The Mössbauer Parameters of the Proximal Cluster of Membrane-Bound Hydrogenase Revisited: A Density Functional Theory Study

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Supporting Information

ABSTRACT: An unprecedented [4Fe-3S] cluster proximal to the regular [NiFe] active site has recently been found to be responsible for the ability of membrane-bound hydrogenases (MBHs) to oxidize dihydrogen in the presence of ambient levels of O2. Starting from proximal cluster models of a recent DFT study on the redox-dependent structural transformation of the [4Fe-3S] cluster, 57Fe Mössbauer parameters (electric field gradients, isomer shifts, and nuclear hyperfine couplings) were calculated using DFT. Our results revise the previously reported correspondence of Mössbauer signals and iron centers in the [4Fe-3S]5+ reduced-state proximal cluster. Similar conflicting assignments are also resolved for the [4Fe-3S]5+ superoxidized state with particular regard to spin-coupling in the broken-symmetry DFT calculations. Calculated 57Fe hyperfine coupling (HFC) tensors expose discrepancies in the experimental set of HFC tensors and substantiate the need for additional experimental work on the magnetic properties of the MBH proximal cluster in its reduced and superoxidized redox states.

1. INTRODUCTION

Membrane-bound [NiFe]-hydrogenases (MBHs) from organisms like Aquifex aeolicus (Aa), Escherichia coli (Ec), Hydrogenovibrio marinus (Hm), or Ralstonia eutropha (Re) are capable of catalyzing the oxidation of H2 at ambient levels of O2, which acts as the terminal electron acceptor.1–4 The oxygen tolerance of these enzymes has been traced to the replacement of a conventional [4Fe-4S] cubane in the O2-inactivated [NiFe]-hydrogenases by an extraordinary [4Fe-3S] cluster at a distance of ∼11 Å to the active site.5–7 Whereas all four iron centers in this [4Fe-3S] “proximal” cluster are still each coordinated by a cysteinate side chain (as in the cubane-type [4Fe-4S] present in O2-sensitive [NiFe] hydrogenases), one inorganic μ3-sulfide is replaced by two “supernumerary” cysteines: Cys19 bridges Fe1 and Fe4 (numbering for Re-MBH and Ec-MBH unless otherwise stated) and Cys120 binds terminally to Fe3 (Figure 1). In the catalytic cycle, the proximal cluster switches between its reduced (RED) and its oxidized state (OX). The RED/OX states, [4Fe-3S]5+/[4Fe-3S]4+, are formally equivalent to the [4Fe-4S]+/[4Fe-4S]2+ redox states of the conventional systems. However, the special constitution of the MBH proximal cluster confers considerable structural flexibility, which allows the attainment of a superoxidized [4Fe-3S]5+ state (S-OX) at physiological redox potentials, a crucial feature for oxygen tolerance. In terms of the electron count, the superoxidized state corresponds formally to the [4Fe-4S]3+ oxidized state of high-potential iron–sulfur proteins and experiences stabilization from the formation of a bond between the deprotonated backbone amide of Cys20 and Fe4 (Figure 1).5 Upon binding of O2 to the bimetallic [NiFe] active site, the quick delivery of two electrons from the proximal cluster6 can thus prevent the formation of detrimental Ni-A oxygen species at the active site that would reactivities very slowly under physiological conditions.10–13 The possible absence of stabilization by spin-dependent delocalization (double-exchange)14 between iron centers of mixed-valent (MV) pairs (i.e., Fe2+-Fe3+) in all three redox states due to the asymmetry of the [4Fe-3S] cluster (“trapped valences”) has been proposed as a complementary explanation for the strikingly small potential difference (<220 mV) between the reduced/oxidized and oxidized/superoxidized redox pairs in MBHs.15

A recent computational study16 rationalized the reversible redox-dependent RED ⇌ S-OX structural transformation of the proximal cluster at the three physiologically accessible [4Fe-3S]3+/4+/5+ redox levels and reproduced the observed 1H NMR hyperfine couplings (HFCs)17–19 of the Fe4-bound Cys20 amide (cf. Figure 1). Two previous combined experimental/DFT studies by Volbeda et al.7 and Pandelia et al.20 have tackled the problem of establishing a correlation between Fe sites from Mössbauer spectroscopy9,20 and the S-OX proximal cluster molecular structure. The cited works disagree on the electronic structure of S-OX, and consequently, there is no consensus on the identity of 57Fe Mössbauer centers with respect to Fe centers in the molecular structure.21,22 The identity of Fe sites and spin coupling has also been addressed
However, severe doubts are in order regarding the DFT-optimized reduced-state structure proposed in ref 20 on account of calculated Mössbauer parameters: the computational model differs for the reduced-state $[4\text{Fe}-3\text{S}]^{3+}$ cluster.  

Figure 1. Optimized models (left) of the Re-MBH proximal cluster from ref 16 and their schematic representations (right). From top to bottom: the $[4\text{Fe}-3\text{S}]^{3+}$ reduced state RED$\text{D}^{3+}$ (a) and the $[4\text{Fe}-3\text{S}]^{5+}$ superoxidized states S-OXD$\text{D}^{5+}$ (b), S-OXD$^{5+}$ (c), and S-OXP$^{5+}$ (d). The inorganic iron–sulfur core atoms and residue numbering corresponds to the PDB 3RGW X-ray structure. Only acidic hydrogen atoms are shown for clarity. In schematic representations, serine-21, histidine-229, and the water molecules W 366 and 447 were omitted for clarity. All of the models were optimized using PBE/lacv3p** for the broken-symmetry state BS13.
fundamentally from available X-ray crystal structures as the bond between Fe3 (in the present Re- and Ec-MBH numbering), one of the supernumerary cysteinate, Cys120, is ruptured, and the Fe3–Fe4 distance is contracted from 3.98 to 2.81 Å.

Here, we take an objective and comprehensive point of view of prior computational and experimental work in reporting a detailed reinvestigation of electronic structure and Mössbauer parameters of the proximal cluster in reduced and superoxidized states.

The disagreement on the S-OX electronic structure is clearly resolved, making the interpretation in terms of a match between Mössbauer spectroscopic and structural Fe centers unambiguous. For the reduced state, we demonstrate that a reasonable match with experimental Mössbauer parameters is possible for a model that agrees excellently with X-ray data. On the basis of our calculations, we suggest that a reevaluation of magnetic Mössbauer spectra with respect to hyperfine structure, or the highly desirable performance of ENDOR experiments, may lead to major revisions of the experimentally reported 57Fe hyperfine tensors for both (paramagnetic) redox states of the proximal cluster.

2. BRIEF SUMMARY OF PREVIOUS EXPERIMENTAL/COMPUTATIONAL STUDIES OF MÖSSBAUER PARAMETERS AND ELECTRONIC STRUCTURE

The elementary features of the electronic structure of multinuclear open-shell transition metal clusters are the distribution of formal metal oxidation (and spin) states and the exchange (and double-exchange4,25,26) interactions between the metal ion spin centers. For elucidating the electronic structure of biological Fe–S clusters, 57Fe Mössbauer spectroscopy is extremely helpful.25–27 As density functional theory (DFT) has been shown to be capable of accurately predicting Mössbauer parameters, most importantly isomer shifts and nuclear quadrupole splittings,28–31 theoretical calculations can help to link “spectroscopic metal centers” to site-specific iron centers in the molecular structure. Using broken-symmetry density functional theory (BS-DFT), the choice of the BS state becomes crucial in the comparison between experiment and computation (see, for example, refs 7, 20, and 32).

Experimental/theoretical Mössbauer parameters of the proximal cluster of MBH published to date are summarized concisely in Table 1.

| Site | S-OX exp. | Fe4 | BS13 PC3 calc | Fe4 |
|------|-----------|-----|---------------|-----|
| δ | 0.60 | 0.26 | 0.21 | N.A. |
| ΔE | 2.41 | 0.46 | 2.36 | 0.38 |
| η | N.A. | N.A. | 0.44 | N.A. |
| ν | 0.12 | 0.60 | 0.60 | 0.60 |
| S | BS13 PC3 calc. | Fe4 | BS13 PC3 calc. | Fe4 |
| δ | 0.74 | 0.22 |
| ΔE | 0.39 | 0.50 |
| η | 0.36 | 0.40 |
| ν | 0.83 | 0.88 |

Table 1. Summary of Published Experimental20,20 and Calculated 57Fe Mössbauer Parameters for the Reduced and Super-Oxidized Aa-MBH Proximal Cluster

Paired experimental and calculated data are arranged to have corresponding Fe sites matching row-wise, as assigned in the respective DFT studies. Calculations of {ΔE0} in ref 7 favored the so-called BS13 PC3(5) model for S-OX (spin alignment corresponding to our BS3, explained in detail in section 3.2), referring to earlier Mössbauer data from ref 9. Pandelia et al. compared calculated Mössbauer parameters with experimental data reported in the same work,20 favoring models Ox2_24 (corresponding to our BS34) for S-OX and Red2_24 (corresponding to our BS34) for RED. Experimental site designations (S = “special”, F = “ferrous”) follow ref 20. The numbering of Fe sites in the DFT results follows the numbering for Ec-MBH and Re-MBH, which are the same. A different numbering for Hm-MBH was used in ref 20 but has been converted in the present table and also in the following work to the numbering of Ec- and Re-MBH as follows: Fe1 → Fe1, Fe2 → Fe4, Fe3 → Fe2, and Fe4 → Fe3. However, naming of the favored models in ref 20, Ox2_24 and Red2_24, has been retained. Signs for nuclear quadrupole splittings {ΔE0} have not been determined in ref 9. In ref 20, the signs of the {ΔE0} could not be determined with full confidence for S-OX; reported signs (given in parentheses) followed the accompanying DFT calculations. We assigned signs to the {Aiso} based on a “classical” spin coupling scheme proposed by Volbeda et al., see Results section. The caption to Table S6 of ref 20 indicates that Mössbauer spectra in applied magnetic fields could not resolve the signs of Aiso for two sites in the reduced-state cluster. Both alternative sets of the signs of Aiso are given in the table; the lower sign option conforms to the DFT results in ref 20, and this sign option was thus chosen for the simulations.20

ment was reached by Pandelia et al. However, the optimized structure (see Table S8 in ref 20) does not resemble published X-ray structures4,26–29 with the proximal cluster in its reduced redox state.

For the superoxidized state, Pandelia et al. and Volbeda et al. deduced different assignments of Mössbauer centers to Fe sites in their X-ray diffraction-based models. The local spin state
of ferric Fe4 in model Ox2_24 (spin coupling corresponding to our BS34, see section 3.2) favored by Pandelia et al. was controversial, and a discussion on the best-suited broken-symmetry state for S-OX and the probable protonation state and conformation of the glutamate residue binding to (proximal) or in the vicinity of (distal) Fe4 ensued.\(^{21,22}\)

We note in passing that a very recent structure determination by X-ray diffraction, vibrational and EPR spectroscopies, and quantum-chemical calculations for the superoxidized state of Re-MBH indicated a somewhat different structure where a hydroxyl ligand is coordinated to Fe1 and additionally accepts a hydrogen bond from His229.\(^{18}\) Consideration of this proposed new species is outside the scope of the present computational Mössbauer study and will be addressed in a separate work.

3. COMPUTATIONAL DETAILS

3.1. Model Structures and Density-Functional Methods. Computational models for the reduced-state cluster were originally constructed\(^{16}\) from the X-ray data for Re-MBH\(^{8}\) (PDB ID: 3RGW). Coordinates of structures employed here for the calculation of Mössbauer and hyperfine parameters have been given partially in the Supporting Information of ref 16. However, for the present study, some additional DFT structure optimizations have been performed.

Full details of the employed optimization protocol were reported previously.\(^{16}\) Briefly, structure optimizations without dispersion corrections, using the lavec3p** basis-set/Fe-pseudopotential combination,\(^{35}\) and a polarizable continuum solvent model (dielectric constant \(\varepsilon = 4.0\)), were performed with the GAUSSIAN 09 program package.\(^{34}\) Polypeptide \(\alpha\)-carbon atoms were fixed in the optimizations except for the \(\alpha\)-carbon of Cys20. Its deprotonated backbone amide binds to Fe4 in the superoxidized state, and it was thus left free to change (cf. ref 16). Structure optimizations including Grimme’s D3 dispersion corrections\(^{35,36}\) were carried out with Jaguar 7.8.\(^{37}\) However, as the small structural changes affected computed Mössbauer parameters only slightly, we will largely report data obtained without dispersion corrections.

When the PBE functional\(^{38,39}\) was used, deviations compared to X-ray structures in important bonding and nonbonding internuclear distances for the reduced-state proximal cluster (computational model RED\(_D\)) amounted to \(\sim 0.1\) Å at most (see also Figure 4 and Table S2 in ref 16). Importantly, the B3LYP\(^{40,41}\) functional overestimated metal–ligand bond lengths compared to the X-ray reference. Here, we therefore discuss only the PBE structures. Calculations of Mössbauer parameters have nevertheless been carried out for some B3LYP structures as well (results are given in the Supporting Information).

For the present study of Mössbauer parameters, accurate single-point calculations at the optimized structures were performed using Turbomole 6.3.\(^{42}\) We used an all-electron (1s8s1p6d)//[9s7p4d]\(^{43–45}\) basis set on Fe sites, shown previously to well-reproduce HFCs in transition metal complexes.\(^{44,45}\) For all other atoms, the flexible IGLO-II\(^{46}\) basis set was used. This basis-set combination will in the following be denoted as “EPRB”. Solvent effects have been taken into account with the COSMO\(^{47}\) conductor-like screening model (\(\varepsilon = 4.0\)). For these calculations, the PBE and the B3LYP functionals were used. Converged orbitals from Turbomole were exported to our in-house program MAG\(^{48}\) to calculate EFGs (\(\Delta E_Q\), \(\eta\)), electron densities at the locations of the Fe nuclei (for the calculation of \(\delta\)), and HFCs. Because of the strong dependence of calculated \(^{57}\)Fe nuclear quadrupole splittings on the density functional used, which is especially pronounced for the reduced-state proximal cluster, certain structures/BS states (see next section) have also been optimized, and single-point calculations were carried out with customized hybrid functionals using 5% Hartree–Fock (HF) exchange (recommended by Szilagyi and Winslow\(^{49}\)) for Fe–S clusters and used by Volbeda et al. in their work on the proximal cluster\(^{2}\) or 10% HF-exchange. The OLYP density functional\(^{40,50}\) which performed well in recent computations of Mössbauer parameters,\(^{31,51}\) has been tested too. Optimizations and single-point calculations using OLYP were performed with GAUSSIAN 09, as the OLYP functional has not been implemented in Turbomole. SCF orbitals were subsequently transferred to MAG for the calculation of spectroscopic properties.

3.2. Broken-Symmetry States. Briefly, in the broken-symmetry approach, states are represented by single Kohn–Sham determinants with maximal local spin projections of the high-spin Fe\(^{2+}\) and Fe\(^{3+}\) centers, i.e., \(|M_s\rangle = S\). When \(\uparrow\) "(net \(\alpha\)) and \(\downarrow\) "(net \(\beta\)) denote \(M_s > 0\) and \(M_s < 0\), there are essentially six configurations \([2Fe\downarrow:\!2Fe\uparrow]\) given that the identity of Fe\(^{3+}/\!Fe^{2+}\) oxidation states is normally either lost during SCF convergence or there is a preference for localizing the “odd electron” on either of the centers of the MV pair. The proximal cluster of MBH is particularly asymmetrical in its superoxidized state. Following ref 16, we denote broken-symmetry states “BS\(ab\)”. Here, \(a\) and \(b\) are the numbers of iron centers that have net \(\beta\) spin when \(\sum M_s = 1/2\). A schematic representation of state BS13 of the superoxidized cluster is shown in Figure 2.

Our convention (indexing net \(\beta\) sites) for naming BS states concurs with that of Pandelia et al.,\(^{20}\) but our numbering for the Fe atoms differs. For the reduced state, our BS designations differ from those of Mouesca et al.,\(^{15}\) who chose to name BS states of the reduced state by the centers that have \(\alpha\)-spin (with \(\sum M_s = 1/2\)). Our numbering of the Fe centers follows that for Re-MBH and Ec-MBH (see Figure 1), as used also in the DFT modeling in ref 16 (starting from X-ray structures of Re-MBH) and refs 7 and 15 (starting from X-ray structures of Ec-MBH; X-ray structures for As-MBH are not available as yet). The numbering of proximal-cluster iron centers for Hm-MBH.
employed in ref 20 can be converted to that for Re-MBH and Ec-MBH as follows: Fe1 → Fe1, Fe2 → Fe4, Fe3 → Fe2, and Fe4 → Fe3.

### 3.3. Calculation of Mössbauer Parameters.

Details on \( \Delta E_Q \) and \( \eta \), and on calibration fits for the calculation of isomer shifts (using the recent test set by Sandala et al.31) are given in section 1 of the Supporting Information. We note that the results of a recent study52 using relativistic calculations with finite-nucleus models likely give a more reliable slope than nonrelativistic point nucleus calculations, but some dependence on the DFT exchange-correlation potential can still be expected. Further, a fit to isomer shift data over multiple systems always provides a check on accuracy, see also ref 53. Computed \( \eta \) values will be disregarded here, as they are influenced too much by small structural inaccuracies to be diagnostic, and their extraction from the spectra is afflicted with more uncertainty than that of \( \{ \Delta E_Q \} \) and \{\( \delta \)\}. The \( \Delta E_Q \) values are most diagnostic for bonding and spin-coupling, whereas the isomer shifts mainly reflect the pattern of formal oxidation states and covalency of the Fe sites. The sign of \( \Delta E_Q \) can change by small perturbations when \( \eta \) is large, i.e., close to 1. 57Fe HFCs in iron–sulfur complexes have been studied computationally for a long time.34 However, quantitative DFT accuracy of the isotropic HFCs (\( A_{iso} \)) of transition metal sites is limited by core–shell spin polarization.44 We have thus applied semiempirical scaling factors to the \( A_{iso} \) values calculated by DFT. The scaling factors were determined from a comparison with experimental isotropic HFCs for a set of 12 distinct Fe sites. Anisotropic HFC contributions depend less on core–shell spin polarization55 and are thus reproduced without any scaling.

Alternatively, a semiempirical scheme developed by Mouesca et al.54 is used, relying on an established approximate proportionality between Fe 3d Mulliken spin populations and 57Fe HFCs.

More details on the calculation of \( A_{iso} \) for 57Fe centers, including the calibration against experimental data, are given in section 5 of the Supporting Information.

### 3.4. Spin Projection.

To compare computed HFCs to experimentally determined effective values, a two-step spin-projection scheme56 has been employed. The \( a_i^{BS}(X) \) raw results are first converted to site tensors (that parametrize the hyperfine interaction of site spin \( S_i \) with nucleus \( X \)) as

\[
a_i(X) = \frac{S_i}{\pm S_i} a_i^{BS}(X) \tag{0.0}
\]

where \( S_i \) is the total spin quantum number (\( S_i = 1/2 \) for RED and S-OX). The sign in the denominator is determined by the projection of the site spin with respect to the projection of the total spin in the Kohl–Sham determinant representing the BS state. Conversion to effective HFCs in the coupled representation is given by the Wigner–Eckart projection theorem

\[
A_i(X) = \frac{\langle S_i S_i \rangle}{\langle S_i S_i \rangle} a_i(X) \tag{0.1}
\]

The ratio \( \langle S_i S_i \rangle/\langle S_i S_i \rangle = \langle S_i S_i \rangle/\langle S_i S_i + 1 \rangle = k_i \) is called the spin-projection coefficient, thus

\[
A_i(X) = k_i a_i(X) \tag{0.2}
\]

The expectation values in eq 0.1 are understood to be taken in any one of the degenerate spin wave functions of the \( S_i = 1/2 \) doublet of interest. These scalar projection coefficients neglect local zero-field splitting (ZFS) interactions and assume that exchange coupling dominates over local ZFS interactions, as is often the case for Fe–S clusters.24 The effective HFC tensors are of course “properties” of a certain doublet only.

A positive spin-projection coefficient indicates that the expectation value of the site-spin projection onto the direction of a weak magnetic field, applied along one of the principal axes of the system’s g-tensor, has the same sign as the expectation value of the projection of the conserved total spin on the field direction. In certain cases, meaningful spin-projection coefficients can be derived by a simple analytical procedure once a formal coupling scheme has been devised (see below).24,57

Because of ambiguities in choosing a coupling scheme, for S-OX, we have instead obtained spin-projection factors from a numerical diagonalization of the Heisenberg–Dirac-van-Vleck (HDV) Hamiltonian, including also a delocalization term (see section 4 in the Supporting Information; eq 0.1 remains approximately valid due to the incipient valence localization). The resulting projection coefficients are very different from sets assumed previously.7,16,20

### 4. RESULTS AND DISCUSSION

#### 4.1. Molecular Structures. a. S-OX State.

Three possible structures of the S-OX proximal cluster (S-OXD\( ^{5+} \), S-OXD\( ^{4+} \), and S-OXP\( ^{3+} \)) differ in the conformation (distal/proximal) and the protonation state of a glutamate residue (Glu76) close to the special iron Fe4. These DFT-optimized structures (in particular S-OX\( ^{5+} \) and S-OX\( ^{4+} \); see Figure 1) agree well16 with the reference X-ray structures.5,7 S-OXD\( ^{4+} \) has protonated Glu76 in a distal position with respect to Fe4 and may represent the primary product of the redox-dependent structural transformation. Recently some of us have postulated a RED\( ^{5+} \) \( \rightarrow \) S-OXD\( ^{5+} \) structural transformation to follow two-electron oxidation of the RED\( ^{4+} \) reduced cluster.16 Following proton transfer off the cluster complex, S-OXD\( ^{5+} \) \( \rightarrow \) S-OX\( ^{4+} \), the deprotonated glutamate residue can shift to bind to the special iron center, Fe4, S-OX\( ^{3+} \) \( \rightarrow \) SOX\( ^{5+} \). This binding is slightly exothermic. Here, we have evaluated S-OX\( ^{5+} \), S-OX\( ^{4+} \), and S-OX\( ^{3+} \) as candidates to represent the HYSCORE-, ENDOR-, and Mössbauer spectroscopically20 detected S-OX species. State BS12 yields the lowest energies for S-OX\( ^{5+} \), S-OX\( ^{4+} \), and S-OX\( ^{3+} \) in structure optimization using DFT.16 The energetic differences between BS12 and BS13 structures however are small for all three S-OX candidates. Searching the S-OX potential energy surface for a minimum with Glu76 protonated at the carboxylate oxygen atom distal to the cluster and attached to Fe4 via the carbonyl oxygen atom had not been successful.16 Our attempt using D3 dispersion corrections in the DFT optimizations also gave no structure analogous to model PC3\( ^{3+} \) by Volbeda et al. (which we would call S-OX\( ^{p-H} \)). Despite differences in modeling (we use a pure QM approach whereas Volbeda et al. employed QM/MM methodology for optimization and extracted smaller QM models for the calculation of Mössbauer parameters), this raises concerns with respect to the stability of a structure like PC3\( ^{3+} \).

b. RED State. The reduced-state model RED\( ^{5+} \) is in good agreement with available crystal structures for all BS states and represents the only structural model for the reduced state evaluated here. Although an alternative conformation (RED\( ^{p-H} \)
had been found to exist on the RED potential energy surface, the RED₄ conformation is energetically competitive with RED₃ only in the OX or S-OX redox states, and it may thus be excluded for the present investigation.

4.2. Broken-Symmetry States and Iron Spin States. Relative energies of all six BS states considered for RED₅⁻³ S-OX₄₁⁻⁷⁻, S-OX₅⁺⁵⁻, S-OX₆⁻⁶⁻, and S-OX₇⁻⁷⁻ are provided in Table S2. The PBE/EPR8 single-point results differ only a little from those obtained directly at the PBE/lacv3p** optimization level, whereas the B3LYP/EPR8 energy differences cover a somewhat larger range (particularly for BS24 and BS34, see below).

All BS states for RED₅⁻³ lie within a rather small energy window of <4 kcal/mol for PBE and <6 kcal/mol for B3LYP. In contrast, the reduced-state models by Pandelia et al. display significantly larger ranges for the relative energies of up to ~18 kcal/mol. The largest “outliers” of this kind can be understood from a comparison of computed Mössbauer parameters (see below). For example, the high relative energy of 18.41 kcal/mol for their Red₁₋₁₃ (PBE structure, B3LYP single point, spin-alignment corresponding to our BS12) may be explained by an unfavorable valence localization in the MV pair. The theoretical Mössbauer parameters calculated by Pandelia et al. show localization according to Fe₃²⁺⁻Fe₄³⁺ in the BS12 MV pair, whereas we obtain a much lower relative energy for BS12 with localization according to Fe₃³⁺⁻Fe₄²⁺. In our experience, the state with unfavorable localization would probably not remain stable during structure optimization.

Conversely, for the S-OX state at the level of structure optimization, BS14 and BS23 have energies ~10 kcal/mol above the lowest state (BS12). We have therefore calculated Mössbauer parameters only for BS12, BS13, BS24, and BS34, which are close in energy. The six different BS configurations for both redox states of the cluster allow in principle different distributions of formal metal oxidation states, as a priori preferences of certain centers (or pairs) for certain oxidation states are not known. In contrast to the situation encountered for the reduced state (see section 4.3), for the superoxidized state the distributions of metal oxidation states within the pairs with the same relative alignments of site spins, BS12/BS34, BS13/BS24, and BS14/BS23, were interestingly found to be identical, as we explain now.

In iron–sulfur clusters, calculated Mulliken spin populations for Fe are often not very diagnostic. This deficiency is due to pronounced delocalization of spin density onto the sulfur atoms (as demonstrated in Table S2). Despite this potential pitfall, the conspicuously low spin population for Fe₄ (almost one unit lower compared to the other sites) in states BS24 and BS34 of the S-OX has been used to assign Fe₄ as a ferrous high-spin site. Localization within the potential MV pairs Fe₂⁻⁻Fe₄ and Fe₃⁻⁻Fe₅ respectively, with Fe₄ being ferric with intermediate spin. This observation is reminiscent of the orbital configurations OS1, OS2, and OS3 for the oxidized [4Fe-4S]³⁺ cluster of high-potential iron–sulfur proteins (HiPIP’s). Notably, a quantum mixture of Sᵢ = 3/2 and Sᵢ = 5/2 local spins for the two sites i and j of the ferric pair has been described for OS1 and OS2. In the present cases, the cluster structure (large separation of the centers of the ferric pair) enforces localization, such that well-defined local spins of Sᵢ = 5/2 for BS34 and Sᵢ = 5/2 for BS24 may be combined for both BS34 and BS24, either with a quantum mixture of Sᵢ = 3/2 and Sᵢ = 5/2 or with a “pure” Sᵢ = 3/2.

Spin canting, i.e., a local Sᵢ = 5/2 with Mᵢ = 3/2 (with the other site spins having maximal projections), cannot be described in the present collinear spin framework, ruling out a pure Sᵢ = 5/2. An interpretation in terms of Sᵢ = 3/2 in BS24 and BS34 is indicated by <S> expectation values. For the BS Kohn–Sham determinants representing BS12 and BS13, <S> has values of ~9.3 in any of the S-OX models (B3LYP single-point), which is close to the “ideal” value of 9.75 for an effective doublet BS state with nine magnetic pairs (ten unpaired α and nine unpaired β electrons). For the BS24 and BS34 models, on the other hand, <S> ≈ 8.4, almost one unit smaller and close to the “ideal” BS value of 8.75 for nine unpaired α and eight unpaired β electrons. In direct product notation, specifying the projections onto the z-axis of the four site spins, where projections are maximal with respect to the site-spin quantum numbers, we thus have l = −4/2, −5/2, +5/2, +4/2) for BS12, l = −4/2, −5/2, +5/2, +3/2) for BS34, l = −4/2, +5/2, −5/2, +3/2) for BS13, and l = −4/2, +5/2, −5/2, +3/2) for BS24.

In addition to these orbital analyses, significantly shorter metal–ligand bond lengths of Fe₄ in states BS34 and BS24 compared to those of experimental structure data, provide evidence of a local spin Sᵢ = 3/2 in those states. The close orbital configuration relationship between BS12 and BS34, and between BS13 and BS24 (each pair only differing by a spin-flip
on Fe4), further leads to Mulliken spin populations and calculated Mössbauer quadrupole splittings for sites Fe1, Fe2, and Fe3, which are generally almost identical between orbital configuration partners, whereas these properties are markedly different between these configurations for Fe4 (see section 4.3).

The described local spin pairing on Fe4 is energetically competitive. BS24 (S4 = 3/2) even represents the lowest state for S-OX5+ at the PBE/EPRB level, which is lower by 0.7 kcal/mol than its orbital configuration partner BS13 (S4 = 5/2). We have to keep in mind, however, that "pure" GGA functionals like PBE tend to overstabilize lower spin states. At the B3LYP level, relative energies of BS24 models with S4 = 3/2 are ~6 kcal/mol higher. Results with the two functionals are expected to bracket the true preferences. To more directly probe the intrinsic preference of Fe4 for either a high-spin S4 = 5/2 or an intermediate-spin S4 = 3/2 or even low-spin S4 = 1/2 state, we diamagnetically substituted Fe12+, Fe23+, and Fe34+ ions, respectively, in the previously optimized BS12, BS13, and BS34 structures. Table 2 gives the computed spin-state energetics.

Table 2. Spin-State Energies (kcal/mol, PBE/B3LYP) Relative to S = 5/2 for Diamagnetically Substituted S-OX5+ Models Optimized in BS States BS12, BS13, and BS34

| S = 1/2 | S = 3/2 | S = 5/2 |
|---------|---------|---------|
| BS12    | +3.57/+14.01 | -3.50/-1.77 | 0.0/0.0 |
| BS13    | +6.45/+17.43 | -0.72/+1.31 | 0.0/0.0 |
| BS34    | -2.73/+3.76 | -6.85/-7.31 | 0.0/0.0 |

“Fe12+ → Zn2+, Fe23+ → Ga3+, Fe34+ → Ga3+, see text. Without reoptimization of the BS structures after diamagnetic substitution.

Even for substitution in the BS12 structure, both functionals provide a slight preference for a local intermediate S = 3/2 spin state. Substitution at the BS34 structure gives a clear preference of ~7 kcal/mol for S = 3/2. That is, the BS34 structure with its shorter Fe4–N bond length is structurally adapted to S4 = 3/2 (even S = 1/2 is below S = 5/2 at PBE level). The strong and unsymmetrical ligand field of Fe4, including hard amide (and carboxylate) ligands, provides an explanation for the close energies of the BS states at this site. Of course, the local spin is coupled to those of the other sites, and more favorable exchange interactions may outweigh local spin-state preferences in some of the BS states. Although this is difficult to disentangle in detail, we nevertheless conclude that Fe4 is relatively close to a local spin-state crossover in all of the energetically competitive BS states. Optimization of S-OX5+ with S4 = 1/2 (without diamagnetic substitution) gives poor agreement with crystallographic data, affording a genuinely square-pyramidal five-coordinate Fe4 with a bond to Cys19 (Fe4–S(Cys19) distance of 2.29 Å) and the terminal cysteinate (Cys20) at the apex. Similarly, metal–ligand bond lengths involving Fe4 are too small in models BS24 and BS34 compared to X-ray references or the optimized BS12 or BS13 models. Another important clue comes from HYSCORE19 and ENDOR data for the 14N HFC of the Cys20 amide binding to Fe4 in the S-OX state. In any conceivable coupling scheme, spin density of low-spin or intermediate-spin Fe4 would be too small to explain the experimentally found strong coupling of the 14N nuclear spin to the S = 1/2 electronic spin in S-OX.

Pandelia et al.20 reported an Fe1–Fe2 MV pair for their favored Ox2_24 model (spin-alignment corresponding to the present BS34), but otherwise, an interpretation of the electronic structure of BS34 and a rationalization of the signs of the spin-projection coefficients used in the calculation of 57Fe HFCs were not provided.

4.3. Mössbauer Parameters of the Super-Oxidized State. a. 57Fe Quadrupole Splittings and Isomer Shifts. In view of the disagreement,9,20–22 between previous interpretations of the Mössbauer parameters of the S-OX state (see section 2), we now computationally reinvestigate the quadrupole splittings and isomer shifts.

Considering energetics, 14N HFCs, and structural data, the above discussion provided arguments against BS14, BS23, BS24, and BS34. Here, we still list calculated Mössbauer parameters for BS34 because this spin-coupling had been preferred in previous computational work (model Ox2_24). However, we need to essentially concentrate on six possibilities for the most likely S-OX species studied by Mössbauer spectroscopy: S-OXp5+, S-OXp6+, and S-OXP5+, each in spin-couplings BS12 or BS13. The computed Mössbauer results at PBE and B3LYP levels are given in Tables 3 and 4.

Table 3. Computed Mössbauer Parameters for the S-OXp5+ Model at PBE/B3LYP Levels in Different BS States As Compared to Experimental Data

| S-OXp5+ | PBE/B3LYP Mössbauer parameters |
|---------|--------------------------------|
| state   | site | ΔE0 (mms−1) | δ (mms−1) | η | Aiso |
| S-OXp5+ | S   | (+)2.45   | 0.46     | 0.5 | 25.7 |
| BS12    | Fe12+ | +1.67/+2.51 | 0.48/0.50 | 0.93/0.70 | + |
| BS13    | Fe12+ | -0.55/-0.66 | 0.37/0.35 | 0.71/0.86 | + |
| BS34    | Fe12+ | +2.51/+3.42 | 0.48/0.55 | 0.21/0.35 | + |

respectively (data for model S-OXp5+ is very similar to S-OXp6+ and is provided in Table S17). Earlier calculated Mössbauer parameters from other groups are found in Table 1.

The consequences of the orbital configuration partner between BS12 and BS34 (see above) are immediately apparent in the strikingly similar Mössbauer parameters predicted for Fe1, Fe2, and Fe3 in these two spin states. The EFG at the position of the Fe4 nucleus is of course very different between BS12 and BS34 due to the different spin state of Fe4 (high-spin for BS12, intermediate-spin for BS34).

Because of the opened cluster conformation in the S-OX state, only two pairs of Fe centers, Fe1–Fe2 and Fe2–Fe3, have crystallographic/calcualted intermetallic distances <2.8 Å (see Table S1). Thus, only BS states BS12 and BS34 may

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exhibit a larger degree of delocalization. A pronouncedly localized MV character of the Fe$^{2+}$–Fe$^{3+}$ pair in BS12 and BS34 is apparent from the calculated quadrupole splittings and isomer shifts, which are both considerably larger for Fe1 than for Fe2 at both PBE and B3LYP levels (with somewhat larger delocalization found for PBE). In models S-OX$_{D}$-$^{5+}$ and S-OX$_{D}$-$^{5+}$, the computed $\Delta E_Q$ of Fe4 in state BS34 is substantially larger when using B3LYP instead of PBE.

For all three BS states, B3LYP gives $\sim$1 mms$^{-1}$ larger $\Delta E_Q$ for Fe1 compared to that of the PBE results (the relative increase is larger for BS12 or BS34, where some delocalization within the Fe1–Fe2 MV pair may play a role, see above). The tendency of the B3LYP functional to overestimate quadrupole splittings in the range $\Delta E_Q > 2.0$ mms$^{-1}$ has been described in a careful DFT calibration study of Mössbauer parameters by Lippard and co-workers$^{30}$ despite B3LYP performing well overall in that work. These differences between PBE and B3LYP prompted us to also evaluate customized B3LYP functionals with S and 10% of exact-exchange admixture for BS13. The resulting $\Delta E_Q$ and $\{\eta\}$ calculated with these functionals are provided in Table S24. Generally, all components of the Cartesian EFG tensors of all centers change monotonically upon increasing the amount of exact exchange from 0% (PBE) via 5% and 10% to 20% (B3LYP). In particular, the largest $\Delta E_Q > 2.5$ mms$^{-1}$ rises with the amount of exact exchange in the functional from +2.51 mms$^{-1}$ with PBE to +3.42 mms$^{-1}$ with B3LYP, whereas the other (smaller) $\{\Delta E_Q\}$ remain comparably unaffected.

Focusing on the PBE results, in the following, we discuss in more detail the different BS states and models. Although we observe quite close agreement between the calculated $\{\Delta E_Q\}$ for our model BS34 S-OX$_{D}$-$^{5+}$ and the corresponding model Ox2_24 favored by Pandelia et al.$^{28}$ in terms of agreement with experimental data, the evidence provided above for a local spin $S_z = 3/2$ leads to far too small $^{14}$N hyperfine coupling.

The B3LYP results for the BS12 state S-OX$_{D}$-$^{5+}$ give Mössbauer parameters only slightly inferior to the PBE data with BS13 as well as reasonable signs of $A_{\text{iso}}$ within a “classical” spin coupling scheme. However, in view of the systematic errors of B3LYP for the largest $\Delta E_Q$ (see above),$^{30}$ which become drastically apparent in the reduced-state results reported below, we arrive at a clear preference for BS13.

Computed Mössbauer parameters for S-OX$_{D}$-$^{5+}$ and S-OX$_{D}$-$^{5+}$ models are very similar, whereas differences with S-OX$_{D}$-$^{5+}$ are more pronounced. In the latter case, deprotonated Glu76 binds to Fe4, which in turn loses its bond to Cys19 (see Figure 1). Cys19 then binds terminally to Fe1 instead of assuming a $\mu_2$-bridging mode between Fe1 and Fe4. Consequently, the Mössbauer parameters of Fe1 and Fe4 are affected much more by these structural changes than those of Fe2 or Fe3. The PBE/EPRB-level Mössbauer parameters (especially $\{\Delta E_Q\}$ for S-OX$_{D}$-$^{5+}$ in BS13 agree well with experimental data for the chosen order of assignment (cf. Table 3).

We may thus assign Fe1 to site “S” and Fe4 to Fe2 (second-largest computed $\Delta E_Q = (-1.34$ mms$^{-1}$) to the observed $\Delta E_Q = (-1.00$ mms$^{-1}$). Uncertainties of DFT calculations as well as spectra simulations leave Fe2 and Fe3 almost indistinguishable in terms of their very similar quadrupole splittings and isomer shifts, and an earlier experimental study treated these two sites as equivalent (cf. Table 1). Notwithstanding this fact, an assignment of Fe2 and Fe3 to the centers with the experimental $\Delta E_Q = (+0.70$ and $\Delta E_Q = (+0.60$ mms$^{-1}$, respectively, leads to a matching pattern of signs for $A_{\text{iso}}$. Interestingly, we did not have to invoke the measured signs of $A_{\text{iso}}$ to single out BS13 as the best approximation. This is a crucial point, because our assessment of $^{57}$Fe HFCs presented in the following section suggests a reevaluation of experimental data pertaining to hyperfine couplings.

Accepting the BS13 state as the best approximation, thus supporting the conclusions of Volbeda et al. (obtained based on computed $\{\Delta E_Q\}$, a clear computational distinction between the three structural models does not seem possible. The “distal” models S-OX$_{D}$-$^{5+}$ and S-OX$_{D}$-$^{5+}$ overall provide slightly better isomer shifts (S-OX$_{D}$-$^{5+}$ gives a value that is somewhat too large for Fe4), but at PBE level, they underestimate the $\Delta E_Q$ of Fe1 (site S). The latter parameter depends very sensitively on the amount of exact exchange in the functional (see Table S24), and single-point calculations with 5%-HF exchange improve the $\Delta E_Q$ of Fe1 for models S-OX$_{D}$-$^{5+}$ and S-OX$_{D}$-$^{5+}$.

b. Hyperfine Couplings. The magnetic hyperfine structure from coupling of $^{57}$Fe nuclear spins ($I = 1/2$) to the electronic spin of paramagnetic clusters in the applied-field Mössbauer spectra of H$_2$-reduced and superoxidized As-MBH was simulated with $^{57}$Fe HFC tensors coaxial to each other.$^{20,61}$ Table 5 reproduces the rhombic $^{57}$Fe HFC tensors for the reduced and superoxidized proximal clusters from ref 20.

For the S-OX state, the quantity $A_{\text{iso}} = -22.4$ MHz, representing the sum over all four $A_{\text{iso}}$ values, lies in a typical range.$^{54}$ However, the dramatically anisotropic tensors for the sites with $|\Delta E_J| = 1.00$ mms$^{-1}$ and $|\Delta E_J| = 0.70$ mms$^{-1}$ (which our calculations show to be ferric) appear odd. Even before going into the spin-projected computed HFC tensors (see below), we can note here that the computed tensors (before or after scalar spin projection) do not exhibit comparably large relative anisotropies. Violation of the strong-exchange limit and a resulting modification of the anisotropies by local zero-field
structure (see Figure 1, and section 2 in the Supporting Information) would favor $S_{13} = 0$, $S_{14} = 1/2$, $S_{1} = 1/2 >$ with $K_{1}^{i} = K_{1}^{i} = 0$. However, simulations indicated that all four Fe sites contributed to hyperfine structure in the experimental spectra.20

To avoid an arbitrary choice of a coupling scheme, we computed spin-projection coefficients by numerical diagonalization of the HDrV + double exchange Hamiltonian (details are given in section 4 of the Supporting Information). Computation of the isotropic exchange-coupling constants $J$ at B3LYP level (preferred here because PBE tends to overestimate isotropic exchange couplings23) for the BS13 S-OXP 54 PBE structure was performed by calculating the energies of eight different relative spin alignments. Double exchange has been accounted for, but its effect is rather small due to the clearly differentiated sites FeI 2+ and Fe2 3+ of the MV pair. Regarding the (unknown) resonance delocalization parameter $B$ for partial valence delocalization between Fe1 and Fe2 as an adjustable parameter, only $J_{12}$ depends on $B$. $J_{12}$ parametrizes the exchange interaction between localized Fe1 2+ ($S = 2$) and Fe2 3+ ($S = 5/2$) centers. Over the considered range for $B$, this model correctly predicts a doublet ground state, and the largest spin-projection coefficients, $K_{1}$ and $K_{2}$, for the ground state are approximately constant over the considered range of realistic values for $B$, whereas $K_{3}$ and $K_{4}$ are in any case very small in contrast to earlier assumptions.18,20 Specifically, a reasonable estimate appears to be $K_{1} = -1.16$, $K_{2} = 0.15$, $K_{3} = -0.25$, and $K_{4} = 2.28$. The spin-projection coefficients calculated explicitly in the present work are relatively close to coupling scheme $S_{23} = 0$, $S_{14} = 1/2$, $S_{1} = 1/2 >$. For Fe1 (which we identify as site $S$), the ratios of the three traceless components and $A_{iso}$ (Table 7) are quite close to the

Table 5. Simulated 57Fe HFC Tensors (in MHz, Converted from Values Given in Tesla in ref 20 by Dividing by 0.724) for the RED and S-OX State of the Au-MBH Proximal Cluster from ref 20a

| site, $\Delta E_{o}$ (mms) b | $A_{iso}$ | $T_{xx}$ | $T_{yy}$ | $T_{zz}$ |
|-----------------------------|--------|--------|--------|--------|
| RED exp.                    |        |        |        |        |
| $S_{1}$ +2.60               | +19.3  | -2.8   | +1.4   | +1.4   |
| $S_{2}$ -0.34               | -43.4  | +13.8  | -6.9   | -6.9   |
| $S_{3}$ +1.52               | +43.2  | +1.8   | ±0.9   | ±0.9   |
| $S_{4}$ +1.23               | +27.6  | +11.1  | +2.8   | +8.3   |
| S-OX exp.                   |        |        |        |        |
| $S_{1}$ +2.45               | +25.7  | -18.6  | +16.2  | +2.4   |
| $S_{2}$ -0.70               | -47.9  | +35.1  | -22.5  | -12.7  |
| $S_{3}$ +0.60               | +33.4  | -5.8   | +1.5   | +4.4   |
| $S_{4}$ -1.00               | -33.6  | +10.1  | +15.7  | -25.8  |

“Effective HFC tensors are decomposed into their isotropic component $A_{iso}$ and the anisotropic traceless part $[T_{xx}, T_{yy}, T_{zz}]$. bSigns of $\Delta E_{o}$ for S-OX state uncertain, see caption to Table 1. For this site, the designations $[T_{xx}, T_{yy}, T_{zz}]$ are not appropriate as the HFC tensor of Fe1 in the RED state was simulated with a principal axis system rotated with respect to the other tensors’ principal axis systems; see ref 61. This is, however, a minor point given the surprisingly small anisotropy of the ferrous site $S$.

splitting interactions (neglected in our calculations) could be considered. However, the agreement between computed relative 14N HFC anisotropies of N20 (cf. Figure 2) with ENDOR data 15,17 at the present level is excellent. For the BS13 S-OXP 54 model, the computed ratios of the three anisotropic 14N HFC tensor components and the isotropic value are $(-0.22, -0.00, +0.22)$ (PBE/EPRB) or $(-0.19, -0.04, +0.23)$ (B3LYP/EPRB). This may be compared to ENDOR data of $(-0.22, -0.03, +0.25). The good agreement suggests strongly that the present scalar spin projection is appropriate for S-OX (notwithstanding possible uncertainties arising from a lack of knowledge of the experimental orientation of the tensor axes). This in fact casts severe doubts on the unusually large 57Fe HFC anisotropies reported for the spectra simulations.

Turning now to the explicit calculation of the 57Fe HFCs for our S-OX models, we need to derive spin-projection coefficients. The localized character of the MV pair of BS13 leaves some freedom in the choice of spin-coupling schemes. From the “pure” coupling schemes (i.e., disregarding the possibility of spin canting) presented in Table 6, the requirement of having $K_{i}^{i} \approx 2$ to correctly predict the N20 14N HFCs 18,19,17 eliminates only scheme $S_{13} = 9/2$, $S_{14} = 2$, $S_{1} = 1/2 >$. Magnetocochemical considerations based on the cluster

Table 6. Possible Spin-Coupling Schemes a for the S-OX State ($S_{1} = 2$, $S_{2} = S_{3} = S_{4} = S/2$)

| $K_{1}$ | $K_{2}$ | $K_{3}$ | $K_{4}$ | $L_{13}$ | $L_{14}$ | $L_{12}$ |
|--------|--------|--------|--------|--------|--------|--------|
| $S_{13} = 1/2$, $S_{14} = 2$, $S_{1} = 1/2 >$ | -8/27 | 14/27 | -14/9 | 7/3 |
| $S_{13} = 9/2$, $S_{14} = 2$, $S_{1} = 1/2 >$ | -88/81 | 10/9 | -110/81 | 7/3 |
| $S_{13} = 9/2$, $S_{14} = 2$, $S_{1} = 1/2 >$ | -88/81 | 7/3 | -110/81 | 10/9 |
| $S_{13} = 9/2$, $S_{14} = 2$, $S_{1} = 1/2 >$ | -4/3 | 2 | -5/3 | 2 |
| $S_{13} = 0$, $S_{14} = 1/2$, $S_{1} = 1/2 >$ | -4/3 | 0 | 0 | 7/3 |

“We employ the notation $S_{1}$, $S_{2}$, $S_{3}$, $S_{4}$ as a symbolical compact notation. The “quantum numbers” $S_{1}$ and $S_{2}$ in general do not need to have sharp values and just denote a coupling scheme that allows for deriving the spin-projection coefficients. Signs of spin-projection coefficients $K_{i}^{i}$ correspond to the BS13 state. The $S_{13} = 9/2$, $S_{14} = 2$, $S_{1} = 1/2 >$ option underestimates the backbone amide 14N HFC.

Table 7. PBE/EPRB-Level Isotropic ($A_{iso}$) Components and Principal Values $T_{ii}$ of the Traceless Symmetric Part of the Effective 57Fe and 14N Hyperfine Coupling Matrices (MHz) for S-OXP 54 Model in BS13 State with Spin-Projection Coefficients $K_{1} = -1.16$, $K_{2} = 0.15$, $K_{3} = -0.25$, and $K_{4} = 2.28$

| site | $A_{iso}$ | $A_{iso}$ | $T_{xx}$ | $T_{yy}$ | $T_{zz}$ |
|------|---------|---------|---------|---------|---------|
| BS13 S-OX 54 | Fe1 2+ | +12.5 | N.A. | -8.3 | +1.5 | +6.8 |
| Fe2 3+ | -2.3 | -2.9 | -0.3 | -0.1 | +0.4 |
| Fe3 3+ | +4.3 | +5.0 | -0.5 | +0.1 | +0.4 |
| Fe4 3+ | -49.7 | -54.5 | -4.7 | -2.6 | +7.2 |
| NCS 54 | +16.0 | - | -3.5 | +0.1 | +3.5 |
| NCN ENDOR | +14.6 | -3.2 | -0.5 | +3.6 |
| NCN HYSCORE | +13.0 | -1.5 | -1.5 | +3.0 |

“Explicit spin-projected DFT results (PBE/EPRB) semieperimentally scaled as described in Computational Details. Values can be compared directly to experimental data. bSemieperimental results from Fe 2+ and Fe 3+ ionic site values and calculated 3d spin populations, see eqs 0.15 and 0.18 in Supporting Information. In the calculations, the principal axis systems are different for the different HFC tensors. Simulations. However, a good match with experiment for this site would require $K_{i}^{i} \approx 2.5$, which is approximately twice which that which appears realistic for this sites’ spin-projection coefficient. We emphasize again that the DFT computed anisotropies for the other (ferric) sites are far from the spectra simulation data, but they appear clearly more reasonable in comparison with literature data for ferric centers in related systems.53,64 Our scalar (strong-exchange) spin-projection
scheme gives very good agreement with the principal components of the $^{14}$N HFC tensor (ENDOR data) of the backbone amide bonding to Fe4 in the S-OX state. It appears unlikely that this agreement is fortuitous because possible spin mixing by local zero-field splitting interactions would then have to alter the experimentally unknown $^{14}$N HFC tensor orientation while keeping the principal components unchanged. Therefore, we suspect the present strong-exchange spin projection to hold to a good approximation. As DFT is known to provide accurate anisotropic $^{57}$Fe HFC components, and the isotropic $^{57}$Fe HFCs have been obtained in two different ways, we suggest that the experimental $^{57}$Fe HFC tensors should be reevaluated, particularly with respect to the uniqueness of the fits.

4.4. Mössbauer Parameters of the Reduced State. 

a. $^{57}$Fe Quadrupole Splittings and Isomer Shifts. Interpretation of the Mössbauer parameters of the six possible BS states of the RED$_{34}$ model (cf. Figure 1) is aided by the molecular structure, particularly by the intermetallic distances. The Fe3–Fe4 and Fe1–Fe3 distances are the only metal–metal distances significantly longer than ~3.8 Å in the experimental reference data and in the DFT-optimized RED$_{34}$ models (Fe1–Fe3: ~3.6 Å, Fe3–Fe4: ~4.0 Å, see also Table S2 in ref 16 for metrics). As states BS12 and BS24 host the MV pair across these distant centers, localization is expected. This is confirmed by the calculations (valence-localization Fe3$^{3+}$-Fe4$^{2+}$ for BS12 and Fe1$^{2+}$-Fe3$^{3+}$ for BS24), which also give well-differentiated quadrupole splittings (and isomer shifts) for the two MV centers (Table 8).

Although BS34 would offer the possibility of delocalization across the Fe1-Fe2 MV pair, Fe1$^{2+}$-Fe3$^{3+}$ localization is also computationally found in this case. Overall, these observations suggest generally ferrous character for Fe1 and Fe4, leaving Fe2 or Fe3 as candidates for the "spectroscopic" site with the largest ferric character ($\Delta \delta_{EQ} = +0.84$ mms$^{-1}$, $\delta = 0.42$ mms$^{-1}$, and a negative $A_{equ}$). BS14 exhibits some valence delocalization for the Fe2-Fe3 MV pair but with mainly Fe3$^{3+}$-Fe3$^{3+}$ character, as suggested by the computed Mössbauer parameters. Similarly, despite some valence delocalization, BS13 exhibits a partially localized Fe2$^{3+}$-Fe4$^{2+}$ MV pair and BS23 a corresponding Fe1$^{2+}$-Fe4$^{2+}$ MV pair. These preliminary considerations suggest Fe2 as the site corresponding to the observed $\Delta \delta_{EQ} = +0.84$ mms$^{-1}$.

To discuss the preferred BS states, we use a spin-coupling scheme (described below) where the MV pair determines the "majority spin." Focusing first on the PBE results (Table 8), BS13 provides comparably good agreement with experimental data as model Red2_24 favored in ref 20 (which corresponds to spin coupling BS34) also when considering the signs of ($A_{equ}$). These signs are different compared to the predictions of model Red2_24 but are not in conflict with spectral simulations per se (see caption to Table 1). More importantly, BS13 RED$_{34}$ and Red2_24 provide different predictions regarding the identity of spectroscopic sites "S" and "F." Although for Red2_24 Fe3 and Fe1 represent sites "S" and "F," respectively, the sites are swapped for our BS13 RED$_{34}$ model. Most notably, BS13 RED$_{34}$ closely resembles the available X-ray structures in contrast to Red2_24 (with deprotonated glutamate bound to Fe4), which differs dramatically in terms of covalent chemical bonding (see Introduction).

B3LYP single-point calculations (Table S12) again have to be viewed with caution (see above for the S-OX case): two $|\Delta E_Q|$ near 3.0 mms$^{-1}$ result for all six BS states. A similar overestimation at B3LYP level is also implicit in the data given in ref 20 (values up to ~4.0 mms$^{-1}$; Table S13). In contrast, B3LYP gives rather similar results as PBE for the ferric Fe2 center of the MV pair (smallest calculated $\Delta E_Q$). This resembles the behavior observed for the S-OX state (see section 4.2). Intermediate exact-exchange admixtures again provide intermediate $\{\Delta E_Q\}$ for the ferrous sites, in particular in those BS states where the ferrous–ferrous pair sites are at a short distance (see Table S14; OLYP results are similar to the PBE values, Table S13).

The PBE data provide the smallest $\{\Delta E_Q\}$ values for the ferrous pair, probably too small, for BS23 and BS24 and to a lesser extent also for BS12 and BS14. BS13 and BS34 provide larger values, as their ferrous pair centers are more distant than in the other BS states. In the overall comparison between computed and experimental Mössbauer parameters, a general shortcoming is that the difference between the two largest $|\Delta E_Q|$ values (sites S and F) is computed to be only ~0.3 mms$^{-1}$ (except for BS24 with PBE, which however exhibits poor overall agreement), whereas it is 1.08 mms$^{-1}$ experimentally. We assume that there are no large species-dependent differences in the structure of the reduced-state proximal cluster (Mössbauer data were obtained for Aa-MBH, our RED$_{34}$ model derives from Re-MBH). Furthermore, given that the too small difference holds for any of the DFT approaches tested, we regard it as possible that the spectral simulations for the reduced state may have to be reconsidered. Indeed, a somewhat larger experimental $|\Delta E_Q|$ of

\[\text{Table 8. Calculated Mössbauer Parameters for RED}_{34}^{+} \text{ in All Six BS States As Compared to Experimental Data}\]

| state  | site | $\Delta E_Q$ (mms$^{-1}$) | $\delta$ (mms$^{-1}$) | $H$ | $A_{equ}$ (MHz) |
|--------|------|--------------------------|-----------------------|-----|----------------|
| RED    | S    | +2.60 | 0.50 | 0.1 | +19.30 |
| exp.   |      | +0.84 | 0.42 | 0.3 | -34.50 |
|        | F    | +1.52 | 0.71 | 0.3 | ±43.24 |
|        |      | +1.23 | 0.44 | 0.9 | ±27.63 |
| BS12   | Fe1$^{2+}$ | +1.87/+2.89 | 0.56/0.65 | 0.40/0.29 | + |
|        | Fe2$^{2+}$ | +1.44/+2.48 | 0.41/0.54 | 0.89/0.88 | + |
|        | Fe3$^{3+}$ | -0.38/-0.66 | 0.43/0.38 | 0.39/0.75 | - |
|        | Fe4$^{2+}$ | +2.04/+3.12 | 0.61/0.68 | 0.62/0.40 | - |
| BS13   | Fe1$^{2+}$ | +2.00/+3.11 | 0.51/0.57 | 0.32/0.29 | + |
|        | Fe2$^{2+}$ | +0.92/+0.79 | 0.43/0.42 | 0.76/0.38 | - |
|        | Fe3$^{3+}$ | +1.77/3.01 | 0.53/0.66 | 0.59/0.90 | - |
|        | Fe4$^{2+}$ | +1.27/+2.23 | 0.55/0.59 | 0.98/0.34 | - |
| BS14   | Fe1$^{2+}$ | +1.50/+2.94 | 0.53/0.59 | 0.45/0.22 | + |
|        | Fe2$^{2+}$ | +0.70/+1.16 | 0.42/0.46 | 0.18/0.34 | - |
|        | Fe3$^{3+}$ | +1.26/+1.58 | 0.48/0.51 | 0.64/0.38 | - |
|        | Fe4$^{2+}$ | -1.34/+3.02 | 0.60/0.68 | 0.90/0.25 | + |
| BS23   | Fe1$^{2+}$ | +1.38/+1.63 | 0.51/0.52 | 0.66/0.98 | - |
|        | Fe2$^{2+}$ | +0.77/+2.99 | 0.47/0.59 | 0.46/0.54 | + |
|        | Fe3$^{3+}$ | +0.72/+2.86 | 0.53/0.61 | 0.30/0.63 | - |
|        | Fe4$^{2+}$ | -0.81/1.09 | 0.55/0.52 | 0.35/0.19 | + |
| BS24   | Fe1$^{2+}$ | +1.86/+3.14 | 0.53/0.61 | 0.07/0.14 | - |
|        | Fe2$^{2+}$ | +0.88/+2.67 | 0.44/0.54 | 0.60/0.98 | + |
|        | Fe3$^{3+}$ | -0.57/-0.83 | 0.43/0.38 | 0.99/0.50 | - |
|        | Fe4$^{2+}$ | +1.20/+2.97 | 0.64/0.71 | 0.11/0.18 | - |
| BS34   | Fe1$^{2+}$ | -1.70/-2.63 | 0.48/0.53 | 0.91/0.80 | - |
|        | Fe2$^{2+}$ | -0.54/0.47 | 0.42/0.43 | 0.86/0.69 | - |
|        | Fe3$^{3+}$ | +1.99/+3.14 | 0.54/0.65 | 0.85/0.67 | + |
|        | Fe4$^{2+}$ | +1.87/+3.07 | 0.56/0.61 | 0.13/0.33 | + |

$^a$PBE/B3LYP data with EPR basis at PBE/lacv3p** structures.
Table 9. PBE/EPRB-Level Isotropic ($A_{iso}$) Components and Principal Values $T_{ii}$ of the Traceless Symmetric Part of the Effective $^{57}$Fe Hyperfine Coupling Tensors (MHz) for RED$_{3}^{3+}$ in the BS13 State with $|S_{MV}| = 9/2, S_{z}/S_{z} = 4, S_z = 1/2 >$ Coupling Scheme

| site               | $A_{iso}$ | $A_{an}$ | $|T_{iso}|, T_{z1}, T_{z2}$ | $|T_{iso}|, T_{z1}, T_{z2}$ |
|--------------------|-----------|----------|----------------------------|----------------------------|
| BS13 RED$_{3}^{3+}$|           |          |                            |                            |
| Fe$^{3+}$          | -12.5     | N.A.     | [-27.9, +6.4, +21.4]       | [-9.3, +2.1, +7.1]         |
| Fe$_{2}$           | -36.6     | -43.0    | [-9.6, -0.5, +10.1]        | [-3.5, -0.2, +3.7]         |
| Fe$_{3}$           | +22.9     | N.A.     | [-29.2, +10.0, +19.2]      | [-9.7, +3.3, +6.4]         |
| Fe$_{4}$           | -36.8     | -44.2    | [-13.2, +0.1, +13.1]       | [-4.8, 0.0, +4.8]          |

“Explicit spin-projected DFT results (PBE/EPRB) semiempirically scaled as described in Computational Details. Values can be compared directly to experimental data. Semiempirical results from Fe$^{2+}$ ionic site values and calculated 3d spin populations, see eq 0.15 in the SI. In the calculations, the principal-axis systems are different for the different HFC tensors. Unrestricted broken symmetry (UBS) designates raw results prior to division by the number of unpaired electrons on the center and prior to multiplication with the spin-projection coefficient; spin-projected (PROJ) values can be compared directly to simulated data.

Figure 3. Summary of the main features of the electronic structure of the proximal cluster of MBH in its S-OX and RED oxidation states in terms of metal oxidation states and spin coupling. The link between “spectroscopic” metal centers and iron centers in the molecular structures is provided in terms of the quadrupole splittings, $\Delta EQ$. Experimental $\Delta EQ$ values are taken from ref 20 (see Table 1 above), and calculated values refer to models BS13 S-OX$_{3}^{3+}$ (Table 3) and BS13 RED$_{3}^{3+}$ (Table 8). Spin projection coefficients $K_i$ (calculated explicitly for S-OX, see Supporting Information) are represented by arrows, where $\uparrow$ and $\downarrow$ denote $K_i > 0$ and $K_i < 0$, respectively, and the length of each arrow is proportional to the magnitude of $K_i$, where $K_i = -1.16$ for S-OX, and so forth (see sections 4.3 and 4.4 for values of spin-projection coefficients; the scaling of arrows is the same for S-OX and RED).

Figure 3. Summary of the main features of the electronic structure of the proximal cluster of MBH in its S-OX and RED oxidation states in terms of metal oxidation states and spin coupling. The link between “spectroscopic” metal centers and iron centers in the molecular structures is provided in terms of the quadrupole splittings, $\Delta EQ$. Experimental $\Delta EQ$ values are taken from ref 20 (see Table 1 above), and calculated values refer to models BS13 S-OX$_{3}^{3+}$ (Table 3) and BS13 RED$_{3}^{3+}$ (Table 8). Spin projection coefficients $K_i$ (calculated explicitly for S-OX, see Supporting Information) are represented by arrows, where $\uparrow$ and $\downarrow$ denote $K_i > 0$ and $K_i < 0$, respectively, and the length of each arrow is proportional to the magnitude of $K_i$, where $K_i = -1.16$ for S-OX, and so forth (see sections 4.3 and 4.4 for values of spin-projection coefficients; the scaling of arrows is the same for S-OX and RED).

Site F and a somewhat smaller $\Delta EQ$ of site S would bring the computed $\{\Delta EQ\}$ for BS13 (PBE or B3LYP-5%) into excellent agreement with the experimental data. The computed isomer shifts at this level are somewhat too small for site F (Fe3) and somewhat too large for the site with $\Delta EQ = +1.23$ mm/s (Fe4) but should still be compatible with the combined uncertainties of the simulations and the calculations. This leaves the fit between theory and experiment for the Mössbauer parameters of the RED state less accurate than for the S-OX state but tends to favor BS13, i.e., a similar spin coupling as for the S-OX state.

b. Hyperfine Couplings. Our calculations of $\{\Delta EQ\}$ and $\{\delta\}$ for the reduced state favor BS13 when using the PBE functional. As there is considerable delocalization within the Fe2-Fe4 MV pair in BS13 (with some predominant ferric character on Fe2), it appears justified to assume the “classical” coupling scheme $|S_{MV}| = 9/2, S_{z}/S_{z} = 4, S_z = 1/2 >$. In this scheme, Fe2 and Fe4 each have positive spin-projection coefficients (“majority spin”) of 11/6, whereas Fe1 and Fe3 both have negative projection coefficients (“minority spin”) of $-4/3$. As the intrinsic HFCs of high-spin $^{56}$Fe$^{3+}$ and $^{56}$Fe$^{3+}$ centers are always negative, multiplication with the spin-projection coefficients gives the signs $\{+, -, +, -\}$ for the effective isotropic $^{56}$Fe HFCs ($A_{iso}$)$_{i=1}$.$4$. These signs correspond to the upper spin option for the $\{A\}$ from the spectra simulation (cf. Table 5) when assuming a correspondence between “spectroscopic” and structural Fe centers as predicted by our BS13 RED$_{3}^{3+}$ model. Although the spectra could be fitted similarly well with both possible sets of signs, Pandelia et al. found satisfactory agreement with theoretical $\{\Delta EQ\}$ and $\{\delta\}$ for model Red2_22 (criticized above) only for the $\{+, -, +, +\}$ option (sequence corresponding to the arrangement of the “RED exp.” entries in Table 1). This assignment of signs $\{+, -, +, +\}$ appears to be supported by a reasonable value $A_{tot} = \sum A_{tot} = -30.8$ MHz (a typical $A_{tot}$ value for Fe–S clusters falls in the range between $-15$ and $-39$ MHz$^2$). However, experimental hyperfine tensors again may have to be regarded with caution. Despite the unusual coordination of Fe sites in the proximal cluster of MBH, the very small simulated hyperfine anisotropy of the ferrous sites S and –even more so– F, appear exceptional for ferrous sites in Fe–S clusters. Comparisons with Mössbauer and ENDOR data of [4Fe-4S]$^2^+$ clusters from ferredoxin$^{65}$ and substrate-free and substrate-bound aconitase$^{66}$ show ferrous sites to generally exhibit larger hyperfine anisotropy.

In Table 9, we report principal values of calculated $^{57}$Fe HFC tensors to illustrate the intrinsically large (and empirically more usual) computed hyperfine anisotropies of ferrous sites S and F, now identified as Fe1 and Fe3, respectively.

Another discrepancy between calculated and simulated parameters concerns the isotropic HFCs and is illustrated by noting that our $A_{tot} = -38.0$ MHz resulting from DFT calculations and spin projection differs appreciably from the estimated experimental $A_{tot} = +0.4$ MHz for the $\{+, +, +, -\}$ option. We thus cautiously supply an alternative set of HFCs in Table 9 that may guide future simulations of hyperfine structure in magnetic Mössbauer or ENDOR spectra of H$_2$-reduced
MBH with the caveat that the strong-exchange limit may not apply here.

We note in passing that the previous DFT (PBE and B3LYP) calculations of Pandelia et al.20 for the RED and S-OX state proximal cluster overestimated the \( A_{iso} \) significantly. We assume that they also used a spin-coupling scheme \( I_{A_{iso}} \approx 9/2, \ S_{A_{iso}} = 4, S_e = 1/2 > \) for the reduced state. This, as well as several aspects of the spin-coupling for the S-OX state, remained unclear in ref 20. However, even application of the largest conceivable magnitudes of spin-projection coefficients for pure coupling schemes cannot explain the extreme overestimation of isotropic \( ^{57} \)Fe HFCs for both S-OX and RED with several sites predicted to have \( | A_{iso} | \approx 60 \text{ MHz} \). Even semiempirical scaling (not detailed in ref 20) of the intrinsic isotropic Fe HFCs56–20 could not account for such large \( A_{iso} \). The HFC anisotropies should not suffer much from deficiencies in the functionals. However, those reported in ref 20 (Table S15 in that work) are also extremely large.

5. CONCLUSIONS

Our broken-symmetry DFT calculations of Mössbauer quadrupole splittings and isomer shifts in combination with the outlined spin-coupling schemes for the proximal 4Fe-3S cluster of membrane-bound hydrogenases provide good agreement with experimental data for the broken-symmetry state BS13 for both the superoxidized and reduced clusters. A concise summary of our present results pertaining to the major features of the electronic structure (metal oxidation states and spin projection coefficients) and the established correspondence between “spectroscopic metal centers” and site-specific iron centers in the molecular structure is provided in Figure 3.

Differences in the computed Mössbauer parameters for different structural models regarding the position and protonation state of the glutamate residue near Fe4 (Glu76 in Re-MBH) are too small to allow identification of the bonding mode of this residue based on Mössbauer spectroscopy alone. However, the present calculations resolve a previous disagreement on the assignment of Mössbauer signals to Fe sites in the superoxidized state of the cluster: they solidify the assignments of Volbeda et al.7 rather than those of Pandelia et al.20 Our results for the reduced state of the cluster disagree with the latter work regarding both preferred choice of broken-symmetry state and assignment of signals to the iron sites. We note that our DFT-optimized reduced-state cluster model (RED34) is structurally much closer to the experimental data (for Re-MBH) than the one used previously. The good agreement of its computed Mössbauer parameters with experiment (in BS13 state) supports its validity (including the assignment of signals to the iron sites), even though the agreement is not as close as for the superoxidized cluster. Interestingly, our calculations suggest the same type of BS state (BS13) for both superoxidized and reduced forms of the cluster, consistent with a conservation of spin coupling during the redox-induced structural transformation of the cluster. The present work supports previous notions that Fe2 remains ferric in both redox states, whereas Fe4 likely is oxidized upon (super)oxidation of the cluster (Fe1 remains ferrous). This allows us to suggest that the “special site” (S), the most distinctive feature in the Mössbauer subspectrum of the proximal cluster in all three redox states (\( \Delta E_Q^{exp} = \pm 2.24 \text{ mms}^{-1} \) in the oxidized state), is Fe1 for all three states, in contrast to an earlier assignment. This has been possible even without using the signs of the hyperfine couplings in singling out the preferred BS state for S-OX (a comparison of computed and experimental \( N_{\text{Oxid}} \) hyperfine tensors provides additional information). We furthermore suggest that, in the superoxidized cluster, the Fe23+-Fe43+ ferric pair determines the majority spin, and the Fe12+-Fe33+ localized mixed-valence pair determines the minority spin, in contrast to the situation encountered for HiPIP’s with the same set of formal metal oxidation states.71,72

An interesting general observation of the present study is the correspondence of pairs of broken-symmetry states regarding the distribution of formal metal oxidation states, thus forming “orbital configuration partners”. Notably, the occurrence of low Fe spin populations on Fe4 in some BS states of the superoxidized cluster could be traced back to a near-degeneracy of high-spin \( (S_e = 5/2) \) and intermediate-spin \( (S_e = 3/2) \) local situations for this site. Although the spectroscopically detected species of the S-OX cluster clearly features an \( S_e = 5/2 \) character, facile \( S_e = 5/2 \rightarrow S_e = 3/2 \) spin-crossover at Fe4 is clearly supported by the present data. It is not excluded that this may be of importance in the role of the proximal cluster in enzyme function (and possibly in oxygen tolerance).

Finally, the DFT computation of Fe hyperfine tensors followed by (scalar) spin projection has provided further insights. In the case of the superoxidized cluster, the hyperfine anisotropies obtained from the spectra simulations of Pandelia et al. appear very large. In addition, our explicit calculation of exchange couplings suggests that a ferric pair is strongly antiferromagnetically coupled, and thus the associated two sites should display only weak hyperfine coupling. Again, this point contrasts with the spectra simulations. Clearly, a reconsideration of the applied-field Mössbauer spectra is warranted. For future refined spectroscopic work addressing the proximal cluster of MBH, biochemical preparation of an enzyme lacking the [Ni-Fe] active site cluster and the [4Fe-4S] distal and [3Fe-4S] medial clusters should allow for enormous simplification of the interpretation of Mössbauer spectra. In addition, \( ^{57} \)Fe ENDOR studies appear desirable; use of the PESTRE technique73 should allow for the determination of the absolute signs of \( ^{57} \)Fe HFCs, possibly with a higher precision and orientation selection compared with magnetic Mössbauer spectroscopy.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jctc.5b00854.

Test set calibrations for the calculation of isomer shifts and isotropic Fe HFCs, experimental and calculated Fe–Fe distances for the S-OX state cluster, relative energies of BS states and Mulliken spin populations of Fe sites, calculations of exchange coupling constants and projection coefficients from diagonalization of the isotropic exchange Hamiltonian + double exchange term, \( ^{57} \)Fe HFC calculation details, Mössbauer parameters for RED and S-OX B3LYP structures, and comparison of different density functionals for the calculation of quadrupole splittings for RED and S-OX models (PDF)

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