

DEVELOPMENT OF DENSITY FUNCTIONAL METHODS FOR IRON-SULFUR
CLUSTERS: APPLICATION TO THE ELUSIVE STRUCTURE OF THE
IRON-MOLYBDENUM COFACTOR OF NITROGENASE

by

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ABSTRACT

Iron-sulfur proteins are found in all forms of life, performing key functions in many of the most important biological processes, such as nitrogen fixation, respiration, DNA repair, and photosynthesis. The functional centers of these metalloproteins are iron-sulfur clusters, which are efficient electron-transfer agents, and provide a range of other functions, from catalysis to iron sensing. The main focus of this dissertation is to eliminate the structural uncertainties of one of the most complex iron-sulfur clusters, the iron-molybdenum cofactor of nitrogenase. The long-term goal of nitrogenase research is to understand all of the molecular-level details that enable biological nitrogen fixation, which may lead to alternatives to the fossil fuel-reliant, high temperature and pressure Haber-Bosch process for industrial nitrogen fixation. The research presented here is all based on computational chemistry, using primarily density functional theory with comprehensive experimental support from the literature. Computational chemistry can help explain experimental findings, and it can be used to probe structural and mechanistic details that cannot be observed through experiment; however, methods for theoretical modeling of iron-sulfur clusters must be developed before accurate results can be obtained. In this work, the best available methods (functionals, basis sets, and population analyses) are developed by comparing calculated properties of a series of model iron-sulfur complexes with experimental reference data. The newly recommended methods are then applied to a protein-embedded cluster, and the effects of the network of hydrogen bonds, dipoles, and electrostatic interactions are systematically probed. In addition to new insights regarding the role of the protein in tuning iron-sulfur cluster properties, an algorithm is derived for constructing computational models that capture all significant environmental effects. Future work will benefit from the foundations developed in these first two studies. The final work is a computational evaluation of the uncertain composition, charge, magnetic structure, and protonation state of the iron-molybdenum cofactor, based on a series of criteria defined by all available experimental data. The results show that the $[\text{Mo}^{\text{IV}}\text{-2Fe}^{\text{II}}\text{-5Fe}^{\text{III}}\text{-9S}^{2-}\text{-C}^{4-}]$ composition with hydroxyl-protonated homocitrate ligand is favored over the $[\text{Mo}^{\text{IV}}\text{-4Fe}^{\text{II}}\text{-3Fe}^{\text{III}}\text{-9S}^{2-}\text{-N}^{3-}]$ composition preferred in the literature, adding new context to the many proposed mechanisms for biological nitrogen fixation.

CHAPTER 1

INTRODUCTION

Iron-Sulfur Clusters

Iron-sulfur proteins are found in all forms of life, from microorganisms to plants and animals. They are involved in such critical biological processes as respiration,¹ photosynthesis,² nitrogen fixation,³ hydrogen metabolism,⁴ and DNA repair.⁵ It is their cofactors, the iron-sulfur clusters, that enable the diverse functions of electron transfer, small molecule activation, catalysis, and sensing of iron and oxygen.⁶ The smallest “cluster,” found in rubredoxins, consists of a single iron ion bound by four cysteine thiolates.⁷ While it is not formally a cluster due to the lack of an Fe $\cdots$ Fe interaction, its redox and iron–thiolate bond properties serve as helpful references for studies of the more typical sulfide-bridged [2Fe-2S], [3Fe-4S], [4Fe-4S], and larger clusters. Cysteines complete the tetrahedral coordination of the irons in clusters that function as electron transfer agents (see Figure 1.1), while clusters with other catalytic functions can have different coordination environments.⁶ For example, there are (Cys)₂(His)₂-ligated clusters in Rieske proteins,⁸ site-differentiated clusters with only three cysteine ligands in, for instance, SAM radical enzymes⁹ and aconitase,¹⁰ and other clusters with non-protein ligands in hydrogenase⁴ and nitrogenase.³ Catalytic functions can also be imparted by

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incorporation of heterometals, such as nickel in CO dehydrogenase and [Ni-Fe]-hydrogenase,¹¹ and molybdenum or vanadium in the nitrogenases.

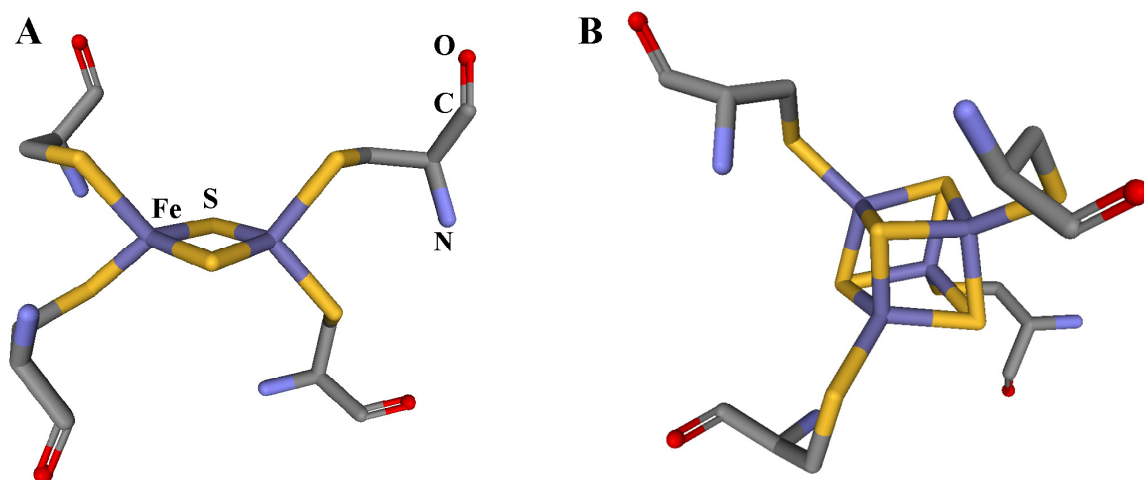


Figure 1.1. Representative cysteine-bound [2Fe-2S] (A) and [4Fe-4S] (B) clusters from [FeFe]-hydrogenase (PDB ID 3C8Y).¹²

Much research has been done on the electronic and magnetic structure of iron-sulfur clusters.¹³ The reduction potentials of iron-sulfur proteins span a remarkable range of -700 mV to 400 mV,¹⁴ due to the large range of cluster charge states and the tuning effects of the protein environment. [2Fe-2S] clusters interconvert between an oxidized 2Fe^{3+} and reduced $\text{Fe}^{3+}\text{Fe}^{2+}$ localized-valence form, which have ground spin states of $S_t = 0$ and $S_t = 1/2$, respectively, as a result of antiferromagnetic coupling of the high-spin irons.^{13, 15} Protein-bound [4Fe-4S] clusters typically access a $1+$ ($S_t = 1/2$ or $3/2$), and $2+$ ($S_t = 0$) charge state, while a $3+$ ($S_t = 1/2$) state is only observed in high potential iron proteins (HiPIP), exemplifying the control of the protein environment (discussed below). The [4Fe-4S] cubanes are structured as two ferromagnetic [2Fe-2S] rhombs that are generally valence-delocalized ($\text{Fe}^{2.5+}$) in the mixed valence state, in contrast to the [2Fe-

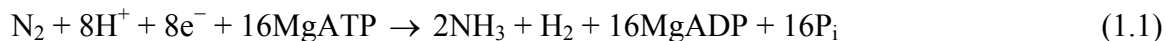
2S] clusters.^{13, 15} These rhombs are antiferromagnetically-coupled to each other through highly covalent iron–sulfur bonds that help mediate the superexchange interactions.¹⁶ The strong overlap between Fe(3d) and S(3p) orbitals also contributes to the electron transfer and catalytic abilities of iron-sulfur clusters (see Chapter 2 for further discussion).

An interesting feature of iron-sulfur proteins is the variation in reduction potentials even within those with the same type of cluster. For example, the reduction potentials of [2Fe-2S] ferredoxins lie in the approximate range of –400 to –200 mV, despite the identical cluster compositions.¹⁴ Clearly, the environment surrounding the cluster, which does vary between proteins, has an important impact on its properties. The various factors affecting iron-sulfur clusters include electrostatic interactions with charged and polar residues,¹⁷⁻²⁰ hydrogen-bonds,^{21, 22} and solvent accessibility²² (see Chapter 3). Research into protein-environmental effects and the properties of iron-sulfur clusters has been greatly aided by the use of synthetic iron-sulfur clusters as model systems.²³ These complexes alleviate the complexity inherent in proteins, providing opportunities to systematically dissect the effects of ligands, hydrogen bonds, and heterometal-substitution. Additionally, in a more practical sense, they allow for higher sample concentrations for spectroscopic studies, and simplify the protein environments in quantum chemical models to homogeneous solvent environments.

Nitrogenase

Nitrogen is an essential component of amino acids and nucleic acids and is often the limiting factor in plant growth.²⁴ Although the atmosphere is largely composed of

dinitrogen, this is not a bioavailable source due to the strength of the $\text{N}\equiv\text{N}$ triple bond; thus, dinitrogen must be first reduced, or “fixed,” to ammonia. Biological nitrogen fixation is carried out by the nitrogenase enzymes. They exist in three forms distinguished by the composition of the active site cofactor, which may contain Mo, V, or Fe.²⁵ The Mo-containing nitrogenase is the most active and most frequently studied, and will be the focus of this discussion. The enzyme consists of two components, the Fe-protein and the MoFe-protein.³ The Fe-protein acts as an MgATP-dependent electron transfer agent to the substrate-binding MoFe protein, and the overall dinitrogen fixation process proceeds with the stoichiometry shown:



The Iron-Molybdenum Cofactor

A significant constraint on our understanding of biological nitrogen fixation is that the structure of the iron molybdenum cofactor (FeMo-co) active site has not been fully determined. FeMo-co has been identified as a $[\text{Mo}-7\text{Fe}-9\text{S}-\text{X}]$ cluster (see Figure 1.2) by the atomic resolution (1.16 Å) crystal structure of the MoFe-protein, where X is an unknown interstitial atom thought to have eight valence electrons (C^{4-} , N^{3-} , or O^{2-}).²⁶ Current knowledge regarding the composition, oxidation states, spin states, and overall charge is reviewed in Chapter 4. From a coordination chemistry perspective, it is remarkable that this 18-atom cluster is linked to the protein matrix by only the Cys275 and His442 residues. An additional non-protein ligand, homocitrate, completes the hexacoordinate environment of the Mo center. Considering that there are only two residues binding FeMo-co, it is not unexpected that it can be extracted into *N*-methyl

formamide (NMF) with homocitrate still bound, an NMF^- anion replacing Cys275, and a neutral NMF molecule replacing His442.²⁷ The structure of extracted FeMo-co (exFeMo-co) has been shown by Mo K-edge extended X-ray fine structure (EXAFS) spectroscopy to be intact, with long range order in the ~ 5.1 Å Mo-Fe interaction, although the structure is slightly relaxed (0.05 Å) in the extracted form. The distances in the first coordination shell of Mo and the distances to the nearby Fe centers were also shown to be nearly identical for extracted and protein-bound cofactor.²⁸

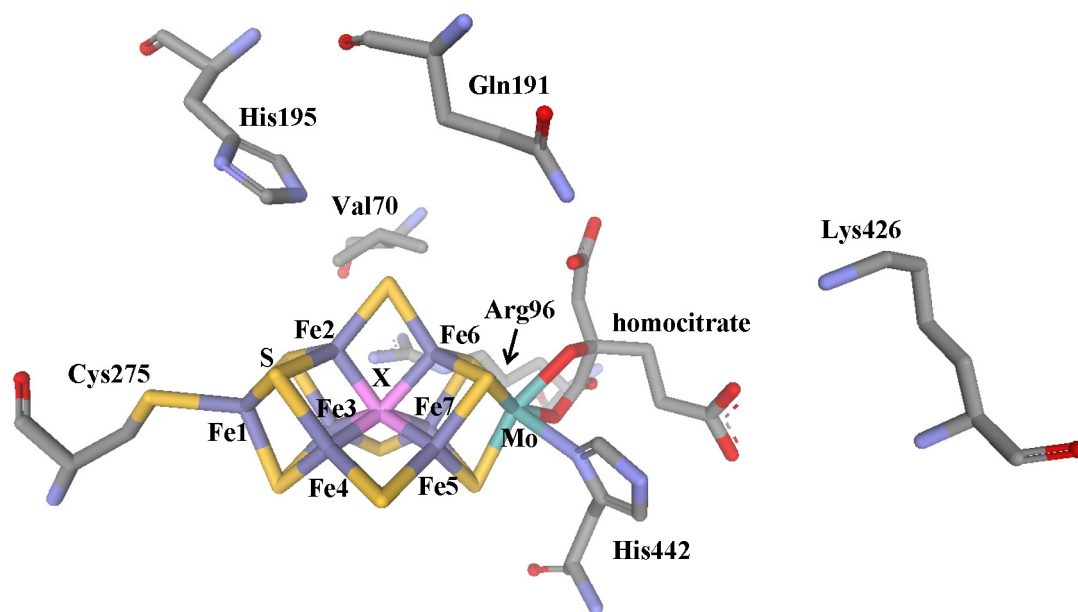


Figure 1.2. The iron-molybdenum cofactor (FeMo-co) of nitrogenase with residues shown by mutagenesis experiments to affect reactivity, as discussed in the text (coordinates from PDB ID 1M1N).²⁶ X is an unknown atom thought to be C^{4-} , N^{3-} , or O^{2-} .

Reactivity and Mechanism. The reactivity of FeMo-co extends beyond dinitrogen fixation. Alternative substrates (generally small unsaturated molecules such as azide (N_3^-) and acetylene (C_2H_2)) and inhibitors (CN^- and CO) have been employed to gain

insights into the nitrogen fixation mechanism. Acetylene is both a substrate and an inhibitor of dinitrogen reduction, and by growing cells in the presence of acetylene, a mutant was discovered with α Gly69 substituted by Ser that had decreased affinity for the larger acetylene substrate, without affecting dinitrogen reduction.²⁹ This was interpreted as a steric effect in which the adjacent residue, α Val70, blocks access to a central 4Fe face of FeMo-co. Subsequent experiments showed that substituting α Val70 with the smaller residues, Ala or Gly, allowed the binding and reduction of larger substrates, such as propargyl alcohol ($\text{HOCH}_3\text{C}\equiv\text{CH}$).³⁰ Preliminary molecular dynamics simulations show conformational changes in the α Val70 position upon approach of an N_2 molecule, consistent with its proposed gate-keeping role.³¹ These results further implicate the 4Fe face composed of Fe2, 3, 6 and 7 (as defined by crystallographic atom labeling, and shown in Figure 1.2) as a likely substrate binding site.

Studies of other MoFe protein variants with substitutions in the vicinity of FeMo-co have offered insights into the role of the protein environment. Variants with a substitution of α Arg96, also located near the same face as α Val70, bind acetylene and cyanide in the resting state, unlike the wild-type protein, indicating that α Arg96 may have a gate-keeping role.³² Substitution of another residue in the same vicinity, α His195, slows or eliminates N_2 reduction, while allowing N_2 to bind, implicating this residue in proton transfer and providing further evidence for the importance of this 4Fe face and the surrounding residues.³³ Furthermore, a putative substrate channel that leads to the same face has also been identified.³⁴ The binding site for alkyne substrates has been narrowed to Fe6 by site-directed mutagenesis studies of α His195, α Val70, and α Gln191;³⁵

however, locating the specific binding site of N_2 has proven to be more difficult. Recently, rapid freeze-quenching of a MoFe variant with a double mutation of α His195 and α Val70 enabled ^{15}N ENDOR studies of trapped intermediates of the N_2 reduction pathway. Diazene (N_2H_2) and hydrazine (N_2H_4) were shown to be trapped in either end-on or in a symmetric bridging coordination mode at the same reduced form of FeMo-co in a $S_t = 1/2$ spin state.^{36, 37} These studies lend support to the “alternating” mechanism, in which alternate N atoms are protonated in successive steps.³⁸ A less reduced intermediate has been trapped using N_2 as a substrate for the wild-type protein, and the ENDOR data were consistent with N_2 bound end-on in a terminal or bridging mode to one or more metal ions with a small spin density, such as a low-spin Fe^{II} or Fe^{III} , Fe^I , or even Mo^{IV} .³⁹

The possibility of dinitrogen binding to Mo cannot be ruled out completely, and such mechanisms are closely linked to the role of homocitrate. A strong chemical argument for the Mo site is that, to date, the only molecular system capable of catalytic N_2 reduction contains Mo.^{40, 41} MoFe protein variants with citrate instead of homocitrate lose the ability to reduce N_2 , but acetylene reduction is unaffected.⁴² Citrate only differs from homocitrate in the length of the $CH_2CH_2COO^-$ arm by removal of a methylene group. ExFeMo-co with either citrate or homocitrate and NMF has an increasing rate of reaction with thiophenolate as $[PhS^-]/[PhSH]$ ratio increases. However, when imidazole replaces NMF at the Mo site of exFeMo-co, the rate decreases with $[PhS^-]/[PhSH]$, while it is unaffected by cyanide, azide, and the proton.⁴³ This was interpreted as evidence for hydrogen bonding between homocitrate and imidazole that is not possible with the shorter arm of citrate. However, even with the longer arm, homocitrate cannot

reach the Mo-bound imidazole of His442 in the wild-type FeMo-co.⁴³ Based on evidence from model complex chemistry,⁴⁴ a mechanism was proposed that homocitrate becomes monodentate during the mechanism, hydrogen bonding to His442.⁴³ With this mechanism, N₂ could bind to the vacant Mo site of the highly reduced FeMo-co, while acetylene reduction would take place away from Mo. An experiment designed to test this hypothesis showed that substitution of α Lys426, a residue proposed to stabilize monodentate homocitrate, affected N₂ reduction without affecting acetylene reduction,⁴⁵ supporting the idea that there are different binding sites. Furthermore, Coucouvanis' model chemistry showed that a [4Fe-4S] cluster with chloride ligands was capable of acetylene reduction,⁴⁶ while Mo is needed to cleave the N₂ single and double bonds.⁴⁷ Whether Mo is the N₂ binding site, or it just modifies the electronic structure of the surrounding Fe sites to allow N₂ binding remains to be determined.

The Thorneley-Lowe Kinetic Model. Our current knowledge of the overall dinitrogen reduction mechanism is derived from the framework provided by the Thorneley-Lowe model, shown in Figure 1.3, that was based on a kinetic analysis of N₂ binding and Fe-protein turnover, i.e., electron accumulation at FeMo-co.⁴⁸ The model defines eight intermediate states for the MoFe protein, E_n, where n is the number of accumulated electrons and protons. When dinitrogen binds, FeMo-co is purported to be in a state that is three or four electrons reduced (E₃H₃ or E₄H₄) relative to the resting state (E₀), and eight electron-transfer events are needed to complete the cycle, whereas reduction of other substrates such as acetylene requires as little as two electrons. Thus, different substrates bind to different states of FeMo-co. However, considering the

accumulation of protons, the transferred electrons may not result in highly reduced Fe sites, because hydrides could form. A new view of the Thorneley-Lowe model based on this concept and the findings from Chapter 4 is presented in Chapter 5.

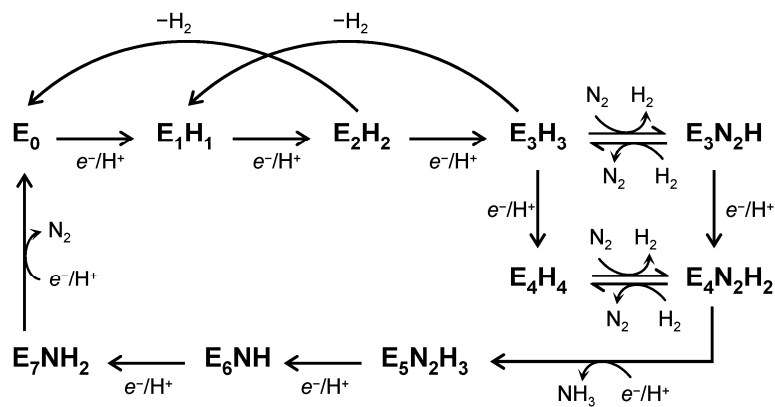


Figure 1.3. The Thorneley-Lowe kinetic model of the dinitrogen reduction mechanism, where E_n indicates the accumulation of n electrons (adapted from ref 48).

Density Functional Theory

Density functional theory (DFT) builds on the premise of the Hohenberg-Kohn theorem⁴⁹ and Kohn-Sham equations⁵⁰ by providing a connection for a chemical system between its electron density distribution and its unique total energy with respect to combinations of nuclear and electron interactions. The electrostatic interactions can be described by the classical Coulomb law or by its Hartree expressions of $J(\rho)$, where J is the Coulomb integral and ρ is the electron density.⁵¹ In practical application of DFT, the electrons are described by a three-dimensional integration grid of the topologically complex electron density. Excluding the numerical nature of handling electron density, the Coulomb interactions are conceptually identical to those considered in wave function-

based molecular-orbital (MO) methods that are often called *ab initio* MO methods. The dividing difference between DFT and *ab initio* MO methods comes from the treatment of the quantum phenomena of electron-electron exchange and correlation interactions.

Exchange Functionals

In *ab initio* MO methods, the exchange term is known exactly in the framework of orbitals and implemented by the Hartree-Fock approximation (Hartree-Fock exchange, HFX), while in DFT this is approximated by mathematical expressions that are functions of spatially dependent electron density, termed exchange functionals (density functional exchange, DFX). Improvement in DFX has been achieved by leading theoretical groups in going from early $X\alpha$ ⁵² and Slater-exchange⁵³ based local DFX functionals, through the modern Kohn-Sham first derivative-corrected semilocal DFX functionals termed generalized gradient approximations (GGA)⁵⁴ and second derivative-corrected functionals (meta-GGA),⁵⁴ to the nonlocal hyper-GGA⁵⁴ or random phase approximation (RPA)^{54, 55} with exact exchange functionals. The new RPA functionals would be attractive for application to transition metal systems if not for the prohibitive computational cost. An alternative scheme introduced by Becke^{56, 57} to correct density functionals uses hybrid exchange functionals to mix exact nonlocal HFX and pure GGA or meta-GGA DFX functionals. From a coordination chemist's point of view, this mixing is a useful tool for empirically tuning metal-ligand bonding in computational models, and thus manipulating the catalytically relevant redox active orbitals at a modest computational cost. For example, GGA functionals, such as BP86^{58, 59} often lead to overly covalent descriptions of ground state electronic structure,⁶⁰ and empirically-

derived hybrid functionals, such as B3LYP,⁵⁷ can improve accuracy. However, no single functional is known to date that works for all transition metal containing systems.

Correlation Functionals

Due to the complex nature of the correlation interaction, there is no quick fix or exact analytical formula in either *ab initio* MO or DFT methods. However, the advantage of using *ab initio* MO methods is the availability of a theoretically converging series of post-HF or post-SCF methodologies that can systematically take into account more complete treatments of dynamic and static correlation effects by approaching the complete configuration interaction limit (full CI), and thus the exact solution of the Schrödinger equation. The significant drawback is that post-HF methods come with very high computational costs (4th-7th order dependence on the size of the system defined by the number of electrons/orbitals). Even considering multi-core compute servers equipped with graphical processing units and recent developments in linear scaling methods,⁶¹ the practical applicability of higher correlation methods is limited to a few transition metal ions coordinated with highly truncated ligand models. By contrast, the density functional correlation (DFC) functionals share the acceptable cost of DFX for realistic systems. Therefore, DFT methods are currently the most practical approach for theoretical calculations involving large bioinorganic systems^{62, 63} up to approximately 300 atoms. Local, semilocal (GGA and meta-GGA), and nonlocal (e.g. hyper-GGA and RPA) DFC functionals comprise an improving series of levels of theory; however, unlike with *ab initio* MO methods, a conceptually converging series for either DFC or DFX functionals is not yet known. The above described features of *ab initio* MO and DFT methods are

graphically illustrated in Figures 1.4A and 1.4B, respectively. It is important to mention that there are exciting recent developments⁶⁴⁻⁶⁶ in DFT with long-range and dispersion correction functions that are applicable for the description of weak interactions, such as π - π stacking, hydrogen bonding, van der Waals and dispersion interactions; however, these address interactions that are at least an order of magnitude weaker than those governing the coordination chemistry of iron-sulfur clusters.

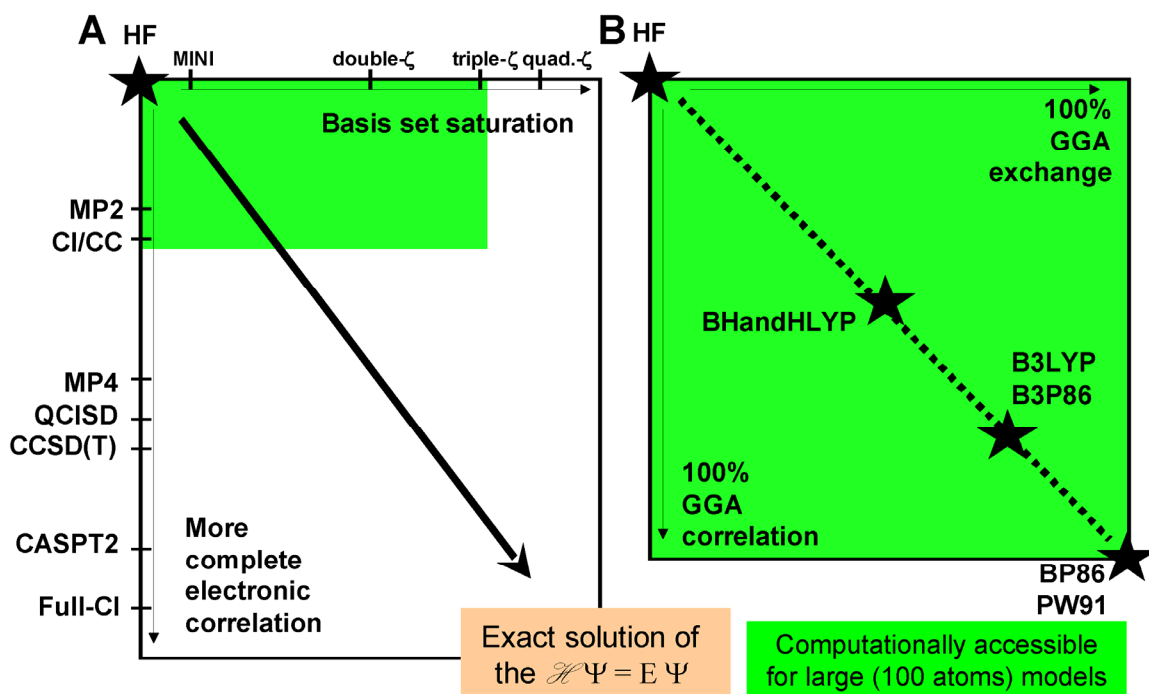


Figure 1.4. (A) Schematic demonstration of theoretically converging series of *ab initio* MO methods and (B) the relationship of hybrid and GGA semilocal DFT approximations.

Basis Sets

The size and quality of the basis set is an important input parameter for DFT calculations. The possible basis sets are Gaussian- (GTO) or Slater-type orbital (STO), numerical, or plane wave. The latter is the preferred choice for calculations with periodic

boundary conditions. Figure 1.4A illustrates on the horizontal axis of the *ab initio* MO chart a converging series of GTO basis sets as the splitting of the valence set and thus the overall basis set size increases. STO-based Kohn-Sham orbitals and other electronic structure descriptors are generally more chemically meaningful due to the hydrogenic radial distribution of STOs; however, they are more computationally expensive to integrate than GTOs. With respect to the quality of a given size basis set, there are some considerable variations, especially when it comes to the description of catalytically important frontier molecular orbitals (FMO). For example, in the case of Fe-S systems, the triple- ζ quality Ahlrich's basis set^{67, 68} with polarization functions provides a more chemically reasonable description of unoccupied FMOs than the Pople's 6-311G^{69, 70} with both polarization and diffuse functions. In order to rigorously evaluate the basis set quality and how well the basis set saturation limit is approached, *ab initio* MO theory is of great practical help. Using a small computational model relevant to a given complex bioinorganic problem up to 20 atoms, this can be readily evaluated using a series of theoretically converging series of *ab initio* MO methods with increasing size of basis set to determine the smallest large basis that reasonably reproduces the available experimental observables. Using any larger basis set would only increase the computational cost without significantly improving the electronic structure description.

Magnetic Interactions

A somewhat controversial issue, in particular for bioinorganic active centers with complex iron-sulfur architecture, is the treatment of magnetic interactions among the high-spin, magnetically-coupled iron centers. A general procedure known as the broken

symmetry approximation was introduced and used to approximate the structure of these systems by Noodleman.⁷¹ This treatment is a quick fix for the limitation of HF *ab initio* MO or DFT calculations. The unrestricted nature of the single-reference wave function or electron density is exploited by allowing different occupation or mixing of the spin-up (α) and spin-down (β) orbitals or densities, respectively. This will result in an electronic structure that is a solution of the HF or KS operator, but not the spin operator.⁷² Although M_s is well-defined, the expectation value of the spin operator ($\langle S^2 \rangle$) deviates from the ideal $S(S+1)$ according to Equation 1.2:

$$\langle \hat{S}^2 \rangle_{\text{BS}} = \left(\frac{N^\alpha - N^\beta}{2} \right) \left(\frac{N^\alpha - N^\beta}{2} + 1 \right) + N^\beta - \sum_i n_i^\alpha n_i^\beta |S_{ii}^{\alpha\beta}|^2 \quad (1.2)$$

where N^α and N^β are the number of α and β electrons, respectively, n_i^α and n_i^β are spin-orbital populations, and $S_{ii}^{\alpha\beta}$ is the overlap integral between the α_i and β_i spin-orbitals.^{73, 74} For example, the BS state of a system with two ferric iron centers ($N^\alpha = N^\beta = 5$) bridged with sulfide ligands has $\langle S^2 \rangle_{\text{BS}} \approx 5$ at the HF level, while $\langle S^2 \rangle = 0$ for the true ground state. For the magnetic interactions between the Fe centers, $|S_{ii}^{\alpha\beta}|^2$ is nearly 0 due to the superexchange pathways through the sulfide ligands. Thus, Equation 1.2 reduces to the true ground state $\langle S^2 \rangle$ plus the number of unpaired β electrons. For a two-spin system, the true ground state energy can be calculated using the total energy and the expectation values of the spin operator of a broken symmetry solution and the ferromagnetic spin state.⁷⁵ Recently, a method has been proposed to use this projected ground state for geometry optimizations, which can result in significantly different structures by more than 0.1 Å deviations in bond lengths.⁷⁶ For a polynuclear system such

as FeMo-co, a more sophisticated approximation of the true ground state based on the Heisenberg Hamiltonian can be used (see Chapter 4). Despite its conceptual limitations, the broken symmetry approach can reliably describe the electronic and geometric structures of iron-sulfur systems given a well-chosen functional and basis set.⁷⁷

Spectroscopic Calibration of DFT

The choice of functional and basis set is of critical importance to any computational study. The adequacy of various levels of theory is typically evaluated through comparison with experimental data. Instead of looking at global electron density-based structural properties, such as molecular geometry or vibrational frequencies, or even UV-vis excitation data, Mössbauer shifts, or g-values for paramagnetic systems, we are a strong proponent of using experimental orbital compositions from the core-excited state spectroscopic technique of multi-edge X-ray absorption spectroscopy (XAS).^{16, 78} The main argument is that those spectroscopic techniques are based on the total electron density, where the differences in individual orbital compositions may be hidden due to cancellations. By contrast, X-ray absorption and emission spectroscopy probe these individual unoccupied and occupied orbitals, respectively. As discussed above, custom functionals based on Becke's hybrid approach^{56, 57} can be adjusted to yield calculated covalencies, in terms of spin densities or frontier orbital compositions, that match those derived from EPR/ENDOR or X-ray absorption spectroscopy, respectively. A complication with calibrating the level of theory in this way is that the theoretical results are also dependent on the method of population analysis. Still, calibrating with experimental covalencies results in the most reasonable and economical mix of "pure"

DFX and HFX for the studied system,^{60, 77} and this method is applied and discussed in more detail in Chapters 2 and 4.

Computational/*in silico* Models

In addition to the level of theory that includes the mathematical formalism for interactions and the basis set considerations, the size and adequacy of the computational model is a key component of electronic structure calculations and theoretical chemical modeling. Truncation, protein environmental effects, and explicit or implicit solvent interactions greatly affect computational cost and accuracy. Assuming the chosen functional, basis set and method of handling magnetic interactions is adequate for describing the chemistry of iron-sulfur clusters, the results will still be inaccurate if the computational model is too small or has incorrect composition. This is particularly relevant for FeMo-co, since the presence of a central ligand was confirmed²⁶ about a decade after obtaining the first crystal structure.⁷⁹ As an example of the consequences of having an unavoidably incomplete model, Lovell et al. initially found preference for the $6\text{Fe}^{\text{II}}\text{-}1\text{Fe}^{\text{III}}$ oxidation state using an early FeMo-co model without the central ligand,⁸⁰ while the $4\text{Fe}^{\text{II}}\text{-}3\text{Fe}^{\text{III}}$ state was favored after inclusion of an anionic central ligand.⁸¹ An additional uncertainty in DFT calculations with FeMo-co is the unknown protonation state of the ligands. Truncation of ligands to save computational time can be reasonable, particularly when the covalent interactions in the cluster are preserved. For example, the uncoordinated carboxylates of homocitrate are not expected to significantly affect the Mo–O bonding, and thus homocitrate can be truncated to a glycolate moiety. Cys275 and His442 can be truncated to an alkyl thiolate and imidazole group, respectively, while

critical covalent interactions would be lost with further truncation to a sulfhydryl and ammonia group.

Protein Environmental Effects. For the most accurate geometry optimizations and calculations of properties such as reduction potentials, the protein environment must also be considered.⁸²⁻⁸⁴ An efficient first-order approximation previously applied in studies of FeMo-co^{85, 86} is to mimic the dielectric environment around the cofactor with an implicit solvent implementation, such as the polarizable continuum model (PCM).⁸⁷⁻⁸⁹ In order to introduce anisotropy, the surrounding protein environment can also be modeled by point charges,⁹⁰ or by using explicit neighboring molecules treated with molecular mechanics.^{91, 92} However, if resources and time permit, expansion of the quantum mechanically treated portion of the model is the preferred way to increase accuracy, as this is the only method that can fully capture, simultaneously, the environmental effects of hydrogen bonding, dipole, electrostatic, and steric interactions of the inner and first-outer-sphere residues in addition to the electronic interactions. We consider a computational model to be converged if additional outer-sphere residues do not significantly affect the electronic interactions within the cluster.⁹³ An alternative method for evaluating the adequacy of a model, demonstrated by Siegbahn,⁹⁴ is to calculate a potential energy profile for a reaction using PCM with various dielectric constants. If the variations between the profiles are small, the model is large enough. This method can be generalized to use any molecular property as a reference, depending on the objectives of the study. Further discussion of protein environmental effects can be found in Chapter 3, which is devoted to this topic.

Research Directions

Computational and experimental methods are synergistic in studies of iron-sulfur clusters. DFT can be used to examine mechanistic pathways, intermediates, and structural details that are difficult to observe experimentally. However, the employed level of theory, method of population analysis, and computational model must accurately describe the chemistry of protein-bound iron-sulfur clusters, and be able to reproduce the available experimental data. One of the goals of the research presented here is to define the most appropriate computational methods for use in studies of iron-sulfur clusters. This is done in Chapter 2, where various DFT functionals, basis sets, and methods of population analysis commonly used in the literature are applied to 1Fe, [2Fe-2S], and [4Fe-4S] model complexes, and the bond covalencies are compared to experimental and *ab initio* wave function-based values. The use of model complexes in Chapter 2 allows for an analysis based purely on iron-sulfur chemistry, without the additional complication of a protein environment.

In Chapter 3, recommended methods from Chapter 2 are applied to the [2Fe-2S] cluster of [Fe-Fe]-hydrogenase, and the effects of the protein environment are systematically probed. An important goal here is to define the types of interactions that must be included in a computational model of a protein-bound iron-sulfur cluster. Through this study we gain new insights into the effects of hydrogen bonds and electrostatic protein-cluster interactions, and an algorithm is derived that selects all of the functional groups in the surrounding environment with significant covalent and electrostatic effects on the cluster.

While the first two studies are focused on method development, the third study, detailed in Chapter 4, is an application of DFT to the identification of the structure of FeMo-co. The remaining structural uncertainties are impeding our progress toward a full understanding of biological nitrogen fixation. This is an important topic because today we use the fossil-fuel-reliant and unsustainable Haber-Bosch process to fix nitrogen for artificial fertilizers at high pressure and temperature. Studies of biological nitrogen fixation have the potential to give alternative means of producing bioavailable nitrogen under ambient conditions. Additionally, uncovering the mechanism of nitrogenase and FeMo-co, one of the most complex iron-sulfur clusters in biology, will enhance our understanding of this ubiquitous class of cofactors. By comparing the geometric and electronic structures of the possible compositions, charge states, and ligand protonation states with all available experimental data, the most likely structure of FeMo-co is determined.

CHAPTER 2

IRON-SULFUR BOND COVALENCY FROM ELECTRONIC STRUCTURE

CALCULATIONS FOR CLASSICAL IRON-SULFUR CLUSTERS

Introduction

Bond covalency is often used to describe the partial covalent character of a metal–ligand (M–L) bond in a coordination complex. It is a fundamental measure of electron distribution that impacts the electronic and geometric structural properties as well as reactivity of transition metal complexes. It can facilitate the formation of M–L–M superexchange pathways for antiferromagnetic coupling of paramagnetic M centers,⁹⁵ mediate long-range, rapid electron transfer,^{96, 97} aid metal centers for binding and activation of small molecules,^{98, 99} and contribute to remarkable ligand-based, non-innocent coordination chemistry.¹⁰⁰ The specific definition of covalency depends on how it was derived or determined from theory or experiment, respectively.

From a historical perspective, M–L bond covalency extended the ionic description of bonding from crystal field theory to the more detailed description from ligand field theory (LFT). The latter accounts for the covalency-induced reduction in the metal d electron-electron repulsion term and the change in the effective nuclear charge (Z_{eff}), which is also known as the nephelauxetic effect.¹⁰¹ The resulting reduction of the Racah parameters, B and C , relative to the free ion values, gives a measure of covalency. Interpretation of electronic absorption spectra and electron paramagnetic spectroscopic measurements further developed the concept of covalency through extraction of

quantitative ground-state bonding descriptions from spectral feature intensities, and the spread of g values or hyperfine coupling constants, respectively. Alternatively, the mixing of metal and ligand orbitals can be determined by valence bond configuration interaction (VBCI)¹⁰² theory in conjunction with transition energies and intensities from, for example, photoelectron spectroscopic (PES)¹⁰³ measurements. The VBCI framework is particularly useful for modeling systems with significant multiplet effects.

In this study, we will mainly focus on experimental covalencies derived from the molecular orbital-based analysis of X-ray absorption spectroscopic (XAS) measurements.¹⁰⁴ The core excited state-based XAS technique has now become a popular method to obtain direct covalency information from pre-edge feature intensities given that the appropriate core electron $\rightarrow$ valence orbital transition dipole integrals have been determined. For an Fe–S bond, the bond covalency defined by the orbital coefficients in the Fe-based, lowest unoccupied molecular orbitals (LUMOs) can be derived semiempirically from S K-edge¹⁶ (3p) and complementary Fe L-edge¹⁰⁵ (3d) X-ray absorption spectroscopic (XAS) measurements. Intense features in S K-edge spectra before the ionization threshold, or edge jump, arise from $1s \rightarrow 3p$ dipole allowed transitions. In the free ligand spectra these transitions are not observed, due to the full occupation of the S(3p) orbitals. However, if the ligand is coordinated to a transition metal with unoccupied d orbitals, a new feature (pre-edge) appears before the rising edge, which gives a direct measure of the ligand character of the LUMOs. An important limitation of this approach is that the experimental covalencies are limited to the extent of S(3p)–Fe(3d) mixing, and do not define the total Fe–S bond covalency. The Fe L-edge

provides complementary information (metal contribution to covalency) by probing the $2p \rightarrow 3d$ transitions, from which the Fe(3d) character in the LUMOs can be determined. Due to complex multiplet and spin-orbit excited state effects in the Fe L-edge spectra, S K-edge XAS is the more direct and commonly applied technique. Since the first quantitative work using S K-edge XAS to obtain bonding information for Cu(II) complexes,¹⁰⁶ there have been numerous studies of biological Fe-S clusters and model compounds,¹⁶ from iron tetrathiolate complexes to the sulfide and thiolate-containing [2Fe-2S], [3Fe-4S], [4Fe-4S], and larger¹⁰⁷ clusters.

M–L bond covalency from electronic structure calculations can be based on the ligand or metal character of orbitals, spin delocalization, Coulomb interactions or energy decomposition. Other theoretical approaches use electron sharing indexes^{108, 109} such as the Mayer¹¹⁰ and fuzzy atom¹¹¹ bond orders, and the delocalization index (DI)^{112, 113} from Bader's theory of atoms and molecules (AIM),^{114, 115} as measures of covalency in terms of shared or exchanged electron pairs. Theoretical orbital coefficient-based bond covalencies can be quantified most directly using either molecular orbital-based wave function theory (WFT) or electron density-based density functional theory (DFT).¹⁶ In addition to the treatment of the exchange and correlation interactions, a pitfall of using modern electronic structure theory to obtain information regarding M–L bond covalencies is the dependence on the method of analyzing the optimized, ground state electronic structure. The most straightforward and commonly employed¹¹⁶ method is the coefficients-squared population analysis (CSPA),¹¹⁷ which directly uses the MO coefficients to quantify the atomic contributions. The CSPA method has serious

limitations if a large Gaussian-type basis set with polarization and diffuse functions is used, due to linear dependence of the basis functions. Mulliken population analysis (MPA),¹¹⁸ which is practically the CSPA method with the addition of overlap is also commonly used, despite its well-known shortcomings of strong basis set dependence and inaccuracy in describing polar bonds due to the equal partitioning of shared electron density. This is expected to be exaggerated for M–L bonding due to the considerable electronegativity difference between the metal and the coordinated main group element. The Minimum Basis Set (MBS)¹¹⁹ Mulliken analysis was developed to overcome the basis set dependence of MPA by first projecting the orbitals from the large basis set onto a minimum basis set (STO-3G). Weinhold's natural population analysis (NPA)¹²⁰⁻¹²² assigns the density to atomic orbitals preferentially, with priority given to core and then valence orbitals. The remaining electron density is then assigned to Rydberg orbitals. Consequently, atomic properties, such as total atomic charge, orbital occupation, and spin densities derived from NPA differ depending on whether the valence or Rydberg sets include the low-lying unoccupied atomic orbitals, such as Fe 4p and S 3d.¹²³ A solely density-based approach is the Hirshfeld population analysis (HPA),¹²⁴ which distributes the density at each point according to weightings defined by the contributions of neutral atom densities. A topological approach to partitioning density is provided by AIM,^{114, 115} in which the density is divided into atomic basins defined by zero-flux surfaces without considering the specific atoms or orbitals contributing to the electron density. The density-based methods, AIM and HPA, have the benefit of being relatively insensitive to basis set effects.¹²⁵

In addition to the dependence of theoretical covalencies on population analysis, the calculated density itself varies according to the employed level of theory. A saturated basis set can be approached by increasing its size until molecular properties such as energy and bond covalency no longer change. However, choosing the most appropriate functional is less straightforward. One method is to use experimental bond covalencies from XAS or EPR measurements as a standard for calibration.⁶⁰ Previously, we carried out this procedure for the prototypical $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$ cluster, using Mulliken spin populations,⁷⁷ which can bias the calculated covalencies due to basis set sensitivity.¹²⁶ Nevertheless, the previously found calibrated level of theory, B(5%HF)P86/6-311+G(d), is preferred due to its good performance in geometry optimizations in comparison to other hybrid functionals, such as B3LYP and smaller basis sets. It is important to highlight that, due to error cancellations, a functional (e.g., B3LYP) that tends to give elongated Fe–S bond lengths, and thus an expanded Fe-S cubane, can be adjusted toward an experimentally sound geometry by using a truncated double- ζ quality basis set.

In this work we focus on the electronic structures of highly covalent iron-sulfur ([Fe-S]) clusters of metalloenzymes found throughout biology.⁶ The Fe–S bond partly derives its unique electronic and magnetic properties from the small energy difference between the Fe d and S p valence orbitals, both of principal quantum number $n = 3$. The positive Z_{eff} of the biologically relevant Fe^{3+} and Fe^{2+} ions stabilize the 3d orbitals, while the negative charges of the thiolate (S^{t}) and sulfide (S^{s}) sulfur centers destabilize the 3p orbitals, and thus reduce the energy gap and increase mixing between the donor ligand orbitals and the vacant metal d-orbitals. Pictorially the Fe–S bond can be considered as

the C-C bond of coordination chemistry. Due to the presence of the S^t-C bond, the $Fe-S^t$ bond is based on two $S(3p)$ orbitals that form pseudo- σ and π -bonds with the Fe center.¹²⁷ By contrast, the formally dianionic bridging sulfides have all three $3p$ orbitals available for bonding and are stronger donors than thiolates.¹²⁸ The μ^2 -bridging sulfides in $[2Fe-2S]$ rhombs form more covalent bonds as defined by per-bond covalency for two $Fe-S$ bonds, but their total electron donation is less than that of μ^3 -bridging sulfides in $[4Fe-4S]$ cubanes,⁹⁵ which donate to three Fe centers. The reduced per-bond covalency of μ^3 -bridging sulfides weakens the superexchange interactions between pairs of Fe centers, leading to the direct-exchange coupled, valence delocalized $Fe^{2.5+}$ pair of a $[2Fe-2S]$ rhombs within $[4Fe-4S]$ clusters, in contrast to the localized electronic structure of Fe^{2+}/Fe^{3+} pair in the mixed-valence $[2Fe-2S]$ clusters.⁹⁵

We start with the mononuclear rubredoxin model, $[Fe(S_2-o-Xyl)_2]^-$ and extend the breadth of functionals, basis sets and population analyses from the earlier study⁷⁷ to perform a more comprehensive evaluation of the sources of errors in determining $Fe-S$ bond covalency. In addition to the experimental reference covalency from XAS measurements, we use a high level, *ab initio* wavefunction method, QCISD, with a saturated basis set, to find theoretically converged reference values for orbital compositions. The favored levels of theory for the mononuclear complex are then applied to larger $[2Fe-2S]$ and $[4Fe-4S]$ clusters to further evaluate the performance of population analysis methods. For each model we also examine how the orbital coefficient-based covalencies most relevant to XAS are related to the total charge transferred from the

ligand to the metal, as well as to the covalencies derived from spin delocalization and the electron sharing index, DI.

Computational Details

Electronic structure calculations were performed with Gaussian 03 Rev E.01¹²⁹ (DFT) and Gaussian 09 Rev A.02¹³⁰ (*ab initio*). The employed functionals are given in Table 2.1. The following basis sets were used as implemented in Gaussian: LANL2DZ,¹³¹⁻¹³⁴ 6-31G,^{135, 136} 6-31+G(d),¹³⁷⁻¹⁴⁰ 6-311G,¹⁴¹⁻¹⁴⁴ 6-311G(d),¹⁴⁵ 6-311+G,^{141, 144} 6-311+G(d), aug-cc-PVTZ,¹⁴⁶⁻¹⁴⁸ aug-cc-PVQZ,¹⁴⁶⁻¹⁴⁸ def-SVP, and def-TZVP, where def refers to the original forms^{67, 68} of the Ahlrichs basis sets. The following revised Ahlrichs basis sets¹⁴⁹ were used as obtained from the EMSL Basis Set Exchange¹⁵⁰: def2-SVP, def2-TZVP, def2-TZVPP, def2-QZVP, and def2-QZVPP. The SDD-VTZ(d) basis set was formed by adding polarization functions for Fe¹⁵¹ and S¹⁵² to the Gaussian implementation of the Stuttgart-Dresden effective core potential (ECP) basis set.^{131, 153, 154} The SCF convergence criterion was 10^{-8} a.u. for DFT and 10^{-6} a.u. for QCISD.

Table 2.1. Definitions of functionals used in this study

symbol	exchange		correlation	ref
	density functional	Hartree-Fock		
B(5%HF)P86	95% Becke88	5%	Perdew86	58, 59
τ HCTHhyb	85 % τ -dependent Boese-Handy	15%	τ -dependent Boese-Handy	155
B3LYP	72% Becke88	20%	Lee-Yang-Parr and Vosko-Wilk-Nusair (III)	59, 156, 157
HSE06	75% screened Perdew-Burke-Ernzerhof	0-25% ^a	Perdew-Burke-Ernzerhof	158
PBE0	75% Perdew-Burke-Ernzerhof	25%	Perdew-Burke-Ernzerhof	159, 160
M06	73% Zhao-Truhlar	27%	Zhao-Truhlar	66
BP86	Becke88		Perdew86	58, 59
BVP86	Becke88		Vosko-Wilk-Nusair (V) and Perdew86	58, 59, 156
HCTH407	Boese-Handy		Boese-Handy	161
τ HCTH	τ -dependent Boese-Handy		τ -dependent Boese-Handy	155
M06-L	Zhao-Truhlar		Zhao-Truhlar	162
OP86	Handy-Cohen (OPTX)		Perdew86	58, 163
PBE	Perdew-Burke-Ernzerhof		Perdew-Burke-Ernzerhof	160
PBEhole	Ernzerhof-Perdew		Perdew-Burke-Ernzerhof	160, 164
PKZB	Perdew-Kurth-Zupan-Blaha		Perdew-Kurth-Zupan-Blaha	165
PW91	Perdew-Wang		Perdew-Wang	166
TPSS	Tao-Perdew-Staroverov-Scuseria		Tao-Perdew-Staroverov-Scuseria	167
VSXC	van Voorhis-Scuseria		van Voorhis-Scuseria	168

^a Distance-dependent Hartree-Fock exchange.

The computational model of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ was constructed using its crystal structure from the Cambridge Structural Database¹⁶⁹ (refcode: OXYSFE10)¹⁷⁰ and symmetrized to C_2 . Some calculations (see below) were conducted using a model truncated to $[\text{Fe}(\text{S}_2\text{C}_4\text{H}_6)_2]^-$, as in Figure 2.1. The effects of symmetrization and truncation on electronic structure properties were negligible according to DFT calculations (see Appendix A). To enable QCISD calculations with large basis sets, the model was further truncated to $[\text{Fe}(\text{SMe})_4]^-$ and $[\text{Fe}(\text{SH})_4]^-$, where the C and H positions were optimized at the BP86/def-TZVP level, while maintaining the C_2 symmetry of the original model. Coordinates for $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-}o\text{-Xyl})_2]^{2-}$ and $[\text{Fe}_2\text{S}_2(\text{SPh})_4]^{2-}$ models were taken directly from the crystal structures (XLDTSF¹⁷¹ and DOMVEG01,¹⁷² respectively). The model of $[\text{Fe}_2\text{S}_2(\text{SEt})_4]^{2-}$ was constructed in GaussView and optimized with B(5%HF)P86/6-311+G(d) in D_2 symmetry. The model of $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$ was derived from the analogous complex with 4-hydroxy-butanethiolate ligands (CESXED),⁴⁴ as in ref 77. Antiferromagnetic coupling in the [2Fe-2S] and [4Fe-4S] models was treated via the broken symmetry (BS) formalism,⁷¹ using the generalized ionic fragment approach⁷⁷ to generate an initial electronic structure. For the [4Fe-4S] model, the lowest energy BS state was chosen from the three possible states in which two high-spin, ferromagnetic, mixed-valence [2Fe-2S] rhombs are antiferromagnetically coupled.

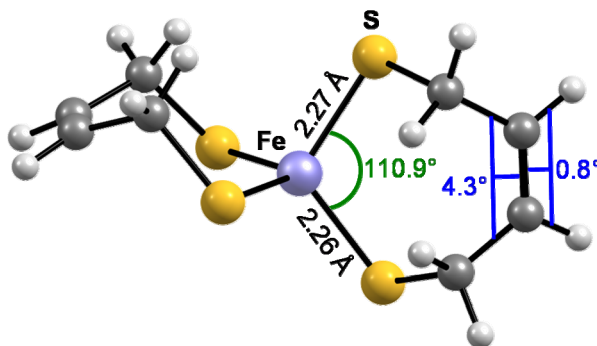


Figure 2.1. $[\text{Fe}(\text{C}_4\text{H}_6\text{S}_2)_2]^-$ -truncated computational model of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ with C_2 symmetry, based on crystallographic coordinates (OXYSE10).¹⁷⁰ Selected Fe–S bond lengths (black), a S–Fe–S angle (green) and C–C–C–C and H–C–C–H torsion angles (blue) are labeled.

Several approaches were used to derive covalency and charge transfer from CSPA, MPA, MBS, NPA, HPA and AIM. The NPA analyses were carried out with two different orbital assignments: the expanded valence set (NPA_x method), in which S(3d) and Fe(4s,4p) were treated as valence orbitals, in addition to S(3s), S(3p) and Fe(3d); and the minimal valence set (NPA_m method), with S(3d), Fe(4s,4p) and higher orbitals treated as part of the Rydberg set. All MPA, MBS and HPA analyses were carried out with Gaussian 09, with the exception of the MPA LUMO analyses, which, along with CSPA, were performed with the QMForge¹⁷³ software. AIM calculations were performed with the AIMAll software.¹⁷⁴

In order to achieve the most direct connection between experimental S K-edge XAS-derived covalencies and theoretical molecular orbital compositions, the thiolate and sulfide 3p characters in the Fe(3d)-based LUMOs were considered. Specifically, the first five LUMOs were summed for the single Fe (β only) and $[\text{2Fe-2S}]$ (α and β) models, and the first nine (or more, depending on Fe(3d) character; see DFT Fe–S Covalency of

[Fe₄S₄(SEt)₄]²⁻) α and β LUMOs for the [4Fe-4S] model. The Fe(3d) contribution was generally 1% or less to the higher energy orbitals that were not analyzed. For the MPA and CSPA LUMO analyses, the total S character was summed for each LUMO. The MBS, NPA and HPA LUMO analyses were performed by separately populating each studied unoccupied orbital without re-optimizing the electronic structure, and then comparing the gross (MBS, HPA) or S(3p) (NPA) population to that of the ground state. The AIM LUMO analyses were performed by generating wave function files for each frozen LUMO, and the atomic charges were used to obtain the total S contribution from the difference between the ground state and the stepwise occupied LUMOs. The atomic basins defined in the interatomic surface files generated from the ground state density were used for integration of the densities described by the LUMO wave function files.

Thiolate sulfur atomic spin densities were used as a separate measure of covalency, as uncompensated α electron spin emerges due to donation of β electron spin from the doubly occupied ligand donor orbitals to the β electron spin deficient, partially unoccupied metal d orbitals. Although ligand spin density is dependent on the degree of M–L bond covalency, it essentially quantifies the unequal donation of α and β spin electrons. For sulfides it was necessary to back-calculate the spin density from the iron and thiolate values, because bridging sulfides have multiple bonding partners of opposing spin polarization. Sulfide donation was also calculated by comparing the sulfur population in the optimized electronic structure to the ionic valence bond limit with a 3p⁶ electron configuration ($\Delta S(3p)$ method) and a –2 charge (Δq method).

Delocalization indexes, DI, were calculated using the AIMAll software. For a Hartree-Fock (HF) wavefunction, DI can be expressed as follows:

$$DI = 2 \sum_{i,j} S_{i,j}(A) S_{i,j}(B) \quad (2.1)$$

where $S_{i,j}$ is the overlap integral of all pairs of spin orbitals over the basin of atom A or B.¹⁷⁵ In the case of DFT, the Kohn-Sham orbitals only allow an approximation of DI, through use of HF formalism.¹⁷⁶ Despite this drawback, trends in DI at HF and post-HF levels of theory are reproduced by DFT.¹⁷⁶

Results and Analysis

Basis Set Evaluation

Finite basis sets impose a cap on the accuracy that can be achieved by a particular functional or *ab initio* MO method, but this error can be minimized by gradually approaching the basis set saturation limit at the cost of increasing computational cost. A diverse array of double- and triple- ζ Gaussian- (GTO) and Slater-type (STO) basis sets have been used in studies of Fe-S clusters and complexes. Previously, we evaluated several all-electron and effective core potential associated basis sets along with a range of density functionals and found that a hybrid density functional with a small amount of HF exchange (B(5%HF)P86) and a triple- ζ quality basis set with both polarization and diffuse functions (6-311+G(d)) are required to reproduce the experimental geometric structures, magnetic coupling, and covalencies for $[\text{Fe}_4\text{S}_4\text{SEt}_4]^{2-}$.⁷⁷ Here, we have applied the B(5%HF)P86 functional and a broader range of basis sets to a simpler Fe-S compound, $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$, in order to find the saturation limit based on total electronic

energy (E_{SCF}) for fixed, experimental molecular geometry, and to evaluate the variability in covalency. As expected, the E_{SCF} values (Figure 2.2, see Table A.1 for numerical results) generally decrease with increasing basis set size, from double- ζ (e.g., 6-31G, def-SVP) to triple- ζ (e.g., 6-311G, def-TZVP, aug-cc-pVTZ) and the largest tested quadruple- ζ (e. g., def2-QZVP, aug-cc-pVQZ). The basis set saturation limit is practically reached at the def2-QZVP level, as the E_{SCF} is almost the same with def2-QZVPP, and it actually regresses to a higher value with the much larger aug-cc-PVQZ basis set. The previous saturation limit, 6-311+G(d),⁷⁷ which was based on geometry and atomic spin densities is 727 kJ/mol higher than the new limit. Weigend and Ahlrichs¹⁴⁹ also noted that def2-QZVP can be considered the basis set limit for DFT. The results here illustrate the good performance of triple- ζ basis sets, with E_{SCF} values approaching those of the quadruple- ζ basis sets at approximately 2% of the computational cost. Based on these results, for large systems, the def-TZVP basis set can be particularly recommended due to its smaller size and lower E_{SCF} relative to 6-311+G(d). With respect to the latter basis sets, we note that polarization functions contribute more to electronic stabilization than diffuse functions, as evidenced by the 367 kJ/mol lower E_{SCF} with 6-311G(d) than with 6-311+G.

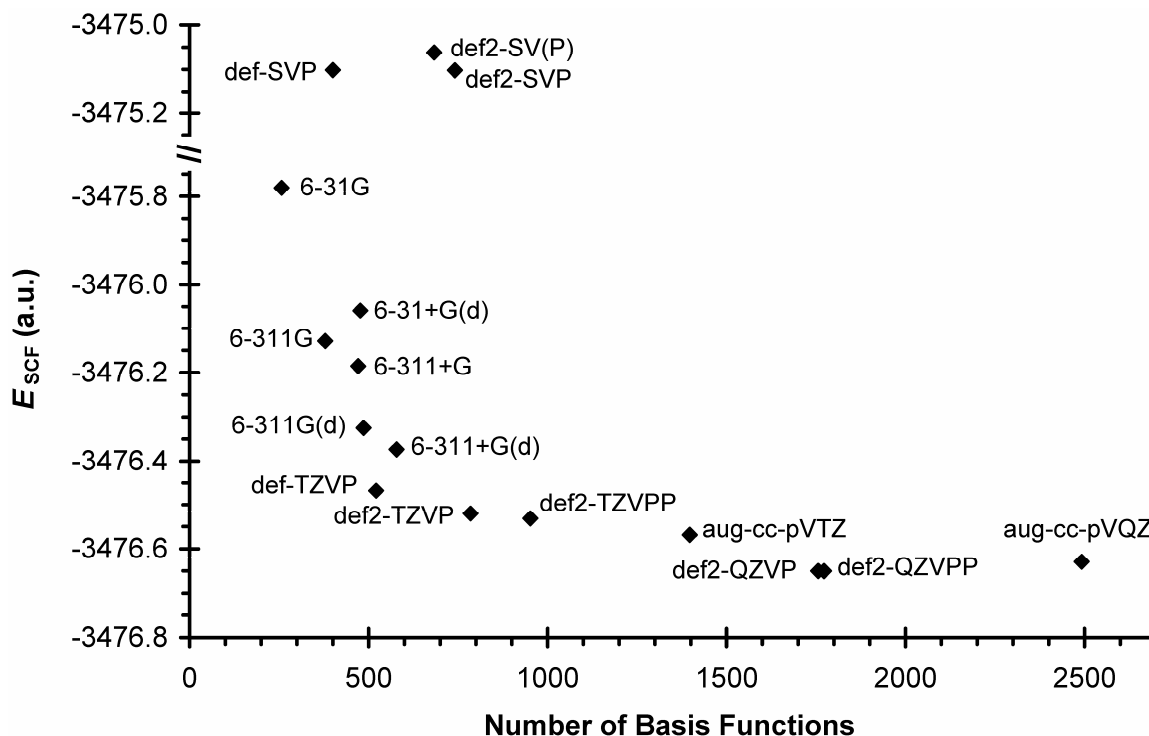


Figure 2.2. Electronic energy (E_{SCF}) of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ using the B(5%HF)P86 functional and various basis sets.

In addition to electronic energy, other properties are expected to converge with basis set saturation. However, for covalencies, the basis set dependence of population analysis methods presents an additional complication. It is well documented that CSPA and MPA analyses are among the most highly basis set-dependent, whereas the AIM method is relatively independent. Since MPA spin densities are a commonly used measure of covalency, we examined first their variation across a series of basis sets (Figure 2.3, see Table A.1 for numerical results) using B(5%HF)P86 for the $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ complex. Unlike with E_{SCF} , the trend of converging thiolate sulfur (S^{I}) atomic spin densities with increasing basis set size is less clear. The diffuse function supplemented 6-311+G and 6-311+G(d) basis sets lead to strikingly high MPA spin

densities ($\sim 0.37 e$), while the values for the double- ζ basis sets ($\sim 0.26 e$) lie significantly lower than those of the larger basis sets, which are generally in the range $0.29\text{--}0.31 e$. These effects can be attributed to the high degree of overlap of diffuse functions with those of neighboring atoms, and the relatively low amount of overlap with double- ζ basis sets. The effects of polarization functions are smaller, but can lead to a few percent increases (e.g., def2-TZVPP or 6-311G(d)) or decreases (e.g., def2-QZVPP) in S^t spin density.

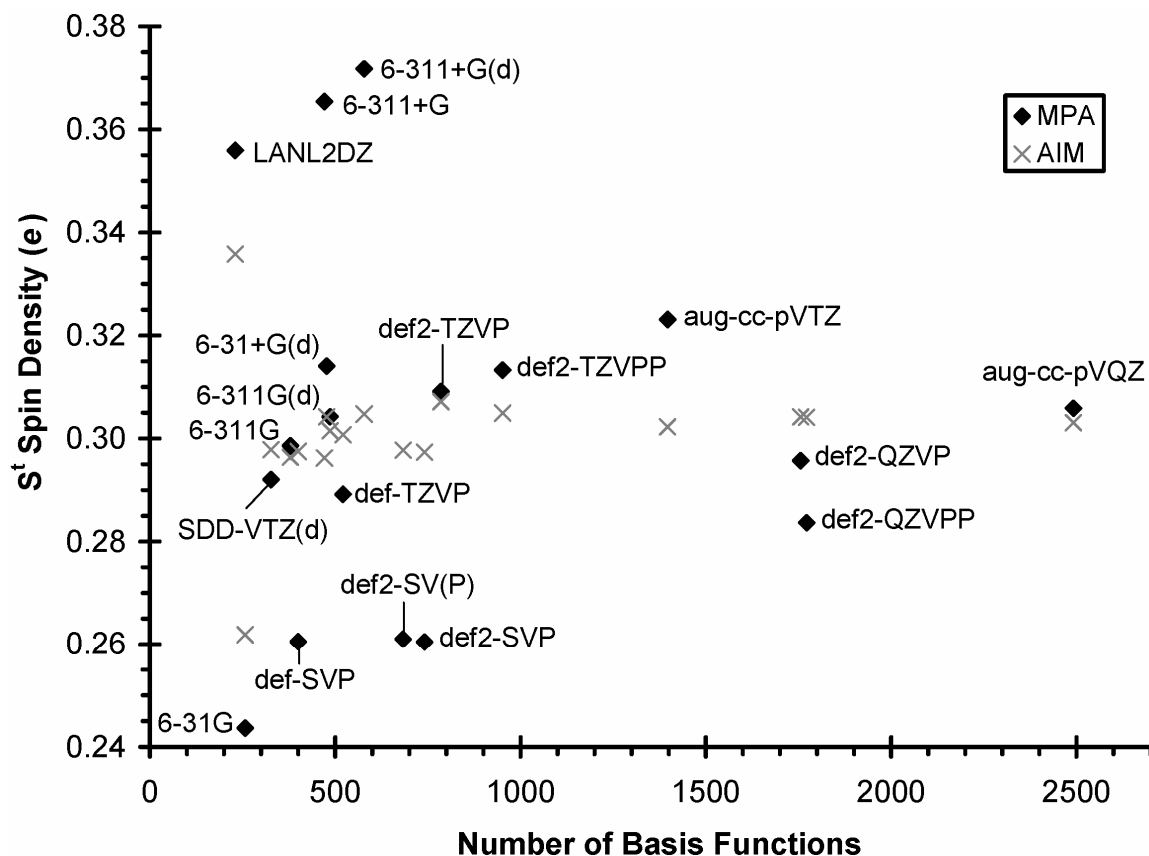


Figure 2.3. MPA and AIM spin density per S^t of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ using the B(5%HF)P86 functional and various basis sets.

It is important to dissect whether differences in spin density reflect real changes in the density and an improved bonding description or, alternatively, the shortcomings of the population analysis method. For example, the $\sim 0.01 e$ change from def2-QZVP to def2-QZVPP is expected to be entirely due to MPA basis set dependence, because the energies are nearly identical (see Figure 2.2). In order to address this, we compare in Figure 2.3 the MPA S^t spin densities to results of AIM analyses. AIM spin densities are derived directly from the electron density, and thus are expected to show a smaller basis set dependence. Indeed, the AIM spin densities for def2-QZVP and def2-QZVPP are the same. Furthermore, with the exception of 6-31G and LANL2DZ, the full series of tested basis sets lie within a small range of $0.01 e$, indicating that for this system, all basis sets larger than 6-31G give a reasonable description of the S^t electron density, including the polarization and diffuse function supplemented double- ζ basis set, 6-31+G(d).

Figure 2.4 graphically illustrates the difference in total electron density of several basis sets from a saturated basis set, def2-QZVPP, using $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ in the orientation shown in Figure 2.4A. Def2-QZVP is not pictured because there is no visible difference. 6-31G clearly provides the poorest electron density throughout the entire molecule, with depletion of electron density in the bonds (blue lobes) and localization at the nuclei (orange lobes) (Figure 2.4B). 6-31+G(d) is vastly improved, with particularly small lobes in the difference isosurface around the S^t centers (Figure 2.4D), and remarkably, the AIM S^t spin density is the same as for def2-QZVP ($0.304 e$). Def-SVP is smaller than 6-31+G(d) and the deviations from the saturated density are thus greater (Figure 2.4c). The Figure 2.4C inset shows that electron density is shifted toward the core

relative to the saturated level. The Fe sites have a more complicated structure, showing layers of deficient or excess electron density, as depicted in the Figure 2.4D inset. At the triple- ζ level there is another significant step toward saturation. C-C and C-H bonds approach the def2-QZVPP level with def-TZVP (Figure 2.4E), while the description is still quite different with 6-311+G(d) (Figure 2.4F); however, the Fe and S sites are similar between the two triple- ζ basis sets. The S^I density does deviate slightly more with def-TZVP, resulting in an AIM spin density of 0.301 e compared to the better 0.305 e with 6-311+G(d).

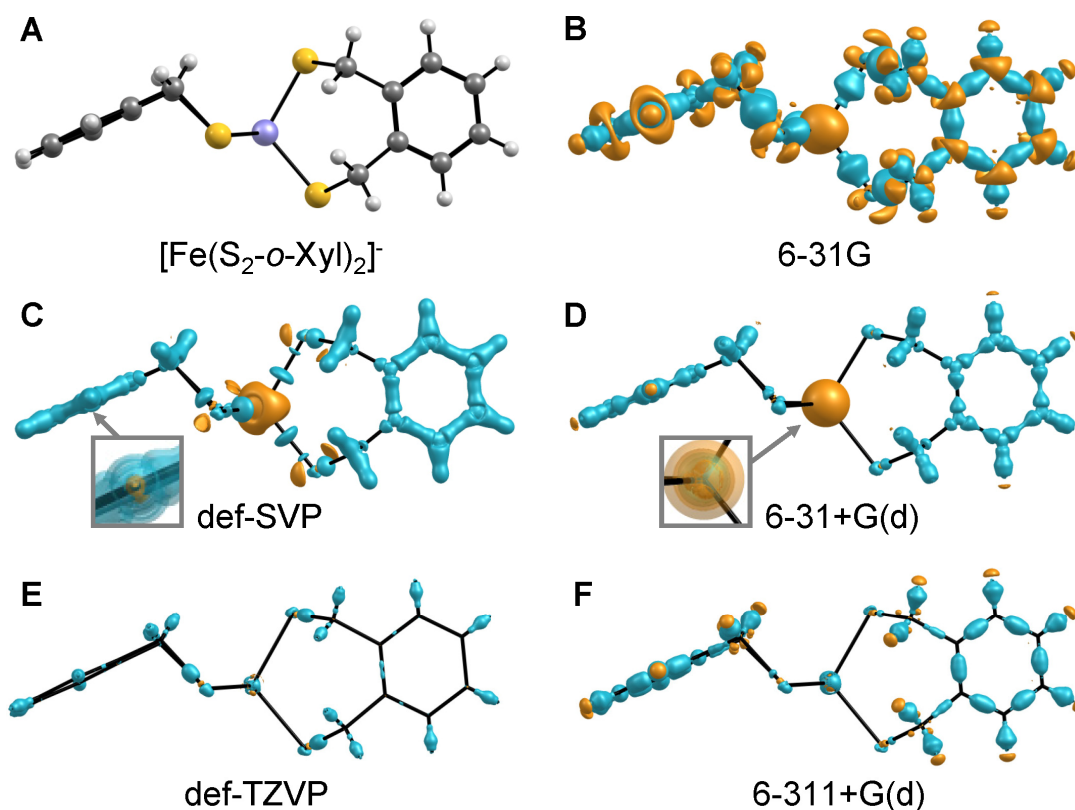


Figure 2.4. Difference electron density isosurface plots for $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ using the def2-QZVPP total density as the reference. A fixed orientation (A) is used in the plots for each basis set (B-F). Blue and orange surfaces represent regions in which the reference basis set has more or less total density, respectively, than the tested basis set. Transparent surfaces are inset for detail.

In summary, based on the electron density analysis and AIM spin densities, as well as E_{SCF} , the triple- ζ Ahlrichs basis sets can be recommended along with the 6-311+G(d) basis set, for which geometric structural error bars have been previously established.⁷⁷ Importantly, the pitfalls of using a basis-set-dependent method such as MPA spin density have been identified and we conclude that AIM results were much more consistent with the actual calculated density.

Functional Evaluation

It has also been discussed in literature^{60, 77} that orbital coefficient-based covalencies are highly dependent on the employed density functional due to changes in the optimized density. To explore the effect of functionals on covalency, we applied a variety of GGA, meta-GGA and hybrid functionals to the $[\text{Fe}(\text{S}_2\text{C}_4\text{H}_6)_2]^-$ truncated model of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ with the theoretically converged def2-TZVP basis set. The results in Figure 2.5 (see Table A.2 for numerical results) show a broad range in S^t spin density (0.24-0.32 e), with pure GGAs generally giving the highest covalencies, followed by the meta-GGAs and finally the hybrids, with some exceptions. For reference, the S^t spin density at the highly ionic Hartree-Fock level is only 0.09 e , which explains the decreasing trend in covalency from pure DFT to hybrid functionals.

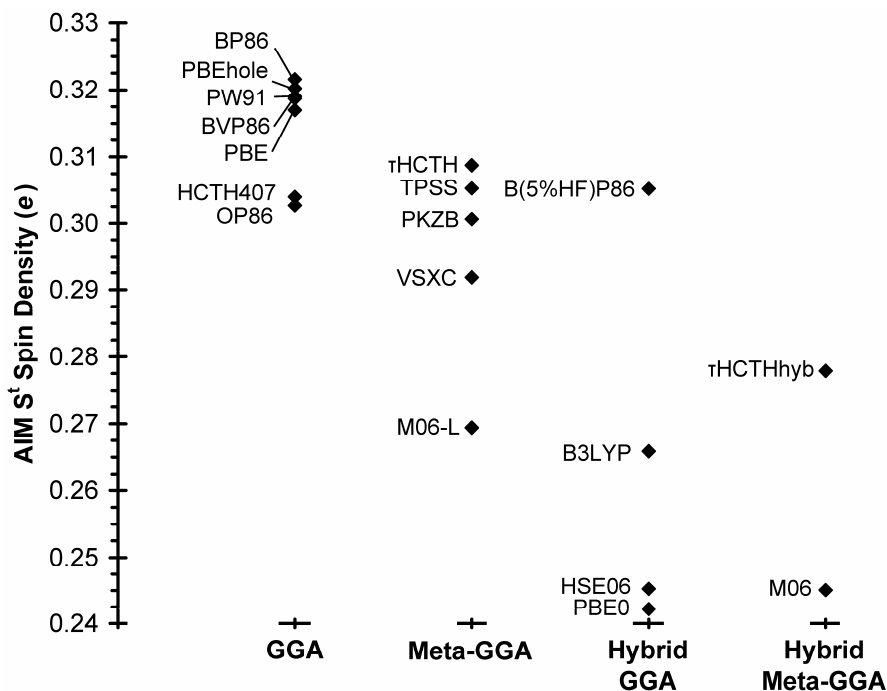


Figure 2.5. AIM spin density per S^t of $[\text{Fe}(\text{C}_4\text{H}_6\text{S}_2)_2]^-$ using the def2-TZVP basis set and various functionals.

Ab Initio Fe–S Covalency of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$

In order to evaluate the performance of density functional theory with respect to atomic spin densities, MO coefficients, and thus covalency of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$, we used the QCISD method at the basis set saturation limit. Other post-HF methods including MP2, MP4(SDQ), and CCSD were tested, but only QCISD yielded a reasonable electronic structure with the five LUMOs being Fe-based. Furthermore, for the less complex electronic structure of Cu(II) complexes, QCISD has been shown to approximate the converged level of theory limit in terms of atomic spin densities.⁶⁰ The basis set saturation limit at the QCISD level with respect to S^t spin density (AIM and MPA) and S^t character of the LUMOs (AIM) was determined using the truncated model, $[\text{Fe}(\text{SH})_4]^-$. To verify the relevance of this highly truncated model, intermediate models,

$[\text{Fe}(\text{SCH}_3)_4]^-$ and $[\text{Fe}(\text{C}_4\text{H}_6\text{S}_2)_2]^-$, were also used when computationally not prohibitive. As shown in Figure 2.6A, the AIM S^t spin densities of $[\text{Fe}(\text{SH})_4]^-$ increase with basis set size until def2-TZVPP, while the S^t characters of the LUMOs plateau at def2-TZVP (Figure 2.6B, see Table A.3 for numerical results). MPA and CSPA analyses display the same general behavior (Table A.3). The spin densities show greater deviations ($\leq 0.03 e$) between models for a given basis set than do the LUMO characters ($\leq 0.01 e$), but in both cases the deviations are constant within $0.01 e$ across the basis sets. Thus, with the assumption that the difference in covalency of $[\text{Fe}(\text{SH})_4]^-$ and $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ at the def2-SV(P) level is the same (within $0.01 e$) at the saturated def2-QZVPP level, we estimated the covalency of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ for each population analysis method. The resulting S^t covalency from AIM and MPA spin densities is $0.30 \pm 0.01 e$, while AIM LUMO analysis gives $0.28 \pm 0.01 e$.

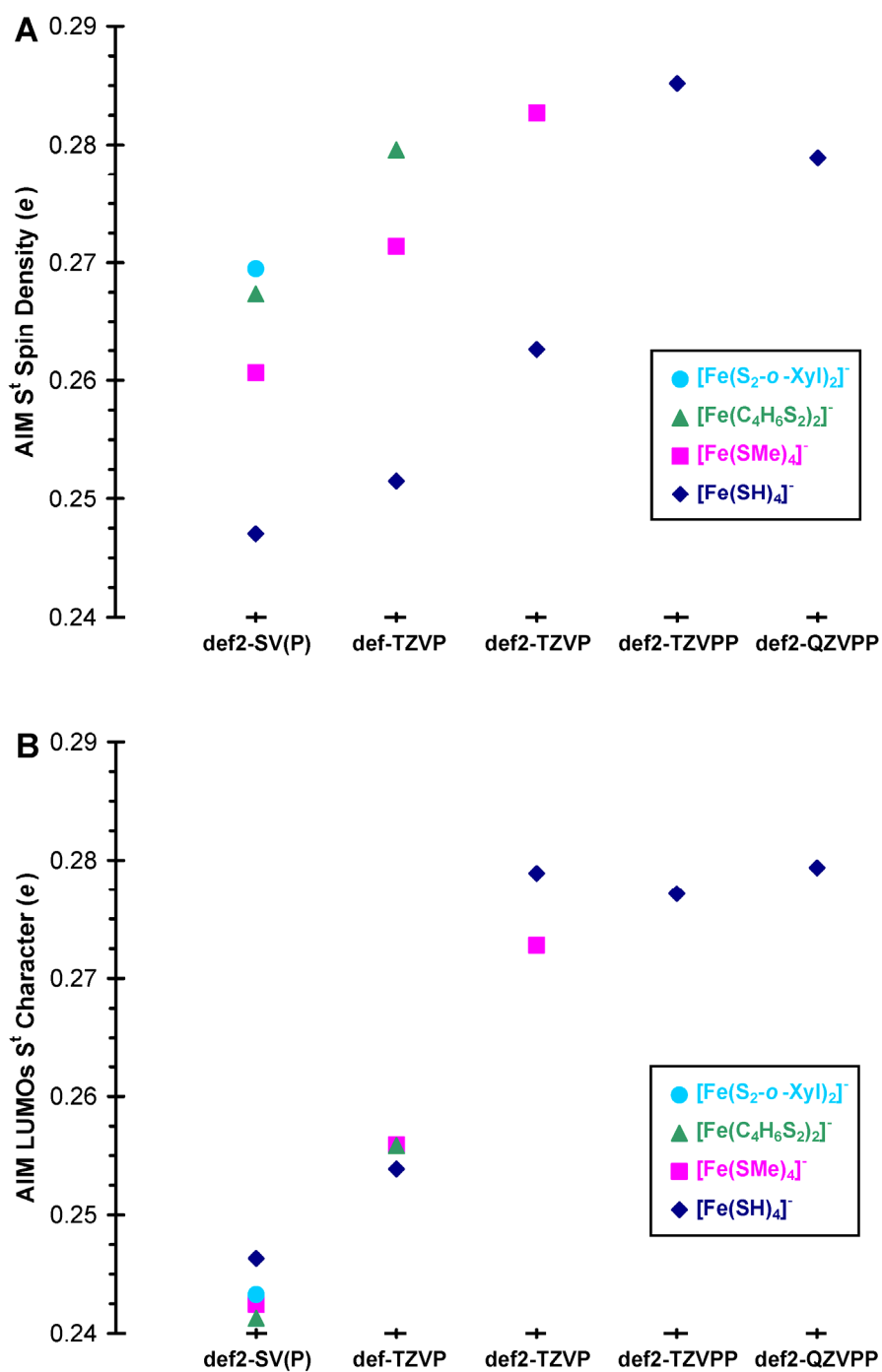


Figure 2.6. Covalency per S^t of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ and three truncated models using QCISD with various basis sets, and analyzing with A) AIM spin densities or B) AIM LUMO compositions.

DFT Fe–S Covalency of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$

Table 2.2 presents DFT S^t spin densities and $S(3p)$ compositions of the LUMOs of the $[\text{Fe}(\text{C}_4\text{H}_6\text{S}_2)_2]^-$ truncated model. The BP86 and B3LYP results with the saturated basis set, def2-QZVP, show the range of covalencies (0.26–0.45 e) that can be found from various population analyses. NPA, HPA and AIM spin densities are somewhat more sensitive to functionals than MPA, with deviations between BP86 and B3LYP of 0.06 e , 0.05 e , and 0.06 e , respectively, compared to 0.04 e with MPA. NPA LUMO compositions are the most sensitive in this respect (0.09 e), followed by CSPA (0.07 e), MPA (0.04 e), MBS (0.03 e), AIM (0.03 e) and HPA (0.02 e) LUMO analyses. Interestingly, the NPA LUMO compositions are significantly more functional-dependent than the corresponding spin densities, while the trend is reversed for the MBS, HPA and AIM methods.

Focusing on the BP86/def2-QZVP results, MPA, MBS, HPA and AIM LUMO analyses (0.38 e , 0.31 e , 0.36 e , and 0.37 e , respectively) give a much more covalent description than the respective spin densities (0.31 e , 0.27 e , 0.32 e , and 0.32 e). This is expected, because spin density increases with β electron donation but decreases with α donation, while in the LUMO analysis only β electrons are considered. The most ionic description is given by MBS, while NPA spin densities (0.43 e) and LUMO compositions (0.45 e or 0.42 e) give the highest covalencies. Switching to the more practical def-TZVP and 6-311+G(d) basis sets exposes the basis set dependence and reliability of the results.

Table 2.2. Covalency per S^t of the [Fe(C₄H₆S₂)₂][−]-truncated computational model of [Fe(S₂-*o*-Xyl)₂][−] using various functionals, basis sets, and population analysis methods defined in the Computational Details

functional	basis set	S atomic spin density						S contribution to LUMO-LUMO+4						
		MPA	MBS	NPA _m	NPA _x	HPA	AIM	CSPA	MPA	MBS	NPA _m	NPA _x	HPA	AIM
BP86	def2-QZVP	0.31	0.27	0.43	0.43	0.32	0.32	0.33	0.38	0.31	0.45	0.42	0.36	0.37
B3LYP	def2-QZVP	0.27	0.22	0.37	0.38	0.27	0.26	0.26	0.35	0.28	0.36	0.34	0.34	0.34
BP86	6-311+G(d)	0.38	0.27	0.43	0.42	0.32	0.32	0.25	0.35	0.31	0.45	0.43	0.36	0.36
B(5%HF)P86	6-311+G(d)	0.37	0.25	0.42	0.41	0.31	0.30	0.24	0.34	0.30	0.43	0.41	0.35	0.35
BP86	def-TZVP	0.30	0.26	0.36	0.33	0.31	0.31	0.25	0.33	0.30	0.36	0.33	0.35	0.35
B(5%HF)P86	def-TZVP	0.29	0.25	0.34	0.32	0.30	0.30	0.24	0.32	0.29	0.34	0.32	0.34	0.34
BP86	SDD-VTZ(d)	0.30	0.26	0.41	0.40	0.31	0.31	0.29	0.32	0.30	0.44	0.41	0.35	0.36
QCISD	extrapolated	0.30					0.30							0.28

As noted above, MPA spin densities are high with 6-311+G(d) (0.38 e ; 0.30 e with def-TZVP), and we now see that MPA and CSPA LUMO compositions are also strongly basis set-dependent. With NPA, the 6-311+G(d) results are within 0.01 e of the def2-QZVP values, while the def-TZVP results are 0.07–0.10 e lower. By contrast, the MBS, HPA and AIM spin density and LUMO methods are close to being basis set independent, each displaying a maximum deviation of 0.02 e between the tested basis sets. Despite the differences in density partitioning of HPA and AIM, the S^t spin densities and LUMO compositions are remarkably similar, likely because these quantities are derived from relative differences (i.e., $\alpha - \beta$, or LUMO – ground state). Notably, the effective core potential basis set, SDD-VTZ(d), can be recommended when higher efficiency is required, as the results differ by less than 0.02 e from those of the tested all-electron basis sets in most cases. In comparison to the QCISD results, BP86 AIM spin densities deviate by 0.02 e , while the more ionic B(5%HF)P86 functional matches QCISD. However, AIM LUMO compositions show at least 0.06 e higher covalency with the tested DFT methods than QCISD.

DFT Fe–S Covalency of [2Fe-2S] Clusters

Table 2.3 presents S^t and S^s covalencies of $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-}o\text{-Xyl})_2]^{2-}$, $[\text{Fe}_2\text{S}_2(\text{SPh})_4]^{2-}$ and $[\text{Fe}_2\text{S}_2(\text{SEt})_4]^{2-}$ represented by spin densities and LUMO compositions at the B(5%HF)P86/6-311+G(d) level. S^s covalencies were additionally derived by comparing the charge (MBS, NPA, HPA or AIM) or the S(3p) population (NPA) to the ionic limit (–2 charge; 6 e S(3p) population). Similar analyses could not be carried out for the S^t due to the presence of a somewhat polar S–C bond that withdraws electron density from the

formally -1 thiolate sulfur. The results from MPA and CSPA LUMO analyses are inconsistent and unreliable, especially for S^t , as they do not give the expected electronic structure of five Fe-based α and β LUMOs that we observe with the other methods. It is important to emphasize that the electronic structure of the $[2Fe-2S]$ cluster is considerably more complex than that of the mononuclear Fe tetrathiolate complex, due to the antiferromagnetic coupling between the two high-spin Fe centers, which we model with a broken symmetry treatment. According to MBS, NPA, HPA, and AIM spin densities and LUMO compositions, the S^t covalency is highest in $[Fe_2S_2(S_2-o-Xyl)_2]^{2-}$ and lowest in $[Fe_2S_2(SPh)_4]^{2-}$, which, in compensation, has the highest S^s covalency. The covalencies are very different depending on the method used, and the trends are not always the same. For example, the MPA S^t spin density is highest for $[Fe_2S_2(SET)_4]^{2-}$ instead of $[Fe_2S_2(S_2-o-Xyl)_2]^{2-}$. The method dependence is different for sulfides and thiolates in that all population analyses, except MPA, show higher S^s covalencies from spin densities than LUMO analyses, while for thiolates the opposite is true. The low S^t spin densities can be attributed to a pitfall of using spin densities to derive covalency that emerges due to donation of both α and β electron density to a different extent. Since the derivation of S^s covalencies in the spin density method involves subtracting the low S^t spin densities from the Fe spin densities, the result is high S^s covalencies.

Table 2.3. Covalency per S^t or per S^s per Fe of three [2Fe-2S] clusters using B(5%HF)P86/6-311+G(d) and various population analysis methods defined in the Computational details

model		S atomic spin density						S contribution to LUMO–LUMO+9						
		MPA	MBS	NPA _m	NPA _x	HPA	AIM	CSPA	MPA	MBS	NPA _m	NPA _x	HPA	AIM
[Fe ₂ S ₂ (S ₂ - <i>o</i> -Xyl) ₂] ²⁻	S ^s	0.64	0.46	0.69	0.70	0.59	0.55	0.26	0.46	0.38	0.62	0.59	0.43	0.44
[Fe ₂ S ₂ (SPh) ₄] ²⁻	S ^s	0.71	0.49	0.73	0.73	0.62	0.59	0.25	0.53	0.39	0.65	0.62	0.45	0.46
[Fe ₂ S ₂ (SEt) ₄] ²⁻	S ^s	0.61	0.45	0.68	0.69	0.58	0.54	0.29	0.46	0.38	0.62	0.59	0.43	0.44
[Fe ₂ S ₂ (S ₂ - <i>o</i> -Xyl) ₂] ²⁻	S ^t	0.28	0.19	0.33	0.32	0.23	0.23	0.08	0.10	0.27	0.36	0.35	0.32	0.32
[Fe ₂ S ₂ (SPh) ₄] ²⁻	S ^t	0.28	0.17	0.30	0.29	0.20	0.20	0.13	0.05	0.23	0.30	0.29	0.27	0.27
[Fe ₂ S ₂ (SEt) ₄] ²⁻	S ^t	0.29	0.19	0.32	0.31	0.22	0.22	0.36	0.32	0.26	0.34	0.33	0.30	0.30

model		Δq					$\Delta S(3p)$	
		MBS	NPA _m	NPA _x	HPA	AIM	NPA _m	NPA _x
[Fe ₂ S ₂ (S ₂ - <i>o</i> -Xyl) ₂] ²⁻	S ^s	0.78	0.63	0.92	0.82	0.61	0.61	0.79
[Fe ₂ S ₂ (SPh) ₄] ²⁻	S ^s	0.80	0.67	0.96	0.84	0.64	0.65	0.82
[Fe ₂ S ₂ (SEt) ₄] ²⁻	S ^s	0.78	0.62	0.91	0.82	0.61	0.61	0.78

The Δq and $\Delta S(3p)$ methods are direct measures of charge transfer which are expected to correlate with S^s covalency, and they provide for insightful comparisons of population analyses, particularly with respect to AIM, which can be considered the conceptually most rigorous method for charges. The total charge transfers (Δq) from MBS (0.78–0.80 e) and HPA (0.82–0.84 e) are similar, while being much higher than the AIM results (0.61–0.64 e). This contrasts sharply with the S^s covalencies from spin densities and LUMO compositions, which are similar with HPA and AIM (0.01–0.04 e deviation) and lowest with MBS (0.06–0.10 e lower than AIM). The differing density partitioning schemes of HPA and AIM have a strong influence on the Δq results because the Δq method is based on total rather than relative populations. AIM and NPA_m charges are remarkably similar, with Δq results differing by less than 0.03 e from each other (this relationship is basis set-dependent; see DFT Fe–S Covalency of $[Fe_4S_4(SET)_4]^{2-}$), while NPA_x results are about 0.30 e higher. NPA_m and NPA_x again differ considerably (0.17–0.18 e deviation) with the $\Delta S(3p)$ method, due to increased allocation to the Fe(4s,4p) AOs when they are treated as part of the valence set in NPA_x . Comparing the Δq and $\Delta S(3p)$ methods, NPA_m results are consistent within 0.02 e , while the NPA_x results differ by about 0.13 e . This indicates that with NPA_m nearly all of the electron donation to the Fe centers is from the $S^s(3p)$, while with NPA_x there is significant involvement of other S^s AOs in bonding, such as $S(3s)$.

DFT Fe–S Covalency of $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$

Table 2.4 presents S^t and S^s covalencies and charge transfer values for $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$ calculated using two functionals (B(5%HF)P86 and B3LYP) and two basis sets (6-311+G(d) and def-TZVP). As with the $[\text{2Fe-2S}]$ clusters, S^s charge transfer from the Δq method is consistent within 0.02 e between AIM and NPA_m when using the 6-311+G(d) basis set; however, this does not hold for def-TZVP. Comparison of the B(5%HF)P86 AIM Δq results for 6-311+G(d) (0.40 e) and def-TZVP (0.40 e) reveals no basis set dependence, while the NPA_m values decrease from 0.42 e with 6-311+G(d) to 0.33 e with def-TZVP, resulting in a 0.07 e deviation from AIM. Spin densities and LUMO compositions again show almost no basis set dependence with AIM (with the exception of S^t LUMO contributions, explained below), while NPA_m S^s and S^t values are at least 0.07 e lower with def-TZVP than 6-311+G(d). In contrast to the Δq results, spin densities and LUMO compositions from AIM and NPA deviate less from each other with def-TZVP than 6-311+G(d). Thus, from this information alone it is difficult to determine the basis set for which NPA produces the most accurate results. MBS and HPA are equally as basis set independent as AIM, and the trends among covalencies and charge transfer values are the same as for the $[\text{2Fe-2S}]$ clusters.

A critical factor influencing the LUMO compositions in Table 2.4 is the deviation in the number of LUMOs with significant Fe(3d) character (>3%) as a function of the employed functional and basis set. According to NPA_m, only def-TZVP produces the expected 9 Fe(3d)-based α and β LUMOs, while there are 10 and 13 orbitals that can be considered Fe-based at the B(5%HF)P86/6-311+G(d) and B3LYP/6-311+G(d) levels,

respectively. NPA_x gives the same number of Fe-based orbitals as NPA_m when using B(5%HF)P86 with def-TZVP or 6-311+G(d), but there are three less at the B3LYP/6-311+G(d) level. The LUMOs shown to be Fe(3d)-based by NPA_m were used in the other population analyses, giving unusually basis set-dependent results for S^t, due to the varying number of LUMOs. This also resulted in higher covalencies with B3LYP than B(5%HF)P86, which is likely an error, since hybrid functionals with more HF exchange are expected to give less covalent bonding. When instead of using all of the LUMOs shown by NPA_m to be Fe(3d)-based, only the first 9 LUMOs are used, AIM gives total S^s contributions of 0.27 *e* and 0.23 *e* for B(5%HF)P86 /6-311+G(d) and B3LYP/6-311+G(d), respectively, instead of 0.29 *e* and 0.33 *e*. While the 9-LUMO-derived S^s covalencies show the expected trend with respect to the HF exchange, the S^t covalencies are still 0.02 *e* higher with B3LYP than B(5%HF)P86. AIM analysis of the Fe contributions to the LUMOs shows that at the B(5%HF)P86/6-311+G(d) level the Fe character averages 0.69 *e* in the first 9 LUMOs before decreasing to 0.14 *e* in LUMO+9 and less than 0.02 *e* thereafter, while with B3LYP the Fe character does not become less than 0.02 *e* until LUMO+16. Due to the orbital mixing with B3LYP, and to a lesser extent, 6-311+G(d), the LUMO-based analysis is only reliable for B(5%HF)P86/def-TZVP. As for the MPA and CSPA LUMO analyses, B(5%HF)P86/def-TZVP is again the only tested method that shows the expected 9 Fe-based LUMOs, and the covalencies from this level of theory are the most consistent with those from the other population analyses.

Table 2.4. Covalency per S^t or per S^s per Fe of [Fe₄S₄(SEt)₄]⁻ using B(5%HF)P86 or B3LYP and 6-311+G(d) or def-TZVP and various population analysis methods defined in the Computational Details

functional	basis set		S atomic spin density						S contribution to LUMOs						
			MPA	MBS	NPA _m	NPA _x	HPA	AIM	CSPA	MPA	MBS	NPA _m	NPA _x	HPA	AIM
B(5%HF)P86	6-311+G(d)	S ^s	0.43	0.31	0.49	0.50	0.41	0.38	0.21	0.24	0.26	0.42	0.38	0.28	0.29
B(5%HF)P86	def-TZVP	S ^s	0.36	0.31	0.41	0.39	0.40	0.37	0.22	0.27	0.23	0.31	0.29	0.27	0.28
B3LYP	6-311+G(d)	S ^s	0.32	0.20	0.39	0.40	0.29	0.27	0.23	-0.27	0.30	0.35	0.28	0.27	0.33
B(5%HF)P86	6-311+G(d)	S ^t	0.22	0.15	0.29	0.26	0.19	0.19	0.49	-0.04	0.34	0.39	0.38	0.41	0.42
B(5%HF)P86	def-TZVP	S ^t	0.16	0.14	0.22	0.19	0.19	0.18	0.28	0.30	0.30	0.32	0.30	0.33	0.33
B3LYP	6-311+G(d)	S ^t	0.22	0.14	0.27	0.26	0.18	0.17	0.59	-0.91	0.39	0.32	0.26	0.60	0.74

			Δq					$\Delta S(3p)$	
			MBS	NPA _m	NPA _x	HPA	AIM	NPA _m	NPA _x
B(5%HF)P86	6-311+G(d)	S ^s	0.57	0.42	0.66	0.57	0.40	0.41	0.56
B(5%HF)P86	def-TZVP	S ^s	0.57	0.33	0.60	0.57	0.40	0.33	0.52
B3LYP	6-311+G(d)	S ^s	0.55	0.38	0.62	0.56	0.37	0.36	0.51

Comparison of Theoretical and XAS Covalencies

Sulfur K-edge XAS gives a S^t covalency of $0.41 \pm 0.03 e$ per Fe– S^t bond for $[Fe(S_2\text{-}o\text{-Xyl})_2]^-$ after taking into account the revised¹⁷⁷ S^t $1s \rightarrow 3p$ transition dipole integral.^{178, 128} From AIM and HPA analyses, the calculated per-thiolate covalencies are too ionic, with the highest spin densities being $0.32 e$ at the BP86/def2-QZVP level (see Table 2.2), although at $0.36\text{--}0.37 e$, the LUMO analyses at the same level of theory approach the experimental value reasonably well. The MBS results are about $0.05 e$ less covalent, and thus further from the experimental result. It is important to realize that basis set dependencies allow a combination of MPA and NPA analyses and levels of theory to produce covalencies that fall within the experimental error. For example, when using diffuse functions, MPA gives spin densities at the BP86/6-311+G(d) level that reach a relatively high value of $0.38 e$. NPA spin densities and LUMO compositions are also favorable with BP86 or B(5%HF)P86 and def2-QZVP, 6-311+G(d) or SDD-VTZ(d) ($0.40 - 0.45 e$) but the results are too ionic with def-TZVP ($0.32\text{--}0.36 e$). The high covalencies from the basis sets other than def-TZVP are likely in error, since the much less basis-set-dependent AIM and HPA spin densities and LUMO compositions are in the range $0.30\text{--}0.37 e$ for BP86 or B(5%HF)P86 and all tested basis sets, which is consistent only with the def-TZVP results from NPA. QCISD gives low covalencies (AIM and MPA) relative to the experiment, which could arise from the limitation of the computational model used and the reliability of the extrapolated values.

The XAS-derived covalencies per S^s per Fe for the $[2Fe\text{-}2S]$ clusters are given in Table 2.5. The S^s covalency of $[Fe_2S_2(SET)_4]^{2-}$ was revised from $0.78 e$ in ref 128 to 0.88

e in ref 179 due to a new background subtraction procedure; thus, we scaled the S^s covalencies of $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-}o\text{-Xyl})_2]^{2-}$ and $[\text{Fe}_2\text{S}_2(\text{SPh})_4]^{2-}$ from ref ¹²⁸ by 1.13. All of the calculated Fe- S^s covalencies (0.44–73 e , see Table 2.3 spin density and LUMO compositions) are much lower than the experimental values (0.88–1.13 e). The Δq and $\Delta S(3p)$ results from MBS, HPA and NPA_x (0.78–0.96 e) are in the range of the experimental covalencies, but these theoretical values need to be taken only as an approximation of the experimental values, since they represent total atomic electron donation and not just the S(3p) or Fe(3d) characters. The experimental covalencies of the S^t centers (0.24–0.31 e) are more readily reproduced by theory, with each population analysis except CSPA giving at least a reasonable agreement. Notably, no single method gives covalencies approaching the experimental values for all three models simultaneously, as the effect of the thiolate composition on the theoretical S^s and S^t covalencies is much smaller than that shown by experiment. Furthermore, $[\text{Fe}_2\text{S}_2(\text{SPh})_4]^{2-}$ has the lowest experimental S^s covalency (0.81 e), but the highest theoretical values of the three models. The dominant factors contributing to the high theoretical S^s covalency of $[\text{Fe}_2\text{S}_2(\text{SPh})_4]^{2-}$ are that (1) the Fe- S^s bond lengths are on average 0.01 Å and 0.02 Å shorter than in the models of $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-}o\text{-Xyl})_2]^{2-}$ and $[\text{Fe}_2\text{S}_2(\text{SEt})_4]^{2-}$, respectively, and (2) the S^s ligands donate more electron density to compensate for the S^t covalency, which is calculated to be weakest for $[\text{Fe}_2\text{S}_2(\text{SPh})_4]^{2-}$, likely due to the electron withdrawing nature of the phenyl group. The calculations do agree with experiment in that $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-}o\text{-Xyl})_2]^{2-}$ has the highest S^t covalency. When S^s and S^t covalencies are summed to give total covalencies per Fe, we find 1.52, 1.48, and

1.47 e for $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-}o\text{-Xyl})_2]^{2-}$, $[\text{Fe}_2\text{S}_2(\text{SEt})_4]^{2-}$, and $[\text{Fe}_2\text{S}_2(\text{SPh})_4]^{2-}$, respectively, from AIM LUMO analysis. Relative to the XAS total covalencies of 2.88, 2.24, and 2.20 e , these computational results are too ionic, and the differences between complexes are too small, but they do follow the same trend.

Table 2.5. Published covalency per S^{t} or per S^{s} per Fe bond of all models examined in this study from S K-edge X-ray absorption spectroscopy

	S^{s}	S^{t}	refs
$[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$		$0.41 \pm 0.03^{a,b}$	178, 128
$[\text{Fe}_2\text{S}_2(\text{S}_2\text{-}o\text{-Xyl})_2]^{2-}$	1.13 ± 0.07^c	0.31 ± 0.02^a	128
$[\text{Fe}_2\text{S}_2(\text{SPh})_4]^{2-}$	0.81 ± 0.07^c	0.29 ± 0.03^a	128
$[\text{Fe}_2\text{S}_2(\text{SEt})_4]^{2-}$	0.88 ± 0.05	$0.24 \pm 0.02^{a,b}$	179
$[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$	0.41 ± 0.02^b	$0.39 \pm 0.03^{a,b}$	180

^a Scaled from published values by 8.05/8.47 due to revised¹⁷⁷ dipole integral.

^b Uncertainty estimated to be minimum of 6% per Fe– S^{t} or Fe– S^{s} bond.

^c Scaled from published values by 0.88/0.78 due to revised¹⁷⁹ background subtraction procedure (see text).

The XAS covalencies of $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$ (bottom of Table 2.5) can be theoretically reproduced more readily by spin densities than LUMO analyses. Among the LUMO analyses, NPA_m with B(5%HF)P86/6-311+G(d) (0.42 e) is the only method that matched the experiment (0.41 ± 0.02 e). However, the 0.11 e lower covalency upon switching to def-TZVP reveals that the agreement with experiment may be attributed to a fortuitous combination of functional, basis set, and population analysis. Spin densities from HPA, MPA and NPA reproduce the experimental S^{s} covalency, but the MPA and NPA results are highly dependent on the basis set. For example, MPA with B(5%HF)P86/6-311+G(d) gives a S^{s} covalency from spin density that lies within 0.02 e of the experimental result, as expected based on our previous work;⁷⁷ however, with def-

TZVP, theory deviates from experiment by 0.05 e . Although the HPA S^s covalencies from spin density are nearly basis set-independent, they likely agree with the experimental result due to the propensity for spin densities to give overly covalent values for S^s , while giving overly ionic values for S^t , as explained in the section titled Reduction Potentials. Thus, the S^t spin densities from all methods are much more ionic than the experimental covalency, deviating by 0.10–0.25 e , similarly to what was found in the earlier study,⁷⁷ and due in part to donation of both α and β electrons from each thiolate. The only methods giving S^t covalencies that lie within the experimental uncertainty are NPA, HPA and AIM LUMO analyses with B(5%HF)P86/6-311+G(d). Again, basis set dependence is prominent in these results; the relatively high covalencies with 6-311+G(d) compared to def-TZVP are attributed to the mixing of significant Fe(3d) character into high-lying LUMOs. With that in mind, the NPA_m LUMO analysis with B(5%HF)P86/6-311+G(d) is the only method that reproduces both the experimental S^s and S^t covalencies.

Delocalization Indexes

The delocalization index, DI, uses the AIM partitioning of density to quantify the number of electrons shared or exchanged between two atoms, thus providing a value for covalency. At the Hartree-Fock (HF) level, the DI gives the bond order for nonpolar bonds, which decreases as charge transfer increases.¹¹³ DFT DI's can be approximated using HF formalism, resulting in consistently overestimated values,¹⁷⁶ but these still allow for meaningful comparisons. DI values calculated for the pairs S^t -Fe and S^s -Fe for the full series of models are compared to the AIM LUMO-derived covalencies in Figure 2.7 (see Table A.4 for numerical results). B(5%HF)P86/6-311+G(d) was used for all

models, except $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$, where the def-TZVP results were used due to the more reasonable LUMO compositions (see DFT Fe-S Covalency of $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$). The DI's are much higher than the LUMO-derived covalencies, but there is a fairly strong correlation ($R^2 = 0.970$) between the two. Considering the sulfides alone, the correlation is excellent ($R^2 = 0.999$), although there are only four data points in this case, two of which are very close together. These empirical relationships, which could be further developed with a larger set of models, can be used to convert DI values into experimentally (XAS) relevant values. With AIM, DI is more efficient to calculate than LUMO-derived covalency, and the use of DI circumvents problems arising from the spread of Fe(3d) into high-lying LUMOs. Furthermore, in this case, the DI values are actually more highly correlated with the XAS data than are the LUMO-derived covalencies, with R^2 values of 0.86 and 0.81, respectively (see Figures A.1 and A.2).

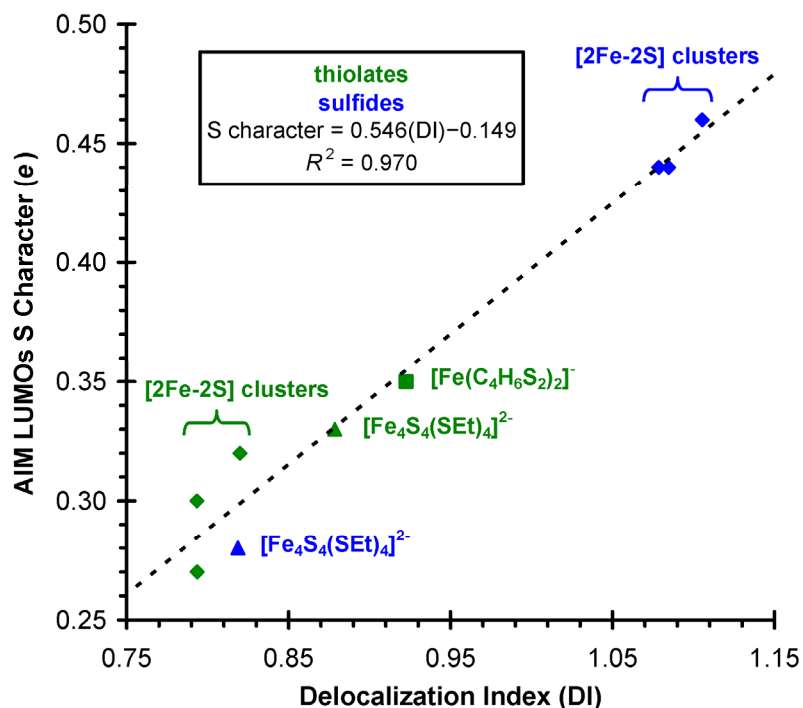


Figure 2.7. Correlation of LUMO-derived covalencies per S^t or per S^s per Fe with corresponding delocalization indexes calculated with B(5%HF)P86/6-311+G(d) for all models, except def-TZVP for $[\text{Fe}_4\text{S}_4(\text{SET})_4]^{2-}$.

Discussion

The calculated S^t and S^s contributions to M–L bonding from various basis sets, functionals and population analyses show strong method dependence. The two commonly used basis sets, def-TZVP and 6-311+G(d), approach the saturation limit based on electronic energy (Figure 2.1), and density topology (Figure 2.4), but they produce very different covalencies when using the population analysis methods CSPA, MPA, or NPA. Due to the lack of significant basis set dependence, we consider the MBS, HPA and AIM methods to provide the most reasonable electronic structure description. The only case where these methods show non-negligible differences ($>0.02 e$) between def-TZVP and

6-311+G(d) is the LUMO compositions of the [4Fe-4S] cluster with the B(5%HF)P86 functional (Table 2.4). This is likely a real difference in the electronic structure description, with def-TZVP producing the expected nine α and β Fe(3d)-based LUMOs, while with 6-311+G(d) the Fe(3d) character appears in the tenth LUMO mixed with Fe(4s). This mixing of Rydberg and valence orbitals of the unoccupied set is characteristic for calculations with diffuse and polarization functions. Thus, we recommend the use of the def-TZVP basis set with a note that 6-311+G(d) can also produce good results from the majority of other population analysis methods. Similarly, B3LYP cannot be recommended for electronic structure analysis due to the spread of Fe(3d) character into higher orbitals, in addition to the previously shown poor performance in geometry optimizations.⁷⁷ B(5%HF)P86 performs well, reproducing the QCISD AIM spin densities for $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$, but all of the DFT methods attribute too much ligand character to the LUMOs relative to QCISD (Table 2.1).

An important disclaimer needs to be made here with regard to comparing experimental and theoretical covalencies. We have found that limitations in basis set saturation and shortcomings of population analysis methods can provide fortuitously experimentally sound covalencies in relation to XAS data, for example. The NPA spin density method with B(5%HF)P86/6-311+G(d) was among the most successful for reproducing S^{I} covalencies of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ and [2Fe-2S] clusters, but the same method fails with def-TZVP. The very high experimental S^{S} covalencies could not be reproduced, and were only approached by the MBS, NPA_{x} , and HPA Δq methods, and the NPA_{x} $\Delta S(3p)$ method, which give high values because they are measures of total or

S(3p) electron donation from the ligand to all available metal orbitals (dominantly 3d, 4s, 4p), rather than just the experimentally probed S(3p) character of the Fe(3d)-based LUMOs. For the $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$ cluster only the HPA, AIM and NPA LUMO analyses reproduced the S^t covalency, although the high covalency was only attained with 6-311+G(d) due to the mixing of Fe(3d) character into a tenth LUMO, while def-TZVP produces the expected 9 Fe(3d)-based LUMOs. Overall, we did not find a single method of calculating orbital and/or atomic compositions that reproduces all of the experimental results.

In general, the NPA, MPA and CSPA cannot be recommended due to basis set dependence, and MBS is disfavored due to highly ionic spin densities and LUMO compositions, leaving HPA and AIM as favored population analyses. By definition, LUMO compositions provide the most direct comparison to XAS covalencies, since XAS probes unoccupied orbitals, but they are also dependent on the employed level of theory. When the level of theory results in the unreasonable spread of Fe(3d) into high-lying unoccupied orbitals (e.g., with B3LYP and/or 6-311+G(d), in some cases), spin densities can be a useful, level of theory independent (for HPA and AIM) measure of M–L bond covalency, particularly for comparing theoretical results among various complexes. However, spin densities tend to give low S^t covalencies and high S^s covalencies compared to LUMO analysis, due to the nonequivalent donation of both α and β electrons from each thiolate. The delocalization index represents a promising alternative to LUMO analysis, correlating well with the LUMO-derived and XAS-derived covalencies, but empirical relationships must be established for direct use of the DI's.

Also correlated with covalency is the total electron donation from the Δq method, but the results are expected to be higher than the solely Fe(3d)–S(3p) bond-based covalency from XAS. Indeed, the HPA and AIM Δq values are always higher than the covalencies derived from just the Fe(3d)-based LUMOs. Notably, the HPA and AIM Δq results differ significantly, but the AIM results are favored because they deviate much less from the spin density and LUMO-derived covalencies, which are expected to represent the majority of the donated charge.

The AIM-derived theoretical covalencies are in accordance with chemical intuition regarding the variation in Fe–S bonding across the series of cluster compositions. The B(5%HF)P86/6-311+G(d) results can be used for comparison except in the case of $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$, where def-TZVP was used to avoid the previously described mixing of Fe(3d) into high LUMOs. The AIM LUMO compositions show decreasing donation per S^t from 0.35 e in $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ to 0.32 e in $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-}o\text{-Xyl})_2]^{2-}$, due to the increased donation from the sulfides relative to a second $\text{S}_2\text{-}o\text{-Xyl}$ ligand. From $[\text{Fe}_2\text{S}_2(\text{SEt})_4]^{2-}$ to $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$, the per-thiolate covalency increases from 0.30 e to 0.33 e , despite the reduction from Fe^{3+} to $\text{Fe}^{2.5+}$, because the number of S^t ligands per Fe is halved, while maintaining a constant ratio of S^s to Fe. The μ^2 -sulfides in $[\text{Fe}_2\text{S}_2(\text{SEt})_4]^{2-}$ are 57% more covalent per Fe than the μ^3 -sulfides in $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$, but the total per-sulfide covalency (0.88 and 0.84 e , respectively) is very similar. We would expect the total covalency per sulfide to be greater in the μ^3 case given equivalent Fe oxidation states, but in this case the reduction from Fe^{3+} to $\text{Fe}^{2.5+}$ leads to decreased donation from the μ^3 -sulfides. The calculated sulfide covalencies are consistent with the

idea⁹⁵ that the strong μ^2 -sulfide bridges promote superexchange interactions, whereas the weaker μ^3 -sulfide bridges lead to direct exchange between the Fe sites.

In conclusion, the AIM population analysis coupled with the B(5%HF)P86 hybrid functional and a triple- ζ basis set with polarization functions provides chemically reasonable covalencies that are not subject to the biases inherent in other methods due to basis set dependence. HPA can also be recommended for calculating spin densities and LUMO compositions, as the results are very similar to those from AIM at a considerably lower computational cost. Furthermore, the results presented here demonstrate potential pitfalls where population analysis methods conspire with functionals and basis sets to produce experimentally sound results due to error cancellations. CSPA, MPA, MBS, or NPA may give results more consistent with a specific experimental value, but this may be fortuitous, rather than being an indication of the quality of the calculated density.

CHAPTER 3

PROTEIN ENVIRONMENTAL EFFECTS ON IRON-SULFUR CLUSTERS: AN
ALGORITHM FOR CONSTRUCTING QUANTUM CHEMICAL MODELSIntroduction

Iron sulfur clusters are found in enzymes throughout biology, enabling such diverse activities as electron transfer, small molecule activation, and iron and oxygen sensing.⁶ They range in composition from the common mono-iron, [2Fe-2S], and [4Fe-4S] clusters, to specialized species like the H-cluster of hydrogenase,¹² and the molybdenum-containing FeMo-cofactor of nitrogenase.²⁶ The properties of Fe-S clusters are critically dependent on the surrounding protein environment. In a classic example, the reduction potentials of the [4Fe-4S] clusters of ferredoxins and high-potential iron proteins (HiPIPs) differ by about 1 V, despite the identical cluster compositions.¹⁸¹ Reduction potentials of the various [2Fe-2S] ferredoxins and rubredoxins also differ between organisms. Much of our understanding of protein environmental effects has come from studies of reduction potentials that have attributed the differences to ligand conformation,¹⁸² hydrogen bonding to the sulfides and Cys(Sγ) ligands,^{21, 22} electrostatic effects from protein dipoles¹⁷⁻¹⁹ and charged residues²⁰ (if not solvated), solvent accessibility,²² and distance from the protein surface.¹⁸³ Theoretical models focusing on electrostatic and solvent interactions, such as the Protein Dipoles Langevin Dipoles (PDLDP) method of Stephens et. al.,¹⁸³ can predict relative differences in reduction potentials within 0.2 V, but quantum calculations are necessary to produce absolute

reduction potentials. A method combining a minimal DFT model (cysteine ligands truncated to SCH₃) with a Poisson-Boltzmann treatment of electrostatic interactions, including cluster and protein partial charges, and the dielectric environments of the protein and solvent, was used by Noodleman and co-workers to calculate absolute reduction potentials of [2Fe-2S],⁸² and [4Fe-4S]¹⁸⁴ clusters in various oxidation states. They found qualitatively correct trends, with errors of less than 0.34 V; thus, electrostatic models of the protein and solvent environment capture some of the most significant contributions to reduction potentials. Further increases in accuracy would come from expanding the quantum region to take into account hydrogen bonding and the effects of ligand geometry at the DFT level.

A simple approach of combining an appropriately sized quantum model with a dielectric continuum to represent the long range interactions can be an effective way to calculate metalloenzyme reduction potentials, spectroscopic properties, and mechanism, but the choice of residues to include in the model is not trivial. Generally, increasing the model size will increase accuracy at the expense of computational time. Noodleman and co-workers¹⁸⁵ recently evaluated the effect of model size and the dielectric constant on geometric parameters, Heisenberg exchange constants, and Mössbauer spectroscopic properties of the diiron active site of ribonucleotide reductase. They found some properties to be more sensitive than others, with quadrupole splittings and Heisenberg exchange constants being strongly affected by the second coordination sphere, and Fe-ligand distances were even dependent on a third coordination sphere of hydrogen-bonding waters. Since the model dependence varies according to the property and the

particular active site, it is often necessary to test several models to avoid missing important protein environmental effects. It would be much more convenient if there were a method to determine which residues should be included in a computational model, without explicitly testing each interaction for each new study.

In this study we systematically evaluate the effects of individual hydrogen bonds, backbone amide dipoles, polar side chains, and charged side chains on the electronic structure of an Fe-S cluster. The [2Fe-2S] cluster of [FeFe]-hydrogenase was chosen as the model system because it is a small cluster, reducing the computational cost, and the coordinates are available from a fairly high resolution crystal structure (1.39 Å).¹² One property used throughout this study to analyze electronic structural perturbations is the covalency of the Fe-S bonds. Interactions with the protein environment result in a redistribution of the electron density within the cluster and changes in bond covalency.^{178, 179, 186} It is important for covalency to be converged in a computational model, because it affects magnetic coupling,⁹⁵ electron transfer pathways,⁹⁶ and reactivity.⁹⁸ Covalency is often measured experimentally with X-ray absorption spectroscopy (XAS), and is generally given in terms of orbital characters.¹⁶ For example, S K-edge XAS gives a direct measure of the S(3p) character of the Fe(3d) manifold, thus quantifying covalent bonding. Many population analyses can be employed to quantify covalency; here, we use the delocalization index (DI) from the quantum theory of atoms in molecules (AIM), which we have found to be proportional to orbital-based covalencies. DI values have the advantage over orbital-based populations of having little model and basis set dependence (see Chapter 2). The other property monitored throughout this study is the vertical

electron affinity (VEA), which was determined using a polarizable continuum model, and thus includes all of the electrostatic terms, but not the structural reorganization that contributes to reduction potentials. The VEA complements the DI by being more sensitive to electrostatic effects from the protein environment. The two properties are not always correlated,¹⁸⁷ and thus provide a more complete analysis of protein effects than if only one were used.

The first section of this paper describes a survey of the surrounding protein environment, and a qualitative map of the first three coordination spheres. Then we provide case studies to demonstrate how the [2Fe-2S] cluster is perturbed by hydrogen bonding and charged side chains, before quantitatively mapping the covalent and electrostatic effects of all interactions using the DI and VEA values. By comparing the magnitude of each effect with the location and orientation of each of the tested functional groups in the protein environment, we determine an algorithm that can be used to *a priori* build a computational model of an Fe-S cluster active site with all important residues included.

Computational Details

Each protein environmental effect was determined by single-point calculations on a set of two models: a reference model, and one that builds upon the reference model by including the truncated residue of interest. The minimal reference model had the Fe-bound cysteines truncated to ethylthiolates ($[\text{Fe}_2\text{S}_2(\text{SEt})_4]^{2-}$), but larger models with full cysteines and additional residues were used in some cases, as described in the results. All

models were based on a pre-publication version of an [FeFe]-hydrogenase X-ray crystal structure, which has since been refined to 1.39 Å (PDB ID 3C8Y).¹² Electronic structure calculations were performed with Gaussian 03 Rev. E.01¹²⁹ (gas phase) and Gaussian 09 Rev. B.01¹³⁰ (gas phase and in the polarizable continuum model,^{87, 88, 188} PCM), using the 6-311+G(d) basis set,^{141, 144, 145} and a gradient-corrected, hybrid functional with 5% Hartree-Fock, 95% Becke88 exchange,⁵⁹ and Perdew86 correlation.⁵⁸ This level of theory has been rigorously validated for use with Fe-S clusters.⁷⁷ Population analyses were carried out with the AIMAll software.¹⁸⁹

Antiferromagnetic coupling was treated by the broken-symmetry (BS) formalism,⁷¹ using the generalized ionic fragment approach (GIFA)⁷⁷ to generate the initial electronic structure. Broken-symmetry states are a weighted average of true spin states with a well-defined M_s . Thus, $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}$ and $\text{Fe}^{\text{III}}\text{Fe}^{\text{II}}$ oxidation states were modeled as $M_s = 0$ and $M_s = 1/2$ broken symmetry states, respectively. The spin states of the oxidized cluster can be described by the Heisenberg Hamiltonian, allowing determination of the true ground state energy.¹⁵ However, since all of the calculations in this study involve comparisons of two models that differ by approximately one residue, the energetic corrections cancel, and the broken symmetry states are sufficient.^a The mixed-valence state is more complicated, being dependent not only on localizing Heisenberg exchange interactions, but also on delocalizing double exchange effects, and a trapping term dependent on site asymmetry, vibronic effects, and the surrounding environment.¹⁹⁰ Nevertheless, most of the corrections to the BS state would cancel when comparing two

^a We found the $S_t = 0$ spin state of $[\text{Fe}_2\text{S}_2\text{SEt}_4]^{2-}$ to be 16 kJ/mol lower in energy than the BS state, and the correction was the same when including Asn41 as *N*-methyl formamide.

models differing solely by a single residue or functional group. Furthermore, Mouesca and co-workers have studied the effects of single hydrogen bonds on a [2Fe-2S] cluster, and found insignificant changes in the double exchange and trapping terms.¹⁹⁰ Therefore, the BS states are sufficient for this study.

Covalent effects were quantified in terms of the AIM delocalization index (DI), which can be expressed as follows:

$$DI = 2 \sum_{i,j} S_{i,j}(A) S_{i,j}(B) \quad (3.1)$$

where $S_{i,j}$ is the overlap integral of all pairs of spin orbitals over the basin of atom A or B.¹⁷⁵ Kohn-Sham orbitals only allow an approximation of DI, through use of HF formalism, but trends found using DFT reproduce those of the HF and post-HF levels of theory.¹⁷⁶ Electrostatic effects were quantified in terms of the vertical electron affinity (VEA_{PCM}) of models embedded in a polarizable continuum with a dielectric constant (ϵ) of 4.0 (unless otherwise stated) to approximate the protein environment. VEA_{PCM} values were calculated from differences in energy of the oxidized and mixed-valence states, according to Equation 3.2 and 3.3.

$$VEA_{PCM} = E_{PCM,oxidized} - E_{PCM,mixed-valence} \quad (3.2)$$

$$E_{PCM} = E_{SCF} + E_{solvation} \quad (3.3)$$

where E_{SCF} is the *in vacuo* electronic energy, and $E_{solvation}$ is the electrostatic solute-solvent interaction energy. Non-electrostatic solvent-solvent interactions were not included because they depend on the cavity geometry, which was the same in the oxidized and mixed-valence states.

Results and Analysis

Qualitative Mapping of Protein Environmental Effects

A two dimensional map of the protein environment within approximately 8 Å of the [2Fe-2S] cluster is presented in Figure 3.1. The first coordination sphere (pink) comprises four cysteine residues attached to the cluster through highly covalent Fe–S^t bonds. Each cysteine and sulfide is involved in hydrogen bonds that potentially perturb the cluster. Thus, the second coordination sphere (orange) is defined as all residues that are involved in hydrogen bonding with the sulfides or cysteine heteroatoms, and all that are covalently linked to the first sphere, yielding four charged residues (Lys45, Glu47, Asp63, Arg86), seven neutral residues (Leu33, Phe35, Asn40, Asn41, Ile48, Thr50, Ala61) and five waters (HOH23, HOH39, HOH55, HOH138, HOH487). All four charged residues are solvent-exposed, three of which have salt-bridge partners, while Arg86 is left unpaired, but forms several hydrogen bonds, including N_εH⁺⋯Cys46(O) (2.95 Å), N_{η2}H⁺⋯Lys45(O) (2.92 Å), and N_{η2}H⁺⋯HOH597(O) (3.36 Å). Glu47(NH) hydrogen bonds to S^s_A (3.32 Å), while Lys45 and Asp63 do not hydrogen bond to the cluster or the first sphere. All second-sphere neutral residues form hydrogen bonds with the cysteines or S^s_B, except Thr50, which is included in the second sphere only because it neighbors Cys49. Even without hydrogen bonding, polar groups (e.g., CO) of neighboring residues have a potentially significant effect on the cluster due to their electric dipoles. Most of the hydrogen bonds from second-sphere neutral residues are formed between backbone NH groups (Leu33, Phe35, Asn41 and Ile48) and Cys–S_γ or S^s_B (3.46, 3.51, 3.41 and 3.39 Å, respectively), with the exceptions being Ala61(NH)⋯Cys49(O) (2.98 Å), and

Asn40(N_{δ2}H)···Cys62(S_γ) (3.25 Å). All of the second shell waters hydrogen bond to Cys(O) atoms (2.59–3.58 Å).

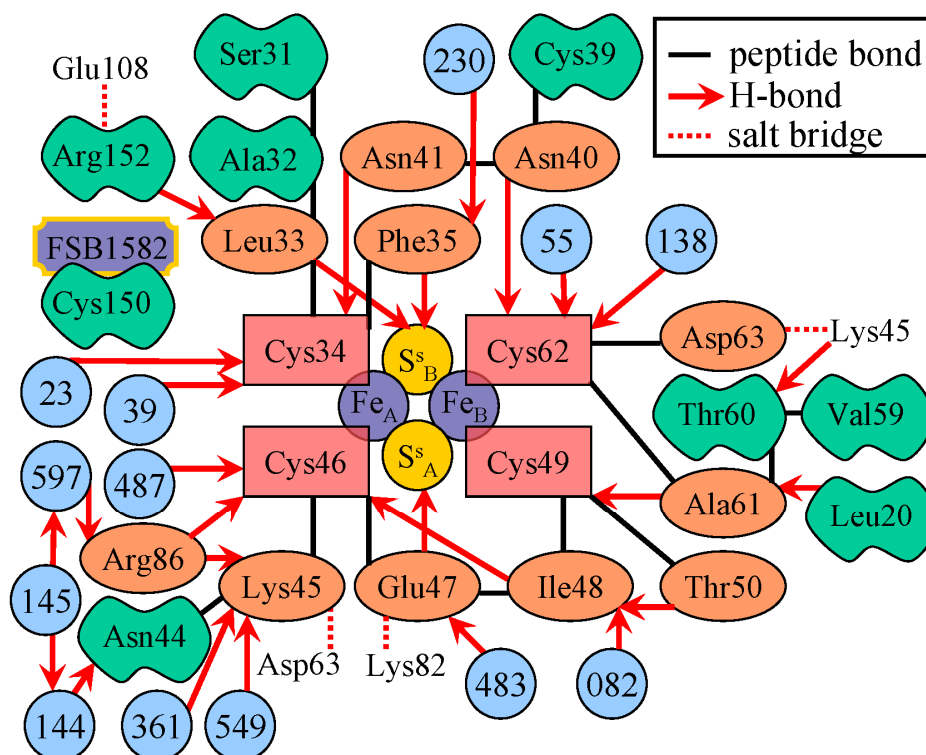


Figure 3.1. Map of the ~ 8 Å environment of the [2Fe-2S] cluster with the first coordination sphere (pink), second coordination sphere (orange), third coordination sphere (green), and waters (blue), salt-bridging residues (colorless) and the [4Fe-4S] cluster FSB1582. Asp63 and Lys45 appear twice for clarity.

The third sphere in Figure 3.1 (green) includes residues that fall into at least one of the following groups: (1) those that hydrogen bond with the NH or CO groups immediately adjacent to the cysteines, and (2) those that have an O···Fe distance of less than 8 Å. The first group includes Leu20 (NH···Ala61(O), 2.95 Å), Thr60 (O···Asp63(NH), 3.32 Å), Arg152 (N_{η1}H···Leu33(O), 2.83 Å), and the waters 82 (OH···Ile48(O), 3.00 Å) 2230 (OH···Leu33(O), 3.22 Å), and 549 (OH···Lys45(NH), 2.95

Å), while the second group is made up of Ser31, Ala32, Cys39, Asn44, Val59, Cys150, and the waters 144, 145, 361, 483, and 597. The only charged residue in the third sphere, Arg152, is not solvent exposed, but it is paired in a salt bridge with Glu108. It is remarkable that the third-sphere residue, Cys150, is coordinated to the [4Fe-4S] cluster, FSB1582, and the shortest Fe $\cdots$ Fe distance between the two clusters is only 10.73 Å. At this distance, the clusters can be expected to affect each other through electrostatic interactions, and based on our findings discussed in the section titled The Effects of Charged Residues on Fe-S Clusters, the effect is not negligible.

Many other schemes can be used to divide a protein environment into coordination spheres. The one used here was chosen because it separates residues by the types of interactions they have with the Fe-S cluster, in addition to generally separating them by their distance from the cluster. Still, all third-sphere residues do not necessarily have weaker effects on the cluster than those of the second sphere. The relative strengths of hydrogen bonding and electrostatic interactions, and factors such as dipole orientation and the specific location of charged or polar groups must be considered to judge the importance of each residue. Thus, a quantitative analysis is required to define rules that can be used to assign the importance of various interactions.

Quantitative Mapping of Protein Environmental Effects

To demonstrate the effects of hydrogen bonding and electrostatic interactions on an Fe-S cluster, we discuss two case studies in the following two sections. These also serve as first examples of how the quantitative measures of covalent (ΔDI) and electrostatic effects ($\Delta\text{VEA}_{\text{PCM}}$) are used in the quantitative mapping analysis.

Subsequently, we quantify the effects of the protein backbone, and the neutral and charged side chains, and present the algorithm that enables classification of residues according to the strength of their interactions with the cluster.

The Effects of Hydrogen Bonds on Fe-S Clusters. The electronic structure of an Fe-S cluster is significantly perturbed by hydrogen bonds between thiolate ligands or sulfides and nearby protein residues. To demonstrate how the cofactor is affected we present a case study using the smallest, most truncated model, $[\text{Fe}_2\text{S}_2(\text{SEt})_4]^{2-}$, shown in Figure 3.2A. In the employed broken-symmetry DFT calculations, the antiferromagnetically coupled $S_t = 0$ state is approximated by an $M_s = 0$ state with localized β (negative) spin density on Fe_A and α (positive) spin density on Fe_B , as shown in Figure 3.2B. The thiolates (S^t) have toroidal-shaped spin density due to spin-polarized donation to the Fe centers through pseudo- σ and π -bonds. The sulfides (S^s) donate dominantly α density to Fe_A and β density to Fe_B in approximately equal quantities, resulting in near-zero spin density. The redistribution of electron density due to a hydrogen bond is significant and will be presented graphically before the full quantitative analysis. In this case study, the $\text{Asn41}(\text{NH}) \cdots \text{S}_A^t$ hydrogen bond was modeled as *N*-methyl formamide by truncating Asn41 at its C_α and including the peptide bond with Asn1040. The density difference isosurface in Figure 3.2C shows the areas of increased (light blue) and decreased (orange) electron density after addition of the *N*-methyl formamide, which appears as the large cloud above S_A^t . The hydrogen bond causes decreased donation from S_A^t to Fe_A , exhibited as an orange cloud along the $\text{S}_A^t\text{--Fe}_A$ bond. The orientations and signs of the remaining isosurface at the Fe centers give evidence for

a series of compensating effects. Specifically, S_A^t and the sulfides increase donation to Fe_A at the expense of sulfide to Fe_B donation, which is compensated by the remaining thiolates. A complementary perspective is given by the spin density difference isosurface in Figure 3.2D, where the decreased donation from S_A^t to Fe_A manifests in a positive spin density difference on S_A^t . The compensating increased sulfide to Fe_A donation is now seen as a negative spin density difference (red clouds) on the sulfides, since they donate dominantly alpha electron density to Fe_A . A small amount of spin density can also be observed on the hydrogen-bonding nitrogen. Such spin density on protein residues can be detected by ^{15}N NMR spectroscopy.^{191, 192}

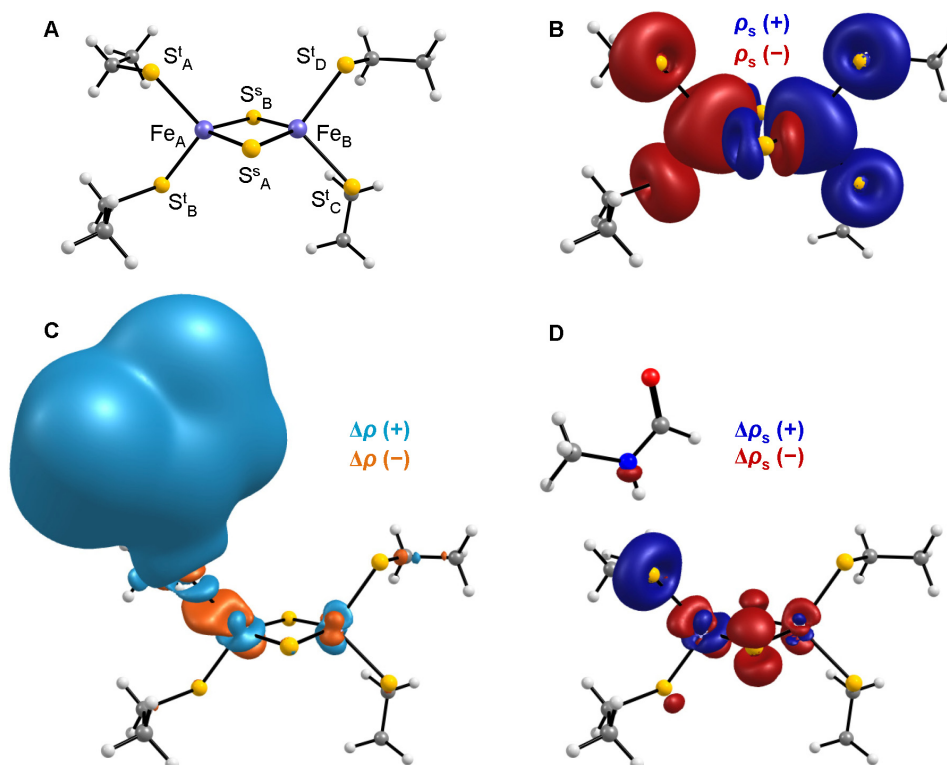


Figure 3.2. The most truncated reference model (A), and its spin-density isofurface (B). Isosurfaces (C) and (D) show change in total density (ρ) and spin density (ρ_s), respectively, due to the addition of the Asn1041-Asn1040 peptide group, modeled with *N*-methyl formamide.

Numerous population analyses are available for quantifying electronic structural properties. Atoms in Molecules (AIM) and Hirshfeld (HPA) population analyses are among the least basis set sensitive and most reliable methods available for studies of [Fe-S] clusters (see Chapter 2), since they are based on partitioning the electron density, rather than orbital coefficients and overlap matrices. Several atomic and bond properties from an AIM analysis of the $[\text{Fe}_2\text{S}_2(\text{SEt})_4]^{2-}$ model with and without the hydrogen-bonding Asn41 (*N*-methyl formamide) are given in Table 3.1. The changes in charge (Δq) due to the hydrogen bond are consistent with those observed graphically in Figure 3.2C. The charge of $\text{S}_\text{A}^\text{t}$ is reduced by $0.02\ e$ since it donates less electron density, and the $0.01\ e$ increase in charge on all the other sulfur species indicates that they all donate more in compensation. As a result of the compensating increases and decreases in donation, the iron charges stay the same, despite the significant perturbation to the overall electron distribution. The spin density changes ($\Delta\rho_\text{s}$) also closely follow those presented in Figure 3.2D, with a $0.03\ e$ change on $\text{S}_\text{A}^\text{t}$ and smaller changes of $-0.02\ e$ and $-0.01\ e$ on the sulfides. The regions of positive and negative change in spin density near the iron centers effectively cancel when the iron atomic basins are integrated, resulting in no overall change, as seen previously for the charge.

Table 3.1. Changes in charge (q), spin density (ρ_s), density at the bond critical point (ρ_{bcp}), and delocalization index (DI), due to the Asn1041(NH)–Asn1040(CO) peptide backbone that forms a hydrogen bond with S_A^{t}

atomic properties			bond properties		
atom	Δq (e)	$\Delta \rho_s$ (e)	bond	$\Delta \rho_{\text{bcp}}$ (au)	ΔDI
S_A^{t}	−0.02	0.03	$\text{S}_A^{\text{t}}\text{--Fe}_A$	−0.0007	−0.05
S_B^{t}	0.01	0.00	$\text{S}_B^{\text{t}}\text{--Fe}_A$	0.0001	0.01
Fe_A	0.00	0.00	$\text{S}_A^{\text{s}}\text{--Fe}_A$	0.0001	0.01
S_A^{s}	0.01	−0.02	$\text{S}_B^{\text{s}}\text{--Fe}_A$	0.0001	0.01
S_B^{s}	0.01	−0.01	$\text{S}_A^{\text{s}}\text{--Fe}_B$	0.0000	−0.01
Fe_B	0.00	0.00	$\text{S}_B^{\text{s}}\text{--Fe}_B$	0.0000	−0.01
S_C^{t}	0.01	0.00	$\text{S}_C^{\text{t}}\text{--Fe}_B$	0.0002	0.01
S_D^{t}	0.01	0.01	$\text{S}_D^{\text{t}}\text{--Fe}_B$	0.0002	0.01

Bond properties are a sensitive probe of electronic structural changes with the advantage over atomic properties of being able to give information about specific pairs of atoms, rather than a single atom that is affected by multiple bonding interactions. The density at a bond critical point can be used to evaluate the strength of hydrogen bonds,¹⁹³ but here we use changes in density at bond critical points ($\Delta\rho_{\text{bcp}}$) to examine perturbations in the Fe–S bonds. As expected, the $\text{S}_A^{\text{t}}\text{--Fe}_A$ bond weakens after addition of the *N*-methyl formamide, with a $\Delta\rho_{\text{bcp}}$ of −0.0007 au, while the remaining bonds to Fe_A are strengthened in compensation (see Table 3.1). The $\Delta\rho_{\text{bcp}}$ values dissect the effects on the sulfide, showing larger changes in the bonds to Fe_A (0.0001 au) than to Fe_B (0.0000 au). The final column in Table 3.1 gives the change in delocalization index (ΔDI), which is a measure of electrons exchanged between two atomic basins, or bond covalency. The ΔDI values show the expected decrease in $\text{S}_A^{\text{t}}\text{--Fe}_A$ covalency (−0.05) with compensating increases in the other bonds to Fe_A (0.01), and the perturbations continue to propagate through the [Fe–S] cluster, with decreases in the sulfide- Fe_B ΔDI values (−0.01)

accompanied by increases in the thiolate-Fe_B values (0.01). Although each of the properties in Table 3.1 shows one aspect of the electronic structure, it is the DI that most directly measures covalent interactions, and it is the property used throughout this study to quantify covalent effects. The interdependence of S^t-Fe and S^s-Fe covalency highlighted in this section is also a feature of larger clusters, as demonstrated by Niu and Ichiye.¹⁸⁷

In addition to evaluating the covalent effects of the protein environment, we quantify the electrostatic effects in terms of the solvated vertical electron affinity (VEA_{PCM}) for the process $[\text{Fe}_2\text{S}_2(\text{SEt})_4]^{2-} + 1 \text{ e}^- \rightarrow [\text{Fe}_2\text{S}_2(\text{SEt})_4]^{3-}$. The VEA_{PCM} values include the most dominant contributions to calculated reduction potentials, differing in the lack of geometric relaxation, and in the neglect of nonelectrostatic solvation effects, but changes in VEA_{PCM} due to the protein environment are expected to be similar to those that would be observed for reduction potentials. Returning to our case study, the $\Delta\text{VEA}_{\text{PCM}}$ is 0.08 eV, demonstrating the significant electrostatic effect of even a single hydrogen-bonding formamide on an [Fe-S] cluster. It is important to note that the 0.08 eV effect cannot be attributed solely to the hydrogen bond, because the CHO group of the formamide also has a significant electrostatic effect that is discussed in the section titled Quantitative Mapping of the Backbone and Neutral Side Chains. Nevertheless, based on previous studies,^{21, 22, 83, 179} the VEA_{PCM} and reduction potential are expected to become more positive with the addition of a hydrogen bond to a thiolate or sulfide, which pulls electron density from the acceptor (S^t_A in this case), preferentially stabilizing the more negatively charged reduced state. In terms of the overall AIM charge of *N*-methyl

formamide, a decrease from $-0.03 e$ to $-0.05 e$ upon reduction of the model indicates a stronger hydrogen bond and greater alleviation of destabilizing electron-electron repulsion within the reduced cluster.

Effects of Charged Residues on Fe-S Clusters. Charged acidic and basic residues polarize the electric field of the protein environment, significantly altering reduction potentials and the electronic structure of Fe-S clusters. As an example, we explore the effects of the negatively charged Asp63 side chain using a larger reference model, $[\text{Fe}_2\text{S}_2(\text{Cys})_3(\text{CysNH}_2\text{CHO})]^{2-}$, where the three cysteines are capped by hydrogens, and CysNH_2CHO is a cysteine with both peptide amide groups. To quantify the effect of the Asp63 side chain, properties of the reference model are compared to those of a larger model constructed by appending the C_α and side chain of Asp63 to the reference, as shown in Figure 3.3A. The isosurface in Figure 3.3B shows the reorganization of electron density that can be attributed to the presence of the carboxylate group, which is displayed as a large light-blue cloud, representing increased density relative to the reference model. The distance and location of the charged group compared to the cluster are key factors that determine its effect. In this case, the closest sulfur species to the carboxylate oxygens are $\text{S}_\text{A}^\text{s}$ and $\text{S}_\text{D}^\text{t}$, at distances of 5.88 Å and 4.49 Å, respectively. Consequently, two of the most prominent regions of depleted electron density (orange) are located on $\text{S}_\text{A}^\text{s}$ and $\text{S}_\text{D}^\text{t}$, and oriented toward the carboxylate. These depletions develop as $\text{S}_\text{A}^\text{s}$ to Fe_A and $\text{S}_\text{D}^\text{t}$ to Fe_B donation increases to minimize repulsion, resulting in regions of accumulated density on the Fe centers. As seen previously in the hydrogen bonding case study, each Fe center is balanced by compensating adjustments in donation from the other sulfur species. In

quantitative terms, the Δq values are 0.03 and 0.07 e for S_A^s and S_D^t , respectively, but less than 0.01 e for the Fe centers. The cysteines more distant from Asp63 are also affected, with S_A^t , S_B^t and S_C^t pushing electron density away from the carboxylate toward the C_β atoms, which in turn push density toward the C_α atoms, and so on. This chain effect is most clearly shown by the alternating orange and light-blue clouds beginning on S_C^t of Cys49.

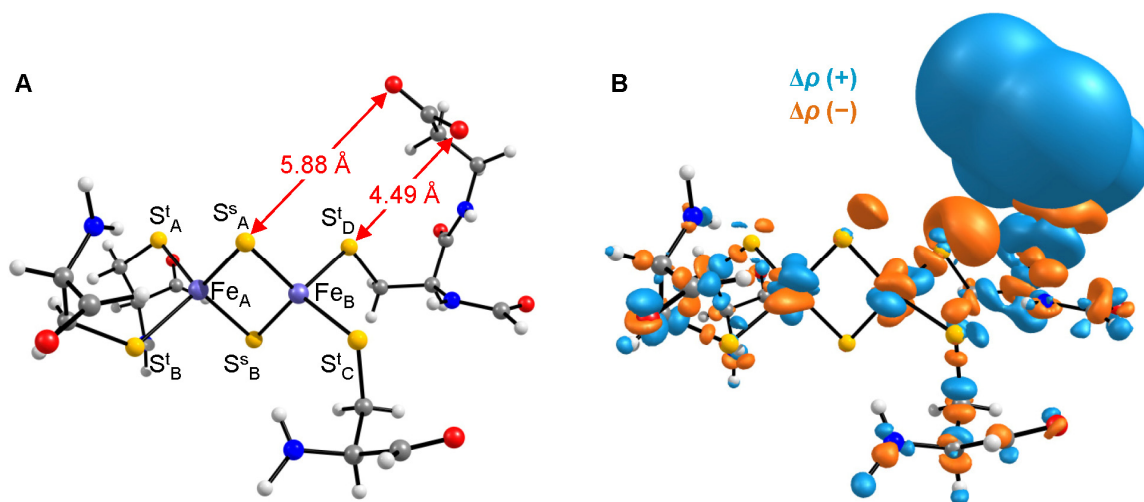


Figure 3.3. A model of the [2Fe-2S] cluster including the side chain of Asp63 (A), and an isosurface depicting the change in total density (ρ) due to addition of the side chain (B).

The electric field polarization caused by a charged side chain affects not only the electrostatic properties of Fe-S clusters, but also the covalent interactions, as evidenced by the changes in DI. Most significantly, the ΔDI for the S_D^t -Fe_B and S_A^s -Fe_A pairs are 0.06 and 0.04, respectively. The remaining S^t -Fe bonds weaken, due to the aforementioned density redistribution toward the cysteine backbones. S_A^s also follows the trend of reducing unfavorable repulsion by donating less to Fe_B, which is closer to the carboxylate, and more to Fe_A, yielding respective ΔDI values of -0.03 and 0.01. The

charge polarization and subsequent covalency changes observed here could have an influence on electron transfer pathways, and for larger, catalytic Fe-S clusters, analogous protein environmental effects would modify pKa's and play a role in determining favorable ligand binding sites. The more obvious effect of charged side chains is that reduction potentials will decrease in the presence of a negatively charged group, as it becomes more unfavorable to gain an electron. Indeed, the VEA_{PCM} of our model decreases by 0.5 eV after addition of the Asp1063 side chain. The effect is large because the carboxylate is quite close to the cluster, with an $Fe \cdots O$ distance of 6.18 Å.

To determine the distance at which electrostatic effects on Fe-S clusters become negligible, we used the interaction between the Lys3021 side chain and the $Fe_2S_2SET_4$ model as our test case. The side chain was truncated to methylammonium and modeled by point charges found through an AIM analysis. In the protein environment the charge of the side chain would be attenuated by the surrounding residues and those lying between the side chain and the cluster. Furthermore, charged residues are commonly located near the protein surface, where their charge would be heavily attenuated by water. In fact, a study involving mutagenesis of charged surface residues of a [4Fe-4S] ferredoxin found no detectable change in reduction potential.¹⁹⁴ In this work we do not consider solvent effects and treat all charged residues as if they were buried in the protein, but in the algorithm presented in the Discussion we recommend not including solvent-exposed charged residues in a computational model. In this case study the protein environment was approximated using PCM with two dielectric constants for comparison: the commonly used $\epsilon = 4.0$ and a higher value of 10.0, recommended by Noodleman and

co-workers for modeling the di-iron site of ribonucleotide reductase.¹⁸⁵ The $\Delta\text{VEA}_{\text{PCM}}$ was calculated for a series of $\text{Fe}_{\text{B}}\text{-N}$ distances, including the native 10.93 Å found in the crystal structure and those from 15–50 Å in 5 Å intervals. Figure 3.4 shows the expected trend of reduced $\Delta\text{VEA}_{\text{PCM}}$ with increasing distance following Coulomb's law, which gives the change in energy due to a distant point charge (q_{protein}) according to Equation 3.4:

$$\Delta E = \frac{1}{4\pi\epsilon_0\epsilon} \frac{(q_{\text{ox}} - q_{\text{red}})q_{\text{protein}}}{r} \quad (3.4)$$

where ϵ_0 is the vacuum permittivity, and q_{ox} and q_{red} are the charges of the oxidized and reduced reference models, respectively. The theoretical potentials from Coulomb's law (black lines in Figure 3.3) fit the data very well, with correlation constants, R^2 , of 0.999 and 0.997 for $\epsilon = 4.0$ and 10.0, respectively. Deviations are smaller at longer distances because the effect of the methylammonium point charges becomes more like that of a single positive unit charge as the distance increases. The $\Delta\text{VEA}_{\text{PCM}}$ values calculated with $\epsilon = 10.0$ are approximately reduced by a factor of 10/4 relative to the $\epsilon = 4.0$ data, but even with the higher dielectric constant, the effect does not drop below 50 mV until 30 Å, highlighting the significance of buried charged side chains. A protein environment with multiple unbalanced charged residues at 30 Å or even farther away could have a large effect on an Fe-S cluster or any other redox-active metallocofactor.

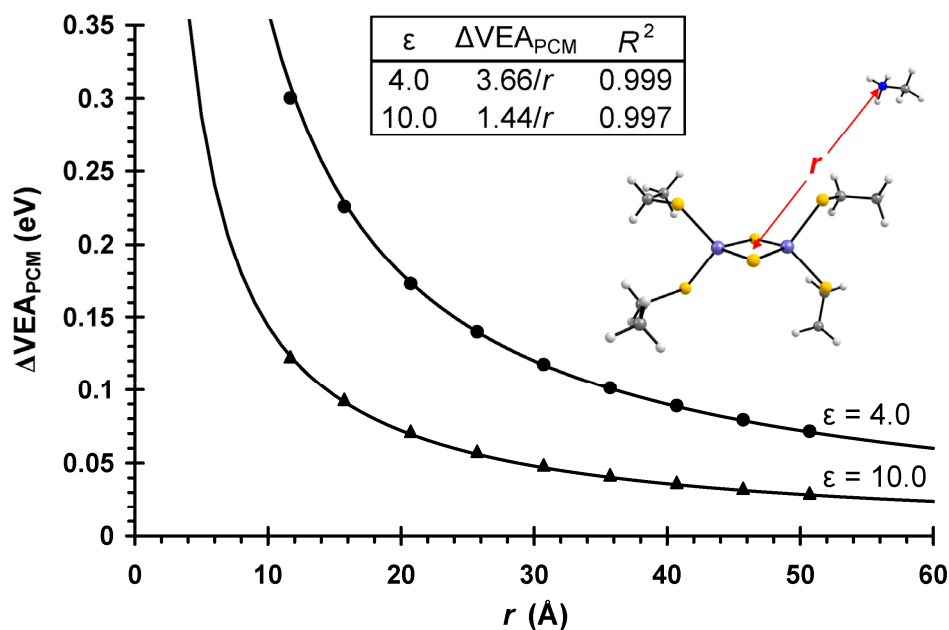


Figure 3.4. The dependence of electrostatic interactions on distance and dielectric constant. The model system and parameters for fits using Coulomb's law are inset.

Quantitative Mapping of the Backbone and Neutral Side Chains. A first step in developing a standard method for building computational models is to determine the appropriate level of truncation for the backbones of the Fe-bound cysteines and their neighboring residues. Using the $[\text{Fe}_2\text{S}_2\text{SEt}_4]^{2-}$ model as reference, the backbone of one Fe-bound cysteine was included at a time, and the effects of the $\text{NH}\cdots\text{S}$ hydrogen bonds and electrostatic polarization from CO-containing groups was quantified in terms of $\Delta\text{DI}(\text{Fe}, \text{S}^{\text{I}})$, $\Delta\text{DI}(\text{Fe}, \text{S}^{\text{S}})$, and $\Delta\text{VEA}_{\text{PCM}}$, which were shown in the preceding sections to be useful measures of covalent and electrostatic perturbations. The effects of the neighboring residue backbones were determined analogously using reference models constructed from three thioethyl groups and one full cysteine with peptide bonds capped by hydrogens. Table 3.2 gives the residue being probed, and the specific atoms that were added to the reference model, along with the resulting effects. ΔDI values were

determined for all Fe-S pairs, but only the one with the greatest magnitude is shown. The results indicate that the ΔDI values of greatest magnitude (0.03), representing the strongest perturbation to the Fe-S covalency, can be attributed to the $\text{NH}\cdots\text{S}^{\text{S}}_{\text{A}}$ hydrogen bond from the residue (Glu47) neighboring Cys46, which is the shortest hydrogen bond in the table (2.5 Å). All of the other hydrogen bonds and dipole interactions contribute small perturbations of 0.02 magnitude or less.

Table 3.2. Changes in delocalization index (DI) and vertical electron affinity (VEA_{pcm}), due to cysteine and adjacent-residue backbone groups, with interactions characterized by differential distance (DD), calculated as $(\text{O}\cdots\text{Fe}) - (\text{C}\cdots\text{Fe})$, and other metric data

residue ^a	model ^b	$\text{O}\cdots\text{Fe}$ (Å)	DD (Å)	H-bond (Å)	H-bond type	$\Delta\text{DI}(\text{Fe}, \text{S}^{\text{t}})^{\text{c}}$	$\Delta\text{DI}(\text{Fe}, \text{S}^{\text{s}})^{\text{c}}$	$\Delta\text{VEA}_{\text{PCM}}$ (eV)
Cys34	–CHO & –NH ₂	5.25	0.96	3.40	$\text{NH}\cdots\text{S}^{\text{t}}_{\text{B}}$	–0.01	0.01	0.10
Cys46	–CHO & –NH ₂	5.41	1.12	3.59	$\text{NH}\cdots\text{S}^{\text{t}}_{\text{A}}$	–0.02	0.01	0.12
Cys49	–CHO & –NH ₂	5.63	0.41			–0.02	0.01	0.05
Cys62	–CHO & –NH ₂	6.05	0.79	3.50	$\text{NH}\cdots\text{S}^{\text{t}}_{\text{C}}$	–0.02	0.01	0.07
Cys34– CHO	–CHO	5.60	0.99			0.01	0.01	0.15
Cys34– NH ₂	–NH ₂			3.51	$\text{NH}\cdots\text{S}^{\text{s}}_{\text{B}}$	0.02	–0.02	0.00
Cys46– CHO	–CHO	5.62	1.03			0.01	0.01	0.15
Cys46– NH ₂	–NH ₂			3.32	$\text{NH}\cdots\text{S}^{\text{s}}_{\text{A}}$	0.03	–0.03	0.02
Cys49– CHO	–CHO	6.55	1.07			–0.02	0.01	0.11
Cys49– NH ₂	–NH ₂					0.01	0.00	–0.03
Cys62– CHO	–CHO	6.54	0.75			0.01	0.01	0.09
Cys62– NH ₂	–NH ₂					0.00	0.00	–0.03

^a Cys–CHO and Cys–NH₂ refer to the CHO and NH₂ groups of adjacent residues.

^b Dashes indicate that the added group was covalently bonded to the reference model.

^c Only the largest-magnitude ΔDI is given, with preference for the positive value if multiple DI's of equal magnitude but opposite sign were found.

Focusing on electrostatic effects, the cysteine backbones increase VEA_{PCM} from 0.05–0.12 eV, with the smallest effect corresponding to the one that does not hydrogen bond (Cys49). However, the lower ΔVEA_{PCM} is more likely due to the CO dipole orientation and the relative distances of the carbon and oxygen from the cluster rather than the lack of a hydrogen bond. When the dipole is oriented with the carbon closer, the ΔVEA_{PCM} is generally positive, as is the case for all of the CO dipoles in Table 3.2. The relative $C\cdots Fe$ and $O\cdots Fe$ distances are strongly correlated with their electrostatic effect. For example, as the difference between the $O\cdots Fe$ and $C\cdots Fe$ distances increase from 0.41 Å (Cys49) to 0.79 Å (Cys62), 0.96 Å (Cys34), and 1.12 Å (Cys46), the corresponding ΔVEA_{PCM} values also increase (0.05 eV, 0.07 eV, 0.10 eV, and 0.12 eV, respectively). To isolate the effects of CHO and NH_2 groups, they were examined individually in the other series of tests detailed in Table 3.2, where the backbone was extended to include the peptide bonds. The electrostatic effect of an additional aldehyde group (0.09–0.15 eV) is much more significant than that of an amine (−0.03–0.02 eV), which can be attributed to the relative charge distributions and dipole moments. The tested amines that do not hydrogen bond (Cys49– NH_2 and Cys62– NH_2) decrease the VEA_{PCM} (−0.03 eV), whereas hydrogen bonds tend to increase VEA_{PCM} , resulting in a net effect of no change (0.00 eV) or a small increase (0.02 eV) for the tested hydrogen-bonding amines (Cys34– NH_2 and Cys46– NH_2 , respectively).

To develop rules for building computational models, cutoffs must be chosen to classify effects as being significant, in which case the group causing that effect must be included in the model. We will use 0.03 for ΔDI and 0.05 eV for ΔVEA_{PCM} as the

minimum level for an effect to be considered significant. Thus, according to the results in Table 3.2, cysteine backbones are important, and they should be extended through the peptide bonds to include the CO groups of their neighbors capped by hydrogens. It is unnecessary to include the neighboring amines if they do not hydrogen bond; however, if they do hydrogen bond they should be included. Although only one of the two hydrogen-bonding neighboring amines (Cys46–NH₂) had a significant effect, the other one (Cys34–NH₂) had a Δ DI of 0.02, despite poor orientation. The hydrogen bond effect would likely strengthen with an optimized geometry and therefore the group can be considered important enough to recommend including in a computational model.

Table 3.3 continues the quantitative mapping of backbone and neutral side chain effects. As in Table 3.2, the groups added to the reference models are listed in the model column. The reference models varied in size from [Fe₂S₂SEt₄]²⁻ to a model containing four cysteines, each extended to include the full peptide groups truncated to CHO and NH₂. The use of larger reference models allowed the covalent connection of distant residues, and a preliminary analysis using Mulliken spin densities showed that the choice of reference model strongly influenced the results. However, the dependence of Δ DI and Δ VEA_{PCM} values on the reference model is negligible. For this reason, Mulliken spin densities are a poor indicator of protein environmental effects, as even relative differences are inconsistent between models.

Table 3.3. Changes in delocalization index (DI) and vertical electron affinity (VEA_{pcm}), due to backbone groups and neutral side chains, with interactions characterized by differential distance (DD), calculated as $(O^{\cdots}Fe) - (C^{\cdots}Fe)$, and other metric data

residue ^a	model ^b	$O^{\cdots}Fe$ (Å)	DD (Å)	H-bond (Å)	H-bond type	$\Delta DI(Fe, S^t)^c$	$\Delta DI(Fe, S^s)^c$	ΔVEA_{PCM} (eV)
Leu20–Ile19	NH ₂ CHO	10.62	0.84			–0.01	0.00	0.03
Ala32–Ser31	NH ₂ CHO	5.98	–0.86			0.02	0.01	–0.04
Leu33–Ala32	–CH ₂ NHCHO	6.77	1.14	3.46	NH $\cdots$ S _B ^s	0.01	–0.04	0.12
Phe35	–CH ₂ Ph					0.00	–0.01	–0.01
Leu36–Phe35	–CH ₂ CONH ₂	6.87	0.43			–0.01	0.00	0.00
Asn40–Cys39	NH ₂ CHO	7.18	–0.23			0.00	–0.01	0.00
Asn40	NH ₂ CHO	6.29	0.84	3.25	NH $\cdots$ S _D ^t	–0.05	–0.01	0.04
Asn41–Asn40	CH ₃ NH ₂ CHO	7.28	1.23	3.41	NH $\cdots$ S _A ^t	–0.05	0.01	0.08
Lys45–Asn44	–CH ₂ NHCHO	6.39	–0.49			0.01	–0.01	–0.01
Ile48–Glu47	NH ₂ CHO	6.15	0.87	3.39	NH $\cdots$ S _B ^t	–0.04	0.01	0.08
Thr50	CH ₃ OH	7.22	–0.55			0.00	0.00	–0.04
Thr60–Val59	NH ₂ CHO	7.38	–0.06			0.00	0.00	0.01
Thr60	CH ₃ OH	5.28	0.94			–0.01	–0.01	0.03
Ala61–Thr60	NH ₂ CHO	5.80	0.54			–0.04	0.01	–0.01
Gly151–Cys150	NH ₂ CHO	6.91	–0.90			0.02	–0.01	–0.07
Cys200–Ala199	NH ₂ COCH ₃	11.78	–1.21			–0.01	0.00	–0.03
HOH (multiple) ^d	(H ₂ O) ₃	7.28 ^d	0.75 ^d	2.69 ^d	OH $\cdots$ Cys(O)	0.01	0.00	0.13

^a Peptide groups are indicated by two residues, with the NH-contributing residue listed first. Side chains are indicated by one residue. ^b Dashes indicate that the added group was covalently bonded to the reference model. ^c Only the largest-magnitude ΔDI is given, with preference for the positive value if multiple DI's of equal magnitude but opposite sign were found.

^d HOH39, HOH138, and HOH487 were tested simultaneously and the metric data are averages.

The ΔDI values in Table 3.3 show that all of the tested groups that form $\text{NH}\cdots\text{S}^{\text{t}}$ or S^{s} hydrogen bonds significantly perturb the Fe-S cluster (-0.04 to -0.05). The remaining ΔDI value of greater than 0.02 magnitude is attributed to the Ala61–Thr60 peptide group, which does have a fairly short $\text{NH}\cdots\text{S}_{\text{C}}^{\text{t}}$ distance of 3.04 Å, and it is the $\text{Fe}-\text{S}_{\text{C}}^{\text{t}}$ bond that is primarily affected, with a ΔDI of -0.04 , but the orientation does not allow hydrogen bonding. Instead, the effect is likely due to the CHO group, which is oriented such that the partial positive charge of the carbon is 3.74 Å from $\text{S}_{\text{C}}^{\text{t}}$, while the oxygen is 4.65 Å away, stabilizing the negative charge at $\text{S}_{\text{C}}^{\text{t}}$, and reducing $\text{S}_{\text{C}}^{\text{t}}-\text{Fe}$ covalency.

Using the ± 0.05 eV cutoff for $\Delta\text{VEA}_{\text{PCM}}$, the hydrogen-bonding groups all have significant effects (0.08–0.13 eV), except the Asn40 side chain, with the borderline value of 0.04 eV. The strongest effect (0.013 eV) is attributed to a set of three water molecules that were tested simultaneously. They form hydrogen bonds with the backbone oxygens of three cysteines, and are oriented with the positive ends (hydrogen atoms) of their dipoles facing the cluster, which induces the positive $\Delta\text{VEA}_{\text{PCM}}$. Of the groups that do not form hydrogen bonds, only the Gly151–Cys150 peptide group has a significant electrostatic effect of -0.07 eV. This effect is negative because the oxygen of the carbonyl is pointing toward the cluster, as indicated by the -0.90 Å difference between $\text{O}\cdots\text{Fe}$ and $\text{C}\cdots\text{Fe}$ distances, which we term the dipole differential distance (DD). The sign of the dipole DD matches that of the corresponding $\Delta\text{VEA}_{\text{PCM}}$ in all instances, with the exception of Ala61–Thr60, which also had the unusual covalent effect described above. The effects of other groups are limited by the long distances from the cluster (Leu20–Ile19, Cys200–Ala99), the orientations of the dipoles (Ala32–Ser31, Leu36–Phe35,

Asn40–Cys39, Lys45–Asn44, Thr60–Val59, Ala61–Thr60), or the lack of a strongly polarizing functional group (Phe35, Thr50, Thr60). Numerical cutoffs in terms of the O $\cdots$ Fe distance and the dipole DD, can be used to classify non-hydrogen-bonding carbonyl-containing groups. A loose set of cutoffs to achieve the goal of eliminating all residues with insignificant effects is 10 Å for the O $\cdots$ Fe distance and ± 0.87 Å for the dipole DD, which only admits Gly151–Cys1150 as a necessary group to include in a model. To take into account important dipole interactions with sulfur species such as the Ala61–Thr60 peptide group, another rule is to include all CO groups with C $\cdots$ S or O $\cdots$ S distances of less than 4 Å. Thus with those criteria, and further recommendations of including all groups that hydrogen bond to sulfur species, and all waters within 8 Å, we have classified all backbone and neutral side-chain groups.

Quantitative Mapping of the Charged Side Chains. The effect of the Asp63 side chain was described in detail above. In this section we additionally quantify the effect of basic side chains and salt-bridging partners, as we explore the effects of all charged residues within 11 Å of the [2Fe-2S] cluster. The Δ DI values in Table 3.4 show that all individual charged residues, except Glu47, have a significant influence (0.03 or greater magnitude) on the covalent bonding in the cluster. This effect is generally diminished by inclusion of a salt-bridging partner, but Lys45 and Asp63 retain significant Δ DI values of ± 0.04 . The Δ VEA_{PCM} results are more straightforward, with large negative values for the acidic residues Glu46 (−0.39 eV) and Asp63 (−0.50 eV), and large positive values for the basic residues Lys45 (0.51 eV), Arg86 (0.40 eV), Arg52 (0.37 eV), and Lys21 (0.30 eV). These effects are distance-dependent and follow Coulomb’s law, as discussed above. For

salt bridges, the $\Delta\text{VEA}_{\text{PCM}}$ values are nearly equal to the sum of individual values, as in, for example, the Lys45 $\cdots$ Asp63 salt bridge that has a -0.01 eV effect produced from individual effects of 0.51 eV and -0.50 eV. As with other dipoles, electrostatic effects of salt bridges depend on the differential distance (DD) of the positively and negatively charged groups from the Fe centers ($(\text{O} \cdots \text{Fe}) - (\text{C or N} \cdots \text{Fe})$). The Lys45 $\cdots$ Asp63 salt bridge has a small effect because the DD is only -0.19 Å. Negative DD values result in negative $\Delta\text{VEA}_{\text{PCM}}$ values because the acidic group is closer to the cluster. The Glu47 $\cdots$ Lys82 pair has the most significant differential distance (-2.18 Å), and, correspondingly, has the strongest electrostatic effect (-0.08 eV).

Table 3.4. Changes in delocalization index (DI) and vertical electron affinity (VEA_{pcm}), due to charged side chains, with interactions characterized by differential distance (DD), calculated as $(\text{O} \cdots \text{Fe}) - (\text{C or N} \cdots \text{Fe})$, and other metric data

residue	model ^a	C or N $\cdots$ Fe (Å) ^b	O $\cdots$ Fe (Å)	ΔDI (Fe,S ^t) ^c	ΔDI (Fe,S ^s) ^c	$\Delta\text{VEA}_{\text{PCM}}$ (eV)
Arg24 & Glu518	$[\text{CH}_3\text{NHC}(\text{NH}_2)_2]^+$ & $[\text{CH}_3\text{COO}]^-$	9.34	10.78	0.01	-0.01	0.05
Glu47	$[-\text{CH}_2\text{CH}_2\text{COO}]^-$		8.41	-0.02	0.02	-0.39
Glu47 & Lys82	$[-\text{CH}_2\text{CH}_2\text{COO}]^-$ & $[\text{CH}_3\text{NH}_3]^+$	10.59	8.41	-0.01	0.01	-0.08
Lys45	$[-\text{NH}_3]^+$	6.37		0.03	-0.05	0.51
Asp63	$[-\text{CH}_2\text{CH}_2\text{COO}]^-$		6.18	0.06	0.04	-0.50
Lys45 & Asp63	$[-\text{CH}_2\text{CH}_2\text{COO}]^-$ & $[\text{CH}_3\text{NH}_3]^+$	6.37	6.18	0.04	-0.04	-0.01
Arg86	$[\text{CH}_3\text{CH}_2\text{NHC}(\text{NH}_2)_2]^+$	8.50		-0.03^b	0.03^b	0.40^b
Arg1152	$[\text{CH}_3\text{CH}_2\text{NHC}(\text{NH}_2)_2]^+$	9.26		-0.03^b	0.03^c	0.37^c
Arg152 & Glu108	$[\text{CH}_3\text{CH}_2\text{NHC}(\text{NH}_2)_2]^+$ & $[\text{CH}_3\text{COO}]^-$	9.26	9.81	-0.02^b	0.01^c	0.04^c
Lys21	$[\text{CH}_3\text{NH}_3]^+{}^d$	10.93		-0.03	0.01	0.30

^a Dashes indicate that the added group was covalently bonded to the reference model.

^b Distances to the nearest Fe are measured from Arg(C_γ) or Lys(N_ε). ^c Only the largest-magnitude ΔDI is given, with preference for the positive value if multiple DI's of equal magnitude but opposite sign were found.

The necessity of including salt-bridging residues in a computational model depends on the DD that yields the 0.05 eV effect we are considering to be significant. The Arg152 $\cdots$ Glu108 and Arg24 $\cdots$ Glu518 salt-bridge pairs have differential distances of 1.44 Å and 0.55 Å, resulting in effects of 0.05 and 0.04 eV, respectively; thus, the 0.87 Å DD cutoff for CO dipoles described above is also expected to be a reasonable standard for evaluating salt bridges. However, the distance from the cluster is also an important factor to consider. At longer distances (20 Å or more), the effect of a salt-bridging pair may become negligible, regardless of the DD, but individual charged residues have a significant effect at distances of up to 60 Å or more, depending on the dielectric constant (see Figure 3.3). Fortunately, these long range interactions are purely electrostatic and are expected to be well-reproduced using point charges. As a test, the Glu47 side chain was modeled with point charges derived from AIM, and the Δ DI values deviated by less than 0.005, while the Δ VEA_{PCM} value was -0.37 eV compared to -0.39 eV in the fully molecular calculation. In summary, we recommend modeling all nearby (<8.00 Å) charged side chains as standard atoms, even if the differential distance is small, because the Δ DI will likely still be significant. All additional charged side chains within at least 60 Å should be modeled with point charges, keeping in mind that charged residues on the surface of the protein should not be included, due to the strongly charge-attenuating solvent. The buried charged residues involved in salt bridges may not have a significant effect, but they can be modeled with point charges with very low computational cost.

Discussion

The results of this study showed that protein environments have strong covalent and electrostatic effects on Fe-S clusters through hydrogen bonding and electrostatic interactions from dipoles and charged groups. Hydrogen bonds that amide NH groups of backbones and side chains form with Fe-bound sulfur species have the effect of decreasing covalency of the adjacent Fe–S bond(s), which is compensated by the other Fe ligands in a ripple effect that spreads throughout the cluster and the cysteines (see The Effects of Hydrogen Bonds on Fe-S Clusters). Electrostatic interactions from charged species cause a similar ripple effect, polarizing and shifting the electron density of the cluster as electron-electron repulsion is reduced (see The Effects of Charged Residues on Fe-S Clusters). Electrostatic effects are remarkably long-range for buried species, with changes in VEA_{PCM} on the order of 0.05 eV at distances of 30–60 Å from the cluster, depending on the dielectric constant of the protein environment, as is shown by Coulombs law (see Figure 3.4). Other charged species in the protein, such as the other Fe-S clusters, will have analogous electrostatic effects that depend on their charge distribution. Amide dipoles and salt bridges also have polarizing effects that depend not only on the distance from the cluster, but on the orientation and the relative distances of the positively and negatively charged ends, which we have termed the dipole differential distance (DD). If the dipole DD is not at least 0.87 Å in magnitude, the dipole is not likely to significantly perturb the cluster, as shown in the two preceding sections.

By quantitatively mapping the covalent and electrostatic effects of the hydrogen bonds, electric dipoles, and charged side chains on the [2Fe-2S] cluster in terms of ΔDI

and $\Delta\text{VEA}_{\text{PCM}}$, the relative importance of each of the surrounding residues and their functional groups was identified. Taking into account the location and orientation of the dipoles and charged residues, we have determined an algorithm that can be used to *a priori* select functional groups that will have a significant effect on the cluster, defined by a ΔDI or $\Delta\text{VEA}_{\text{PCM}}$ value of 0.03 or 0.05 eV, respectively. A minimal computational model of a protein-embedded Fe-S cluster should contain the following list of residues and functional groups:

- (1) Fe-bound cysteines including the peptide bond to the neighboring CO group,
- (2) groups that hydrogen bond to an Fe-bound sulfur species,
- (3) CO groups with C or O within 4 Å of an Fe-bound sulfur species,
- (4) CO groups with an O $\cdots$ Fe distance of less than 10 Å and a dipole DD of at least 0.87 Å magnitude,
- (5) waters with an O $\cdots$ Fe distance of less than 8 Å,
- (6) buried charged groups within 7 Å of an Fe site, and
- (7) additional buried charged side chains and other charged species (e.g., an Fe-S cluster) within 60 Å of an Fe site, which can be modeled with point charges

The algorithm for selecting significant functional groups was used to construct the new computational model of the [2Fe-2S] site that is presented in Figure 3.5 in the same style as the qualitative map (Figure 3.1, see Quantitative Mapping of Protein Environmental Effects), except the specific groups to be included are now specified, and those that can be modeled with point charges are depicted as unfilled shapes. Both figures do not show the charged residues beyond the third coordination sphere, which would all

be modeled with point charges. By comparing the two maps, we see that all of the second-coordination-sphere residues in Figure 3.1 had a significant effect on the cluster, except Thr50, which was not selected by the algorithm. In the third coordination sphere, five of the nine residues (Ser31, Cys39, Val59, Leu20, Asn40) did not have a significant effect and do not need to be included in a computational model. The NH group of Gly151 was added to the third coordination sphere to complete the peptide bond to Cys150(CO), producing a more accurate dipole than if Cys150(CO) were modeled as formaldehyde. All the waters except 82 and 23 were included, due to the long-range electrostatic effect of their dipoles. The computational model based on Figure 3.5 contains 167 atoms, which would be expensive with 6-311+G(d), but very feasible with the smaller, but still well-performing,^{77, 195} triple- ζ Stuttgart-Dresden effective core potential basis set.^{131, 153, 154}

The protein environmental interactions described and quantified in this paper are expected to be very similar to those affecting Fe-S clusters of all sizes, and also other transition metal sites in metalloenzymes. Electrostatic interactions, in particular, are transferable to any metalloenzyme active-site, since the electric potential at a metal site due to the protein environment is dependent on the location of the metal, not its charge. The algorithm developed in this paper is a useful tool for constructing computational models with an optimal balance of accuracy and efficiency. Larger models would have diminishing gains in accuracy, while smaller models would miss significant interactions that tune active-site properties.

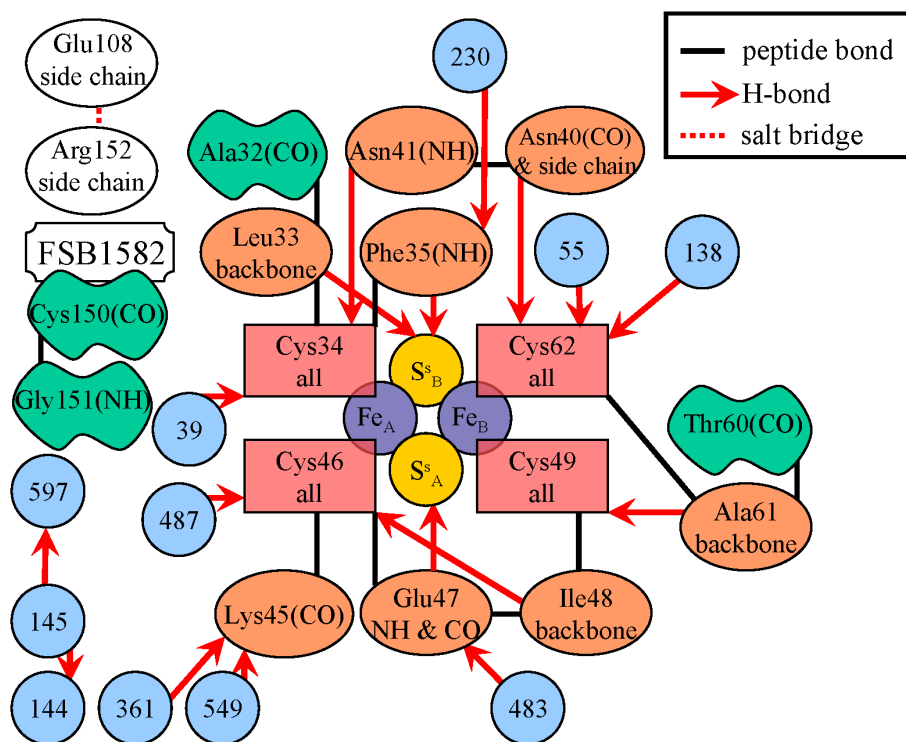


Figure 3.5. Map of the ~ 8 Å environment of the [2Fe-2S] cluster showing residues selected by the algorithm developed in this study, with the first coordination sphere in pink, second coordination sphere in orange, third coordination sphere in green, waters in blue, and groups to be modeled by point charges in colorless shapes. FSB1582 is a [4Fe-4S] cluster that can be modeled by point charges. The atoms of each residue that must be modeled to capture all significant interactions are indicated.

CHAPTER 4

COMPARATIVE ASSESSMENT OF THE COMPOSITION AND CHARGE STATE
OF NITROGENASE FeMo-COFACTORIntroduction

Biological nitrogen fixation is catalyzed by the two-component nitrogenase metalloenzyme.²⁵ The catalytically active [M-7Fe-9S] cluster of nitrogenase occurs in three forms distinguished by its composition, where M is Mo, V, or Fe. The Mo-containing nitrogenase is the most active and most studied and thus the focus of this research. A significant limitation in understanding the molecular basis of biological nitrogen fixation is the lack of electronic structural and compositional knowledge of the catalytically active iron-molybdenum-sulfur cluster, FeMo-co. FeMo-co has been identified as a [Mo-7Fe-9S-X] cluster from the nearly atomic resolution (1.16 Å) crystal structure of the MoFe protein of nitrogenase.²⁶ While the composition of the central ligand (X) could not be unambiguously established experimentally, crystallographic analysis of the resolution-dependent electron-density profile and the interaction of nitrogenase with dinitrogen and ammonia, it was tentatively assigned to be N. The two neighboring elements of C and O have also fit well the experimental electron density profiles. In FeMo-co, these atoms would correspond to carbido (C^{4-}), nitrido (N^{3-}), or oxo (O^{2-}) ligands. Other 10-electron ions as alternative ligands, such as B^{5-} , F^{-} , Na^{+} , or Mg^{2+} can likely be excluded due to charge incompatibility (see below) or limited

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biochemical availability (B^{5-} , F^-). Cys275 and His442 residues on the terminal Fe and Mo, respectively, and a nonprotein ligand, homocitrate, complete the inner-sphere coordination environment of FeMo-co.

The MoFe-protein has been isolated with three forms of FeMo-co: resting or native ($FeMo-co^N$), one-electron reduced ($FeMo-co^{Red}$), and one-electron oxidized ($FeMo-co^{Ox}$).³ Electron nuclear double resonance (ENDOR) data suggest the Mo center is diamagnetic with a formally +IV (d^2) oxidation state in the resting form.¹⁹⁶ X-ray absorption spectroscopic (XAS) measurements further support the presence of the Mo^{IV} oxidation state in all three forms.^{107, 197} $FeMo-co^N$ shows a characteristic $S_t = 3/2$ EPR signal, while the reduced form is EPR silent with integer spin, $S_t \geq 1$. The oxidized form is diamagnetic as observed at 4 K,¹⁹⁸⁻²⁰⁰ but a low-lying excited $S_t \geq 1$ state is populated between 240 and 320 K.²⁰¹ The reduction potential for the $FeMo-co^{Ox}/FeMo-co^N$ couple has been determined to be -42 mV,²⁰² while the second reduction step for $FeMo-co^N/FeMo-co^{Red}$ has only been estimated to be -465 mV.³

FeMo-co can be extracted (exFeMo-co) into *N*-methyl formamide (NMF) solution with the homocitrate ligand remaining coordinated, a deprotonated solvent anion (NMF^-) substituting the negatively charged thiolate of Cys275, and a neutral NMF molecule replacing the imidazole of His442.²⁷ A cation binding study indicated that $exFeMo-co^{Ox}$ has an overall charge of -2 .²⁰³ With a plausible assumption that the protonation state of homocitrate is the same in the protein-bound and extracted form, the charge of $FeMo-co^{Ox}$ can be considered to be the same as the $exFeMo-co^{Ox}$. Thus, the charge of $FeMo-co^N$ is taken to be -3 , which provides a criterion for evaluating the formal oxidation states

of the unknown Fe centers. Moreover, the $S_t = 3/2$ resting form and a diamagnetic Mo^{IV} center limit the formal Fe oxidation states to $6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}$, $4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}$, $2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}$, and a 7Fe^{III} state that is unlikely due to the required Fe^{IV} site in the oxidized form. ^{57}Fe ENDOR experiments led to the suggestion of the $6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}$ assignment for the CO-bound FeMo-co ,²⁰⁴ while Mössbauer isomer shifts were more consistent with the $4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}$ state for the resting form.²⁰⁵ An electrochemical study of exFeMo-co linked the above Mössbauer and ENDOR assignments by implying that the CO-bound form is more consistent with a two-electron-reduced form relative to the resting state.²⁷

Earlier computational studies employing density functional theory (DFT) also favored the $4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}$ oxidation state for the resting form. Vrajmasu *et al.* calculated Mössbauer isomer shifts for all possible Fe oxidation states and $\text{X} = \text{C}^{4-}$, N^{3-} , and O^{2-} ; only the $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}]$, $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{C}^{4-}]$, and $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{N}^{3-}]$ oxidation state assignments were consistent with experiments.²⁰⁶ As an indication for the interconnectedness of the formal oxidation states of the Fe centers and the nature of the interstitial ligand, Lovell, et al. initially found preference for the $6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}$ state using an early FeMo-co model without the presence of an interstitial atom.⁸⁰ However, inclusion of an anionic central ligand gave better agreement between the calculated and the experimental Mössbauer isomer shifts for the $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}]$ state than that of $[6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}-\text{N}^{3-}]$.⁸¹ Furthermore, structural optimizations favored the $4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}$ state with either N^{3-} or C^{4-} while disfavoring O^{2-} . The calculated reduction potentials indicated that $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}]$ was the most likely oxidation state and composition. Notably, the results for the $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{N}^{3-}]$ state were not reported. By comparing

HOMO energies of FeMo-co and Fe-S clusters, Dance found preference for $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}]$, or $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{C}^{4-}]$ with a protonated μ^2 -sulfide or Fe site.^{207, 208} Comparison of the three lowest energy spin states showed that $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{N}^{3-}]$ had an $S_t = 3/2$ ground state, consistent with experiment, while $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}]$ had an $S_t = 5/2$ ground state. The $S_t = 3/2$ state was found to be about 20 kJ/mol higher for the latter. By contrast, using noncollinear spin calculations, Shimpl et al. reported $S_t = 3/2$ to be the ground state for $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}]$, while the ground states for $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{N}^{3-}]$ and $[6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}-\text{N}^{3-}]$ were $S_t = 1/2$ and $S_t = 0.24$, respectively, with the optimized $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}]$ giving the best agreement with experiment.⁹¹ In summary, previous computational work favors the $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}]$ state, although other states and compositions could not be unequivocally ruled out.

It is important to note that density functional theory calculations can be greatly influenced by the nature and the amount of exchange and correlation functionals, the completeness of employed basis sets and the level of truncation of the employed computational model. In addition, due to the presence of high-spin Fe centers within weak-field ligand environments, the method of describing ferro- and antiferromagnetic interactions is critical. Mössbauer²⁰⁵ and ENDOR²⁰⁹ measurements indicate four parallel spins and three antiparallel to the majority spin (spin-up or α) direction. While theoretically not exact,⁷² a commonly used practical method to treat spin-coupling interaction within the formalism of DFT is the broken symmetry (BS) approach.⁷¹ For FeMo-co^N, Noodleman defined 10 major BS spin-coupling patterns⁸⁰ with numerous alternative spin configurations. In addition to the challenge of obtaining and maintaining

the correct spin state, the lowest energy BS state was found to depend on the chemical composition of the computational model and the employed density functional.⁸⁵ As discussed below, the atomic coordinates of computational models are also critical, particularly when the coordinates are based on a protein crystal structure with modest resolution. Furthermore, the protonation states of coordinated residues and ligands are often unknown, even for a close to atomic resolution protein structure. The protonation state of the homocitrate ligand is a key aspect that has not yet been systematically evaluated. This is important because homocitrate contributes to the overall charge of FeMo-co. Furthermore, the protonation state can affect the bonding interactions around the Mo center and thus have implications for a proposed mechanism in which homocitrate becomes monodentate, opening up a coordination site for N₂.²¹⁰ The deprotonated homocitrate ligand with a charge of -4 (or a truncated version with a charge of -2) has been chosen for many theoretical studies,^{81, 208, 211, 212} while some assume a -3 charge for the complete ligand.^{204, 206} An earlier computational work already suggested the possibility that the coordinated hydroxyl oxygen is protonated in the resting form.²¹³ Others proposed that protonation of the coordinated carboxylate group may be the first step of the catalytic mechanism.^{45, 210}

In this study we carried out a comprehensive evaluation of all available experimental data with respect to spin-coupling schemes, spin state energies, iron oxidation states, compositions of the interstitial atom, protonation states of the coordinated groups of the homocitrate ligand, reduction potentials, isomer shifts, quadrupole splittings and hyperfine coupling parameters. On the basis of a series of

structural, energetic, electronic, and magnetic criteria, we can rule out certain compositions and oxidation states, and we find that only the $[\text{Mo}^{\text{IV}}\text{-}2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}\text{-}9\text{S}^{2-}\text{-}\text{C}^{4-}]^+$ composition with hydroxyl-protonated homocitrate ligand (HC^{3-}) is favored consistently for the resting form of FeMo-co.

Computational Details

The computational model of FeMo-co (Figure 4.1) was based on the highest resolution (1.16 Å) crystal structure of the MoFe protein available to date from *Azotobacter vinelandii* (PDB ID 1M1N)²⁶. The ligands Cys275, His442, and homocitrate were truncated to methylthiolate, imidazole, and glycolate, respectively. We consider glycolate to be an acceptable computational model of homocitrate for evaluating the electronic and geometric structures of FeMo-co due to the limited role of the omitted distal carboxylate arms of homocitrate in polarization of the FeMo-co electron density similarly to weakly bound, solvated counterions.

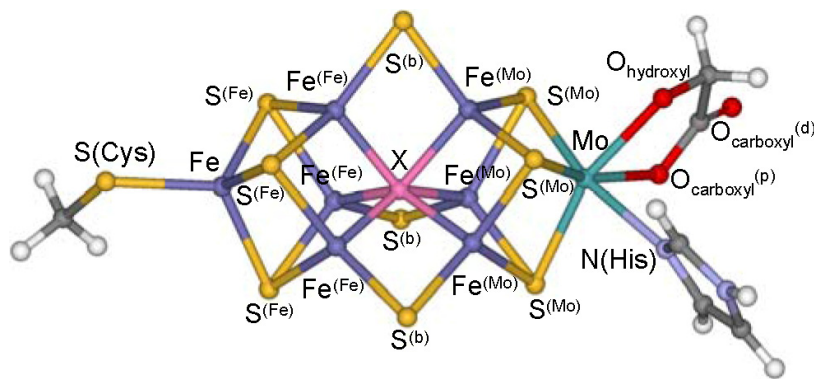


Figure 4.1. Computational model of FeMo-co based on the 1.16 Å crystal structure of the MoFe protein from *Azotobacter vinelandii* (PDB ID 1M1N)²⁶.

The majority of this work (see below for exceptions) was carried out using Gaussian03 Rev. E.01¹²⁹ employing the gradient-corrected Becke88⁵⁹ exchange and Perdew86⁵⁸ correlation functionals (BP86) and the Stuttgart–Dresden effective core potentials and valence basis set (SDD-VTZ(d))^{131, 153, 154} for all atoms, with additional polarization functions for Fe¹⁵¹, Mo¹⁵¹ and S¹⁵². This choice was rigorously validated for [4Fe-4S] clusters in a previous work,⁷⁷ and to ensure consistency, representative calculations (see below) were also carried out with hybrid functionals such as B3LYP,⁵⁷ B5HFP86,²¹⁴ and/or a larger basis set (6-311+G(d)).²¹⁵ Isomer shifts, quadrupole splittings and the hyperfine coupling constant for interstitial ligand X were calculated using ADF2009²¹⁶⁻²¹⁸ at the BP86/STO-TZP level, with an integration accuracy of 5. The Conductor-like Screening Model (COSMO)²¹⁹⁻²²² with $\epsilon = 30$ was used to approximate the polar crystal or solvent environments of the complexes in the isomer shift training set, while the less polar constant, $\epsilon = 10$, was used to approximate the dielectric environment of the protein matrix around FeMo-co. The spin-coupling calculations were all carried out using the oxidation state assignment and composition [4Fe^{II}-3Fe^{III}-N³⁻] without geometry optimizations for *in vacuo* computational models. Notably, gas-phase calculations for models with -4 or greater negative overall charge showed unreasonable spin densities; thus, these highly charged models were embedded in a polarizable continuum model (PCM)⁸⁷⁻⁸⁹ with $\epsilon = 10$. Relative energies of spin states calculated using PCM were within 2 kJ/mol of the corresponding energy differences from gas-phase calculations. However, for consistency, all geometry optimizations were performed in the presence of PCM. Optimizations at the computationally more expensive B5HFP86/6-

311+G(d) level resulted in geometric structural deviations of only 0.01 Å relative to BP86/SDD-VTZ(d). This is similar to previously determined experimental vs calculated structural deviations of less than 0.03 Å (rms value) for the model system $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$ at the B5HFP86/6-311+G(d) level.⁷⁷

Ferro- and antiferromagnetic spin-coupling schemes were treated using the broken symmetry (BS)⁷¹ approach.^a The initial localized spin states were constructed by the ionic fragment approach.⁷⁷ For example, the $\text{FeMo-co}^{\text{Ox}}$ state has been described in the literature as diamagnetic ($S_t = 0$).¹⁹⁸⁻²⁰⁰ Thus, the initial electronic structure was constructed from Fe^{II} ($m_s = \pm 2$) and Fe^{III} ($m_s = \pm 5/2$) centers to result in an $M_s = 0$ state, where the total number of spin-up (α) and spin-down (β) electrons was equal. The S_t and M_s spin quantum numbers are used to differentiate among the pure spin states and the calculated broken symmetry (BS) spin states. The initial ionic electronic structure was allowed to relax to the broken-symmetry solution. The order of Fe^{II} and Fe^{III} centers in the ionic fragments had no effect on the converged broken-symmetry state. Pure antiferromagnetically coupled spin states were considered for reduction potential calculations by using a spin projection procedure⁷⁵ based on the Heisenberg Hamiltonian with FeMo-co modeled as a two-spin system. This approach is conceptually incorrect due to the presence of seven paramagnetic centers; however, this can be considered as a first-order approximation due to the formal topology of FeMo-co being composed of a $[\text{Mo-3Fe-3S}]$ and a $[\text{4Fe-3S}]$ cluster that are coupled through three bridging sulfide and the

^a In the BS method, paramagnetic centers have majority spin-up or spin-down electrons, and the total system has a well-defined total spin quantum number (M_s). Although BS states are not pure spin states and do not represent a conceptually correct solution of the spin operator, their single-reference representation can be conveniently accessed by the DFT formalism and can accurately predict the geometric and electronic structural features of Fe-S clusters.

central ligands. With this simplified model, there is a linear relationship between energies and expectation values of the spin operator ($\langle S^2 \rangle$), which was determined from the broken symmetry and the pure ferromagnetically coupled (maximal S_i) state. Then the energy of the pure ground spin state was obtained using the known $\langle S^2 \rangle$ value (e.g., 15/4 for an $S_t = 3/2$ state). A more rigorous approach to spin projection,²²³ in which the Hamiltonian included coupling between the individual Fe sites, was carried out for $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}]$, and the projected ground state energy only deviates by 4 kJ/mol between the two approaches (see Results). Double-exchange²²⁴ effects were not included in the spin state analysis, but are the subject of ongoing work, as an extensive treatment adds substantial complexity, even to two-center systems such as $[2\text{Fe}-2\text{S}]$ clusters.¹⁹⁰ A recent work considered double-exchange interactions for a multinuclear spin system on top of the Heisenberg exchange and an effective Coulomb intercenter repulsion;²²⁵ however, due to computational limitations the size of the system was limited to six Fe centers.

In order to evaluate the adequacy of the selected level of theory, the comparison of experimental¹⁸⁰ and calculated sulfur 3p orbital characters ($S(3p)$) of metal–sulfide bonds was carried out. Sulfur K-edge X-ray absorption spectroscopic measurements¹⁸⁰ for a prototypical model complex of $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$ define a total of 1.23 and 0.41 e donation from the bridging sulfides and thiolate sulfurs to the irons. The theoretical sulfur covalency values were derived from the difference between the $S(3p)$ atomic populations of the covalent electronic structure and the theoretical ionic limit of six electrons. $S(3p)$ characters were obtained from the Natural Population Analysis (NPA)²²⁶ with a minimal valence set, where $S(3d)$, $\text{Fe}(4s, 4p)$, $\text{Mo}(5p)$, and higher orbitals were treated as part of

the Rydberg atomic orbital set. The BP86/SDD-VTZ(d) level gives 1.19 and 0.40 e S(3p) character for the Fe–sulfide and Fe–thiolate bonds that already well approximate the experimental values. Employing a larger all-electron basis set (6-311+G(d)) and an experimentally validated hybrid functional (B5HFP86)²¹⁴ improves only slightly this agreement to 1.22 and 0.42 e . A detailed analysis of errors in calculated parameters for the same model system has already been published.⁷⁷

To determine reduction potentials that include contributions from solvation effects, free energy changes ($\Delta G^\circ_{\text{reduction}}$) were calculated^a according to the following equations:

$$\Delta G^\circ_{\text{reduction}} = \Delta G^\circ_{\text{solute}} + \Delta \Delta G^\circ_{\text{solvation}} \quad (4.1)$$

$$\Delta G^\circ_{\text{solute}} = \Delta \varepsilon_{\text{SCF}}^{\text{s.p.}} + \Delta \text{ZPE}_{\text{solute,corr}} + \Delta H^\circ_{\text{solute,corr}} - T \Delta S^\circ_{\text{solute,corr}} \quad (4.2)$$

$$\Delta \Delta G^\circ_{\text{solvation}} = \Delta \Delta G_{\text{electrostatic solute/solvent}} + \Delta \Delta G_{\text{nonelectrostatic solvent/solvent}} \quad (4.3)$$

where $\Delta \varepsilon_{\text{SCF}}^{\text{s.p.}}$ is the difference in the gas-phase spin-projected (indicated by the superscript ‘s.p.’) ground state electronic energy of the PCM-optimized reduced and oxidized forms. The enthalpy, entropy, and zero-point energy (ZPE) corrections were calculated as changes between the reduced and the oxidized forms of the solute. $\Delta G_{\text{electrostatic}}$ refers to the electrostatic solute–solvent interactions, and $\Delta G_{\text{nonelectrostatic}}$ refers to the nonelectrostatic cavitation, dispersion, and repulsion energies of solvent–solvent interactions. Overall, in this approach we take into account both inner- and outer-sphere

^a The second derivative effect of the nonelectrostatic interactions on $\Delta G^\circ_{\text{solute}}$ were not included, but we expect this contribution to be negligible due to small structural changes upon redox. The $\Delta G^\circ_{\text{solute}}$ and $\Delta \Delta G^\circ_{\text{solvation}}$ terms were calculated for BS states without spin-projection correction to solvation terms.

relaxation effects including the geometric relaxations upon solvation. The standard one-electron reduction potentials ($n = 1$) were then calculated using the Nernst equation:

$$\Delta G^\circ_{\text{reduction}} = -nFE^\circ \quad (4.4)$$

where F is the Faraday constant, $23.06 \text{ kcal mol}^{-1} \text{ V}^{-1}$. A -4.43 eV correction was applied as a reference for the absolute reduction potential of the standard hydrogen electrode (SHE).²⁰² Using only gas-phase-optimized structures and exclusion of the corrections due to geometric changes upon solvation can introduce a considerable 60 mV error.^a With the above approach, gas-phase optimizations actually can be omitted, since each contribution mentioned in Equations 4.1–4.3 can be derived from PCM-optimized structures and related energy analysis.

Mössbauer quadrupole splittings (ΔE_Q) were calculated from the electric field gradient tensors (V) in a coordinate system where $V_{zz} \geq V_{xx} \geq V_{yy}$, using Equation 4.5, where e is the elementary charge, Q is the nuclear quadrupole moment of ^{57}Fe (0.158 barn)²²⁷ and η is the asymmetry parameter ($(|V_{xx} - V_{yy}|)/V_{zz}$).

$$\Delta E_Q = \frac{1}{2} eQV_{zz} \sqrt{1 + \frac{\eta^2}{3}} \quad (4.5)$$

Hyperfine coupling constants (HFCC) depend critically on the spin-polarized electron density at the nucleus ($\rho_s(0)$), which is difficult to calculate accurately for transition-metal systems.²²⁸ For oligonuclear complexes, magnetic interactions and the use of the BS approach further complicate the calculations. Neese et al. have developed a method based on the Heisenberg Hamiltonian,²²⁹ which derives scaling factors for the

^a Gas-phase optimizations were only performed for one representative model, $[\text{4Fe}^{\text{II}}\text{-3Fe}^{\text{III}}\text{-N}^{\text{3-}}]$ with protonated $\text{O}_{\text{hydroxyl}}$ of homocitrate.

calculated HFCCs at transition metal centers. However, a recent study of the Fe₆S₆ “H-cluster” of [FeFe]-hydrogenase demonstrates that even with the scaling factors the absolute accuracy of calculated HFCCs is low, although the ratios of HFCCs can be qualitatively useful.²³⁰ In this work we employ the semiempirical method developed by Mouesca et al.,^{231, 232} which avoids calculation of the problematic $\rho_s(0)$ values. The procedure has previously been used to analyze spin coupling in FeMo-co⁸⁰ and the P-cluster²³¹ of nitrogenase.

The isotropic contributions to the observed HFCCs (a_i^{exp}) for Fe sites in a spin-coupled system are proportional to the projections (K_i) of the local site spins (S_i) onto the total spin. Thus, the hyperfine parameters (a_i^{calc}) were determined using the following equation:

$$a_i^{\text{calc}} = K_i(|\rho_s(\text{Fe}_i)|/2S_i)a_i^{\text{ionic}} \quad (4.6)$$

In Equation 4.6, the spin densities, $\rho_s(\text{Fe}_i)$, scale the empirically derived²³² intrinsic Fe site parameters, a_i^{ionic} , which are -34.0 MHz, -32.5 MHz, and -31.0 MHz for Fe²⁺, Fe^{2.5+} and Fe³⁺ with $S_i = 2$, $9/4$ and $5/2$, respectively. K_i factors (see Appendix C) were calculated according to Equation 4.7 developed by Noodleman and co-workers.^{80, 85}

$$K_i = \langle \mathbf{S}_i \cdot \mathbf{S}_i \rangle / \langle \mathbf{S}_i \cdot \mathbf{S}_i \rangle \quad (4.7)$$

Calculations of hyperfine couplings for X = ¹⁴N or ¹³C were also performed according to Equation 4.8 by Noodleman and co-workers,⁸⁵ where $a_{\text{iso}}^{\text{BS}}(\text{X})$ values were obtained from the broken symmetry calculation and scaled by the P_x factors 0.40, 0.46 and 0.51 for the 6Fe^{II}-1Fe^{III}, 4Fe^{II}-3Fe^{III} and 2Fe^{II}-5Fe^{III} oxidation state distributions, respectively.

$$a_{\text{iso}}(\text{X}) = \frac{1}{6} \sum_{i=2,3,\dots,7} (|K_i / S_i| S_t) a_{\text{iso}}^{\text{BS}}(\text{X}) = P_{\text{x}} a_{\text{iso}}^{\text{BS}}(\text{X}) \quad (4.8)$$

Due to the local symmetry of the central 6 Fe belt and the bridging sulfide ligands, we considered the same hyperfine coupling interaction between the Fe and the interstitial atom, X. The electron density at X takes into account the individual effects of the neighboring Fe sites, and the projection of the calculated hyperfine coupling of X onto the total spin $S_t = 3/2$ takes into account the individual projection coefficients and spin of each Fe site.

The ^{57}Fe isomer shifts (δ) from Mössbauer spectroscopy are directly proportional to the electron density at the nuclei, $\rho(0)$. Calculated $\rho(0)$ values are dependent on the employed functional and basis set;²³³ thus, to calculate the ^{57}Fe isomer shifts for FeMo-co, the relationship between $\rho(0)$ and δ was obtained for a training set, which is composed of [Fe-S] and [Mo-Fe-S] clusters of various charge and spin states. The calculated and experimental values were fit to Equation 4.9, where a constant, A (in $e a_0^{-3}$), was chosen to reduce the magnitude of $\rho(0)$ (in $e a_0^{-3}$), and the fit parameters are α (in $e^{-1} a_0^3 \text{ mm s}^{-1}$) and C (in mm s^{-1}).

$$\delta = \alpha(\rho(0) - A) + C \quad (4.9)$$

Results and Discussion

Evaluation of Broken-Symmetry Spin-Coupling Schemes

Assuming weak-field ligand environments, such as those from thiolate and sulfides, the formally Fe^{III} and Fe^{II} centers adopt high-spin $S = 5/2$ and 2 states, respectively. The total spin ($S_t = 3/2$) of $\text{FeMo-co}^{\text{N}}$ is a result of ferro- and

antiferromagnetic coupling between the Fe centers of which four have parallel and three have antiparallel (4+3) spin orientations according to the interpretations of ENDOR²⁰⁹ and Mössbauer²⁰⁵ data. When FeMo-co's electronic structure is treated with broken symmetry (BS) formalism, a given charge and oxidation state distribution with high-spin Fe centers can have 35 non-equivalent BS states with $M_s = 3/2$ that are also consistent with the above-mentioned experimental 4+3 spin orientation. These states can be divided into 10 groups based on the number of antiferromagnetically coupled Fe pairs that are: (1) bridged by two sulfides, and (2) bridged by one sulfide and X (see representative states in Figure 4.2).⁸⁰ The first type is exclusive to pairs that include the terminal Fe. A third type of Fe pair is bridged by only X, and the significance of this type is discussed below. In order to build up a reference base for *a priori* choosing the lowest energy spin state, we evaluated all 35 spin states (see Table C.1). We also evaluated the dependence of the relative energies of spin-coupling states on the applied functional (BP86 vs B3LYP) and basis set (SDD-VTZ(d) vs 6-311+G(d)).

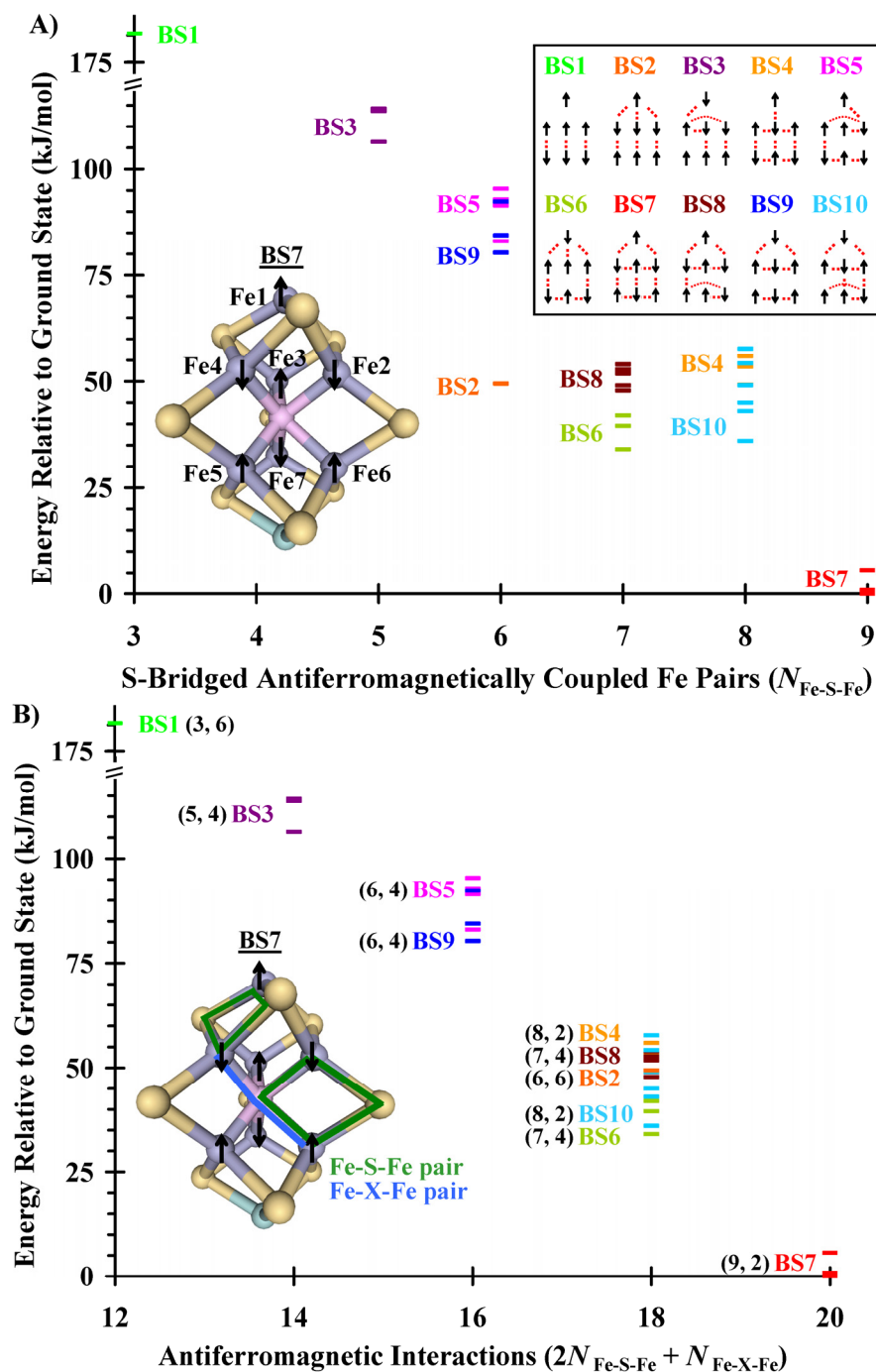


Figure 4.2. Relative energies of the 35 individual spin states to the lowest energy state, BS7, plotted against (A) the number of sulfide-bridged antiferromagnetically coupled Fe pairs and (B) the total number of antiferromagnetic interactions with $N_{\text{Fe-S-Fe}}$ and $N_{\text{Fe-X-Fe}}$ given in parentheses for each group of BS states. Spin distributions (A, inset) show representative examples from each group. The nomenclature for the BS states was adapted from ref 80.

Figure 4.2 summarizes the results obtained for the relative energies of various spin-coupling schemes of $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}]$.^a The results in Figure 4.2A display a clear trend of lower energy with increasing numbers of sulfide-bridged antiferromagnetically coupled pairs of Fe centers ($N_{\text{Fe-S-Fe}}$) at the BP86/SDD-VTZ(d) level. The individual states within each of the 10 major groups depicted in the inset of Figure 4.2A are similar in energy. The BS2 state is strikingly (>25 kJ/mol) lower in energy than the BS9 and BS5 states, even though they all have the same $N_{\text{Fe-S-Fe}}$ (6). This is because BS2 has six X-bridged antiferromagnetically coupled Fe pairs ($N_{\text{Fe-X-Fe}}$), while BS9 and BS5 have four. Similarly, BS4 and BS10 are almost equivalent in energy to BS8 and BS6 because the former have one more $N_{\text{Fe-S-Fe}}$ (8), but two less $N_{\text{Fe-X-Fe}}$ (2). From this relationship we see that the stabilization due to an X-bridged antiferromagnetic interactions is approximately half that of sulfide-bridged interactions. Note that the relative strength of the interactions is not due to the nature of X versus sulfide, but rather the number of bridging ligands. All of the Fe pairs bridged by at least one sulfide have two bridging ligands in total (two sulfides, or a sulfide and X), while the X-bridged pairs have only one. Thus, the primary factor affecting the stability of BS states is the number of individual M–L–M antiferromagnetic interactions ($2N_{\text{Fe-S-Fe}} + N_{\text{Fe-X-Fe}}$), as shown in Figure 4.2B.

Figure 4.3 shows a representative state for each number of antiferromagnetically coupled Fe pairs calculated at the different levels of theory (see Table C.2 for numerical results). Although the overall trend is the same in each case, there is a notable functional dependence for the first excited state among the representative states in Figure 4.3. With

^a Calculations involving other oxidation state distributions or compositions revealed that trends in the relative energies of spin coupling schemes are independent of the cluster composition and charge state.

the pure DFT (GGA) functional, BP86, the lowest energy spin state other than BS7 in Figure 4.3 is BS6, consistent with the findings of Noodleman and co-workers,⁸⁵ who examined the same representative states using a GGA functional, PW91. However, we find that using the popular hybrid functional, B3LYP, BS10 is lower in energy than BS6. Most importantly, the ground state for each tested level of theory is BS7, and the overall trend is the same. A similar trend was also observed by Noodleman and co-workers using an early FeMo-co model without the interstitial atom, although BS6 was found to be the ground state.⁸⁰ With $X = N^{3-}$ and a change in functional, they found BS7 to be the ground state for $X = N^{3-}$,⁸⁵ in agreement with results from Blöchl and co-workers,⁹¹ who used a different theoretical approach. Additionally, ESEEM and ENDOR measurements indicate that if $X = N$ or C the hyperfine coupling to X is weak, which, as shown by DFT, favors a spin state with symmetric spins in the central ‘6Fe-belt’, such as BS7.⁸⁵

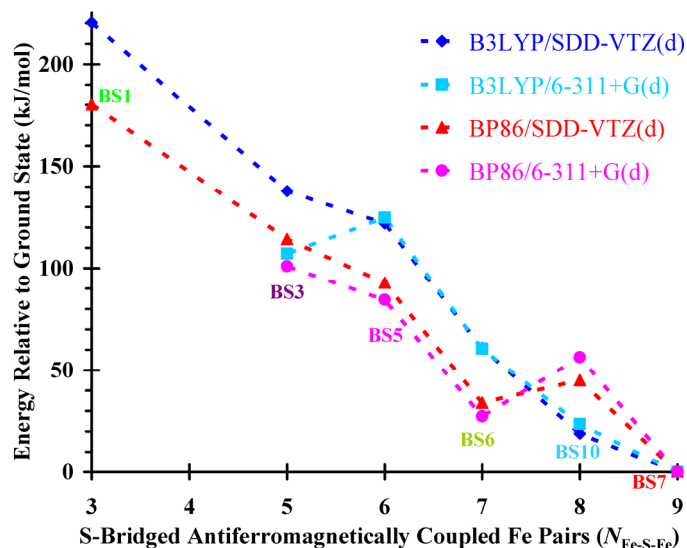


Figure 4.3. Relative energies of representative BS states calculated with various functionals and basis sets. The spin distributions are shown in the inset of Figure 4.2A.

Within BS7, the oxidation states $6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}$, $4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}$ and $2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}$ can be described by several formal distributions that all satisfy $M_s = 3/2$. Three such distributions are given for the $2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}$ state in Figure 4.4. Although the formal subcluster M_s values vary, due to delocalization, calculations converge to a single electronic structure (Figure 4.4, far right). Thus, the oxidation state, M_s value and spin-coupling scheme are enough to uniquely describe an electronic structure, without specifying the specific oxidation state distribution.

Formal oxidation state distributions						Calculated $\rho_s(\text{Fe})$ (e)	
	$\text{Fe}^{\text{III}}\downarrow \text{Fe}^{\text{II}}\uparrow$		$\text{Fe}^{\text{III}}\downarrow \text{Fe}^{\text{II}}\uparrow$		$\text{Fe}^{\text{III}}\downarrow \text{Fe}^{\text{III}}\uparrow$	-2.81	+2.78
	$\text{Fe}^{\text{III}}\uparrow \text{Fe}^{\text{III}}\uparrow \text{Fe}^{\text{III}}\downarrow$		$\text{Fe}^{\text{III}}\uparrow \text{Fe}^{\text{II}}\uparrow \text{Fe}^{\text{III}}\downarrow$		$\text{Fe}^{\text{II}}\uparrow \text{Fe}^{\text{II}}\uparrow \text{Fe}^{\text{III}}\downarrow$	+2.80	+2.81 -2.77
	$\underbrace{\text{Fe}^{\text{III}}\downarrow \text{Fe}^{\text{II}}\uparrow}_{M_s: 0} \quad \underbrace{\phantom{\text{Fe}^{\text{III}}\downarrow \text{Fe}^{\text{II}}\uparrow}}_{+3/2}$		$\underbrace{\text{Fe}^{\text{III}}\downarrow \text{Fe}^{\text{III}}\uparrow}_{-1/2} \quad \underbrace{\phantom{\text{Fe}^{\text{III}}\downarrow \text{Fe}^{\text{III}}\uparrow}}_{+2}$		$\underbrace{\text{Fe}^{\text{III}}\downarrow \text{Fe}^{\text{III}}\uparrow}_{-1} \quad \underbrace{\phantom{\text{Fe}^{\text{III}}\downarrow \text{Fe}^{\text{III}}\uparrow}}_{+5/2}$	-2.91	+2.94

Figure 4.4. Three of the formal oxidation state distributions for the $2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}$ state, with subcluster M_s values indicated below. Calculated spin densities $\rho_s(\text{Fe})$ for $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{N}^{3-}]$ are given on the right.

Spin State Dependence on Oxidation States and Compositions for $\text{FeMo-co}^{\text{N}}$ and $\text{FeMo-co}^{\text{Ox}}$

A commonly considered criterion for evaluating the likeliness of a given oxidation state and composition for FeMo-co is that the ground spin state must be consistent with the experimentally observed $S_t = 3/2$. It is important to realize that the consideration of only weak-field, high-spin Fe^{II} and Fe^{III} centers limits the possible oxidation states of the resting form to $6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}$, $4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}$, $2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}$ and 7Fe^{III} , all of which can access the $S_t = 3/2$ state. The 7Fe^{III} state is unlikely, as discussed in the

introduction, and it was not evaluated. In order to assess the ground and excited spin states, the energies of $S_t = 5/2$ and $1/2$ for each oxidation state with $X = C^{4-}$, N^{3-} or O^{2-} were compared to $S_t = 3/2$ using the lowest energy BS7 spin-coupling scheme with high-spin Fe^{III} and Fe^{II} centers at atomic coordinates derived from the crystal structure.²⁶ Due to the limitation of the BS approach, only the M_s value is well-defined in the FeMo-co calculations (see Computational Details).

The relative spin state energies presented in this section have been calculated by assuming that the BS7 state with a given M_s value best represents the spin state of corresponding S_t , i.e., the $S_t = 3/2$ state is modeled by an $M_s = 3/2$ BS state. To estimate the validity of this assumption we considered the Heisenberg Hamiltonian. As a first-order approximation, FeMo-co can be considered as a two-spin system, where a single exchange coupling constant, J , couples the $[4Fe-3S]$ subcluster to the $[Mo-3Fe-3S]$ subcluster. With this model, the spin-projected⁷⁵ ground state is -22 kJ/mol lower in energy than the ground BS state for $[4Fe^{II}-3Fe^{III}-N^{3-}]$, and excited spin states are similarly corrected by about -20 kJ/mol. Thus, the two-spin model will not affect the relative ordering of the ground and the closest BS state energies. As an improvement to the single J model, the coupling of all 7 spin sites with 18 J constants was carried out using the Yamaguchi generalized spin projection method,²²³ and spin state energies were obtained by diagonalizing a sizable Hamiltonian.²³⁴ For $[4Fe^{II}-3Fe^{III}-N^{3-}]$ the resulting ground state has $S_t = 3/2$ and is 18 kJ/mol lower in energy than the $M_s = 3/2$ BS7 state. The lowest energy $S_t = 1/2$ and $S_t = 5/2$ states lie about 5 kJ/mol above the ground state, while the excited BS states (see Figure 4.5A) are 40–50 kJ/mol above the ground BS

state. Overall, the BS and the Heisenberg approaches both give qualitatively correct results with respect to the experimental $S_t = 3/2$ ground state and the same relative ordering of excited states. Furthermore, from a thorough analysis of the eigenvectors of the Heisenberg Hamiltonian we find that the BS7 state with high-spin Fe centers and a particular M_s value (3/2, for example) has the highest weighting (11%) among all of the more than 10000 BS states contributing to the lowest energy pure spin state with a corresponding S_t value (3/2, accordingly).

In contrast to other work,^{91, 207} Figure 4.5A shows that $S_t = 3/2$ is the lowest lying spin state regardless of the oxidation state and composition. The preferred $4\text{Fe}^{\text{II}}\text{-}3\text{Fe}^{\text{III}}$ assignment has a large energy gap (>30 kJ/mol) between the ground and the excited spin states; this becomes considerably less (~10 kJ/mol) for $6\text{Fe}^{\text{II}}\text{-}1\text{Fe}^{\text{III}}$ and $2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}$. Overall, an $S_t = 3/2$ ground state does not allow for discriminating between compositions, charges, or formal oxidation states (for numerical results see Table C.3).

The oxidized form of FeMo-co ($\text{FeMo-co}^{\text{Ox}}$) has an even-electron $S_t = 0$ ground spin state according to Mössbauer^{198, 199} and MCD²⁰⁰ measurements, providing another means of evaluating oxidation state and composition. The energies of three spin states, $S_t = 0, 1$, or 2 , were calculated for the $5\text{Fe}^{\text{II}}\text{-}2\text{Fe}^{\text{III}}$, $3\text{Fe}^{\text{II}}\text{-}4\text{Fe}^{\text{III}}$ and $1\text{Fe}^{\text{II}}\text{-}6\text{Fe}^{\text{III}}$ oxidation states, which are the corresponding one-electron-oxidized forms of $6\text{Fe}^{\text{II}}\text{-}1\text{Fe}^{\text{III}}$, $4\text{Fe}^{\text{II}}\text{-}3\text{Fe}^{\text{III}}$, and $2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}$ (with an $S_t = 3/2$ ground state), respectively. As shown in Figure 4.5B, when using the $\text{FeMo-co}^{\text{N}}$ crystal structure for oxidized calculations, no combination of Fe centers and interstitial atom has an $S_t = 0$ ground state.

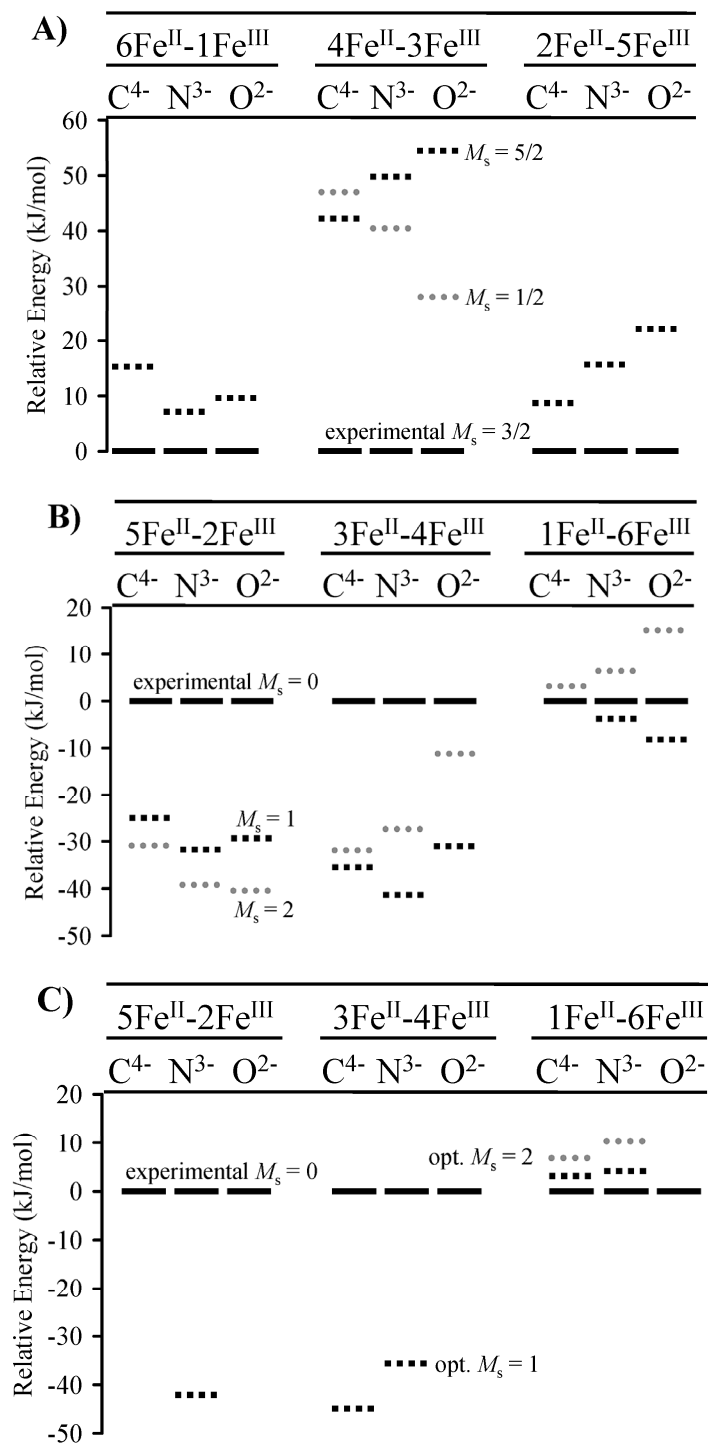


Figure 4.5. Relative energies of (A) the $M_s = 1/2$ (dotted gray line) and $5/2$ states (dotted black line) to the $M_s = 3/2$ ground state (solid black line) of $\text{FeMo-co}^{\text{N}}$, and the $M_s = 1$ (dotted gray line) and 2 states (dotted black line) relative to the $M_s = 0$ state (solid black line) of $\text{FeMo-co}^{\text{Ox}}$ using the experimental crystal structure (B) and optimized geometries (C).

Importantly, using structurally optimized FeMo-co^{Ox} models, we find that the $S_t = 0$ state becomes the ground spin state for 1Fe^{II}-6Fe^{III} (Figure 4.5C) with a low-lying $S_t = 1$ state (~ 3 kJ/mol), showing a close agreement with experiment²⁰¹. For 3Fe^{II}-4Fe^{III} and 5Fe^{II}-2Fe^{III}, geometry-optimized structures show the same experimentally inconsistent high energy $S_t = 0$ state as in Figure 4.5B (for numerical results see Table C.4). However, if the 3Fe^{II}-4Fe^{III} state is considered to be the reduced form of 2Fe^{II}-5Fe^{III}, rather than the oxidized form of 4Fe^{II}-3Fe^{III}, then the results are consistent with the $S_t \geq 1$ state shown by experiment. Independently from the composition of the interstitial atom, these results indicate a strong preference for the 1Fe^{II}-6Fe^{III} oxidation state assignment for FeMo-co^{Ox}, which in turn corresponds to 2Fe^{II}-5Fe^{III} for the resting FeMo-co^N form.

Geometric Differences with Various Oxidation States and Compositions

As shown for the relative energies of FeMo-co^{Ox} spin states, electronic structure is intimately linked to geometric structure. Comparison of the interatomic distances in selected optimized FeMo-co^N structures to those in the nearly atomic resolution (1.16 Å) crystal structure²⁶ and Extended X-ray Absorption Fine Structure analyses^{197, 235} can provide another criterion for evaluating different compositions, charges, and iron oxidation state distributions, as these properties are expected to affect the overall size of the cluster and the Fe–X bond lengths. Table 4.1 compares the structural optimization results for the FeMo-co^N form in the most probable states, [2Fe^{II}-5Fe^{III}-N³⁻], [2Fe^{II}-5Fe^{III}-C⁴⁻], [4Fe^{II}-3Fe^{III}-O²⁻], [4Fe^{II}-3Fe^{III}-N³⁻], and [4Fe^{II}-3Fe^{III}-C⁴⁻], with experimental metric data. The optimized structure of the highly oxidized state [2Fe^{II}-5Fe^{III}-O²⁻] is not

reported due to cluster dissociation. The results for the unlikely, highly reduced $[6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}-\text{N}^{3-}]$ are shown in Table C.5. Looking at the S(Cys)-Fe through $\text{Fe}^{(\text{Fe})}\cdots\text{Fe}^{(\text{Fe})}$ distances, the $2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}$ structures are generally contracted by up to 0.07 Å relative to the crystal structure, while the $4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}$ structures are expanded up to 0.07 Å, the notable exception being the $\text{Fe}^{(\text{Fe})}\cdots\text{Fe}^{(\text{Fe})}$ distances in $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{O}^{2-}]$, which are 0.14 Å expanded. The $\text{Fe}^{(\text{Fe})}\cdots\text{Fe}^{(\text{Fe})}$ distances are expected to be among the most sensitive to the composition of X, along with the $\text{Fe}^{(\text{Fe})}-\text{X}$, $\text{Fe}^{(\text{Fe})}\cdots\text{Fe}^{(\text{Mo})}$, $\text{Fe}^{(\text{Mo})}\cdots\text{Fe}^{(\text{Mo})}$ and $\text{Fe}^{(\text{Mo})}-\text{X}$ distances. For $\text{X} = \text{O}^{2-}$, these distances are 0.10–0.16 Å expanded relative to the crystal structure compared to deviations of -0.02–0.06 Å for $\text{X} = \text{N}^{3-}$ and C^{4-} . The stronger donation from the higher charged and less electronegative N^{3-} or C^{4-} interstitial atoms seems to be necessary to maintain short distances between X and the Fe centers.^a Similar to previous studies,^{81, 85, 236} we find that O^{2-} is likely not the interstitial atom based on geometric criteria. At this level of experimental information and theoretical treatment, the differences between the structures with N^{3-} versus C^{4-} are too small (less than 0.03 Å) to be able to unambiguously rule out either composition.

^a To test the relationship between covalency, electronegativity and Fe–X bond length, we optimized the $4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}$ state with $\text{X} = \text{F}^-$, and the low covalency and high electronegativity led to bonds overly expanded by more than 0.40 Å, indicating the lack of ability to share sufficient electron density for covalent Fe–X bonds.

Table 4.1. Interatomic distances in optimized FeMo-co^N for selected oxidation states^a

X =	calculated distances (Å)									experiment
	2Fe ^{II} -5Fe ^{III} -X				4Fe ^{II} -3Fe ^{III} -X					refs 26, 197, 235
	C ⁴⁻		N ³⁻		C ⁴⁻		N ³⁻		O ²⁻	
HC(O _{hydroxyl})	O ⁻	OH	O ⁻	OH	O ⁻	OH	O ⁻	OH	O ⁻	
S(Cys)-Fe	2.22	2.20	2.20	2.18	2.30	2.28	2.27	2.26	2.25	2.27
Fe-S ^(Fe)	2.26	2.25	2.25	2.25	2.32	2.32	2.31	2.31	2.32	2.28
Fe···Fe ^(Fe)	2.63	2.62	2.63	2.63	2.73	2.72	2.73	2.72	2.73	2.66
S ^(Fe) -Fe ^(Fe)	2.28	2.27	2.28	2.27	2.32	2.31	2.31	2.30	2.30	2.27
Fe ^(Fe) -S ^(h)	2.19	2.19	2.19	2.19	2.24	2.23	2.23	2.23	2.22	2.23
Fe ^(Fe) ···Fe ^(Fe)	2.65	2.64	2.69	2.68	2.66	2.65	2.67	2.67	2.80	2.65
Fe ^(Fe) -X	2.01	2.00	2.03	2.03	2.01	2.00	2.01	2.01	2.10	1.98
Fe ^(Fe) ···Fe ^(Mo)	2.59	2.57	2.58	2.57	2.62	2.61	2.62	2.61	2.76	2.59
Fe ^(Mo) ···Fe ^(Mo)	2.66	2.66	2.68	2.68	2.61	2.60	2.63	2.63	2.74	2.61
Fe ^(Mo) -S ^(b)	2.20	2.19	2.20	2.18	2.24	2.22	2.23	2.21	2.23	2.22
Fe ^(Mo) -X	2.00	2.00	2.00	2.00	2.01	2.00	2.02	2.01	2.12	2.02
Fe ^(Mo) ···Mo	2.76	2.72	2.78	2.74	2.74	2.70	2.75	2.71	2.76	2.69
S ^(Mo) -Fe ^(Mo)	2.28	2.28	2.29	2.29	2.29	2.28	2.29	2.28	2.28	2.23
S ^(Mo) -Mo	2.40	2.37	2.40	2.37	2.39	2.37	2.40	2.38	2.41	2.35
Mo-N(His)	2.25	2.22	2.24	2.21	2.26	2.24	2.26	2.22	2.24	2.30
Mo-O _{carboxyl}	2.12	2.11	2.10	2.08	2.16	2.16	2.14	2.13	2.11	2.18
Mo-O _{hydroxyl}	2.01	2.22	1.98	2.20	2.05	2.26	2.03	2.23	2.01	2.20
avg. Mo-O/N	2.13	2.19	2.11	2.16	2.16	2.22	2.14	2.19	2.12	2.23
Fe···Mo	7.02	6.94	7.02	6.95	7.17	7.11	7.17	7.10	7.22	7.00
avg. error	-0.01	-0.02	-0.01	-0.01	0.02	0.02	0.02	0.02	0.05	
rms dev.	0.06	0.04	0.07	0.05	0.06	0.04	0.07	0.04	0.11	
core avg. error	0.01	0.00	0.02	0.01	0.03	0.02	0.03	0.02	0.07	
core rms dev.	0.04	0.03	0.05	0.04	0.04	0.03	0.04	0.03	0.09	

^a Experimental distances are given for the average of four FeMo-co structures from the crystal structure²⁶ along with those from two EXAFS studies.^{197, 235} Superscripts (Fe) and (Mo) refer to the [4Fe-3S] and [Mo-3Fe-3S] subclusters, respectively; (b) denotes the bridging sulfides. HC(O_{hydroxyl}) refers to the deprotonated (O⁻) or protonated (OH) hydroxyl group of the homocitrate ligand.

In the [Mo-3Fe-3S] subcluster there are modest contractions and expansions of 0.02 Å in the Fe^(Mo)-S^(b) bond length for the 2Fe^{II}-5Fe^{III} and the 4Fe^{II}-3Fe^{III} structures, respectively. These trends reverse for the Fe^(Mo)...Fe^(Mo) distances, which are about 0.05 Å longer in 2Fe^{II}-5Fe^{III} than in the 4Fe^{II}-3Fe^{III} structures or crystal structure. The remaining distances in the Mo-containing subcluster (Fe^(Mo)...Mo, S^(Mo)-Fe^(Mo), and S^(Mo)-Mo) are 0.04–0.09 Å expanded relative to the experimental structures regardless of the

composition and oxidation state. The core metrics without the terminal ligand distances have acceptably low overall rms deviations of 0.04–0.05 Å for FeMo-co^N relative to the experimental structures; however, the [Mo-3Fe-3S] subcluster expands in 4Fe^{II}-3Fe^{III} and 2Fe^{II}-5Fe^{III}, while the [4Fe-3S] subcluster expands in 4Fe^{II}-3Fe^{III} structure, but contracts in 2Fe^{II}-5Fe^{III}. The end-to-end distance of FeMo-co (Fe^{III}–Mo) shows a large difference. In 4Fe^{II}-3Fe^{III}, the longest dimension of the cluster is at least 0.17 Å expanded, while in 2Fe^{II}-5Fe^{III}, this distance deviates by only 0.01 Å from the experimental value of 7.00 Å. The significance of the 0.17 Å expansion may be diminished by considering the percent difference from 7.00 Å, yet the preference of the Mo^{IV}-2Fe^{II}-5Fe^{III} assignment for FeMo-co^N cannot be ignored, since the magnitude of this deviation is considerably larger than the uncertainty in the crystallographic coordinates.

As proposed earlier,^{26, 81} we also considered protonated interstitial ligands, such as HN²⁻, HC³⁻ and H₂C²⁻ ions, for the 2Fe^{II}-5Fe^{III} oxidation state. When X = HC³⁻, the Fe centers closest to the H atom on the interstitial ligand are repelled, resulting in overall expansion of the cluster. The average and rms deviations are very similar (0.11 Å) to those observed for X = O²⁻ composition. The expansion is more extreme with HN²⁻ and H₂C²⁻ due to considerably reduced *e* donation to the Fe centers and in the case of H₂C²⁻ the larger size. On the basis of these structural findings, we can rule out protonated interstitial ligands as filling the central cavity of FeMo-co.

The Protonation State of the Homocitrate Ligand

A large deviation of 0.15–0.22 Å from the experimental structure is found between the Mo center and the O_{hydroxyl} group of the homocitrate ligand (Table 4.1,

columns marked with OH for the homocitrate hydroxyl group, HC(O_{hydroxyl})). This is an important difference because the protonation state of this ligand can significantly affect the electronic structure and thus the redox and coordinative properties of the Mo site. Due to the lack of a strong secondary coordination sphere effect involving this hydroxyl group, the long experimental Mo–O_{hydroxyl} bond indicates that the hydroxyl arm of the homocitrate is likely protonated. To test the correlated effects of ligand protonation and coordination geometry, a set of structural optimizations was carried out for the [2Fe^{II}-5Fe^{III}-N³⁻] state in which either the uncoordinated N_δ of imidazole was deprotonated (imidazolato ligand) or the glycolate ligand was protonated at the proximal carboxyl oxygen, the distal carboxyl oxygen, or the hydroxyl oxygen. The variations in the Mo···Fe and Mo–L distances are summarized in Table 4.2. The presence of an imidazolato ligand results in a slight increase of the Mo–O bond lengths; however, the Mo–N bond length becomes unreasonably short (2.17 Å) relative to the experimental values of 2.30 Å (crystallography) and 2.29 Å (EXAFS). Protonation of either oxygen atoms of the carboxyl group results in an expanded Mo–O_{carboxyl} bond (2.26 and 2.24 Å) without significantly improving the Mo–O_{hydroxyl} bond length (2.01 Å). However, the hydroxyl-protonated model, Mo–O_{hydroxyl}, shows excellent agreement with the crystallographic data (both 2.20 Å). Furthermore, the Mo···Fe and Mo–S distances decrease by 0.03–0.04 Å to 2.74 and 2.37 Å, respectively, which are closer to the crystallographic values (2.69 and 2.34 Å) than in the deprotonated form. As expected, the conjugate Mo–O_{carboxyl} and Mo–N(His) bonds to the Mo–O_{hydroxyl} bond decrease by 0.02–0.03 Å to compensate for the decreased donation from O_{hydroxyl} to the Mo center compared to the deprotonated model.

However, this decreases the level of agreement in these bond lengths relative to the crystal structure to 0.10 and 0.09 Å, respectively. Overall, the improved Mo^{III}-Fe/Mo-S distances and the good agreement in the Mo-O_{hydroxyl} bond length favor the O_{hydroxyl}-protonated homocitrate ligand model with an rms deviation of 0.06 Å compared to 0.12 Å without protonation relative to the crystal structure. We rationalize the disagreement in other Mo-ligand bond lengths by the absence of second sphere coordination sphere interactions in our computational model, such as a network of hydrogen-bonded water molecules around the coordinated short carboxyl arm of the homocitrate^{34, 213, 237, 238} or the N_ε of His442.

Table 4.2. Effect of homocitrate and imidazole ligand protonation states on the optimized Mo^{III}-Fe and Mo-L distances (Å) in the [2Fe^{II}-5Fe^{III}-N³⁻] state^a

homocitrate imidazole	deprot. prot.	deprot. deprot.	HO _{carb.} ^(d) prot.	HO _{carb.} ^(p) prot.	HO _{hyd.} prot.	XRD ref 26	EXAFS ref 197	EXAFS ref 235
Mo ^{III} -Fe ^(Mo)	2.78	2.80	2.77	2.77	2.74	2.69	2.70	2.68
Mo ^{III} -S ^(Mo)	2.40	2.41	2.38	2.38	2.37	2.34	2.37	2.34
Mo-N(His)	2.24	2.17	2.23	2.23	2.21	2.30	2.21 ^b	2.29
Mo-O _{carboxyl}	2.10	2.12	2.26	2.24	2.08	2.18	2.21 ^b	2.14
Mo-O _{hydroxyl}	1.98	1.98	1.99	2.01	2.20	2.20	2.21 ^b	2.31
Avg. Mo-O/N	2.11	2.09	2.16	2.16	2.16	2.22	2.21	2.25
rms dev.	0.12	0.13	0.11	0.10	0.06			

^a HO_{carb.}^(d) and HO_{carb.}^(p) represent protonation of the distal and proximal O_{carboxyl} center, respectively. HO_{hyd.} indicates protonation of the hydroxyl oxygen. Prot. and deprot. refer to protonated and deprotonated states, respectively. The rms deviations are given relative to the crystal structure. ^b Mo-O/N distances were reported only as an average, due to inherent difficulty in distinguishing between light atoms.

Using the O_{hydroxyl}-protonated state, the best agreement with experimental data is achieved for the [2Fe^{II}-5Fe^{III}-C⁴⁻], [4Fe^{II}-3Fe^{III}-N³⁻], and [4Fe^{II}-3Fe^{III}-C⁴⁻] states (Table 4.1, columns HC = OH). For 2Fe^{II}-5Fe^{III}, the X = C⁴⁻ model is more consistent with the

experimental structures than $X = N^{3-}$ in nearly all tabulated dimensions, and the rms deviations for the core distances slightly improve from 0.04 Å to 0.03 Å upon protonation. At the same time, the $[2Fe^{II}-5Fe^{III}-N^{3-}]$ state cannot be definitively eliminated based on the interatomic distances alone. Comparing the best structures obtained with protonated homocitrate ligand, the $[2Fe^{II}-5Fe^{III}-C^{4-}]$ model is somewhat contracted relative to the crystal structure, with an average deviation of -0.02 Å, while the $4Fe^{II}-3Fe^{III}$ oxidation state models are slightly expanded, with an average deviation of 0.02 Å; however, the overall rms deviations are the same. To avoid overlooking compositions and oxidation states due to fortuitous error cancellation, we evaluated the possibility of $X = O^{2-}$ with the protonated model, but the rms deviation for the core distances remains too high (0.09 Å), with considerable deviations in the $Fe^{(Fe)}-X$ (0.13 Å) and $Fe^{(Fe)}\cdots Fe^{(Mo)}$ (0.16 Å) distances. Also, the results for the $O_{hydroxyl}$ -protonated $6Fe^{II}-1Fe^{III}-N^{3-}$ state (see Table C.5) show unreasonable large geometric differences relative to the experimental structure.

Reduction Potentials

Reduction potentials were considered as energetic criteria for evaluating the oxidation state and composition of FeMo-co. The reduction potential of the $FeMo-co^{Ox}/FeMo-co^N$ pair was calculated for the $2Fe^{II}-5Fe^{III}$, $4Fe^{II}-3Fe^{III}$ and $6Fe^{II}-1Fe^{III}$ states with C^{4-} , N^{3-} , or O^{2-} , covering a large range of charges (from -5 to -1 for our model), in addition to two of the $O_{hydroxyl}$ -protonated models favored in the structural studies described above. For $FeMo-co^{Ox}$, we used the spin states shown in this work to be lowest in energy: $S_t = 0$ for $1Fe^{II}-6Fe^{III}$, and $S_t = 1$ for $3Fe^{II}-4Fe^{III}$ and $5Fe^{II}-2Fe^{III}$. The

ligand protonation state is assumed to be unchanged during the reduction process, and this is supported by an EXAFS study showing a 0.03 Å increase in the average Mo-O/N distance upon reduction,²³⁹ which is reproduced in our calculations.

Table 4.3 summarizes the calculated reduction potentials along with a decomposition analysis according to Equations 4.1–4.3. The reduction potentials for the models with deprotonated homocitrate range from -2.68 V to +0.59 V for the most and least negatively charged states, $[6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}-\text{N}^{3-}]^{5-}$ and $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{N}^{3-}]^{-}$, respectively, while the experimental value lies between these extremes at -0.042 V.²⁰² Examining the intermediately charged states, $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}]^{3-}$ is still too negatively charged ($E^{\circ} = -0.83$ V), but $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}]^{2-}$ gives $E^{\circ} = 0.00$ V, which is close to the experimental value. However, as discussed above, models with protonated $\text{O}_{\text{hydroxyl}}$ groups (denoted with +H) need to be considered. Thus, by evaluating protonated models we find that the reduction potentials for $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}+\text{H}]^{2-}$ and $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]^{-}$ are -0.34 V and 0.38 V, respectively, nearly equidistant from the experimental value in opposite directions, and both can be considered favored by this criterion.

Table 4.3. Reduction potentials (in V) relative to the standard hydrogen electrode (SHE)^a

model	gas phase ^b			solute corrections				$\Delta\Delta G^\circ_{\text{solv}}$		E° vs SHE
	$\Delta\epsilon_{\text{SCF}}$	s.p.	$\Delta\epsilon_{\text{SCF}}^{\text{s.p.}}$	ΔZPE	ΔH°	$T\Delta S^\circ$	ΔG°	$\Delta\Delta G_{\text{elec}}$	$\Delta\Delta G_{\text{non}}$	
[6Fe ^{II} -1Fe ^{III} -N ³⁻] ⁵⁻	-6.37							8.14	-0.02	-2.68
[4Fe ^{II} -3Fe ^{III} -C ⁴⁻] ⁴⁻	-4.96							7.79	-0.02	-1.62
[4Fe ^{II} -3Fe ^{III} -C ⁴⁻ +H] ³⁻	-2.81							6.07	-0.03	-1.19
[4Fe ^{II} -3Fe ^{III} -N ³⁻] ³⁻	-2.33			-0.01	0.00	0.04	-0.04	6.00	-0.03	-0.83
[4Fe ^{II} -3Fe ^{III} -N ³⁻ +H] ²⁻	0.30	-0.02	0.28	-0.02	-0.02	-0.09	0.05	3.78	-0.02	-0.34
[4Fe ^{II} -3Fe ^{III} -O ²⁻] ²⁻	0.77			-0.01	-0.04	-0.13	0.08	3.58	-0.04	-0.05
[4Fe ^{II} -3Fe ^{III} -O ²⁻ +H] ¹⁻	3.45			-0.01	-0.02	-0.06	0.03	1.31	-0.04	0.31
[2Fe ^{II} -5Fe ^{III} -C ⁴⁻] ²⁻	0.68	-0.03	0.65	0.06	-0.03	-0.12	0.15	3.66	-0.03	0.00
[2Fe ^{II} -5Fe ^{III} -C ⁴⁻ +H] ¹⁻	3.46	-0.04	3.43	0.04	0.00	-0.03	0.07	1.34	-0.03	0.38
[2Fe ^{II} -5Fe ^{III} -N ³⁻] ¹⁻	3.81	-0.03	3.77	0.04	0.03	0.06	0.01	1.25	-0.01	0.59
experiment (ref 202)										-0.042

^a Column “s.p.” indicates the spin projection correction to ionization potential (IP). The contributions under $\Delta G^\circ_{\text{solute}}$ reflect the difference between the zero-point energetic (ZPE), enthalpic and entropic corrections to the reduced and oxidized solvated species as a result of structural optimizations. ϵ° was calculated from $\text{IP}^{\text{s.p.}}$, $\Delta G^\circ_{\text{solute}}$ and $\Delta\Delta G^\circ_{\text{solv}}$ as described in Eqs. (1)–(3). The models labeled “+H” are the O_{hydroxyl}-protonated models. All numbers are given in V. ^b *In vacuo* electronic energy ($\langle \Psi(0) | \hat{H} | \Psi(0) \rangle$)⁸⁷ with PCM-optimized geometry.

Although reduction potentials alone do not rule out either of the $\text{O}_{\text{hydroxyl}}^-$ protonated models, they exhibit a general trend that allows for the elimination of models on the basis of overall cluster charge. For example, we can readily rule out the $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{N}^{3-}+\text{H}]$ state for $\text{FeMo-co}^{\text{N}}$, because its neutral total charge would render its reduction potential even more positive than that of $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{N}^{3-}]^-$ (0.59 V) and $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]^-$ (0.38 V). The calculated reduction potentials indicate the overall charge of the truncated $\text{FeMo-co}^{\text{N}}$ model is likely -2 or -1 . Considering the -3 charge of the full homocitrate ligand compared to -1 for the glycolate with protonated hydroxyl groups in both, we expect the overall charge of $\text{FeMo-co}^{\text{N}}$ to be -4 or -3 . Previous work indicated the charge of $\text{exFeMo-co}^{\text{Ox}}$ is -2 ,²⁰³ corresponding to a total charge of -3 for $\text{FeMo-co}^{\text{N}}$ (-1 for our model). Thus, these charge considerations show a preference for the $2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}$ oxidation state distribution with $\text{X} = \text{C}^{4-}$ and protonated homocitrate.

By analyzing each component of the reduction potential calculations, we find that the major contributions are from the ionization potential (IP or $\text{IP}^{\text{s.p.}}$) and the electronic solute–solvent interactions ($\Delta G_{\text{electronic}}$). Corrections accounting for differences in protein-embedded structures upon reducing FeMo-co ($\Delta G_{\text{solute,corr.}}^{\circ}$) improve the reduction potential by up to 150 mV,^a while spin projection (s.p., using two-spin projection method) and nonelectrostatic solute–solvent interactions each account for less than 40 mV. While none of these corrections are negligible, the first approximation of using the SCF energies ($\text{IP} + \Delta G_{\text{electrostatic}}$) to calculate E° results in an error of only 10 mV for the $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ state and even less for the $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}+\text{H}]$ state due to error

^a The corrections accounting for changes in gas phase structure upon reducing FeMo-co , and those accounting for structural relaxation due to embedment in the PCM model partially cancel, leaving only one term, $\Delta G_{\text{solute}}^{\circ}$.

cancellations. However, this cancellation is not universal, since we find a larger error in certain cases (e.g., 90 mV for the $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}]$ model). Importantly, the overall conclusions derived from reduction potential calculations remain the same regardless of how the reduction potentials are calculated.

Spectroscopic Properties

In addition to composition, geometric structure, and relative energies of spin and oxidation states, we also examined the electronic structural information from Mössbauer and ENDOR spectroscopic measurements. These have been used in the literature as dominant criteria to differentiate among various proposals for the structure and composition of FeMo-co, since they are highly sensitive to oxidation state, ligand environment, and geometric structure.²⁴⁰ We find that the calculated Mössbauer isomer shifts, quadrupole splittings, and ENDOR hyperfine coupling constants are greatly affected by the methodological details including the composition of the isomer shift training set and the employed level of theory.

Mössbauer Isomer Shifts. The currently accepted process of calculating Mössbauer isomer shifts requires the derivation of an empirical relationship between calculated electron density values at the nucleus ($\rho(0)$) and experimental isomer shifts for a set of model compounds at a given level of theory. For our training set we employed a variety of [Fe-S] and [Mo-Fe-S] clusters that are the closest models for the coordination environment of the Fe centers in FeMo-co. Since most of the published isomer shifts for the training set are for solid state samples (see Tables C.6 and C.7), it was most

appropriate to use the crystallographic structures in the calculations (see Table C.6 method 1), yielding a best fit to Equation 4.9 of $\delta = -0.620 e^{-1} a_0^3 \text{ mm s}^{-1} (\rho(0) - 11833 e a_0^{-3}) + 0.563 \text{ mm s}^{-1}$ (Figure C.1A) with a standard deviation of 0.06 mm s^{-1} . From this equation, the isomer shifts were derived using the calculated average $\rho(0)$ of the Fe centers in FeMo-co in the structurally optimized $2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}$, $4\text{Fe}^{\text{II}}\text{-}3\text{Fe}^{\text{III}}$ and $6\text{Fe}^{\text{II}}\text{-}1\text{Fe}^{\text{III}}$ states with C^{4-} or N^{3-} .

As shown by the δ_{I} values in Table 4.4 (see Table C.8 for individual site δ values), isomer shift decreases by an average of 0.13 mm s^{-1} with oxidation along the series from $6\text{Fe}^{\text{II}}\text{-}1\text{Fe}^{\text{III}}$ to $4\text{Fe}^{\text{II}}\text{-}3\text{Fe}^{\text{III}}$ to $2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}$ with $\text{X} = \text{N}^{3-}$. Interestingly, the isomer shift decreases by about 0.05 mm s^{-1} in going from $\text{X} = \text{N}^{3-}$ to C^{4-} due to an increase in $\rho(0)$, which is, in turn, likely caused by increased overlap with the Fe(4s) as discussed by Neese²⁴¹ for a series of Fe halides. The average isomer shift of the $[2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}\text{-}\text{N}^{3-}]$ state (0.41 mm s^{-1}) agrees best with the experimental²⁰⁵ value of 0.41 mm s^{-1} , followed by the $[4\text{Fe}^{\text{II}}\text{-}3\text{Fe}^{\text{III}}\text{-}\text{C}^{4-}\text{+H}]$ state (0.45 mm s^{-1}), both of which were shown above to be disfavored by other criteria. As with the reduction potentials, the average isomer shift of the most likely hydroxyl-protonated states, $[2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}\text{-}\text{C}^{4-}\text{+H}]$ and $[4\text{Fe}^{\text{II}}\text{-}3\text{Fe}^{\text{III}}\text{-}\text{N}^{3-}\text{+H}]$, lie nearly equidistant from the experimental value, at 0.32 mm s^{-1} and 0.51 mm s^{-1} , respectively.

Table 4.4. Mulliken spin densities ($|\rho_s(\text{Fe})|$), quadrupole splittings ($|\Delta E_Q|$) and isomer shifts (δ) averaged over the Fe sites; hyperfine couplings of Fe (a^{test}) and X ($a(\text{X})$); and rms deviations of the individual site hyperfine couplings (a_i) for a series of oxidation states and compositions, where +H indicates O_{hydroxyl}-protonated models

level of theory	model	δ_1 (mm s ⁻¹) ^a	δ_2 (mm s ⁻¹) ^a	δ_3 (mm s ⁻¹) ^a	$ \rho_s(\text{Fe}) $ (e)	$ \Delta E_Q $ (mm s ⁻¹)	a^{test} (MHz)	rms dev. a_i (MHz)	$a(\text{X})$ (MHz)
BP86 ^b	2Fe ^{II} -5Fe ^{III} -N ³⁻	0.41	0.29 ^c	0.45	2.92	1.13	-25.1	4.2	1.1
	4Fe ^{II} -3Fe ^{III} -N ³⁻	0.53	0.38 ^c	0.54	3.06	1.03	-27.3	5.6	-0.3
	6Fe ^{II} -1Fe ^{III} -N ³⁻	0.68	0.52 ^c	0.66	2.98	0.95	-29.6	5.0	-0.3
	2Fe ^{II} -5Fe ^{III} -C ⁴⁻	0.35	0.24 ^c	0.40	2.73	0.90	-24.3	3.5	3.7
	4Fe ^{II} -3Fe ^{III} -C ⁴⁻	0.48	0.34 ^c	0.50	2.89	0.94	-25.1	4.7	-0.7
	4Fe ^{II} -3Fe ^{III} -O ²⁻	^d		^d	3.27	^d	-29.5	6.8	^d
	2Fe ^{II} -5Fe ^{III} -C ⁴⁻ +H	0.32	0.22	0.38	2.68	0.86	-23.8	3.3	2.7
	4Fe ^{II} -3Fe ^{III} -C ⁴⁻ +H	0.45	0.32 ^c	0.48	2.85	0.93	-24.4	4.5	-1.0
	4Fe ^{II} -3Fe ^{III} -N ³⁻ +H	0.51	0.45	0.52	3.03	0.97	-27.0	5.5	-0.4
B3LYP ^b	2Fe ^{II} -5Fe ^{III} -C ⁴⁻ +H				3.42	1.16	-25.4	5.9	6.1
	4Fe ^{II} -3Fe ^{III} -N ³⁻ +H				3.50	1.43	-31.8	8.0	-0.7
B5HFP86/	2Fe ^{II} -5Fe ^{III} -C ⁴⁻ +H				2.44		-24.1	2.7	
6-311+G(d)	4Fe ^{II} -3Fe ^{III} -N ³⁻ +H				2.77		-25.1	4.5	
	experiment	0.41 ^e		0.41 ^e		0.69 ^e	-17.9 ^e		<0.10 ^f

^a Subscripts refer to the methods employing a training data set with (1) crystal structures, (2) BP86/STO-TZP optimized structure (3) crystal structures without outliers. ^b SDD-VTZ(d) basis set for $|\rho_s(\text{Fe})|$ and a^{test} , STO-TZP for $|\Delta E_Q|$, $a(\text{X})$ and δ . ^c Estimated on range of δ for [2Fe^{II}-5Fe^{III}-C⁴⁻+H]. ^d Calculations did not converge. ^e Ref 205. ^f Ref. 85.

Alternatively, the training set structures can be optimized in order to use the minimum energy structure for the employed computational method, as is often preferred.^{206, 227, 242} The best fit for the set of optimized structures (see Table C.6 method 2) is $\delta = -0.744 e^{-1} a_0^3 \text{ mm s}^{-1} (\rho(0) - 11833 e a_0^{-3}) + 0.593 \text{ mm s}^{-1}$ (Figure C.1B), with a standard deviation of 0.04 mm s^{-1} . This fit appears to be significantly different than the previous fit; however, the effect on the calculated average isomer shift of FeMo-co is only 0.01 mm s^{-1} on average over the eight listed states. For consistency, the FeMo-co structure should also be optimized at the BP86/all-electron STO-TZP level as used for the training set. With the optimized structures, the average isomer shifts of the $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ and $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}+\text{H}]$ states are 0.22 mm s^{-1} and 0.45 mm s^{-1} , respectively; thus, the latter state is favored with this method.

Importantly, a significant drawback of the isomer shift fitting procedure is the dependence on the choice of model compounds for the training set. The slopes of $\alpha = -0.620 e^{-1} a_0^3 \text{ mm s}^{-1}$ or $-0.744 e^{-1} a_0^3 \text{ mm s}^{-1}$ from method 1 and method 2 above, respectively, are steeper than the published values ranging between -0.3 and $-0.5 e^{-1} a_0^3 \text{ mm s}^{-1}$.^{81, 206, 243} We note that the two models with the largest deviation from the correlation line in Figure C.1A are $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ and $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-}o\text{-Xyl})_2]^{2-}$, and their experimental isomer shifts of 0.13 mm s^{-1} and 0.17 mm s^{-1} , respectively,^{244, 245} are much lower than values typically used for $[\text{Fe}^{\text{III}}-\text{S}]$ complexes in training sets (for example, see refs 80, 206, and 243). Rao and Holm used 0.28 mm s^{-1} as the lowest isomer shift for an $[\text{Fe}^{\text{III}}-\text{S}]$ complex in their evaluation of the linear relationship between isomer shifts and oxidation state.²³ Notably, their fit predicts 0.40 mm s^{-1} for the $2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}$ oxidation state

and 0.51 mm s^{-1} for $4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}$. Thus, if we remove these outliers (see Table C.6 method 3), the new correlation line is described by $\delta = -0.491 e^{-1} a_0^3 \text{ mm s}^{-1} (\rho(0) - 11833 e a_0^{-3}) + 0.568 \text{ mm s}^{-1}$ (Figure C.1C), with a standard deviation of 0.04 mm s^{-1} , and the slope is more consistent with the aforementioned published values. With this method, the average isomer shifts of the $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ and $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}+\text{H}]$ states become 0.38 mm s^{-1} and 0.52 mm s^{-1} (δ_3 values in Table 4.4), respectively, favoring the former.

The three described methods produce significantly different sets of data, resulting in a large error bar for the calculated isomer shifts. For example, the isomer shifts of the $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ state were calculated within a range of 0.16 mm s^{-1} . The second method yields the smallest isomer shifts. In order to estimate the δ_2 values for other states than just explicitly calculated, we subtract 0.16 mm s^{-1} from the maximum calculated isomer shift for each state, producing an assumed lower limit for δ_2 (see Table 4.4), because the range used is much larger than that observed for $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ (0.07 mm s^{-1}). Then, after taking into account the standard deviations of each method (e.g., δ_3 values carry an error bar of 0.04 mm s^{-1}), we find that all tested states except $[6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}-\text{N}^{3-}]$ have theoretical isomer shifts spanning a range that includes the experiment value. However, we consider the third method to be most appropriate, due to the exclusion of training set complexes with unusually low isomer shifts, and the resulting improvement in the correlation line. Furthermore, the third (and first) method uses structures optimized with our standard, previously validated procedure and is thus expected to be more accurate than the second method. The δ_3 values provide a stringent

criterion for selecting only the $2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}$ oxidation state with C^{4-} or N^{3-} central ligands as preferred assignments, with or without protonation.

Mössbauer Quadrupole Splitting. First, we carried out of an evaluation of the calculated Mössbauer quadrupole splitting parameters (ΔE_Q) using the same training set as for the isomer shift calculations (see above). The absolute values of ΔE_Q for the selected Fe sites are found to have an rms deviation of 0.31 mm s^{-1} from the experiment when using crystallographic coordinates, and 0.32 mm s^{-1} for the corresponding optimized structures (see Table C.6), which is a typical level of accuracy.^{227, 240, 243} The calculated ΔE_Q values for the $2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}$, $4\text{Fe}^{\text{II}}\text{-}3\text{Fe}^{\text{III}}$ and $6\text{Fe}^{\text{II}}\text{-}1\text{Fe}^{\text{III}}$ states with C^{4-} or N^{3-} are presented in Table 4.4 (see Table C.9 for individual site ΔE_Q values). The sign of ΔE_Q is difficult to calculate reliably,²⁴⁶ and thus not surprisingly, none of the oxidation states or compositions match the experimental distribution of six negative and one positive ΔE_Q in our calculations. To facilitate comparison of the theoretical and experimental quadrupole splitting values, we take the mean of $|\Delta E_Q|$ over the Fe sites and find a range of 0.86 mm s^{-1} ($[2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}\text{-}\text{C}^{4-}\text{+H}]$) to 1.13 mm s^{-1} ($[2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}\text{-}\text{N}^{3-}]$) for the various states. These are all overestimated relative to the experimental value of 0.69 mm s^{-1} , although considering the error bar of 0.32 mm s^{-1} , all tested states except the deprotonated $[2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}\text{-}\text{N}^{3-}]$ and $[4\text{Fe}^{\text{II}}\text{-}3\text{Fe}^{\text{III}}\text{-}\text{N}^{3-}]$ give results consistent with experiment, indicating the difficulty of distinguishing between states by this criterion.

The lowest four calculated mean $|\Delta E_Q|$ values are attributed to states with $\text{X} = \text{C}^{4-}$ and the highest four to those with $\text{X} = \text{N}^{3-}$, while there is no obvious correlation with oxidation state. However, there is a strong correspondence between the ordering of states

by increasing mean absolute Fe spin density ($|\rho_s(\text{Fe})|$) and by increasing $|\Delta E_Q|$, with only one state ($2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{N}^{3-}$) breaking the trend (see Table C.10 for individual site $\rho_s(\text{Fe})$ values). This correlation indicates that the dominant factors affecting spin densities including bond covalency and the employed level of theory also strongly influence ΔE_Q values. Indeed, a change in functional that increases Fe spin density also increases the $|\Delta E_Q|$ values for the two most favored states from the compositional, structural and energetic considerations, $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ and $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}+\text{H}]$, from 0.86 mm s^{-1} and 0.97 mm s^{-1} (BP86) to 1.16 mm s^{-1} and 1.43 mm s^{-1} (B3LYP). The $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ state is consistently the closest to the experiment, but the accuracy of these calculations, as demonstrated with the training set, is not high enough to give strong preference to this state.

Hyperfine Couplings. Due to the limitations for describing accurate hyperfine couplings of transition metal nuclei from *ab initio* calculations, the ^{57}Fe hyperfine coupling constants (a_i^{calc}) were determined through a semiempirical procedure (see Computational Details) as previously applied by Noodleman and co-workers⁸⁵ to the $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}]$ state. We extended these calculations to the $2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}$, $4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}$ and $6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}$ states with C^{4-} , N^{3-} , or O^{2-} . The resulting a_i^{calc} values (see Table C.11) are summed over the seven Fe sites to give a^{test} , a straightforward parameter introduced by Mouesca and co-workers²³² for comparison with experimental data. The results in Table 4.4 show that the a^{test} value of the $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ state (-23.8 MHz) is closest to that of the Mössbauer experiment (-17.9 MHz),²⁰⁵ while the $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{O}^{2-}]$ (-29.5 MHz) and $[6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}-\text{N}^{3-}]$ (-29.6 MHz) states have the largest deviations. Although the spin

projection coefficients, K_i , are dependent on the oxidation state assignments of each iron site (see Table C.12), spin densities are the dominant factors contributing to differences in a^{test} in this series. As with the quadrupole splittings, there is a nearly perfect correspondence between the ordering of FeMo-co states arranged by a^{test} values from least to most negative, and the ordering by increasing $|\rho_s(\text{Fe})|$, with the only exception being the $[6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}-\text{N}^{3-}]$ state, which has an intermediate $|\rho_s(\text{Fe})|$. Due to the reliance on atomic spin density values, the a^{test} values are highly dependent on the employed functional and basis set. In addition to the BP86/SDD-VTZ(d) level of theory used for the series, the $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ and $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}+\text{H}]$ states were evaluated with B3LYP/SDD-VTZ(d) and B5HFP86/6-311+G(d), which has been found previously⁷⁷ to be excellent for [Fe-S] cluster spin densities. With B3LYP/SDD-VTZ(d), a^{test} becomes more negative for both states and the difference between them increases from -3.1 MHz to -6.4 MHz, while with B5HFP86/6-311+G(d), the gap decreases to -0.9 MHz. Despite strong computational method dependence, the $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ state consistently has the closest a^{test} to experiment.

As a further analysis of the hyperfine couplings, and to avoid possible error cancellation caused by the summation of positive and negative values in determining a^{test} , the rms deviation of the individual site a_i^{calc} values were also compared (Table 4.4). Since the specific Fe site corresponding to an experimental a_i is unknown, the rms deviations were calculated by ordering the a_i values numerically. Here, again, the $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ state has the smallest rms deviation of 3.3 MHz, compared to 5.5 MHz for $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$, and the largest value of 6.8 MHz for $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{O}^{2-}]$. The ordering of

states is generally the same as for a^{test} with some minor rearrangements. With B3LYP the rms deviations increase by about 2.5 MHz, while with B5HFP86/6-311+G(d) the results improve, yielding 2.7 MHz for $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$. Overall, the hyperfine coupling calculations favor both protonation states of $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}]$. Since the error is difficult to estimate, other states cannot be excluded based on this criteria alone.

Advanced EPR methods, such as ENDOR and ESEEM techniques, are ideal for detecting weakly coupled spin systems, such as an interstitial atom within the central 6Fe belt in the BS7 state (see Evaluation of Broken-Symmetry Spin-Coupling Schemes for details). On the basis of the lack of a central ^{13}C , or $^{14/15}\text{N}$ atom signal it was suggested that if the interstitial ligand X is C^{4-} or N^{3-} then the $a_{\text{iso}}(^{13}\text{C})$ or $a_{\text{iso}}(^{14}\text{N})$ coupling constants must be less than 0.05–0.1 MHz or 0.03–0.07 MHz, respectively.⁸⁵ Table 4.4 shows the calculated $a_{\text{iso}}(\text{X})$ values for the same series of FeMo-co states used for the Mössbauer parameters. In each state $a_{\text{iso}}(\text{X})$ is higher than the experimental detection limit with the lowest absolute value of 0.3 MHz for the $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}]$ and $[6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}-\text{N}^{3-}]$ states, while the $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ state has a higher value of 2.7 MHz. The dependence on the computational method is again strong, as a switch from BP86 to the B3LYP functional approximately doubles $a_{\text{iso}}(\text{X})$. The use of a hybrid functional such as B5HFP86 gives the best agreement with XAS data and geometric parameters (see Computational Details), but inclusion of the HF exchange component increases the already too high $a_{\text{iso}}(\text{X})$ values further away from the experimental detection limit. Thus, we conclude that the required level of accuracy to reproduce a very small hyperfine

coupling for X is out of reach with current DFT functionals, even when employing a level of theory calibrated to experimental metal–ligand bonding information.

Conclusions

In this work, we applied broken symmetry DFT calculations with spectroscopically (XAS) calibrated functionals and saturated basis sets to provide a comprehensive evaluation of all available experimental information about the electronic, geometric, energetic, and magnetic properties of the protein-bound nitrogenase FeMo-co. We found that the relative energies of spin-coupling schemes are dominantly governed by the number of antiferromagnetic interactions, including those mediated by X. The ground state spin-coupling scheme, BS7, has the maximum number of antiferromagnetic M–L–M interactions within the 7Fe-cluster of FeMo-co. Using the BS7 spin-coupling scheme, we calculated the ground spin state for oxidized and resting FeMo-co, geometric structures, reduction potentials, and Mössbauer and hyperfine parameters for all possible oxidation states ($6\text{Fe}^{\text{II}}1\text{Fe}^{\text{III}}$, $4\text{Fe}^{\text{II}}3\text{Fe}^{\text{III}}$, $2\text{Fe}^{\text{II}}5\text{Fe}^{\text{III}}$) and interstitial atoms (C^{4-} , N^{3-} , O^{2-}), assuming a weak-field ligand environment for the Fe centers and an 10-electron interstitial atom.

Table 4.5 summarizes the preferred oxidation states and compositions for the iron centers and the interstitial atom based on independent experimental criteria and homocitrate protonation states. The critical findings for each criterion follow:

- (1) We are not able to rule out any possibilities based on the ground spin state of resting FeMo-co, as each is consistent with the experimental $S_{\text{t}} = 3/2$ state.

- (2) However, only the $2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}$ oxidation state is consistent with the $S_t = 0$ ground oxidized form.
- (3) The optimized geometries with deprotonated homocitrate favor the $2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}$ states with C^{4-} or N^{3-} , while strongly disfavoring O^{2-} and protonated forms of the central ions due to an unreasonable expansion of the central ‘6Fe-belt’ relative to the crystal structure. Contrary to what is expected based on size, a highly charged, larger interstitial atom with lower electronegativity is preferred over a smaller, more electronegative ion to maintain the cluster core geometry. The optimized $\text{FeMo-co}^{\text{N}}$ structures consistently show that the $\text{Mo-O}_{\text{hydroxyl}}$ bond length is far too short (0.14 to 0.21 Å) relative to its experimental value for all oxidation states and compositions. This discrepancy was eliminated by protonating the coordinated $\text{O}_{\text{hydroxyl}}$ group. With the $\text{O}_{\text{hydroxyl}}$ -protonated model, the $[2\text{Fe}^{\text{II}}\text{-}5\text{Fe}^{\text{III}}\text{-}\text{C}^{4-}\text{+H}]$, $[4\text{Fe}^{\text{II}}\text{-}3\text{Fe}^{\text{III}}\text{-}\text{C}^{4-}\text{+H}]$ and $[4\text{Fe}^{\text{II}}\text{-}3\text{Fe}^{\text{III}}\text{-}\text{N}^{3-}\text{+H}]$ states show the closest agreement with the experimental structures.
- (4) The states with the most reasonable reduction potentials for $\text{FeMo-co}^{\text{Ox}}/\text{FeMo-co}^{\text{N}}$ all carry a total charge of -1 or -2 . It is important to emphasize that a solely reduction potential-based evaluation of composition can be misleading, since the reduction potential for the $[4\text{Fe}^{\text{II}}\text{-}3\text{Fe}^{\text{III}}\text{-}\text{O}^{2-}]$ state with -2 total charge only deviates 10 mV from experiment, despite the significantly expanded geometry. Alternative models such as $[4\text{Fe}^{\text{II}}\text{-}3\text{Fe}^{\text{III}}\text{-}\text{N}^{3-}\text{+H}]$ with a protonated bridging sulfide ligand are also expected to have

favorable reduction potentials based on the overall charge despite the already reported⁸¹ cluster expansion. The reduction potentials of $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ (0.38 V) and $[4\text{Fe}^{\text{II}}-3\text{Fe}^{\text{III}}-\text{N}^{3-}+\text{H}]$ (−0.34 V) are the closest to the experimental value of −0.042 V.

- (5) The $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$ state is preferred based on the experimentally consistent -2 charge in the $\text{FeMo-co}^{\text{Ox}}$ or $\text{exFeMo-co}^{\text{Ox}}$ form.
- (6) Mössbauer isomer shift calculations are strongly dependent on the employed training set composition and structural optimization state, but with our favored method only the $2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}$ states with C^{4-} or N^{3-} yield experimentally consistent values.
- (7) The quadrupole splittings are closest to experiment with $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}+\text{H}]$, but the large error bars overlap the experimental value for nearly all tested states.
- (8) Current DFT methods do not have the required accuracy for calculating unambiguously hyperfine couplings in a multinuclear, delocalized mixed-valence, spin-coupled system, such as FeMo-co . Nevertheless, the $2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}$ oxidation state with C^{4-} has ^{57}Fe hyperfine couplings most consistent with experiment with respect to both a^{test} and rms deviation of the individual site parameters, a_i .
- (9) The hyperfine coupling for X are larger than the limits set by experiment, regardless of composition and oxidation state, likely due to limited accuracy.

Table 4.5. Magnetic, electronic, geometric, and energetic preference for oxidation states of the iron centers and composition of the interstitial ligand^a

criterion	oxidation state	interstitial atom	
		HC(O ⁻ _{hydroxyl})	HC(HO _{hydroxyl})
resting $S_t = 3/2$	no preference	no preference	no preference ^b
oxidized $S_t = 0$	Mo ^{IV} -2Fe ^{II} -5Fe ^{III}	C ⁴⁻ , N ³⁻	C ^{4-b} , N ^{3-b}
geometry	Mo ^{IV} -2Fe ^{II} -5Fe ^{III}	C ⁴⁻ , N ³⁻	C ⁴⁻
	Mo ^{IV} -4Fe ^{II} -3Fe ^{III}		C ⁴⁻ , N ³⁻
charge	Mo ^{IV} -2Fe ^{II} -5Fe ^{III}	N ³⁻	C ⁴⁻
	Mo ^{IV} -4Fe ^{II} -3Fe ^{III}		O ²⁻
reduction potential	Mo ^{IV} -2Fe ^{II} -5Fe ^{III}	C ⁴⁻	C ⁴⁻
	Mo ^{IV} -4Fe ^{II} -3Fe ^{III}	O ²⁻	N ³⁻ , O ²⁻
isomer shift (all)	Mo ^{IV} -2Fe ^{II} -5Fe ^{III}	C ⁴⁻ , N ³⁻	C ⁴⁻ , N ^{3-c}
	Mo ^{IV} -4Fe ^{II} -3Fe ^{III}	C ⁴⁻ , N ³⁻	C ⁴⁻ , N ³⁻
isomer shift (δ_3)	Mo ^{IV} -2Fe ^{II} -5Fe ^{III}	C ⁴⁻ , N ³⁻	C ⁴⁻ , N ^{3-c}
	Mo ^{IV} -2Fe ^{II} -5Fe ^{III}	C ⁴⁻	C ⁴⁻
quadrupole splitting	Mo ^{IV} -4Fe ^{II} -3Fe ^{III}	C ⁴⁻	C ⁴⁻ , N ³⁻
	Mo ^{IV} -6Fe ^{II} -1Fe ^{III}	N ³⁻	N ^{3-c}
Fe hyperfine coupling	Mo ^{IV} -2Fe ^{II} -5Fe ^{III}	C ⁴⁻	C ⁴⁻
X hyperfine coupling	no preference	no preference	no preference

^a HC(O⁻_{hydroxyl}) and HC(HO_{hydroxyl}) indicate the deprotonated and protonated states of homocitrate, respectively. ^b Estimated based on results for deprotonated models. The $S_t = 0$ and 1 states of [2Fe^{II}-5Fe^{III}-C⁴⁻+H] were tested, and the results were consistent with the deprotonated case. ^c [2Fe^{II}-5Fe^{III}-N³⁻+H] and [6Fe^{II}-1Fe^{III}-N³⁻+H] results were estimated.

In conclusion, only [2Fe^{II}-5Fe^{III}-C⁴⁻+H] is consistently favored, and we consider it to be the most likely oxidation state and composition. The [4Fe^{II}-3Fe^{III}-N³⁻+H] state is the second best, as it passed the structural (with only the protonated homocitrate) and reduction potential criteria; however, it failed with respect to the relative energies of oxidized spin states, the overall charge, the isomer shifts, and the hyperfine parameters.

The more oxidized electronic state, the protonation state of the homocitrate, and the presence of a carbide central ligand open up exciting further directions for investigations of the molecular mechanism of biological nitrogen fixation and the biosynthesis of the FeMo-cofactor of nitrogenase

CHAPTER 5

CONCLUSION

DFT calculations are a powerful tool to probe structural and mechanistic details of iron-sulfur proteins that cannot or have not yet been experimentally described. However, this must be done within the boundaries of a systematically and critically compiled experimental background. Even if the DFT results make sense within the context of previous experiments, they are only trustworthy if great care is taken in choosing the level of theory, type of population analysis, and computational model. Our approach is to calibrate the theoretical method using experimentally well-defined model clusters. The B(5%HF)P86/6-311+G(d) level was previously shown to accurately reproduce the geometric structures of [4Fe-4S] clusters.⁷⁷ In Chapter 2, the method analysis was extended to take into account the electronic structure of 1Fe, [2Fe-2S], and [4Fe-4S] clusters with various ligands. In the case of electronic properties such as bond covalency, the population analysis method also has a significant effect, and several popular methods were analyzed. The B(5%HF)P86 hybrid functional combined with a triple- ζ basis set with polarization functions was confirmed as a reliable method for treating all types of iron-sulfur clusters, and the AIM population analysis proved to be the least biased in terms of basis set dependence.

The computational methods recommended in Chapter 2 can also be applied to protein-embedded clusters, where the structures are perturbed by hydrogen bonds, and

This section contains text coauthored by Harris, T. V. and Szilagyi, R. K. Nitrogenase structure and function relationships by density functional theory. In *Nitrogen fixation: methods and protocols*; Ribbe, M. W., Ed.; Humana Press: New York, 2011; pp 267-292.

electrostatic and steric interactions with the surrounding residues. Methods of constructing appropriate computational models of metalloenzyme active sites are not well established. Large models produce more realistic results, but the computational cost can be prohibitive. In Chapter 3, the [2Fe-2S] cluster of [FeFe]-hydrogenase was used as a test system to probe the individual interactions with the surrounding residues, affording a deeper understanding of the influence of the protein environment on the electronic structure of iron-sulfur clusters. An important outcome was a standard method for constructing computational models that captures all significant environmental effects. This study has broad impact because the principles of electrostatic interactions are the same for any metalloenzyme active site, and thus the algorithm is expected to be transferable to other bioinorganic systems.

An application of the calibrated DFT methods to the long-standing uncertainties in the structure of the nitrogenase FeMo-co was presented in Chapter 4. DFT-based mechanistic studies will be important for understanding biological nitrogen fixation, but they cannot be accurate if the cluster model does not have the correct composition, charge, spin state, and protonation state. By comparing the geometric and electronic properties of all possible structures to the available metric data and reduction potentials, as well as EPR, Mossbauer, and ENDOR parameters, many structures were ruled out, and $[2\text{Fe}^{\text{II}}-5\text{Fe}^{\text{III}}-\text{C}^{4-}]$ with protonated homocitrate was found to be the most likely oxidation state and composition. The structure of FeMo-co has been the subject of considerable controversy, and has been revised several times. The study presented here is as comprehensive as possible, and uses well-founded methods; thus, the findings add

context to mechanistic proposals, and will be a starting point for future DFT studies using a realistic virtual chemical model of the nitrogenase active site.

Future Directions

An Extended Thorneley-Lowe Model

In this section, a new molecular mechanism of biological nitrogen fixation is proposed for consideration in future DFT and experimental studies. All proposed mechanisms must be based on the framework of the Thorneley-Lowe kinetic model, which defines eight intermediate states, E_n , for the MoFe protein, where n is the number of accumulated electrons and protons. The mechanism proposed here is a more detailed description of the Thorneley-Lowe model, taking into account the results of Chapter 4, and the many experimental studies of trapped intermediates. To determine the structure of the active site in each intermediate state, the most likely binding sites of the protons/hydrides must be defined, as the reduction is generally delocalized over the entire FeMo-co cluster. The E_4 state was recently shown by ENDOR measurements to contain two hydrides, each bound in a bridging mode between iron centers of FeMo-co,²⁴⁷ with the other two protons presumably bound to nearby residues ($H^+_{(out)}$). Due to the lack of spectroscopic evidence, it is assumed that intermediates with protonated bridging sulfides are only short-lived species. This is a departure from previous computational studies (reviewed in refs 25 and 248), since it is anticipated that protons can migrate from the sulfides ($>SH^-$) to the reduced iron centers ($Fe^{II}\cdots Fe^{II}$) to form hydride ligands ($Fe^{III}-H^- - Fe^{III}$ and $>S^{2-}$) that can aid in the accumulation of additional electrons on the cluster.

Figure 5.1 shows an extended Thorneley-Lowe scheme for the first four electron- and proton-transfer steps that was constructed with the assumption that from a given E_n state there are two possible paths to reach the E_{n+1} state: (1) a nearby residue is protonated ($H^+_{(out)}$) and the FeMo-co is reduced, or (2) a hydride (initially $H^+_{(in)}$) forms and the metals are oxidized. Using these steps a few remarkable observations can be made. The EPR silent E_1 state can be either reduced or oxidized by one electron relative to FeMo-co^N, depending on whether outer-sphere protonation or hydride formation occurs. Initially, there are three possibilities for E_2 , but FeMo-co^{SupRed} would be short-lived with its high pK_a , and the doubly hydride-coordinated FeMo-co^{SupOx} is unlikely because it does not have a path for the H_2 evolution observed during kinetics experiments.²⁴⁹ The hydride-bound FeMo-co^N is the most attractive candidate for the E_2 state, as it would likely display the observed²⁴⁹ $S_t = 3/2$ signal, and be ready for H_2 -evolution when the hydride becomes protonated from an outer-sphere source. Consequently, the E_3 state, prior to possible N_2 binding, must have the same oxidation state assignment as E_1 and also be EPR silent. For E_4 , the trihydride state is unlikely (not shown in Figure 5.1), and the high- pK_a FeMo-co^{SupRed} H^- state is expected to gain at least an additional hydride from the outer sphere, leaving FeMo-co^N with two hydrides as the only possibility. The $S_t = 1/2$ spin state likely arises from structural perturbations by the two bridging hydrides that only have sufficient space within two faces of the 6Fe belt. This is attractive since it would leave the third face available for N_2 binding. Similarly, the E_3 state that most likely binds N_2 (FeMo-co^{Ox}(H^-)₂) has two bridging hydrides. The alternate E_3 state of hydride-

bound $\text{FeMo-co}^{\text{Red}}$ would not be able to bind N_2 because the resulting structure would be equivalent to that of the E_1 state, which does not interact with N_2 .

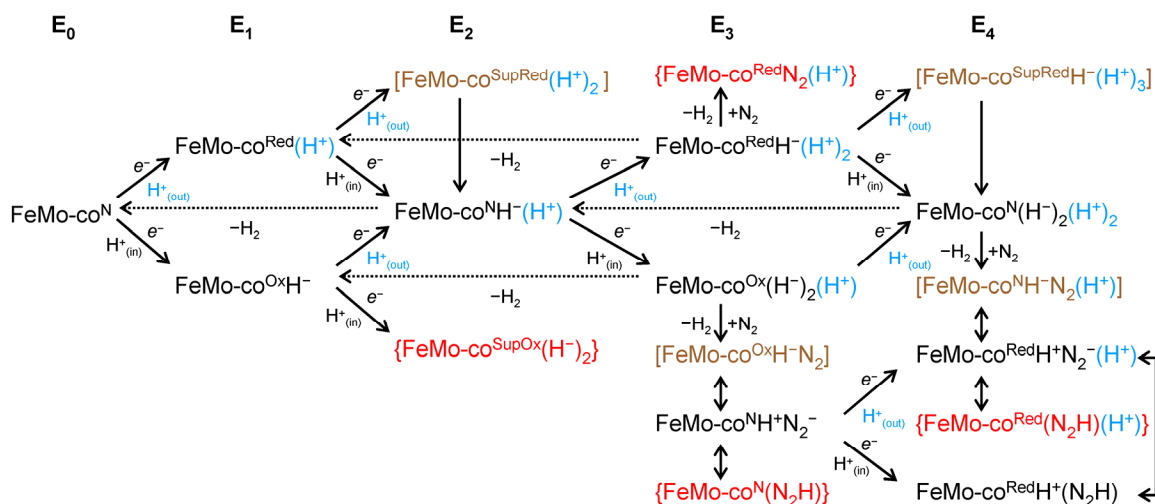


Figure 5.1. Modified Thorneley-Lowe scheme showing possible speciation of electrons and protons/hydrides for each state, E_n , where n is the number of proton-coupled electron transfers to the MoFe protein. Protons that will bind or are already bound to nearby residues are colored blue, short-lived states are in brown, and less likely states are in red.

Remarkably, the molecular kinetic scheme in Figure 5.1 suggests that N_2 binds to a formally equivalent oxidation state level to $\text{FeMo-co}^{\text{Ox}}$ in E_3 , or $\text{FeMo-co}^{\text{N}}$ in E_4 . It is likely that a modified cluster relative to the crystallographically characterized resting form makes N_2 binding possible. Due to the lack of an observed exchangeable hydride in the N_2 -bound intermediate,³⁹ the initial state with a single hydride and physisorbed N_2 in E_3 and E_4 must be short-lived, with a hydride electron reducing N_2 to N_2^- . The leftover proton after reduction must bind to a bridging sulfide or nearby residue that is not detectable by ENDOR spectroscopy. At this point there are only two N_2 -bound structures: $\text{FeMo-co}^{\text{NH}^+\text{N}_2^-}$ of E_3 , and $\text{FeMo-co}^{\text{RedH}^+\text{N}_2^-}$ of E_4 , which also has an additional outer proton. It is expected that the E_3 state is the experimentally observed N_2 -

bound intermediate with non-integer total spin and without experimentally observable exchangeable protons, because the E_4 state is diamagnetic or an integer spin system. In both states, inner proton migration to N_2^- is not expected because the resulting states would be essentially the same as E_0 and E_1 , which do not bind N_2 . From the E_3 state, an additional outer- or inner-proton-coupled electron transfer will lead to the N_2^- or N_2H -bound E_4 state, respectively, from which reduction to ammonia will occur. A final consideration for the actual structure of the reduced and bound N_2 (chemisorbed) is that it is likely to be bridging to prevent the formation of an N_2H_x state at the E_3 or E_4 level. Terminal coordination would attract spontaneous protonation, resulting in exchangeable protons attached to a paramagnetic center that have not been detected experimentally by ENDOR measurements.

A Computational Model of FeMo-co for Future Mechanistic Studies

The actual molecular steps of biological nitrogen fixation will only be revealed through studies that use a computational model encompassing all major inner- and outer-sphere-coordination effects to FeMo-co, evaluate access to electron and proton transfer pathways, and consider reactant and product channels. These calculations are yet to be performed using realistic virtual chemical models of the nitrogenase active site. A computational model based on the algorithm defined in Chapter 3 combined with the level of theory calibrated in Chapter 2 would have remarkable potential for exploring the proposed mechanism. A two-dimensional and corresponding three-dimensional

representation of the network of weak interactions around FeMo-co that can affect the electronic structure of the cluster is presented in Figure 5.2.

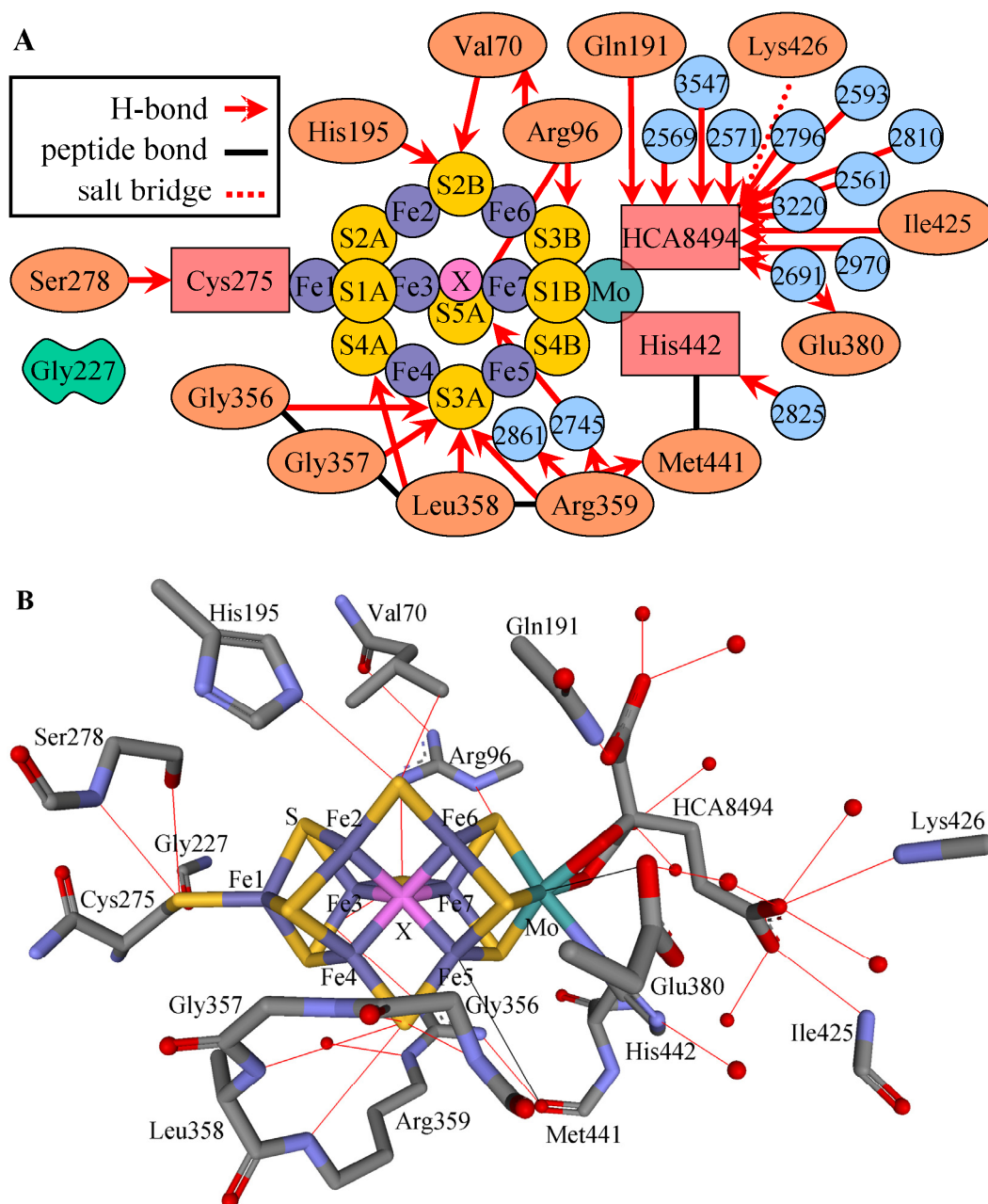


Figure 5.2. Protein environment of FeMo-co. (A) First, second, and third-coordination-sphere residues are shown in pink, orange, and green, respectively, while waters are in blue. (B) The three-dimensional representation includes the residues from (A), truncated for use as a computational model. Hydrogen atoms are omitted for clarity.

The above model is derived using the new algorithm, and it also includes the residues shown experimentally to affect the FeMo-co structure and nitrogen fixation mechanism. The waters (blue), second-sphere residues (orange), and third-sphere residues (green) would need to be treated explicitly, while all other outer-sphere charged residues can be approximated by point charges. The steric and electrostatic effects of the remaining protein environment can be modeled with molecular mechanics and PCM, respectively. Preliminary results with the model shown in Figure 5.2B show surprisingly small changes in reduction potentials compared to those calculated using the minimal model embedded in a dielectric continuum, as in Chapter 4; however, the surrounding residues are still expected to play an important role in the substrate reduction mechanism.

Outlook

As shown by the Thorneley-Lowe scheme, the N_2 reduction mechanism involves a large number of intermediate states, many of which are difficult to trap experimentally. DFT allows examination of every intermediate, and assessment of the feasibility of proposed mechanistic pathways, including N_2 binding modes, and protonation states. Currently, high-quality theoretical calculations have the greatest potential to explain the specific electronic tuning by molybdenum, the role of weak interactions with the protein environment, the importance of magnetic coupling among the Fe centers, the possibility of the molybdenum center being the catalytically active site, and the complete molecular mechanism including specific transition states. This combined information would map the potential energy surface of biological nitrogen fixation, providing feedback for the design of future site-directed substitutions and other biochemical experiments, as well as

an invaluable blueprint for rationalized design of biomimetic compounds. The synergism between computational chemistry and biochemical research design is well exemplified by the recent work on the biosynthesis of the FeFe-hydrogenase catalytic cluster.²⁵⁰⁻²⁵³

Beyond understanding nitrogen fixation, FeMo-co provides a challenging case study for theoretical method development and application, as it is one of the most complex iron-sulfur clusters in biology. For example, treatments and approaches learned by studying the structure of FeMo-co and its chemical function are expected to be readily applicable to other bioinorganic systems, particularly those containing heterometal-substituted iron-sulfur clusters. In addition, we have proposed universal computational tools for understanding the intimate molecular details of the complex protein environmental effects. The numerous site-directed mutagenesis studies provide an excellent training set for the development of multi-layered computational models that include DFT-, *ab initio* wave function-, and molecular mechanics-based regions.²⁵⁴ With the continually increasing accessibility of larger computational resources and more accurate levels of theory for many-electron systems, the already indispensable DFT techniques will soon have even greater ability to complement experiment and increase our understanding of bioinorganic systems. It is the mission of computational bioinorganic chemists to perform the highest quality simulations in order to facilitate scientific advancement and mutual discourse in the general metalloenzymology community.

APPENDICES

APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER 2

Checkpoint Files and Coordinates

Formatted checkpoint files and XYZ coordinates for all models are provided online at <http://computational.chemistry.montana.edu/covalency>.

Effect of Model Truncation

Truncating the $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ model to $[\text{Fe}(\text{C}_4\text{H}_6\text{S}_2)_2]^-$ had little effect on the results of DFT calculations. Specifically, with BP86/def2-QZVP, truncation resulted in changes in covalency per S^{t} of $-0.003 e$, $-0.002 e$, and $0.001 e$, from MPA spin densities, AIM spin densities, and AIM LUMO analyses, respectively.

Supporting Tables and Figures

Table A.1. Electronic energy (E_{SCF}), and MPA and AIM spin density per S^{t} of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ using the B(5%HF)P86 functional and various basis sets

	basis functions	E_{SCF} (a.u.)	S^{t} spin density (e)	
			MPA	AIM
LANL2DZ	230	-783.32	0.356	
SDD-VTZ(d)	327	-2336.14	0.292	
6-31G	257	-3475.78	0.244	0.262
6-31+G(d)	477	-3476.06	0.314	0.304
6-311G	379	-3476.13	0.299	0.296
6-311+G	471	-3476.19	0.365	0.296
6-311G(d)	486	-3476.33	0.304	0.301
6-311+G(d)	578	-3476.37	0.372	0.305
def-SVP	400	-3475.10	0.261	0.297
def2-SV(P)	683	-3475.06	0.261	0.298
def2-SVP	741	-3475.10	0.260	0.297
def-TZVP	521	-3476.47	0.289	0.301
def2-TZVP	785	-3476.52	0.309	0.307
def2-TZVPP	952	-3476.53	0.313	0.305
def2-QZVP	1756	-3476.65	0.296	0.304
def2-QZVPP	1772	-3476.65	0.284	0.304
aug-cc-pVTZ	1397	-3476.57	0.323	0.302
aug-cc-pVQZ	2492	-3476.63	0.306	0.303

Table A.2. MPA spin density per S^t of $[\text{Fe}(\text{C}_4\text{H}_6\text{S}_2)_2]^-$ using the def2-TZVP basis set and various functionals

functional	S^t spin density (e)
B(5%HF)P86	0.305
τ HCTHhyb	0.278
B3LYP	0.266
HSE06	0.245
PBE0	0.242
M06	0.245
BP86	0.322
BVP86	0.319
HCTH407	0.304
τ HCTH	0.309
M06-L	0.269
OP86	0.303
PBE	0.317
PBEhole	0.320
PKZB	0.301
PW91	0.319
TPSS	0.305
VSXC	0.292

Table A.3. Covalency per S^t of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$ and three truncated models using QCISD with various basis sets and population analyses

model	basis set	spin density		S contribution to LUMO– LUMO+4
		AIM	MPA	AIM
$[\text{Fe}(\text{SH})_4]^-$	def2-SV(P)	0.247	0.214	0.246
$[\text{Fe}(\text{SH})_4]^-$	TZVP	0.252	0.237	0.254
$[\text{Fe}(\text{SH})_4]^-$	def2-TZVP	0.263	0.257	0.279
$[\text{Fe}(\text{SH})_4]^-$	def2-TZVPP	0.285	0.289	0.277
$[\text{Fe}(\text{SH})_4]^-$	def2-QZVPP	0.279	0.273	0.279
$[\text{Fe}(\text{SMe})_4]^-$	def2-SV(P)	0.261	0.228	0.242
$[\text{Fe}(\text{SMe})_4]^-$	TZVP	0.271	0.253	0.256
$[\text{Fe}(\text{SMe})_4]^-$	def2-TZVP	0.283	0.274	0.273
$[\text{Fe}(\text{C}_4\text{H}_6\text{S}_2)_2]^-$	def2-SV(P)	0.267	0.234	0.241
$[\text{Fe}(\text{C}_4\text{H}_6\text{S}_2)_2]^-$	TZVP	0.280	0.261	0.256
$[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$	def2-SV(P)	0.269	0.236	0.243
$[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$	def2-QZVPP ^a	0.301 ^a	0.295 ^a	0.276 ^a

^a Extrapolated as explained in the section titled *Ab Initio* Fe–S Covalency of $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$.

Table A.4. Delocalization indexes (DI) calculated with B(5%HF)P86/6-311+G(d) for all models, except def-TZVP for $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$

model	DI(Fe, S ^s)	DI(Fe, S ^t)
$[\text{Fe}(\text{C}_6\text{H}_4\text{S}_2)_2]^-$		0.923
$[\text{Fe}_2\text{S}_2(\text{S}_2\text{-}o\text{-Xyl})_2]^{2-}$	1.079	0.820
$[\text{Fe}_2\text{S}_2(\text{SPh})_4]^{2-}$	1.105	0.794
$[\text{Fe}_2\text{S}_2(\text{SEt})_4]^{2-}$	1.085	0.793
$[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$	0.819	0.879

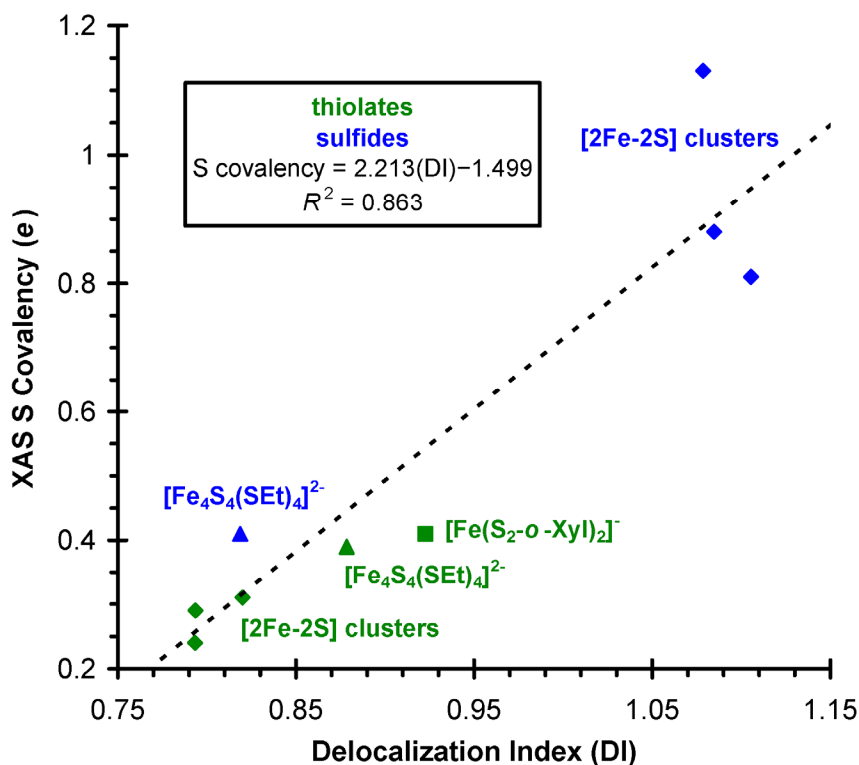


Figure A.1. Correlation of experimental (XAS) covalencies per S^t or per S^s per Fe with corresponding delocalization indexes (DI) calculated with B(5%HF)P86/6-311+G(d) for all models, except def-TZVP for $[\text{Fe}_4\text{S}_4(\text{SEt})_4]^{2-}$. The DI of $\text{Fe}(\text{C}_4\text{H}_6\text{S}_2)_2]^-$ was used for $[\text{Fe}(\text{S}_2\text{-}o\text{-Xyl})_2]^-$.

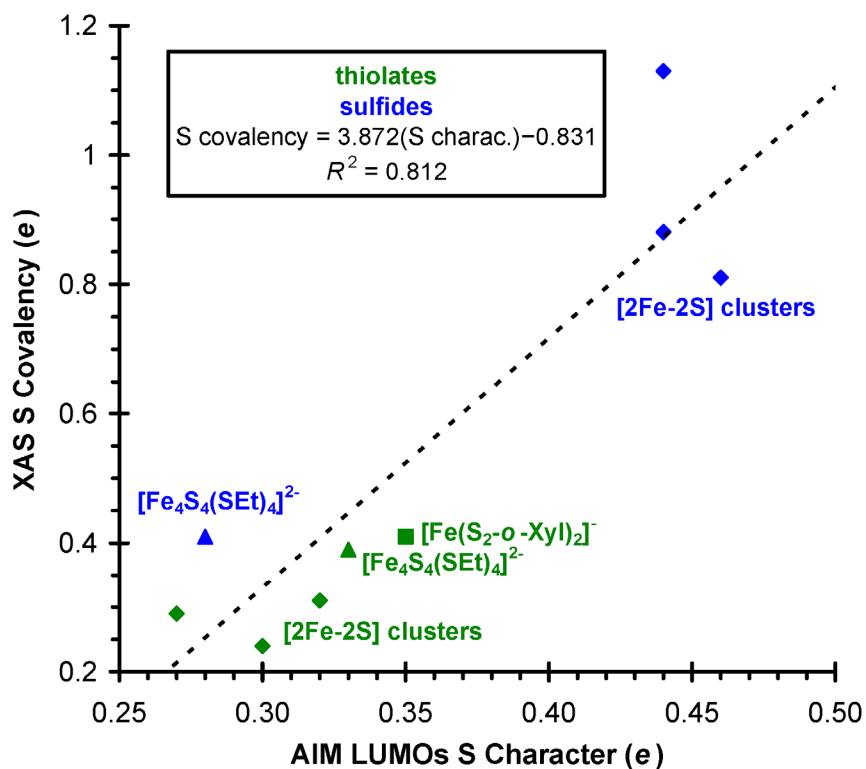


Figure A.2. Correlation of experimental (XAS) covalencies per S^t or per S^s per Fe with LUMO-derived covalencies calculated with B(5%HF)P86/6-311+G(d) for all models, except def-TZVP for [Fe₄S₄(SET)₄]²⁻. The truncated model, Fe(C₄H₆S₂)₂⁻, was used for [Fe(S₂-o-Xyl)₂]⁻.

APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER 3

Checkpoint Files and Coordinates

Formatted checkpoint files and XYZ coordinates for all models are provided online at http://computational.chemistry.montana.edu/protein_environment.

APPENDIX C

SUPPORTING INFORMATION FOR CHAPTER 4

Checkpoint Files and Coordinates

Formatted checkpoint files and XYZ coordinates for all models are provided online at <http://computational.chemistry.montana.edu/FeMo-co>.

Supporting Tables and Figures

Table C.1. Spin distributions and energies of the 35 spin states and the ferromagnetic state of FeMo-co^N (4Fe^{II}-3Fe^{III}-N³⁻) relative to BS7-1^a

spin state	relative energy (kJ/mol)	antiferromagnetically coupled Fe pairs	majority spin of Fe1, 2, 3, 4, 5, 6, 7
Ferro.	582.8	0	ααααααα
BS1	180.3	3	ααααβββ
BS2	49.4	6	αβββααα
BS3-1	114.3	5	βββαααα
BS3-2	106.4	5	βαββααα
BS3-3	113.7	5	ββαβααα
BS4-1	49.2	8	ααβαββα
BS4-2	56.0	8	αβααβαβ
BS4-3	53.4	8	αααβαββ
BS5-1	92.9	6	ααβαβαβ
BS5-2	83.0	6	αβααββα
BS5-3	91.5	6	αααββαβ
BS5-4	92.1	6	αααβββα
BS5-5	91.9	6	αβαααββ
BS5-6	95.4	6	ααβααββ
BS6-1	34.1	7	βαααβαβ
BS6-2	42.0	7	βαααββα
BS6-3	39.6	7	βααααββ
BS7-1	0.0	9	αβαβααβ
BS7-2	5.6	9	ααββαβα
BS7-3	0.8	9	αββαβαα
BS8-1	51.9	7	ααββααβ
BS8-2	54.0	7	αβαββαα
BS8-3	52.8	7	αβαβαβα
BS8-4	51.8	7	ααβββαα
BS8-5	49.0	7	αββαααβ
BS8-6	47.9	7	αββααβα
BS9-1	92.4	6	ββαααβα
BS9-2	80.3	6	βαβαααβ
BS9-3	118.6	6	βααββαα
BS10-1	45.0	8	ββααααβ
BS10-2	43.0	8	βαβαβαα
BS10-3	49.1	8	βαβααβα
BS10-4	57.8	8	ββααβαα
BS10-5	36.0	8	βααβααβ
BS10-6	54.3	8	βααβαβα

^a The calculations were carried out with BP86/SDD-VTZ(d) using crystallographic coordinates.

Table C.2. Energies of a representative spin state for each number of antiferromagnetically coupled Fe pairs calculated with various functionals and basis sets

spin state	SDD-VTZ(d)		6-311+G(d)		S-bridged antiferromagnetically coupled Fe pairs
	BP86	B3LYP	BP86	B3LYP	
BS7-1	0	0	0	0	9
BS10-1	45.0	18.9	56.3	23.6	8
BS6-1	34.1	60.9	27.4	60.5	7
BS5-1	92.9	122.1	84.5	124.8	6
BS3	114.3	137.7	101.0	107.2	5
BS1	180.3	220.2			3

Table C.3. Relative energies of the $S_t = 1/2$ and $5/2$ states to the $S_t = 3/2$ ground state of FeMo-co^N

	6Fe ^{II} -1Fe ^{III}		4Fe ^{II} -3Fe ^{III}		2Fe ^{II} -5Fe ^{III}
	$S_t = 5/2$		$S_t = 1/2$	$S_t = 5/2$	$S_t = 5/2$
C ⁴⁻	14.8		47.1	42.4	8.6
N ³⁻	7.0		40.7	49.8	15.7
O ²⁻	9.8		27.7	54.7	22.1

Table C.4. Relative energies of the $S_t = 1$ and 2 states to the $S_t = 0$ ground state of FeMo-co^{Ox} using crystallographic coordinates (XRD) or optimized (opt.) geometries for each state

	5Fe ^{II} -2Fe ^{III}		3Fe ^{II} -4Fe ^{III}		1Fe ^{II} -6Fe ^{III}	
	$S_t = 1$	$S_t = 2$	$S_t = 1$	$S_t = 2$	$S_t = 1$	$S_t = 2$
C ⁴⁻ (XRD)	-24.6	-30.7	-35.4	-32.1		3.5
N ³⁻ (XRD)	-31.4	-39.3	-41.3	-27.4	-3.7	6.5
O ²⁻ (XRD)	-29.2	-39.9	-31.1	-11.2	-8.3	15.5
C ⁴⁻ (opt.)			-45.1		3.4	6.8
N ³⁻ (opt.)	-42.2		-35.6		4.2	10.4

Table C.5. Interatomic distances in the optimized $[6\text{Fe}^{\text{II}}-1\text{Fe}^{\text{III}}-\text{N}^{3-}]$ state of FeMo-co^a

HC(O _{hydroxyl})	calculated distances (Å)		exp
	O ⁻	OH	
S(Cys)–Fe	2.32	2.30	2.27
Fe–S ^(Fe)	2.34	2.34	2.28
Fe [⋯] Fe ^(Fe)	2.75	2.74	2.66
S ^(Fe) –Fe ^(Fe)	2.35	2.34	2.27
Fe ^(Fe) –S ^(b)	2.27	2.27	2.23
Fe ^(Fe) ⋯Fe ^(Fe)	2.67	2.67	2.65
Fe ^(Fe) –X	2.02	2.01	1.98
Fe ^(Fe) ⋯Fe ^(Mo)	2.64	2.63	2.59
Fe ^(Mo) ⋯Fe ^(Mo)	2.63	2.64	2.61
Fe ^(Mo) –S ^(b)	2.27	2.25	2.22
Fe ^(Mo) –X	2.02	2.02	2.02
Fe ^(Mo) ⋯Mo	2.73	2.71	2.69
S ^(Mo) –Fe ^(Mo)	2.31	2.30	2.23
S ^(Mo) –Mo	2.40	2.38	2.35
Mo–N(Imi)	2.26	2.25	2.30
Mo–O _{carboxyl}	2.18	2.17	2.18
Mo–O _{hydroxyl}	2.08	2.28	2.20
avg. Mo–O/N	2.17	2.23	2.23
Fe [⋯] Mo	7.19	7.14	7.00
avg. error	0.04	0.04	
rms dev.	0.07	0.06	
core avg. error	0.05	0.04	
core rms dev.	0.05	0.05	

^a Experimental distances are given for the average of four FeMo-co structures from the crystal structure²⁶ along with those from two EXAFS studies.^{197, 235} Superscripts (Fe) and (Mo) refer to the [4Fe-3S] and [Mo-3Fe-3S] subclusters, respectively; (b) denotes the bridging sulfides. HC(O_{hydroxyl}) refers to the deprotonated (O⁻) or protonated (OH) hydroxyl group of the homocitrate ligand

Table C.6. Calculated and experimental Mössbauer parameters of model complexes^a

	δ_{exp}	δ_{calc}			$\Delta E_{\text{Q,exp}}$	$\Delta E_{\text{Q,calc}}$	
		m. 1	m. 2	m. 3		m. 1	m. 2
$[\text{Fe}(\text{SPh}_4)]^{2-}$	0.66	0.70	0.65	0.67	3.24	-2.70	-2.67
$[\text{Fe}(\text{SEtOH})_4]^{2-}$	(0.73)		0.71		(3.48)		2.91
$[\text{Fe}(\text{SEt})_4]^{2-}$	0.74		0.70		3.5		2.92
$[\text{Fe}(\text{SEt})_4]^-$	0.25	0.22	0.20	0.29	0.62	-0.37	-0.48
$[\text{Fe}((\text{S}_2\text{-}o\text{-xyl})_2)]^-$	0.13 (0.13)	0.20	0.23		0.57 (0.57)	-0.50	-0.56
$[\text{Fe}_2\text{S}_2(\text{S}_2\text{-}o\text{-xyl})_2]^{2-}$	0.17 (0.19)	0.24	0.23		0.57 (0.36)	-0.51	-0.44
$[\text{Fe}_4\text{S}_4(\text{SEtOH})_4]^{2-}$	0.46 (0.47) ^b	0.39	0.40	0.43	1.14 (1.15) ^b	0.90	0.87
$[\text{Fe}_4\text{S}_4(\text{SPh}_4)]^{2-}$	0.46	0.40	0.43	0.43	1.07	-0.92	-0.76
$[(\text{Tp})\text{MoFe}_3\text{S}_4\text{Cl}_3]^{-c,d}$	0.49 ^b	0.41	0.40	0.44	0.61 ^b	0.64	0.43
	0.54 ^b	0.49	0.54	0.51	1.09 ^b	-1.21	-1.04
$[(\text{Tp})\text{MoFe}_3\text{S}_4\text{Cl}_3]^{2-c,d}$	0.65 ^b	0.64	0.64	0.63	1.15 ^b	1.75	1.78
	0.62 ^b	0.61	0.68	0.60	0.62 ^b	0.94	0.81

^a Experimental isomer shifts (δ_{exp}) and quadrupole splittings ($\Delta E_{\text{Q,exp}}$) were collected for solid samples at 4.2 K, except where noted. Values in parentheses are for solution samples, which were employed in method 2 (m.2). Isomer shifts are relative to Fe metal at room temperature. Calculations were performed with crystallographic coordinates for the isomer shift training set (m.1) or optimized structures (m.2). In method 3 (m.3) crystallographic coordinates were used and $[\text{Fe}((\text{S}_2\text{-}o\text{-xyl})_2)]^-$ and $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-}o\text{-xyl})_2]^{2-}$ were omitted from the training set. References for the structures and experimental parameters are in Table C.7. ^b Experimental temperature was 77 K. The tabulated isomer shifts are corrected to 4.2 K based on a 0.12 mm/s linear increase from 300 K to 4.2 K.²⁴³

^c Tp = hydrotris(pyrazolyl)borate(1-) ^d Two spectroscopically unique Fe sites.

Table C.7. References for crystallographic structures and Mössbauer parameters of model complexes

	structure ID ^a	structure refs	Mössbauer refs
[Fe(SPh ₄)] ²⁻	PTHPFE10	255	255
[Fe(SEtOH) ₄] ²⁻			256
[Fe(SET) ₄] ²⁻			257
[Fe(SET) ₄] ⁻	CANDAW10	258	257
[Fe((S ₂ - <i>o</i> -xyl) ₂)] ⁻	OXYSFE10	170	244
[Fe ₂ S ₂ (S ₂ - <i>o</i> -xyl) ₂] ²⁻	XLDTSF	171	259, 260
[Fe ₄ S ₄ (SEtOH) ₄] ²⁻	CESSEY	44	44
[Fe ₄ S ₄ (SPh ₄)] ²⁻	FEMJAI02	261	262
[(Tp)MoFe ₃ S ₄ Cl ₃] ^{-b}	MIGGOY	263	263
[(Tp)MoFe ₃ S ₄ Cl ₃] ^{2-b}	MIGHEP	263	263

^a Cambridge Structural Database refcode ^b Tp = hydrotris(pyrazolyl)borate(1-)

Table C.8. Calculated isomer shifts for FeMo-co^N using method 1^a

X=	2Fe ^{II} -5Fe ^{III} -X			4Fe ^{II} -3Fe ^{III} -X				6Fe ^{II} -1Fe ^{III} -X	exp ^{b, c}
	C ⁴⁻		N ³⁻	C ⁴⁻		N ³⁻		N ³⁻	
	O ⁻	OH	O ⁻	O ⁻	OH	O ⁻	OH	O ⁻	
HC(O _{hydroxyl})									
Fe1	0.35	0.30 (0.22; 0.36)	0.30	0.61	0.59	0.55	0.52 (0.46; 0.53)	0.70	0.39
Fe2	0.37	0.35 (0.19; 0.40)	0.46	0.48	0.46	0.55	0.54 (0.47; 0.55)	0.64	0.33
Fe3	0.33	0.29 (0.22; 0.35)	0.40	0.50	0.48	0.55	0.54 (0.51; 0.55)	0.83	0.41
Fe4	0.38	0.35 (0.17; 0.40)	0.47	0.49	0.46	0.55	0.54 (0.46; 0.55)	0.64	0.33
Fe5	0.39	0.38 (0.29; 0.42)	0.46	0.46	0.44	0.55	0.53 (0.46; 0.54)	0.64	0.48
Fe6	0.37	0.34 (0.24; 0.39)	0.43	0.43	0.39	0.52	0.48 (0.42; 0.51)	0.61	0.39
Fe7	0.30	0.25 (0.19; 0.32)	0.37	0.39	0.35	0.46	0.41 (0.36; 0.45)	0.68	0.50
average	0.35	0.32 (0.22; 0.38)	0.41	0.48	0.45	0.53	0.51 (0.45; 0.52)	0.68	0.41

^aResults from method 2 and 3 are in parentheses. See the section titled Mössbauer Isomer Shifts for details. ^b 4.2 K relative to Fe at room temperature. ²⁰⁵ ^c Assignment of experimental values to Fe sites is based on the hyperfine couplings (see Table C.10)

Table C.9. Calculated quadrupole splittings (ΔE_Q) for FeMo-co^N using BP86/STO-TZP^a

HC(O _{hydroxyl})	2Fe ^{II} -5Fe ^{III} -X			4Fe ^{II} -3Fe ^{III} -X				6Fe ^{II} -1Fe ^{III} -X	exp ^{b, c}
	C ⁴⁻		N ³⁻	C ⁴⁻		N ³⁻		N ³⁻	
	O ⁻	OH	O ⁻	O ⁻	OH	O ⁻	OH	O ⁻	
Fe1	-0.83	-0.86 (1.45)	-0.86	1.18	1.16	1.10	1.1 (1.56)	1.25	-0.56
Fe2	0.82	0.8 (1.08)	-0.71	1.08	0.95	0.67	-0.63 (1.11)	0.97	-0.66
Fe3	-0.91	-0.88 (-1.18)	-1.20	-0.97	-1.02	1.50	1.57 (2.02)	1.08	0.68
Fe4	0.84	0.81 (1.07)	-0.72	1.09	0.95	0.66	-0.62 (1.07)	0.97	-0.66
Fe5	-1.04	-0.97 (-1.19)	1.58	0.69	0.78	-1.00	-0.95 (-1.36)	0.65	-0.94
Fe6	-0.91	-0.91 (-1.11)	1.45	0.69	0.74	-0.90	-0.83 (-1.25)	0.70	-0.69
Fe7	-0.93	-0.79 (-1.06)	1.42	-0.91	-0.90	1.37	-1.1 (1.61)	1.07	-0.65
average $ \Delta E_Q $	0.90	0.86 (1.16)	1.13	0.94	0.93	1.03	0.97 (1.43)	0.95	0.69

^a Results for B3LYP/STO-TZP are in parentheses. ^b 4.2 K.²⁰⁵ ^c Assignment of experimental values to Fe sites is based on the hyperfine couplings (see Table S10).

Table C.10. Calculated spin densities ($\rho_s(\text{Fe})$) for FeMo-co^N using BP86/SDD-VTZ(d)^a

X=	2Fe ^{II} -5Fe ^{III} -X			4Fe ^{II} -3Fe ^{III} -X					6Fe ^{II} -1Fe ^{III} -X	
	C ⁴⁻		N ³⁻	C ⁴⁻		N ³⁻		O ²⁻	N ³⁻	
	O ⁻	OH	O ⁻	O ⁻	OH	O ⁻	OH	O ⁻	O ⁻	
HC(O _{hydroxyl})										
Fe1	2.68	2.61 (3.67, 2.23)	2.62	3.26	3.24	3.25	3.23 (3.58; 2.91)	3.24	3.24	
Fe2	-2.74	-2.70 (-3.53, -2.38)	-2.97	-2.91	-2.88	-3.09	-3.08 (-3.53; -2.79)	-3.33	-3.15	
Fe3	2.66	2.58 (3.47, 2.51)	2.92	3.06	3.04	3.19	3.17 (3.57; 3.02)	3.42	2.92	
Fe4	-2.76	-2.70 (-3.53, -2.37)	-2.98	-2.92	-2.89	-3.10	-3.09 (-3.54; -2.80)	-3.34	-3.16	
Fe5	2.85	2.82 (3.29, 2.70)	3.07	2.65	2.59	2.89	2.86 (3.40; 2.64)	3.18	2.94	
Fe6	2.86	2.81 (3.15, 2.63)	3.07	2.63	2.56	2.87	2.84 (3.39; 2.57)	3.16	2.91	
Fe7	-2.57	-2.52 (-3.30, -2.24)	-2.78	-2.80	-2.74	-2.99	-2.95 (-3.51; -2.67)	-3.26	-2.54	
avg. $ \rho_s(\text{Fe}) $	2.73	2.68 (3.42, 2.44)	2.92	2.89	2.85	3.06	3.03 (3.50; 2.77)	3.27	2.98	

^a Values in parentheses correspond to B3LYP/SDD-VTZ(d) and B(5%HF)P86/6-311+G(d).

Table C.11. Semiempirical hyperfine couplings for FeMo-co^N and assignment of spectroscopic sites to Fe sites

HC(O _{hydroxyl})	X=	2Fe ^{II} -5Fe ^{III} -X			4Fe ^{II} -3Fe ^{III} -X					6Fe ^{II} -1Fe ^{III} -X		site type
		C ⁴⁻		N ³⁻	C ⁴⁻		N ³⁻		O ²⁻	N ³⁻		
		O ⁻	OH	O ⁻	O ⁻	OH	O ⁻	OH	O ⁻	O ⁻	exp ^b	
Fe1		-9.1	-8.9 (-12.5; 7.6)	-8.9	-7.1	-7.0	-7.0	-7.0 (-7.8; -6.3)	-7.0	-7.3	-11.8	A3
Fe2		10.2	10.0 (13.1; 8.8)	11.1	7.2	7.1	7.7	7.6 (8.8; 6.9)	8.3	8.3	11.7	B1
Fe3		-9.1	-8.8 (-11.8; -8.5)	-9.9	-6.6	-6.6	-6.9	-6.9 (-7.7; -6.5)	-7.4	-6.6	-3.7	A4
Fe4		10.3	10.0 (13.1; 8.8)	11.1	7.2	7.2	7.7	7.7 (8.8; 6.9)	8.3	8.4	11.7	B1
Fe5		-21.2	-21.0 (-24.5; -20.1)	-22.8	-22.6	-22.0	-24.6	-24.3 (-28.9; -22.4)	-27.0	-25.0	-17.1	A2
Fe6		-21.3	-20.9 (-23.5; -19.5)	-22.9	-22.4	-21.8	-24.4	-24.1 (-28.8; -21.8)	-26.8	-24.7	-18	A1
Fe7		15.9	15.6 (20.5; 13.9)	17.2	19.0	18.7	20.3	20.1 (23.8; 18.1)	22.2	17.3	9.3	B2
<i>a</i> ^{test}		-24.3	-23.8 (-25.4; -24.1)	-25.1	-25.1	-24.4	-27.3	-27.0 (-31.8; -25.1)	-29.5	-29.6	-17.9	

^a Hyperfine couplings were derived from BP86/SDD-VTZ(d) spin densities for all states. Values in parentheses correspond to B3LYP/SDD-VTZ(d) and B(5%HF)P86/6-311+G(d). ^b 4.2 K.²⁰⁵

Table C.12. Fe site oxidation assignments for FeMo-co^N based on spin densities (see Table C.11) with corresponding spins (S_i) and derived spin projection factors (K_i)

	2Fe ^{II} -5Fe ^{III} -X			4Fe ^{II} -3Fe ^{III} -X			6Fe ^{II} -1Fe ^{III} -X		
	ox. state	S_i	K_i	ox. state	S_i	K_i	ox. state	S_i	K_i
Fe1	2+	2	+2/5	2.5+	9/4	+3/10	2+	2	+4/15
Fe2	3+	5/2	-3/5	3+	5/2	-2/5	2.5+	9/4	-11/30
Fe3	2+	2	+2/5	2.5+	9/4	+3/10	2+	2	+4/15
Fe4	3+	5/2	-3/5	3+	5/2	-2/5	2.5+	9/4	-11/30
Fe5	3+	5/2	+6/5	2+	2	+1	2+	2	1
Fe6	3+	5/2	+6/5	2+	2	+1	2+	2	1
Fe7	3+	5/2	-1	2+	2	-4/5	2+	2	-4/5

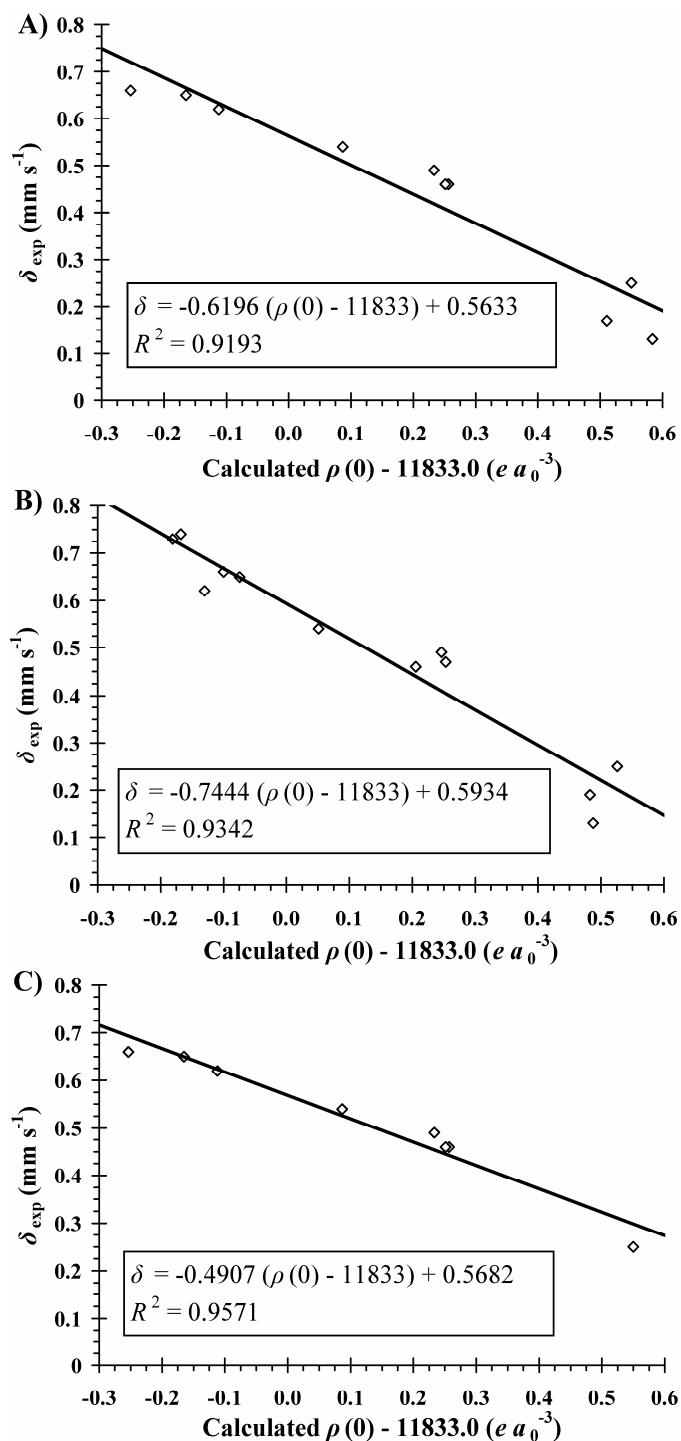


Figure C.1. Correlation between experimental isomer shifts (δ_{exp}) and calculated electron densities at the nuclei ($\rho(0)$) of a set of [Fe-S] and [Mo-Fe-S] clusters. The calculations were carried out with crystallographic coordinates (A) or optimized structures (B). The data points in plot (C) are the same as (A), except those corresponding to $[\text{Fe}((\text{S}_2\text{-}o\text{-xyl})_2)]^-$ and $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-}o\text{-xyl})_2]^{2-}$ were omitted.

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