4]</td>
</tr>
<tr>
<td>( T )</td>
<td>[+13.2, 0.0, +13.2]</td>
<td>[+13.2, −13.2, 0.0]</td>
</tr>
<tr>
<td>( a_{\text{anis}} )</td>
<td>+24.8</td>
<td>+22.4</td>
</tr>
<tr>
<td>([\alpha, \beta, \gamma])</td>
<td>0, 20, 20</td>
<td>10, 30, 0</td>
</tr>
</tbody>
</table>

\(^{\text{a}}\)Euler angles \([\alpha, \beta, \gamma]\) defined according to the zyx convention describe the rotation of the A eigenframe away from the g reference frame; with an uncertainty of \(\lesssim 2^\circ -3^\circ\).
Because the magnitude of $\nu$ through the application of a Davies ENDOR multisequence at a nucleus in question (Figures 6 and S5), but these fields also have the lowest ENDOR signal intensity, and as a result, the PESTRE response at these fields is not readily obtained. However, the excellent resolution and precise simulation of the new $^1$H ENDOR data allow us to identify ENDOR features associated with an individual hydride even in spectra at intermediate $g$ values, where the overall patterns of the two hydrides overlap extensively (Figures 6 and S5 and S10). Thus, at $g = 1.977$ $^1$H exhibits an isolated sharp peak with $\nu_\gamma = 65.5$ MHz whose $^1$H Davies ENDOR response (Figure 7A) is strong enough to obtain a $^1$H PESTRE trace. In the corresponding $^1$H PESTRE trace, shown in Figure 7B, the $\nu_\gamma$ intensity in the PESTRE multisequence relaxes to the BSL level from below, which for $^1$H ($g_\parallel > 0$) implies $A > 0$. Because the magnitude of $A$ is governed by $a_{\perp\perp}$, this means that $a_{\perp\perp} > 0$ for $^1$H1.

In the $^1$H ENDOR spectrum at $g_\parallel = 2.008$, (A) $35$ GHz $^1$H stochastic CW (thicker black line) and Davies (thinner black line) ENDOR responses around the $\nu_\gamma$ (black arrow) and $\nu_\delta$ (purple arrow) branches of the $^1$H2 doublet and sum (red) and separate $^1$H1 (blue) and $^1$H2 (green) simulations. Simulation parameters (B/B model) are listed in Table 1. (B) $^1$H PESTRE traces collected from the $\nu_\gamma$ (black) and $\nu_\delta$ (purple) branches of the $^1$H2 doublet. The following experimental conditions were used. (A) $^1$H stochastic CW ENDOR: the same as in Figure 4. $^1$H Davies ENDOR: microwave frequency, 34.757 GHz; $\pi = 80$ ns; $\tau = 600$ ns; $t_{\pi} = 35$ $\mu$s; repetition time, 100 ms; $t_{\text{rep}} = 5$ $\mu$s; temperature, 2 K. (B) The same as in A ($^1$H Davies) except for the 150 ms repetition time and the 41.1 ($^1$H) MHz rf values.

was confirmed by an additional $^1$H PESTRE measurement of the $\nu_\gamma = 70.8$ MHz signal from $^1$H2 at $g = 1.970$ (Figure S11).

With the hyperfine signs as determined from the $^1$H PESTRE measurements, the present ENDOR measurements and analysis provide signed hydride hyperfine tensors with extraordinary precision. The result is hyperfine tensors for $^1$H1 and $^1$H2 whose anisotropic components $T$ not only are fully rhombic, namely, with each exhibiting a null component $[\tau, 0, -\tau]$ but also exhibiting identical, although permuted, principal values within the precision of these measurements (Table 1). We emphasize that the tabulated couplings naturally arise from the fitting rather than being imposed as a constraint on the fitting. The hydrides are not, however, chemically identical; the isotropic components, although similar, are not identical: $a_{\parallel\parallel} \approx +22$ and $+25$ MHz for $^1$H1 and $^1$H2, respectively. The availability of both magnitudes and signs of the hydride couplings provides a strong constraint on computations of the geometric and electronic structure of the hydride-bound FeMo-co of E4(4H), as will be seen below.

Figure 7. Absolute hyperfine sign determination for hydride H1 by PESTRE at $g_\parallel = 1.977$. (A) $35$ GHz $^1$H stochastic CW (thicker black line) and Davies (thinner black line) ENDOR responses around the $\nu_\gamma$ branch of the $^1$H1 doublet, and sum (red) and separate $^1$H1 (blue) and $^1$H2 (green) simulations. Simulation parameters (B/B model) are listed in Table 1. (B) $^1$H PESTRE trace collected from the $\nu_\gamma$ branch of the $^1$H1 doublet. The following experimental conditions were used. (A) $^1$H stochastic CW ENDOR: the same as in Figure 4. $^1$H Davies ENDOR: microwave frequency, 34.757 GHz; $\pi = 80$ ns; $\tau = 600$ ns; $t_{\pi} = 35$ $\mu$s; repetition time, 100 ms; $t_{\text{rep}} = 5$ $\mu$s; temperature, 2 K. (B) The same as in A ($^1$H Davies) except for the repetition time of 200 ms and the rf frequency of 65.5 MHz.

Relative Orientations of the H1/H2 Hyperfine Tensors. The small values for the Euler angles relating the hyperfine tensor of each hydride to the $g$-tensor axis frame (Table 1) imply that the hyperfine (A), and thus the anisotropic dipole-coupling (T), tensors of both hydrides are essentially coaxial with $g$ and thus are themselves coaxial; indeed, Table 2 (below) explicitly shows that each of the three corresponding pairs of principal axes for the hyperfine couplings of the two hydrides subdents an angle of only a few degrees. However, although the anisotropic tensors for the two hydrides have components with identical values, as noted above, these are permuted with respect to each other.

Figure 8. Absolute hyperfine sign determination for hydride H2 by PESTRE at $g_\parallel = 2.008$. (A) $35$ GHz $^1$H stochastic CW (thicker black line) and Davies (thinner black line) ENDOR responses around the $\nu_\gamma$ (black arrow) and $\nu_\delta$ (purple arrow) branches of the $^1$H2 doublet and sum (red) and separate $^1$H1 (blue) and $^1$H2 (green) simulations. Simulation parameters (B/B model) are listed in Table 1. (B) $^1$H PESTRE traces collected from the $\nu_\gamma$ (black) and $\nu_\delta$ (purple) branches of the $^1$H2 doublet. The following experimental conditions were used. (A) $^1$H stochastic CW ENDOR: the same as in Figure 4. $^1$H Davies ENDOR: microwave frequency, 34.757 GHz; $\pi = 80$ ns; $\tau = 600$ ns; $t_{\pi} = 35$ $\mu$s; repetition time, 50 ms; $t_{\text{rep}} = 5$ $\mu$s; and temperature, 2 K. (B) The same as in A ($^1$H Davies) except for the 150 ms repetition time and the 41.1 ($^1$H) MHz rf values.

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orientations of the components of the coaxial tensors provide through an analysis presented below, the permuted relative primarily because of this permutation. Of central importance, 

![Image](https://example.com/image.png)

**Table 2. Angles between Corresponding H1/H2 Components of the H1, H2 Rhombic Dipolar Tensors**

| Experiment | \{-(H1)\}||+(H2)\} | \{0(H1)\}||-(H2)\} | \{+(H1)\}/0(H2)\} | \{0(H1)\}||0(H2)\} |
|------------|-----------------|-----------------|-----------------|-----------------|
| BP86\footnote{BP86} | 12              | 11              | 11              | 84              |
| B3LYP      | 16              | 11              | 16              | 81              |
| BP86\footnote{BP86} | 47              | 45\footnote{BP86} | 78\footnote{BP86} | 100\footnote{BP86} |
| B3LYP      | 46              | 46\footnote{BP86} | 78\footnote{BP86} | 100\footnote{BP86} |

**Chart 1. 1H Tensor Permutations**

![Chart 1](https://example.com/chart.png)

According to the kinetic model for nitrogenase catalysis,\footnote{The calculated angles are the complement; an angle and its complement are experimentally indistinguishable.} \footnote{These angles are fixed by the \(\mp\) signs to equal the dihedral angle between [Fe–H–Fe] planes.} (i) They exhibit large isotropic couplings that for the high-spin ions of FeMo-co would primarily arise from spin delocalization into the M–H bond(s), exactly as seen here. (ii) The anisotropic hyperfine coupling tensor of a terminal hydride exhibits a "dipolar" form, \(T \approx -t_1 - t_2 r_2\), where the unique direction corresponds to the M–H vector. (iii) The anisotropic coupling to a hydride interacting with two metal ions depends jointly on the distances to the two anchor M ions and on their spin. When the spin on the second metal is small and/or its distance to the hydride is much larger than to the first, the anisotropic coupling can approach the dipolar form of a terminal M–H. However, in sharp contrast, a rhombic coupling, \(T \approx [t_1, 0, t_1]\) is a definitive signature of a hydride bridge that has significant interactions with both anchor metal ions.

**Point-Dipole Approaches.** Our studies have further shown that we can accurately calculate the dipolar tensor of a hydride, \(T\), bound to ions of a multinuclear center by summing the through-space point-dipole interactions with each paramagnetic center \(M\) (Fe, Mo),

\[
T = \sum T_i = i M_i, t_i = (K_d) \left[ \frac{2g_i \beta_i \beta_i}{r_i^3} \right]
\]

where the \(3 \times 3\) matrices \(M_i\) incorporate the orientation of the \(M_i\)--H vector relative to the FeMo-co molecular frame. The weight, \(t_i\), of an individual \([M_i, -H]\) point dipole is determined by three factors: (1) a dipolar term, which depends on fundamental constants and the inverse cube of the \(M_i\)--H distance, \(r_i\); (2) the covalency parameter \(d_i\) (\(0 < d_i \leq 1\)) (i.e., the fraction of the unpaired spin density, \(\rho_u\), of an ion of spin \(S_i\) that remains on the ion after it undergoes covalent interactions with its ligands), as obtained from a BS-DFT computation, \(d_i = |\rho_i|/2S_i\); and (3) the vector-coupling coefficient, \(K_i\) for ion \(i\) obtained from the spin-coupling model for FeMo-co in which its total spin is determined by exchange coupling between metal ions. The point-dipole approach is particularly appropriate for nitrogenase and other enzymes that involve high-spin Fe ions because, to a first approximation, they should be spherical “balls of spin” — exactly so for Fe\(^{11+}\) \((S = 5/2)\) and very nearly so even for Fe\(^{11+}\) \((S = 2)\) — and thus behave like point dipoles when interacting with distant nuclei.

For FeMo-co, one can numerically diagonalize the matrix \(T\) to obtain predicted principal values for the observed cluster anisotropic coupling tensor,

\[
T = [T_1, T_2, T_3]
\]
along with the orientation of that tensor relative to the molecular frame. Instead, in the anchor approach, this information is obtained from the dominant contributions to eqs 2 and 3 (i.e., the interactions of an [Fe−H−Fe] hydride with its two anchoring FeMo-co Fe ions). The anchor model is reliable for a multimetallic cluster such as FeMo-co because it exhibits an r⁻³ dependence on the distance r between the metal ion and hydride: the longer distances between a hydride and the nonanchor metal ions minimize the interactions with these other ions and, consequently, their contribution to the full sum over ions (eqs 2). This expectation was supported by a comparison of the calculations with the anchor-model equations of eqs 4 to an implementation of eq 3 for test-case, spin-projected (S = 1/2) DFT-derived states for E₁(4H)⁹.

**[Fe−H−Fe] Bridge.** A signal benefit of the anchor approach to an [Fe−H−Fe] moiety is that the sum of dipolar interactions between two Fe atoms and a bridging hydride at an arbitrary relative position, Chart 2, yields analytic equations for the components of the tensor, T, and the tensor orientation, eqs 4,²⁰,²¹,⁴⁸

\[
T = t_1M_1 + t_2M_2 = \left( \frac{t_1}{t_1} \right) M_2 
\]

\[
T_1 = \frac{1}{2} \left( t_1 \left( \frac{t_1}{2} \right) + \frac{1 + \left( \frac{t_1}{2} \right)}{2} \right) + \frac{3}{2} \cos 2\beta_1 \left( \frac{t_1}{t_1} \cos 2\beta_1 \right) \right) 
\]

\[
T_2 = -\frac{1}{2} \left( t_1 \left( 1 + \left( \frac{t_1}{t_1} \right) \right) \right) \]  

\[
T_3 = -(T_3 + T_2) 
\]

\[
\tan 2\lambda = \frac{\sin 2\beta_1 \left( \frac{t_1}{t_1} \sin 2\beta_1 \right)}{\cos 2\beta_1 + \left( \frac{t_1}{t_1} \right) \cos 2\beta_1} 
\]

Components T₁ and T₃ lie in the [Fe−H−Fe] plane, rotated away from the Fe−Fe axis by an angle γ about the normal to that plane (Tᵢ). (T₃ lies along that axis for γ = 0.) These equations reveal that both the relative magnitudes of the components of the anisotropic coupling tensor and the tensor orientation (eq 4e) depend on the geometry of the center (expressed here in terms of the two internal angles β₁ and β₂) and a single spin/structure-dependent parameter—the ratio of tᵢ for the anchor metal ions, tᵢ/tᵢ—while the absolute signs and values of the components scale with the tᵢ parameter.

It is straightforward to solve the analytical equations for the anchor model for conditions under which the dipolar tensor, T, becomes fully rhombic, namely, has one null component. As illustrated in Figure 9, which plots the “reduced” tensor components given by eqs 4,

\[
a_i = T_i/t_i 
\]

for a typical DFT-determined bridging hydride geometry, there are two distinct scenarios in which the signature rhombic symmetry of the hyperfine tensors of the two (B/B) E₁(4H) hydrides (Table 1) can be precisely matched by the tensor arising from the interactions of a hydride bridge with the spins of its two anchor Fe ions.

(i) When t₁ and t₂ have opposite signs and T is rhombic because t₁ = −t₂ (eq 4c and Figure 9), the null tensor element is T₂, oriented along the normal to the [Fe−H−Fe] plane (eq 4c, Chart 3). This imposes an exact constraint on the ratios of the Kᵣdᵣ products in terms of the ratio of the Fe−H distances, rᵣ:

\[
T_2 = 0 \Rightarrow \left( \frac{t_1}{t_1} \right) = 1, \frac{|K_d|}{|K_d|} = \left( \frac{r_2}{r_1} \right)^3 
\]

The two nonzero components T₃ and T₁ have opposite signs and equal magnitudes and are rotated around the normal/T₂ by the angle γ (eq 4d)

(ii) If t₁ and t₂ have the same sign, t₁/t₁ > 0, then solving eqs 4 for a null tensor element is often possible (but not always; see Figure 9). For all plausible FeMo-co [Fe−H−Fe] geometries and ratios t₁/t₁, there are at most two values of t₂/t₁ that yield a null T₁ component (e.g., Figure 9), which lies in the Fe−Fe plane (Chart 4). The two nonzero components T₂ and T₃ have opposite signs and equal magnitudes (eq 4d), T₂ = −T₃, determined by specifying

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**Figure 9.** Reduced dipolar tensor components, aᵢ = Tᵢ/tᵢ, calculated as a function of t₂/t₁ for a hydride bridge with R = 2.52 Å, r₁ = 1.65, and r₂ = 1.69. The two aᵢ nulls give similar angles, γ.

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**Chart 2**

**Chart 3**
either of the two \( t_i \) values. Finally, \( T_1 \) are \( T_3 \) are rotated by an angle \( \gamma \) (eq 4e). Such a situation has been seen for delocalized mixed-valence Fe\(_2\) complexes.\(^{23,25}\)

**\([\text{Fe}_2\text{H}_4]\) Triangle.** The P-7 structure recently proposed by Siegbahn\(^{25}\) has two hydrides, each lying roughly in the plane of three Fe ions (Figure 3, Chart 5). Although we show below that the question of whether this model can describe E\(_4\)(4H) is ultimately decided by other arguments, for completeness we note that the anchor model can even provide guidance in considering the hyperfine couplings of such a moiety. Thus, if \( t_3 = -(t_1 + t_2) \), then the dipolar tensor is rhombic with \( T_2 = 0 \), and if \( t_3, t_2 > 0 \) (as shown), then \( T_3 = -T_3 > 0 \) and \( 0 \leq \gamma \leq 15^\circ \). However, if \( (t_1, t_2) \) are of opposite sign, then \( \gamma \) approaches \( \pm 45^\circ \) depending on which one is greater in magnitude.

**Testing the Janus Structural Models.** The central goal of this study is the discrimination among computationally proposed structures of the E\(_4\)(4H) (\( S = 1/2 \)) Janus intermediate (Figure 3). We do so by integrating BS-DFT-derived structures with the key characteristics of the E\(_4\)(4H) intermediate obtained from high-resolution ENDOR experiments. These key characteristics shown in this report are the following: (i) There are two metal hydrides bound to the FeMo-co cluster, and they exhibit nearly identical hyperfine tensor components. (ii) The tensors are very closely coaxial (and coaxial with g). (iii) The two hyperfine tensors are dominated by large, nearly equal isotropic couplings. (iv) The anisotropic hyperfine interaction tensors, \( T \), for the two hydrides are perfectly rhombic within experimental error (B/B 2D ENDOR pattern). (v) Although the components of the two anisotropic tensors have the same magnitudes, the signed tensor components of one hydride are “permuted” with respect to each other, with the correspondence between the components of the two hydride tensors summarized in Table 2 and Chart 1.

The analysis presented below focuses primarily on the relative orientations of the signed hyperfine components of the rhombic H1 and H2 hyperfine tensors. With this approach, we need not employ fine details of the BS-DFT spin densities to calculate tensor components, a task that approaches, and indeed likely passes, their limits of reliability. Instead, to determine whether a BS-DFT state with a given arrangement of two hydrides (Figure 3) is capable of reproducing the permuted rhombic tensors of experiment, one need only incorporate into the anchor equations the \([\text{Fe}−\text{H}−\text{Fe}]\) geometries for each hydride, which are robustly determined, solve for the \( t_i \) that yield a rhombic dipolar tensor for each, and then compare the calculated relative orientations with experiment (Figure 9 and Table 2). Calculations with this protocol first show that it is robust against quite substantial variations in the structure of the Fe−H−Fe fragment. Second, the protocol eliminates any residual uncertainties caused by the limitations of the BS-DFT energetics and the secondary contributions from spins on the other Fe ions: the experimental tensors indeed are rhombic, and this procedure reproduces their symmetry by construction. Finally, as noted above, the anchor equations are supported by comparisons with anisotropic DFT tensors obtained by summing the dipolar interactions with all of the metal ions. The relevant metric and electronic structure parameters of the FeMo-co E\(_4\) states visualized in Figure 3 and used in the protocol are listed in Table S1, which includes structural data obtained from DFT calculations using both BP86 and B3LYP exchange and correlation functionals;\(^{14}\) the comparisons with experiment are carried out for structures generated with both functionals.

**E\(_4\)(4H)\(^{[a]}\) with Parallel Hydride Bridges.** For both BP86 and B3LYP functionals, the lowest-lying structure is E\(_4\)(4H)\(^{[a]}\), which features two bridging hydrides, \([\text{Fe}−\text{H}−\text{H}−\text{Fe}]\) and \([\text{Fe}−\text{H}−\text{H}−\text{Fe}]\) (Figure 3). In E\(_4\)(4H)\(^{[a]}\), the planes of the \([\text{Fe}−\text{H}−\text{Fe}]\) and \([\text{Fe}−\text{H}−\text{Fe}]\) hydride bridges are essentially orthogonal to the Fe2,3,6,7 face and nearly parallel to each other, with the angle between their normals (dihedral angle between \([\text{Fe}−\text{H}−\text{Fe}]\) planes) being only \( \tau \approx 10^\circ \) (Table S1). Both functionals predict a cluster of BS states with essentially the same geometry and only slightly different energy (within 20 kJ/mol from the lowest-energy solution) yet differing in the orientations of the anchor Fe spins.\(^{14}\)

For each functional, there are states within this cluster of BS solutions that cannot satisfy the experimental restrictions on the dipolar interaction tensors of the two (Chart 1; see also Tables 1 and 2). For example, we find anchor spin patterns \((t_3; t_6)(t_3; t_5) = (-/-)(-/-), \) where each hydride bridges Fe atoms with parallel spins (as in Chart 4), and \((\mp)(\mp), \) where each hydride bridges Fe atoms with antiparallel spins (as in Chart 3). These BS states yield two nearly identical hydride hyperfine tensors, with positive \( \delta_{\text{iso}} \) and coaxial rhombic dipolar tensors, but the corresponding tensor components are nearly parallel rather than permuted (Chart 1). However, the cluster of low-lying BS solutions also includes states where the \([\text{Fe}−\text{H}−\text{H}−\text{Fe}]\) hydride has antiparallel anchor spins, implying opposite signs of their dipolar coupling parameters, \((t_3; t_6) \leftrightarrow (-/+), \) while the \([\text{Fe}−\text{H}−\text{Fe}]\) hydride has parallel anchor spins, with \((t_3; t_5) \leftrightarrow (+/+)\), as schematized in Chart 6. When this spin arrangement exhibits two hydrides with tensors of rhombic symmetry, the \([\text{Fe}−\text{H}−\text{H}−\text{Fe}]\) hydride has its null tensor component normal to its \([\text{Fe}−\text{H}−\text{Fe}]\) plane \((T_2 = 0)\) with \( T_3 \leq 0; \) the \([\text{Fe}−\text{H}−\text{Fe}]\) (Figure 3) hydride has \( T_1 = 0 \) and \( T_3 > 0 \) (both in-plane). The tensors are rotated about the normal to their \([\text{Fe}−\text{H}−\text{Fe}]\) normal \((T_1)\) direction by an angle, \( \gamma \), that ranges from \( 31 \) to \( 54^\circ \) for the two functionals (Table 2).

Thus, by examining the patterns of signs of the interaction parameters \( t_i \) in combination with DFT-computed E\(_4\)(4H)\(^{[4]}\) geometries as inputs into the anchor equations, we can predict the relative orientations of rhombic \([\text{Fe}−\text{H}−\text{Fe}]\)/\([\text{Fe}−\text{H}−\text{H}−\text{Fe}]\)
Fe7] hydride tensors that are possible for a structure, thereby determining whether a model (Figure 3) exhibits tensors that match the experimentally determined angles between the corresponding tensor components of H1 and H2. As can be seen from the results reported in Table 2, tensors for E4(4H)(a) as computed using the structure from the DFT functional and the anchor signs (−/+−) for [Fe2−H1−Fe6] and (+−/) for [Fe3−H2−Fe7] accurately reproduce the experimentally observed coaxial, rhombic dipolar coupling tensors with nearly parallel permuted components, and near-orthogonal nulls, as drawn in Chart 6.

E4(4H) Dihydride Dibridge. E4(4H) (Figure 3) features a dihydride dibridge with both hydrides anchored by Fe2 and Fe6. Again, both functionals give a cluster of BS solutions with essentially the same geometric structure differing only slightly in energy,14 with members of the cluster exhibiting both parallel [e.g., (+/+)/] and antiparallel [e.g., (∓/−−)] Fe2/Fe6 anchor spins determining the signs of the ti. In the parallel case, a rhombic tensor for each hydride must have the null component lying in its [Fe−H−Fe] plane, while in the antiparallel case, the null is normal to its plane. With both BP86 and B3LYP functionals, the anchor-model dipolar tensor for each hydride is rotated around the normal to its [Fe−H−Fe] plane by an angle of γ ~ 45°; in the computational models, the two planes are rotated relative to each other about the Fe−Fe vector with large dihedral angles (BP86, 77.7°; B3LYP, 99.7°). As above for the E4(4H)(a) structure, we have calculated the angles between the near-coaxial permuted component of the calculated rhombic hydride dipolar tensors for both parallel (+/+) and antiparallel (∓) spin orientations (signs of ti). As can be seen from the results reported in Table 2, the calculated angles between corresponding components of the permuted tensors for the E4(4H)(a) structure, whether considering states with (+/+) or (∓) anchor Fe, strongly disagree with the experimentally determined angles, and in addition, the null components are not orthogonal for the (+/+) configuration. This violation of the experimental constraints on the relative tensor orientations provides strong evidence against such a structure for E4(4H).46

E4(3H;CH). The E4(3H;CH) structure is no less readily dismissed by comparison to the present experiments. This structure contains only one hydride, in a nearly symmetrical bridging [Fe2−H−Fe6] arrangement (average Fe−H distance, 1.65 Å). The central carbide of the resting FeMo-co is protonated, with the resulting C−H fragment nearly symmetrically coordinated “above” a square plane of the four Fe atoms, Fe2, 6, 4, 5, on the surface of the “exploded” FeMo-co cluster [Fe4(CH)], with the [Fe2−H−Fe6] hydride roughly lying in this plane.

To begin, the DFT computations show that the C−H fragment has essentially zero spin density. Thus, this 1H atom would not have the large isotropic coupling associated with H1 and H2 seen experimentally (Table 1). The C−H hydrogen will undergo through-space anisotropic dipolar couplings to the four pseudoanchor Fe ions that bind the carbon. However, a consideration of a pseudoanchor model that incorporates only couplings to the Fe of the Fe4 plane (or of the more complete of eq 3) indicates that it would be impossible for the diagonalized sum of these couplings to yield a rhombic tensor whose components are equal to those of the hydride bridge, but permuted. In fact, the dipolar coupling components for the C−H1 hydrogen will be much less than to the hydride bridge. The [Fe2−H−Fe6] bridge is nearly symmetric, with an average Fe−1H distance of 1.65 Å, whereas the average distance between the 1H bonded to the C and Fe ions of the Fe4 anchor base is 2.64 Å. Thus, for sets of Fe d, spin coefficients (eq 2a) for the two hydrides with comparable values needed to achieve rhombic dipolar tensors, the difference in distances would make the magnitudes of the Fe−H dipolar coupling ti parameters for the pseudoanchors of the C−H much smaller than those for the anchors of the bridging hydride, li(Fe4)/li(bridge) ≈ 1/4, and the resulting nonzero dipolar tensor components for C−H would be correspondingly smaller. Indeed, with both a small aii and a small dipolar coupling for such a C−H moiety, if one were present then it would be expected to give ENDOR signals that fall within the signals from the −5H protons rather than correspond to one of the H1 or H2 Fe-bound hydrides.

Thus, we conclude that 1H of a E4(3H;CH) C−H fragment cannot give a hyperfine tensor—neither isotropic nor dipolar contributions—that matches that of the hydride bridge as seen in experiment and that E4(3H;CH) and similar models with a singly protonated carbide do not describe the Janus E4(4H) intermediate.

P-7. This model can be dismissed on the basis of only the properties of E4(4H) and its reactivities. (i) E4(4H) has accumulated 4[e−/H+] as cleanly shown by its return to E0 by two sequential steps of hydride protonation with the overall loss of two H2 molecules. Instead, P-7 has accumulated 7[e−/H+] and cannot return to E0 by the loss of two H2 molecules. (ii) E4(4H) is an odd-electron system with S = 1/2 whose EPR spectrum is well established13 and whose ENDOR properties we report here in detail; P-7 has an even number of electrons and thus an integer spin (proposed as S = 0), which is incompatible with the experimental evidence. (iii) E4(4H) undergoes an experimentally observed, kinetically facile, near-thermoneutral conversion to the EPR-active (S = 1/2) E4(2N2H) through re loss of H2 and the binding/reaction of N2; re by P-7 would create an even-electron state (also proposed as S = 0), which is again incompatible with experiment.
The relaxation of \( E_7(4H) \) to \( E_0 \) needs further elaboration. The proposal that the P-7 state, with seven accumulated \([e^-]/[H^+]\), is the Janus intermediate (the state that binds \( N_2 \) upon \( re \) of \( H_2 \)) instead of \( E_5(4H) \), with four accumulated \([e^-]/[H^+]\), is embedded in a mechanism that involves the activation of the true \( E_0 \) through the accumulation of \( 5[e^-]/[H^+] \) to generate a state, which we will denote as P-5 (referred to as \( A_5^0 \) or \( E_5^0 \) in ref 29), after which the enzyme cycles through an 8\([e^-]/[H^+]\) catalytic process that begins and ends with P-5 and produces \( 2NH_3 \). However, no such preactivation process has been observed in the many pre-steady-state measurements carried out over the years.\(^2,4\) Moreover, a two-step relaxation of the even-electron P-7 with the release of two \( H_2 \) molecules would produce the even-electron P-3, whereas the measurements of the relaxation of the odd-electron \( S = 1/2 \) \( E_5(4H) \) with the release of \( 2H_2 \) unambiguously show that it relaxes directly to the EPR-active odd-electron \( E_0 \) \((S = 3/2)\) resting state. The structure of this state has been beautifully revealed as having a trigonal-prismatic Fe\(_6\)C core with intercalated carbide,\(^30\) not the EPR-silent (or integer-spin) P-5 state with a peripheral iron-bound methyl group as would be required by such a mechanism. And there is no doubt that \( E_5(4H) \) indeed relaxes to the \( E_0 \) state. That the crystalline \( E_0 \) and the putative EPR-silent \( E_0 \) formed by \( E_5(4H) \) relaxation are identical is established by the identity of the EPR spectra of the solution \( E_0 \) formed by relaxation and that of the crystalline \( E_0 \), both of which precisely match the long-established EPR spectrum of as-isolated Mo-nitrogenase.

## SUMMARY AND CONCLUSIONS

Exceptionally high resolution 2D field-frequency 35 GHz \(^1\)H ENDOR patterns confirm and highly refine our previous reports that the \( E_5(4H) \) nitrogense Janus intermediate contains two \([Fe−H−Fe]\) bridging hydrides and in addition characterizes the protons bound to sulfides that accompany the bridging hydrides. A detailed analysis of 2D field-frequency ENDOR patterns shows that the hydrides exhibit nearly identical hyperfine tensor components (Table 1). The tensors are dominated by large, nearly equal isotropic couplings, and the signs of the isotropic couplings have been established for the first time. The components of the anisotropic hyperfine interaction tensors determined here for the two \( E_5(4H) \) hydrides are identical and perfectly rhombic within experimental error (B/B 2D ENDOR pattern). Such rhombic dipolar interactions are a signature of bridging hydrides, as established in studies of enzyme active sites\(^6\) and model studies.\(^23−25\) \(^{95}\)Mo ENDOR studies showed that the \(^{95}\)Mo hyperfine coupling for this intermediate is about 5-fold too small for Mo to be an anchor metal ion of either hydride, leaving two Fe atoms as the only possible anchors for each of two \([Fe−H−Fe]\) hydride bridges.

The anisotropic tensors of the two hydrides are nearly coaxial and have identical components, but the tensor components of one hydride are permuted with respect to those of the other, a property captured by noting the near-orthogonality of the equivalent tensor components for the two hydrides and corresponding, nearly exact coaxiality of the permuted components (Table 2). The determination of the absolute signs of the tensor components for each of the two hydrides, as presented in the table, is of particular importance in relating these tensors to structure.

These experimental findings were complemented by a careful theoretical analysis of structural models of the \( E_5(4H) \) state. The broken-symmetry DFT calculations of \( E_5(4H) \) identified \( E_5(4H)\(^{(a)}\) \), where two hydrides bridge the Fe2/Fe6 and Fe3/Fe7 pairs of atoms, as the lowest-energy isomer. However, variations between different flavors of DFT exchange and correlation functionals, while having a small impact on geometries, can appreciably affect the energy ordering.\(^14,16,17\) These considerations, along with the inability of DFT to accurately describe multireference electronic systems, such as FeMo-co, make it difficult to determine the nature of \( E_5(4H) \) based on DFT-derived relative energies alone. To overcome these limitations, the present spectroscopic properties have been combined with DFT calculations to test five possible structures for the Janus intermediate (Figure 3: \( E_5(4H)\(^{(a)}\), \( E_5(4H)\(^{(b)}\), \( E_5(4H)\(^{(g)}\), \( E_5(3H;CH) \), and P-7), primarily through use of a model hyperfine Hamiltonian based on a point dipolar interaction with the electron spins of the anchoring Fe ions. The spectroscopic signatures for each of these structures have been assessed and compared with experiment.

Of the five structures in Figure 3, three can be dismissed as qualitatively in disagreement with the ENDOR results: P-7, \( E_5(3H;CH) \), and \( E_5(4H)\(^{(b)}\). Indeed, P-7 has the wrong electron count \((7 \not= 4[e^-]/[H^+])\) and is not even an odd-electron, EPR-active state. Neither of the other two contains two bridging hydrides, much less two that can exhibit the signature rhombic, coaxial, and permuted anisotropic hyperfine coupling tensors, while the \(^1\)H of the protonated carbide of \( E_5(3H;CH) \) and other such models would fail to show the large measured isotropic coupling. A third structure, \( E_5(4H)\(^{(a)}\), does exhibit two hydrides, both of which bridge the same pair of Fe ions (Fe2 and Fe6), but the resulting relative orientations of the two hydride dipolar tensors are in strong quantitative disagreement with those found experimentally.

Only the \( E_5(4H)\(^{(a)}\) structure can accurately account for the observed presence of two bridging hydrides with rhombic anisotropic tensors that are coaxial but with permuted tensor components (Table 2). Taken together, these results allow us to assign \( E_6(4H)\(^{(a)}\) \), with \([Fe2−H−Fe6]\) and \([Fe3−H−Fe7]\) hydride bridges whose planes are essentially perpendicular to the Fe2,3,6,7 FeMo-co face and parallel to each other (Figure 3), as the lowest-energy FeMo-co state of the Janus intermediate, trapped during the relatively slow hand-freezing process used to generate the samples for ENDOR measurements. Although these measurements were performed at 2 K, EPR spectra indicate that no other isomer of Janus becomes populated by \( \sim 25 \text{ K}\)\(^{13,14}\) (above which the signal becomes relaxation-broadened). However, because calculations have indicated the presence of isomers, such as \( E_5(4H)\(^{(b/c)}\) (Figure 3), very close in energy, we can envision that at ambient temperature the \( E_5(4H) \) hydrides may become fluxional, with a dynamic population of multiple isomers.

### ASSOCIATED CONTENT

Supporting Information

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ENDOR spectra, simulations, and related parameters (PDF)

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Identification of the N2 Binding State.
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(48) Equation 4a was suggested for clarity by a knowledgeable reviewer. Equations 4b–4d are from ref 20, with $\beta_2$ here being the complement of $\beta_2$ defined there, noting, however, that for a given geometry one can express tensor components in terms of any two geometric parameters and the law of cosines, as expressly noted in ref 21.

(49) For completeness, we note that no state in which the two hydrides share a common vertex (cv), such as one bridging Fe2 and Fe6 and the second bridging Fe2 and Fe3, has been found in BS-DFT computations even at extremely high energies, so such structures are not considered.