Introduction

The need for a fixed form of nitrogen is a common factor among all life forms. Ubiquitous in biomolecules, such as nucleic acids and proteins, nitrogen is one of the most important and essential elements for life. Nitrogen comprises roughly 80% of earth's atmosphere in the relatively inert form of dinitrogen (N_2; N≡N). Dinitrogen cannot be metabolized by most organisms. In order to survive, organisms must rely on sources of reduced nitrogen such as ammonia (NH_3), nitrite (NO_2^-), nitrate (NO_3^-), and amino acids.

Reduced nitrogen, in the form of ammonia, is obtained through the process of nitrogen fixation. Playing a central figure in the global nitrogen cycle, nitrogen fixation occurs through both biological and industrial processes. Nitrogen reduction by the industrial Haber-Bosch process requires an iron catalyst, the consumption of non-renewable fossil fuels, and pressure and temperature as high as 300 atm and 723 K, respectively (40). In 1995, approximately 8×10^{10} kg of ammonia were manufactured via the Haber-Bosch process (40). However, this energy intensive process accounts for only 25% of the global production of fixed nitrogen. Conversely, biological nitrogen fixation occurs at ambient temperature and pressure. That nitrogen fixation is performed in nature at relatively low levels of temperature and pressure is indicative of the sophistication of the biocatalytic mechanism. It is through biological nitrogen fixation that approximately 60% of reduced nitrogen is obtained, at an estimated yield of 1.7×10^{11} kg per year (40). The small remainder of the global fixed nitrogen occurs as a result of electrical storms (12).

This universal demand by living organisms for a fixed source of nitrogen is satisfied by a group of nitrogen fixing organisms called diazotrophs. These organisms utilize nitrogenase, an enzyme complex that performs the six-electron reduction of atmospheric dinitrogen to ammonia. Found in both the *Eubacteria* and *Archae* kingdoms, diazotrophs exist in a variety of environmental niches ranging from free living soil organisms to symbiotic bacteria, in both aerobic and anaerobic conditions. Despite

the differing physiological conditions among the various nitrogen fixing bacteria, the nitrogenase complex is evolutionarily conserved (46).

The genetic conservation and the fact that all reported diazotrophs possessed a molybdenum (Mo) dependent nitrogenase system made it difficult to accept Mo-independent nitrogenase systems when first proposed by Bishop *et al*. in 1980. The idea of alternative nitrogenases became a reality when it was shown that strains of *Azotobacter vinelandii* with their structural genes for Mo-nitrogenase removed were still capable of reducing N_2. Two alternative systems to the Mo-nitrogenase have been found; the vanadium and iron nitrogenases. These three systems, although related to each other, are genetically distinct systems and are not merely the same protein complex with a metal substitution (23). In this study, the focus of research was on the Mo-nitrogenase system.

Enzymology of Nitrogenase

The nitrogenase complex has two component metalloproteins called the iron (Fe) protein (component 2, dinitrogenase reductase) and the molybdenum iron (MoFe) protein (component 1, dinitrogenase)(40). The Fe protein is a γ_2 homodimer ($M_r \approx 63{,}000$; encoded by *nif*H) and contains four Fe atoms organized into a Fe_4S_4 cluster that bridges its two subunits. The Fe protein is the obligate electron donor to the MoFe protein (6), harbors the sites at which MgATP is bound and hydrolyzed (6, 92), and has an as yet undetermined role in iron molybdenum cofactor (FeMo-co) biosynthesis (27, 57).

Due to high conservation of amino acid sequences from approximately 20 different nitrogenases (69), the crystal structure of the Fe protein from *A. vinelandii* (29) is considered as representative of all of the nitrogenase Fe proteins. The Fe_4S_4 center of the Fe protein is covalently linked to both subunits by cysteine 97 and cysteine 132 from each of the two subunits (29, 32, 40). The Fe protein also has a sequence characteristic of nucleotide binding proteins. Such identifying markers include a protein sequence called a Walker A motif consisting of a G-X-X-X-X-G-K-S sequence (95), as well as a β-strand •loop • α-helix structure (81), common to nucleotide binding proteins.

Upon binding of MgATP, the Fe protein undergoes a conformational change that increases accessibility of the Fe_4S_4 center to chelators such as α,α'-bipyridyl (55, 94). These changes appear to be necessary for successful electron transfer to occur. The nucleotide-induced changes also have an effect on the electronic properties of the Fe_4S_4 cluster, including altered electron paramagnetic resonance (EPR) and circular dichroism spectra (66, 78). MgATP binding to the Fe protein also induces a significant lowering of the redox potential of the Fe_4S_4 cluster from -300 to -420 mV (6, 78, 96, 99).

However enticing it may be simply to assign the function of MgATP binding to lowering the redox potential of the metal center and altering the protein conformation, it should be understood that MgADP behaves in a similar fashion. However, Fe protein with MgADP bound is unable to perform electron transfer to the MoFe protein. This suggests that MgATP binding results in changes that are critically different from MgADP induced changes and it is these changes that are important for proper electron shuttling to the MoFe protein (51, 78).

The function of MgATP and how it helps in electron transfer within the nitrogenase complex is still unclear. Recently it has been suggested that hydrolysis of MgATP is not necessary to transfer electrons from the Fe protein to the MoFe protein (51). By deleting an amino acid (Leu 127) in the signal transduction pathway of the Fe protein, the Fe protein retains a conformation resembling that of the MgATP bound state. This altered Fe protein (L127Δ) binds to the MoFe protein with high affinity and is shown to transfer a single electron without MgATP hydrolysis. This indicates the conformational reconfiguration of the Fe protein upon nucleotide binding is important in electron transfer and that if in the proper configuration, nucleotide hydrolysis is not necessary for the transfer of at least a single electron (51, 52).

The larger of the two component proteins, the MoFe protein, is a $\alpha_2\beta_2$ heterotetramer ($M_r \approx 240{,}000$; encoded by *nif*D and *nif*K, respectively) which contains two metal centers. While the Fe protein possesses a typical Fe_4S_4 cluster bridged between its two subunits, the metal centers within the MoFe protein are unique. The P-cluster spans the junction of pseudosymmetry between the α and β subunits of the MoFe

protein. The P-cluster is comprised of a Fe_8S_7 cluster in the form of two Fe_4S_3 subclusters bridged by a common inorganic sulfur (3, 71) and is thought to accept electrons from the Fe protein. During protein-protein docking, the P-clusters were predicted to be positioned squarely between the Fe_4S_4 center of the Fe protein and the FeMo-cofactor of the MoFe protein (11, 40, 47, 48). Schindelin *et al.* have crystalized an Fe:MoFe complex that was stabilized by the binding of ADP-AlF_4^- to the Fe protein's nucleotide binding region. The Fe_4S_4 cluster and the P-cluster lie along the pseudo-two-fold axis of the protein complex. This placement positions the P-cluster equidistant between the Fe_4S_4 cluster and FeMo-co as originally predicted. This suggests the P-cluster mediates intramolecular transfer of electrons to the FeMo-cofactor (80), the second metal center in the MoFe protein and site of substrate binding and reduction (33). This evidence and studies with altered MoFe proteins (60, 70), altered Fe proteins (51) and kinetic measurements (56) are consistent with the P-clusters being the primary electron acceptors in the MoFe protein.

Nitrogenase's catalytic cycle begins with the reduction of the Fe protein by flavodoxin or ferredoxin *in vivo* or by $Na_2S_2O_4$ *in vitro* (7). The reduced protein binds two MgATP and undergoes a conformational change that allows its Fe_4S_4 cluster to become more solvent-exposed. Nucleotide binding also reduces the midpoint potential of the metal center (51, 52). The Fe protein then complexes with the MoFe protein and transfers a single electron to the MoFe protein (54). Eady and coworkers (1978) demonstrated that electron transfer is coupled to MgATP hydrolysis; however, the exact order of events is still not understood. Following the transfer of a single electron and MgATP hydrolysis, the Fe:MoFe protein complex dissociates in what is the rate-limiting step of this cycle (91). The process is repeated for eight cycles until substrate is reduced, product is released and the MoFe protein returns to a resting state (11, 56).

In 1954 it was observed that nitrogenase could reduce substrates other than dinitrogen when Mozen demonstrated the reduction of nitrous oxide (67). In the years that followed it was shown that nitrogenase could reduce various substrates including protons and other triple-bonded molecules including: azide (22), cyanide and methylisocyanide (31), cyclopropene (61), and acetylene (22). To date, nitrogenase is the

only enzyme capable of reducing acetylene. The reduction of acetylene to ethylene has provided a convenient assay of nitrogenase activity using gas chromatography (72); so much so that acetylene reduction has since become a standard method in determining nitrogenase function *in vivo* and *in vitro.*

FeMo-cofactor

FeMo-cofactor was first isolated from acid denatured MoFe protein by Shah and Brill in 1977. Shah was able to restore nitrogenase activity by the addition of isolated cofactor to crude extracts containing cofactor-less MoFe protein (apo-MoFe protein)(84). The holo-MoFe protein has a characteristic $S = 3/2$ EPR signal that is absent in the apo-protein preparations. Isolated FeMo-cofactor also exhibits a $S = 3/2$ EPR signal. This $S = 3/2$ signature was seen in apo-MoFe preparations that had isolated cofactor added to them linking the EPR spectroscopic signature to the cofactor.

The structure of FeMo-co was not completely known for many years after its initial isolation (10). This novel prosthetic group consists of a metal-sulfur core composed of a Fe_4S_3 cluster bridged by three inorganic sulfides to a $MoFe_3S_3$ cluster (47). The organic acid homocitrate is coordinated to the Mo atom through its 2-carboxy and 2-hydroxy groups (47, 48), yielding a stoichiometry of Mo:Fe_7:S_9:homocitrate.

The biosynthesis and insertion of FeMo-co into the MoFe protein has become the subject of intense study. As a result of this research, many *nif* specific genes have been implicated in the development and maturation of the cofactor and its placement in the apo form of the MoFe protein, which is synthesized separately. The six specific gene products that have been implicated in FeMo-cofactor biosynthesis are NifH, NifE, NifN, NifB, NifQ and NifV.

NifH

In addition to its role as the obligate electron donor to the MoFe protein, the Fe protein (NifH), also has an obligate, yet undetermined role in cofactor biosynthesis (27, 77, 85). It is known that the Fe protein's role in cofactor biosynthesis is separate from its role in catalysis (42, 93). The observation that certain strains of *A. vinelandii* (77) and *Klebsiella pneumoniae* (27) which were altered in *nifH* failed to accumulate FeMo-co indicated the possible need for NifH in cofactor synthesis or maturation. The

requirement for NifH in the synthesis of FeMo-co was first demonstrated using the *in vitro* FeMo-cofactor synthesis assay (77, 85). Shah and coworkers found that NifH is not a sulfur donor to the cofactor (85) and it has even been suggested that NifH does not have to contain a FeS center in order to participate in cofactor biosynthesis (74). One of the possible roles is that the Fe protein, in a manner similar to its role in catalysis, may be needed for a specific redox reaction prior to MoFe protein maturation (2). NifH may help to promote an association of an additional set of subunits with apo-MoFe (1). This subunit has been identified as *nifY* in *K. pneumoniae*. A non-*nif* regulated protein called gamma protein, has been proposed to be a *nifY* analogue in *A. vinelandii* (34).

NifE and NifN

As with strains that are altered in *nifH,* strains with mutations/deletions in *nifE* and *nifN* do not accumulate FeMo-co. NifEN is a $\alpha_2\beta_2$ heterotetramer with a high degree of sequence homology between NifE and NifD and between NifN and NifK (5). The high degree of primary sequence similarity between NifEN and the MoFe protein has led to the proposal that NifEN's role in cofactor biosynthesis is as an assembly site on which FeMo-co is built upon (4, 19, 30). NifEN has two Fe_4S_4 clusters per tetramer. It has been proposed that there is one metal center located at each $\alpha\beta$ interface, in a position analogous to the P-cluster in the MoFe protein. However, the location of these metal centers is thought to be too far from the proposed assembly site of the cofactor. Therefore their incorporation into the FeMo-cofactor would be unlikely (30) making the NifEN complex simply the scaffold for, but not a donor to, the cofactor during assembly.

NifB

NifB was implicated in cofactor biosynthesis because mutations to *nifB* result in the lack of FeMo-co and that *nifB* altered strains are reactivated upon addition of isolated FeMo-co (76). While attempting purification of NifB, Shah and coworkers isolated the product of NifB, a low molecular weight FeS precursor to the FeMo-cofactor, which they termed NifB-co (87). Through the addition of B-co, the requirement for NifB in the *in vitro* cofactor synthesis assay was fulfilled. The only metal observed in NifB-co is iron, suggesting a role as an Fe donor to FeMo-co (87) and that Mo and homocitrate are

associated in later steps (30, 87). In strains of *A. vinelandii* that were altered in other biosynthetic genes (*nifH*, *nifE*, or *nifN*), NifB-co accumulated in the cell (1, 2, 87), providing additional evidence linking NifB and NifB-co with FeMo-co biosynthesis. Primary sequence analysis of the *Clostridium pasteurianum nif* system showed that the *nifN* and *nifB* genes are fused (16), indicating the possibility that NifB directly interacts with NifEN and during this interaction donates its FeS precursor to the NifEN complex.

NifB's role in FeMo-cofactor assembly is not limited to the Mo-nitrogenase system. In the two Mo-independent systems, *nifB* is required. That *nifB* has a role in the alternative nitrogenase systems leads in the direction that there is a common cofactor assembly pathway (2, 23).

NifQ

The processing of Mo prior to incorporation onto the FeMo-cofactor is associated with NifQ (41). Strains of *K. pneumoniae* deficient for *nifQ* show a Nif^- phenotype when Mo is below nanomolar concentrations but become Nif^+ upon addition of exogenous MoO_4^{2-} (41). This was also shown to be true for *A. vinelandii* (45) and *Rhodobacter capsulatus* (65). In *nifQ*$^-$ strains, the uptake of Mo is not prevented or inhibited, just lowered (41).

Sequence analyses of *nifQ* in *K. pneumoniae*, *A. vinelandii*, and *R. capsulatus* show a conserved cysteine-rich region near their carboxy-termini (20). These cysteines may provide a potential metal binding site whose location is near the surface of the NifQ protein. These data are consistent with the theory that NifQ is involved with the synthesis of a Mo-S intermediate that, in combination with the NifB product, NifB-co, would provide the bulk of metal and sulfur for cofactor synthesis (20).

NifV

Initially identified in *Klebsiella pneumoniae*, *nifV* altered strains were first isolated by their inability to grow anaerobically on nitrogen-free medium (62). They were later characterized by their ability to reduce acetylene but their inability to reduce nitrogen (62). Unlike the wild type nitrogenase, H_2 evolution by the enzyme complex of *nifV* altered strains is inhibited by carbon monoxide (62).

It was hypothesized that these altered strains were unable to fix nitrogen due to an inability to incorporate nitrogen into the cell (62). This hypothesis was tested by derepressing cultures of *K. pneumoniae* under an atmosphere of $^{15}N_2$ and then examining cell material for ^{15}N. These strains failed to incorporate ^{15}N into cell material, but were able to reduce acetylene, indicating that derepression of nitrogenase had occurred and that the altered strains could not reduce nitrogen (62).

Upon demonstrating that the altered strains had an undamaged Fe protein component, it was proposed that any defect in the altered enzyme was due to a problem within the MoFe protein. McLean and coworkers concluded that perhaps the *nifV* gene product has a role in the processing of the MoFe protein and that perhaps this processing enabled wild-type nitrogenase to be resistant to CO inhibition (62). CO binds to the enzyme but is not itself reduced (63).

The level of H_2 evolution decreased due to CO's inhibitory role, thereby increasing the ATP/2e$^-$ ratio for the altered strains (63). This increase, however, was not observed in the wild-type enzyme. In uninhibited NifV$^-$ nitrogenase and wild-type nitrogenase, there is no difference in the ATP/2e$^-$ ratio, implying that the normal coupling of ATP hydrolysis and electron transfer was not affected by the alteration in the NifV$^-$ enzyme (63). This observation led McLean and coworkers to propose that CO induced an uncoupling of ATP hydrolysis and electron transfer in the nifV strains (63). By lowering the product output of H^+ reduction but not altering the rate of ATP hydrolysis, the ATP/2e$^-$ ratio increased in the mutant strains, indicating that a futile cycling of electrons from the Fe protein to the MoFe protein was occurring (63).

Hawkes *et al.* examined the FeMo-cofactor of both of wild-type and *nifV*$^-$ *K. pneumoniae* strains. FeMo-co extracted from a *nifV* strain was added to the apo-MoFe of a *nifB* mutant. The resulting holo-enzyme reduces acetylene, but reduced nitrogen only poorly exhibits CO-dependent inhibition of H^+ reduction (33). The resulting holo-dinitrogenase exhibited a similar phenotype to the *nifV*$^-$ altered nitrogenase indicating that perhaps the defect was not in the MoFe protein, but rather, that the decrease in nitrogenase activity was due to an alteration in the cofactor (33). This provided strong evidence implicating the FeMo-cofactor as the active site of nitrogenase (33).

The metal and sulfur content of the wild type cofactor and the *nifV*$^-$ cofactor are the same (33, 64), as were EPR spectroscopic signatures (64). The search to determine the difference between the wild type and altered MoFe protein's cofactor led investigators to propose that an organic component was associated with the wild type cofactor and was presumed missing from the *nifV*$^-$ cofactor. The reasoning was three-fold: 1) the overall net negative charge of isolated cofactor was not in agreement with the metal and sulfur content of FeMo-co (10); 2) X-ray absorption studies revealed that the Mo atom was coordinated by three S atoms and three N or O atoms (25, 64); and, 3) the resistance of FeMo-co to iron-chelating reagents (10). Hoover and coworkers showed that homocitrate was the organic component physically associated with FeMo-co (33, 39).

Electron paramagnetic resonance was performed on both wild-type and NifV$^-$ MoFe protein as well as FeMo-co that had been extracted from both proteins. The EPR spectra showed that both proteins and both cofactors exhibited similar if not the same spectroscopic profile (33). If any differences did exist, they were subtle enough not to effect the $S = 3/2$ center. Both proteins possess the same ratio of Mo:Fe. Because the EPR spectra of proteins were not particularly different, it was proposed that any modification to the cofactor seemed likely to involve the Mo atom rather than any of the Fe atoms present (33). Hawkes *et al.* concluded NifV is not involved in processing the MoFe protein, but processes FeMo-cofactor. In Δ*nifV* strains, an incompletely processed cofactor results in altered substrate reducing properties (33).

Eidsness *et al.* employed extended X-ray absorption fine structure (EXAFS) spectroscopy to investigate the possibility of the involvement of Mo. The Mo K absorption edge regions for both wild-type and altered MoFe proteins were nearly identical, suggesting that Mo was in the same oxidation state in both proteins. The Mo-S and Mo-Fe distances were the same in both proteins (25). Only by performing electron nuclear double resonance (ENDOR) spectroscopy could the first physical evidence of a difference between the two proteins be obtained; the Mo was perturbed in the altered protein's cofactor (64). It was proposed that the alteration was due to either an addition, subtraction, or replacement of a non-sulfur metal ligand (64).

Hoover *et al*. had demonstrated the presence of a low molecular weight factor, termed V-factor, present in the media of actively growing *K. pneumoniae*. V-factor accumulated in all *nif* mutants except those defective in *nifV* (*nifA* mutants were not considered) (35, 36). Hoover *et al*. isolated and purified the compound and potentially identified it as homocitric acid (36). Using nuclear magnetic resonance (NMR) and mass spectroscopy (MS) the structure of V-factor was determined to be homocitrate. Further proof was obtained after performing the *in vitro* FeMo-co synthesis assay (85). During the assay, samples were given either V-factor or homocitrate in addition to all the other necessary components required for cofactor assembly. Both sets achieved the same level of activation. These experiments marked the first report of the presence and function of homocitrate in a prokaryotic organism (36).

If V-factor was indeed homocitrate, then it could be extrapolated that *nifV* encodes for homocitrate synthase. In the yeast *Saccharomyces cerevisiae*, homocitrate, an intermediate in lysine biosynthesis, is formed upon the condensation of acetyl-CoA and α-ketoglutarate (90). Sequence comparison between *nifV* and acetyl-CoA binding synthases (20) supported the hypothesis that the *nifV* gene product was a homocitrate synthase. Purification of the *nifV* gene product (98) showed that NifV was indeed a homocitrate synthase and that it not only catalyzed the condensation of acetyl-CoA and α-ketoglutarate, but it would catalyze reactions between acetyl-CoA and various other keto acids (98).

Addition of homocitrate to the medium of actively growing *K. pneumoniae* deficient for *nifV* cured the Δ*nifV* phenotype, but the mechanism was not known (38). In contrast, the addition of homocitrate to a final concentration of 1 mM to a derepressed *A. vinelandii* NifV$^-$ strain did not stimulate diazotrophic growth (44). That it was required for the *in vitro* biosynthetic assay indicated a role beyond Fe^{3+} or MoO_4^{2-} transport (38). Using ^{3}H-labeled homocitrate, Hoover *et al*. showed that homocitrate is a component of FeMo-co. The organic acid extracted from the cofactor was shown by NMR to be homocitrate and that it was in a ratio of 1:1 with Mo (39). The crystal structure of FeMo-co supports the physical association and implies that the carboxyl and hydroxyl groups on C2 are the ligands to the Mo atom (47, 48).

Homocitrate

Homocitrate is coordinated to the Mo atom of the FeMo-cofactor by its 2-carboxyl and 2-hydroxyl groups (39). It is surrounded by buried water molecules and positioned on the side of the cofactor that is nearest the P-cluster (47). Two possible functions can immediately be attributed to homocitrate. These are to mediate the transfer of protons to reduced intermediates on the cofactor and/or to function in electron transfer from the P-cluster to FeMo-cofactor during substrate reduction (47).

To clarify the role of homocitrate, Ludden and Shah developed *in vitro* methods for substituting various organic acids on the cofactor (43). The resulting dinitrogenases exhibited altered substrate specificity and inhibitor susceptibility (43). The structural characteristics of homocitrate or its substituents required for the synthesis of a cofactor that is catalytically competent are: 1) the 2-hydroxyl and 2-carboxyl groups; 2) the R-configuration around the chiral carbon; and, 3) a carbon chain length of four to six carbon atoms that contains two terminal carboxyl groups (43).

The specific function(s) of homocitrate in nitrogenase however, is still unknown. Various propositions regarding homocitrate's role in both processing of the cofactor and its role in catalysis have been theorized. In cultures of N_2-fixing *K. pneumoniae*, homocitrate was excreted into the medium in concentrations as high as 0.2 mM, possibly to outcompete and avoid incorporation of any other organic acids (2, 36). It also suggests a possible role in chelation of Fe and/or Mo uptake into the cell, and/or initial processing of the FeMo-cofactor (2, 37). This hypothesis was given strength when it was shown that when no organic acids were used in the *in vitro* FeMo-cofactor synthesis assay, there was a significant lack of ^{99}Mo accumulation in any protein-bound form. Thus homocitrate may very well be involved in early steps of cofactor biosynthesis and/or maturation (37).

Experimental Procedures

Construction of Azotobacter vinelandii strains

Throughout the course of this work, two strains of *A. vinelandii* were used. They were: (i) a strain that has seven histidine codons inserted between codons 481 and 482 of the wild-type *nifD* gene (DJ995) and (ii) a strain derived from DJ995 with a significant portion of the *nifV* gene removed (DJ1009). The construction of DJ995 was engineered by transforming a wild-type *A. vinelandii* strain designated DJ200 with the plasmid pDB827. Through double reciprocal recombination, the plasmid DNA was incorporated into the *A. vinelandii* chromosome, resulting in DJ995. Plasmid pDB827 contains a 2.9 kb *Sal*I restriction enzyme fragment with *nifD* in its entirety and portions of *nifH* and *nifK*, which flank *nifD*. This plasmid was then cloned into the pUC119 cloning vector. DJ995 was crossed with pDB312, a plasmid with a kanamycin resistance cartridge inserted into the *nifV* gene. This strain, DJ1005, was crossed with pDB107. Plasmid pDB107 contains a 0.5 kb deletion in *nifV*, removing the Km^R cartridge and much of *nifV*. This cross produced DJ1009, an *A. vinelandii* strain deleted for *nifV* and with a poly-histidine labeled MoFe protein.

Cell Growth.

A. vinelandii cultures were grown in a 150 L custom-built fermenter (W. B. Moore, Inc. Easton, PA) at 30ºC in modified Burk medium (89) which contained 10 mM Na_2MoO_4 and 10 mM urea as a fixed nitrogen source. Cultures were sparged with pressurized air (80 L/min at 5 psi) and agitated at 125 rpm. When the cell density reached ≈220 Klett units (red filter), derepression for *nif* gene expression was begun by concentrating the cell culture (6-fold) using a custom-built AG Technologies tangential-flow concentrator. Upon concentration, cells were resuspended in Burk medium with no added nitrogen source (82). Once harvested, cells were stored at -80ºC until needed.

Crude Extract Preparation

All protein manipulations were performed under anaerobic conditions maintained using a Schlenk apparatus (8). *A. vinelandii* crude extracts were prepared using an osmotic shock method (83) in degassed 25 mM Tris-HCl (pH 7.9) with 1 mM sodium dithionite added after degassing. Approximately 360 g of cells (wet weight) were processed for each purification. To reduce protease activity, phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 0.2 mM.

Purification of Poly-Histidine Tagged MoFe Protein

Prior to purification, crude extracts were brought to 500 mM NaCl by the addition of degassed, granular NaCl. Immobilized-metal-affinity-chromatography (IMAC) and DEAE-Sepharose anion-exchange chromatography (Amersham-Pharmacia, Piscataway, NJ) were used for purification of poly-histidine tagged proteins. Column eluents were monitored by absorbance at 405 nm using a Pharmacia UV-1 optical detector and control unit (Amersham-Pharmacia).

Cell extracts were loaded onto a Zn(II)-charged IMAC column (100 mL of resin in a 2.5 cm x 30 cm column) with a peristaltic pump. The column was then washed with three column volumes of Buffer A (25 mM Tris-HCl, pH 7.9, 500 mM NaCl, 1 mM Na dithionite) containing 40 mM imidazole-HCl. Any protein that remained bound to the column was then eluted using Buffer A containing 250 mM imidazole-HCl. The eluted protein was collected and diluted 8-fold in degassed 25 mM Tris-HCl buffer, pH 8.0, containing 1 mM Na dithionite. Diluted protein was then loaded onto a DEAE-Sepharose column (30 mL of resin in a 1.5 cm x 15 cm column) and eluted using a linear NaCl gradient (100 mM to 750 mM NaCl over 8 column volumes). Eluted proteins were collected at approximately 300 mM NaCl. The eluted protein was concentrated using an Amicon concentrator (Beverly, MA) fitted with a YM100 filter. Concentrated, enriched product was pelleted and stored in liquid nitrogen until needed.

Protein Quantitation

Protein was quantitated by a modified biuret method using bovine serum albumin as the standard (18).

Gel Electrophoresis

Derepression of nitrogenase in whole cells and protein enrichment were monitored via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (50). Samples were separated in polyacrylamide gels comprised of a 12% polyacrylamide (1.35% cross linker) separating gel and a 4% stacking gel. Prior to electrophoresis, samples were treated with SDS sample buffer composed of 0.06 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS (w/v), 0.05% β-mercaptoethanol, and 0.002% bromophenol blue. Samples were boiled for three minutes and electrophoresed at 25 mA/gel on a Hoefer Mighty Small apparatus (Hoefer, San Francisco, CA). Proteins were stained with 0.1% Coomassie Blue R-250 (Sigma).

Nitrogenase Assays

Nitrogenase activity of enriched wild type and altered MoFe proteins was determined by assays designed to monitor acetylene reduction and/or hydrogen evolution. Assays were performed in a 1 mL reaction mix in 9.25 mL serum vials fitted with butyl rubber stoppers and aluminum crimp seals under the appropriate atmosphere. Reaction mixtures contained 25 mM HEPES, pH 7.4, 2.5 mM ATP, 5 mM $MgCl_2$, 30 mM phosphocreatine, 27.5 units creatine phosphokinase, and 20 mM sodium dithionite. Reaction vials containing deionized distilled water and ATP regeneration solution were sealed and then degassed and flushed with the appropriate gas on an automatic fill and evacuation gassing manifold. Each vial underwent 4 cycles of 100 second evacuation

and 15 second gassing. Upon degassing, sodium dithionite was added to each sample to a final concentration of 20 mM. During acetylene reduction assays, the appropriate amount of acetylene was added following dithionite addition. Acetylene reduction assays were performed under a 10% acetylene atmosphere. Following degassing and reductant addition, 0.05 mg MoFe protein was added. Assays were initiated by the addition of 0.5 mg component 2 (Fe protein). Samples were incubated for 8 minutes with gentle shaking in a 30°C water bath and the reaction was terminated by addition of 250 μL of 0.4 M EDTA, pH 8.0. H_2 evolution was monitored by injection of 200 μL of the gas headspace into a Shimadzu GC-14 equipped with a Supelco 80/100 molecular sieve 5A column (Supelco, Bellefont, PA) and a TCD detector. Ethylene and ethane production were monitored upon injection of 200μL of the gas phase using a Hewlett-Packard 5890A gas chromatograph equipped with an Al_2O_3 capillary column and a FID detector. Standard gases of 1% H_2, 1 ppm C_2H_4, or 1 ppm C_2H_6 (Scott Specialty Gases, Inc., Plumsteadville, PA) were used to calibrate the gas chromatographs prior to assay analysis. All injections were performed using a 500μL Pressure-Lok Series A-2 gas tight syringe (Supelco, Bellefont, PA).

Preparation of Gas Mixtures

Pre-purified Ar and N_2 were purchased from Industrial Gas (Radford, VA) and pre-purified CO was purchased from Matheson (East Rutherford, NJ). Acetylene was made by adding deionized distilled water to solid CaC_2. The acetylene gas evolved was collected and sealed in a 1L separatory funnel (Kimax) and gas was withdrawn as needed. All gas transfers were performed using gas tight Hamilton syringes.

ATP Hydrolysis

MgATP hydrolysis was determined by a colorimetric assay (26). Thirty microliters of liquid assay reaction mix was passed over a Dowex AG 1-X8 analytical

anion exchange resin (BioRad, Richmond, CA) to remove inhibitory components and washed with ddH_2O to give a final volume of 3.5mL. One mL α-naphtol (1% (w/v)) and 0.5mL 1% diacetyl (v/v) were added simultaneously to the sample flow through. Samples were incubated at room temperature for 20 minutes and then sample absorbance at 520nm was determined using creatine as the standard (0-500 nmole creatine).

Ammonia Assay

Nitrogen reduction assays were performed the same conditions as H_2 evolution and C_2H_2 reduction assays but 100% N_2 was substituted for C_2H_2. The liquid reaction mix (0.5 mL) was passed over a Dowex AG 1-X8 analytical strong anion exchange resin. Column eluent was treated with 0.3mL sodium phenotide (5% (w/v) phenol plus 2.5% (w/v) sodium hydroxide) followed by 0.45 mL nitroprusside (0.02% (w/v)) and 0.45mL hypochlorite (10% (v/v)) added simultaneously. The assay mixture was incubated at room temperature for 40 minutes. Ammonia production was assayed by the indophenol method (15) with $(NH_4)_2SO_4$ as the standard (0-250 nmole NH_3). Ammonia production was measured via indophenol absorbance at 630nm.

Organic Acid Analysis

Isolated MoFe protein was heated at 100°C for ten minutes. The denatured sample was passed over a Dowex 50 chromatography column (2.0cm x 0.5cm). The eluted sample was evaporated to dryness and then incubated with 1M HCl in methanol for 24 hours at room temperature. The sample was evaporated to dryness and dissolved in methylene chloride. Once resuspended, the sample was washed with sodium bicarbonate (half saturation). The methylene chloride was removed and the sample was concentrated by evaporation under N_2. The concentrated sample was then loaded onto a gas chromtograph-mass spectrophotometer for analysis of organic acid constituents.

Metal Analysis

Fe was quantitated by the α,α' bipyridyl method (28).

Chemical Modification of Cysteines

The alkylating reagent, N-Iodoacetyl-N'-(5-sulfo-1-naphthyl) ethylenediamine (I-AEDANS), was used to determine if the purified MoFe protein had solvent exposed sulfhydryl groups, indicative of the presence of apo-MoFe protein. Six time points were considered in this experiment: 0 sec, 5 sec, 15 sec, 30 sec, 60 sec and 300 sec. For this analysis, 100 μg of protein was brought up to a volume of 40 μL by the addition of 0.5 M Tris-HCl, pH 8.0, with 1 mM Na-dithionite. The alkylation reaction was initiated by the addition of 10 μL of a 10 mM I-AEDANS solution in 0.5 M Tris-HCl, pH 8.0 to the protein sample at room temperature. Individual alkylation reactions were terminated at the designated time points by the addition of 10 μL of 1.0 M DTT. Approximately 10 μg of I-AEDANS treated protein was then loaded onto a SDS-PAGE gel (50) to separate individual protein bands and to remove excess I-AEDANS. Protein alkylation was visualized by UV illumination of the PAGE gel prior to staining with Coomassie brilliant blue.

Results

The majority of previous work with Δ*nifV* strains has been in *Klebsiella pneumoniae*. The results of that research demonstrated that the *nifV* gene product was of great significance to the formation of intact FeMo-cofactor. Due to the evident importance of this gene, we thought it prudent to investigate the effects of a *nifV* deletion in *Azotobacter vinelandii* and relate our results to those of the earlier research performed in *K. pneumoniae.* The strain used throughout this research was DJ1009. DJ1009, as previously described in Experimental Procedures, is a strain of *A. vinelandii* with a significant portion of the *nifV* gene removed, resulting in an altered MoFe protein.

Growth of DJ1009 and purification and initial characterization of the altered MoFe Protein

DJ1009 is able to grow on nitrogen-depleted media, however its growth rate is extremely slow. For this reason, cultures of DJ1009 are treated as Nif^- and are derepressed to initiate the production of nitrogenase proteins, a process performed for strains completely incapable of fixing N_2. Protein was purified via immobilized metal affinity chromatography. Altered MoFe protein extracts appeared brown in color compared to wild-type extracts which were dark brown in color. A slight red color was evident in the purified altered MoFe protein. Protein purity was assayed via SDS-PAGE and was consistent with wild-type MoFe protein. This procedure yields approximately 1.5g enriched wild-type MoFe protein (17) and approximately 500mg altered MoFe protein for every 360g of cell culture processed. To ensure that the addition of the histidine tag did not interfere with the catalytic activity of the MoFe protein, MoFe protein isolated via traditional purification protocols has been compared to poly-histidine-tagged MoFe proteins (17, Zheng, personal communication). These comparisons indicated that there are no appreciable differences between poly-histidine-tagged MoFe protein and non-tagged MoFe protein.

During purification of the altered MoFe protein it was noticed that the color of the extract while on an immobilized metal affinity column (IMAC) was slightly red and very

similar to that of apo-MoFe protein from a *nifB* deletion strain rather than a dark brown color. We used the chelator α,α' bipyridyl to quantitate the moles Fe present per mole altered MoFe protein. We calculated approximately 14.5± 1.8 Fe: altered MoFe protein. MoFe protein contains 30 Fe per mole of MoFe protein while cofactorless MoFe protein (apo-MoFe protein) possesses 16 Fe per MoFe tetramer. The presence of 14.5±1.8 moles Fe per MoFe protein in the altered strain led us to assume that a significant portion of the altered MoFe protein purified from DJ1009 was actually apo-MoFe protein.

To further investigate the presence of apo-MoFe protein in enriched samples, an alkylation experiment using the flourescent compound I-AEDANS was performed with the appropriate wild-type controls (Figure 1). Alkylations were performed to determine the presence of solvent-exposed cysteines in the peptide backbone. In wild-type MoFe, αCys275 is coordinated via its sulfhydryl group to a Fe atom of the FeMo-cofactor (49, 59). In apo-MoFe protein, it is thought that a conformational change exposes the thiol group of αCys275 to solvent (59). In samples of altered MoFe protein, alkylation occurred within 15 seconds while the intact wild-type MoFe protein did not indicate any modification. These results are consistent with those reported by Magnuson, *et al.* (59) and Christiansen, *et al.* (17).

It was reasoned that if apo-MoFe protein was present in the enriched altered MoFe protein sample, then the addition of purified FeMo-cofactor should increase the level of activity. Altered MoFe protein samples were incubated with isolated FeMo-cofactor. Acetylene assays were performed and the production of both ethylene and ethane were monitored. There was no significant increase in ethane production but the amount of ethylene produced by sample incubated with cofactor increased over 4-fold compared to protein with no additional cofactor (data not shown).

Determination of organic acid content

In *K. pneumoniae* citrate instead of homocitrate has been found to be coordinated to the FeMo-cofactor produced by Δ*nifV* strains. Knowing that deleting *nifV* prevents homocitrate from being synthesized and coordinated to the FeMo-cofactor (43), we investigated the possibility that another organic acid may be present. Gas chromatography coupled to mass spectrometry could not detect homocitrate, citrate or any other

organic acid in appreciable concentrations in the altered MoFe samples. This indicates that in *A. vinelandii*, when *nifV* is deleted, no organic acid is substituted on the FeMo-cofactor.

Electron paramagnetic resonance (EPR)

The EPR spectra for wild-type MoFe protein and altered MoFe protein are similar to each other (Figure 2). A slight broadening of the signal in the g = 3.6-4.4 region in the altered protein's spectrum could represent alterations in the positioning of the cofactor or in its environment but there are no other significant differences in the overall spectrum. In the low field region both spectrum are characteristic of the FeMo-co $S = 3/2$ spin state. These data are consistent with that found by McLean *et al*. in the *Klebsiella* system. Around the g = 2 region of the altered protein's EPR spectrum there is a separate signal which is similar to signals previously reported for inactive apo-MoFe proteins (33,Christiansen, personal communication)**.**

Nitrogenase catalytic activities

Despite an inability to effectively reduce nitrogen, altered MoFe protein is able to catalyze the reduction of acetylene and protons. The levels of activity for acetylene- and proton-reduction are both approximately 10% of wild type (Tables 1 and 2). Remarkably, altered MoFe protein from *K. pneumoniae* exhibits specific activity levels almost equivalent to wild-type MoFe protein (62). Upon analysis of acetylene reduction by the altered MoFe protein, it can be seen that the altered protein performs a four-electron reduction to produce ethane as well as a two-electron reduction to ethylene. It has been previously demonstrated that other altered strains of MoFe protein can catalyze the reduction of acetylene by both two and four electrons to yield ethylene and ethane, respectively (82). This activity is not observed in wild type strains.

Wild-type nitrogenase is insensitive to inhibition of proton reduction by CO (7, 31). In contrast, altered MoFe protein performs CO sensitive catalysis of proton reduction (Table 1). This is not novel to the *Azotobacter* system. Similar inhibition has

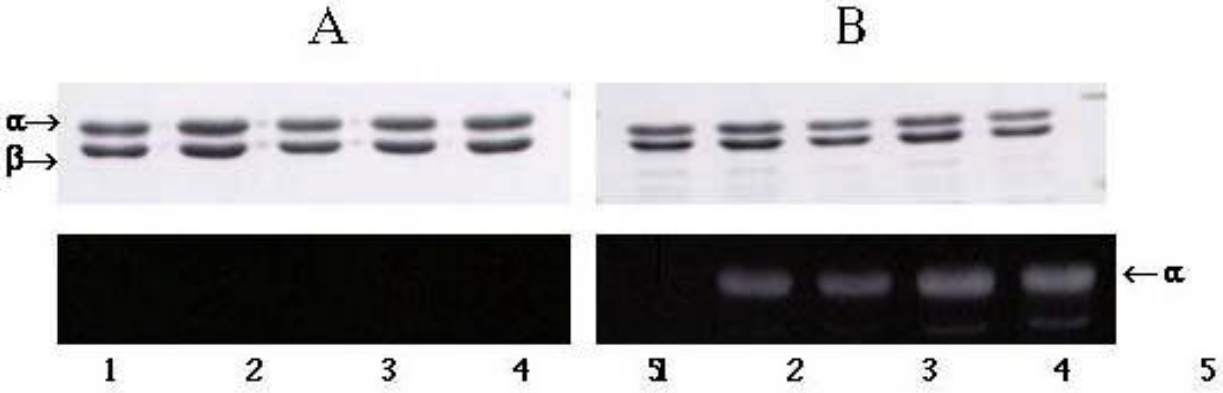

Figure 5: SDS-PAGE and alkylation of purified wild type MoFe protein (Column A) and purified NifV⁻ MoFe protein (Column B). Time course alkylations were performed by addition of 10mM I-AEDANS and quenched by addition of 1M DTT. Lane 1: 0 sec; Lane 2: 15 sec; Lane 3: 30 sec; Lane 4: 60 sec; Lane 5: 300 sec.

been noted in *K. pneumoniae* altered MoFe protein. However, inhibition by CO in *K. pneumoniae* is over 70% (33, 62) compared to a less sensitive *A. vinelandii* which exhibits roughly 50% inhibition.

It has been shown that in high enough concentrations, N_2 acts as an inhibitor of proton reduction (75). To investigate if N_2 would inhibit the performance of the altered protein, we performed nitrogenase assays under a 100% N_2 atmosphere and measured the amount of H_2 evolution. N_2 inhibited wild-type catalysis of H^+ reduction is greater than 75%. The altered protein, however, was not as inhibited by N_2, exhibiting less than 25% inhibition (Table 1).

Determination of kinetic parameters

Altered MoFe protein and wild-type MoFe protein are both capable of acetylene reduction. To determine the affinity of the enzyme for substrate, acetylene reduction to ethylene was monitored as a function of acetylene concentration. Using a Lineweaver-Burk plot the K_m for acetylene was determined to be 0.007 atm for wild-type and 0.021 atm for the altered MoFe protein (Table 3).

Strains defective in *nifV* in both *K. pneumoniae* and *A. vinelandii* exhibit little if any N_2 reduction (data not shown); however, N_2 acts as an inhibitor to both proton and acetylene reduction. To determine the pattern of N_2 inhibition of acetylene reduction, the rates of acetylene reduction were measured as a function of acetylene concentration in the presence of various fixed concentrations of N_2. Lineweaver-Burk plots were generated and from them it was determined that N_2 acts as a competitive inhibitor of acetylene reduction in both the wild-type nitrogenase and the altered enzyme, exhibiting K_i values of 0.111 atm for the wild-type and 0.625 atm for the altered strain (Table 3). CO also is an inhibitor of acetylene reduction. To determine the pattern of CO inhibition of acetylene reduction, similar assays to those measuring N_2 inhibition were performed. Measuring acetylene reduction as a function of acetylene concentration in the presence of various fixed concentrations of CO and developing Lineweaver-Burk plots, it was determined that CO behaves as a competitive inhibitor of acetylene reduction in the wild-type and as a non-competitive inhibitor of the altered enzyme.

Table 1: CO and N_2 inhibition of H_2 evolution.

Strain	Specific Activity of H_2 evolution under[3]		
	100% Ar	**10% CO/ 90% Ar**	**100% N_2**
DJ995[1]	2150±184	2090±182	550±35.2
DJ1009[2]	270±61.4	131±6.74	200±44.7

[1]Wild-type MoFe protein with poly-histidine tag
[2]Altered MoFe protein with poly-histidine tag
[3]Specific activity is expressed as nmoles H_2 produced per min per mg MoFe protein assayed.

Table 2: Specific activity[1] of acetylene reduction.

Strain	H_2	C_2H_4	C_2H_6	Total
DJ995[2]	189±17.6	1520±53.8	0.38±0.225	1710±61.8
DJ1009[3]	69±9.92	102±21.7	1.63±0.389	173±30.8

[1]Specific activity is expressed as nmoles H_2, C_2H_4, or C_2H_6 produced per min per mg MoFe protein assayed.
[2]Wild-type MoFe protein with poly-histidine tag
[3]Altered MoFe protein with poly-histidine tag

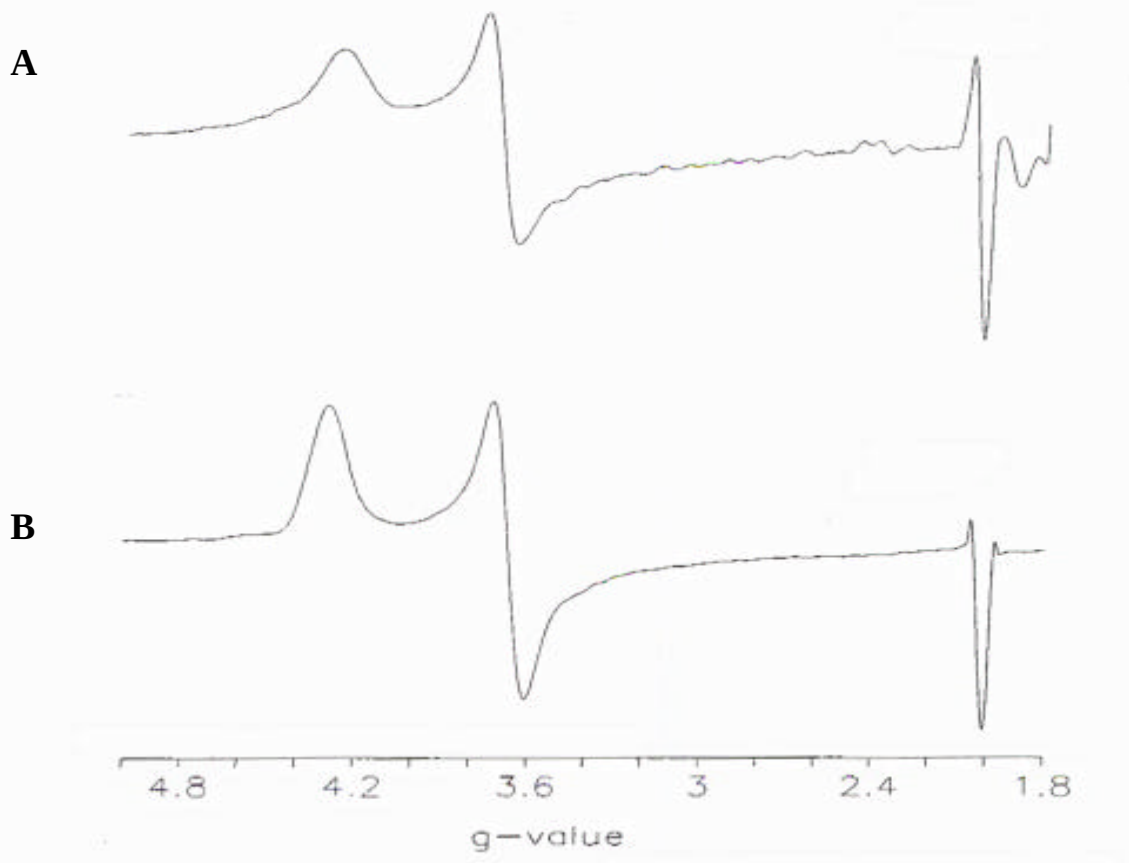

Figure 2: Electron paramagnetic resonance spectra of (A) altered MoFe protein (DJ1009) and (B) wild-type MoFe protein (DJ995).

The inhibition constant for wild-type has a K_i value of 1.5 x 10^{-4} atm. With regards to the altered MoFe protein, CO has a K_{ii} value of 8.6 x 10^{-3} atm and a K_{is} value of 4.7 x 10^{-4} atm. K_{ii} and K_{is} values were determined using secondary plots. In *K. pneumoniae*, CO also acts as a non-competitive inhibitor. Its affinity for the enzyme is higher however, exhibiting a K_{ii} value of 2.5 x 10^{-5} atm and a K_{is} value of 9.5 x 10^{-5} atm.

Another kinetic measurement performed was an assay to determine the affinity of the Fe protein for the wild-type and altered MoFe proteins. This assay involves titration of various concentrations of Fe protein and a constant MoFe protein concentration and measuring the H_2 evolution. This assay essentially treats the Fe protein as a substrate for the MoFe protein. Using Lineweaver-Burk plots, we determined the amount of Fe protein required to achieve half maximum velocity. For wild-type MoFe this value was between 2.0-7.0 μM Fe protein per 0.42 μM MoFe protein. Altered MoFe protein required 0.8-1.0 μM Fe protein per 0.42 μM MoFe protein to achieve half maximum velocity.

MgATP hydrolysis accompanying reduction of alternative substrates

Measuring the amount of MgATP hydrolyzed for each electron pair delivered to the MoFe protein is a way of determining the efficiency of the nitrogenase complex. Under an Ar atmosphere, wild-type nitrogenase hydrolyzes approximately 4.5 MgATP for every electron pair delivered by the Fe protein. Nitrogenase with the altered MoFe protein hydrolyzes more than twice the number of MgATP with a ratio of 10 MgATP/$2e^-$ (Table 4). When measured under an acetylene atmosphere, the wild-type nitrogenase consumes a similar amount of MgATP as it does under Ar. Nitrogenase with the altered MoFe protein however hydrolyzes more than twice the concentration of MgATP under acetylene than it does under an Ar atmosphere resulting in a ratio of almost 22 MgATP per electron pair. The addition of CO does not affect the wild-type's efficiency of MgATP hydrolysis and the addition of N_2 only increases the ratio to 6.3 MgATP/$2e^-$. Nitrogenase with the altered enzyme however, exhibits increases in MgATP hydrolysis when CO or N_2 is present relative to wild-type nitrogenase.

Nitrogenase with the altered MoFe protein hydrolyzes a comparable amount of MgATP under Ar or N_2; however, under an acetylene atmosphere the ratio of MgATP/$2e^-$ is remarkably high. The amount hydrolyzed is similar to conditions when N_2 is behaving as an inhibitor. This similarity may be anticipated considering that both acetylene and N_2 inhibit proton reduction.

Table 3: Kinetic parameters of wild-type MoFe protein (DJ995) and altered MoFe protein (DJ1009).

Strain	K_m (C_2H_2)	K_i (CO)			K_i (N_2)
			K_{ii}	K_{is}	
DJ995	0.007	0.00015			0.111
DJ1009	0.021		0.0086	0.00047	0.625

All values were obtained using Lineweaver-Burk plots in figures 3-6 or secondary plots (not shown) derived from figures 3-6. All values are expressed in atmospheres.

Table 4: ATP/2e$^-$ ratio of poly-histidine tagged wild-type MoFe (DJ995) protein and altered MoFe (DJ1009) protein.

Strain	**Ar**			**10% C_2H_2**	**100% N_2**
	- CO	**+CO**	**+N_2**		
DJ995	4.41±0.279	4.18±0.326	6.31±0.156	4.68±0.431	6.79±0.68
DJ1009	10.10±0.968	16.30±1.38	20.40±0.162	21.80±1.94	8.72±0.51

The values represent the ratio of MgATP hydrolyzed per 2e$^-$ transferred. In instances where a second gas was added, 10% of final volume was used.

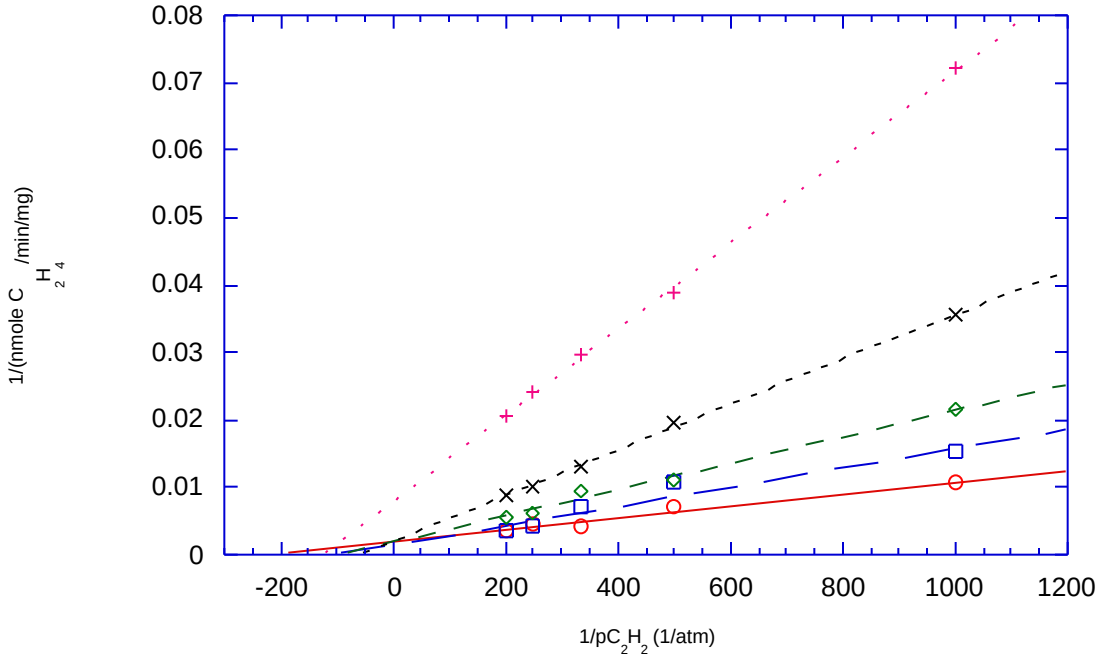

Figure 3: CO inhibition of acetylene reduction catalyzed by purified wild-type MoFe (DJ995) protein. The graph shows a series of Lineweaver-Burk plots where the reciprocal of the acetylene concentration is plotted against the reciprocal of the acetylene-reduction specific activity. Each plot was determined in the presence of fixed levels of CO. (○) 0.00025 atm CO; (□) 0.0005 atm CO; (◊) 0.0007 atm CO; (×) 0.001 atm CO; (+) 0.0025 atm CO.

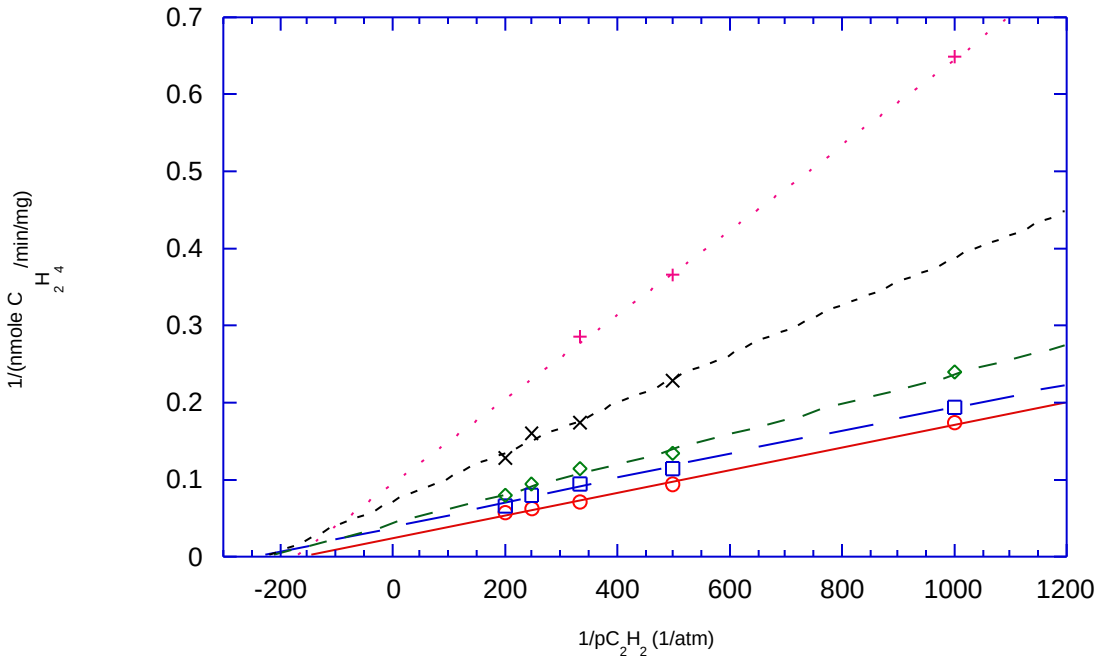

Figure 4: CO inhibition of acetylene reduction catalyzed by purified altered MoFe (DJ1009) protein. The graph shows a series of Lineweaver-Burk plots where the reciprocal of the acetylene concentration is plotted against the reciprocal of the acetylene-reduction specific activity. Each plot was determined in the presence of fixed levels of CO. (○) 0.00025 atm CO; (□) 0.0005 atm CO; (◊) 0.0007 atm CO; (×) 0.001 atm CO; (+) 0.0025 atm CO.

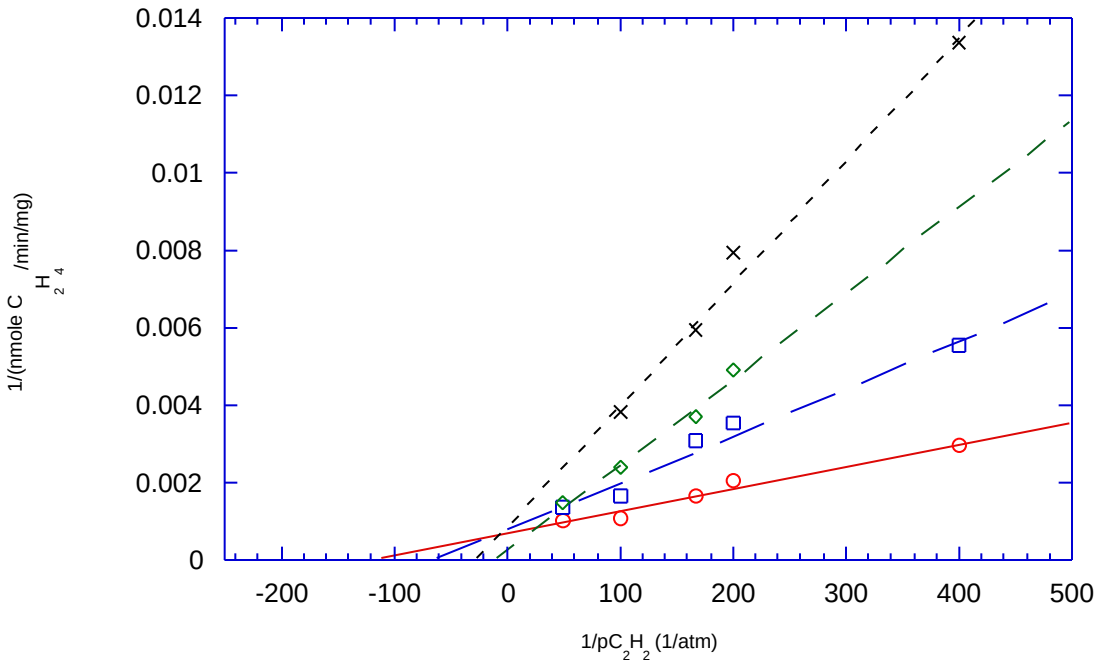

Figure 5: N_2 inhibition of acetylene reduction catalyzed by purified wild-type MoFe (DJ995) protein. The plot shows a series of Lineweaver-Burk plots where the reciprocal of the acetylene concentration is plotted against the reciprocal of the acetylene-reduction specific activity. Each plot was determined in the presence of a fixed level of N_2. (○) 0 atm N_2; (□) 0.2 atm N_2; (◊) 0.4 atm N_2; (×) 0.6 atm N_2.

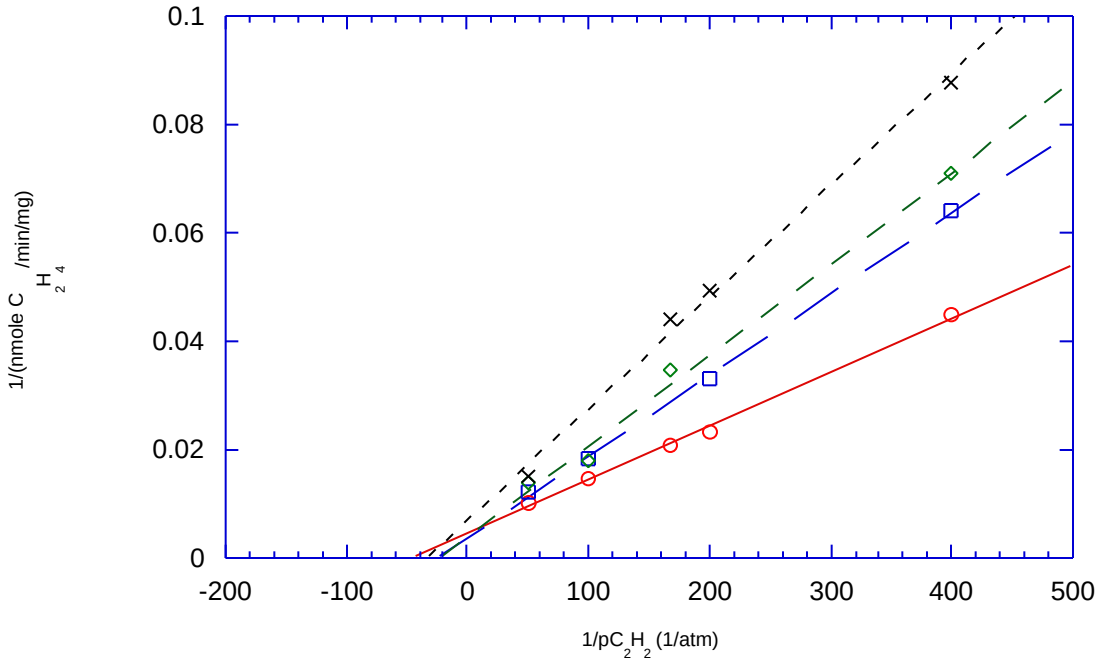

Figure 6: N_2 inhibition of acetylene reduction catalyzed by purified altered MoFe (DJ1009) protein. The plot shows a series of Lineweaver-Burk plots where the reciprocal of the acetylene concentration is plotted against the reciprocal of the acetylene-reduction specific activity. Each plot was determined in the presence of a fixed level of N_2. (○) 0 atm N_2; (□) 0.2 atm N_2; (◊) 0.4 atm N_2; (×) 0.6 atm N_2.

Discussion

The results from this work provide insight to the importance of *nifV* and homocitrate for a fully functional FeMo-cofactor. The MoFe protein from Δ*nif*V strain of *A. vinelandii* possesses altered substrate reduction and CO inhibition properties. Biophysical characterization has demonstrated that there is little difference between the MoFe proteins and FeMo-cofactors of wild-type and Δ*nif*V strains. However, in *A. vinelandii* the ability of the altered strain to perform catalysis has been compromised. Much of the difference can be attributed to a lack of the organic acid homocitrate on the FeMo-cofactor.

During purification of altered MoFe protein, the lighter color of the altered MoFe protein cell free extract was attributed to having a lower concentration of MoFe protein relative to the concentration of MoFe protein in wild-type cell free extract. The altered protein's resemblance to apo-MoFe protein when applied to an IMAC column led us to believe our extracts were a mixture of altered MoFe protein and apo-MoFe protein (17). The results of Fe quantitation determined there to be roughly half the quantity of Fe in holo-MoFe protein; however, this is only suggestive of the presence of apo-MoFe protein. Alkylation experiments further suggested the presence of apo-MoFe protein. In holo-MoFe protein there is no association of the alkylating reagent with the MoFe protein. However, in apo forms of the MoFe protein, I-AEDANS has been shown to coordinate with the thiol group of Cysα275, a ligand to the FeMo-cofactor (59).

Insertion assays showed that the activity of the altered strain increased when isolated FeMo-co was added to purified MoFe protein from a Δ*nif*V strain (data not shown). Our cofactor insertion assays with our altered MoFe protein samples did not exhibit any ethane evolution. This is not surprising because the FeMo-cofactor used was isolated from wild-type MoFe protein, which still maintains an intact homocitrate moiety.

EPR results provided interesting aspects of the altered MoFe protein. The overall spectra showed that the FeMo-cofactor in altered MoFe protein was intact. Zumft and Mortenson have shown that inactive MoFe protein will display a peak in the $g \approx 2$ region (99) similar to the peak displayed in the EPR spectrum for altered MoFe protein. Work by Christiansen, et al. have shown that apo-MoFe protein does contribute a signal in the g

≈ 2 region of the EPR spectra (17). The presence of this inactive species does not interfere with activity assays but rather data analysis and calculations where concentration of active protein is necessary. The broadening of the signal in the g = 3.6-4.4 region indicates a possible repositioning of the cofactor in the polypeptide environment. ENDOR spectroscopy has previously indicated a disturbance to the Mo on the cofactor from Δ*nif*V strains, but does not reveal any information regarding the orientation of the cofactor (64). A repositioning of the cofactor is very probable. Three amino acids, Glnα191, Gluα440 and Gluα427 normally interact with homocitrate either directly or via water molecules (48) possibly helping to lock the cofactor into position. In altered MoFe protein, these interactions do not occur due to a lack of homocitrate. In Δ*nif*V *K. pneumoniae*, similar interactions are likely to occur with citrate. However, in Δ*nif*V *A. vinelandii* citrate is not present. The interactions of these amino acids with homocitrate possibly provided an anchor for the cofactor. A homocitrate-less cofactor would have more free rotation in the cofactor-binding pocket resulting in reorientation. This reorientation, if it occurs, would not necessarily be the same throughout the sample, resulting in a non-homogeneous mixture and thereby contributing to a broadening of the EPR spectra.

Altered MoFe protein is catalytically active; however, the levels are far below that for wild-type MoFe protein. We determined the altered protein's level of both acetylene and proton reduction activity to be roughly 10% of wild-type (Table 2). This value is not necessarily an accurate representation of the altered protein's ability to perform catalysis. The presence of apo-MoFe in the altered MoFe preparations inflates the determined concentration of protein, thereby decreasing specific activity values. Assuming 50% of the MoFe preparations from DJ1009 is apo-protein, activity of the altered MoFe protein would still be significantly lower than wild-type MoFe protein. What may be said is MoFe protein with a FeMo-cofactor possessing no organic acid, performs proton, acetylene reduction and nitrogen reduction poorly relative to wild-type MoFe protein. The differences in the pattern of inhibition exhibited by CO on the altered protein versus the wild type may be explained by the idea that a second binding site is more accessible to the inhibitor that is not present on the wild type enzyme. The plot in Figure 3, representing the wild-type enzyme under conditions of increasing CO indicate

competitive inhibition except for the final concentration of CO measured. At this level it would appear that a non-competitive pattern is indicated. Since this is not seen in the altered enzyme and wild-type is known to be inhibited via a competitive mechanism, this result is likely due to intrinsic error.

In addition to a lowered level of catalytic activity, other differences in catalysis between wild-type and the altered MoFe protein occur. The production of ethane from a four-electron reduction of acetylene by the altered MoFe protein is not performed by wild-type. Other MoFe proteins with altered amino acids surrounding the cofactor are able to reduce acetylene to ethane (82). These amino acids are involved with hydrogen bonding to the cofactor and their absence may provide a secondary binding site for substrates and/or inhibitors previously unavailable, similar to what may occur when homocitrate is absent. With homocitrate missing and a possible reorientation of the cofactor, there could be more room on the cofactor to allow for the ethylene intermediate to bind and a second two-electron reduction to be performed. The missing organic acid may also expose a binding site normally not available allowing a molecule such as CO to bind to the cofactor and disrupt H^+ reduction. The lowered ability to reduce nitrogen may also be attributed to the lack of homocitrate. Perhaps dinitrogen simply falls out of the binding pocket due to a reorientation of the cofactor.

Fe protein titration assays indicated that there is an appreciable difference in the affinity of the wild-type MoFe protein for the Fe protein relative to the affinity of the altered protein for the Fe protein. The results of these assays indicate that a higher concentration of Fe protein is needed for catalysis with the altered MoFe protein (data not shown) relative to the concentration required for catalysis with the wild-type MoFe protein.

The amount of MgATP hydrolysis is greater in nitrogenase with the altered protein than in wild-type nitrogenase with the various substrates tested. This could be attributed to a futile cycling of electrons. Another possible reason that may be attributed to the higher ratio of MgATP hydrolysis per pair of electrons transferred involves the presence of apo-MoFe protein. It has been demonstrated the nitrogenase comples of apo-MoFe protein with Fe protein is able to perform nucleotide hydrolysis without performing catalysis (17). If this is true, then it would mean that more protein species is

hydrolyzing nucleotide while a significantly lower concentration of protein is actually performing catalysis. This would contribute to the higher MgATP/$2e^-$ ration. Under inhibitory conditions an uncoupling of nucleotide hydrolysis and electron transfer can be associated with the increased ratio of MgATP hydrolysis to electron transfer.

The deletion or alteration to the *nifV* gene results in a MoFe protein that is unable to reduce nitrogen in sufficient amounts under physiological conditions. The drastic changes to catalysis that have been reported in the altered strains, such as ethane evolution and CO inhibition of H^+ reduction, are strongly suggestive of the importance of the NifV protein and the presence of an organic acid, namely homocitrate. McLean and Dixon have suggested that *nifV* might have been involved in the evolutionary development of nitrogenase from a cyanide-detoxifying enzyme (62). The importance of *nifV* and its gene product cannot be understated.

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Appendix: Preparation of Acetylene Gas

The enzyme nitrogenase has also been shown to be an acetylene reductase (22). This has allowed researchers to develop fast, efficient, and easily employable acetylene reduction assays to both characterize new diazotrophic organisms as well as nitrogenase systems already known.

Our laboratory uses freshly prepared acetylene prepared in the laboratory. Stored in a separatory funnel, the gas can be withdrawn for use at the convenience of the experimenter. Although the preparation of acetylene is a simple one, it should be remembered that acetylene is an extremely hazardous compound and all care should be taken when handling it and any compounds utilized in its making. Acetylene is the product of a reaction between calcium carbide and ddH_2O. Care should be taken so as to keep the work area as dry as possible when calcium carbide is present.

The holding apparatus is comprised of a tear-drop shaped separatory funnel and a large cylindrical separatory funnel (Kimax) connected to each other by rubber tubing. The funnels in our apparatus are 500 mL and 1000 mL respectively. The holding stands should be positioned such that the tear-drop flask is in a higher position relative to the cylindrical flask. Both flasks are filled with water, making sure that the connecting tube also is filled and all air bubbles are removed. When the two flasks are filled, the stopcock on the cylindrical flask is closed.

The cylindrical flask must be filled to completion, with no headspace whatsoever. Parafilm is used to wrap tightly over the neck and top of the flask such that no air bubbles are trapped. If any air is trapped under the parafilm sheet, the film is removed and water is added to the flask before reapplying the parafilm. Once applied, the cylindrical flask is inverted.

In a low rimmed container, tap water is added to three quarters volume. We utilize a 10” x 10” x 4” plastic container. The bowl is placed under the inverted cylindrical flask. The flask head and neck are lowered into the water. Before removing the parafilm cover make sure that the head and neck are fully submerged and no air is trapped in the parafilm wrap. The parafilm sheet is carefully removed. Any air bubbles caught in the flask must be released through the connection tube. The stopcock can be

opened to allow for release of any trapped air, however, before doing so the stop cock on the tear-drop flask must be closed. At this time a suba-seal rubber stopper is submerged in the water pan, removing any air trapped in the frets or openings.

The holding apparatus can be left as is while the next set of steps is performed. A 125 mL vacuum flask is connected to rubber surgical tubing approximately 18 in. in length. Approximately 2 grams of calcium carbide are carefully added to the 125 mL flask. The canister is closed and removed from the immediate working area. To the 125 mL flask 10-15 mL of water are added and the flask is immediately plugged. The acetylene gas is allowed to vent through the tubing for a few seconds to remove any air from the surgical tube. The tube is put into the water pan up into the head and neck of the cylindrical flask. The gas enters the flask and displaces the water from the cylinder. When the flask is half to three-quarters filled with gas the tubing is removed. The 125 mL flask is unplugged and submerged into a bucket of water to quench the reaction. The rubber stopper is carefully inserted into the head of the cylindrical flask, as to not introduce air. Once the cylinder is sealed, it is re-inverted and placed onto its holding stand. The cylinder's stopcock is opened. Acetylene is withdrawn with a gas-tight syringe as needed.

VITA

Leonard Michael Comaratta was born at Camp LeJeune, NC in December, 1970. The son of a Marine, he moved around the country (with a brief stint on the island of Okinawa) during his childhood until coming to college. He completed his undergraduate degree in biology in May 1993 from Virginia Polytechnic Institute and State University. In the fall of 1994 he returned to Virginia Tech to pursue a graduate degree in biochemistry. Upon completion of his degree, he will take a position as associate scientist for CropTech Development, Inc. in Blacksburg, VA.