The Electronic Structure of the Iron-Molybdenum Cofactor of Nitrogenase:
A Historical Perspective

Introduction

Our atmosphere is composed of 80% dinitrogen, yet none of it is bioavailable. Before the invention of the Haber-Bosch process in 1913, only bacteria were known to be capable of fixing dinitrogen. Ammonia produced through the costly, fossil-fuel reliant Haber-Bosch process is an essential raw material of artificial fertilizers that are responsible for supporting 40% of the world population.\(^1\) Beyond the environmental and economic benefits of an alternative means of fixing dinitrogen, it is also exciting from a fundamental science point of view.

Biological nitrogen fixation is catalyzed by the heterodimeric metalloenzyme nitrogenase, composed of the Fe-protein and the MoFe-protein (Figure 1A). The Fe-protein is involved in electron transfer to the P-cluster ([Fe\(_8\)S\(_7\)]) of the MoFe-protein, from which electrons flow to the active site metallocofactor of formula [MFe\(_7\)S\(_9\)X], where M is Mo, V or Fe, and X is an unknown interstitial atom (thought to be C, N or O).\(^2\) This paper will focus on the molybdenum containing FeMo-co (Figure 1B), which is the most frequently studied and the most active of the three cofactor varieties. FeMo-co binds, activates and reduces dinitrogen, but the substrate binding mode and mechanistic details are yet to be understood.\(^3\) Electronic structural elements including the charge and spin coupling of the cluster are crucial to our understanding of nitrogenase, yet they, too, are not well defined. I will discuss the evolution of our knowledge of the electronic structure of FeMo-co.

\[\text{FeMo-co} \quad \text{P-cluster} \quad \text{A)} \]

\[\text{unknown (C,N,O)} \quad \text{His} \quad \text{B)} \]

\[\text{Mo} \quad \text{homocitrate} \]

\[\text{Cys} \]

\[\text{Figure 1. Crystal structure of nitrogenase (A) and FeMo-co (B) with coordinating ligands.}^4\]

Methods

Many spectroscopic methods have been applied to the study of FeMo-co. EPR, which probes the splitting of energy levels in an external magnetic field of species with one or more unpaired electrons, was used to find the spin state of FeMo-co. ENDOR, a combination of NMR and EPR, is a method of observing hyperfine couplings between nuclei and unpaired electrons. This is useful for identifying the spin states of metal centers in complex clusters. Mössbauer spectroscopy is a gamma-ray-based, isotope-
specific technique from which information can be obtained regarding oxidation state (isomer shift), and the symmetry of the absorber’s environment (quadrupole coupling). Hyperfine coupling information from Mössbauer has complemented ENDOR in the study of FeMo-co. XAS is an element-specific spectroscopy based on core electron excitation into frontier orbitals and beyond. It has been used here to probe the oxidation state of Mo. X-ray crystallography is single crystal x-ray diffraction, which provides excellent geometric information. However, the resolution depends on the nature of the crystal, and because perfect crystals are difficult to grow, sometimes crucial information can be missing from a crystal structure.

Density functional theory-based calculations have also been important in the study of FeMo-co. Spectroscopy is an important complement to DFT, as the calculated geometries and electronic properties such as hyperfine coupling parameters can be compared to experimental data. Then the calculations can be used to explore possible spin couplings and oxidation state assignments by searching for the lowest energy structure.

**Results and Discussion**

Long before the first crystal structure of nitrogenase was published, researchers began to elucidate the electronic properties of FeMo-co through various spectroscopies. In 1977, extracted FeMo-co was shown by EPR and Mössbauer spectroscopies to have a total spin of $S = 3/2$, with the unpaired electrons centered on the irons, rather than the molybdenum.\(^5\) An ENDOR study of isotopically enriched MoFe protein in 1988 served to further elucidate the electronic structure. The $^{57}$Fe hyperfine couplings of the $S = 3/2$ spin state showed three sites, labeled $A^1$-$A^3$, with negative coupling constants, and two sites, labeled $B^1$ and $B^2$, with positive coupling constants indicating an antiferromagnetically coupled system.\(^6\) A previously observed $^{95}$Mo quadrupole coupling indicated a $+4$ oxidation state for Mo in the resting protein, and this assignment was supported by a Mo L-edge XANES study.\(^7,8\) Additionally, the small hyperfine coupling constant of Mo pointed to a diamagnetic spin state. At this point, further details of the electronic structure such as the lowest energy spin coupling configuration were difficult to determine due to the lack of a well-resolved structure.

![Figure 2. Crystal structure of FeMo-co as of 1992.](image)
In 1992, the first X-ray crystal structure of nitrogenase was published providing much-needed information about the geometry of the FeMo-co cluster. Unfortunately, the resolution was not high enough to elucidate the interstitial atom, leading to many incorrect conclusions based on the highly unusual structure with trigonally coordinated irons (Figure 2). Nevertheless, this era brought many important insights regarding the electronic structure, including two key proposals for the valence assignments of the irons. Since the spin states for Fe$^{3+}$ and Fe$^{2+}$ are $S = 5/2$ and $S = 2$, respectively, the $S = 3/2$ state of the cluster must have an odd number of Fe$^{3+}$ centers. Hoffman and coworkers argued that there must be only one or three Fe$^{3+}$ centers because the cofactor is in a reducing environment, and their ENDOR data supported the assignment of $[\text{Mo}^{4+}, \text{Fe}^{3+}, \text{Fe}^{2+}, S^{2-}]^{3+}$, with the Fe$^{3+}$ coupled to a Fe$^{2+}$ as a 2Fe$^{2.5+}$ pair.

An alternative valence assignment was proposed by Münck and coworkers based on Mössbauer data that identified the two missing irons from the previously described ENDOR study in which five sites were observed (A$^1$-A$^3$, B$^1$, B$^2$). The new analysis showed that B$^1$ actually represents two irons, while the seventh site, now labeled A$^4$, has very small hyperfine interactions that eluded previous detection. By comparison of isomer shift values with those from a model trigonally coordinated Fe$^{2+}$ complex, it was determined that the FeMo-co core is $[\text{Mo}^{4+}, \text{Fe}^{3+}, \text{Fe}^{2+}, S^{2-}]^{3+}$.

Through density functional calculations, Noodleman and coworkers assessed several spin states for each of the two valence assignments to determine the most likely electronic structure. The Mo$^{4+}$4Fe$^{2+}$3Fe$^{3+}$ state was ruled out because this electron deficient core draws an electron from the homocitrate ligand, leaving a radical which is not observed in EPR studies, and resulting in a state better described as Mo$^{4+}$5Fe$^{2+}$2Fe$^{3+}$. Calculated isomer shifts of the Mo$^{4+}$6Fe$^{2+}$Fe$^{3+}$ oxidation state were in reasonable agreement with experiment, and calculated hyperfine parameters of the lowest energy spin state agreed with experiment if the spins were canted from a purely parallel or antiparallel arrangement. Out of 12 tested spin coupling arrangements for Mo$^{4+}$6Fe$^{2+}$Fe$^{3+}$, BS2 had the lowest energy in gas phase, but when considering protein and solvent effects, BS6 becomes the lowest energy configuration (Figure 3). The geometry of BS6 also showed the best agreement with that of the crystal structure. Even within the BS6 configuration, several spin coupling arrangements are possible, but Fe$^{2+}$ is always Fe$^{3+}$. Although calculations at this time favored Mo$^{4+}$6Fe$^{2+}$Fe$^{3+}$, the theory of the FeMo-co electronic structure was about to be turned upside-down by a surprising discovery.

Figure 3. Two possible spin coupling arrangements for Mo$^{4+}$6Fe$^{2+}$Fe$^{3+}$.13
In 2002, a 1.16 Å resolution X-ray crystal structure of the MoFe-protein resolved a previously unrecognized interstitial ligand in FeMo-co (Figure 1). The identity of this ligand was proposed to be $C^4-$, $N^3-$ or $O^{2-}$, and subsequent studies have explored these possibilities. Noodleman and coworkers\textsuperscript{14} revised their earlier work\textsuperscript{13}, and on the basis of calculated redox potentials, the preferred ligand is $N^3-$ with the $Mo^{4+}4Fe^{2+}3Fe^{3+}$ oxidation state. Also, with $N^3-$ included the calculated isomer shifts are now closer to the experimental value with the $Mo^{4+}4Fe^{2+}3Fe^{3+}$ state than with the $Mo^{4+}6Fe^{2+}Fe^{3+}$ state.

Münck and coworkers\textsuperscript{15} proposed a spin coupling scheme consistent with the $Mo^{4+}4Fe^{2+}3Fe^{3+}$ state that involves two ferromagnetically coupled $[2Fe^{2+}Fe^{3+}]$ triangles delocalized as $[3Fe^{2.33+}]$ (Figure 4). One triangle is antiferromagnetically coupled to the terminal $Fe^{3+}$ with canted spin, resulting in a $S = 5$ state, rather than $S = 4$. This four iron system is coupled to the other triangle to produce the $S = 3/2$ resting state. Although this coupling assignment is consistent with hyperfine parameters, other configurations are still possible.

![Figure 4](image-url) Proposed spin coupling in $Mo^{4+}4Fe^{2+}3Fe^{3+}$. Large block arrows represent $Fe(d5)$ spins, while small block arrows represent minority $Fe(d6)$ spins. Single line arrows show hole delocalization. The triangles are ferromagnetically coupled $[2Fe^{2+}Fe^{3+}]$ delocalized as $[3Fe^{2.33+}]$, and with spin $S = 13/2$.\textsuperscript{15}

Blöchl and coworkers\textsuperscript{16} carried out DFT calculations in which the spins could take any direction (as opposed to uniaxial), and under the assumption that the central ligand is $N^3-$ they proposed a new oxidation state and coupling scheme. They added electrons that are involved in metal-metal bonding interactions (according to their calculations) to $Mo^{4+}7Fe^{3+}9S^{2-}N^3-$, and came up with an oxidation state assignment of $Mo^{4.5+}5Fe^{2.5+}2Fe^{2+}9S^{2-}N^3-$, where the two ferrous irons are located next to the Mo site. The proposed spin state involves three pairs of ferromagnetically coupled irons, antiferromagnetically coupled to each other, and a lone iron near the Mo which is antiferromagnetically coupled to its neighbors.

The Achilles’ heel of recently proposed structures is that they rely on $N^3-$ as the central ligand, when in fact Hoffman and coworkers have provided spectroscopic evidence (ENDOR) that $N^3-$ is not involved.\textsuperscript{17} More certain than the proposed spin couplings is that the oxidation state of the cluster as a whole is $[MoFe_7S_2X]^0$, where $X$ is the unknown ligand. Deviations from a neutral oxidation state result in poor reproduction of redox potentials\textsuperscript{14}, and calculated HOMO energies that do not correlate well with the HOMO energy of similar reference compounds.\textsuperscript{18} Thus, the oxidation state of individual iron sites will depend greatly on the identity of the interstitial ligand.
Conclusions

Over the years, spectroscopic and density functional studies have provided valuable evidence regarding the electronic structure of FeMo-co. These studies have led to many proposed spin coupling and oxidation state assignments, but they have all been built upon uncertain foundations, namely, X-ray crystal structures. The first crystal structure brought fundamental knowledge of the geometry and the number of metal centers, from which many DFT studies were based. Unfortunately, these studies used the incomplete structure with trigonally coordinated irons. Now, the identity of the interstitial ligand, shown through many calculations to be N, rather than C or O has come under scrutiny due to recent evidence opposed to the presence of N. Finding the identity of this ligand is of crucial importance, and it may be time to broaden the search to more than just C, N and O.

References