Spectroscopic investigation of nitrogenase: EPR and MCD studies of the FeMo cofactor and the P-cluster

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SPECTROSCOPIC INVESTIGATION OF NITROGENASE:
EPR AND MCD STUDIES OF THE
FEMO COFACTOR AND THE P-CLUSTER

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Chemistry

by
Robyn Broach
B.S., Philadelphia College of Pharmacy and Science, 1998
May 2004
ACKNOWLEDGEMENTS

I would like to extend a big thanks to my parental units. I am grateful for the support, encouragement, and strength you gave me, it helped me to complete such an endeavor.

Dr. Brian “The Big Guy” Hales thank you for the opportunity to do this work as well as your support and wisdom that keep me on a straight path. I would also like to express my appreciation to Dr. Kresimir Rupnik for sharing his time so unselfishly.

And thank you to MF, my friends, and the rest of my family for being so supportive and caring, especially when it came to the Colt 45.

I also appreciate all of my committee members, Dr. Hales, Dr. Stanley, Dr. LiCata, Dr. Poliakoff, and Dr. Sterling for donating their time and sharing their expertise.
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LIST OF SYMBOLS

MoFe protein  Molybdenum-iron protein
Fe protein  iron protein
Av1  Molybdenum-iron protein from *Azotobacter vinelandii*
Av2  iron protein from *Azotobacter vinelandii*
P-cluster  metallocluster of MoFe protein
P², P⁰  P-cluster in the native or all-ferrous state
P⁺¹  P-cluster oxidized by one equivalent
P⁰², P⁺²  P-cluster oxidized by two equivalents
P⁺³  P-cluster oxidized by three equivalents
L127Δ Fe protein  altered Fe protein, lysine at position 127 deleted
L127Δ Fe-MoFe protein  complex of altered Fe protein and wild-type MoFe protein
α-195⁶⁄₉ MoFe protein  MoFe protein with glutamine substituted by histidine
ΔnifB MoFe protein  apo-MoFe protein with *nifB* deleted
ΔnifH MoFe protein  apo-MoFe protein with *nifH* deleted
MgATP  magnesium adenosine triphosphate
Eₙ  reduction state of nitrogenase in Lowe-Thorneley Scheme
LT  Lowe-Thorneley Scheme
DEAE  diethylaminoethyl cellulose anion exchanger
EPR  Electron Paramagnetic Resonance
MCD  Magnetic Circular Dichroism
ENDOR  Electron Nuclear Double Resonance
CD  Circular Dichroism
NMR  Nuclear Magnetic Resonance
TCD-GC  Thermal Conductivity Detector Gas Chromatography
FID-GC  Flame Ionization Detector Gas Chromatography
S_{EPR1}, S_{EPR2}, S_{EPR3}  S = ½ EPR signals observed under C₂H₂
1b, 1c  S = ¾ EPR signals observed under N₂ or Argon
µ  vector quantity magnetic moment of an electron
β  Bohr magneton constant
βₐ  nuclear Bohr magneton constant
g  g factor, the electronic splitting factor
g┴  g component for H perpendicular to axis of symmetry
g║  g component for H parallel to axis of symmetry
A  hyperfine coupling constant
D  axial zero field splitting parameter
E/D  rhomobic distortion
zfs  zero-field splitting parameter
J  total angular momentum
Mₗ  quantum number of total angular momentum
ΔΔ  MCD absorption difference
A_{LCP}  absorption with left circularly polarized light
A_{RCP}  absorption with right circularly polarized light
A  A term, contribution to MCD spectra
B term, contribution to MCD spectra
C term, contribution to MCD spectra
effective transition moment products
scaling parameter
ABSTRACT

Little is known about substrate binding and reduction of nitrogenase. EPR spectroscopy is used here to observe intermediate states generated by different substrates. Two different spin states (S=3/2 and S=1/2) were exhibited for each substrate, which may result from different binding of the substrate to the cofactor (side-on or terminal binding) or the difference of the substrate binding to either Fe or Mo of the cofactor. Parallel studies were performed on a variant MoFe protein, alpha-195Gln, which exhibited different signals from the wild-type suggesting that the substituted amino acid maybe necessary to reach some mechanistic states that the wild-type MoFe protein can reach.

Electron transfer between the Fe protein and the MoFe protein was investigated to help determine the initial electron transfer pathway in nitrogenase. The altered Fe protein, L127-deletion Fe protein, is permanently in the complex-ready conformation and complexes with the MoFe protein to allow one electron transfer. The MCD studies suggest the presence of a second paramagnetic center in addition to the resting state cofactor. The second paramagnetic center may result from an electron delocalized over the entire P-cluster or its return to the Fe protein.

The P-cluster is suggested to play a role in the electron transfer from the Fe protein to the cofactor. Apo-proteins were used to provide information about the function and the maturation of the P-cluster. One apo-protein, nifB-deletion MoFe protein, exhibits redox characteristics analogous to the wild-type MoFe protein, i.e., both as-isolated proteins have the P-cluster in the state P⁰ and could be oxidized to P⁺². The second apo-protein, nifH-deletion MoFe protein, demonstrated different characteristics. The as-isolated form appears to be in the P⁺¹ state and can be oxidized to a previously unobserved state now suggested to be S=2.0. These results indicate that nifH-deletion MoFe protein P-clusters electronically differ from the mature fully
functioning P-cluster in the *nifB*-deletion and wild-type MoFe proteins suggesting that NifH is necessary for the maturation of the P-cluster.
CHAPTER 1. INTRODUCTION

1.1 Introduction

Nitrogen is essential for all living things. It is a necessary building block for amino acids and nucleic acids. Nitrogen gas is plentiful making up ~78% of the earth’s atmosphere but is extremely stable and unreactive. The triple bond of N$_2$ accounts for the high activation energy that must be reached to reduce N$_2$ to a more reactive form (NH$_3$). It is theorized that the first method of the conversion of nitrogen to ammonia was performed by inorganic iron sulfide (FeS), possibly allowing amino acids and nucleic acids to form in the primordial soup of chemicals during the prebiotic era of earth [6]. Today, nature’s process of converting N$_2$ to its bioavailable form, ammonia, is through nitrogen-fixing bacteria. The industrial process currently converting nitrogen to ammonia is the Haber-Bosh process, which uses an Fe catalyst, high temperatures (723 K) and high pressure (500 atm) utilizing a tremendous amount of energy.

Nitrogen-fixing bacteria or diazotrophic microorganisms contain the enzyme nitrogenase, which catalyzes N$_2$ to ammonia at ambient temperature and atmospheric pressure using ATP as its source of energy. Under optimal in vitro conditions nitrogenase follows the conversion reaction below

\[
N_2 + 16\text{MgATP} + 8e^- + 8H^+ \rightarrow 2\text{NH}_3 + H_2 + 16\text{MgADP} + 16P_i \quad (1)
\]

Nitrogenase is an important enzyme to study structurally and mechanistically. The information obtained through the investigation of nitrogenase presented here will assist in the process of creating a mimic of the enzyme as well as benefit the fundamental understanding of biology.

1.2 Structure

Nitrogenase consists of two purifiable components called the molybdenum iron protein (MoFe protein) and the iron protein (Fe protein). The enzyme purified and investigated here
comes from the bacterium *Azotobacter vinelandii*. The MoFe protein can be viewed as two identical enzyme halves that do not communicate (i.e. no enzyme cooperativity) with each other (Figure 1.2.1). This protein (MW ≈ 230,000) is an $\alpha_3\beta_2$ tetramer containing two different metal clusters in each half of the protein, the FeMo cofactor and the P-cluster. The FeMo cofactor is a [Mo-7Fe-9S-homocitrate] metalloccluster totally engulfed within the $\alpha$ subunits, and the P-cluster is a [8Fe-7S] cluster that bridges each $\alpha$ and $\beta$ subunit. The FeMo cofactor is coordinated to the $\alpha$ subunit through the side chains of two residues, a histidine ($\alpha$-442$^{\text{His}}$) bound to Mo and a cysteine ($\alpha$-275$^{\text{Cys}}$) bound to Fe at opposing ends of the cluster. The FeMo cofactor is believed to be the site at which substrates bind and are reduced.

The most recent crystal structure of the MoFe protein by Rees et al. [4] at 1.16 Å resolution reveals that there is a central atom that is 6-coordinated to the six central Fe atoms of the FeMo cofactor (Figure 1.2.2). The central atom completes an approximate tetrahedral coordination instead of the distorted trigonal coordination proposed from the previous, less resolved crystal structures [7]. The central atom is proposed to be a single nitrogen atom. Subsequent theoretical calculations [8, 9] agree that the single nitrogen atom is the most probable element in the center of the FeMo cofactor. Currently, several groups are investigating how the N atom is inserted into the FeMo cofactor. The options offered are either through biosynthesis of the cofactor or during the catalytic process of reducing N$_2$ to ammonia. Through spectroscopic techniques and isotopic labeling [10] it was deduced that the interstitial N atom in the FeMo cofactor is not exchangeable during turnover, but it is not ruled out that a non-exchanging N could participate in catalysis. This is supported by earlier studies of pre-steady-state kinetic data [11] where a burst of NH$_3$ production is not observed, meaning the interstitial N is not likely reduced to NH$_3$ (in other words, the lag in NH$_3$ production would not be observed).
Figure 1.2.1: Crystal structure of wild-type MoFe protein\(^1\) from \textit{Azotobacter vinelandii}

\(^1\) Figure generated from the coordinates for the MoFe protein (PDB entry 1M1N) [4] using the program Discovery Studio Viewer Pro 5.0.
**Figure 1.2.2:** FeMo cofactor 1.16Å resolution²

² Figure generated from the coordinates for the MoFe protein (PDB entry 1M1N) 4. Einsle, O., et al., *Nitrogenase MoFe-protein at 1.16Å Resolution: Central Ligand in the FeMo Cofactor.* Science, 2002. **297:** p. 1696-1698. using the program Discovery Studio Viewer Pro 5.0.
The P-cluster is an [8Fe-7S] cluster. A more complete description is a [4Fe-4S] cubane bridged to a [4Fe-3S] cluster by a sulfide bond. The function of the P-cluster is believed to be electron transfer between the Fe protein and the FeMo cofactor. This is supported by (1) its position between the [4Fe-4S] metal cluster of the Fe protein and the FeMo cofactor and (2) the fact that P-clusters can achieve many oxidation levels. Two redox states of the P-cluster have been crystallized, the P\textsuperscript{N} and the P\textsuperscript{OX}. P\textsuperscript{N} is the resting state form of the P-cluster, also called P or P\textsuperscript{0}, where all Fe atoms are ferrous while P\textsuperscript{OX} is the 2-equivalent oxidized form of the P-cluster (P\textsuperscript{2+}). The structures of the two redox states of the P-clusters, shown in Figure 1.2.3 [3], show a conformational change upon oxidation. P\textsuperscript{N} is coordinated to the α-subunit by three cysteines and by two cysteines to the β-subunit with the central sulfur coordinated to six Fe atoms, three in from each cubane. P\textsuperscript{OX} coordination differs from P\textsuperscript{N} in that the central sulfur loses coordination of two Fe atoms from the cubane in the β-subunit, and is replaced with two additional coordinations to the polypeptide, one from each subunit. Figure 1.2.3(b) shows that one of the Fe atoms coordinates to β-Ser\textsuperscript{188} and the other Fe atom forms a second coordination to α-Cys\textsuperscript{88} bridging the cluster to the residue. Since the ligands in the free state may be protonated and then deprotonated in the bound state to the metal cluster, the exchanging of the ligands and coordination of the central sulfur may insure unidirectional electron transfer as well as coupling electron and proton transfer.

Studies on different residue substituted MoFe proteins have also helped confirm the role of the P-cluster. The residue substitution was undertaken within 4 parallel helices of the polypeptide between the P-cluster and the FeMo cofactor. These substitutions do not affect the environment of either of the metalloclusters and possibly could affect the electron pathway [12].
**Figure 1.2.3:** Crystal structures of the P-cluster in different oxidation states [3] (a) $\text{P}_N$ (b) $\text{P}_{\text{OX}}$

The color representation is Green (Fe), yellow (S), blue (N), red (O), and gray (C).
The residue $\beta$-98$^{\text{Tyr}}$ was substituted by phenalanine, leucine, and histidine. $\beta$-98$^{\text{His}}$ is the only variant MoFe protein that exhibits reduced specific activity for $\text{H}^+$, $\text{N}_2$ and $\text{C}_2\text{H}_2$ reduction. Parallel titrations of component–protein ratio with the wild-type and $\beta$-98$^{\text{His}}$ MoFe protein revealed that both the maximum specific activity and maximum component ratio decreased for $\beta$-98$^{\text{His}}$. This suggests that the substitution may cause an alteration in the electron pathway between the Fe protein and the MoFe protein. The source of the reduced activity was examined further through kinetic experiments that indirectly measure the dissociation rate of the protein complex. The results of these experiments show that the $\beta$-98$^{\text{His}}$ MoFe protein initially exhibits electron transfer at the same rate as the wild type. The rate later decreases suggesting that the P-cluster is able to accumulate electrons but is unable to efficiently transfer them to the FeMo cofactor. These kinetic experiments also showed the uncoupling of MgATP hydrolysis and electron transfer, suggesting a back donation of an electron from the MoFe protein to an oxidized Fe protein, a consequence due to an alteration in the electron transfer pathway between the P-cluster and the FeMo cofactor. Thus, it was determined that the region between the P-cluster and the FeMo cofactor does contribute to intramolecular electron transfer.

FeMo cofactor deficient MoFe protein, also referred to as apo-MoFe protein, has been useful to the investigation of the MoFe protein. Apo-MoFe protein can be achieved by deleting certain genes to disrupt the biosynthesis of either the Fe or MoFe proteins. In vivo, the full functioning MoFe protein requires the participation of a large number of nitrogen fixation (nif) specific gene products. The genes products of $\text{nifD}$ and $\text{nifK}$ are the $\alpha$ and $\beta$ subunit sof the MoFe protein, and the gene products of $\text{nifE}$, $\text{nifN}$, $\text{nifB}$, and $\text{nifH}$ each play a role in the formation and insertion of the FeMo cofactor. Deletion of any one of these genes results in a FeMo cofactor deficient MoFe protein.
Two different forms of apo-MoFe protein will be discussed. Each form resulted from the deletion of one gene required to synthesize the complete MoFe protein. These genes are \textit{nifB} or \textit{nifH} and the deletion of them produces the $\Delta nifB$ and $\Delta nifH$ MoFe proteins. In both proteins the FeMo cofactor is absent while the P-cluster is intact. The \textit{nifB} gene product is involved in the synthesis of an iron and sulfur-containing precursor of the FeMo cofactor called NifBco. When this gene is deleted, the resultant apo-MoFe protein is incapable of reducing substrates, but it can complex with Fe protein (MgATP)$_2$ and carry out 60\% of normal MgATP hydrolysis. This suggests that a protein complex is formed but it is different from that with the wild-type MoFe protein [13]. Isolated FeMo cofactor can be inserted into the $\Delta nifB$ MoFe protein resulting in a reconstitution of 80\% of the activity [13]. This information, along with the crystal structure, reveals that the polypeptides of the $\Delta nifB$ MoFe protein are in an open conformation awaiting the cofactor insertion but different from that observed for the wild-type MoFe protein [14].

Deletion of $\Delta nifH$ results in a different apo-MoFe protein. $\Delta nifH$ MoFe protein is very oxygen sensitive and unstable, which has hindered the production of a crystal structure. However, gel electrophoresis exhibits an $\alpha_2\beta_2$ tetramer of $\Delta nifH$ MoFe protein indistinguishable from the wild-type MoFe protein tetramer [15]. The $\Delta nifH$ MoFe protein can complex with the Fe protein with MgATP like $\Delta nifB$ MoFe protein can carry out MgATP hydrolysis but not substrate reduction. Unlike $\Delta nifB$ MoFe protein, isolated cofactor insertion in $\Delta nifH$ MoFe can only occur in the presence of Fe protein and MgATP [15]. In conclusion, the $\Delta nifB$ MoFe protein exhibits an open conformation awaiting FeMo cofactor insertion while the $\Delta nifH$ MoFe protein is a closed conformation requiring the Fe protein with MgATP bound for FeMo cofactor insertion.
These two cofactor deficient proteins are also useful in the investigation of the function of the P-cluster, its redox properties, and how electrons are accepted from the Fe protein. Spectroscopic techniques, which will be discussed later, show different redox properties for each variant protein.

The Fe protein (MW ≈ 63,000) is a $\gamma_2$ dimer. The crystal structure of the Fe protein shows a presence of a single [4Fe-4S] cluster coordinated by four cysteine residues, two from each subunit, symmetrically bridging the subunits (Figure 1.2.4) [2]. The exposure of the cluster is exposed to the medium may be responsible for the sensitivity of the Fe protein to oxygen and solvents. Amino acid sequences of more than 20 different Fe proteins are known and show that this protein is highly conserved in both the conventional and alternate forms of nitrogenase. The Fe protein is the only known redox-active agent that is capable to transfer electrons to the MoFe protein. The Fe protein is also known to be required for the biosynthesis of the FeMo cofactor and the insertion of the preformed FeMo cofactor into deficient MoFe protein [16].

Electron transfer to the MoFe protein is coupled with the hydrolysis of ATP. The Fe protein can bind one MgATP or MgADP molecule to each subunit. Positive cooperativity is observed when MgATP or MgADP molecules bind, such that binding after the first molecule binds the protein’s affinity increases for the second molecule. MgATP has a greater affinity to oxidized Fe protein than the reduced suggesting a conformational change between the oxidation states. On the other hand, MgADP, binds more tightly than MgATP, is a competitive inhibitor of MgATP, and is an inhibitor of enzymatic turnover.

There is approximately a 19 Å distance between the [4Fe-4S] metal cluster of the Fe protein and the bound MgATP, contributing to the unlikelihood that hydrolysis of MgATP and electron transfer are directly coupled. However, after two molecules of MgATP bind to the Fe
Figure 1.2.4: Crystal Structure of a pair of Fe proteins complexed by MgADP from Azotobacter vinelandii

Gamma subunits – purple
Metal clusters – red
MgADP - yellow

Figure was generated from the coordinates for the Fe protein (PDB entry 1FP6) [2] using the program Discovery Studio Viewer Pro 5.0.
protein, the protein is capable of complexing with the MoFe protein, suggesting that binding of MgATP induces a conformational change in the protein environment, which allows the Fe protein to complex with the MoFe protein.

The enzyme complex consists of a MoFe protein and two Fe proteins, where one Fe protein is bound to each $\alpha\beta$ half of the MoFe protein. Figure 1.2.5 shows the crystal structure of the complex of MoFe protein and Fe protein stabilized by MgADP-AlF$_4^-$ . The aluminum tetrafluoride serves as a transition state analogue for phosphate during MgATP hydrolysis. This structure illustrates the large conformational change the Fe protein experiences relative to the MgATP-Fe protein-MoFe protein complex compared to free Fe protein.

A MgATP-free protein complex was discovered as a result of protein engineering. Site directed mutagenesis of the Fe protein created an Fe protein that mimics the conformation of the wild-type Fe protein with two MgATP bound [17]. This was achieved by the deletion of the leucine residue at position 127 in the Fe protein. The L127$\Delta$ Fe protein can complex with MoFe protein with or without nucleotide binding and is illustrated in Figure 1.2.6 (a & b) [5]. The L127$\Delta$ Fe protein resembles wild-type Fe protein with MgATP bound in other structural ways. For example, the redox potential of the [4Fe-4S] cluster decreases 120 mV and two molecules of MgATP can still bind [17]. Although MgATP can bind to the L127$\Delta$ complex, it is inactive (no ATP hydrolysis or electron transfer occurs). However, the ADP-AlF$_4^-$ stabilized complex is capable of slow MgATP hydrolysis.

Comparing the wild-type complex stabilized by ADP-AlF$_4^-$ and the L127$\Delta$ Fe protein complex with and without MgATP bound yields insight on the importance of the conformational change induced by MgATP hydrolysis. The interfacing of the MoFe protein and the Fe proteins in all three complexes are extremely similar, but differences appear in the Fe protein and
Figure 1.2.5: Crystal Structure of the MoFe - Fe protein complex\textsuperscript{5} stabilized by MgADP-AlF\textsubscript{4}\textsuperscript{−} from \textit{Azotobacter vinelandii}

\textsuperscript{5}Figure was generated from the coordinates for the MoFe-Fe protein complex with MgADP-AlF\textsubscript{4}\textsuperscript{−} (PDB entry 1M34) \cite{1} using program Discovery Studio Viewer Pro 5.0.
Figure 1.2.6 (a): Crystal Structure of L127Δ Fe protein-MoFe protein complex without MgATP from *Azotobacter vinelandii*

*Figure was generated from the coordinates for the L127Δ Fe-MoFe protein complex without MgATP (PDB entry 1G20) [5] using program Discovery Studio Viewer Pro 5.0.*
**Figure 1.2.6 (b):** Crystal Structure of L127Δ Fe protein MoFe complex with MgATP from *Azotobacter vinelandii*.

Figure was generated from the coordinates for the L127Δ Fe-MoFe protein complex with MgATP (PDB entry 1G21) [5] using program Discovery Studio Viewer Pro 5.0.
nucleotide interactions. Surprisingly, the ADP-AlF$_4^-$ stabilized complex is least like the complex of L127Δ Fe protein with MgATP bound. A more open conformation is observed in the complex of L127Δ Fe protein with MgATP bound [5]. This most likely is due to the missing interactions between the nucleotide and the Fe protein caused by the residue deletion. This lack of interaction can also account for the mutant’s inability to carry out MgATP hydrolysis.

Site directed mutagenesis or residue substitution also enables an examination of cofactor peptide interactions, which may be crucial for effective substrates binding and/or reduction. Observing and comparing results of an altered MoFe protein and a wild-type protein under identical conditions can give a better understanding on the nature of site(s) of reduction and any intermediates that may form during turnover.

The $\alpha$-195Gln MoFe protein is an example of altered MoFe protein that has yielded useful insight to the role of the protein environment around the FeMo cofactor on substrate binding and reduction. $\alpha$-195Gln represents a residue substitution of the histidine residue to a glutamine residue at position 195 in the $\alpha$ subunit [18] as illustrated in Figure 1.2.7. This substitution was chosen because of the hydrogen bond to the bridging sulfur of the FeMo cofactor provided by the $\epsilon$–nitrogen of the imidazole group from the histidine residue. Many substitutions have been performed on the $\alpha$-195 position, but the $\alpha$-195Gln MoFe protein demonstrated the properties with the most potential to answer substrate related questions.

The $\alpha$-195Gln MoFe protein is capable of efficiently reducing C$_2$H$_2$ and protons to C$_2$H$_4$ and H$_2$ with respect to the wild-type MoFe protein. However, the $\alpha$-195Gln MoFe protein is only capable of reducing 1-2% of N$_2$. The $\alpha$-195Gln MoFe protein’s lack of ability to reduce N$_2$ is attributed to the alterations induced in the MoFe protein from the substitution.
Figure 1.2.7: Crystal structures of the wild type FeMo cofactor and the $\alpha^{-195}\text{Gln}$ FeMo cofactor with surrounding amino acid residues$^8$

$^8$ Figure was generated from the coordinates for the wild-type and $\alpha$-His$^{195}$ MoFe proteins (PDB entry 1M1N and 1FP4). 4. Ibid, 18. Kim, C.H., W.E. Newton, and D.R. Dean, Role of the Mofe Protein Alpha-Subunit Histidine-195 Residue in Femo-Cofactor Binding and Nitrogenase Catalysis. Biochemistry, 1995. 34(9): p. 2798-2808. using programDiscovery Studio viewer Pro 5.0.
Since N₂ is a substrate yet inhibits C₂H₂ and proton reduction, it was determined by Dean et al. [18] that N₂ is a competitive inhibitor suggesting that all three substrates compete for the same active site. It is possible that the different substrates bind to the active site at different redox states. It was further demonstrated that N₂ binds to α-195Gln MoFe protein, uncouples MgATP hydrolysis and electron transfer, implying the substrate plays a role in controlling the electron flow. In other words, the rate of MgATP hydrolysis is unchanged even though the variant protein is unable to reduce N₂, possibly due to a hindered electron pathway or an electron sink created in the MoFe protein.

1.3 Fe Protein Cycle

In Section 1.2 the MoFe – Fe protein complex was described. The Fe protein can only complex with MoFe protein after it undergoes a conformational change induced by the binding of two MgATP molecules, also described in Section 1.2. The Fe protein cycle (Figure 1.3.1) begins once the proteins complex, allowing electron transfer and MgATP hydrolysis to occur, resulting in oxidized Fe protein with two MgADP molecules bound. The Fe protein is oxidized from [4Fe-4S]⁺ to [4Fe-4S]²⁺ electron transfer to the MoFe protein. The oxidized form of the Fe protein with bound MgADP molecules then dissociate from the one-electron reduced MoFe protein. The oxidized Fe protein completes the Fe cycle by releasing the MgADP molecules and undergoing a one electron reduction by the reductant sodium dithionite. The Fe protein then repeats the Fe cycle upon binding two new MgATP molecules. The dissociated one-electron reduced MoFe protein is also free to repeat the cycle with another reduced Fe protein leading to the existence of a mixture of several reduced forms of the MoFe protein. The frequency at which the MoFe protein can undergo the Fe protein cycle is limited by the availability of reduced Fe protein and is referred to as electron flux.
Figure 1.3.1: The Fe protein cycle

1.4 Electron Flux

Nitrogenase activity depends on electron flux. The electron flux is determined by the ratio of Fe protein : MoFe protein. As the ratio increases the electron flux increases until the Fe protein becomes saturating, normally at a ratio of 4:1. Dependent of substrate, electron flux influences the distribution of electrons to products. This is illustrated with N\textsubscript{2} reduction. N\textsubscript{2} reduction is favored over H\textsubscript{2} production at high electron flux while the opposite occurs at low electron flux. In fact, at very low electron flux (1:100), only H\textsubscript{2} is produced and no N\textsubscript{2} is reduced.

1.5 MoFe Protein Cycle

The MoFe protein cycle, otherwise known as the Lowe-Thorneley Scheme, is presently accepted as the substrate reduction scheme, by nitrogenase. The MoFe protein cycle (Figure
1.5.1) consists of 8 Fe protein cycles resulting in 8 electron and 8 protons transfers to ½ of the independently functioning MoFe protein in order to reduce one N₂ to 2NH₃ and H₂. This protein cycle is based on the reaction in eq (1) and illustrates the binding of various substrates to the MoFe protein. Different substrates bind to the MoFe protein at different sites or possibly the same site at a different redox state [16]. Three different cases of substrate binding have been observed and can be classified as competitive and noncompetitive. If more than one substrate binds to the same site on the MoFe protein, they are referred to as competitive, while if they bind at different sites they are referred to as noncompetitive. The case where different substrates bind to the same site but at different redox states are also considered to be classified as noncompetitive.

Substrates competing for different redox states of the MoFe protein are controlled by electron flux. For example, substrates that have the propensity to bind to more reduced levels of the MoFe protein are preferentially reduced under high electron flux. This is observed during N₂ reduction. N₂ binds after 3 or 4 electrons have been transferred to the MoFe protein, equivalent to states E₃ and E₄ in Figure 1.5.1. These states are generated during high electron flux [11, 19-21]. On the other hand, under low electron flux the electrons are diverted to H₂ evolution at states E₂ or E₃. This suggests that N₂ displaces H₂ during high electron flux, and the reduction of N₂ becomes irreversible after it has been protonated once.

### 1.6 Substrates and Inhibitors of the Wild-Type MoFe Protein

Nitrogenase reduces many substrates other than nitrogen (N₂), such as acetylene (C₂H₂), cyanide (HCN), azide (HN₃), carbon disulfide (CS₂), and protons (H⁺). Virtually all substrates of nitrogenase contain a double or triple bond between different combinations of carbon, nitrogen, or oxygen molecules. It is beneficial that nitrogenase reduces more than one substrate, because
Figure 1.5.1: The Lowe-Thorneley MoFe protein cycle. $E_n$ represents one of the two halves of the MoFe protein, where $n$ refers to the number of electrons and protons transferred or the number of Fe protein cycles performed.
the number and nature of active site(s) can be determined through examining nitrogenase during turnover under different conditions.

Nitrogen is the physiological substrate of nitrogenase, and for each nitrogen molecule reduced two ammonia molecules and one hydrogen molecule are produced, as in eq (1). Hydrogen evolution is an intimate part of the mechanism of nitrogen reduction as described the section above. The Lowe-Thorneley scheme suggests that N₂ displaces H₂ on the enzyme prior to its being reduced. Therefore, nitrogen is an inhibitor of H₂ evolution, but is incapable of eliminating it 100%, unlike most of the alternate substrates.

In turn, nitrogen reduction is inhibited by the alternate substrates. Acetylene undergoes a two-electron reduction that yields ethylene (C₂H₄). Ethylene is not reduced further but does inhibit electron flux and MgATP hydrolysis. H₂ is an inhibitor of only nitrogen reduction, while hydrogen evolution is inhibited by all other substrate reductions. From this information, it is possible to begin to assign binding sites. N₂ is suggested to bind to nitrogenase when it is reduced by 3 or 4 electrons (E₃ or E₄) by displacing H₂, but acetylene, a noncompetitive inhibitor of nitrogen reduction, must bind to another site or at a less reduced form of the enzyme (E₀ or E₁), which prevents N₂ from binding to the enzyme. This also explains how C₂H₂ completely inhibits H⁺ reduction, since they compete for the same reduction state of the enzyme.

Cyanide and azide illustrate a different class of substrates than N₂ or C₂H₂, in that they exist in solution as two species. The relative concentration of the two species is dependent on the pH. Azide exists as HN₃ and N₃⁻ with a pKₐ = 4.6. At the pH range normally studied (6.0 - 8.5) the dominant species is N₃⁻. Both species are substrates [22]. HN₃ can be reduced by 6 electrons to form hydrazine (N₂H₄) and ammonia, while N₃⁻ is reduced by 2 electrons to form nitrogen and ammonia. The N₂ produced can be reduced further to ammonia. HN₃ is a
competitive inhibitor of N\textsubscript{2} reduction, including the inhibition of the further reduction of N\textsubscript{2} produced by the reduction of N\textsubscript{3}\textsuperscript{-}. Acetylene inhibits HN\textsubscript{3} reduction while H\textsubscript{2} evolution is inhibited by HN\textsubscript{3} reduction. Thus, HN\textsubscript{3} appears to bind to a less reduced state of nitrogenase, similar to protons and acetylene, preventing N\textsubscript{2} binding [23]. The other substrate, N\textsubscript{3}\textsuperscript{-}, is inhibited by N\textsubscript{2}, and C\textsubscript{2}H\textsubscript{2}, but not by H\textsubscript{2} or HN\textsubscript{3}, suggesting that it binds to multiple redox states of the enzyme.

The enzymology of cyanide reduction is different than that of azide. Cyanide exists in solution as HCN and CN\textsuperscript{-} with a pKa = 9.11, meaning the HCN is the more dominant species at neutral pH. HCN is the substrate while CN\textsuperscript{-} is an inhibitor of total electron flow. HCN can undergo a 6-electron reduction to produce methane and ammonia. A partial reduction is also observed to methylamine [24] (CH\textsubscript{3}NH\textsubscript{2}). In the presence of nitrogen, HCN reduction is unaffected, but H\textsubscript{2} evolution is decreased and electrons are diverted to excess ammonia production via nitrogen reduction. HCN reduction favors low electron flux and can completely inhibit H\textsubscript{2} production. This suggests that HCN is reduced at a level less reduced than that is need for H\textsubscript{2} evolution [25]. CN\textsuperscript{-} inhibits electron flux while the MgATP hydrolysis rate is unchanged, thus increasing the ATP/2 electron ratio. The CN\textsuperscript{-} induced inhibiton is relieved only by the inhibitor carbon monoxide (CO). Although, CO is isoelectronic with N\textsubscript{2}, it only acts as a noncompetitive inhibitor. CO inhibits reduction of all substrates except protons thus diverting all electrons to H\textsubscript{2} evolution.

1.7 Substrates and Inhibitors of the Altered MoFe Protein \(\alpha\)-195Gln

The altered MoFe protein \(\alpha\)-195Gln gives added insight into the number and nature of active sites of nitrogenase. This variant MoFe protein is unique in that N\textsubscript{2} binds but is only 1-2% reduced to NH\textsubscript{3}, while protons and acetylene are reduced similar to that in the wild-type MoFe
protein [26]. CO inhibition of the $\alpha$-195$^{\text{Gln}}$ MoFe protein is also very similar of the wild-type MoFe as described above [18]. Therefore, this variant protein demonstrates how the polypeptide environment surrounding the cofactor plays a role in effective substrate binding and reduction.

Cyanide and azide reduction by the altered MoFe protein are also different. For example, the $\alpha$-195$^{\text{Gln}}$ MoFe protein is unaffected by the inhibitor, CN$^-$, while HCN is reduced to CH$_4$ and NH$_3$ but with an altered product ratio compared to the wild-type protein. The $\alpha$-195$^{\text{Gln}}$ MoFe protein generates only 6-electron products [27], CH$_4$ and NH$_3$, without any CH$_3$NH$_2$. Also, the $\alpha$-195$^{\text{Gln}}$ MoFe protein produces equal amounts of CH$_4$ and NH$_3$, unlike the wild-type MoFe protein, which produces an excess of NH$_3$. In the presence of acetylene $\alpha$-195$^{\text{Gln}}$ MoFe protein exhibits inhibition of HCN reduction. In contrast, the wild-type MoFe protein shows an increase in CH$_4$ production in the presence of C$_2$H$_2$. The increase in CH$_4$ production is not a result of C$_2$H$_2$ reduction, but a result of complete HCN reduction. This suggests that the protein’s affinity for HCN is lowered by the bound C$_2$H$_2$ therefore, inhibiting the production of intermediates and only exhibiting 6 electron products CH$_4$ and NH$_3$. The $\alpha$-195$^{\text{Gln}}$ MoFe protein is capable of reducing azide at 10% of the rate of the wild-type MoFe protein [28, 29]. Further analysis revealed that the reduced rate of azide reduction is a result of electron flux inhibition induced by azide bound to the $\alpha$-195$^{\text{Gln}}$ MoFe protein not exhibited by the wild-type MoFe protein.

Altogether, it can be speculated that the hydrogen bond from the residue 195 to the central sulfide of the FeMo cofactor in $\alpha$-195$^{\text{Gln}}$ MoFe protein (Figure 1.2.6) is sufficient for HCN and H$^+$ reduction, but inadequate for N$_2$ and azide reduction. This suggests that the mechanism for the protonation of N$_2$ may differ from that for the protonation of H$^+$ or HCN, possibly from (1) utilizing a different proton pathway or (2) the reduction site of N$_2$ is situated in
the vicinity of residue 195 while the other substrate reduction sites are situated somewhere else
in the vicinity of the FeMo cofactor.

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CHAPTER 2. EXPERIMENTAL METHODS

2.1 Cell Growth

Azotobacter vinlandii was grown at Virginia Tech, the University of Wisconsin, and the University of California, Irvine. Harvested cells were stored at -80°C until use. All protein manipulations were performed under anaerobic conditions maintained using either a Schlenk line apparatus [1] or an anaerobic glovebox (VAC).

2.2 Wild-Type MoFe Protein Purification

The wild-type MoFe proteins are purified using a combination of chromatography columns. A crude extract of 300g of Azotobacter vinelandii cells is prepared by osmotic shock in an anaerobic glovebox. The cells are suspended in 5 volumes of 4 M glycerol in 0.050 M Tris-HCl buffer at pH=8.0 for 45 min then centrifuged at 10,000 RPM (Sorvall® Model: RC-5B with a GSA rotor) for 15 min. The glycerol is decanted, the pellet is blended by an upright mixer with 5 volumes of 0.050 M Tris-HCl buffer at pH=8.0 containing deoxyribonuclease I and ribonuclease A. After the blended mixture sits for 45 min, the lysate is heated treated to 56°C for 5 min in a water bath. Once the lysate is cooled, it is centrifuged at 13,000 RPM for 90 min, then the crude extract is decantanted and the pellet is discarded. Prior to use, all buffers are degassed under argon, checked for reducibility (2 mM sodium dithionite) by methyl viologen paper, and rechecked for the appropriate pH by pH paper before use unless otherwise stated [2]. Tris buffer, dithionite deoxyribonuclease I, and ribonuclease A are from Sigma Aldrich where glycerol and sodium chloride are from Fisher Scientific.

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9 Dithionite is concentrated Na₂S₂O₄ neutralized in 0.25 M NaOH and added to all buffer solutions to scavenge residual O₂.
10 Methyl viologen paper is prepared by soaking strips of chromatography or filter paper in 1% methyl viologen then drying. A blue color indicates the presence of residual dithionite and is used determining the reduce ability of the solution applied to the paper.
The crude extract is then loaded onto a DEAE column (~500 mL of resin from Sigma Aldrich in a 5.0 cm x 25 cm column), previously washed with 3 column volumes of Buffer A solution. All columns are verified for reduce ability and appropriate pH prior to the loading of the crude extract. Once the crude extract is loaded, 500 mL of Gradient Buffer B is used to wash the column followed by a linear gradient (Buffer B to Buffer C). The gradient roughly separates the components of nitrogenase, which is collected in fractions. The MoFe protein elutes approximately at 0.25 M NaCl and the Fe protein elutes at approximately 0.35 M NaCl. The presence of each component of nitrogenase in the eluant is demonstrated by the activity assays described in Section 2.5.

Table 2.2.1 Solutions for wild type MoFe protein purification

<table>
<thead>
<tr>
<th>Name</th>
<th>Tris Buffer (M)</th>
<th>NaCl (M)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer A</td>
<td>0.025</td>
<td>0.08</td>
<td>7.9</td>
</tr>
<tr>
<td>Gradient Buffer B</td>
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<td>0.10</td>
<td>7.9</td>
</tr>
<tr>
<td>Gradient Buffer C</td>
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<td>0.50</td>
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</tr>
<tr>
<td>Buffer D</td>
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<td>0.08</td>
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<td>Gradient Buffer E</td>
<td>0.025</td>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Gradient Buffer J</td>
<td>0.020</td>
<td>0.60</td>
<td>7.6</td>
</tr>
</tbody>
</table>

The MoFe protein fraction is diluted with 4 volumes of 0.50 M Tris-HCl at pH=8.0 then purified further by a second DEAE column (~100 mL of resin in a 2.5 diameter column). The second DEAE column is prewashed with 4 column volumes of Buffer D. The diluted MoFe protein fraction is loaded onto the column and washed with Gradient Buffetr E (500 mL)

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11 All solutions’ parameters for the wild type purification procedure are described in Table 2.2.1
12 All solution contained 2mM Sodium Dithionite
followed by a linear gradient Buffer E to Buffer F. The MoFe protein elutes approximately at 0.25 M NaCl.

The Fe protein fraction is also purified similar to that of the MoFe protein. Diluted Fe protein is loaded onto a Q-sepharose column (~100 mL of resin from Sigma Aldrich in a 2.5 diameter column), previously washed by 4 column volumes of Buffer G. After loading the protein is washed by 500 mL of Gradient Buffer H followed by a linear gradient (Buffer H to Buffer J). The Fe protein elutes around 0.35 M NaCl. The purification is illustrated in a flow chart Figure 2.2.1.

![Flow chart of the isolation of wild-type MoFe and Fe protein](image)

**Figure 2.2.1** Flow chart of the isolation of wild-type MoFe and Fe protein
2.3 Poly-Histidine-Tagged MoFe Protein Purification

Site-directed mutagenesis and gene-replacement techniques are used to construct MoFe proteins that contain poly-histidine sequences near the N- or C-terminus regions of their $\alpha$ subunits [3]. The poly-histidine MoFe protein and the untagged Fe protein are purified using a combination of chromatography columns similar to the wild-type MoFe protein. A crude extract of 300 g of cells is prepared by osmotic shock in an anaerobic glovebox as describe above for the wild type *Azotobacter vinelandii* cells excluding the heat treatment.

The crude extract is then loaded onto a DEAE column (~500 mL of resin in a 5.0 cm x 25 cm column), previously washed with 3.5 column volumes of Buffer $\alpha$ solution$^{13}$. The column is washed with Gradient Buffer $\beta$ (500 mL) followed by a linear gradient of Buffer $\beta$ to Buffer $\chi$. The poly-histidine-tagged MoFe protein elutes approximately at 0.25 M NaCl and the Fe protein elutes approximately at 0.40 M NaCl. The MoFe and Fe protein are roughly separated by this step and the protein and collected into fractions (three or more). Each fraction is tested for activity as described in Section 2.5.

The MoFe protein fractions are then loaded onto an immobilized metal-affinity chromatography column$^{14}$ (IMAC)(135 mL of resin in a 2.5 cm x 25 cm column). The IMAC (Amersham Pharmacia) is prepped by a series of solutions. The first step is to load 3.5 column volumes of non-reducing 0.40 M ZnSO$_4$ in dH$_2$O at 4 mL/min, then 3 column volumes of Binding Buffer. The IMAC column preparation is verified using methyl viologen paper, a phosphate buffer, and pH paper to confirm that the eluting solution is reducing, contains no Zn$^{2+}$, and the column is at a pH =7.9. The second fraction, from the previous DEAE column is loaded first while collecting the eluant, which contains Fe protein. This ensures maximum yield of the

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$^{13}$ All solutions’ parameters for the poly-histidine tagged MoFe and untagged Fe protein purification procedure are described in Table 2.3.1.

$^{14}$ The IMAC needs to be an iminodiacetic acid based bead instead of a nitilotriacetic acid based bead.
Table 2.3.1 Solutions for the poly-histidine tagged MoFe protein purification and untagged Fe protein

<table>
<thead>
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<th>Name</th>
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<th>NaCl (M)</th>
<th>Imidazole (M)</th>
<th>pH</th>
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</tr>
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<td>0.10</td>
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<td>0.50</td>
<td>0.0</td>
<td>7.9</td>
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<tr>
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<td>0.0</td>
<td>7.9</td>
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</tr>
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<td>0.0</td>
<td>7.6</td>
</tr>
</tbody>
</table>

poly-histidine tagged MoFe protein. This step is repeated for the fraction containing mainly MoFe protein and then the column is washed with 4 column volumes of Washing Buffer at 4.0 mL/min. The poly-histidine tagged MoFe protein, indicated by the single dark brown band on the column, is released by applying the Eluting Buffer to the column at 4.0 mL/min.

To further purify the poly-histidine tagged MoFe protein, it is diluted 7 times with 0.007 M EDTA (Sigma Aldrich) in 0.50 M Tris-HCl at pH = 8.0 and loaded onto a DEAE column (~100 mL of resin in a 2.5 cm diameter column). The DEAE column is pre-washed with 3.5 column volumes Buffer δ. When the column is reducing the poly-histidine MoFe is loaded and washed by Gradient Buffer ε (500 mL) followed by a linear gradient of Buffer ε to Buffer φ. The poly-hisitidine tagged MoFe protein elutes around 0.025 M of NaCl.

The Fe protein fraction collected after the DEAE column is diluted with 0.50 M Tris-HCl at pH=8.0 before loading onto a Q-sepharose column (~100mL of resin in a 2.5 cm diameter column)
Polyhistidine tagged MoFe protein crude extract after lysing procedure

Sediment discarded
Supernant

DEAE cellulose column
(5 cm dia.-400 ml of resin)
Prep with Buffer $\alpha$
then run gradient $\beta$ to $\chi$

Av2 fraction
Av1 fraction

IMAC column
(2.5 cm dia.-135 ml of resin)
Prep with 0.40 M ZnSO$_4$ in dH$_2$O
and Binding Buffer then
load each fraction separately

Av2 does not bind collect
fraction

Dilute fraction 4X with
0.5 M Tris-HCl (pH=8.0)

Q-Sepharose column
(2.5 cm dia.-100 ml of resin)
Prep with Buffer $\gamma$
run gradient $\eta$ to $\phi$

Concentrate fraction with an Amicon YM30
and dilute NaCl concentration

Wash bound Av1 with
Washing Buffer then
release with Eluting Buffer

Dilute fraction with 7 mM EDTA
in 0.5 M Tris-HCl (pH=8.0)

DEAE cellulose column
(2.5 cm dia.-100 ml of resin)
Prep with Buffer $\delta$
run gradient $\varepsilon$ to $\phi$

Concentrate fraction with an Amicon
YM30 and dilute NaCl concentration

Figure 2.3.1 Flow chart of the isolation of poly-His tagged MoFe and untagged Fe protein
column). The Q-sepharose column is pre-washed with 3.5 columns volumes of Buffer $\gamma$. Once the column is reducing the Fe protein is loaded and washed by Gradient $\eta$ (500 mL) and eluted with a linear gradient of Buffer $\eta$ to Buffer $\phi$. The Fe protein elutes in the region of 0.040 M of NaCl [3]. This last purifying step for the Fe protein can be repeated for the Fe protein collected after the IMAC column for a maximum yield of Fe protein. Figure 2.3.1 is a flow chart that illustrates the procedure of the purification of poly-histidine tagged MoFe protein and untagged Fe protein.

If only the poly-histidine tagged MoFe protein is desired the purification procedure is simplified by deleting the first DEAE column. Once the crude extract is prepared, it is loaded directly to a prepped IMAC column. The poly-histidine tagged MoFe protein is released from the IMAC column, diluted and further purified on a prepped DEAE column as described above.

2.4 Protein Concentration and Determination

An ultrafiltration process is used to concentrate each component of nitrogenase. Ultrafiltration is the selective rejection of solutes by solvent flow through an anisotropic membrane. YM30 and YM100 Amicon Diaflo® membranes were selected for the molecular weight cut offs of 30,000 and 100,000 which rejects the nitrogenase proteins and allows water, Tris buffer, and salt to pass through, thus concentrating the protein. Since protein recovery is essential, a hydrophilic membrane is required which is designed for non-specific protein binding properties and resistance to biochemical solvents. Once the volume of the protein solution has decreased in half, dilute the protein solution by one volume of 0.50 M Tris–HCl at pH=7.6 to decrease the salt concentration then reconcentrate the protein to approximately 40 mg/mL or higher.
A concentration determination is carried out on both component of nitrogenase. The colorimetric method used (Bio-Rad Bradford protein assay) [4], involves the binding of the Coomassie brilliant blue G-250 dye to the protein. The absorbance (595 nm) is measured on a Varian spectrophotometer (Model: Cary 219). A 10 µL sample of concentrated protein is diluted to 500 µL with distilled water and vortexed. The solution (10-60 µL) is combined with 600 µL of Bio-Rad (Bradford) protein assay and diluted with distilled water to a final volume of 3 mL, vortexed and allowed to stand for 20 min. The concentration is then determined from the protein standard curve obtained using bovine serum albumin.

### 2.5 Specific Activity Assays

Specific activity is measured in terms of acetylene reduction or H2 evolution [1]. A reaction vial (14.5 mL) is stoppered with a rubber septum and evacuated and flushed with the appropriate gas (1 atm of C2H2 or Argon) by means of a Schlenk line. The flushed vials are filled with 1 mL of degassed regenerating solution consisting of, 38 mM Tes-KOH pH=7.4, 2.5 mM ATP, 5.0 mM MgCl2, 30 mM creatine phosphate, 2 mM dithionite, and 0.125 mg creatine phosphokinase (all from Sigma Aldrich). After preincubation of 2 min in a 30°C water bath all vials are verified for reducing ability by methyl violgen paper. Then the desired amount of Av1 is added giving a protein concentration of ~1mg/mL. Finally, the reaction is initiated by the addition of Av2 in the appropriate molar ratio. The specific activity of each component is determined by titrating one nitrogenase component protein against the other.

Following the initiation of the reaction, the turnover mixture is shaken in a 30°C water bath. After 6 min the reaction is terminated by the addition of 0.1 mL of 30% trichloroacetic acid (Sigma Aldrich). An aliquot (0.3 mL) of headspace gas is transferred by way of a pressure-lock syringe into a gas chromatograph. A Varian (Model 3700) GC fitted with a Porapak N
column (He) (Altech) and a flame ionization detector (FID) is used to analyze the headspace gas of the acetylene reduction assays, and a Shimadzu GC-14 equipped with a 80/100 molecular sieve 5A column (He) and a thermal conductivity detector (TCD) is used to analyze the headspace gas of the hydrogen evolution assays.

A standard curve for each GC is used to calculate the number of nanomoles of gas (ethylene or hydrogen) generated. The hydrogen peak elutes at 0.70 min on the TCD GC while the ethylene and acetylene peaks elute at 0.65 and 0.9 min on the FID GC. All gases are from BOC.

2.6 Protein Storage

The protein is frozen into 50 µL pellets by liquid nitrogen (BOC) and kept in storage dewars at 77 K.

2.7 EPR Sample Preparation

All samples are prepared using a Schlenk line. As-isolated Av1 samples are prepped under argon in reducing Tes-KOH buffer at pH=7.4. For these samples, Av1 is diluted by Tes-KOH buffer to a final concentration of 10-20 mg/mL in the final volume of 350 µL.

Turnover samples using protons (under Ar), N₂ or C₂H₂ as the substrates are prepared by degassing individual vials containing regenerating solution, dithionite, Av1, Av2, Tes-KOH buffer (pH=7.4) under argon. For each sample, an EPR tube, the vials for regenerating solution and the protein components are degassed under the appropriate gas (argon, nitrogen, or 10% acetylene). Degassed protein is stored on ice when not being used. The dithionite is checked for reducing ability by methyl viologen paper and added to the degassed regenerating solution along with the Tes buffer for a final concentration of 0.023 M and re-checked for reducing ability. Syringes are pre-washed before use with reducing Tes-KOH buffer. Appropriate amounts of
regenerating solution and Tes-KOH buffer are put into an incubation vial and Av1 and Av2 into separate vials. All vials are incubated at 30°C in a shaker water bath for 15 min. The incubated regenerating solution/Tes-KOH buffer is injected into an EPR tube and turnover is initiated by adding the incubated protein mixture. Complete mixing is ensured by drawing the reaction mixture out of the EPR tube into the syringe, reinjecting the sample back into the tube, and shaking it down to the bottom of the tube. After the appropriate time interval the sample is frozen in a liquid N2/hexane slurry.

The turnover samples final volume is 350 µL with the final concentration of the regenerating solution at 23 mM Tes-KOH (pH=7.4), 6 mM ATP, 7 mM MgCl2, 25 mM Na2S2O4, 30 mM creatine phosphate, and 0.125g/L creatine kinase. The final concentration of Av1 is 10-20 mg/mL. The final concentration of Av2 depends on the molar ratio of the turnover sample.

Preparation of turnover samples using either NaCN or NaN3 as the substrates begins with degassing individual vials containing one of the following: regenerating solution, dithionite, 3 M HCl, Av1, Av2, the three-buffer mixture at pH= 7.4, 0.2 M stock solution of NaCN or NaN3 in the three-buffer mixture at pH=10.5, incubation vials, or EPR tubes. The three-buffer mixture consists of 0.075 M Bis-Tris 0.038 M HEPPS and 0.038 M CHES buffers. The three-buffer mixture is preferred to Tes buffer due to the wide pH range studied (all from Sigma Aldrich). As described above, the degassed protein is stored on ice when not being used, the dithionite is checked for reducing ability prior to adding it to the regenerating solution, the three-buffer mixtures, and the stock solution of NaCN or NaN3 which are then re-checked for reducibility. The degassed 3 M HCl is added to the stock solution until the desired pH. The pH is read anaerobically by puncturing the rubber septa top with a 5 mm diameter, no glass stainless steel
pH probe (IQ Scientific Instruments, Inc. Model: IQ240). The solution is diluted to 0.1 M with the three-buffer mixture at pH=7.4. The regenerating solution, the three-buffer mixture at pH=7.4, and the pH modified NaCN or NaN₃ are put into an incubation vial while Av1, Av2, and the pH modified NaCN or NaN₃ are put into a separate incubation vial. The vials are incubated in a 30°C water bath for 15 min. The regenerating mixture is injected into a degassed EPR tube followed by the protein mixture to initiate turnover. Mixing is ensured as stated above. After the proper time interval elapsed the sample is frozen in a liquid N₂/hexane slurry.

2.8 MCD Sample Preparation

The ∆nifH, and ∆nifB MoFe proteins were grown and purified at University California, Irvine [3]. The modified MoFe proteins are stored at 77 K until use. Buffers containing 10% glycerol are normally used for the stability of ∆nifH and ∆nifB MoFe proteins. MCD spectroscopic technique requires glass formation, therefore, these proteins were stored in a 50% glycerol solution. As-isolated or resting state samples are defrosted and degassed in a 3.5 mL vial on a Schlenk line. A sample cell is flushed with helium gas, which the degassed protein is injected and immediately frozen by lowering into liquid helium.

Oxidized and reduced ∆nif MoFe proteins were prepared with indigo disulfonate and Ti (III) citrate respectively, at University of California, Irvine [5]. Oxidized ∆nifB samples were prepared with thionine in a anaerobic glovebox. To oxidize this protein, as-isolated ∆nifB MoFe protein is applied to a Sephadex G-25 column equilibrated with degassed, non-reducing 50% glycerol in 0.050 M Bis-Tris at pH=7.1, which removes the reductant dithionite from the protein. Once the dithionite is removed, the ∆nifB MoFe protein is titrated with 0.001 M thionine until the endpoint (i.e. faint blue color) is reached.
2.9 References


CHAPTER 3. EXPERIMENTAL TECHNIQUES

3.1 Introduction to EPR

Electron Paramagnetic Resonance (EPR) spectroscopy studies molecules and ions containing unpaired electrons. The technique detects paramagnetic substances by their resonant absorption of microwave radiation in an external magnetic field. EPR studies provide structural or environmental information, electron distribution, and geometry of paramagnetic molecules.

Metalloproteins are frequently investigated by EPR because these proteins often contain free radicals, transition metal ions, or metal clusters that are involved in enzymatic catalysis and/or electron transfer. EPR is also a practical method for studying metalloproteins. It can characterize structure of the paramagnetic cofactor in the resting state as well as intermediates states and product complexes. Furthermore, quantification of the EPR signal intensity can be used to determine the number of EPR active centers present. Finally, EPR spectra of metalloproteins can illustrate the spin state as well as magnetic coupling between two or more paramagnetic centers, which may indicate the existence of metal clusters or an electron transfer chain.

3.2 Basic EPR Instrumentation

An EPR spectrometer (Bruker: Model 300) used in this study requires an external magnetic field, a source of radiation and detection system of the absorption by the sample. Figure 3.2.1 is a picture of many of the necessary components of an EPR spectrometer the waveguide that guides the irradiating microwave light from the klystron to the detector, the electromagnet, the sampler holder and cavity, and the vacuum line and cryo port, which are essential to work at low temperatures. An electromagnet produces the external magnetic field (0-8000 Gauss) while monochromatic microwave radiation is generated by a klystron. In
Figure 3.2.1: Picture of electromagnet and sample holder of the Bruker EPR spectrometer
general, the microwave tuning is very sensitive. The output frequency of a klystron can only be varied 5-10% above or below its centered frequency to produce a linear response. On the other hand, it is possible to linearly vary the external magnetic field from zero to several times the resonant magnetic field strength linearly. The most common frequency used for studies is around 9.5 GHz, referred to as X-band. Since it is easier to scan the magnetic field than the frequency, the klystron is tuned to the sample cavity and held at a constant frequency while the magnetic field is scanned linearly through the resonant condition. Furthermore, modulation of magnetic field enhances sensitivity and resolution resulting in the detection of a first-derivative spectrum. This is important since first derivative spectra are narrower and more resolved than absorption spectra, which often accentuates structure [1].

### 3.3 EPR Theory (S = ½)

Electrons possess a permanent magnetic moment termed the electron spin. The spin of an unpaired electron can sense the immediate environment. This capability allows EPR spectroscopy to provide information about biological molecules containing free radicals or metal ions.

The classical expression that describes the energy an electron experiences when it comes into contact with an external magnetic field ($\mathbf{H}$) is shown in equation (1) [2]

$$ E = -\mu \cdot \mathbf{H} $$

(1)

where $\mu$ is the vector quantity magnetic moment of an electron and the resultant of the dot product is in the direction of the external magnetic field or otherwise referred to as the z-axis (letters in bold represent a vector). The magnetic moment is defined as

$$ \hat{\mu} = -g\beta \hat{S} $$

(2)
where $\beta$ is the Bohr magneton constant, $g$ is the electronic splitting factor, and $\hat{S}$ is the total spin operator of the electron. The negative sign is necessary to cancel the negative charge of the electron. The Bohr magneton constant, $\beta$, is the relationship between an orbiting charged particle relating the magnetic moment of a classical particle and the quantum mechanical angular momentum. The electronic splitting factor, $g$, represents the relationship between the electron’s magnetic moment, its orbital motion about the nucleus, and its intrinsic magnetic moment about its own axis. For a free electron $g = 2.0023$. Replacing eq. 2 into eq.1 produces the Hamiltonian or the quantum mechanical expression

$$\hat{H} = g\beta \hat{S} \bullet \mathbf{H}$$

and is simplified to

$$\hat{H} = g\beta \hat{S}_z \mathbf{H}$$

by displaying the projection of $\mathbf{S}$ onto $\mathbf{H}$, the z-axis, and replacing the dot product with the quantum operator, $\hat{S}_z$, multiplied by $H$. [2].

For an $S = \frac{1}{2}$ system, $\hat{S}_z$ has eigenvalues $m_s = \pm \frac{1}{2}$, therefore, at the resonance condition, a transition is induced when the photon energy, $\hbar \nu$, equals the energy split due to the Zeeman effect defined as

$$\Delta E = E(m_s = \frac{1}{2}) - E(m_s = -\frac{1}{2}) = \hbar \nu = g\beta \mathbf{H}$$

The Zeeman effect (or the Zeeman interaction) is described as the splitting of degenerate energy levels of an electron in a magnetic field. A simplified example of the Zeeman interaction for a free electron is illustrated in Figure 3.3.1, where $S = \frac{1}{2}$ and $m_s = \pm \frac{1}{2}$.

As mentioned above, the presence of both photon energy and a magnetic field are necessary to induce a transition. In spite of this, all transitions are not observable by EPR.
spectroscopy due to thermal energy or spin-lattice relaxation time. Thermal energy dictates the population of each spin orientation in a magnetic field. The relative population of each orientation can be calculated by the Boltzmann distribution formula, eq. 6 is the ratio of populations of the spin up and spin down orientations

$$\frac{N^+}{N^-} = \exp\left(-\frac{\Delta E}{kT}\right)$$

(6)

This means that the population difference between the $m_s = \frac{1}{2}$ and $m = -\frac{1}{2}$ levels increases as the temperature decreases. Thus, as the sample temperature decreases the intensity of the EPR signal increases.

![Zeeman levels when S = ½](image)

**Figure 3.3.1:** Zeeman levels when S = ½

This trend is counteracted by the spin-lattice relaxation time, which also increases as the temperature decreases. In general, after an electron is in its excited state, it loses energy to its
surroundings and returns to its lower energy orientation. The average time for the deactivation is called the spin-lattice relaxation time. The spin-lattice relaxation time is based on the Heisenberg uncertainty principle, thus, if the relaxation time is small the change in energy is large leading to line broadening and lack of signal. This is due to the mean lifetime of the spin state and for transition metals this is usually small. Therefore, decreasing the temperature of the sample increases the spin-lattice relaxation time resulting in an observable EPR signal.

In the case of a free electron, the g-factor is isotropic with a value of 2.0023. However, when an unpaired electron is in an atom or a molecule it is affected by electrons from other orbitals and spin-orbit interactions must be taken into consideration. These interactions induce a deviation of the g-factor from the free electron value. Furthermore, the g-factor becomes anisotropic or direction dependent, dramatically changing the EPR spectrum, indicating structural and environmental interactions.

The deviation from $g = 2.0023$ is best illustrated by the example of a crystal. To observe a complete EPR spectrum of a single crystal, the crystal is rotated so that the external magnetic field is parallel to each axis, x, y, and z of the crystal. If all of the g-factors observed in each orientation are equal the environment of the paramagnet is symmetrical or isotropic, but if one or more of the g-factors differ from the other the paramagnet is considered anisotropic. In the case when a single crystal is unavailable a powder sample can be examined. In a powder sample the molecules are randomly oriented and all orientations are observed in one spectrum. This is comparable to the addition of the spectra collected in each direction of a single crystal. For the mechanistic nitrogenase protein studies described later neither a single crystal nor a powder is available and, therefore, a frozen solution is examined. However, a frozen solution is the same as a powder sample, since the molecules are frozen in random orientations.
Spectroscopic g-factors are classified into four groups. As previously stated, an isotropic g-factor corresponds to an environment of the unpaired electron, which is symmetrical (Figure 3.3.2(a)). On the other hand, there are three descriptive patterns associated with anisotropic g-factors. Two patterns describe axial symmetry, where two axes have the same g-factor ($g_x = g_y = g_\perp$) and a different third g-factor ($g_z = g_\parallel$). The two axial cases are subdivided by $g_\parallel > g_\perp$ and $g_\parallel < g_\perp$ which one can be visualized as a discus or a football, respectively (Figure 3.3.2(b) & (c)). The final anisotropic classification is rhombic, where all three g-factors are unique (Figure 3.3.2(d)).

Additional structural information can often be observed from an EPR spectrum. For example, hyperfine interactions give environmental information through the interaction of the unpaired electron with the nuclear spin of a neighboring nucleus. The nuclear magnetic moment, similar to an electron magnetic moment, is defined by

$$\hat{\mathbf{I}} = g_n \beta_n \hat{\mathbf{I}}$$

where $\beta_n$ is the nuclear Bohr magneton constant, $g_n$ is the nuclear g-factor (which is a characteristic of the nucleus) and $\mathbf{I}$ is the nuclear spin. The value of $\beta_n$ is approximately 2000 times smaller than the Bohr magneton constant for an electron due mainly to the differences in the masses of the nucleus and the electron. The result of this difference is a smaller relative energy of the nuclear orientations.

The presence of nuclear hyperfine interaction modifies the energy of the electron by

$$E = g\beta m_s H + hA m_1 m,$$

where $m_s$ is the spin state of the electron, $m_1$ is the spin state of the nucleus, and $A$ is the hyperfine coupling constant. Hyperfine interactions obviously change the energy requirement of the resonant condition. This occurs because the nuclear spin orientations split the original
Zeeman energy levels of the spin. In general, the nuclear spin will cause the signal to split into \((2I+1)\) lines. Figure 3.3.3 illustrates the hyperfine interaction of a hydrogen atom where \(S = \frac{1}{2}\) and \(I = \frac{1}{2}\).

### 3.4 EPR Theory of \(S > \frac{1}{2}\)

Multiple unpaired electrons induce a zero-field splitting (zfs). The zfs is the energy separation of various \(m_s\) states in the absence of an external magnetic field, a result of interelectronic interactions. The Hamiltonian in equation (9) represents the energy of the spin

---

\[ 
\frac{1}{2} \hbar A + \frac{1}{2} + \frac{1}{2} \\
\frac{1}{2} g \beta H \\
\frac{1}{2} \hbar A + \frac{1}{2} - \frac{1}{2} \\
\frac{1}{2} g \beta H \\
-\frac{1}{2} \hbar A + \frac{1}{2} + \frac{1}{2} \\
\frac{1}{2} g \beta H \\
-\frac{1}{2} \hbar A + \frac{1}{2} - \frac{1}{2} \\
\frac{1}{2} g \beta H \\
-\frac{1}{2} \hbar A + \frac{1}{2} + \frac{1}{2} \\
\]

**Figure 3.3.3:** The hyperfine interaction of a hydrogen atom where \( S = \frac{1}{2} \) and \( I = \frac{1}{2} \)

states when more than one electron is present. The first term in the expression is the Zeeman interaction as discussed in Section 3.3. The second term is the expression of the spin-spin interaction \( (S \geq 1) \), which results in a zfs,

\[
\hat{H} = g \beta \hat{S}_z H + D \left[ \hat{S}_x^2 - \frac{1}{3} \hat{S}_z^2 + \frac{E}{D} \left( \hat{S}_x^2 - \hat{S}_y^2 \right) \right]
\]

where \( D \) is the axial zfs parameter and \( E/D \) quantifies the degree of rhombic distortion of the electronic environment \( (0 \leq E/D \leq \frac{1}{3}) \). For a transition metal, the zfs is viewed as a correction to the energy of the individual spin states arising from spin-orbit coupling.
3.4.1 Kramers Spin Systems

Half-integer systems are called Kramers spins systems. In these systems, the D parameter is a quantity that characterizes the magnitude and direction of the axial distortion. For example, in a system $S = \frac{5}{2}$ three cases are presented with different values of D while $E = 0$. The first condition is when $D = 0$, there is no zfs and all of the spins diverge linearly with magnetic field (Figure 3.4.1(a)). Energy separations between the $\Delta m_s = \pm 1$ pairs are equal and resonate at the same external magnetic field resulting in a single EPR line. In the second case, the $\pm m_s$ pairs are split in the absence of the external magnetic field, but less than the microwave radiation ($0 < D < h\nu$; Figure 3.4.1(b)) again allowing transitions to occur between each $\Delta m_s = \pm 1$ pairs, but at a different magnetic field to yield an EPR line containing 5 lines for one transition. The third case occurs when $D >> h\nu$. Transition metal ions and metal ion clusters usually fall in this category. Here the energy separation between each $m_s$ pair is greater than the microwave radiation and, thus, transitions cannot be induced between spin states (Figure 3.4.1(c)). Consequently, the only transitions observed are between pairs of different $|m_s| \pm m_s$ levels. In this situation, the sign of D, positive or negative, determines whether $m_s = \pm \frac{1}{2}$ or $m_s = \pm \frac{5}{2}$ is the ground state.

The E parameter characterizes off-axis asymmetry called rhombic distortion. This parameter allows a mixing of states such that the EPR signal can no longer be described by the $m_s$ states alone [2]. In general, when $D >> h\nu$, the g-factor of an EPR spectrum depend both on D and E/D. To aid in the predictions of g-factors, a rhombogram is used which is a graphical representation of g-factors as a function of E/D. Rhombograms illustrate the extent of mixing between the $m_s$ states as a function of the rhombicity, E/D. Figure 3.4.2 is an example of a rhombogram of a $S = \frac{3}{2}$ system. As the value of E/D increases from 0 to $\frac{1}{3}$ the mixing between...
Figure 3.4.1: The affects of three different values of the D parameter on the energy levels of a $S = \frac{5}{2}$ spin system
Figure 3.4.2: Rhombogram for $S = \frac{3}{2}$ EPR signal
states increases making it possible to observe EPR signals in excited $m_s$ states previously EPR silent.

### 3.4.2 Non-Kramers Spin Systems

Integer spins, or non-Kramers spin systems, occur when there is an even number of unpaired electrons present. These systems often cannot be detected by conventional EPR spectrometers. Integer spins have a zfs between each $\pm m_s$ level as well as, a zfs separating the $\pm m_s$ pairs when $E > 0$. Figure 3.4.3 illustrates the zfs for a $S = 2$ system. When $E/D = 0$ the $m_s$ pairs are degenerate, but the $\Delta m_s > \pm 1$, prohibiting observation of a signal. A transition can be observed when the external magnetic field is large enough to reduce the energy gap between $m_s = 0$ and $m_s = \pm 1$. However, this is beyond the capabilities of most EPR magnets, thus, making $S = 2$ system EPR silent.

![Energy levels of an integer spin system](image)

**Figure 3.4.3:** Energy levels of an integer spin system$^{17}$ ($S = 2$) with $D > 0$, $H = 0$

---

It is possible to see some integer spin states when the EPR spectrometer is reconfigured; changing the orientation of the plane the microwave magnetic field oscillates does this. In a conventional EPR spectrometer, the microwave magnetic field oscillates perpendicular to the external magnetic field. By switching the microwave magnetic field to oscillate parallel to the external magnetic field, the selection rules change from $\Delta m_s = \pm 1$ to $\Delta m_s = 0$ making previously EPR-silent signal observable. An example will be discussed later for the P$^{+2}$ cluster of the nitrogenase protein.

3.5 Introduction to MCD

Magnetic Circular Dichroism (MCD) spectroscopy is an experimental technique that investigates the geometric and electronic structures of transition metal complexes. MCD is the measurement of the differential absorption of left and right circularly polarized light induced in a magnetic field parallel to the propagation of light.

MCD spectroscopy provides analogous information to EPR spectroscopy in which electronic ground states of paramagnetic centers are studied, but MCD provides additional information. MCD spectroscopy simultaneously observes ground and excited electronic states for paramagnetic as well as non-paramagnetic centers. MCD is also site selective, where a system containing multiple chromophores, each chromophore generates a distinct absorption band making it possible to study the individual centers. Together the complementary information of MCD and EPR spectroscopy can create a more detailed picture of the electronic structure of a bioinorganic system consisting of multiple chromophores and multiple spin states making it an exceptionally useful technique to study nitrogenase.
3.6 Basic MCD Instrumentation

A MCD spectropolarimeter consists of a Circular Dichroism (CD) instrument and a superconducting magnet. The CD (JASCO: Model J-710) produces alternating right and left circularly polarized light through a photoelastic modulator (PEM), the superconducting magnet (Oxford: Model Spectromag 400-7T) induces the magnetic field (0-7 T) parallel to the propagation of the light, and the photomultiplier tube (PMT) is the detector (Figure 3.6.1).

**Figure 3.6.1**: Picture of MCD spectropolarimeter
Sample temperatures are determined with a thin film resistance temperature sensor (Lakeshore: Cernox: Model CX1050-Cu-1-4L). The temperature sensor was attached to the sample holder directly above the cuvette and has a temperature range of 1.4 - 325 K. This temperature sensor was added to increase accuracy of the temperature readings. The original temperature sensor supplied with the Oxford magnet is placed far above the sample (~ 2 cm) and reads a different temperature than the temperature of the sample due to a temperature gradient created by the simultaneous flooding of the sample cell with liquid helium and pumping of the vacuum, which is the procedure required to reach the appropriate temperature of the experiment. The additional temperature sensor was also chosen for the increase of capabilities: low magnetic field induced errors, fast thermal response time, and high sensitivity at low temperatures. To further assess the accuracy of the MCD a Hall generator (Lakeshore: Model HGCA-3020) is placed outside the superconducting magnetic to confirm the linearity of the magnetic field.

Sample cells were constructed of optical quality quartz (Buck Scientific: Model BS-1-Q-1 or Spectrocell: Model SUV R-2001). Each cuvette is cut to the appropriate dimensions to fit inside the sample holder (1.8 cm x 0.45 cm x 1mm), resulting in a cuvette with a sample volume of ~160 ul. The cuvette is sealed on three sides allowing them to be flushed with helium gas and top loaded with the sample while the cuvette is secure in the sample holder. Figure 3.6.2 is a picture of the sample holder and the injection port where the cuvette is flushed with gas and top loaded with the sample.

During low-temperature studies, the sample must form an optical glass upon freezing to permit the recording of an absorption spectrum. The formation of glass for aqueous solutions is aided by the addition of 50 % (v/v) glycerol or ethylene glycol. Glasses formed at low-temperature tend to depolarize circularly polarized light, producing a glass-induced CD. The
a. Sample holder

![Image of sample holder with label and scale]

b. Injection Port

![Image of injection port with label and scale]

Figure 3.6.2: Picture of the sample holder and injection port
problem is eliminated in MCD data by subtracting the right circularly polarized spectra from the left circularly polarized spectra, which removes the naturally occurring CD. However, it is still necessary to run a background CD after the sample is frozen due to the occasion of a strain-induced signal when the glass is formed. When this happens, it is essential to remove the sample and defrost it anaerobically in a glovebox followed by the preparation of a new cuvette before reloading the sample.

3.7 MCD Theory

MCD is based on the Faraday effect, which describes how matter, in the presence of a magnetic field parallel to the direction of propagation of light, appears optically active [3]. The Faraday effect operates on the Zeeman interactions described in the prior Section 3.3 where an applied magnetic field induces the splitting of electronic states previously degenerate in zero field.

\[
\begin{align*}
J & \quad M_J \\
1 & \quad +1 \\
& \quad 0 \\
& \quad -1 \\
0 & \quad 0 \\
H = 0 & \quad H \neq 0
\end{align*}
\]

**Figure 3.7.1:** Selection rules for left and right circularly polarized light
The selection rules of MCD govern the total angular momentum, $J$. $M_J$ is the quantum number that describes the $z$-component of total angular momentum in the direction of the magnetic field. A $(2J+1)$-fold degeneracy exists in the absence of a magnetic field, but the degeneracy is lifted in the presence of a magnetic field by Zeeman interactions allowing $\Delta M_J = \pm 1$ transition to occur. Figure 3.7.1 illustrates that left and right circularly polarized light correspond to an electric dipole photon having $J = 1$ and $M_J = -1$ and $+1$ where the absorption of these photons cause the system to change $+1$ for left circularly polarized light and $-1$ for right circularly polarized light. MCD transitions, unlike EPR, are spin forbidden ($\Delta m_s = 0$); MCD observes electronic transitions not magnetic transitions.

### 3.8 Components of MCD Spectra

MCD is a multidimensional spectroscopy. It has many experimental variables that play a crucial role in characterizing the chromophore being examined. Each variable has a wide range: temperature (1.5 K to ambient temperature), magnetic field (0-7 Tesla), and wavelength of the incident radiation (200-800 nm). Two types of spectra are collected through MCD one in which the intensity is dependent on wavelength and the second in which intensity is dependent on magnetic field. In addition, each of these spectra can also be collected at various temperatures to give more information on the transitions.

The MCD absorption $\Delta A = A_{LCP} - A_{RCP}$ for a transition from a ground state to an excited state is defined by

$$\Delta A = \gamma \beta H \left[ - \frac{\partial f(E)}{\partial E} \right] A + \left( B + \frac{C}{kT} \right) f(E) \right] \Delta \epsilon$$ (10)
where $\gamma$ is the spectroscopic constant, $\beta$ is the Bohr magneton constant, $H$ is the magnetic field, $k$ is the Boltzmann constant, $f(E)$ is the line shape function (Gaussian), $E = h\nu$ the energy of the incident radiation, $d$ is the path length, $c$ is the concentration of the molecular species [4].

$$M_J$$

$$M_J = +1, 0, -1$$

**Figure 3.8.1**: Example of the origin of the A-term for the atomic transition ($^1S\rightarrow^1P$)

Three additive contributions to MCD spectra are the A, B, and C-terms. The A-term arises when excited states are degenerate. A simple example is given by the atomic transition ($^1S\rightarrow^1P$), where the excited states are degenerate, illustrated in Figure 3.8.1. In the presence of a magnetic field, $H$, the excited states experience Zeemen splitting where the energy levels are $M_J= +1, 0, -1$. The transitions corresponding to left and right circularly polarized light will be
equal in intensity, but vary in frequency resulting in a derivative shaped absorption band that is temperature independent.

The C-term unlike the A-term requires ground state degeneracy. The atomic transition ($^1P \rightarrow ^1S$) is the example given to demonstrate a transition responsible for the C-term to arise (Figure 3.8.2). The ground state experiences Zeeman interaction where Boltzmann distribution has an affect on the population of the ground states. This changes the intensity of the left and right circularly polarized light according to temperature ultimately leading to a temperature dependent C-term.

\[
\begin{align*}
M_J & \\
0 & \\
+1 & \\
0 & \\
-1 & \\
\end{align*}
\]

\[
\begin{align*}
H = 0 & \\
H \neq 0 & \\
\end{align*}
\]

**Figure 3.8.2:** Example of the origin of the C-term for the atomic transition ($^1P \rightarrow ^1S$)
The B-term contribution to the MCD spectra does not require degeneracy; it is the result of the mixing of electronic states. The B-term is induced by ground and excited states mixing with all other relatively close excited states. Thus, all molecules exhibit this effect, accounting for the universality of the Faraday effect. The B-term is temperature independent.

3.9 Magnetization Saturation Curves

Magnetization curves monitor the saturation properties of discrete MCD bands as a function of increasing or decreasing of magnetic field or temperature. This adds information to resolving and assigning electronic transitions such as estimations of the ground state spin state, g-factors, and zero field parameters (zfs).

Magnetization curves are dominated by the temperature dependent C-term of a paramagnetic transition. As previously stated, the C-term is temperature dependent due to the Boltzmann distribution population of the Zeeman splittings of the ground state, thus the C-term is linearly dependent on H/T which is commonly referred to as the Curie law. Although this is only true when the Zeeman splitting << kT, H can increase and/or T can decrease until the C-term becomes independent of H/T meaning the lowest Zeeman splitting becomes saturated by the magnetic field, this is illustrated in Figure 3.9.1.

The magnitude of the magnetic saturation curve is the normalized magnetization vs. $\beta B/2kT$ where the MCD intensity is normalized relative to the intensity at saturation. The magnitude of the low-temperature magnetic saturation curve is governed by g-factors of the ground state. These values can be extracted for isolated doublets by extrapolating the initial slope of the saturation curve to the MCD intensity at saturation where the intercept, $I$ on the x-axis is dependent on the g-factor. For an isotropic doublet system $I = 1/g$ and for a complete axial doublet $I = \frac{3}{2}g_{\parallel}$ if $g_{\perp} = 0$. The value of the g-factor increases with the initial slope of the
Figure 3.9.1: Temperature dependence of saturation magnetization curves of MoFe as-isolated ($S = \frac{3}{2}$)

Magnetization curve. Figure 3.9.2 illustrates a series of theoretical magnetic curves with the corresponding x-intercept values. The g-factors determined from MCD are not as accurate as determined from EPR but are used to assign EPR signals to a specific electronic transition.

Magnetization curves of spin system $S > \frac{1}{2}$ are identified by the nesting of curves obtained at different temperatures, large deviations from theoretical data for $S = \frac{1}{2}$ with $g = 2$ a change in the initial slope occurs as temperature increases. These systems ($S > \frac{1}{2}$) have zero field splitting (zfs) discussed previously in Section 3.4, which is the absence of ground state degeneracy in the absence of a magnetic field.
In a Kramers spin system ($S > \frac{1}{2}$) the ground state zfs results in $(2S+1)/2$ doublets. If the zfs of the doublets is much greater than the Zeeman interaction then each doublet can be treated as a separate $S = \frac{1}{2}$ system. If this occurs the lowest doublet will be significantly populated at helium temperatures allowing the estimation of the spin state and effective g-factors of the lowest lying ground state doublet through the intercept procedure described above.

A non-Kramers spin system ($S > \frac{1}{2}$) becomes more complex. In an axial system ($D \neq 0$, $E=0$) zfs is defined by $M_s = \pm S$, $\pm(S-1),...,0$ and the lowest lying doublet is determined by the

---

Figure 3.9.2: Theoretical magnetization saturation curves\(^\text{18}\) $S = \frac{1}{2}$ and $g = 2.0$

---

sign of $D$. The value of $D$ is assumed to be much larger than the Zeeman splitting as stated previously, then each doublet is considered as a separate system. In a system when $D < 0$ the lowest lying doublet is $M_s = \pm S$ and the ground state is populated at helium temperatures and the spin state and effective g-factor can be determined through the intercept method. In a system when $D > 0$ the lowest lying doublet is $M_s = 0$ which is non-degenerate, thus only when the thermal energy is large enough to overcome the zfs and populate higher lying doublets where anomalous temperature dependence are observed. At low temperature it is also possible to see a normal saturation which is due to a temperature dependent B-term. B-terms may become a major contributor, like the C-term, when field-induced mixing occurs in closely spaced zfs components.

### 3.10 MCD Saturation Magnetic Curve Analysis Program

The analysis program written by Neese and Solomon [5] can calculate saturation magnetization curves using experimental data as a basis set. The program’s model is valid for any ground spin states, as well as, the entire range of zfs and Zeeman interactions.

Experimental data are analyzed by fitting the Spin Hamiltonian parameters and the effective transition moment products $M_{xy}^{\text{eff}}, M_{xz}^{\text{eff}}, M_{yz}^{\text{eff}}$, with a scaling parameter $A_{\text{satlim}} = \gamma/4\pi S$. The spin Hamiltonian parameters $g, D, E/D$ are based on

$$\hat{H} = \beta \mathbf{H} g \hat{S} + D \left[ S_z^2 - \frac{1}{3} \hat{S}(\hat{S} + 1) + \frac{E}{D} \left( S_x^2 - S_y^2 \right) \right]$$

is described beforehand in Sec 3.4 as the expression for energy of the Zeeman interaction and the correction to the energy of the individual spin states arising from spin-orbit coupling. The effective transition moment products are the plane of polarization that reflect the anisotropy of
the g-factors. Since the initial slope of the magnetization curve is proportional to the g-factors, the transition polarizations relate the transition dipole to the g-factor axes of a powder or randomly oriented sample. Figure 3.10.1 illustrates the dependence of the magnetization curves on of the transition moment products of the as-isolated MoFe protein \( (S = \frac{3}{2}) \) as discussed later.

![Figure 3.10.1: Polarization effect on fitting experimental magnetization saturation curves of as-isolated MoFe protein \((S = \frac{3}{2})\)](image)

The fitting model is valid for the entire range of zfs, large, small, and intermediate. For all studies performed here it is assumed that the zfs parameter, \( D \), is much larger than the
Zeeman splittings, thus it is possible to treat the degenerate systems as separate $S = \frac{1}{2}$ systems.

As previously discussed, at low temperature (~1.6 K) studies only the lowest lying levels are populated determining the behavior of the magnetization curve.

### 3.11 References


CHAPTER 4. TURNOVER INDUCED EPR SIGNALS

4.1 Introduction to Turnover Induced EPR Signals

Nitrogenase reduces many substrates such as nitrogen, acetylene, cyanide, azide, carbon disulfide and protons. In this chapter, EPR spectroscopy will be used to probe the enzyme during turnover in order to observe intermediate states generated by different substrates. This is important because little is known about substrate binding and reduction. Several intermediate states have been observed in the past and have given insight to changes at the FeMo cofactor during turnover. To further probe substrate binding altered MoFe proteins will be employed to probe the roles of various amino acids during turnover.

The first turnover induced EPR signals were observed with the wild-type MoFe protein during turnover in the presence of the inhibitor, CO. CO generates two different $S = \frac{1}{2}$ EPR signals dependent on the pressure of CO termed hi-CO (0.5 atm) and lo- CO (0.08 atm). The CO signals appear only during turnover, and their appearances coincide with the bleaching of the $S = \frac{3}{2}$ FeMo cofactor resting state signal. Further studies [1, 2] determined that lo-CO is a result of one CO molecule bridged between two Fe atoms of the FeMo cofactor while the hi-CO is a result of two CO molecules terminally bound to two different Fe atoms of the FeMo cofactor. Although, hi and lo-CO signals require turnover conditions, the interconversion between the two signals do not require turnover conditions [3]. This is demonstrated by quenching hi-CO samples with ethylene glycol with the subsequent removal of CO from the gas phase. This led to the conversion of the hi-CO signal into the lo-CO signal. The presence of the lo-CO signal sustained after the complete removal of CO gas, while readdition of CO gas led to the return of the hi-CO signal. The interconversion of hi and lo-CO signals in the absence of turnover indicate that the hi and lo-CO signals are a result of the same structural or redox state of the FeMo cofactor.
Studies at low electron flux (Av2:Av1 = 1:100) revealed a two-minute lag in the appearance of the hi-CO signal with no detection of the lo-CO signal [3], demonstrating that lo-CO is not an intermediate step to hi-CO. At moderate to high electron flux (Av2:Av1 = 1:3 to 1:1) both signals are observed under the appropriate CO pressure. However, two new signals (g = 1.95, 1.81 and g = 5.78, 5.15) were detected under hi-CO conditions. The EPR signal (g = 1.95, 1.81) was determined to be the previously observed P⁺ signal of the oxidized P-cluster [4] while the other EPR signal (g = 5.78, 5.15) termed hi(5)-CO is not associated with any known oxidation state of the P-cluster or [4Fe-4S] cluster of the Fe protein. Therefore, the origin of hi(5)-CO was speculated to be associated to the FeMo cofactor.

Turnover dependent EPR signals have also been observed with the wild-type MoFe protein is in the presence of the substrate [5] CS₂. The CS₂ signal is (g = 2.21, 1.99, 1.97). The signal was not detected when CS₂ was replaced with H₂S, a product of CS₂ reduction. Therefore, the signal is a result of an intermediate state and not a product of the reduction of CS₂. Further studies [6], demonstrated two additional EPR signals resulting from the reduction of CS₂. These signals formed sequentially suggesting three distinct intermediates, where each intermediate was determined to be a result of a carbon-containing fragment of CS₂ bound to the FeMo cofactor.

Variant proteins have also exhibited turnover dependent EPR signals. The study of these proteins allows the investigation of the roles different amino acids play during turnover. One example is α-195Gln MoFe protein, which is unique in that N₂ binds but is only reduced 1-2% to NH₃ while proton and C₂H₂ reduction is unaffected. The structure of α-195Gln MoFe protein is discussed in Section 1.2. During turnover under C₂H₂ two new EPR signals were observed with α-195Gln MoFe protein, SEPR1 (g = 2.12, 1.98, 1.95) and SEPR2 (g= 2.00) [7]. SEPR1 has been shown to arise from to the cofactor with either C₂H₂ bound bridging between two Fe atoms or
two terminaly bound C$_2$H$_2$ molecules to two different Fe atoms of the cofactor [8]. The $S_{\text{EPR2}}$ signal was originally assigned to a radical, but recently it has been shown to be associated with an FeS cluster$^{19}$. 

The $\alpha$-70$^{\text{Ala}}$ MoFe protein is another example of variant proteins demonstrating substrate dependent EPR signals. The $\alpha$-70 amino acid is located near one of the three [4Fe-4S] faces in the first shell of amino acids surrounding the FeMo cofactor. The substitution of $\alpha$-70 valine to alanine demonstrates that amino acids near the cofactor affect enzyme activity. The $\alpha$-70$^{\text{Ala}}$ MoFe protein expands the substrate range by allowing the reduction of larger alkynes [9]. For example, the $\alpha$-70$^{\text{Ala}}$ MoFe protein is capable of reducing propargyl alcohol (HC≡CCH$_2$OH) while the wild-type MoFe protein cannot. During turnover with propargyl alcohol the $\alpha$-70$^{\text{Ala}}$ MoFe protein exhibits an $S = 1/2$ EPR signal ($g = 2.123, 1.998, 1.986$). As with the other substrate induced signals, this signal appears as the $S = 3/2$ resting state signal diminishes. The $S = 1/2$ signal is unaffected by electron flux, but disappears as the substrate is removed. It was determined that the $S = 1/2$ signal is a result of the alcohol binding directly to the FeMo cofactor. Therefore, the substitution of the $\alpha$-70 amino acid with an amino acid with smaller side chains (alanine) than the original amino acid (valine) is capable of reducing larger alkynes, which suggests that side chains near the cofactor control reactivity by restricting access to the cofactor.

Turnover signals also have been identified at low magnetic field ($g = 5$ region). Two $S = 3/2$ signals were observed with the wild-type MoFe protein during turnover under argon, nitrogen, or acetylene ($g(b) = 4.21, 3.79$ and $g(c) = 4.69$) and termed 1b and 1c [10]. Since changing the substrate did not affect the formation of the signals, the signals are assumed to be independent of substrate. Electron flux studies suggest that these signals arise from reduced states of the MoFe

$^{19}$ Hales, unpublished results
protein. To determine which reduced state of the MoFe protein that induces signal 1b and 1c, a steady state of the E₀ and E₁ electronic states was established under low electron flux (1:100). The signals 1b and 1c were not detected. However, increasing the electron flux produced signals 1b and 1c suggesting that the signals must be associated with a more reduced form of the MoFe protein than either E₀ or E₁. Kinetic simulations suggested that 1b is associated with E₃, which should be EPR silent. If signal 1b is a representation of E₃ then all three electrons are not located on the FeMo cofactor but result from two electrons at the FeMo cofactor and the third in a location or a spin state not detectable. Because signal 1c is formed after 1b, it is likely that it represents a state even more reduced than E₃.

The wild-type MoFe protein does not bind any substrates unless it is undergoing turnover. However, a recently constructed variant α-96Leu MoFe protein has been shown to bind substrates without turnover conditions [11]. The α-96Leu MoFe protein exhibited new signals when incubated in the presence of acetylene (g = 4.50, 3.50) or cyanide (g = 4.06). A decrease in the intensity of the S = 3/2 resting state signal observed was correlated with the appearance of the substrate dependent signals. Further studies determined that the substrate dependent signals were a result of cyanide and acetylene directly binding to the FeMo cofactor. This suggests the role for the α-96 amino acid substituted would be a side chain acting as a gatekeeper moving during turnover in order to permit accessibility of acetylene or cyanide to a specific [4Fe-4S] face of the FeMo cofactor.

Several examples of previously determined turnover induced EPR signals have been presented to illustrate how intermediates have begun to determine the number and nature of substrate binding and reduction at the FeMo cofactor. The work presented here is a continuation of determining intermediate states. The wild-type and α-195Gln MoFe protein are incubated
under multiple substrates nitrogen, acetylene, cyanide, azide and protons to identify other intermediate states and to determine their environmental changes and the role amino acids play during turnover.

4.2 Result of Turnover Induced EPR Signals

$S = \frac{1}{2}$ Signals

Results of the past ten years discussed above demonstrate that substrates and inhibitors can bind to the FeMo cofactor during turnover and often induce $S = \frac{1}{2}$ EPR signals. It has also become evident that most of these signals are structurally similar (Table 4.2.1), having near-axial symmetry with $g_\perp \approx 2$. Recent $^{57}$Fe ENDOR studies confirm this and show near identical hyperfine coupling when either CO (lo-CO) or C$_2$H$_2$ ($S_{EPR1}$) binds to the FeMo cofactor, strongly suggesting binding to the same face of the cofactor. To further investigate these signals, we undertook an extended study of $S = \frac{1}{2}$ signals generated under turnover conditions in the presence of H$^+$, N$_2$, HCN and HN$_3$.

H$^+$ and N$_2$: During turnover in the presence of either 100% Ar (i.e., $2H^+ + 2e^- \rightarrow H_2$) or 100% N$_2$ (i.e., $8H^+ + 8e^- + N_2 \rightarrow 2NH_3 + H_2$) nitrogenase induced nearly identical EPR spectra in the $g = 2$ region (Figure 4.2.1). These spectra consist of two overlapping signals with low-field inflections $g = 2.14$ and $g = 2.10$ at nearly a constant ratio where the latter signal is dominant. This dominance has assigned the full $g$-factor profile of this signal to $g = [2.10, 2.01, 1.98]$. Similarities and differences are observed when comparing these signals with the $S = \frac{1}{2}$ signals induced under CO, C$_2$H$_2$ or HC≡CCH$_2$OH. Like many of the previously observed signals, the symmetry of these signals is near axial with $g_\perp \approx 2$. These signals are more intense at 4K than 12K and thus are more analogous to the signals observed under C$_2$H$_2$ than those under CO or HC≡CCH$_2$OH. This temperature dependency is atypical for FeS cluster $S = \frac{1}{2}$ signals, which
usually saturate easily at liq. He temperatures. The dependency of the intensity of these signals upon electron flux is also different from many of the previous systems. Since electrons are donated by the Fe protein to the MoFe protein, electron flux, the rate electrons move through

\[ S = \frac{1}{2} \text{ signals detected during turnover of nitrogenase} \]

<table>
<thead>
<tr>
<th>Substrate/Inhibitor</th>
<th>g-factors</th>
<th>Comments</th>
<th>Reference</th>
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<td>hi</td>
<td>2.17, 2.06, 2.06</td>
<td>0.5 atm CO</td>
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<tr>
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<td></td>
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the enzyme, is dependent on the ratio of Av2:Av1. The hi-CO, C₂H₂ and H⁻C≡CCH₂OH induced signals are observable under all electron flux conditions, suggesting that they are produced
Figure 4.2.1: EPR spectra of wild-type MoFe protein during turnover under (a) Argon and (b) N₂ at T = 4 K and P = 16 mW
during the initial reduction of the enzyme and do not require a "highly reduced" form of the enzyme. This is not the situation under Ar or N2, where the signals are only observable at a Av2:Av1 component ratio of 1:1 or higher (Figure 4.2.2). In other words, these signals not only require turnover but also require a moderate electron flux. A similar situation exists for lo-Co and hi(5)-CO.

The spectra of both signals are identical whether generated under Ar or N2. This suggests that these gases do not influence the ability of the wild-type enzyme to generate either signal. To further test this, the $\alpha$-195$^{Gln}$ MoFe protein was investigated. As discussed above, this variant protein has the interesting phenotype that N2 binds but is only reduced at a very low level (majority of electrons continue to go into dihydrogen production). During turnover under N2 the $\alpha$-195$^{Gln}$ MoFe protein only exhibited the $g = 2.14$ signal (Figure 4.2.3). For some reason, the phenotype of this variant protein inhibits its ability to generate the $g = 2.10$ signal. Therefore, the absence of the $g = 2.10$ signal for the variant protein suggests that the two signals are generated independent of each other even though both signals are simultaneously observed with the wild-type protein. The generation of only the $g = 2.14$ signal by the variant protein allows the assignment of the full principal $g$-factors of that signal to be $g = [2.14, 2.02, 1.98]$.

Finally, the time course for the generation of the two signals by the wild-type enzyme was investigate at 25°C and a molar ratio of Av2:Av1 = 3:1. Under these conditions, a relatively slow rate of formation of the pseudo steady-state signal was observed, maximizing at 30-40 sec (Figure 4.2.4). During this generation, both signals are simultaneously generated in approximately the same ratio, independent of time. This is similar to the C$_2$H$_2$ induced signals previously investigated, where both $S_{EPR1}$ and $S_{EPR2}$ signals were detected in the same ratio.
Figure 4.2.2: EPR spectra of the wild-type MoFe protein during turnover under N₂ and varying component ratio (Av2:Av1) at T = 12 K and P = 16 mW
Figure 4.2.3: EPR spectra of (a) the \(\alpha-195\text{Gln}\) MoFe protein and (b) wild-type MoFe protein during turnover under \(N_2\) at \(T = 4\) K and \(P = 16\) mW.
**Figure 4.2.4:** The time dependence of the intensity of the wild-type MoFe protein N$_2$ induced EPR signal ($g = 2.10$ peak)

independent of time or component protein ratio and supports the suggestion that one signal is not a mechanistic precursor of the other.

**HCN:** Cyanide is an interesting substrate of nitrogenase because it has the ability to undergo either 4- or 6-electron reductions

\[
\text{HCN + 4e}^- + 4\text{H}^+ \rightarrow \text{CH}_3\text{NH}_2 \tag{1}
\]

\[
\text{HCN + 6e}^- + 6\text{H}^+ \rightarrow \text{CH}_4 + \text{NH}_3 \tag{2}
\]
with a possible additional 2-electron reduction side reaction

\[
HCN + 2e^- + 2H^+ + H_2O \rightarrow HCHO + NH_3
\]  

(3)

The mechanistic importance of each of these reactions depends on the Av2:Av1 component ratio. At medium component ratios (Av2:Av1 = 2:1), reaction (1) dominates while reaction (2) becomes significant when the component ratio is increased (Av2:Av1 = 8:1) [14]. Cyanide is also interesting because, while HCN is a substrate, CN\(^-\) is a potent inhibitor of electron transfer in the enzyme.

Under turnover conditions in the presence of 4mM cyanide and pH 7.5, an \( S = \frac{1}{2} \) signal is observed (Figure 4.2.5), which is similar to one of the signals (\( g = 2.14 \)) observed under Ar or N\(_2\) except with slightly different \( g \)-factors, \( g = [2.14, 2.02, 1.99] \). Other than this small shifts in the two high-field \( g \)-factors, the most significant difference between this signal and the \( g = 2.14 \) signal observed under Ar is that the HCN-induced signal is best observed at elevated temperature (25K) while suppressed at 4K, just the opposite of the previous two signals. This means that the electronic environment of the signal under HCN is different from that under Ar or N\(_2\).

HN\(_3\): As with cyanide, azide reduction can be represented by concurrent multi-electron reduction reactions. However, during reduction both HN\(_3\) and N\(_3\)^- act as substrates where each induces a different set of reactions by the enzyme:

\[
HN_3 + 6e^- + 6H^+ \rightarrow N_2H_4 + NH_3
\]  

(4)

\[
N_3^- + 2e^- + 2H^+ \rightarrow N_2 + NH_3
\]  

(5)

\[
N_3^- + 8e^- + 8H^+ \rightarrow 3NH_3
\]  

(6)

Under turnover conditions with the azide concentration at 6 mM and pH 6.9 (substrate form is predominantly HN\(_3\)), no EPR signal is observed at 4K in the \( g = 2 \) region with a microwave power of 20 mW. However, when the temperature is raised above 20K a sharp
Figure 4.2.5: EPR spectrum of the wild-type MoFe protein during turnover under 4mM NaCN (pH = 7.5) at T = 25 K and P = 16 mW. Underlying broad signal is due to the Fe protein.
Figure 4.2.6: EPR spectrum of the wild-type MoFe protein during turnover with 2 mM NaN₃ (pH = 6.9) at T = 60 K and P = 16 mW
isotropic signal appears with \( g = 2.007 \) and line width 2.69 mT (Figure 4.2.6) and persists to higher temperature (at least 150K). These spectral parameters strongly suggest that this signal originates from a radical center rather than a metal cluster.

\[ \text{C}_2\text{H}_2: \] Other than dinitrogen, acetylene is probably the most investigated nitrogenase substrate. While we have observed acetylene-induced \( S = \frac{1}{2} \) EPR signals with the \( \alpha-195\text{Gln} \) MoFe protein, past attempts by various research groups to generate turnover signals with wild-type nitrogenase have been unsuccessful. Using our current protocol for generating turnover signals, a weak \( S = \frac{1}{2} \) is now observed (Figure 4.2.7). This axial signal with \( g = [2.13, 2.00, 2.00] \) is observable at 4K and differs from either of the two \( \text{C}_2\text{H}_2 \)-induced signals (SEPR1 and SEPR2) previously detected when the \( \alpha-195\text{Gln} \) variant MoFe protein was used.

\[ S = \frac{3}{2} \text{Signals} \]

Our previous studies on the CO-induced signals of nitrogenase now clearly establishes that, in addition to the two \( S = \frac{1}{2} \) signals (lo-CO and hi-CO) described above, a FeMo-cofactor centered \( S = \frac{3}{2} \) signal is also generated, which is strictly dependent on the presence of high concentration (> 0.05 atm) of CO in the turnover mixture. Although no high-spin \( \text{C}_2\text{H}_2 \)-induced states were observed with the \( \alpha-195\text{Gln} \) MoFe protein, the results presented here suggest that other high-spin states may be induced during turnover that are substrate dependent.

\[ \text{H}^+ \text{ and N}_2: \] Figure 4.2.8 shows a series of inflections detected for wild-type nitrogenase undergoing turnover in the presence of either Ar or \( \text{N}_2 \). All low field signals described below have the \( g \)-factors in the range of 4-6 and most likely arise from the lowest-field inflections of \( S = \frac{3}{2} \) spin states. While identical \( S = \frac{1}{2} \) spectra are generated in the \( g = 2 \) region under Ar (\( \text{H}^+ \)) or \( \text{N}_2 \) for the wild-type enzyme (Figure 4.2.1), the high-spin spectra differ for the two substrates. Both spectra have inflections at \( g = 5.45 \) while the inflection at \( g = 5.80 \) under Ar appears to shift
Figure 4.2.7: EPR spectrum of the wild-type MoFe protein during turnover under C$_2$H$_2$ at $T = 4$ K and $P = 16$ mW. Underlying broad signal is due to the Fe protein.
Figure 4.2.8: Low field EPR spectra of the wild-type MoFe protein during turnover under (a) N$_2$ and (b) Argon at T = 4 K and P = 50 mW
to 5.73 under N$_2$. Two additional inflections observed at 4.82 and 5.11 in the spectrum under N$_2$ are absent in the spectrum under Argon.

When the $\alpha$-195$^{\text{Gln}}$ MoFe protein is substituted for the wild-type protein, a new series of inflections is detected (Figure 4.2.9), which, unlike those observed for the wild-type enzyme, are now identical under Ar and N$_2$. Two of the inflections ($g = 4.22$ and 4.67) are similar to those ($g = 4.21$ and 4.69) detected by others during turnover with the wild-type enzyme. The other two inflections ($g = 5.43$ and 5.75) appear to be the same two inflections observed with the wild-type enzyme ($g = 5.45$ and 5.80), but shifted slightly to new $g$-factors in the variant protein.

**$\text{HN}_3$:** Azide is the only other substrate tested which yielded turnover inflections attributed to $S = \frac{3}{2}$ states with the wild-type MoFe protein (no low-field inflections were detected when either cyanide or acetylene when used as substrates). The inflections observed under azide ($g = 4.83$ and 5.17) occur in a region of the spectrum previously void of turnover inflections with other substrates or inhibitors and are obviously dependent on the presence of this substrate (Figure 4.2.10). Both of these inflections are absent when $\alpha$-195$^{\text{Gln}}$ MoFe protein undergoes turnover with azide, however, a very minor inflection at $g = 4.80$ may represent a spectral shift of the 4.83 inflection.

### 4.3 Discussion of Turnover Induced EPR Signals

The best current mechanistic and kinetic model for nitrogen fixation is that developed by Lowe and Thorneley. This model evolved from stopped-flow and rapid-quench studies and assumes a cycling of single electrons into the MoFe protein from the Fe protein with up to eight cycles for N$_2$ reduction, i.e.,

$$\text{N}_2 + 8\text{H}^+ + 8\text{e}^- \rightarrow 2\text{NH}_3 + \text{H}_2$$
**Figure 4.2.9:** Low field EPR spectra of the $\alpha$-195$^{Gln}$ MoFe protein during turnover under (a) $N_2$ and (b) Argon at $T = 4\, K$ and $P = 50\, mW$
Figure 4.2.10: Low field EPR spectra of the (a) the $\alpha$-195$^{\text{Gln}}$ and (b) wild-type MoFe protein during turnover with 2 mM NaN$_3$ (pH = 6.9) at $T = 4$ K and $P = 50$ mW
In this model, the MoFe protein is considered to be a dimer of substrate reduction sites (E) and contains two FeMo cofactors. During each cycle there is a one-electron reduction of each site from \( E_n \) to \( E_{n+1} \), where \( n \) represents the number of reducing equivalents that have been transferred (\( 0 \leq n \leq 7 \)). Therefore, the as-isolated form would be represented as \( E_0 \) while the form following reduction by one electron would be designated as \( E_1 \), etc.

At early stages in the cycle, the protein can "relax" back to a lower E level with the concomitant evolution of \( \text{H}_2 \) (\( E_n \rightarrow E_{n-2} + \text{H}_2 \)). However, it is assumed that relaxation can occur from levels \( E_2 \), \( E_3 \) or \( E_4 \), but not higher levels. According to the Lowe/Thorneley (LT) theory, once the enzyme has reached \( E_4 \) it has "committed" to nitrogen fixation and must complete the cycle and produce \( \text{NH}_3 \).

The LT scheme predicts the cyclic one-electron reduction of the MoFe protein, thus, the electron count of this protein should alternate between odd and even. In the steady state, about half of the \( E_n \) states would contain an odd number of electrons while the other half possesses an even number. Since odd-electron states are almost always observable by EPR spectroscopy, the EPR spectrum of a turnover sample in steady state should integrate to half the resting-state spectrum. Early investigations of nitrogenase turnover samples, however, showed spectra containing a large (> 90%) decrease of the \( S = \frac{3}{2} \) FeMoco signal without the obvious generation of any new signals. Subsequent studies at 10°C did illustrated the generation of additional signals [12], albeit in low concentration (2-3% of MoFe protein). One exception is a pair of intense \( S = \frac{1}{2} \) signals (lo-CO and hi-CO) observed during turnover in the presence of the potent noncompetitive inhibitor CO. Detailed analysis of these CO-induced signals using \(^{13}\text{C}\) and \(^{57}\text{Fe}\) ENDOR spectroscopy has revealed direct evidence that these signals represent one or two CO molecules, respectively, binding to Fe in the FeMo cofactor. Recent studies now show similar binding of the substrates \( \text{C}_2\text{H}_2 \), \( \text{CS}_2 \) and propargyl alcohol discussed above.
All of these systems exhibit substrate or inhibitor induced $S = \frac{1}{2}$ signals. The majority of the signals are near axial with $g_\perp \leq 2.0$ and $g_\parallel$ in the range 2.09 - 2.21, suggesting similar molecule-cofactor interactions (Table 4.2.1). This suggestion is strengthened by $^{57}$Fe ENDOR spectroscopy, which reveals nearly identical iron hyperfine patterns for lo-CO generated with CO and the $S_{\text{EPR1}}$ signals induced during reduction of $\text{C}_2\text{H}_2$ with the $\alpha$-$\text{195Gln}$ variant MoFe protein\textsuperscript{20}. The similarity between these spectra is interpreted to mean that both CO and $\text{C}_2\text{H}_2$ bind to the same 4Fe4S "face" of the FeMo cofactor.

The $g$ 2.14 signal observed by us under Ar and N$_2$ with either the wild-type or $\alpha$-$\text{195Gln}$ variant MoFe protein from *A. vinelandi* is identical to a signal previously observed under Ar with the wild-type enzyme from *Klebsiella pneumonia*. This signal, however, differs in high-field $g$-factors from a signal reported by Davis, et al. [13] under N$_2$ with the *A. vinelandi* enzyme (Table 4.3.1). Despite these small differences, because this signal is detected under Ar and/or N$_2$, these results suggest that the $g$ 2.14 signal is a turnover signal of nitrogenase associated with proton reduction.

The second signal observed at $g$ 2.10 under Ar or N$_2$ is only seen with the wild-type enzyme from *A. vinelandii* and has only been reported by us. This signal has $g$-factors significantly different from the signal ($g = 2.093, 1.974, 1.933$) previously reported for the *K. pneumonia* enzyme under Argon [15]. It is highly unlikely that this difference arises from a species-induced spectral shift of the same signal. Of greater interest is the absence of the $g$ 2.10 signal when the $\alpha$-$\text{195Gln}$ variant MoFe protein is used under N$_2$. In other words, while the $g$ 2.14 signal is observed in the presence of either Ar or N$_2$ for both the wild-type and $\alpha$-$\text{195Gln}$ MoFe protein, the generation of the $g$ 2.10 is both substrate and species dependent. As

\textsuperscript{20} Hales and Hoffman manuscript in preparation
previously mentioned the α-195Gln MoFe protein does not significantly reduce N₂. The reason for this poor level of reduction may be the inability of the variant enzyme to achieve a mechanistic state necessary for N₂ reduction. We propose that this state is the one associated with the g 2.10 signal.

The detection of both the g 2.14 and g 2.10 states requires moderate electron flux conditions. This can be contrasted with the hi-CO- and C₂H₂-induced signals previously investigated by us. These latter signals were observable under all electron flux conditions. The moderate flux requirement for the generation of the g 2.14 and 2.10 signals suggests that they are formed at a more reduced state of the enzyme than either the hi-CO or C₂H₂ signals. This is consistent with the identification of the g 2.10 signal as a mechanistic state necessary for N₂ reduction.

The maximum detection of both the g 2.14 and g 2.10 signals occur at very low temperature (4 K). This can be contrasted with most S = ½ signals of typical FeS clusters, where maximum detection often occurs at temperatures greater than 10 K. The only previously detected g 2 turnover signals best observed at 4K are S₁EPR₁ and S₂EPR₂ induced under acetylene with the α-195Gln MoFe protein. The temperature for best detection of an EPR signal is related to its relaxation properties. Fast, efficient relaxation of a signal usually corresponds to a low temperature for maximum detection. The g 2.14 and g 2.10 signals are also similar to the S₁EPR₁ and S₂EPR₂ signals in that both pairs of signals are formed simultaneously. Whether the simultaneous formation of these pairs of signals is related to their efficient relaxation is unknown. However, it is interesting to note that only the g 2.14 signal is observed under HCN turnover and that this isolated signal is best observed at high temperatures (25 K) rather than the low-temperature requirement g 2.14/2.10 pair. This suggests that there may be a relationship
between efficient relaxation and signal pair formation. For this relationship to exist the pair of signals must be structurally or kinetically linked. The source of this linking is currently unknown.

The only radical signal detected during nitrogenase turnover is the isotropic $g = 2.007$ signal observed under HN$_3$. While it is not absolutely known that this signal originates from a radical center, the narrow line width (2.69 mT) and high temperature detection (150K) of this signal are consistent with this characterization. If this it true, it represents the first radical intermediate observed with nitrogenase. (Signals $S_{EPR2}$, the C$_2$H$_2$-induced turnover signal with the $\alpha$-195$^{Gln}$ MoFe protein, was previously characterized as possibly originating from a radical, but is now known to be associated with an FeS center). The molecular origin of this radical is unknown. Obvious possible candidates are components in the electron transfer pathway, such as amino acids (e.g., tyrosine or tryptophan) or the molybdenum-ligating molecule, homocitrate. Because the $g$-factor of the radical signal (2.007) is close to that of the free electron (2.002), the origin is probably a hydrocarbon and is highly unlikely a derivative of azide.

We also present here the first C$_2$H$_2$-induced signal from wild-type nitrogenase. Our success in observing this signal (where others have failed) may be due to the optimized turnover conditions used in this study. This signal differs significantly from the $S_{EPR1}$ and $S_{EPR2}$ signal previously observed under C$_2$H$_2$ with the $\alpha$-195$^{Gln}$ MoFe protein. With the $\alpha$-195$^{Gln}$ MoFe protein, a third poorly resolved signal ($S_{EPR3}$) was also detected with a minimum at $g = 1.99$. Unfortunately, the full spectrum of $S_{EPR3}$ could not be characterized since it is obscured by the more intense $S_{EPR1}$ signal. The C$_2$H$_2$-induced signal described here with the wild-type enzyme could be the same as $S_{EPR3}$.

---

$^{21}$ Hales unpublished results
Even though a C$_2$H$_2$-induced signal has never been reported in turnover samples with the wild-type enzyme, an identical spectrum was previously detected under C$_2$H$_4$ with the wild-type enzyme from *K. pneumonia*. It may seem contradictory that the same signal can be generated with either C$_2$H$_2$ or C$_2$H$_4$. However, this situation is not unique. An identical situation exists with the α-195$^{\text{Gln}}$ MoFe protein where the three C$_2$H$_2$-induced signals (S$_{\text{EPR1}}$, S$_{\text{EPR2}}$ and S$_{\text{EPR3}}$) are also generated under an atmosphere of C$_2$H$_4$. At present there is no mechanistic explanation for this phenomenon.

Our work clearly shows that, in addition to the S = ½ signals described above, several S = 3/2 substrate-dependent signals are also generated during turnover. The first identified high-spin turnover signal is hi(5)-CO, observed in the presence of high concentrations of CO. It is now known that, while hi(5)-CO is generated in parallel with the S = ½ hi-CO signal, it is structurally different from the latter signal. Also, using rapid-freeze techniques, Newton and coworker [10] recently observed two high-spin turnover signals at $g$ 4.21 and 4.69 with the wild-type enzyme and labeled the signals 1b and 1c, respectively. These signals were not associated with any distinct substrates.

It is interesting to note that all of the high-spin signals observed to date arise from S = 3/2 states, the same spin state of the resting FeMo cofactor (Table 4.3.1). Figures 4.2.8 and 4.2.9 show the spectra of turnover samples in the presence of either Ar or N$_2$ using the wild-type enzyme or the α-195$^{\text{Gln}}$ MoFe protein. In all of these spectra the pair of inflections at $g$ 4.31/3.76 for the wild-type protein, and 4.39/3.64 for the variant protein, arise from the FeMo cofactor signal in the resting state. All other inflections are turnover induced. In the spectra of the α195$^{\text{Gln}}$ MoFe protein the inflections at $g$ 4.22 and 4.67 correspond to signals 1b and 1c, respectively, previously identified by Newton and coworkers under N$_2$ with the wild-type
Table 4.3.1: $S = \frac{3}{2}$ signals detected during turnover of nitrogenase

<table>
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<th>Reference</th>
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<td>CO      hi(5)-CO</td>
<td>5.78</td>
<td>0.5 atm CO</td>
<td>many</td>
</tr>
<tr>
<td>C$_2$H$_2$</td>
<td>None detected</td>
<td>Lowe[12], this work</td>
<td></td>
</tr>
<tr>
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<td>Sorlie [7]</td>
<td></td>
</tr>
<tr>
<td>H$^+$</td>
<td>1b</td>
<td>Lowe, Fisher [10]</td>
<td></td>
</tr>
<tr>
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<td></td>
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<td>4.69</td>
<td></td>
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<td></td>
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enzyme (at g 4.21 and 4.69). According to that work, these signals are rapidly generated (~ 262 msec for 1b and ~470 msec for 1c) following initiation of turnover. The time delay prior to their initial detection suggests that these signals are generated by reduced states achieved following several electron-transfer events by the Fe protein (see Lowe/Thorneley scheme above). The absence of these signals in our spectra with the wild-type enzyme is unexpected. However, our
spectra were recorded on samples frozen 20 seconds following initiation of turnover. As such, signals 1b and 1c may have decayed by that time and/or been replaced by the new signals at $g$ 5.45 and 5.73 (observed at 5.80 under Ar). On the other hand, signals 1b and 1c are clearly observed in the turnover spectra with the $\alpha$-195Gln MoFe protein as well as the new signals (now shifted to $g$ 5.43 and 5.75 in the variant protein). The simultaneous detection of all four inflections argues against 1b and 1c being mechanistic or structural precursors of the new inflections.

The new inflections in the $g$ 5.4 and 5.7 regions are most likely the same signals previously detected by others under turnover with the wild-type enzyme from *K. pneumoniae*. The previous publications described those inflections as arising from (a) one signal, (b) a ground-state P cluster signal or (c) the excited-state $S = \frac{7}{2}$ signal of $P^{3+}$. Our results suggest that those interpretations are incorrect. The two inflections obviously are associated with two separate $S = \frac{3}{2}$ signals having different rhombicities. Furthermore, these signals originate from the FeMo cofactor and not the P cluster (this is confirmed by the $g$-factor shift when the $\alpha$-195Gln MoFe protein is used). Finally, the temperature dependency of these signals unambiguously shows them to be associated with the ground-state. Because these two signals have always been observed together, their formation is most likely linked. It is possible that each inflection represents a small structural variation of the same state.

In addition to the 5.4/5.7 pair of signals, there is another pair of signals observed around $g$ 4.8/5.1 with only the wild-type enzyme under N$_2$ or azide. As with the previous pair of signals, this pair always appears together and may similarly represent a small structural variation of the same state. The substrate and species dependence of these signals strongly suggests that they are associated with mechanistic states common to both N$_2$ or azide reduction. It has been shown
(Eq. 4) that the reduction of HN$_3$ generates N$_2$H$_4$, which is also a proposed intermediate of N$_2$ reduction. The pair of g 4.8/5.1 signals may correspond to a N$_2$H$_4$-bound state.

In conclusion, we have now clearly shown that both S = $\frac{1}{2}$ and S = $\frac{3}{2}$ intermediate states are generated during turnover with most nitrogenase substrates and inhibitors. Several of the S = $\frac{1}{2}$ signals have already been investigated with ENDOR spectroscopy and shown to represent substrates or inhibitors bound to a specific FeS “face” of the FeMo cofactor. Due to low signal intensity, the structural origin of the S = $\frac{3}{2}$ states cannot similarly be investigated with ENDOR techniques. However, the variation of the intensity with substrate concentration for many of these signals as well as the dependency of their spectral parameters on the form of the MoFe protein used, clearly show that these signals also arise from substrate interacting with the FeMo cofactor. Why some turnover states are S = $\frac{1}{2}$ while others are S = $\frac{3}{2}$ is unknown. The total spin of the FeMo cofactor is dependent on the metal ion valencies of the Fe and Mo atoms as well as the type of coupling of the substrate with the metal cluster. The two different spin states may represent different turnover reduction states (i.e., different E$_n$ states in the Lowe/Thorneley scheme) and/or different modes of substrate binding (e.g., side-on or end-on). Another possibility is that one spin state represents substrate binding to Fe while the other arises from Mo binding. Recent theoretical modeling studies suggest substrates bind to Fe at low reduction states (E$_1$ or E$_2$) and to Mo at higher reduction states (E$_3$ and E$_4$). ENDOR results on the resting state of the enzyme show only small hyperfine coupling with Mo. If this is correct, substrate binding to Mo would produce a smaller perturbation on the cluster's wave function than would Fe binding. As such, Fe binding may correspond to the intermediate S = $\frac{1}{2}$ states while Mo binding would be related to the S = $\frac{3}{2}$ intermediate states.
4.4 References


CHAPTER 5. L127Δ Fe PROTEIN AND L127Δ Fe – MoFe PROTEIN COMPLEX

5.1 Introduction of L127Δ Fe Protein and L127Δ Fe – MoFe Protein Complex

Site-directed mutagenesis has been used to create an Fe protein that mimics the conformation of wild-type Fe protein with two MgATP molecules bound. This was achieved by the deletion of leucine residue 127 in the Fe protein. The altered Fe protein (L127Δ Fe protein) can complex with MoFe protein with or without MgATP bound. The midpoint potential of the [4Fe-4S] cluster in the isolated L127Δ Fe protein is shifted by -120 mV compared to the wild-type Fe protein, analogous to the midpoint potential shift the [4Fe-4S] cluster experiences in the wild-type Fe protein when two MgATP molecules bind [1]. Further shifts in the midpoint potential occur in both the [4Fe-4S] cluster of the Fe protein and the [8Fe-7S] cluster P-cluster when the L127Δ Fe protein complexes with the MoFe protein. In the complex, the midpoint potential of the [4Fe-4S] cluster of the L127Δ Fe protein shifts -200 mV while the P-cluster shifts –80 mV. Consequently, both metal clusters becoming stronger reductants [2].

The binding of the L127Δ Fe protein with the MoFe protein is at least 300 times tighter than the wild-type Fe - MoFe protein complex [3]. After binding, the L127Δ Fe protein is capable of transferring one electron to the MoFe protein without MgATP present, but is incapable of reducing substrates even when MgATP is present. The one electron transfer can be monitored by an increase in absorption at 430 nm, which indicates an oxidation from [4Fe-4S]⁺ to [4Fe-4S]⁺² for the Fe protein [3]. The primary electron transfer rate of the wild-type Fe protein to the MoFe protein is 700 times faster than of the L127Δ Fe protein to the MoFe protein. Addition of MgATP to L127Δ Fe protein accelerates the electron transfer 25 times, suggesting that MgATP not only plays a role in the conformational change of Fe protein to complex with the MoFe protein, but also in electron transfer between the component proteins. The electron transfer from
the L127Δ Fe protein to the MoFe protein also can be monitored by EPR. EPR spectroscopy shows that an electron transfers from the Fe protein by the disappearance of the \( S = \frac{1}{2} \) EPR signal \( (g = 2.05, 1.93, 1.87) \) of the \([4\text{Fe}-4\text{S}]^{1+}\) cluster, upon oxidation to an EPR silent state. Following the electron transfer there was no appearance of a new signal or bleaching of the existing resting state signal of the FeMo cofactor. Furthermore, if an electron had transferred to the P-cluster a new signal is expected to appear, since the P-cluster prior to an electron transfer is all ferrous and EPR silent. Similarly, if the electron had transfer to the FeMo cofactor, the \( S = \frac{3}{2} \) signal of that cluster would diminish. Although no new signals appeared, this does not rule out the possibility that the electron transferred is delocalized over the entire P-cluster or between the P-cluster and the Fe protein.

The conformational change of the Fe protein upon the binding of MgATP is necessary for nitrogen reduction to occur. MgATP binding also induces change of the EPR [4] and CD spectra [5]. The EPR spectrum of the wild-type Fe protein is a rhombic \( S = \frac{1}{2} \) signal \( (g = 2.05, 1.93, 1.87) \). In the presence of MgATP the signal becomes axial \( (g = 2.05, 1.92, 1.92) \) indicating a structural or environmental change of the \([4\text{Fe}-4\text{S}]^{1+}\) cluster induced by MgATP. The CD spectra of Fe protein with and without MgATP also demonstrate changes of the \([4\text{Fe}-4\text{S}]\) cluster through distinct spectral differences.

The Fe protein was the first protein shown to exhibit a mixed spin state. The \([4\text{Fe}-4\text{S}]^{1+}\) cluster exists as a \( S = \frac{1}{2} \) or a \( S = \frac{3}{2} \) spin state. The two cluster forms can be interconverted by the addition of certain agents to the Fe protein solution. The Fe protein is vulnerable to medium effects because the \([4\text{Fe}-4\text{S}]\) cluster is exposed to solvent. Medium effects of the \([4\text{Fe}-4\text{S}]\) cluster have been demonstrated by EPR studies of the Fe protein in buffer containing 50% ethylene glycol or 4 M urea, where both additives shift the percentage of two co-existing spin
states [6]. In the presence of 50% ethylene glycol the Fe protein exhibits 90% in the $S = \frac{1}{2}$ spin state and 10% in the $S = \frac{3}{2}$ spin state while in the straight buffer the Fe protein exists 35% in the $S = \frac{1}{2}$ spin state and 65% in the $S = \frac{3}{2}$ spin state. A 4 M urea solution shifts the cluster to $>90\%$ $S = \frac{3}{2}$. Furthermore, the addition of ethylene glycol to Fe protein with MgATP revealed that the $S = \frac{1}{2}$ EPR signal reverts from the axial signal back to the rhombic signal suggesting that the ethylene glycol interferes with MgATP binding to Fe protein.

The L127Δ Fe protein now opens opportunities to study the Fe protein in the MgATP bound conformation. The EPR signal of the L127Δ Fe protein exhibits a broader version of the EPR signal in the presence of MgATP [7] which supports the idea that L127Δ Fe protein mimics the Fe protein in the MgATP bound conformation without MgATP. The CD spectrum of L127Δ Fe protein suggests the same effects. Addition of MgATP to L127Δ Fe protein results in no changes in either spectra [7].

The use of the L127Δ Fe protein eliminates some of the problems experienced in studying the wild-type Fe protein. For one thing, since the L127Δ Fe protein is in the MgATP bound conformation without MgATP, the MgATP bound structure can be investigated in the presence of ethylene glycol, which inhibits MgATP binding. Furthermore, because the L127Δ Fe protein forms a stable complex with the MoFe protein in the absence of turnover, the environment of the metallocluster in the complex can be studied spectroscopically.

5.2 Results and Discussion of L127Δ Fe Protein

EPR spectra of the wild-type and the L127Δ Fe protein were recorded in the presence and absence (wild-type only) of 50% ethylene glycol (Figure 5.2.1). The spin quantification of the wild-type Fe protein without ethylene glycol exhibited a signal of 35% $S = \frac{1}{2}$ and 65% $S = \frac{3}{2}$ while with 50% ethylene glycol a 90% $S = \frac{1}{2}$ and 10% $S = \frac{3}{2}$ signal was exhibited. These
results concur with the previous studies [6]. The spin state mixture of the L127Δ Fe protein in 50% ethylene glycol differs from the wild-type, where the variant protein exhibits a 15% S = ½ and 85% S = 3/2 spin quantification. It has been suggested that the S = 3/2 spin state of the Fe protein is induced by freezing because room temperature NMR studies performed on the protein exhibit a S = ½ spin state only. Our result do not discount the suggestion that the S = 3/2 spin state is a freezing artifact. If this is true, the Fe protein in the MgATP bound conformation is affected more by freezing, maybe as a consequence of the subunits pushing the [4Fe-4S] cluster outward in order to complex with the MoFe protein.

![EPR spectra comparison](image)

**Figure 5.2.1:** Comparison of EPR spectra of the wild-type (1.56 mM) and the L127Δ Fe protein (1.00 mM) in 50% ethylene glycol at T = 5 K and P = 15 mW
Comparisons of the ground state properties of the [4Fe-4S] cluster in the L127Δ Fe protein to those of the wild-type Fe protein were extended to MCD. The MCD spectrum of the wild-type Fe protein in 50% glycerol was repeated and agreed with the previous studies [8]. Figure 5.2.2a shows MCD spectra at multiple temperatures (T = 1.68, 4.24, 10.8 K) for the wild-type Fe protein illustrating positive bands at 730 nm and 520 nm and a negative band from 590 – 670 nm. This spectrum is characteristic of a [4Fe-4S]+ cluster, as previously seen in several ferrodoxins [9]. Figure 5.2.2b are the MCD spectra for the L127Δ Fe protein at T = 1.68, 4.23, 10.8 K and exhibits similar positives bands at 730 nm and 520 nm and a negative band in the 600 – 680 nm region. The negative band centered at $\lambda = 420$ nm is probably due to a minor cytochrome impurity. Both Fe proteins show an increase in intensity as the temperature decreases. The intensity difference between T = 1.6 K and T = 4.2 K in the L127Δ Fe protein is much less than the observed difference in the wild-type at the positive bands. This suggests that the $S = \frac{3}{2}$ state makes a larger contribution to the variant protein spectrum. Figure 5.2.3 compares the spectra of the wild-type and L127Δ Fe proteins at T = 1.6 K, demonstrating a small spectral shift and broadening of both the positive and negative bands for the latter protein. These differences may be associated with the high content of the $S = \frac{3}{2}$ [4Fe-4S] +1 cluster in the L127Δ Fe protein. In other words, the spectra may indicate some of the environmental differences between the wild-type and L127Δ Fe proteins. For instance, the subunits of the L127Δ Fe protein are in a more open conformation than those of the wild-type Fe protein, which may affect the electronic environment of the [4Fe-4S] cluster of the L127Δ Fe protein.

MCD magnetization curves provide information on the nature of the ground state of the paramagnet. Figure 5.2.4(a) & (b) presents magnetization plots of the wild-type and L127Δ Fe protein at $\lambda = 520$ nm and multiple temperatures. (Magnetization curves at $\lambda = 730$ nm yields
Figure 5.2.2: MCD spectra of the (a) wild-type and (b) L127Δ Fe protein in 50% ethylene glycol at multiple temperatures
The wild-type Fe protein exhibits no nesting of the magnetization curves at different temperatures while the L127Δ Fe protein exhibits some nesting. “Nesting” is the term used to describe a series of magnetization curves whose initial slope increases or decreases as the temperature is increased. Nesting is a characteristic of a spin state $> \frac{1}{2}$. Therefore, the curves in Figure 5.2.4(b) indicate that the L127Δ Fe protein has a larger contribution from the $S = \frac{3}{2}$ spin state.

**Figure 5.2.3:** Comparison of the wild-type and the L127Δ Fe protein normalized by concentration at $T = 1.6$ K

similar information and are shown in the Appendix)
Figure 5.2.4: Magnetization curves of (a) the wild-type and (b) the L127Δ Fe protein at \( \lambda = 520 \) nm and multiple temperatures
Parameters for Simulation at $S = 3/2$:  $D = -2.00; E/D = 0.21; g = 2.0023$; polarization $(xy, xz, yz) = 0.503, -0.966, 2.108$

Parameters for Simulation at $S = 1/2$:  $D = 0.0; E/D = 0.0; g = 2.0023$; polarization $(xy, xz, yz) = 0.700, 1.00, 0.300$

Simulation 80% $S = 1/2$:  20% $S = 3/2$ Simulation + 20% $S = 1/2$ Simulation

**Figure 5.2.5:**  Magnetization curve of the wild-type Fe protein and simulations at $\lambda = 520$ nm and $T = 1.64$ K
Parameters for Simulation at $S = 3/2$: $D = -2.00$; $E/D = 0.21$; $g = 2.0023$; 
  
polarization $(xy, xz, yz) = 0.740, -0.240, 1.28$

Parameters for Simulation at $S = 1/2$: $D = 0.0$; $E/D = 0.0$; $g = 2.0023$ 
  
polarization $(xy, xz, yz) = 0.720, -0.411, 1.777$

Simulation 35% $S = 1/2$: 65% $S = 3/2$ Simulation + 35% $S = 1/2$ Simulation

**Figure 5.2.6:** Magnetization curve of the L127Δ Fe protein and simulations at $\lambda = 520$ nm and $T = 1.64$ K
Figures 5.2.5 and 5.2.6 further illustrate the deviation the Fe proteins exhibit from a pure S = ½ system at T = 1.6 K. The scatter plot represents the experimental data and line plots represent the theoretical curves. Theoretical plots of 100% S = \(3/2\) (blue) and 100% S = ½ (green) and best fit of the experimental data which is a percentage of S = \(3/2\) and S = ½ (red) are plotted. The best fit of the wild-type Fe protein is 80% S = ½, in agreement with the previous work by Onate et al. [8] and the EPR spin quantification above, and the best fit of the L127Δ Fe protein is 35% S = ½, which also agrees with the EPR spin quantification above.

The parameters determined from the simulations for the Fe proteins for S = ½ are \(g = 2.0023\) and for the isotropic S = \(3/2\) system they are E/D = 0.21, D = -2.00 cm\(^{-1}\), and \(g = 2.0023\). Simulations were carried out independent of the previous studies. The only disagreement with the previous work, thus far, is that it was reported that with the addition of MgATP to Fe protein the percent of S = ½ spin state did not change. In the present work with L127Δ Fe protein, which mimics the MgATP conformation, shows a definite decrease in the S = ½ spin state. This could mean the mutation affects the heterogeneity of the spin state.

The simulations were performed for multiple temperatures, which was not done in the previous work on the wild-type Fe protein. Figure 5.2.7(a) & (b) show the magnetization curves at T = 1.6, 4.2, and 10 K for the experimental data along with the percent of S = ½ simulation determined as the best fit at T = 1.6 K. As the temperature increases excited states become populated, complicating the data and the simulation. The excited-state electronic structure of [4Fe-4S]\(^{+}\) clusters is not well understood. The best fit of the experimental data is at T = 1.6 K, where mainly the ground state is populated, while higher temperature magnetization curves represent ground and excited states and are not simulated as well.
Figure 5.2.7: Magnetization curves of (a) the wild-type and (b) the L127Δ Fe protein and simulations at $\lambda=520$ nm and multiple temperatures
5.3 Results and Discussion of L127Δ Fe – MoFe Protein Complex

As mentioned above, the L127Δ Fe protein forms a tight complex with the wild-type MoFe protein where it is capable of transferring one electron to the MoFe protein without MgATP hydrolysis. This is of interest because the one electron transfer mimics turnover, so with the complex it is possible to see a partial pathway of the electron from the Fe protein to the MoFe protein. EPR and MCD spectroscopic studies were performed to gain greater insight into the electronic structure of the metalloclusters in the complex and observe any new spin states resulting from the one electron transfer. A comparison of the L127Δ Fe – MoFe protein complex and the wild-type MoFe protein EPR spectra is shown in Figure 5.3.1.

**Figure 5.3.1:** Comparison of EPR spectra of the wild-type MoFe protein and L127Δ Fe – MoFe protein complex at $T = 5$ K and $P = 15$ mW
The EPR spectrum of the L127Δ Fe – MoFe complex is essentially the same signal as that of the MoFe protein in the resting state, showing the $S = \frac{3}{2}$ signal attributed to the FeMo cofactor ($g = 4.31, 3.67, 2.01$). The absence of the EPR signal of the $[4\text{Fe}-4\text{S}]^{+1}$ cluster indicates that the one electron has transferred from the L127Δ Fe protein. Following this transfer, the EPR spectrum should change. Either a new EPR signal should appear as a result of the reduction of the P-cluster or the $S = \frac{3}{2}$ signal should bleach as a result of the reduction of the FeMo cofactor. Neither happens. Obviously, the FeMo cofactor is not reduced. However, even though there is no formation of a new P-cluster signal, this does not rule out the possibility that the electron is delocalized over the entire P-cluster or that the P-cluster and Fe protein are in an EPR silent state.

MCD spectra of the wild-type MoFe protein and the L127Δ Fe – MoFe protein complex at $T = 1.6, 4.2, 10\text{ K}$ (Figure 5.3.2(a) & (b)) reveal positive bands at the wavelengths 520 nm, 580 nm, and 730 nm. The positive bands of the MoFe protein and the L127Δ Fe - MoFe protein complex show an increase in intensity as the temperature decreases, indicating paramagnetic centers. The direct comparison of the MoFe protein and the complex at $T = 1.6\text{ K}$ (Figure 5.3.3) illustrates that the positive bands of the complex are more intense at the wavelengths 520 nm and 730 nm and that they are slightly shifted compared to the MoFe protein. These differences suggest an environmental change in the FeMo cofactor in the complex. The intensity increase suggests that more than one paramagnetic center maybe contributing to the spectra. Therefore, magnetization curves were recorded to provide information on the spin state(s) present.

The magnetization curves of the MoFe protein and the L127Δ Fe – MoFe protein complex at $\lambda = 520\text{ nm}$ and multiple temperatures ($T = 1.6, 4.2, 10\text{ K}$) are similar but not identical (Figure 5.3.4 a & b). (Magnetization curves at $\lambda = 730\text{ nm}$ and multiple temperatures...
Figure 5.3.2: MCD spectra of (a) the wild-type MoFe protein and (b) the L127Δ Fe – MoFe protein complex at multiple temperatures
**Figure 5.3.3:** Comparison of wild-type MoFe protein and L127Δ Fe – MoFe protein complex at T = 1.6 K

are shown in the Appendix). Magnetization curves of both proteins exhibit nesting, indicating that the paramagnetic signal is greater than $S = \frac{1}{2}$. The major difference between the magnetization curves is observed at $T = 1.6$ K where the MoFe protein exhibits a steeper slope compared to the complex, possibly suggesting a higher spin state or a small change in the FeMo cofactor environment due to complexing, there is also the possibility of a small spin contribution from a second paramagnetic center formed from the one electron transfer in the complex.
Figure 5.3.4: Magnetization curves of (a) the wild-type MoFe protein and (b) the L127Δ Fe - MoFe protein complex at $\lambda = 520$ nm and multiple temperatures
Simulations of the magnetizations curves were performed at multiple temperatures. The parameters, E/D and g for the MoFe protein and the FeMo cofactor center in the L127Δ Fe – MoFe protein were known from analysis of the S = 3/2 EPR signal work above (E/D = 0.055 and g = 1.990). The experimental data, simulations, and parameters for the proteins at λ = 520 nm are shown in the Figures 5.3.5 and 5.3.6. These simulations predicted the spin state for the L127Δ Fe – MoFe protein complex to be 100% S = 3/2. The D parameters from the simulations vary slightly for MoFe protein (D = 7.50) and the complex (D = 6.50). The simulations at higher temperatures do not fit as well as at T = 1.6 K this maybe a consequence of not having a well-developed electronic structure for the excited states or the presence of multiple signals. The agreement of the parameters calculated from the simulations of the magnetization curves of the L127Δ Fe – MoFe protein complex at λ = 520 and 730 nm rules out any contribution from the P\textsuperscript{OX} form of the P-cluster after an electron transfer because another spin state would have been characterized.

The simulations of the magnetization curves and the EPR data suggest that the MCD signals of the complex contain a contribution from the FeMo cofactor in the resting state. The intensity difference of the positive band centered at 520 nm between the MoFe protein and the complex is still a concern (Figure 5.3.3). Obviously another paramagnetic FeS center is present. The addition of the MCD spectrum of the L127Δ Fe protein to the MoFe protein spectrum, according to the spin concentration from EPR, is compared to the MCD spectra of the L127Δ Fe – MoFe complex in Figure 5.3.7. The similarities of these spectra suggest that the spectrum of the complex may contain a contribution from an FeS cluster such as the [4Fe-4S]\textsuperscript{+1} cluster of the L127Δ Fe protein. Furthermore, because the L127Δ Fe protein is mainly in a S = 3/2 state, as discussed in Sec 5.2, the contribution of this center to the magnetization curves of the complex
Figure 5.3.5: Magnetization curves of the wild-type MoFe protein and simulation at $\lambda = 520$ nm and multiple temperatures.
Figure 5.3.6: Magnetization curve of L127Δ Fe – MoFe protein complex and simulation at $\lambda = 520$ nm and multiple temperatures

Parameters for Simulation at $S = 3/2$: $D = 6.50$; $E/D = 0.0.056$; $g = 1.990$; polarization = -1.881, -0.205, 2.750
Figure 5.3.7: Sum of L127Δ Fe protein and wild-type MoFe protein MCD spectra compared to L127Δ Fe – MoFe protein complex at \( T = 1.6 \) K

would best fit a simulation at 100% \( S = \frac{3}{2} \), in agreement with our data. Moreover, the second paramagnetic FeS cluster contributing to the MCD spectrum of the complex, maybe the \([4\text{Fe}-4\text{S}]\) cluster of the Fe protein and would have to be EPR silent.

The MCD spectrum of the L127Δ Fe – MoFe complex consists of a \( S = \frac{3}{2} \) contribution from each protein. Our results suggest that this maybe a result of futile electron cycling, which is the back donation of electron transfer from the MoFe protein to the Fe protein. In other words, the electron the Fe protein initially donated is returned after a certain time lag. Thus, the L127Δ Fe protein is in its original spin state while contributing to the MCD spectrum of the complex.
The $S = \frac{3}{2}$ EPR signal of the [4Fe-4S] cluster may not be observed because it is broadened due to the futile electron cycle. Thus, the L127A Fe protein in the complex may still be paramagnetic, but in an EPR-silent state.

The absence of new signals has made it difficult to pinpoint the position of the transferred electron. If the electron were successfully transferred to the P-cluster another spin-state would have been characterized, but this does not rule out the possibility that the electron has further reduced the P-cluster creating the extra intensity of the MCD signal yet in a state previously unobserved or EPR-silent. The position of the transferred electron is inconclusive, but the suggestion that a second paramagnetic center is present along with the FeMo cofactor resting state does promote the position of the electron transferred to be one of two places the P-cluster of the [4Fe-4S] cluster of the Fe protein. This new information from the MCD studies will aid in future studies of the electronic turnover states of nitrogenase.

5.4 References


CHAPTER 6. THE \(\Delta nifB\) and \(\Delta nifH\) MoFe PROTEINS

6.1 Introduction of the \(\Delta nifB\) and the \(\Delta nifH\) MoFe Proteins

FeMo cofactor deficient MoFe protein, also referred to as apo-MoFe protein, has been useful to the investigation of the MoFe protein. Apo-MoFe protein is be produced by deleting certain genes associated with the biosynthesis of either the Fe protein or the MoFe protein. Two forms of apo-MoFe protein are presented here. Each form is a result of the deletion of one gene required to synthesize the complete MoFe protein.

Genes \(nifB\) or \(nifH\) were deleted, which resulted in the production of the \(\Delta nifB\) and \(\Delta nifH\) MoFe proteins. In both proteins the FeMo cofactor is absent while the P-cluster or its precursor is present. Metal analysis of the wild-type MoFe protein demonstrates that 53% of the Fe content is associated with the P-cluster. The \(\Delta nifB\) and \(\Delta nifH\) exhibited 55% of the total Fe content of the wild-type MoFe protein with no detectable molybdenum [1, 2]. The absence of the \(S = \frac{3}{2}\) FeMo cofactor resting state EPR signal in the \(\Delta nifB\) and \(\Delta nifH\) MoFe proteins confirms the absence of a normal FeMo cofactor. SDS gel electrophoresis has determined that there is no difference in the molecular weights of the subunits in the apo-MoFe proteins compared to the subunits in the MoFe protein [1, 2].

The \(\Delta nifB\) and \(\Delta nifH\) MoFe proteins are incapable of reducing substrates in the presence of Fe protein and MgATP, but their activity can be reconstituted if the FeMo cofactor is inserted. Isolated cofactor can be added directly to \(\Delta nifB\) MoFe protein with 80% of activity reconstituted. However, isolated cofactor alone cannot reconstitute the activity of the \(\Delta nifH\) MoFe protein; it also requires the presence of the Fe protein and MgATP. The crystal structure of the \(\Delta nifB\) MoFe protein has been determined and demonstrates that the \(\alpha\) subunits are in an open
conformation awaiting the insertion of the FeMo cofactor, therefore, suggesting that the Fe
protein with MgATP bound are needed for a conformational change in the \( \Delta nifH \) MoFe protein
or for transporting the FeMo cofactor to the \( \Delta nifH \) MoFe protein for insertion.

The colors of the two apo-MoFe proteins differ. The \( \Delta nifB \) MoFe protein is red-brown
while the \( \Delta nifH \) MoFe protein is brown. This indicates electronic differences with possible
differences in the structural or redox properties of the P-clusters. The EPR signals of the each
apo-MoFe protein give further insight on the differences of the P-clusters. The \( \Delta nifB \) MoFe
protein exhibits no EPR signal indicative of the diamagnetic \( P^N \) state of the P-cluster in the
resting state of the MoFe protein. In contrast, the \( \Delta nifH \) MoFe protein is paramagnetic,
exhibiting a \( S = \frac{1}{2} \) EPR signal (\( g = 2.05, 1.98, 1.90 \)). The presence of an EPR signal suggests
that the P-cluster of the \( \Delta nifH \) MoFe protein is in a different oxidation state and may represent a
precursor to the mature P-cluster in the MoFe protein.

The P-clusters of both apo-MoFe proteins can be oxidized by indigo disulfonate (IDS) to
the \( P^{\text{OX}} \) (or \( P^{2+} \)) state. When the \( \Delta nifB \) MoFe protein is oxidized to \( P^{\text{OX}} \) an EPR signal appears (\( g = 11.8 \)). This same signal has been observed following a 6 equivalent oxidation of the MoFe
protein, 2 equivalents for each P-cluster and one equivalent for each FeMo cofactor. The
resultant MoFe protein has diamagnetic FeMo cofactors and paramagnetic P-clusters in the \( P^{\text{OX}} \)
state [3, 4]. However, when \( \Delta nifH \) MoFe protein is oxidized to \( P^{\text{OX}} \) the \( S = \frac{1}{2} \) signal of the as-
isolated protein disappears, while the EPR signal (\( g = 11.8 \)) of the normal \( P^{\text{OX}} \) state is not
detected. UV-visible experiments have confirmed the oxidation of both apo-MoFe proteins.
While these results indicate that the P-clusters of both apo-MoFe proteins can be oxidized, the
oxidized form of the P-clusters in the \( \Delta nifH \) MoFe protein are obviously different from the
oxidized P-clusters in the wild-type or the \( \Delta nifB \) MoFe proteins.
The $\Delta nifB$ and $\Delta nifH$ MoFe proteins can complex with MgATP bound Fe protein and perform 60% of wild-type MgATP hydrolysis. However, no substrate reduction occurs [1, 2]. The $\Delta nifB$ MoFe protein also exhibits a one-electron transfer while $\Delta nifH$ MoFe protein does not. The one electron transfer to oxidized $\Delta nifB$ MoFe protein by the Fe protein was monitored by the reduction of both the $P^{OX}$ and [4Fe-4S]$^{+1}$ EPR signals and the concomitant formation of the $P^{+1}$ signal ($g = 2.05, 1.93, 1.81$) [2, 5]. The $\Delta nifH$ MoFe protein does not demonstrate electron transfer. It is possible that the $\Delta nifH$ MoFe protein may be incapable of electron transfer due to a structural difference in the P-cluster arising from an unusual conformation or oxidation state making it unable to accept electrons. These previous studies of $\Delta nifB$ and $\Delta nifH$ MoFe proteins illustrate several differences between their P-clusters. The MCD studies presented here will provide further information on the different oxidation states of the P-cluster of each protein.

6.2 Results and Discussion of $\Delta nifB$ and $\Delta nifH$ MoFe Proteins

EPR spectra of as-isolated $\Delta nifB$, $\Delta nifH$ MoFe proteins and oxidized $\Delta nifB$, and $\Delta nifH$ MoFe proteins were recorded (Figure 6.2.1). The as-isolated $\Delta nifH$ MoFe protein exhibits a $S = \frac{1}{2}$ EPR signal which disappears upon oxidation while the as-isolated and oxidized $\Delta nifB$ MoFe proteins are EPR-silent. Spin quantification of the as-isolated $\Delta nifH$ MoFe protein shows that the signal represents 70% of the protein in the $S = \frac{1}{2}$ spin state. All of these results agreed with the previous work [1, 2].

The MCD spectroscopic study of the as-isolated $\Delta nifB$ MoFe protein confirms that its P-cluster is diamagnetic. Figure 6.2.2 illustrates the temperature independent MCD spectra of the $\Delta nifB$ MoFe protein characteristic of a $S = 0$ spin state. The MCD spectrum suggests that the
P-cluster is in an all-ferrous state, $P^N$, as observed in the wild-type MoFe protein. Figure 6.2.3 shows the MCD spectrum of the wild-type MoFe protein, exhibiting positive bands at 520 nm and 580 nm that increase in intensity as the temperature decreases. These bands arise from the FeMo cofactor, as discussed in Chapter 5.

The MCD spectrum of the as-isolated $\Delta nifH$ MoFe protein (Figure 6.2.4) exhibits an increase in intensity as the temperature decreases (data not shown) with positive bands at 520 nm and 800 nm and a negative band centered at 680 nm. As previously discussed (Chapter 5) this spectrum is characteristic of an $S = \frac{1}{2}$ [4Fe-4S]$^{1+}$ cluster, possibly indicating the presence of 1 or
Figure 6.2.2: MCD spectra of the as-isolated Δ*nif*B MoFe protein at multiple temperatures

Figure 6.2.3: MCD spectra of the as-isolated wild-type MoFe protein at multiple temperatures
2 \text{[4Fe-4S]}^{+1} \text{ clusters which could be a precursor to the mature P-cluster seen in } \Delta nifB \text{ and wild-type MoFe proteins.}

The MCD spectrum of oxidized } \Delta nifB \text{ MoFe protein (Figure 6.2.5) is markedly different from that of the as-isolated protein. The spectrum of oxidized } \Delta nifB \text{ MoFe protein is no longer temperature independent with the intensity increasing with decreasing temperature. The oxidized } \Delta nifB \text{ MoFe protein exhibits positive bands at 520 nm and 590 nm and a major positive band at approximately 700 nm. The MCD spectrum of the } \Delta nifH \text{ MoFe protein also experiences a significant spectral change upon oxidation (Figure 6.2.6(a)). The positive band at 520 nm and the negative band at 680 nm seen in the spectrum of the as-isolated protein disappear and give}
way to the appearance of a positive band beginning at 700 nm similar to that observed in the oxidized \( \Delta nifB \) MoFe protein. Figure 6.2.6(a) & (b) compares the oxidized \( \Delta nifB \) and oxidized \( \Delta nifH \) MoFe proteins indicating the beginning of a strong positive band in the 700-800 nm region in both apo-proteins [4]. Johnson et al., along with previous studies of the oxidized MoFe protein [3] demonstrated that the strong positive band centered around 800 nm is a result of the oxidized P-cluster, \( P^{\text{OX}} \), in the MoFe protein.

The magnetization curves of \( \Delta nifB \) MoFe protein (Figure 6.2.7(a) & (b)) were nearly linear and temperature independent again confirming that its P-cluster is in a diamagnetic state comparable to the \( P^{\text{N}} \) state of the P-cluster in the wild-type MoFe protein. As stated previously,
Figure 6.2.6: Comparison of the MCD spectra of oxidized (a) $\Delta nifH$ and (b) $\Delta nifB$ MoFe proteins at multiple temperatures (600-800 nm region)
Figure 6.2.7: Magnetization curves of the as-isolated $\Delta nifB$ MoFe protein at $\lambda = 420$ nm and multiple temperatures (a) temperature dependent and (b) magnetic field dependent.
the P-cluster of the ΔnifB MoFe protein is in a diamagnetic state and because of this the signal is a result of the B-term only, meaning the signal is induced by ground and excited state mixing with all other relatively close excited states. The magnetization curve of the as-isolated ΔnifH MoFe protein in Figure 6.2.8 illustrates a paramagnetic system and the best-fit by a simulation of a \( S = \frac{1}{2} \) spin state. The down slope of the magnetization curve after saturation may be due to the presence of a small amount of a high spin state. This state is not observable in the EPR spectrum, but is implied because the observable \( S = \frac{1}{2} \) signal represents only 70\% of the protein.

![Normalized MCD](image)

**Figure 6.2.8:** Magnetization curve and simulation of the as-isolated ΔnifH MoFe protein at \( T = 1.6 \) K

Magnetization curves of oxidized ΔnifB MoFe protein in Figure 6.2.9 and 6.2.10 were recorded at \( \lambda = 790 \) nm and multiple temperatures (\( T = 1.58, 4.24, 9.44 \)). The magnetization
Figure 6.2.9: Magnetization curves of the oxidized ΔnifB MoFe protein and simulation (S = 3.0) at λ = 790 nm and multiple temperatures (a) D > 0 and (b) D < 0
Figure 6.2.10: Magnetization curves of the oxidized \( \Delta nifB \) MoFe protein and simulation \( (S = 4.0) \) at \( \lambda = 790 \) nm and multiple temperatures (a) \( D > 0 \) and (b) \( D < 0 \)
curves exhibit an initial steep slope and a rapid approach to saturation and nesting, which suggests a high spin state. Nesting occurs when the slope of the magnetization curves increase or decrease as the temperature increases. Nesting is a result of a zero field splitting with low lying excited states or a large deviation from a theoretical $S = \frac{1}{2} g = 2$ system, which causes the absence of ground state degeneracy in the absence of a magnetic field. Since these states have been predicted to be integer spin states, the simulations required a multitude of parameters.

Figures 6.2.9 and 6.2.10 illustrate the experimental magnetization curves and the best-fit simulations performed at $S = 3.0$ and $S = 4.0$ for oxidized $\Delta$nifB MoFe protein. Multiple simulations are shown to illustrate the differences in fit. The simulations performed at $S = 3.0$ are deemed closest to the experimental data due to the best-fit of the initial slope of the curve at $T= 1.6 \, \text{K}$. This is consistent with the previous Mössbauer data [6]. That suggests the state $P^{\text{OX}}$ is an $S = 3.0$ or $S = 4.0$ spin state with the zero field splitting parameter, $D$, less than zero. However, our simulations with $S = 3.0$ can have the zero field splitting parameter either greater than or less than zero (Figure 6.2.9). The previous work suggesting $D < 0$ was based on the assumption that the ground spin state would be $M_s = \pm 3.0$. This is an appropriate suggestion because if $D < 0$ $M_s = \pm 3.0$ would be responsible for the observed low temperature EPR and MCD signal. On the other hand, if $D > 0$ the ground state would be diamagnetic ($M_s = 0$), which would be less likely to yield the EPR or MCD signal. The simulations performed here for $D < 0$ suggest the magnitude of $D$ is an intermediate value, meaning the zero field splitting and the zeeman interaction energies become comparable. Therefore, when the value of $D$ is a negative intermediate value (in this case $D = -3.00$ with low rhombicity, $E/D = 0.08$) the ground state $M_s$ level ($M_s = \pm 3.0$) makes the major contribution to the magnetization curve with minor contributions from the other $M_s$ levels. However, when the value of $D$ is an intermediate
positive value (D = 4.00 with high rhomobicity, E/D = 0.33) the Ms levels mix and even though the ground state is diamagnetic (Ms = 0.0), a signal is observed because all Ms levels contribute resulting in a mixing of states. Magnetization curves and simulations for oxidized ΔnifB MoFe protein were also performed at λ = 520 nm (Data shown in Appendix). The parameters of the best-fit simulations agreed with the parameters simulated at λ = 790 nm, confirming that the MCD signal is a result of the P-cluster only and that the value of D can be less than or greater than zero when D is an intermediate value. While the values of D and E/D are inconclusive for oxidized ΔnifB MoFe protein, our simulations better define the range of the acceptable values and show the possibility of D > 0, which had previously been discounted by others.

The magnetization curve of oxidized ΔnifH MoFe protein at λ = 790 nm and multiple temperatures (T = 1.58, 4.24, 9.44) exhibit an initial steep slope and a rapid approach to saturation, which is a characteristic of a high spin state (Figure 6.2.11). This is also consistent with the nesting of the curves, which is also characteristic of S > ½. The nesting observed for the oxidized ΔnifH MoFe protein differed from the nesting observed for the oxidized ΔnifB MoFe protein in that the nesting is negative, meaning the intensity of the magnetization curves intensity decreases as the temperature increases. As done with the oxidized ΔnifB MoFe protein, simulations were performed using many parameters. The best-fit was achieved with S = 2.0, D = -3.00, E/D = 0.08, and g = 2.0023. Figure 6.2.11 illustrates the simulations at S = 2.0 and S = 3.0 magnetization curves and demonstrates the difference in fit for the different spin state assignments of ΔnifH MoFe protein. The difference in parameters of the oxidized ΔnifH and the oxidized ΔnifB MoFe proteins obtained from the simulations provide an explanation of the differences observed in the EPR spectra, with an observable EPR signal for oxidized ΔnifB MoFe protein while the oxidized ΔnifH MoFe protein is EPR-silent. Since, the zero field
Figure 6.2.11: Magnetization curves of the oxidized ΔnifH MoFe protein and simulations at λ = 790 nm and multiple temperatures (a) S = 2.0 and (b) S = 3.0
splitting between a $\text{Ms} = \pm 3.0$ pair is smaller than the splitting between a $\text{Ms} = \pm 2.0$ pair, the microwave energy used in X-band EPR spectrometer is incapable of inducing a signal between the $\text{Ms} = \pm 2.0$ pair. This would explain why a parallel mode EPR signal is not observed for the oxidized $\Delta nifH$ MoFe protein but is for the oxidized $\Delta nifB$ MoFe protein.

To summarize our results so far, both apo-MoFe proteins can be oxidized even though the P-cluster in each as-isolated protein exists in a different spin state. The as-isolated $\Delta nifB$ MoFe protein exists in a $S = 0$ spin state and the as-isolated $\Delta nifH$ MoFe protein exists in a $S = \frac{1}{2}$ spin state. Both P-clusters of the apo-MoFe proteins can be oxidized to integer states. The $\Delta nifB$ MoFe protein is oxidized to $P^{\text{OX}}$, most likely a $S = 3.0$ spin state, which is similar to that proposed for the wild-type MoFe protein P-cluster and is supported by similar EPR and MCD spectra. However, the $\Delta nifH$ MoFe protein is most likely oxidized to an $S = 2.0$ spin state. The $S = 2.0$ spin state is typically unobserved by EPR due to the large zero field splitting, suggesting why an EPR signal is observed for the oxidized wild-type or $\Delta nifB$ MoFe proteins but not for oxidized $\Delta nifH$ MoFe protein. The difference in the spin state of the P-clusters of the two as-isolated apo-MoFe proteins may be a result of (1) the $\Delta nifH$ MoFe protein P-clusters are structurally constrained or (2) the $\Delta nifH$ MoFe protein P-clusters are present in a different oxidation state. Most likely both explanations are correct. In fact, the difference in structure and oxidation state of the P-clusters in the $\Delta nifH$ MoFe protein compared to the $\Delta nifB$ and the wild-type MoFe protein is very likely the reason electron transfer does not occur in the former protein.

$$\alpha \beta + \text{FeP} \rightarrow \Delta nifH \xrightarrow{\text{nifH}} \Delta nifB(?) \xrightarrow{\text{FeMo cofactor}} \text{MoFeP} \quad (1)$$

The P-cluster has been suggested to play a role in electron transfer from the Fe protein to the FeMo cofactor. The P-cluster of the wild-type MoFe protein can be oxidized one equivalent at a to four oxidation states $P^N$, $P^{+1}$, $P^{\text{OX}}$ ($P^{+2}$), and $P^{+3}$. As discussed above $P^N$ is the $S = 0$ spin
state, P^{+1} is a mixed S = \frac{1}{2} and \frac{5}{2} spin state, P^{OX} is a S = 3 or 4 spin state, and P^{+3} is a mixed S = \frac{1}{2} and \frac{7}{2} spin state. Comparing the P-cluster of the wild-type MoFe protein with the results presented here, the \Delta nifB MoFe protein contains a P-cluster analogous to the wild-type MoFe protein and can exist in states P^N, P^{+1}, P^{OX}, while the P-cluster of the \Delta nifH MoFe protein has demonstrated different characteristics. The \Delta nifH MoFe protein can be reduced past the as-isolated state by Ti (III) citrate to an EPR silent-state analogous to the wild-type MoFe protein is an all-ferrous state (P^N). This suggests that the as-isolated \Delta nifH MoFe protein is not in the P^N state and may be in a P^{+1} state. This assignment is consistent with the S = \frac{1}{2} EPR signal observed in the as-isolated \Delta nifH MoFe protein. Even though the \Delta nifH MoFe protein, the \Delta nifB MoFe protein and the wild-type MoFe protein all exhibit S = \frac{1}{2} signals in the P^+ state, this signal is different in the \Delta nifH MoFe protein. Furthermore, the later two proteins exhibit an additional S = \frac{5}{2} signal in the P^+ state. These spectral differences, like those described above for the P^{+2} state, reinforce the contention that the P-clusters in the \Delta nifH MoFe protein are structurally different from those of the \Delta nifB MoFe protein and the wild-type MoFe protein. Obviously, during the in vivo synthesis of the holo-MoFe protein, NifH interacts with the \Delta nifH MoFe protein converting its P-cluster into the mature P-cluster observed in both the \Delta nifB and wild-type MoFe proteins.

6.3 References


APPENDIX. ADDITIONAL RESULTS

(1) Magnetization curves of the wild-type Fe protein at λ = 730 nm and multiple temperatures
Parameters for Simulation at $S = 3/2$: $D = -2.00$; $E/D = 0.21$; $g = 2.0023$; polarization = 0.650, -0.310, 1.322

Parameters for Simulation at $S = 1/2$: $D = 0.0$; $E/D = 0.0$; $g = 2.0023$; polarization = 1.380, -0.458, 1.05

Simulation 80% $S = 1/2$: 20% $S = 3/2$ Simulation + 80% $S = 1/2$ Simulation

Magnetization curve of wild-type Fe protein and simulations at $\lambda = 730$ nm and $T = 1.64$ K
Parameters for Simulation at $S = \frac{3}{2}$: $D = -2.00$; $E/D = 0.21$; $g = 2.0023$; polarization = 0.650, -0.310, 1.322

Parameters for Simulation at $S = \frac{1}{2}$: $D = 0.0$; $E/D = 0.0$; $g = 2.0023$; polarization = 1.380, -0.458, 1.05

Simulation 80% $S = \frac{1}{2}$: 20% $S = \frac{3}{2}$ Simulation + 80% $S = \frac{1}{2}$ Simulation

Magnetization curves of the wild-type Fe protein and simulation at $\lambda = 730$ nm and multiple temperatures
Magnetization curves of the L127Δ Fe protein at $\lambda = 730$ nm and multiple temperatures

(4)
Parameters for Simulation at $S = 3/2$: $D = -2.00$, $E/D = 0.21$, $g = 2.0023$

polarization = 1.94, 2.831, -3.023

(5) Magnetization curve of L127Δ Fe protein and simulation at $\lambda = 730$ nm and $T = 1.68$ K
Magnetization curves of the L127Δ Fe–MoFe complex at $\lambda = 730$ nm and multiple temperatures.
Magnetization curves of L127ΔFe – MoFe protein complex and simulation at \( \lambda = 730 \text{ nm} \) and multiple temperatures

Parameters for Simulation at \( S = 3/2 \):  
- \( D = 6.50 \)
- \( E/D = 0.056 \)
- \( g = 1.990 \)
- polarization = 1.191, 1.608, -0.190
Magnetization curves of the oxidized $\Delta nifB$ MoFe protein and simulation ($S = 3.0$) at $\lambda = 520$ nm and multiple temperatures (a) $D > 0$ and (b) $D < 0$

- **a. Simulation at $S = 3.0$ and $D > 0$**
  
  Parameters for simulation at $S = 3.0$
  $D = 4.00$; $E/D = 0.33$; $g = 2.0023$
  polarization = $0.510$, $1.100$, $0.460$

  Simulation for $S = 3.0$ all temperatures - Black lines
  - Oxidized $\Delta nifB$ MoFe protein data $T = 1.58$ K - Blue circles
  - Oxidized $\Delta nifB$ MoFe protein data $T = 4.24$ K - Green circles
  - Oxidized $\Delta nifB$ MoFe protein data $T = 9.44$ K - Red circles

- **b. Simulation at $S = 3.0$ and $D < 0$**

  Parameters for simulation at $S = 3.0$
  $D = -3.00$; $E/D = 0.08$; $g = 2.0023$
  polarization = $1.200$, $1.800$, $-0.850$

  Simulation for $S = 3.0$ all temperatures - Black lines
  - Oxidized $\Delta nifB$ MoFe protein data $T = 1.58$ K - Blue circles
  - Oxidized $\Delta nifB$ MoFe protein data $T = 4.24$ K - Green circles
  - Oxidized $\Delta nifB$ MoFe protein data $T = 9.44$ K - Dk. Pink circles

(8) Magnetization curves of the oxidized $\Delta nifB$ MoFe protein and simulation ($S = 3.0$) at $\lambda = 520$ nm and multiple temperatures (a) $D > 0$ and (b) $D < 0$
Magnetization curves of the oxidized ΔnifB MoFe protein and simulation (S = 4.0) at λ = 520 nm and multiple temperatures (a) D > 0 and (b) D < 0

Parameters for simulation at S = 4.0
D = 4.00; E/D = 0.33; g = 2.0023
polarization = 0.420, 1.120, 0.500

Parameters for simulation at S = 4.0
D = -3.00; E/D = 0.08; g = 2.0023
polarization = 1.200, 1.800, -0.850
Magnetization curves of the oxidized $\Delta nifB$ MoFe protein and simulation ($S = 2.0$) at $\lambda = 520$ nm and multiple temperatures

Parameters for simulation at $S = 2.0$

- $D = -3.00$; $E/D = 0.08$; $g = 2.0023$
- polarization = 1.200, 1.800, -0.850
VITA

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