A COMPARATIVE STUDY OF THE STRUCTURAL FEATURES
AND KINETIC PROPERTIES OF THE MoFe AND VFe PROTEINS FROM

*AZOTOBACTER VINELANDII*

by

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ABSTRACT

A Comparative Study of the Structural Features and Kinetic Properties of the MoFe and VFe Proteins from *Azotobacter vinelandii* by Miguel Alejandro Pabón Sanclemente, Master of Science Utah State University, 2009

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Biological nitrogen fixation is accomplished in the bacterium *Azotobacter vinelandii* by means of three metalloenzymes: The molybdenum, vanadium, and iron-only nitrogenase. The knowledge regarding biological nitrogen fixation has come from studies on the Mo-dependent reaction. However, the V- and Fe-only-dependent reduction of nitrogen remains largely unknown.

By using homology modeling techniques, the protein folds that contain the metal cluster active sites for the V- and Fe-only nitrogenases were constructed. The models uncovered similarities and differences existing among the nitrogenases regarding the identity of the amino acid residues lining pivotal structural features for the correct functioning of the proteins. These differences, could account for the differences in catalytic properties depicted by these enzymes.
The quaternary structure of the dinitrogenases also differs. Such component in the Mo-nitrogenase is an $\alpha_2 \beta_2$ tetramer while for the V- an Fe-only nitrogenase is an $\alpha_2 \beta_2 \delta_2$ hexamer. The latter enzymes are unable to reduce N$_2$ in the absence of a functional $\delta$ subunit, yet they reduce H$^+$ and the non-physiological substrate C$_2$H$_2$. Therefore, the $\delta$ subunit is essential for V- and Fe-only dependent nitrogen fixation by a mechanism that still remains unknown. In attempt to understand why the $\delta$ subunit is essential for V-dependent N$_2$ reduction from a structural stand point, this work presents the strategy followed to clone the vnfG gene and purify its expression product, the $\delta$ subunit. The purified protein was subjected to crystallization trials and used to stabilize a histidine-tagged VFe protein that would otherwise purify with low Fe$^{2+}$ content and poor H$^+$ and C$_2$H$_2$ reduction activities. The VFe preparation was used to conduct substrate reduction assays to assess: i) The electron allocation patterns to each of the reduction products of the substrates C$_2$H$_2$, N$_2$, N$_2$H$_4$, and N$_3^-$; and ii) Inhibition patterns among substrate and inhibitor of the nitrogenase reaction. This work also reports on the effect N$_2$H$_4$ and N$_3^-$ has on the electron flux to the products of the C$_2$H$_2$ reduction.

The work presented herein provides information with which to compare and contrast biological nitrogen fixation as catalyzed by the Mo- and V-nitrogenases from *Azotobacter vinelandii*. 

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<td>bovine serum albumin</td>
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<td>Da</td>
<td>Dalton</td>
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<td>DFT</td>
<td>density functional theory</td>
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<tr>
<td>e−</td>
<td>electron</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EESEM</td>
<td>electro-spin echo envelope modulation</td>
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<td>ENDOR</td>
<td>electron-nuclear double resonance</td>
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<td>EPR</td>
<td>electron paramagnetic resonance</td>
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<td>EXAFS</td>
<td>extended x-ray absorption fine structure</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>immobilized-metal affinity chromatography</td>
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<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
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<td>K_m</td>
<td>Michaelis-Menten constant</td>
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<td>K_i</td>
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<td>log MW</td>
<td>logarithm of the molecular weight</td>
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<td>MCD</td>
<td>magnetic circular dichroism</td>
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<td>MgATP</td>
<td>magnesium adenosine triphosphate</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut-off</td>
</tr>
<tr>
<td>Na$_2$S$_2$O$_4$</td>
<td>sodium dithionite</td>
</tr>
<tr>
<td>NCBI</td>
<td>national center for biotechnology information</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDB</td>
<td>protein data bank</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<td>PMSF</td>
<td>phenylmethanesulphonylfluoride</td>
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<td>SDS-PAGE</td>
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Nitrogen (N) is an ubiquitous element in living organisms \(^1,2\), being part of macromolecular scaffolds such as proteins and nucleic acids. Since nitrogen oxides trapped on the earth’s lithosphere are not accessible to living organisms, almost, if not all, of the nitrogen assimilated by the biosphere comes from the vast stock of nitrogen gas (N\(_2\), or dinitrogen) found on the earth’s atmosphere (~ 80 \%). The process by which dinitrogen is transformed to a species with a higher reduction state is called nitrogen fixation.

Nitrogen fixation occurs in the upper atmosphere, where electrical discharges provide the driving force to overcome the high energy barrier needed to break the dinitrogen triple bond, which with a dissociation energy of 946 kJ/mol \(^3\), makes the dinitrogen molecule probably the most stable diatomic molecule known. Only ~ 1 \% of the total nitrogen fixed per year can be attributed to atmospheric processes that combine N\(_2\), H\(_2\)O, and O\(_2\) to form HNO\(_3\). The gross of the total amount of nitrogen fixed per year is accomplished on the biosphere by means of two processes: biological nitrogen fixation and the Haber-Bosch industrial process. Biological nitrogen fixation transforms atmospheric nitrogen into ammonia – a nitrogenous species that is readily taken up by living organism – through a biosynthetic chemical event that is thought to be only comparable in importance with photosynthesis.
The organisms that carry out the reduction of dinitrogen are called diazotrophs. These are bacteria and cyanobacteria that contain the genetic make-up to manufacture protein complexes called nitrogenases, which are metallo-enzymes that bind the triple bonded N₂ molecule and catalyze its conversion into two moles of ammonia (2NH₃) under cellular conditions (1 atm, ~ 25 °C), utilizing a reaction that requires an electron source. The human-devised version of biological nitrogen fixation, the Haber-Bosch chemical process, also utilizes the vast stock of inert atmospheric N₂ to transform it into NH₃ in the presence of H₂ with the aid of an Fe-based catalyst.

Even though the Haber-Bosch process has mimicked what nature has devised over millions of years of evolution, it is still an expensive and polluting alternative since it requires high pressures (~ 200 atm) and temperatures (~ 500 °C) - conditions generated at the expenses of fossil fuel burning - to accomplish what microorganisms otherwise do at ambient conditions. Of relevance during these days of energy concerns is the fact that ~ 1 % of the total energy used by humans is consumed by the Haber-Bosch process which, since the time of its development (in the early 20th century), has significantly contributed to the growth of the human population, from ~ 1 billion, to the current estimates of ~ 6 billion. Since the human population is expected to double within the next 50 years, it is imperative to find an alternative to the energy-demanding Haber-Bosch process to fulfill the nitrogen needs of a populated earth with ~ 10 billion human beings, if we are to maintain our current eating habits. Therefore, during the last 50 years researchers have turned their eyes toward nature to look for inspiration to
aid in the development of small molecular-weight chemical catalysts capable of achieving the high yields of nitrogen fixation obtained by the Haber-Bosch process but under the mild conditions in which the nitrogenase systems operate.

BIOLOGICAL NITROGEN FIXATION

Biological nitrogen fixation is accomplished by each of three distinct oxidoreductase complexes that differ in the metal content of their active sites, the subunit composition of their protein assembles and the structural genes that code for each of the three enzymatic systems \(^6\). The three nitrogenase complexes are expressed, just to name a few examples, in the aerobic, Gram-negative bacteria *Azotobacter vinelandii*, as well as in *Azotobacter paspali* and *Azomonas agili*, organisms that belong to the family Azotobacteracea \(^9\).

The nitrogenases (EC 1.18.6.1) are classified according to their metal content as follows: molybdenum, vanadium, and iron-only nitrogenase. The protein component of each of the nitrogenases is encoded by the *nif* operon \(^10\), the *vnf* operon \(^11\) and the *anf* operon \(^12\), for the Mo-, V- and Fe-only nitrogenases, respectively. These protein assemblies provide the coordination environments to metal scaffolds composed of iron, sulfur, and molybdenum, vanadium, or only iron. The names given to each enzymatic complex derives from the composition of the metal clusters located at their respective active sites.

The genes required for Mo-based nitrogen fixation are within the *nif* operon whereas the V- and Fe-based nitrogen fixation systems require the products of certain *nif* genes for the correct functionality of the *vnf-* and *anf-*
encoded nitrogenases\textsuperscript{13,14}. The expression of the operons that encode each protein complex depends on the metal content of the growth medium\textsuperscript{15}. Under diazotrophic growth conditions (in the absence of a “fixed” form of nitrogen), the \textit{nif} operon is expressed when concentrations of molybdenum higher than 25 nM are present in the growth medium; the genes that encode the alternative nitrogenases remain repressed at that Mo concentration. The \textit{vnf} operon is expressed when the bacteria are grown diazotrophically in a medium deficient in molybdenum but containing vanadium. On the other hand, the \textit{anf} operon is only derepressed in media deficient of both molybdenum and vanadium. Thus, the V- and Fe-only nitrogenases are referred to as the “alternative” nitrogenases because they only play a role in N\textsubscript{2} fixation when Mo is scarce. Under diazotrophic growth conditions, the Mo-nitrogenase is the preferred system to be expressed when proper concentrations of Mo are available in the media even if vanadium is present.

All three nitrogenase systems share a structural feature: they are composed of two proteins that purify separately: dinitrogenase and dinitrogenase reductase\textsuperscript{5,6}. The dinitrogenases, the protein components that harbor the enzyme active sites, are called MoFe, VFe, and FeFe proteins, being each one of them part of the Mo-, V- and Fe-only nitrogenase complexes, as their names suggest. The MoFe protein is an $\alpha_2\beta_2$ heterotetramer of $\sim$ 240 kDa encoded by the \textit{nifDK} genes (\textit{nifD} encodes the $\alpha$ subunit and \textit{nifK} encodes the $\beta$ subunit)\textsuperscript{10}; both VFe and FeFe proteins (encoded by \textit{vnfDGK} and \textit{anfDGK} genes, respectively) has been reported to purify as protein complexes of composition $\alpha\beta_2$, $\alpha_2\beta_2$, and $\alpha_2\beta_2\delta_2$\textsuperscript{16-}. 
A second protein component common to all three nitrogenases is the ~64 kDa homodimer (γ₂) dinitrogenase reductase – also called Fe protein – which is the obligate electron donor to the dinitrogenases.

The three dinitrogen reductase components are encoded by the nifH, vnfH and anfH genes. These genes share a high degree of similarity: The sequence identity between nifH and vnfH is 100% whereas between nifH/vnfH and anfH is 63%. The nifH and vnfH genes are phylogenetically indistinguishable, indicating that they have originated from a gene duplication event. This idea has been corroborated multiple times by experimental work in which VnfH has been effectively replaced by NifH as the electron donor for the reduction of the VFe protein.

**Mo-dependent Nitrogen Fixation.** The most studied nitrogenase complex, the Mo-nitrogenase, ideally catalyzes the reduction of N₂ according to the following balanced equation:

\[
N_2 + 8H^+ + 8e^- + 16\text{MgATP} \rightarrow 2\text{NH}_3 + H_2 + 16\text{MgADP} + 16\text{PO}_4^{3-} \quad (\text{Eq. 1})
\]

Figure 1-1 shows the X-ray derived model of 1/2 (αβγ₂) of the Mo-nitrogenase complex isolated from *A. vinelandii*. As previously stated, the complex is composed of two metal-containing proteins: i) the Fe protein, a homodimer (γ₂) with two MgATP binding pockets and a [4Fe-4S] cluster coordinated between the two γ subunits and, ii) the MoFe protein, a protein that purifies as a heterotetramer (α₂β₂) that contains two unique metal clusters: the P-
cluster, an \([8\text{Fe}-7\text{S}]\) cluster and the FeMo-cofactor (Figure 1-3), a \([7\text{Fe}-9\text{S}-\text{X}-\text{Mo-homocitrate}]\) cluster\(^{26}\), which is the site for substrate reduction (experimental evidence suggest that X could be C or O, but not N\(^{27}\)). There is a P-cluster located at the interface of each \(\alpha\beta\) heterodimer and an FeMo-cofactor embedded within each \(\alpha\) subunit.

**Figure 1-1. Ribbon scheme of the X-ray derived structure of the Mo-containing nitrogenase.** Depicted is one half of the complex (left) as well as the relative locations of the two bound MgATP, the \([4\text{Fe}-4\text{S}]\) cluster, the P-cluster and the active site FeMo-cofactor (right) (PDB access number 1G21).
For $N_2$ reduction to occur, the Fe protein must first bind two MgATP molecules. This causes a conformational change on the Fe protein that allows it to dock onto the MoFe protein. In an ATP-hydrolysis-driven event, the Fe protein conformational state is changed, which position the [4Fe-4S] cluster in the optimal distance and orientation for the transfer of a single electron to the P-cluster and into the MoFe protein active site. Dinitrogen is converted into ammonia when a sufficient number of electrons are accumulated at the FeMo-cofactor active site, which occurs when multiple cycles of protein docking, MgATP hydrolysis, electron transfer, and protein dissociation are repeated.

_A Glimpse at Substrate Reduction._ The Mo-nitrogenase is a versatile enzyme that is not only able to reduce $N_2$ and $H^+$; it also reduces a number of small unsaturated compounds such as acetylene ($C_2H_2$), carbonyl sulfide (COS), nitrous oxide ($N_2O$), hydrazine ($N_2H_4$), azide, cyanide, diazirine, and diazene, among many others. Thus, depending on the species being reduced, a total of two to eight electrons and protons are shuttled to the active site to accomplish substrate reduction: substrate reduction by an odd number of electrons has never been reported.

The Thorneley-Lowe cycle for the sequential reduction of the MoFe protein has been used since its proposition to explain how the sequential accumulation of protons and electrons readies the enzyme active site for reduction of substrates that are able to accept from two to up to eight electrons.
Figure 1-2. The Thorneley-Lowe cycle for the sequential reduction of the MoFe protein by multiple rounds of Fe protein docking, MgATP hydrolysis, and electron transfer. Dotted arrows denote one electron transfer event.

The cycle (Figure 1-2) starts with the MoFe protein in its dithionite-reduced state (E0). Multiple cycles of Fe protein docking to the MoFe protein sequentially transform the active site of the enzyme to more reduced states (E1, E2, E3,…E7). The different nitrogenase substrates bind and are reduced at the enzyme active site as it becomes more reduced, which implies that more reduced substrates bind to more oxidized active sites. The scheme implies H+ and C2H2 bind to the enzyme and are reduced when the active site is “mildly” reduced (E2), which occurs during the early stages of the cycle, whereas N2 can only bind and be reduced when the enzyme active site has accumulated what is believed to be 3 to 4 [H+/e−], being these oxidation states represented in the cycle as E3 and E4, respectively.
It is not known whether the electrons are successively accumulated in the active site (or P-cluster) to then reduce the substrates in one multi-electron transfer step or whether the electrons are added in a step-wise manner to the substrates as they arrive to the active site-substrate adduct. In spite of this uncertainty, the Thorneley-Lowe cycle provides a framework that can be used to explain otherwise striking observations regarding substrate-inhibitor interactions at the Mo-nitrogenase active site. For example: i) N2 is a non-competitive inhibitor of C2H2 reduction whereas C2H2 competitively inhibits the reduction of N2; ii) H2 is a competitive inhibitor of the reduction of N2 but it does not inhibit the reduction of C2H2, N2H4, or N3; iii) hydrogen evolution can be suppressed by excess C2H2 but not by excess N2; and iv) N2 is the only substrate that triggers the exchange of D2 with protons from solution to form HD.

Insight on N2 Binding at the Mo-nitrogenase Active Site. An overall understanding of the sequence of macromolecular steps needed to accomplish substrate reduction by the nitrogenase complexes has been reached and a detailed mechanism describing N2 reduction at the atomic level is being sketched based on new spectroscopy data obtained by conducting research on altered Mo-nitrogenases turning-over isotopically-labeled substrates.

A multidisciplinary approach that combines molecular biology, kinetic, spectroscopy and computational chemistry techniques have rendered evidence...
that strongly suggests the FeMo-cofactor iron belt composed of iron atoms 2, 3, 6, and 7 (Figure 1-3) as the place where substrate reduction takes place.

Figure 1-3. Stereo representation of the FeMo-cofactor modeled from X-ray data obtained from A. vinelandii Mo-nitrogenase crystals (PDB access number 1M1N). Two amino acid residues, α-275Cys and α-442His, bind the cluster to the MoFe protein catalytic pocket. The hydroxyl and carboxyl groups from R-homocitrate ligate the molybdenum atom of FeMo-cofactor. The central atom is suspected to be C or O, but not N (17). Also shown are the amino acids that provide the first shell of non-covalent interactions to the cofactor face comprised of Fe atoms 2, 3, 6 and 7 (numbering is as on 19). The color code for atoms is: Fe in green, S in yellow, Mo in magenta, C in black, N in blue, and O in red.

Substitution of the MoFe protein α-70Val residue by alanine (Figure 1-3) expands the putative enzyme active site such that larger, unsaturated molecules become substrates or even better substrates of the Mo-nitrogenase 41. Among these are alkynes such as propyne and propargyl alcohol 41, butyne 41, and more reduced forms of dinitrogen, such as hydrazine 42 and diazene 33, and methylidiazene 40.

Propargyl alcohol, hydrazine, and methylidiazene can be freeze-trapped on the enzyme active site under turnover conditions (in the presence of the Fe protein
component, MgATP, and a source of reducing equivalents, i.e. sodium dithionite) 33, 42, 43. EPR and ENDOR studies of the substrate-FeMo-cofactor interactions taking place as altered enzymes are turning over fully and partially labeled N₂, hydrazine, and methyldiazene have aided in the identification of binding geometries of N₂ reduction intermediates that are believed to arise during the course of the chemical reaction 39, 40. These trapped states only occur if the enzyme has amino acid substitution in the right combination. For example, hydrazine can only be freeze-trapped interacting with the FeMo-cofactor if a double mutation (α-70Val→Ala/α-195His→Gln) is introduced on the MoFe protein. As previously mentioned, these amino acid substitutions alter the FeMo-cofactor region comprised of Fe atoms 2, 3, 6, and 7 (Figure 1-3). This piece of experimental evidence, along with the documented N₂ chemistry on low-coordinate iron complexes 44-48, strongly support the hypothesis that advocates the FeMo-cofactor central iron atoms as the metals where N₂ binds and the N-N bond cleaved, a plausible idea considering the unsaturated coordination sphere of the iron atoms that compose the 2, 3, 6, and 7 iron belt region of the FeMo-cofactor 26.

V-DEPENDENT NITROGEN FIXATION

It is known since the 1930s that Mo and V stimulate nitrogen fixation in Azotobacter sp. 49. Half a century later, in 1980, Bishop et al showed that A. vinelandii possesses an alternative N₂-fixation system that is expressed during conditions of molybdenum deficiency 50. The community responded skeptically
to this finding – since all of the nitrogenases that had so far been purified contained molybdenum – but was then convinced by the idea of Mo-independent N₂ fixation when an *A. vinelandii* strain that specifically had the *nifHDK* operon deleted grew diazotrophically in media to which no molybdenum had been added \(^{51,52}\). During the same year, a vanadium-containing nitrogenase was isolated from *A. vinelandii* cells grown in media supplemented with vanadium \(^{17,18}\).

Unexpectedly, and two years after the isolation of the V-nitrogenase, a third nitrogenase system was isolated from *A. vinelandii* grown under combined molybdenum and vanadium limitations \(^{19}\).

The operons that code for these newly isolated nitrogenases were identified and sequenced after the enzymes were purified \(^{11,12,53}\). The genes *vnfDK* and *anfDK* showed sequence similarity to the *nifDK* genes and, unexpectedly, a small open reading frame was identified and located between the genes encoding the α and β subunits of both V- and Fe-only nitrogenases. The genes that code for the δ subunit of both VFe and FeFe proteins has since been identified in organisms that are phylogenetically related to *A. vinelandii* and *A. chroococcum*. However, the δ subunit primary structure apparently has only been used by Nature as part of the alternative nitrogenase machineries since there is no counterpart that can be identified by a BLAST search using the microbial genome collection deposited in the Blast Assembled Genome databank.

*The vnfG Gene.* The newly discovered open reading frame was called *vnfG* and *anfG*, and the product of its expression, the δ subunit, would reproducible co-purify with the VFe protein though its significance was not
known at the time\textsuperscript{18}. The role the $\delta$ subunit plays in Mo-independent nitrogen fixation has been scarcely assessed since its gene was discovered and a study demonstrated that mutant strains containing alterations in both $\text{vnfG}$ and $\text{anfG}$ genes were unable to grow in N-free, Mo-deficient medium\textsuperscript{54}. The study sought to determine the effect that a substitution of a conserved cysteine residue in both VnfG and AnfG had on cell diazotrophic growth. The cysteine residue ($\delta$-$36\text{Cys}$ on AnfG and $\delta$-$17\text{Cys}$ on VnfG) was substituted for either an alanine or a serine, without significantly affecting the generation time of the modified strains, as compared to the wild-type counterpart. These results indicated that the conserved cysteine, which was hypothesized to participate in cluster binding, was not necessary for nitrogen fixation as catalyzed by the alternative nitrogenases. However, introducing a stop codon at the cysteine position of either $\text{anfG}$ or $\text{vnfG}$ did stop diazotrophic growth of the altered cells. Likewise, deletion of $\text{anfG}$ or $\text{vnfG}$ from a strain which $\text{nifHDK}$ operon had also been deleted rendered cells unable to grow in N-free media. Interestingly, cell-free extracts of these mutant strains showed $\text{C}_2\text{H}_2$ reduction activities, which indicated that the altered $\delta$ subunits (or VFe and FeFe proteins without $\delta$ subunits) were not essential for reduction of $\text{C}_2\text{H}_2$.

It is yet unknown how the $\delta$ subunit acts to support $\text{N}_2$ reduction. Kirn and Rees suggested that it might be involved in the stabilization of the dinitrogenase 2 (VFe protein) quaternary structure\textsuperscript{55}. This hypothesis inspired experimental designs to elucidate the function of the $\delta$ subunit. Chatterjee \textit{et al.}\textsuperscript{56} reported the purification of an apodinitrogenase 2 of composition $\alpha_2\beta_2\delta_2$ which substrate
reduction activity was restored upon addition of FeV-cofactor, the V-nitrogenase metal active site. The study also demonstrated that the $\alpha_2\beta_2\delta_2$ hexamer dissociates under certain conditions to yield free $\delta$ subunit and a form an apodinitrogenase 2 with no $H^+$, $C_2H_2$, or $N_2$ reduction activities after FeV-cofactor reconstitution assays. However, the activity could be restored upon addition of VnfG to the FeV-cofactor activation assay system. The $\delta$ subunit has also been reported to bind FeV-cofactor, and in an MgATP-dependent process, activate the $C_2H_2$ reduction activity of apodinitrogenase 2 \cite{57}. Taken together, these \textit{in vitro} observations has led to a hypothesis that suggest the $\delta$ subunit is a molecular chaperon which function is to strengthen the interactions among the different VFe protein components so as to support $N_2$ reduction \cite{57,58}.

It has been well documented the effect that protein environments have on the midpoint potential of redox active centers such as heme groups and Fe:S clusters \cite{59-62}. Therefore, another plausible hypothesis to explain how the $\delta$ subunit readies the VFe protein metal active site for $N_2$ reduction is that the interactions between the $\delta$ subunit and the rest of VFe protein component position the amino acid envelope surrounding the FeV-cofactor (or even the P-cluster), such that the metal cluster can reach the high reduction potential values needed to bind and subsequently activate the $N_2$ molecule for the $6[H^+/e^-]$ reduction. A corollary of this hypothesis is that in the absence of the $\delta$ subunit, the FeV-cofactor is still capable of reaching a reduction potential sufficiently high to perform the $2[H^+/e^-]$ reduction of protons and acetylene. Experimental evidence exploring the different oxidation states that the VFe protein can access in the
absence and in the presence of the δ subunit are needed to support the previously proposed hypothesis. Likewise, a comparison of structural models for the VFe protein complex in its hexameric ($\alpha_2\beta_2\delta_2$) and tetrameric ($\alpha_2\beta_2$) state could trace changes in the hydrogen bond networks that surround the active site or even reveal the creation or destruction of covalent bonds between the protein and the metal cluster active site. Both events have been recognized to be modulators of the midpoint potential of redox-active metal centers. Biochemical and structural data would provide the elements to understand how a polypeptide with no associated catalytic activity primes the metal centers within the alternative nitrogenase active sites such that N$_2$ reduction can be carried out.

The VFe Protein Metal Active Site. The V-nitrogenase has been isolated from bacterial species such as *A. vinelandii* and *A. chroococcum* by purification procedures that, unfortunately, have resulted in protein preparations that vary in metal content, enzymatic activities, and quaternary structures. However, and in spite of the caveats that represent heterogeneous protein preparations, both proteins have been scrutinized using the techniques developed to study the Mo-nitrogenase.

Iron and vanadium EXAFS studies of purified VFe protein and extracted FeV-cofactor indicate that the latter is a metal cluster analogous to the MoFe protein FeMo-cofactor but with vanadium replacing molybdenum. The V:Fe:S cluster has been called FeV-cofactor. Several lines of evidence indicate that the FeV-cofactor is a V:Fe:S assembly akin to the Mo-nitrogenase FeMo-cofactor. For example: i) the FeMo-cofactor anchors to the MoFe protein, $\alpha$-275Cys and $\alpha$-
$^{442}\text{His}$, are conserved on the primary structure of the VFe protein $\alpha$ subunit $^{11,53}$; ii) the $nifV$ gene product, homocitrate synthase—enzyme that condensates $\alpha$-ketoglutarate and acetyl-CoA to form the FeMo-cofactor $R$-homocitrate moiety $^{64}$—is required for the proper function of the V-nitrogenase $^{14}$; iii) insertion of FeMo-cofactor into apo-VFe protein results in a hybrid enzyme with catalytic activity, which indicates that both metal clusters are structurally and electronically similar $^{65}$; and iv) V and Fe $K$-edge EXAFS spectral lines of purified VFe protein and isolated FeV-cofactor from $R$. capsulatus and $A$. vinelandii share a high degree of similarity to those from Mo and Fe in the FeMo-cofactor center of MoFe proteins. Fe $K$-edge EXAFS of extracted FeV-cofactor are dominated by Fe-Fe and Fe-S interactions similar to those of FeMo-cofactor $^{66,67}$; V $K$-edge EXAFS of purified VFe protein can be simulated by a three component fit with Fe, S, and O as the nearest neighbors to the V atoms $^{68,69}$. These assignments and the distances of the V atom from the scattering atoms are very similar to those for Mo in the FeMo-cofactor. A structural model for the V:Fe:S cluster has been proposed (Figure 1-4) based on the previously presented data, DFT and BS calculations $^{70}$, and the chemical structure of the FeMo-cofactor determined from the crystal structure of the MoFe protein $^{26}$; the only structural difference between the metal centers is the replacement of the FeMo-cofactor terminal, octahedral-coordinated Mo for a V atom at the terminal position of the FeV-cofactor.
Whereas the above observations suggest that the FeMo- and the FeV-cofactors share structural features, a closer look at the paramagnetic properties of both systems embedded in their respective protein environments reveals differences in terms of magnetic behavior. This difference in magnetic behavior is most likely linked to having different transition metals in similar Fe, S, O, and N scaffolds. The EPR spectrum of the VFe protein, in its reduced, ground state (preparations in the presence 2 mM dithionite) is significantly different and more complex than that of the MoFe protein under the same conditions, since several paramagnetic species are present, some of which have been hypothesized to arise from damaged metal centers\textsuperscript{71}. However, the spectra is still consistent with a $S = 3/2$ ground state, centered at $g = 5.5$ (spin quantitation of 0.9 spins per V)\textsuperscript{16}, with an additional $S = 1/2$ signal centered at $g = 1.94$ that, upon quantitation, yields approximately 0.4 spins per protein\textsuperscript{17}. The intensity of the signal arising from the $S = 3/2$ center has been reported to be 20-fold weaker in intensity than that of the $S = 3/2$ center of the MoFe protein, even tough the VFe protein preparations analyzed in these studies were fully active at reducing H\textsuperscript{+}, N\textsubscript{2}, and C\textsubscript{2}H\textsubscript{2}\textsuperscript{18}. 

**Figure 1-4.** A) The FeMo-cofactor and B) the proposed model of the FeV-cofactor. The suggested amino acids anchoring the FeV-cofactor to the VFe protein are included in the Figure.
Another difference is observed during enzymatic turnover in the presence of CO. Under these conditions, the Mo nitrogenase generates two different $S = 1/2$ EPR signals while neither signal is detected when the V nitrogenase from *A. vinelandii* is used.

Wild-type and altered Mo-nitrogenases have been freeze-trapped turning over isotopically-labeled $N_2$, hydrazine, methylidene, and other non-physiological substrates such as propargyl alcohol. The concomitant substrate-metal center interactions have been surveyed using EPR and ENDOR spectroscopy. This strategy has successfully provided insights into binding modes and possible mechanisms for $N_2$ reduction as catalyzed by wild-type and altered Mo-nitrogenases. In contrast, the V-nitrogenase has yet to be freeze-trapped turning over $N_2$ or any other $N_2$ analog. The realization of these series of experiments might enable to visualize several different intermediates along the $N_2$ reduction pathway as catalyzed by the V-nitrogenase. With these set of data, it could then be possible to compare and contrast biological $N_2$ reduction as catalyzed by similar protein assemblies having metal clusters which are structurally similar but possessing either molybdenum or vanadium within their structural scaffolds.

*Substrate Reduction by the V-nitrogenase.* The Mo- and V-nitrogenases are catalytic units with dissimilar catalytic properties. It has long been recognized that differences in their active site cofactor atomic compositions and the interaction of these cofactors with their respective protein environments most likely modulate the reactivity and selectivity of these systems towards $N_2$ and
non-physiological substrates. Two chemical equations have been proposed for
the reduction of N\textsubscript{2} as catalyzed by the V-nitrogenase:

\[
\text{N}_2 + 12\text{H}^+ + 12\text{e}^- + 40 \text{ (or 24)}\text{MgATP} \rightarrow \\
2\text{NH}_3 + 3\text{H}_2 + 40 \text{ (or 24)}\text{MgADP} + 40 \text{ (or 24)}\text{PO}_4^{3-} \quad (\text{eq. 2})
\]

The fact that there is a discrepancy in the number of ATP (24 or 40)\textsuperscript{71,77} needed to reduce 1 mol of N\textsubscript{2} reflects the difficulties associated with preparing
and purifying the V-nitrogenase\textsuperscript{78}. Researchers who investigate the V-
nitrogenase mode of action have acknowledged on multiple occasions that
heterogeneous protein preparations, which display inconsistent metal content
values and rates of substrate reduction, have slowed the initial, rapid progress
seen in this area during the 1980’s.

In spite of these caveats, research on V-dependent biological nitrogen
fixation has been carried on. Activity studies have shown that the V-nitrogenase
is less efficient than the Mo-nitrogenase at reducing the physiological substrate
N\textsubscript{2}. When compared with the Mo-nitrogenase, the enzyme hydrolyses at least
twice as many phosphodiester bonds to accomplish the reduction of 1 mol of N\textsubscript{2}\textsuperscript{6}. Another difference between the two systems is that seen regarding the distribution
of electrons under N\textsubscript{2}: the Mo-nitrogenase delivers \~ 25 \% of the total electron
flux to produce H\textsubscript{2} and \~ 75 \% for N\textsubscript{2} reduction\textsuperscript{5} whereas the V-nitrogenase
delivers \~ 50 \% of the total electron flux to H\textsuperscript{+} reduction and \~ 50 \% for N\textsubscript{2}
reduction\textsuperscript{6}. These observations indicate that the V-nitrogenase is a better
hydrogenase and a worse nitrogenase than the Mo-nitrogenase. As pointed out in 6, this difference might not be due to different affinities for N₂, since the apparent $K_m$ for the substrate N₂ is similar for Mo- and V-nitrogenases. The same trend is observed when the enzyme is under a C₂H₂ atmosphere: C₂H₂ is a poor substrate for the V-nitrogenase since ~ 60 % of the total electron flux is diverted to H⁺ reduction whereas the Mo-nitrogenase delivers ~ 15 % of the total electron flux to H⁺ reduction under the same conditions 5,6.

A catalytic feature that has been recognized to be a hallmark of the alternative nitrogenases is the reduction of C₂H₂ by 4[H⁺/e⁻] to yield a small but readily detectable amount of ethane (C₂H₆); ~ 2 % of the total electron flux is diverted to the formation of this product. Dilworth et al. 79 found that, for the VFe protein isolated from A. chroococcum: i) the ratio of electron flux yielding C₂H₆ to that yielding C₂H₄ is increased by raising the ratio of Fe protein to VFe protein and by increasing the assay temperature up to at least 40 ºC, and ii) pH values above 7.5 decrease the C₂H₆/C₂H₄ ratio. Two altered MoFe proteins have been reported to yield a significant amount of C₂H₆ when turning over C₂H₂ 28,80. In one of the enzymes, $\alpha$-191Gln was replaced by a lysine ($\alpha$-191Gln$\rightarrow$Lys); this enzyme deviated to the product C₂H₆ 13 % of the total electron flux. The second altered MoFe protein possesses a mutation in which $\alpha$-195His was replaced by asparagine ($\alpha$-195His$\rightarrow$Asn); this modification altered the electron flux such that 23 % of the total electron flux was diverted to the formation of C₂H₆. Interestingly, neither enzyme reduces N₂.
It is believed that both wild-type Mo- and V-nitrogenases carry out the reduction of N₂ through similar mechanisms of reaction. A few experimental observations support this thought: i) As with the Mo-nitrogenase, the V-nitrogenase catalyzes the highly stereospecific reduction of C₂H₂, giving cis-C₂D₂H₂ when the reaction is carried out in D₂O \(^{79}\); ii) During the reduction of N₂ by the Mo-nitrogenase, N₂H₄ can be detected in solution when the reaction is quenched with acid or alkali \(^{74}\); likewise, the V-nitrogenase readily produces a small but detectable amount of N₂H₄ when it is turning over N₂ \(^{81}\). These results indicate that N₂ reduction is likely to follow a mechanism of reaction in which a metal-bound dinitrogen-hydride reduction intermediate arises as the reaction proceeds. In spite of these observations, more experimental data is clearly needed to conclude that both Mo- and V-nitrogenase catalyze the reduction of N₂ by similar mechanisms of reactions. A piece of experimental evidence would be that indicating that N₂H₄ is a substrate of the V-nitrogenase. Previous experiments have indicated that N₂H₄ is a substrate of the Mo-nitrogenase \(^{30}\), thereby suggesting that this semi-reduced form of N₂ can enter the route for N₂ reduction at a latter stage of the N₂ reaction coordinate. No such assumption can yet be made for the V-nitrogenase.

*Inhibition of Substrate Reduction.* As previously mentioned in the *A Glimpse at Substrate Reduction* section, the Thorneley-Lowe cycle for the sequential reduction of the Mo-nitrogenase provides a framework to understand otherwise striking observation regarding substrate-inhibitor interactions at the MoFe protein active site. The cycle can accommodate the non-reciprocity in the
inhibition patterns observed between C\textsubscript{2}H\textsubscript{2} and N\textsubscript{2}, the fact that H\textsubscript{2} inhibits competitively N\textsubscript{2} reduction but it has no effect on the reduction of semi-reduced nitrogenous species such as hydrazine\textsuperscript{30}, azide, and methylisocyanide\textsuperscript{36}, and the H/D exchange phenomena only seen when the MoFe protein is turning over N\textsubscript{2} in the presence of D\textsubscript{2}\textsuperscript{38}.

Surprisingly, none of these kinetic behaviors, which are relevant to the mechanism of Mo-dependent N\textsubscript{2} reduction, has been assessed for the V-nitrogenase, thus weakening the proposition for a common route for N\textsubscript{2} reduction as catalyzed by both enzymes. Thus far it has been reported that H\textsubscript{2} does not inhibits the reduction of C\textsubscript{2}H\textsubscript{2}\textsuperscript{79} but it completely inhibits the reduction of N\textsubscript{2}, although kinetic data identifying the nature of the inhibition has yet to be presented. The effect CO exerts on the reduction of C\textsubscript{2}H\textsubscript{2} has also been investigated, showing surprising differences in the effect this small molecule has on the reduction of C\textsubscript{2}H\textsubscript{2}\textsuperscript{82}. In brief, at low electron flux (at a Fe protein:VFe protein ratio of 1:5) and at low CO partial pressure, the CO molecule doubles the rate of C\textsubscript{2}H\textsubscript{4} formation instead of inhibiting the reaction. Only under high CO partial pressures C\textsubscript{2}H\textsubscript{2} reduction is inhibited. Low partial pressures of CO also changes the electron distribution pattern to C\textsubscript{2}H\textsubscript{2}, resulting on an increase in the formation of C\textsubscript{2}H\textsubscript{4} and C\textsubscript{2}H\textsubscript{6} (in an equimolar manner), under either high or low electron flux.

*Towards Understanding V-based Biological Fixation.* The investigation of the V-based biological reduction of nitrogen has represented a challenge for the scientific community due to the nature of the V-nitrogenase. This metallo-
enzyme has been shown to be extremely oxygen-sensitive: exposure to air at 30 ºC in the absence of dithionite results in rapid loss of activity ($t_{1/2} = 40$ sec), in contrast with the MoFe protein, which under similar conditions has a $t_{1/2}$ of 8 min.

Moreover, the fact that the VFe protein purifies as a mixture of multimeric species presenting different stoichiometries has been recognized to be a hurdle in the studies of the V-nitrogenase, definitively contributing to the difficulties in obtaining crystals for structural studies, as mentioned in 71.

Chapter 2. This thesis presents an alternative approach to circumvent the problems associated with obtaining crystals of the VFe protein for X-ray diffraction analysis. Chapter 2 in this thesis reports on the protein folds for the dinitrogenase components of the V- and Fe-only nitrogenases obtained in silico using homology modeling techniques. “Low resolution” models of the VFe and FeFe protein folds provide so far the best approximation of the spatial arrangement of the atoms that compose these proteins. The presented models hold essential information about the spatial location of amino acid residues conserved throughout the primary structure of the nitrogenase peptide components. The structural models were built by virtue of: i) primary structure alignments of the $\alpha$ and $\beta$ subunits from the VFe, FeFe, and MoFe proteins known to be expressed in eight phylogenetically-related microorganism, and ii) model building using a molecular graphic software (Deep View, 83) which “threaded” the VFe and FeFe protein $\alpha$ and $\beta$ subunits (targets) onto the X-ray-derived MoFe protein $\alpha$ and $\beta$ subunit folds (templates) modeled at 1.16 Å resolution 84. The sequence identity existing between the target and the template
primary structures is ~ 30 %. However, after the modeling process, the resulting protein folds reveal that the amino acid identity increases as much as 80 % in those areas that are predicted to be pivotal for the function of the proteins, such as the amino acid environment surrounding the P-cluster and the FeV- and FeFe-cofactor metal active sites. This is an example of an evolutionary trend in Nature: structure is more conserved than sequence because evolution tends to conserve function and function depends more directly on structure than on sequence \(^{85}\). Therefore, since the nitrogenases share functional traits, the structure of the MoFe protein active site provides a good model for its counterparts in the VFe and FeFe proteins even if the overall sequence homology between the MoFe proteins and the alternative nitrogenases is low.

In any case, the models uncover the identities of the functional groups that most likely dictate the reactivity and the selectivity of the alternative nitrogenases, as well as the identity of the amino acid side chains that could be potentially involved in: i) the coordination of the metal clusters to the protein medium; ii) defining the selectivity and reactivity toward substrates; iii) the lining of the protein cavities from which substrates and products could enter to an exit the active site; and iv) the Fe protein docking surfaces.

*Chapter 3.* However, these structural models present a caveat: the absence of the \(\delta\) subunit protein fold. As previously mentioned, this polypeptide has been shown to be essential for the V- and Fe-only-dependent reduction of \(N_2\). Therefore, an understanding of the enzymatic properties of these enzymes from a structural stand point will be partially hindered by the lack of information
regarding the effect of the δ subunit on the overall structure of the VFe and FeFe protein folds. An approach to sidestep this problem would involve modeling a protein fold for the δ subunit using as a template an experimentally-derived structure of a protein homolog so as to then conduct in silico docking experiments between the modeled VFe and FeFe proteins and the δ subunit. However, a search on the protein data back (PDB) repository yields a structural hit from a bacterial protein transcriptional regulator (PDB accession code 2H99) that does not seem to have functional or physiological relationship with the δ subunit. Since the latter approach did not yield a positive result, the next logical step is to determine experimentally the protein fold for the δ subunit.

To this end, Chapter 3 presents a description of the cloning procedure for the vnfG gene from A. vinelandii, and the expression, purification, and crystallization trials conducted on its gene product, the δ subunit. The goal is to obtain highly ordered crystals in a reproducible manner to eventually conduct X-ray diffraction experiments. Crystallization trials have been performed and the results are described; a series of recommendations are outlined so as to guide future efforts on the necessary steps to yield protein crystals for structural analysis.

Chapter 4. As previously mentioned, the V-nitrogenase has proven to be a challenging system to study because of its sensitivity to oxygen exposure and because of the heterogeneity in metal content and subunit composition. The lack of consistency among protein preparations has probably discouraged researchers to undertake work on an otherwise interesting investigation topic: oxido-reduction
reactions carried out by a biological system with the aid of an exotic transition metal in biology, vanadium. Therefore, a quick, reproducible protocol for purifying large amounts of VFe protein could aid with the efforts invested into this area of research.

Christiansen et al. \cite{59} constructed an \textit{A. vinelandii} strain that expresses large amounts of histidine-tagged MoFe protein. A purification protocol that involves a metal-affinity chromatographic column easily and quickly separates all the soluble cellular components from the histidine-tagged protein, yielding large amounts of highly pure, active enzyme. Knowledge regarding the mechanism of Mo-dependent nitrogen fixation at a atomic scale has definitively been accelerated by the fact that highly concentrated wild-type and altered MoFe protein preparations, obtained by means of the mentioned protocol, have been used to make a variety of EPR, EESEM, and ENDOR samples of the proteins as they turn over isotopically-labelled N$_2$ and other non-physiological nitrogenous substrates with isotopically-labeled atoms at specific positions within the molecules.

Since a better understanding of the Mo-dependent reduction of nitrogen emerged in part thanks to the above mentioned strategy, a logical step that could quicken the progress in the field of V-based nitrogen fixation is to use a molecular biology strategy to construct a bacterial strain that expresses a histidine tagged VFe protein amenable for selective purification using metal-affinity chromatography. This strain was constructed in the laboratories of Dr. Dennis Dean at Virginia Tech and Chapter 4 in this thesis describes the purification strategy that leads to highly pure histidine-tagged VFe protein in large amounts.
Unfortunately, this protein preparation has low specific activities, most likely related the fact that the protein preparation exhibits low Fe$^{2+}$ content. However, upon addition of recombinant $\delta$ subunit to the cell supernatant containing the histidine-tagged VFe protein, the resulting enzymatic preparation displays higher Fe$^{2+}$ content and improved H$^+$ and C$_2$H$_2$ reduction activities. An indirect link between the $\delta$ subunit and V-based catalytic activity is presented in this chapter.

The histidine tagged VFe protein readily reduces N$_2$, H$^+$, and C$_2$H$_2$ to C$_2$H$_4$ and C$_2$H$_6$, the latter being the hallmark of the alternative nitrogenases, as previously mentioned. This chapter reports on the electron allocation patterns of the VFe protein as it reduces the above substrates, N$_2$H$_4$ (which has not been previously reported as a substrate for the enzyme), and N$_3^-$.

Likewise, a study of the inhibitory effect of N$_2$, C$_2$H$_2$, N$_2$H$_4$, and H$_2$ on the reduction of substrates is presented. These studies seek to provide experimental information with which to compare and contrast the mechanism of action of similar protein systems possessing active sites with dissimilar atomic compositions, i.e. vanadium or molybdenum as integral constituents of molecular scaffolds where substrate binding and activation occur. Lastly, Chapter 4 reports on the stimulatory effect of N$_2$H$_4$ and N$_3^-$ on the production of C$_2$H$_6$ when the enzyme is turning over C$_2$H$_2$. This effect has been previously observed when altered MoFe proteins from *A. vinelandii* turn over C$_2$H$_2$ at 30 ºC or when wild-type MoFe protein from *A. chroococcum* does so at temperatures as high as 50 ºC. However, chemical species extraneous to the nitrogenase protein folds are observed for the first time.
to dramatically re-direct the flux of electrons to augment the formation of C$_2$H$_6$, this being an effect that could not be reproduced by the wild-type Mo-nitrogenase.

Finally, this thesis lays information on biological nitrogen fixation that attempts to contribute to the field of Mo- and V-based biological reduction of nitrogen from a structural and kinetic stand point. Structural models of the VFe and FeFe protein folds are presented as well as a strategy to clone the $\textit{vnfG}$ gene, overexpress it, and purify its product, the $\delta$ subunit. Information regarding the kinetic properties of a histidine-tagged VFe protein is also presented.

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CHAPTER 2

THE FeFe AND VFe PROTEINS FROM AZOTOBACTER VINELANDII:
STRUCTURAL FEATURES REVEALED BY HOMOLOGY MODELING

ABSTRACT

In Azotobacter vinelandii, biological reduction of dinitrogen into ammonia can be accomplished by three different nitrogenase complexes: The Mo-nitrogenase and the V- and Fe-only nitrogenases. These isoenzymes differ in the metal composition of their active sites, the subunit composition of their protein assemblies, and the structural genes that code for each of the three enzymatic systems. Structural information derived from X-ray diffraction experiments has been used to generate a model of the Mo-nitrogenase protein fold and its active site, which has helped expand the basic knowledge of the mechanism of N₂ reduction at the atomic level. However, no such structural information is available for the V- and Fe-only nitrogenases, hampering efforts to establish structure/function relationships in Mo-devoid nitrogenases. In an attempt to reveal structural information that will lead to future research on the V- and Fe-only nitrogenase systems, this work present “low resolution” models of the VFe and FeFe protein folds constructed by homology modeling.
INTRODUCTION

The Molybdenum nitrogenase, the Vanadium nitrogenase and the Iron-only nitrogenase complexes are isoenzymes that reduce atmospheric N\textsubscript{2} by six electrons and six protons to form two molecules of NH\textsubscript{3} \textsuperscript{1-3}. Thus the nitrogenase complexes accomplish biological nitrogen fixation, a chemical catalysis on which life on earth depends. These metal-containing, oxygen-labile enzymes also catalyze the reduction of two H\textsuperscript{+} to H\textsubscript{2}, C\textsubscript{2}H\textsubscript{2} to C\textsubscript{2}H\textsubscript{4}, and other small, unsaturated substrates. The nitrogenases are genetically distinct biological catalysts: The Mo-nitrogenase is encoded by the \textit{nifHDK} \textsuperscript{4} operon whereas the V- and Fe-only nitrogenase complexes are encoded by the \textit{vnfHDGK} \textsuperscript{5} and \textit{anfHDGK} \textsuperscript{6}, respectively.

The nitrogenase operons have only been found in prokaryotic genomes. The cells that express these operons possess regulatory mechanisms that direct the expression of the nitrogenase gene clusters in a process that depends on the metal content of the environment where the cells inhabit \textsuperscript{7}. Thus the \textit{nif} operon, which encodes the Mo-nitrogenase, is expressed when the concentration of molybdenum is higher than 25 nM even if vanadium is present in the growth medium. On the other hand, the \textit{vnf} operon is expressed when the cells grow diazotrophically in a medium deficient in molybdenum but containing vanadium. The Fe-only nitrogenase, encoded by the \textit{anf} operon, is only manufactured when the cells grow diazotrophically in a medium deficient of both molybdenum and vanadium.

Complex biochemical machineries are required for the process of biological nitrogen fixation \textsuperscript{8}; the nitrogenases are a two-component metal-protein
assembles that purify separately: the dinitrogenase reductase and the
dinitrogenase (MoFe, VFe, and FeFe, for molybdenum, vanadium, and iron-only
dinitrogenase, respectively). The reductase components, also called Fe proteins,
are ferredoxin-like homodimers of approximately 60,000 Da\textsuperscript{9-11}. The Fe protein
harbors a [4Fe-4S] metal cluster coordinated between its subunits and two Mg-
ATP binding sites, one on each subunit. This protein serves as the obligate
electron donor to the dinitrogenase component, in a process coupled to the
hydrolysis of Mg-ATP. In contrast with the Fe proteins, which share a high
degree in sequence identity, the MoFe, VFe, and FeFe proteins are distinct from
each other in that their sequence identities are rather low\textsuperscript{4-7}, they differ in the
metal content of their active sites\textsuperscript{2}, and the quaternary structure that define them.
The MoFe protein invariably purifies as a heterotetramer (α\textsubscript{2}β\textsubscript{2}) of approximately
240,000 Da. It harbors two unique metal clusters: i) the P-cluster, which consist
of two 4Fe-4S cluster bridged by two cysteine thiol ligands, and ii) the FeMo-
cofactor, a [7Fe-9S-X-Mo-homocitrate] cluster\textsuperscript{12} (Figure 2-1), which is the site
for substrate reduction. There is a P-cluster located at the interface of each αβ
dimer and a FeMo-cofactor embedded within each α subunit.

The VFe and FeFe proteins have so far been purified as multimeric
proteins depicting different subunit stoichiometries (αβ\textsubscript{2}, α\textsubscript{2}β\textsubscript{2}, and α\textsubscript{2}β\textsubscript{2}δ\textsubscript{2}) and
degrees of enzymatic activity\textsuperscript{13-17}. Encoded by \textit{vnfG} and \textit{anfG}, the δ subunit is a
small peptide (14,000 Da) exclusively associated with Mo-independent nitrogen
fixation. This polypeptide has no known catalytic activity but it has been proven
to be essential for diazotrophic growth of \textit{A. vinelandii} cells in the absence of
molybdenum. Iron and vanadium EXAFS studies of the VFe and FeFe proteins indicate that they harbor metal clusters analogous to the MoFe protein FeMo-cofactor but with vanadium or iron replacing molybdenum (Figure 2-1). They are the so-called FeV-cofactor and FeFe-cofactor. The existence of the P-cluster within the VFe protein has been inferred from Mössbauer, MCD, and EPR spectroscopic analysis of both isolated holo-protein and FeV-cofactor. The Mössbauer spectral signature of the P-cluster was also seen in the FeFe protein.

![Figure 2-1](image_url) **Figure 2-1. The FeMo-cofactor (A) and the proposed models of the VFe- (B) and FeFe-cofactors (C).** The numbering of the Fe atoms in (A) is as described earlier (12). The suggested amino acids anchoring the VFe- and FeFe-cofactor to the VFe and FeFe protein α subunits are included in the Figure.

It is not fully known the mechanism of dinitrogen reduction as catalyzed by the nitrogenases. However, the elucidation of the three-dimensional (3D) structure of the *Azotobacter vinelandii* MoFe protein fold and its metal centers together with biochemical data collected in the previous 40 years, has permitted the elaboration of hypothesis to address important questions regarding the mechanism of Mo-dependent N₂ reduction. The structural model of the MoFe protein FeMo-cofactor center suggested where and how the different nitrogenase substrates could bind on the enzyme active site. Inspection of the
protein envelope surrounding the FeMo-cofactor allowed to pin-point the amino acid residues that would likely dictate substrate reactivity and selectivity. A combination of gene manipulation techniques, kinetic studies, and spectroscopic analysis of altered Mo-nitrogenases turning over N₂ and non-physiological substrates provides insightful observations that have led to the proposition of reduction intermediates and binding modes for different substrates on the Mo-nitrogenase FeMo-cofactor active site \(^{25-31}\). The elucidation of the 3D structure of the *A. vinelandii* MoFe protein and its metal centers has definitively helped expand the basic knowledge of the mechanism of N₂ fixation at the atomic level.

Such progress in understanding the mechanism of N₂ reduction as catalyzed by a Mo-based biological catalyst has not occurred for the V- and Fe-only nitrogenases, due in part to the lack of a detailed kinetic characterization of the reduction of N₂ and other substrates and to the fact that the structural nature of the V- and Fe-only nitrogenases protein folds and metal centers are still unavailable. To circumvent this latter problem, the protein folds of both FeFe and VFe proteins from *A. vinelandii* were constructed using homology modeling techniques \(^{32}\).

These models provide “low resolution” protein frameworks that could unravel differences regarding the identity and spatial arrangement of the amino acid residues surrounding MoFe, VFe, and FeFe protein active site clefts and P-cluster cavities that would otherwise be impossible to visualize based solely on amino acid sequence alignments. Protein architectural features such as hypothetical proton-transfer pathways could then be calculated from the predicted
VFe and FeFe protein folds. Inspection of the modeled protein folds could also reveal the amino acid residues that delineate hypothetical electron transfer pathways. This chapter discusses structural features of the modeled VFe and FeFe protein folds and how they compare with the X-ray crystal structure of the *A. vinelandii* MoFe protein 1M1N. The implications that the structural similarities and differences may have on the catalytic activities of both V- and Fe-only nitrogenases as compared with the Mo-nitrogenase will be discussed.

**METHODS**

Homology modeling is the process by which a 3D model of a target sequence is built based on a homologue experimentally solved structure (experimental processes include X-ray crystallography and solution nuclear magnetic resonance)\(^3^3\). The method relies on the observation that in nature the structural conformation of a protein is more highly conserved than its amino acid sequence, and that small or medium changes in sequence typically result in only small changes in the 3D structure\(^3^4\). All homology-modeling methods consist of the following four steps: i) template selection (known 3D structure of related protein); ii) target-template primary structure alignment; iii) model building; and iv) evaluation\(^3^2,3^5\). Following is a description of the methodology employed to generate the predicted models of the VFe and FeFe protein folds.

*Template Selection.* The template used to perform the homology modeling was the nitrogenase MoFe protein αβ pair from *A. vinelandii* (protein data bank accession code 1M1N)\(^3^6\). From hereon, the amino acid numbering is...
that of *A. vinelandii* α-subunit and β-subunit, unless otherwise indicated. A specific amino acid residue will be called α- or β-, to identify the chain where it is located, followed by the position where it is located within the chain, and the amino acid three letter code in superscript, e.i. α-70Val refers to a valine residue located in the 70th position of the *A. vinelandii* MoFe protein α-subunit.

**Multiple Sequence Alignment.** The MoFe protein α-subunit and β-subunit amino acid sequences from eight randomly selected bacteria were aligned using the web tool Multalin ([http://prodes.toulouse.inra.fr/multalin/multalin.html](http://prodes.toulouse.inra.fr/multalin/multalin.html)) Multalin uses an algorithm which approach is based on the dynamic-programming method of pairwise alignment, a computational tool that performs hierarchical clustering alignments using the matrix of the pairwise alignment scores. The protein sequence databank codes for each of the aligned nitrogenase α-subunit/β-subunit sequences are: NIFD_AZOVI/NIFK_AZOVI (*Azotobacter vinelandii* MoFe protein α/β subunits), ANFD_AZOVI/ANFK_AZOVI (*A. vinelandii* FeFe protein α/β subunits), VNFD_AZOVI/VNFK_AZOVI (*A. vinelandii* VFe protein α/β subunits), NIFD_BRAJA/NIFK_BRAJA (*Bradyrhizobium japonicum* MoFe protein α/β subunits), NIFD_KLEPN/NIFK_KLEPN (*Klebsiella pneumoniae* MoFe protein α/β subunits), NIFD_CLOPA/NIFK_CLOPA (*Clostridium pasteurianum* MoFe protein α/β subunits), B29042/PN0681 (*Rhodobacter capsulatus* MoFe protein α/β subunits), and A23874/B23874 (*Rhizobium* sp. MoFe protein α/β subunits).

**Model Building.** Deep View (Swiss-PDB Viewer) was the software used to generate the predicted 3D models for the αβ pairs of the FeFe and VFe
proteins. An αβ pair from the X-ray derived model of the A. vinelandii MoFe protein was used as the template onto which the VFe and FeFe protein α and β subunit pair were threaded. To generate the core of the model, the backbone atom positions of the template structure and the targets were averaged. The template was weighted by its sequence similarity to the target sequences; significantly deviating atom positions were excluded. Model side chain reconstruction of the target sequences was based on the weighted positions of corresponding residues in the template structure. Possible side chain conformations were selected from a backbone dependent rotamer library. Since the sequence identity between the template and the target sequences is ~ 30%, inaccurate target-template superimposition was expected; this problem was corrected by manually editing the target-template alignment using as a guide the result obtained in the multiple sequence alignment. Deviations in the protein structure geometry were regularized by steepest descent energy minimization using GROMOS96 force field.

**Water and Gas Channel Predictions.** The program CAVER, installed as a plug-in in the molecular modeling software PyMOL, was used to identify the likely water and gas channel paths on the MoFe protein and the predicted VFe and FeFe protein folds. CAVER uses an algorithm that performs a search within a protein skeleton which has been modeled on a discrete 3D grid space. In this study the grid side dimension was specified to be 0.8 Å. Initially, the user specifies the starting search point; the specified starting point for CAVER search was the X-atom within the FeMo-cofactor iron cage as modeled in the MoFe
protein αβ pair from *A. vinelandii* 1M1N. CAVER works by traversing grid points in an empty space of a pocket/tunnel (starting the calculations at the specified starting point), preferring those points that have more empty space around. A cost-function evaluates and gives higher penalties to points that are closer to protein atoms; points with low cost-function values are preferred, and thus connected to define the path of the tunnel. The calculation ends when the searching process reaches the protein convex boundaries as computed by the program Qhull 44.

*Electrostatic Potential Maps.* The electrostatic potential maps of the Fe protein docking interface for the experimentally-determined MoFe protein fold, and the predicted VFe and FeFe protein folds were calculated with the APBS plug-in installed in PyMOL. Originally written by Baker *et al.* 45, APBS is a software package for modeling biomolecular solvation through solution of the Poisson-Boltzmann equation. The electrostatic potential maps calculation was performed using the default PyMOL parameter values for solvent dielectric constant of 80 and protein dielectric constant of 2. The experiments were run at 298K and the ionic strength was at its physiological value of 0.145 M.

**RESULTS AND DISCUSSION**

*Amino Acid Sequence Alignment.* The primary structures of the *A. vinelandii* MoFe, VFe, and FeFe protein α subunits and β subunits, including those from five randomly selected nitrogen-fixing microbes, were aligned as a preparatory step to undertake the construction of the αβ, VFe, FeFe protein folds.
The alignment results (Figure 2-S1) identified the amino acid residues that have been conserved along the course of the nitrogenase complexes evolution and indicate that the MoFe protein α subunit shares 31% sequence identity with the VFe protein α subunit and 29% with the FeFe protein α subunit whereas both VFe and FeFe protein α subunits show 56% sequence identity. Amino acids such as α-275Cys and α-442His – both of which anchor the FeMo-cofactor to the Mo-nitrogenase α subunit – are thoroughly conserved in the α subunit sequences examined in this and other studies 2. α-70Val is a residue located over a specific FeMo-cofactor Fe face 46; when this residue is substituted by alanine or glycine the putative enzyme active site is expanded such that larger, unsaturated alkynes – such as propyne, propargyl alcohol 47, and butyne 47 – and more reduced forms of dinitrogen – such as hydrazine 26, methyl diazene 25, and diazene 29 – become substrates or better substrates of the Mo-nitrogenase. Therefore, α-70Val plays a role in dictating substrate selectivity within the Mo-nitrogenase active site. Not surprisingly, this amino acid residue has been conserved within the nitrogenase α subunits and has as counterpart α-56Val in both VFe and FeFe protein α subunits. Likewise, residues such as MoFe protein α-195His and α-191Gln have as counterparts α-179His and α-175Gln, in the VFe and FeFe protein α subunits, respectively. Biochemical and spectroscopy data indicate that that the mentioned amino acids residues may function as the reactive groups that donate protons to the substrates and may also dictate substrate reactivity and selectivity 48.

A similar level of sequence identity is present among the MoFe, VFe, and FeFe protein β subunits: The sequence alignment shows that the primary structure
of the MoFe protein β subunit shows 29% identity with both VFe and FeFe protein β subunits whereas both VFe and FeFe protein β subunits show 56% sequence identity (Figure 2-2S). A series of cysteine residues that compose the protein envelope of the Mo-nitrogenase P-cluster are conserved on both VFe and FeFe protein β and α subunits: Amino acid residues such as β-153Cys, β-70Cys, and β-188Ser, which coordinate the P-cluster to the protein matrix along with α-154Cys, α-62Cys, and α-88Cys, are all conserved in the MoFe, VFe, and FeFe α/β subunit sequences that were selected to perform this study.

Model Building. The sequence similarities between the MoFe protein α/β subunit and the VFe/FeFe protein α/β subunit (~30%) fall within a homology modeling band that has been called the “twilight zone” because the quality of homology modeling may vary widely. Thus the strategy to follow in order to construct a reliable model is to manually edit the sequence alignment without perturbing the highly-conserved core regions located in both α and β subunits. Deep View features a tool that allows users to manually adjust the alignment while visually verifying the structural implications, e.g. the placement of insertions or deletions in the correct structural context or the conservation of structural features with a functional role. Simultaneously, amino acid side chains that make clashes with neighboring atoms are replaced after consulting the Deep View rotamer library. In such manner the predicted VFe and FeFe αβ pair folds were built. These models were then submitted for automated refinement to the SWISS-MODEL server via the “Submit Modeling Request” tool in Deep View. The final models are shown in Figure 2-2, in which ribbon diagrams depicting the
VFe and FeFe $\alpha\beta$ pair folds have been superimposed over the structure of a MoFe $\alpha\beta$ pair. The RMS values for the VFe and FeFe $\alpha\beta$ pairs superimposed over the MoFe protein counterpart are 0.92 Å and 1.16 Å, respectively, as calculated using the “Align” command in the software PyMOL. This last batch of refined models was used throughout this work to make structural comparisons among the MoFe and the predicted VFe and FeFe protein folds.

Several structural features can be highlighted upon examination of an overlay of the MoFe protein and the modeled VFe and FeFe protein $\alpha\beta$ pairs (Figure 2-2): i) An open space exists in the interface of the modeled $\alpha\beta$ pairs. This tunnel connects the surface of the proteins with the putative active site cavity, as previously identified in $^{46,51,52}$; ii) A $\sim$ 15 Å long alpha helix segment that stretches at the N-terminus of the MoFe protein $\alpha$ subunit is absent on the VFe and FeFe $\alpha\beta$ pair models; iii) Deep View modeled an extended loop with a stretch of 12 amino acids located at the N-terminus of the VFe protein $\alpha$ subunit; in the MoFe protein X-ray structure the stretch of amino acids that is structural equivalent to the loop was modeled as an alpha helix spanning 31 amino acid residues on the C-terminus of the $\beta$ subunit; iv) No steric impediments that would disrupt the putative pockets that allocate both P-cluster and the FeMo-cofactor metal centers were found in the predicted VFe and FeFe protein $\alpha\beta$ pairs.

However, notice that on the previous protein folds the $\delta$ subunit has not been included, which could affect the reliability of the models. The $\delta$ subunit of both VFe and FeFe proteins has no counterpart that can be identified by a BLAST search using the microbial genome collection deposited in the Blast Assembled
Genome databank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). However, a BLAST search using the Protein Data Bank database (ftp://ftp.wwpdb.org) retrieved a structure hit, a transcriptional regulator from the bacteria genera *Acinetobacter* sp. (PDB accession code 2H99), for which the δ subunit has a primary structure similarity value below 25%. A model of the δ subunit using as a template the mentioned structure hit was not constructed because both proteins share low primary structure identity and have no known functional or physiological relationship. Thus the δ subunit protein fold cannot be constructed (unless de novo methods are used) until the crystal structure of a protein homolog (that could be used as a template on a homology modeling experiment) is deposited in the PDB repository. Therefore, questions addressing the essentiality of the δ subunit in Mo-independent nitrogen fixation will remain unanswered from a structural standpoint in view of the availability of neither predicted nor experimentally solved protein fold for the δ subunit.
Figure 2-2. Ribbon diagrams of the polypeptide fold for nitrogenase proteins. Shown is the Fe protein docking face from the MoFe protein. (panel A) The ribbon diagram for one αβ subunit pair of the MoFe protein is shown with the α-subunit in magenta and the β subunit in cyan (PDB file 1M1N). (panel B) The predicted ribbon diagram for an αβ subunit pair of the FeFe protein (with the α-subunit in light red and the β subunit in dark red) is shown superimposed on the MoFe protein αβ-subunit pair. (panel C) The predicted ribbon diagram for an αβ subunit pair of the VFe nitrogenase (with the α subunit in light blue and the β subunit in dark blue) is shown superimposed on the MoFe protein αβ subunit pair.
The VFe and FeFe Protein Putative Active Site Cavities. The predicted models of the VFe and FeFe protein folds depict a cavity within their α subunits that could harbor the FeV- and FeFe-cofactors. The FeMo-cofactor, with a radius of approximately 3.3 Å and 7 Å in length, occupies a cavity that is 10.86 Å in length and 9.31 Å in width (measured from the MoFe protein 1M1N structure). The predicted VFe protein fold shows a space with a length of 10.89 Å and a width of 9.52 Å; this space, which would make up the putative FeV-cofactor cavity, is sufficiently ample to allocate a FeV-cofactor with a predicted length of 7.62 Å. The putative FeFe-cofactor cavity is 10.89 Å in length and 9.87 Å in width, which is also sufficiently ample to be occupied by a FeFe-cofactor with a predicted length of 7.82 Å. Thus, the models contain a cavity with no spatial constrains that could be occupied by the metal active sites.

Isolated FeMoco-cofactor is unable to reduce dinitrogen. Hence, the FeMo-cofactor must be protein-bound to support substrate reduction, indicating that the polypeptide environment around the cofactor must contribute to its substrate binding and reduction properties. The amino acids that form part of the FeMo-cofactor protein envelope are highly conserved in the VFe and FeFe protein α subunits. These amino acid residues are shown in Figure 2-3; they would compose the first shell of non-covalent interactions between the VFe and FeFe protein α subunits and their corresponding metal active sites. The stereo image representations of the predicted amino acid that compose the metal cluster cavity within the VFe and FeFe protein folds provide a spatial framework to pinpoint conserved and non-conserved amino acid residues and to position them
relative to the metal cluster active site. Table 2-1S catalogs these amino acids as identical, similar, and non-similar. Invariable amino acid residues, such as $\alpha$-275$^{\text{Cys}}$, $\alpha$-442$^{\text{His}}$, $\alpha$-195$^{\text{His}}$, $\alpha$-191$^{\text{Gln}}$, and $\alpha$-70$^{\text{Val}}$, find their corresponding residues in the VFe and FeFe $\alpha$ subunit models. A total of 60\% of the surveyed amino acids are identical and are spatially equivalent, according to the models. Thus, the amino acid identity among $\alpha$ subunits increases from an overall of $\sim$ 30\% to as much as 60\% in the space that encompasses the protein part of the enzyme active sites. A difference is $\alpha$-96$^{\text{Arg}}$, which in spite of being conserved in the primary structure of the VFe and FeFe protein $\alpha$ subunits, the predicted models consider $\alpha$-82$^{\text{Lys}}$ as the $\alpha$-96$^{\text{Arg}}$ counterpart and place its side chain at a distance of 5 Å from where a hypothetical FeV-cofactor 4Fe-4S face would be located; the side chain of $\alpha$-96$^{\text{Arg}}$ is $\sim$ 4 Å above any atom that is part of the FeMo-cofactor 4Fe-4S face composed of Fe atoms 2, 3, 6, and 7 (Figure 2-1). The guanidino group of $\alpha$-96$^{\text{Arg}}$ has been proposed to be part of a hydrogen bonding network placed above the mentioned 4Fe-4S face since it is within hydrogen bonding distance to the carbonyl groups of $\alpha$-70$^{\text{Val}}$ and $\alpha$-69$^{\text{Gly}}$, which also approach the same 4Fe-4S face. MoFe proteins with $\alpha$-96$^{\text{Arg}}$ substituted by either glutamine or alanine depict altered substrate reduction capabilities, indicating its importance in enzymatic catalysis. Thus, it can be reasonable to consider that $\alpha$-82$^{\text{Lys}}$, having a different side chain and located farther from the metal center as compared with $\alpha$-96$^{\text{Arg}}$, could confer both V- and Fe-only nitrogenases novel substrate reduction capabilities, such as their property to yield
Figure 2-3. Stereoview of the amino acid environment around the FeMo-cofactor.  (panel A) Shown are selected amino acid residues in the MoFe protein located within 5 Å of the FeMo-cofactor (with atom colors Fe in green, S in yellow, Mo in magenta, C in black, N in blue, and oxygen red).  Shown in red are the predicted positions of corresponding amino acids in the FeFe protein.  (panel B) Shown in blue are the predicted positions of corresponding amino acids in the VFe protein.  The top amino acid labels correspond to the MoFe protein, while the bottom labels correspond to the FeFe (panel A) and VFe proteins (panel B).
four electron reduction products upon \( \text{N}_2 \)
\(^{57} \) and \( \text{C}_2\text{H}_2 \)
\(^{58} \) reduction. There are other amino acid residues lining the putative VFe and FeFe protein metal cluster cavity that have no counterpart in the FeMo-cofactor cavity (see Table 2-1S). Therefore, subtle differences in the amino acid environment that delineates the putative VFe and FeFe protein active site cavity, in addition to the replacement of Mo for V or Fe in the metal cluster active site, could account for the differences in catalytic properties displayed by the Mo-, V-, and Fe-only nitrogenases.

*The VFe and FeFe Protein Putative P-cluster Cavities.* The P-cluster is located at the interface between the MoFe protein \( \alpha \) and \( \beta \) subunits \(^{46,51} \). Its two 4Fe-4S clusters are bridged by two cysteine thiol ligands (from residues \( \alpha -88\text{Cys} \) and \( \beta -95\text{Cys} \)) \(^{12} \). The remaining four Fe atoms are coordinated each by a single cysteine thiol ligand (from residues \( \alpha -154\text{Cys} \) and \( \alpha -62\text{Cys} \), \( \beta -70\text{Cys} \) and \( \beta -153\text{Cys} \)). Figure 2-3 shows a stereo view of the amino acid residues that compose the putative P-cluster cavity in the VFe and FeFe protein models, in which it can been seen that the non-bridging residues and the residues that coordinate the P-cluster to the MoFe protein \( \alpha \) and \( \beta \) subunits are conserved and overlap well over the modeled VFe and FeFe protein \( \alpha \) and \( \beta \) subunits (average RMS of 0.8 Å). Only three of the sixteen surveyed VFe protein side chains (all located 5 Å away from the P-cluster) are not conserved. Thus, the sequence identity increases from \( \sim 30 \) % for the overall \( \alpha \) and \( \beta \) subunits to \( \sim 80 \) % for the putative P-cluster protein envelope. The same trend is seen for the FeFe protein P-cluster cavity: fourteen of the sixteen amino acids surveyed are identical, raising the sequence identity among the amino acids that compose the putative FeFe-cofactor cavity to \( \sim 90 \) %.
Table 2-2S list the amino acids that are located 5 Å away from the P-cluster and catalogs them as identical and non-conserved.

Figure 2-4. Stereoview of the amino acid environment around the P-cluster. (panel A) Shown are selected amino acid residues in the MoFe protein located within 4 Å of the P-cluster (see legend to Figure 2-3 for colors). Shown in red are the predicted positions of corresponding amino acids in the FeFe protein. (panel B) Shown in blue are the predicted positions of corresponding amino acids in the VFe protein. The top amino acid labels correspond to the MoFe protein, while the bottom labels correspond to the FeFe (panel A) and VFe proteins (panel B).
The P-cluster, which is ~ 7.7 Å in length and ~3.8 Å in width, could occupy the pocket (with dimension of 11.6 Å in length and ~ 6 Å in width) delineated by the surveyed side chains, which are located at the interface of the proposed VFe and FeFe α and β subunit folds.

*Putative Proton-Transfer Pathway.* The Mo-nitrogenase metal cluster active site is buried ~ 10 Å away from the surface of the protein. Because substrate reduction requires the addition of protons, there must be a proton-transfer pathway from the surface of the protein to the active site of the enzyme. Such channel has been proposed to exist and a promising candidate was revealed upon examination of the MoFe protein 1M1N structure \(^{46,52}\). For this study, the software CAVER calculated and displayed the channel, which is located on the interface of the MoFe protein αβ pair (Figure 2-5A). This channel, which in the MoFe protein structure is water-filled, is lined by amino acid residues which side chains are predominantly charged or hydrophilic (~ 75 %). The “bucket-brigade” mechanism for proton transfer propose that the transfer could occur via a shuttling mechanism in which protons are transferred from one amino acid side chain to another, or to water, until they reach the substrate \(^8\). This channel is also thought to be a dynamic path from which substrates and products diffuse in and out of the protein. CAVER was used to search for channels that would connect the surface of the modeled VFe and FeFe proteins to their putative active site cavities. The software found an interstice between the α and β subunits of both VFe and FeFe protein folds (Figure 2-5B, C) that is also delineated by predominantly hydrophilic amino acid side chains (~ 85 %).
This aperture connects the putative active site cavities with the surface of the modeled proteins following a path that resembles that found on the X-ray structure of an αβ pair from the MoFe protein 1M1N structure. However, a hypothetical loop modeled as part of the VFe protein fold crosses the channel on the protein surface boundaries; the channel within the modeled FeFe protein is not disrupted by any modeled structural feature. However, it must be noticed that the tunnel calculations were performed in models that do not include the δ subunit protein fold. Thus, the location of the hypothetical channels could be incorrect because it is unknown where the δ subunit, binds on the VFe and FeFe proteins.

The amino acid side chains that delineate the proposed proton-transfer path are listed on Table 2-3S. A total of 23 spatially equivalent side chains were surveyed, 8 of which have not been conserved, 7 were similar, and the remaining 8 were identical. Since more than half of the amino acids that define the walls of the hypothetical path have been conserved among the nitrogenases, it seems plausible to believe that there could be similarities among the proposed MoFe protein proton-transfer path and the hypothetical VFe and FeFe protein counterparts, even though the δ subunit was not included in the calculation to find channels within the modeled VFe and FeFe protein folds.
Figure 2-5. **The proton-transfer pathway.** The proposed proton transfer pathway that connects the protein surface to the FeMo-cofactor is shown for the MoFe protein (panel A), and the modeled FeFe (panel B) and VFe protein (panel C). The view is perpendicular to the αβ subunit interface.
Electrostatic Potential Map of the Fe Protein Docking Face. The Fe protein docking face on the MoFe protein has a surface area that ranges from ~2,000 Å² to ~3,500 Å², depending on the Fe protein nucleotide state. The Fe protein from the V-nitrogenase (VnfH) can dock onto this surface and transfer electrons to the MoFe protein, forming a fully functional nitrogenase. This cross-reactivity is possible if the contact surfaces of the different Fe proteins (NifH, VnfH, and AnfH) are structurally similar and if there are similarities in the distribution of electronic charges on the Fe protein docking face of the MoFe, VFe, and FeFe proteins. As for structural similarities, the primary structure of NifH shows 100% identity with VnfH, and both NifH and VnfH show 62% identity with AnfH. In terms of the distribution of electronic charges, Figure 2-6 shows the electrostatic potential maps that correspond to the MoFe protein and the modeled VFe and FeFe protein Fe protein docking faces.

Figure 2-6. Electrostatic potential maps of the Fe protein docking interface. The atomic partial charges and Van der Waals radii for the MoFe (panel A), FeFe (panel B) and VFe (panel C) proteins were assigned using the CHARMm parameter set. The calculations were performed through the DS DelPhi module (DS Modeling 1.1, Accelrys), which uses a finite difference solution to the non-linear Poisson-Boltzmann equation on a cubic grid. Positive charges are colored red, negative charges are colored blue, and neutral charges are colored white.
These electrostatic potential maps show two marked features that are common to the MoFe and the predicted VFe and FeFe protein folds: i) A positively charged surface on the center of the two-fold axis that approximately relates the αβ subunit pairs, which is the region where the P-cluster is located within the MoFe protein fold, and ii) A negatively charged ring around the center of the αβ pair two-fold axis. Overall, the MoFe protein and the modeled VFe and FeFe proteins present similar distribution of electrostatic charges on their Fe protein docking faces. Therefore, the compatibility among the nitrogenase components can be attributed to the fact that the Fe proteins are structurally equivalent analogs, and that these proteins interact with protein surfaces with almost identical distribution of charges. Finally, differences in substrate electron allocation and substrate selectivity and reactivity among the nitrogenases is probably not a consequence of the interactions between the MoFe, VFe, and FeFe proteins with their matching Fe protein components but rather a consequence of the metal variability and the structural differences in the MoFe, VFe, and FeFe proteins active sites.

CONCLUSION

The sequence identity among the *A. vinelandii* MoFe, VFe and FeFe protein α and β subunits was found to be sufficiently high to undertake the construction, using homology modeling techniques, of αβ pair folds for the VFe and FeFe proteins. An αβ pair from the *A. vinelandii* MoFe protein 1M1N structure was selected as the template onto which the primary structure of both
VFe and FeFe protein α and β subunits were threaded. After model building and refinement, the RMS values for the VFe and FeFe αβ pairs superimposed over the MoFe protein counterpart were 0.92 Å and 1.16 Å, respectively. Attempts to model the VFe and FeFe protein δ subunit were hampered by the lack of an available, experimentally solved template, affecting the reliability of the generated models.

The modeled protein folds present structural features that are shared with the template. Common, important architectural motifs, relevant for protein function, include a cavity within the α subunit that was generated as a result of the modeling process. This cavity is where the putative metal cluster active site could be located. Interestingly, the sequence identity among the amino acid residues that line the proposed cavity increased to ~ 60 %. In contrast, the sequence identity among the α subunits is ~ 30 %. Subtle differences in the spatial location and side chain composition of the putative active site cavity were located. Slight modifications in the protein environment that composes the active site of the examined models, as compare with that of the MoFe protein, could result in proteins having very distinctive catalytic properties. A similar trend was observed for the putative P-cluster cavity: the sequence identity among the amino acid residues that line the proposed P-cluster cavity increased to as much as ~ 90 %. Another shared structural feature is the proposed proton-transfer path. This interstice connects the surface of the proteins to the putative active site cavity. The modeled channel had a high degree of hydrophilic character: ~ 80 % of the
side chains that delineate the walls of both VFe and FeFe protein model channels are hydrophilic.

The predicted VFe and FeFe protein αβ pair folds provide molecular models with which to guide mutagenesis efforts that seek to identify: i) amino acid residues involved in electron transfer and active site reactivity and selectivity; ii) amino acid residues that line the route from which substrates gain access to the enzyme active site cavity and products exit to the surface of the protein; iii) amino acid residues that are essential for Fe protein docking. Structural, kinetic, and spectroscopic studies of modified enzymes should be conducted in order to validate the predictions based on the V- and Fe-only nitrogenase models generated by homology modeling.

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http://www.pymol.org


CHAPTER 3

THE VNFG GENE FROM AZOTOBACTER VINELANDII: CLONING, EXPRESSION, PURIFICATION, AND CRYSTALLIZATION OF ITS PRODUCT, THE VFe PROTEIN δ SUBUNIT

ABSTRACT

The V-nitrogenase is one of three phylogenetically-related nitrogenases found in the bacterium *Azobacter vinelandii*. The *vnfDGK* operon codes for the dinitrogenase component of the V-nitrogenase (the VFe protein). When expressed, the *vnfDGK* operon provides the information to manufacture the VFe protein α, β, and δ subunits. Mutant strains containing alterations in the *vnfG* gene are unable to grow in N-free, Mo-deficient medium. Interestingly, the strains are still able to reduce H⁺ and C₂H₂. This indicates that the δ subunit is essential for the V-dependent reduction of nitrogen but not for H⁺ or C₂H₂ reduction. Currently, the mechanistic role the δ subunit plays in nitrogen reduction as catalyzed by the VFe protein still remain largely unknown. The 3D structure of the δ subunit could provide clues as to how it primes the active site of the VFe protein for N₂ reduction. Unfortunately, such information is not available. As a way to initiate studies addressing the essentiality of the δ subunit for V-dependent N₂ reduction, this work present the strategy followed to clone the *vnfG* gene and purify its expression product, the δ subunit. The purified protein was studied
using gel filtration chromatography and subjected to crystallization trials. The results of these experiments are shown and discussed briefly.

INTRODUCTION

The biological reduction of atmospheric nitrogen (N₂) into ammonia (NH₃) is accomplished by metal-containing enzymes called nitrogenases. In addition to reducing N₂, these enzymes also reduce H⁺ and the non-physiological substrate C₂H₂, among other small, unsaturated substrates. Thus far three genetically distinct nitrogenase systems have been characterized in *Azotobacter vinelandii*: the molybdenum, vanadium, and iron-only nitrogenases. The Mo-, V-, and Fe-only nitrogenase structural genes are clustered in three different operons: *nif* (for nitrogen fixation), *vnf* (for vanadium-dependent nitrogen fixation), and *anf* (for alternative nitrogen fixation) operons, respectively. The expression of the operons that encode for these enzymes is strictly regulated by the metal availability in the growth medium: in the presence of molybdenum the *nif* operon is the preferred system to be expressed even if vanadium is present in the growth medium; the *vnf* operon is derepressed in the absence of molybdenum in medium containing vanadium. Lastly, the *anf* operon is expressed in medium depleted of both molybdenum and vanadium.

The nitrogenases are oxygen-labile enzymatic complexes composed of two protein components: the dinitrogenase reductase and the dinitrogenase (1, 2, and 3 for molybdenum, vanadium, and iron-only dinitrogenase, respectively). The dinitrogenase reductase component is a homodimer of approximately 60,000
Da that contains a 4Fe-4S cubane cluster bridging its subunits and two Mg-ATP binding sites (one on each subunit) \(^7,^8\). This protein serves as the obligate electron donor to the dinitrogenase component in a process coupled to Mg-ATP hydrolysis and a source of reduction equivalents (ferredoxins or flavodoxins in \textit{vivo} and sodium dithionite in \textit{vitro}). Each of the nitrogenase operons possesses on their 5′ ends a copy of the gene that encodes for the dinitrogenase reductase. These genes (\textit{nifH}, \textit{vnfH}, and \textit{anfH}) share a high degree of similarity: The sequence identity between \textit{nifH} and \textit{vnfH} is 100\% whereas between \textit{nifH}/\textit{vnfH} and \textit{anfH} is 63\%. These proteins have proven to be functionally interchangeable: They serve as electron donors to the “wrong” dinitrogenase component \(^9\).

The dinitrogenase components (MoFe, VFe, and FeFe proteins, for molybdenum, vanadium, and iron-only dinitrogenases, respectively) contain the site for substrate binding and reduction. These proteins are multimeric complexes with molecular weights of approximately 240,000 Da. The mature MoFe protein purifies as a heterotetramer of composition \(\alpha_2\beta_2\) whereas mature VFe and FeFe proteins has been reported to purify as protein complexes of composition \(\alpha\beta_2\), \(\alpha_2\beta_2\), and \(\alpha_2\beta_2\delta_2\)\(^{10-14}\). The genes that encode the MoFe protein \(\alpha\) and \(\beta\) subunits (\textit{nifD} and \textit{nifK}, respectively) are contiguous (\textit{nifDK}) whereas the genes that encode the VFe and FeFe proteins \(\alpha\) (\textit{vnfD} and \textit{anfD}) and \(\beta\) (\textit{vnfK} and \textit{anfK}) subunits are disrupted by \textit{vnfG} and \textit{anfG}. This has been recognized to be a genetic feature that distinguishes the organization of the \textit{Azotobacter vinelandii} \textit{vnf} (\textit{vnfHDGK}) and \textit{anf} (\textit{anfHDGK}) operons from the organization of the \textit{nif} (\textit{nifHDK}) operon.
The δ subunit, the product of the expression of the *vnfG* and *anfG* genes, has no homologous within the bacterial genera. It is a 14,000 Da, non-catalytic protein peptide only associated with Mo-independent nitrogen fixation. The *vnfG* gene has been genetically proven to be essential for diazotrophic growth of *A. vinelandii* cells in the absence of molybdenum. Mutant strains containing alterations in both *vnfG* and *anfG* genes were unable to grow in N-free, Mo-deficient medium. As mentioned in the introduction of this thesis, Waugh *et al.* sought to determine the effect that a substitution of a conserved cysteine residue in both VnfG and AnfG had on cell diazotrophic growth. The cisteine residue (δ-36Cys on AnfG and δ-17Cys on VnfG) was substituted for either an alanine or a serine, without significantly affecting the generation time of the modified strains, as compared to the wild-type counterpart. These results indicated that the conserved cysteine, which was hypothesized to participate in cluster binding, was not necessary for nitrogen fixation as catalyzed by the alternative nitrogenases. However, introducing a stop codon at the cysteine position of either *anfG* or *vnfG* did stop diazotrophic growth of the altered cells. Likewise, deletion of *anfG* or *vnfG* from a strain which *nifHDK* operon had also been deleted rendered cells unable to grow in N-free media. Interestingly, cell-free extracts of these mutant strains showed C₂H₂ reduction activities, which indicated that the altered δ subunits enabled reduction of C₂H₂ by both V- and Fe-only nitrogenases but could not sustain the reduction of N₂.

It is yet not known what the mechanistic role of the δ subunit is in the V- and Fe-only-dependent reduction of N₂. Kirn and Rees suggested that it might be
involved in the stabilization of the VFe and FeFe quaternary structures\(^{16}\). This hypothesis inspired experimental designs to elucidate the function of this non-catalytic peptide. Chatterjee et al. reported the purification of a VFe protein of composition \(\alpha_2\beta_2\delta_2\) which substrate reduction activity was restored upon addition of FeV-cofactor\(^ {17}\), the V-nitrogenase metal active site analogous to the MoFe protein FeMo-cofactor. Chatterjee et al. demonstrated that the \(\alpha_2\beta_2\delta_2\) hexamer dissociates under certain conditions to yield free \(\delta\) subunit and a form of apo-VFe protein with no \(H^+\), \(C_2H_2\), or \(N_2\) reduction activities even after FeV-cofactor reconstitution assays. However, the activity could be restored upon addition of \(\delta\) subunit to the FeV-cofactor activation assay system. The \(\delta\) subunit has also been reported to bind FeV-cofactor, and in an Mg-ATP dependent process, activate the \(C_2H_2\) reduction activity of the VFe protein\(^ {18}\). Taken together, these \textit{in vitro} observations has led to hypothesis that categorize the \(\delta\) subunit as a molecular chaperon which function is to strengthen the interactions among the different VFe protein subunits, yielding a VFe protein capable of supporting \(N_2\) reduction\(^ {19}\).

In spite of the previous research recount, direct observations explaining at the molecular level the mechanistic contribution of the \(\delta\) subunit to V- and Fe-only nitrogenase \(H^+\), \(C_2H_2\), and \(N_2\) reduction activities are yet scarce. The \(\delta\) subunit could provoke conformational changes within the VFe and FeFe proteins such that the metal cluster active site access the high redox potential needed to bind and activate \(N_2\) for reduction. It is envisioned that the formation of the proper hydrogen bond networks or even the creation or destruction of covalent bonds between the metal cluster active site and their protein environments could
affect the physical and electronic structure of the enzyme active site. There are examples that illustrate the effect of modifying the hydrogen bonds networks or the coordination environment on the midpoint potential of metal centers such as Fe-S clusters\textsuperscript{20} and heme groups\textsuperscript{21}. A thorough investigation of the structural modifications undertaken by the VFe protein when in its different quaternary conformations could provide insights regarding the role the $\delta$ subunit plays in nitrogen fixing systems devoid of molybdenum. Thus, as a first step to elucidate the mode of action of the $\delta$ subunit and how it relates to $V$-dependent substrate specificity and reduction, a biochemical and structural investigation was initiated to better understand how the VFe protein third subunit primes the $V$-nitrogenase active site for substrate binding and reduction. This chapter describes the cloning and overexpression of the $\textit{vnfG}$ gene and the purification to homogeneity of its expression product, the $\delta$ subunit. It will also be described the conditions necessary to obtain a single oligomeric species of the purified protein and the crystallization trials conducted so far.

MATERIALS AND METHODS

\textit{Bacterial Strains and Growth Conditions.} \textit{Azotobacter vinelandii} strain Trans (wild-type) was grown at 30 $^\circ$C in liquid Burk medium prepared with deionized water as previously described. When \textit{A. vinelandii} was grown on plates, the Burkes medium was solidified with 1.6\% agar. \textit{Escherichia coli} strains JM109 (New England Biolabs) and BL21 (Novagen) were grown in liquid Luria-Bertani (LB) medium at 37 $^\circ$C and when grown on plates the growth medium was
solidified with 1.6% agar. When required, antibiotics were added to the following final concentrations: ampicillin, 100 μg/mL; kanamycin, 50 μg/mL. The stock concentration of IPTG and X-gal used in this study were 25 mg/mL and 20 mg/mL, respectively.

**Genomic and Plasmidic DNA Isolation.** Plasmidic, circular DNA was isolated from *E. coli* using the GenElute plasmid miniprep kit from Sigma-Aldrich. Genomic DNA from *A. vinelandii* was isolated as follows: Approximately 200 mg of cell paste that had been suspended in 400 μL of sterile water was mixed with 150 μL of lysis buffer (Triton X-100 10%, Lysozyme 5 mg/mL, Tris-HCl 0.1M pH 8). The suspended cells were boiled for 10 minutes and centrifuged at maximum speed on a bench-top centrifuge for 2 minutes. The brown-yellow supernatant containing the genomic DNA was collected and mixed with 700 μL of isopropyl alcohol. The mixture was incubated at room temperature for 5 minutes after which time it was spun at maximum speed for 1 minute. The pellet containing the genomic DNA was resuspended with 500 μL of isopropyl alcohol and incubated for 3 minutes at room temperature. The solution was then spun at maximum speed for 1 minute and the resulting pellet was resuspended in 1 mL of 190-proof ethanol, followed by a centrifugation for 1 minute. The supernatant was discarded and the pellet was then allowed to air-dry for approximately 30 minutes. The crude preparation of genomic DNA was finally suspended in 200 μL of sterile water and 20 μL of GenElute plasmid miniprep kit ES buffer (Sigma-Aldrich).
vnfG Cloning and Insertion of Poly-Histidine Tag. The restriction enzymes, T4 DNA polymerase, and DNA ligase used throughout the manipulation of vnfG were purchased from New England BioLabs. Fail-Safe DNA polymerase (Epicentre Technologies) was used to perform PCR experiments following the manufacturer instructions. Site-directed mutagenesis experiments were performed with PfuTurbo Polymerase (Stratagene) following the manufacturer recommendations. Primers were custom-made by Integrated DNA Technologies. A stretch of vnfDGK (~2.3kb), including a segment of vnfD, the entire vnfG gene, and a segment of vnfK, was PCR-amplified using as a template isolated, genomic DNA from A. vinelandii strain Trans. Forward primer BBP324 (GACATAAGCTTCTCGAGAAGAGCATGCACGAAGCCTTCGACG) introduced a HindIII restriction site (underlined) on the PCR product 5’ end whereas reverse primer BBP325 (GACCAGGGCATTCGGCACCGAAGGTCTTCTGC) annealed on the 3’ end of the vnfDGK stretch containing a PstI restriction site. The vnfDGK fragment was then inserted into pUC19 plasmid cloning sites HindIII and PstI. This plasmid (pPCRVFe1) was used to transform E. coli strain JM109. The transformed cells were subjected to blue/white screening in solid, ampicillin-containing LB medium to which 50μL of IPTG and 50μL of X-gal had been spread. Plasmidic DNA was then extracted from white colonies to confirm the correctness of the inserted sequence. Primers BBP345 (GTCAAAGCTTGATGGTGGATGGTGGATGGTGGATGG)
GCGGCCCGCACGATGACCGG) and BBP346 (GTCAAAGCTTTGATGAGCCAGTCCCATCTCGACGATCTGTTC) were used to incorporate 7 histidines codons and a HindIII restriction site on the 3’ end of vnfD, thus creating plasmid pPCRVFe3. This plasmid had a XbaI restriction site that had to be removed before adding seven histidine codons on the 3’ end of vnfG. The removal involved digestion of pPCRVFe3 with XbaI, generation of blunt ends with T4 DNA polymerase, and ligation with T4 DNA ligase. A PCR experiment introduced 6 histidine codons, followed by a XbaI restriction site, on the 3’ end of vnfG using primers BBP347 (GTCATCTAGAGTGGATGATGGTGATGATGGTGAGGTGGTGGTTGAGTTCGCGGTTGG) and BBP348(GTCATCTAGATGAGTCATCGGCGCGGAGACGGCCGGAACG). This resulted in pPCRVFe7, a plasmid containing the vnfG gene with a HindIII restriction site on its 5’ end and 6 histidine codons on its 3’ end followed by a XbaI restriction site. pPCRVFe7 was then digested with HindIII and XbaI restriction enzymes. The resulting 365bp long fragment encompassing the vnfG gene was inserted into pBB052 HindIII and XbaI cloning sites. pBB052 is a pUC-based cloning vector which ampicillin resistant cassette has been replaced by a kanamycin resistant cassette. The resulting plasmid (pPCRVFe10) was used to transform E. coli JM109. The transformed cells were subjected to blue/white screening on solid LB medium containing kanamycin. Plasmidic DNA was extracted from white colonies to confirm the correctness of the vnfG sequence. Thus was constructed the vnfG gene with 6 histidines coding-codons located at its 3’ end. This gene was then moved to the NdeI and EcoRI restriction sites of the
pET-based expression vector pPCRWE48. A site-specific mutagenesis experiment was designed to incorporate a \(NdeI\) restriction site into the start sequence of \(vnfG\). Primers BBP396

\[(GAACCATGATTACGCCAAGCTCATATGAGCCAGTCCC ATCTCGACGA TCTG)\] and BBP397

\[(CAGATCGGTAGATGGGACTGGCTCATATGAGCTTGGCATGGAAT CATGGTTC)\] were used for this purpose, thus creating pPCRVFe13. A second site-specific mutagenesis experiment was designed to add a stop codon and to remove the \(XbaI\) restriction site at the 5’ end of \(vnfG\). Primers BBP398

\[(CATCACCATCACTCTTGAGGATCCCCGGGTACCGAG)\] and BBP399

\[(CTCGGTACCCGGGGATCCTCAAGAGTGATGGTGATG)\] were used for this purpose, thus creating pPCRVFe15. This plasmid was digested with \(NdeI\) restriction enzyme and ligated with T4 DNA ligase. This step was necessary to remove an unwanted \(NsiI\) restriction site, creating in the process pPCRVFe16.

Lastly, pPCRVFe16 and pPCRWE48 were double-digested with \(NdeI\) and \(EcoRI\) and the resulting plasmid (pPCRVFe19) was used to transform \(E.\ coli\) strain BL21 (BL21- pPCRVFe19) which, upon IPTG induction, produced C-terminal, histidine-tagged VnfG.

**Expression and Purification of Histidine-tagged VnfG.** T7 polymerase initiated the transcription of \(vnfG\) when a 1L cell culture of BL21- pPCRVFe19 that had been grown to an \(OD_{600\mu}\text{m} \) of ~0.6 was induced with 260 µM IPTG. After induction for three hours the cells were harvested by centrifugation at 10,000 \(\times\) g for 10 minutes at 4 °C (SLA-3000 rotor, Sorvall) and suspended in 30
mL of lysis buffer (NaCl, 0.2 M; DNase 1 mg; PMSF, 0.1 mM; Tris-HCl 50 mM pH 8). The suspended cells were then passed three times through a chilled French pressure cell at 1200 psi and 4 °C. The cell lysate was clarified by centrifugation (8,000 × g for 20 minutes at 4 °C in a SS-34 Sorvall rotor). Approximately 30 mL of the resulting supernatant was applied on a Ni\(^{2+}\)-loaded IMAC column (6 cm × 1.7 cm) that had been previously equilibrated with 50 mL of wash buffer (Tris-HCl 50 mM pH 8, NaCl 0.2 M;). Unbound proteins and other cellular components were washed off the column with 50 mL of wash buffer. This was followed by a stepwise elution of unwanted bound proteins using 30 mL of each 50 mM and 100 mM imidazole solutions in wash buffer. The column was then washed with 50 mL of a 200 mM imidazole solution in wash buffer, which resulted in the elution of histidine-tagged VnfG as a pure protein, as estimated by 18% SDS-PAGE. A 1L cell culture typically yielded ~30 mg of histidine-tagged VnfG. The protein solution was then concentrated using a centrifugal filter devise (10,000 MWCO; Amicon Ultra) to a final volume of 3 mL. Protein dialysis was carried out using 5 cm of Snake Skin Pleated Dialysis Tubing (Pierce). The protein was dialyzed overnight (twice) at 4 °C against 2L of buffer Tris-HCl 50 mM pH 8, 0.2 M NaCl. Protein concentration was determined using the Biuret method, with bovine serum albumin as a standard.

**Gel Filtration Chromatography.** It was of interest to determine the different oligomeric states that the recombinant VnfG can adopt when dissolved in buffers at different pH’s and under different ionic strengths. In order to conduct the investigation, a 0.5 mL aliquot of recombinant, purified VnfG containing 4
mg of total protein was loaded on a Sephacryl S-200 molecular sieve (Pharmacia Biotech) packed on a XK16 column (1.7 cm × 53 cm) (Pharmacia). The column was calibrated with the molecular weight markers Aldolase (158 kDa), BSA (66 kDa), Carbonic Anhydrase (29 kDa), and Cytochrome C (12.5 kDa). The solvent flow was set at 0.5 mL/min. Before each pass of the protein, the column was equilibrated with ~ 100 mL of buffer. Immediately after equilibrating the column, 0.5 mL of a solution containing the molecular weight standards (each at 3 mg/mL and dissolved into the equilibration buffer) was loaded a run in the column to obtain a calibration curve. The buffers tested were: i) Tris-HCl 50 mM pH 8, NaCl 0.5 M, and ii) MOPS 50 mM pH 7, NaCl 0.5 M.

RESULTS

Cloning VnfG. The entirety of vnfG was successfully amplified from A. vinelandii genomic DNA (in conjunction with stretches of vnfD and vnfK) and inserted into the cloning vector pUC19 restriction sites HindIII and PstI. After multiple molecular biology steps that involved elimination of restriction sites, site-directed mutagenesis, and PCR amplification, the vnfG gene (with six histidines coding-codons on its 3’ end) was inserted into the pET-based expression vector (pPCRWE48) restriction sites NdeI and EcoRI, thus creating the plasmid pPCRVFe19. The sequence of vnfG inserted into pPCRVFe19 was 100 % identical to the DNA sequence deposited in the NCBI microbial genome collection. A BLAST search located the vnfG gene on the A. vinelandii AvOP ctg60 (accession number AAAU03000001). The δ subunit, the product of the
expression of \(\text{vnfG}\), consists of 113 amino acid residues having a theoretical molecular mass of \(\sim 13.4\) kDa and a pI of 5.01.

*Expression and Purification of Histidine-Tagged VnfG.* Production of histidine-tagged VnfG was accomplished by inducing the expression of the gene that encodes T7 polymerase. This gene, which is located in the chromosome of *E. coli* strain BL21, is under the control of the *lac* promoter and operator, which is located on the 5’ end of the *vnfG* gene inserted into a pET-based expression vector (the plasmid containing the *vnfG* gene for expression in *E. coli* is PCRVFe19). The induction of the promoter was achieved with IPTG, which resulted in the expression of a protein with an experimental molecular weight of \(\sim 13.2\) kDa (Figure 3-1, lane 2).

Upon cell rupture, which was accomplished by passing the cells through a French press, VnfG was solubilized and the resulting solution was loaded on a \(\text{Ni}^{2+}\)-loaded IMAC. Almost all of the histidine-tagged VnfG binds on the column, as judged by examining the column flow-through (Figure 3-1, lane 4). After doing a step-wise elution of contaminant proteins with increasing concentrations of imidazole (Figure 3-1, lane 5-6), pure histidine-tagged VnfG readily eluted off the column with 200 mM of imidazole (Figure 3-1, lane 7).
Figure 3-1. SDS-PAGE (18%) showing the induction of \textit{vnfG} in \textit{E. coli} BL21-pPCRVFc19 and the purification steps that yielded pure $\delta$ subunit. Lane 1, un-induced cells; lane 2, induced cells; lane 3, cell lysate; lane 4, column flow-through; lane 5, 50 mM imidazole wash; lane 6, 100 mM imidazole wash; lane 7, 200 mM imidazole wash. Pageruler$^\text{TM}$ molecular weight marker (MW) was from Fermentas.

Crystallization Trials. The crystallization experiments were conducted with the aid of the automated crystallization facility at the Hill laboratory (University of Utah). The protein used throughout these experiments was dissolved in buffer Tris-HCl 50 mM pH 8, NaCl 0.2 M, and a concentration of $\sim$ 4 mg/mL. Using the sitting drop vapor diffusion method, over 1500 crystallization conditions were tested at room temperature with the as-isolated protein, two of which yielded what seemed to be protein crystals (Figure 3-2, Figure 3-3). In both cases, the crystals took 60 days to grow. Attempts to reproduce the crystal growth at USU using one of the two conditions are currently been conducted.
Figure 3-2. Time-course captions of VnfG crystals growing at pH 6.5. 
Panel 1, day 1; panel 2, day 60; panel 3, day 75; and panel 4, day 90. The crystallization cocktail consisted of Tris-Bis 0.1 M pH 6.5, PEG 3350 25% w/v.
Figure 3-3. Time-course captions of VnfG crystals growing at pH 7.5. Panel 1, day 1; panel 2, day 60; panel 3, day 75; and panel 4, day 90. The arrow points at what most likely is a protein crystal. The crystallization cocktail consisted of HEPES 0.1 M pH 7.5, PEG 3350 25% w/v.

The tested conditions are a modification of the following: Bis-Tris 0.1 M pH 6.5, PEG 3350 25% w/v. The different concentrations of PEG used were 19%, 22%, 25%, 28%, 31%, and 34% whereas the Bis-Tris buffer concentration and pH was kept unmodified. The protein concentrations used during the trials were 5 mg/mL, 7.5 mg/mL, 10 mg/mL, and 15 mg/mL. The crystals that grew at the Hill laboratory were brought to USU and used as “seeds” to accelerate crystals
growth. The crystals grown in Bis-Tris 0.1 M pH 6.5, PEG 3350 25% w/v were crushed and added to each crystallization well. A second set of crystallization trials was left “unseeded”. As of the time of writing this thesis, the crystallization trials using just one of the crystallization cocktails have so far yielded negative results. The second crystallization cocktail, which consist of HEPES 0.1 M pH 7.5, PEG 3350 25% w/v, remains to be tested.

Analysis of the VnfG Oligomeric States Using Gel Filtration Chromatography. Partially purified apo-VFe protein has a subunit composition of $\alpha_2\beta_2\delta_2$. Upon passing of the protein through a Superose 12 column the apoprotein was reported to dissociate to yield dimers of the $\delta$ subunit with a molecular mass of ~ 26.3 kDa. Chatterjee et al. observed that, in vitro, FeV-cofactor associates with the $\delta$ subunit dimer and activates the C2H2 reduction activity of the apo-VFe protein. In view of these observations, it was of concern to characterize the oligomeric state of the purified, recombinant $\delta$ subunit so as to assess whether this version of the protein could form protein species with different oligomeric states. A sephacryl S-200 was used for the purpose of identifying the different polymers of the $\delta$ subunit that could exist in two different solutions: i) buffer Tris-HCl 50 mM pH 8, NaCl 0.1 M, and ii) buffer MOPS 50 mM pH 7, NaCl 0.1 M. As seen in Figure 3-4, the oligomeric state depends on the pH of the solution in which it is dissolved. At pH 7, the recombinant $\delta$ subunit seems to be mostly present as an ~ 79,000 Da hexamer (as calculated with the aid of the calibration curve shown in Figure 3-5), whereas at pH 8 a mixture
of species exist, the most predominant being that one corresponding to an
hexamer of the δ subunit.

![Graph](image1.png)

**Figure 3-4. Analysis of recombinant δ subunit by gel filtration chromatography.** A Sephacryl S-200 resin (Pharmacia) was use to conduct this study. *Dotted line*, elution profile of recombinant δ subunit at pH 8, NaCl 0.5 M (buffer Tris-HCl 50 mM). *Straight line*, elution profile of recombinant δ subunit at pH 7, NaCl 0.5 M (buffer HEPES 50 mM)

![Graph](image2.png)

**Figure 3-5. Calibration curve for the protein standards run on a Sephacryl S-200 (1.7 cm × 53 cm).** The void volume of the column was 55 mL. The $K_{av}$ value for each of the protein standards was calculated according to the following equation: $K_{av} = \frac{V_e - V_o}{V_e - V_o}$, where $V_e$ is the volume required for the protein to elute, $V_o$ is the void volume of the column, and $V_c$ is the volume of the column. The molecular weight markers at listed in the *Materials and Methods* section.
REFERENCES


CHAPTER 4
ELECTRON ALLOCATION AND SUBSTRATE-INHIBITOR
INTERACTIONS AT THE AZOTOBACTER VINELANDII
VFe PROTEIN ACTIVE SITE

ABSTRACT

Azotobacter vinelandii accomplishes the reduction of atmospheric nitrogen by means of three phylogenetically-related nitrogenases: The Mo-, V-, and Fe-only nitrogenases. As their name suggest, each contain a unique transition metal, which is associated to the active site of the enzymes. The nitrogenases are a two-component protein complex: the dinitrogenase reductase and the dinitrogenase. This work deals with the dinitrogenase component from the V-nitrogenase, the VFe protein, which was genetically modified and it now contains seven histidine residues in tandem at the N-terminus of the β subunit. The protein readily purifies with the aid of an IMAC column, although with low Fe$^{2+}$ content and poor reduction activities. However, upon addition of recombinant, pure δ subunit (the VFe protein third subunit), metal content of the enzyme increases and the reduction activity is improved 10-fold. A detailed activity and kinetic characterization was undertaken with this enzyme preparation and a histidine-tagged MoFe protein in order to reveal the effect on the catalytic properties of the enzymes upon substituting Mo for V in active sites that present slight modifications on their amino acid residues. Several observations were made: i) The histidine-tagged VFe protein reduces H$^+$, C$_2$H$_2$, N$_2$, and N$_3^-$ as previously
reported; ii) The $K_m$ values for the reduction of C$_2$H$_2$ and N$_2$ as catalyzed by the VFe protein are in agreement with those previously reported in the literature; iii) The $K_m$ value for the substrate N$_3^-$ was similar to its $K_m$ value when the examined enzyme is the Mo-nitrogenase; iv) The enzyme also reduces N$_2$H$_4$, with a $K_m$ that resembles that of its Mo-dependent counterpart; v) The nonreciprocal response of N$_2$ and C$_2$H$_2$ is observed in both enzymes; vi) For the VFe protein, H$_2$ inhibits N$_2$ reduction but not C$_2$H$_2$, N$_2$H$_4$, or N$_3^-$ reduction; and vii) For the first time, it is seen that N$_2$H$_4$ and N$_3^-$, but not H$_2$ or N$_2$, re-direct the electron flux to increase the production of C$_2$H$_6$ when the enzyme turns over C$_2$H$_2$. The histidine-tagged VFe protein depicts catalytic and inhibition patterns that resemble those of the MoFe protein, but it also possesses unusual catalytic properties that can be attributed to having V and an unique amino acid environment forming its active site.

INTRODUCTION

The biological reduction of atmospheric nitrogen into ammonia, a process called biological nitrogen fixation, is a chemical catalysis performed by the nitrogenases $^1,^2$, which are oxygen-labile, metal-containing enzymes expressed in organisms that belong to the bacterial and archaeal domains $^3$. The nitrogenases reduce dinitrogen (N$_2$) according to the following equation:

$$\text{N}_2 + n\text{e}^- + n\text{ATP} + n\text{H}^+ \rightarrow 2\text{NH}_3 + n\text{H}_2 + n\text{ADP} + n\text{P}_i$$

(Eq. 1)

where $n$ varies depending on the nitrogenase being studied.

*Azotobacter vinelandii* harbors structural genes that encode three genetically distinct nitrogenase systems: the molybdenum, vanadium, and iron-
only nitrogenases, which are encoded by the \( nifHDK \)^4, \( vnfHDGK \)^5, and \( anfHDGK \) operons \(^6\), respectively. Not only are these enzymes genetically distinct; as their name suggests, the first nitrogenase is a molybdenum-containing protein, and the second and third nitrogenases contain vanadium or only iron, respectively \(^1\). The latter two nitrogenases are collectively called alternative nitrogenases. All the nitrogenases are multimeric complexes composed of two proteins that purify separately: The Fe protein (NifH, VnfH, or AnfH) is a \( \sim \) 60,000 Da ferredoxin-like homodimer bridged by a 4Fe:4S cluster. This protein possesses two MgATP binding sites, one on each subunit \(^7\). The Fe protein is the only known electron donor to the nitrogenase active sites, in a process dependent on the hydrolysis of MgATP \(^2\). The second component of the Mo-, V-, and Fe-only nitrogenases, wherein substrate binding and reduction occurs, is an \( \alpha_2\beta_2 \) heterotetramer of \( \sim \) 240,000 Da, called MoFe protein, or an \( \alpha_2\beta_2\delta_2 \) heterohexamers of similar molecular weights, called VFe or FeFe proteins \(^1\). X-ray diffraction data collected from crystals of MoFe proteins from different organisms confirmed the presence of two different types of metals clusters embedded within their protein folds: the P-cluster, an \([8\text{Fe-7S}]\) cluster and the FeMo-cofactor, a \([7\text{Fe-9S-X-Mo-homocitrate}]\) cluster, which is the site for substrate reduction (experimental evidence suggest that \( X \) could be C or O, but not N \(^8\)). In the MoFe protein, there is a P-cluster located at the interface of each \( \alpha\beta \) heterodimer and a FeMo-cofactor embedded within each \( \alpha \) subunit. No such structural information is available for the alternative nitrogenases. However, biochemical and physicochemical studies conducted on the V-nitrogenase indicate
that its metal centers are structurally homologous to the MoFe protein metal centers 9-13.

The alternative nitrogenases are believed to be hexamers of composition ($\alpha_2\beta_2\delta_2$). Only do the alternative nitrogenases co-purify with the $\delta$ subunit, a small polypeptide (~14,000 Da) encoded by $\text{vnfG}$ with no known catalytic activity. The $\delta$ subunit has been proven to be essential for Mo-independent N$_2$ fixation: Mutant strains containing alterations in the $\text{vnfG}$ gene have been shown to be unable to grow in N-free, Mo-deficient medium 14. However, cell-free extracts of these mutant strains showed C$_2$H$_2$ reduction activities, which indicated that the altered $\delta$ subunits enabled reduction of C$_2$H$_2$ by both V- and Fe-only nitrogenases but could not sustain the reduction of N$_2$ into NH$_3$. The $\delta$ subunit is probably a structural factor that arranges that metal active site, modifying hydrogen bond networks or even creating or destroying covalent bonds between the protein and the metal cluster active site such that it reaches the high reduction potential needed for N$_2$ binding and reduction.

In addition to reducing N$_2$, it is well known that all the nitrogenases are also capable of reducing H$^+$, C$_2$H$_2$, and other small, unsaturated substrates 2, even though the metal composition within their active sites, the subunit composition of their protein assemblies, and the structural genes that code for each of the three enzymatic systems are unique for each nitrogenase. Definitively during the course of the nitrogenase evolution its structural genes have been redesigned to code for specific protein frames that can accommodate the environmentally-available transition metals that have the reactivity to bind and reduce the almost
inert N$_2$ molecule. Unsurprisingly, the structural heterogeneity among the nitrogenases is translated into functional heterogeneity. For example, i) the efficiency of N$_2$ reduction decreases for V-nitrogenase, which channels $\sim$ 50 % of the total electron flux to N$_2$ compared to $\sim$ 75 % for Mo-nitrogenase $^{1,2}$; ii) the preference for C$_2$H$_2$ reduction over H$^+$ reduction is much weaker for V-nitrogenase than for Mo-nitrogenase $^{15}$; iii) during N$_2$ reduction only, the V-nitrogenase requires twice the amount of MgATP hydrolysis compared to the Mo-nitrogenase $^1$; iv) another difference is observed during enzymatic turnover in the presence of CO. Under these conditions, the Mo nitrogenase generates two different $S = 1/2$ EPR signals $^{16,17}$, while neither signal is detected when the V nitrogenase from *A. vinelandii* is used $^{11}$; and v) the V-nitrogenase releases significant amounts of four-electron-reduced products, i.e., N$_2$H$_4$ during N$_2$ reduction $^{18}$, and C$_2$H$_6$ during C$_2$H$_2$ reduction $^{15}$, whereas wild-type Mo-nitrogenase releases neither under normal conditions [and exception being altered MoFe proteins that can accomplish the 4e$^-$ reduction of C$_2$H$_2$ to form C$_2$H$_6$ at 30 $^\circ$C $^{19,20}$].

A common requirement for the reduction of substrates as catalyzed by these enzymes is the shuttling of electrons from the Fe protein to the protein metal active sites, in a process driven by the hydrolysis of MgATP. At least for the MoFe protein, it has been determined that a round of Fe protein docking, MgATP hydrolysis, electron transfer, and Fe protein dissociation, is required to send a single electron from the Fe protein to the site of substrate reduction $^2,21$. Therefore, the reduction of H$^+$, C$_2$H$_2$, N$_2$, and the many other nitrogenase non-
physiological substrates require the accumulation of two or more electrons before substrate reduction can occur. The Thorneley-Lowe kinetic scheme for the sequential reduction of the MoFe protein \(^2,21,22\) is so far the best model that links the 2, 4, and 6 electron reduction of \(H^+\), \(C_2H_2\), and \(N_2\) with the successive rounds of electron transfer events needed to prime the enzyme active site for substrate reduction.

The proposed cycle starts with the dithionite-reduced form of the MoFe protein \((E_0)\) and is followed by eight successive rounds of \(H^+/e^-\) additions \((E_1, E_2, E_3,\ldots E_7)\) to give further reduced states of the MoFe protein. Each successive round of MoFe protein reduction requires one round of Fe protein cycle and electron transfer into the MoFe protein.

A series of experimental observations regarding substrate-inhibitor interactions taking place at the MoFe protein active site can be explained on the basis of the Thorneley-Lowe, which original proposition was based on pre-steady state kinetics of the \textit{Klebsiella pneumoniae} Mo-nitrogenase using stopped-flow spectrophotometry and rapid-quench EPR to detect reduction intermediates \(^22\). For example: i) \(N_2\) is a non-competitive inhibitor of \(C_2H_2\) reduction whereas \(C_2H_2\) competitively inhibits the reduction of \(N_2\) \(^23\); within the context of the Thorneley-Lowe scheme, this can be seen as \(N_2\) binding to more reduced states of the MoFe protein (i.e. \(E_3\) and \(E_4\)) whereas \(C_2H_2\) would bind to a less reduced MoFe protein (i.e. \(E_1\) and \(E_2\)); ii) \(H_2\) is a competitive inhibitor of the reduction of \(N_2\) but it does not inhibit the reduction of \(C_2H_2\) \(^23\), \(N_2H_4\) \(^24\), or \(N_3^-\) \(^25\), thus suggesting that \(H_2\) and \(N_2\) share binding sites an that the latter substrates bind earlier on the Thorneley-
Lowe reduction scheme proposed for the MoFe protein; iii) hydrogen evolution can be suppressed by excess C2H2 but not by excess N2; and iv) N2 is the only substrate that triggers the exchange of D2 with protons from solution to form HD. The steady-state kinetic characterization of the non-reciprocal inhibition patterns for the MoFe protein, as seen for the C2H2/N2 interaction, can be accommodated on the framework provided by the Thorneley-Lowe scheme and has definitively contributed to the validation of the model for the successive reduction of the MoFe protein by sequential addition of H+/e\(^{-}\). However, for the V-nitrogenase, the Thorneley-Lowe scheme for the reduction of H\(^{+}\), C2H2, and N2 still remains obscure since no kinetic data accounting for substrate-inhibition patterns is yet available. It is unknown what the nature of the inhibition of N2, N2H4, and C2H2 reduction by H2 is, or what type of inhibitor C2H2 is to N2 (and N2H4) reduction and vice versa.

As previously stated, each of the nitrogenase systems is a distinct biological complex capable of catalyzing the same chemical reactions. However, due to the lack of experimental data, it is yet unclear whether they share a common route for substrate reduction. This chapter presents, for a histidine-tagged V-nitrogenase, kinetic data that seeks to provide insights into the mechanism of substrate reduction within the kinetic framework embodied in the Thorneley-Lowe scheme for N2 reduction. Substrate-inhibitor patterns have been determined using classic enzyme steady-state kinetics. Additional data is also presented that reports on the effects that N2H4 and N3\(^{-}\) have on the distribution of electrons to the substrate C2H2.
MATERIALS AND METHODS

*Construction of Histidine-Tagged VFe Protein.* The histidine-tagged VFe protein was constructed in the laboratory of Dr. D. Dean by inserting a histidine-coding sequence within the *vnfK* restriction site *Mfe*I (located at the 3’ end of the gene) using molecular biology techniques as described in 28. The linker sequence was AATTTAAATCAC CACCATCACCACCATCACCAT and the parent strain was *A. vinelandii* DJ1254. This strain possesses a tungsten-tolerant phenotype and a Δ*nifHDK* genotypic background. The resulting *A. vinelandii* strain was named DJ1258.

*Bacterial Strain and Growth Condition.* *A. vinelandii* strain DJ1258 was the strain used for this study to express and purify the V-nitrogenase. *A. vinelandii* DJ1258 expresses a VFe protein containing a poly-histidine tail at the N-terminus of its β subunit. This strain contains deletions within the *nif* operon (Δ*nifKD*), rendering it incapable of expressing the Mo-nitrogenase. It also possesses a WO$_4^{2-}$ tolerance mutation that permits the expression of the V-nitrogenase in the presence of Mo. When *A. vinelandii* DJ1258 was grown the Burk media was supplemented with vanadium at a final concentration of 10 μM. Three 10 mL seed cultures of *A. vinelandii* DJ1258 were started to produce a large-scale growth of this strain. The seed culture consisted of Burk medium containing a nitrogen source (urea), V, and Mo. After overnight growth, the seed cultures were used to inoculate three 1 L Burk media containing a nitrogen source, V, but no Mo. After overnight growth, these were used as the inoculums to start a large-scale growth, which was carried out in a 100 L fermentor filled
with Mo-free Burk medium with added V$_2$O$_5$ to a final concentration of 10 μM. The cells were subjected to a derepression step as previously described $^{28}$. Harvested cells were frozen with liquid nitrogen and kept at $-80$ °C.

**Purification of Histidine-Tagged MoFe Protein, Histidine-tagged VnfG, and Wild-Type Fe Protein.** Histidine-tagged MoFe protein and wild-type Fe protein were expressed by *A. vinelandii* strains DJ995 and DJ884, respectively, and purified as described earlier $^{28}$. Histidine-tagged VnfG was expressed in *Escherichia coli* and purified as described in Chapter 2 in this thesis.

**Purification of Histidine-Tagged VFe Protein.** Since the V-nitrogenase is extremely susceptible to oxidation, all the solutions used throughout the purification procedure were made anoxic through repeated cycles of degassing and flushing with argon gas. After degassing, Na$_2$S$_2$O$_4$ was added to each solution to a final concentration of 2 mM. In brief, approximately 240 g of wet cell paste was broken open by an osmotic shock procedure as described in ($^{28}$). The cell-free extract was centrifuged in a Beckman Ti-19 rotor at 30,000 $\times$ g for 45 min. After centrifugation, approximately 3 μM of degassed, purified VnfG was added to the resulting supernatant. This solution was then transferred to a degassed flask and applied to an IMAC column (20 cm $\times$ 3 cm) which had been previously loaded with Ni$^{2+}$ ions (NiCl$_2$ 0.1 M, 400 mL) and equilibrated with a buffer containing 50 mM Tris-HCl pH 8, 0.5 M NaCl, 2 mM Na$_2$S$_2$O$_4$, and 15 % glycerol. Loosely bound proteins and other unbound cell components were removed from the resin with two volumes of equilibrating buffer followed by one volume of equilibrating buffer to which imidazole had been added to a final
concentration of 12 mM. To elute bound VFe protein, the direction of the buffer flow was reversed and the column washed with a 300 mM imidazole solution prepared in equilibration buffer. The VFe protein migrated through the column as a dark, well resolved band. The resulting dark-colored protein solution was concentrated to a final volume of ~ 10 mL in an Amicon concentration chamber fitted with a 100,000 MWCO filtration membrane (44.5 mm in diameter) under an argon pressure of 20 psi. Frozen pellets of the histidine-tagged VFe protein were obtained by dripping into liquid nitrogen the protein solution in a drop-wise manner. The protein pellets were stored at liquid nitrogen temperature until further use. The homogeneity of the VFe protein preparation was assessed using 18 % SDS-PAGE. Protein concentration was determined using the Biuret method, with bovine serum albumin as a standard.

**Protein N-terminal Sequencing.** Protein N-terminal sequencing was performed by automated Edman degradation at the ASU Proteomic and Protein Chemistry Laboratory.

**Enzymatic Assay Conditions.** Enzymatic assays to monitor substrate reduction rates for the Mo- and V-nitrogenase were performed at 30 °C in a water bath under constant shaking (150 strokes/min). Each enzymatic assay contained a total of 50 μg of MoFe protein or 125 μg of VFe protein (unless otherwise stated), together with a 35-fold molar excess of wild-type Fe protein (NifH). Each 1.1-mL reaction mixture contained 66 μmol of MOPS (pH 7.0), 22 μmol of MgCl₂, 10 μmol of ATP, 60 μmol of phosphocreatine, creatine phosphokinase (0.5 mg/mL), BSA (0.6 mg/mL), and, when necessary, the appropriate amount of aqueous
substrate. All the enzymatic assays were carried out in 10 mL reaction vials capped with butyl rubber stoppers and aluminum seals. The head-space and reaction mixture in the vials were made anoxic through repeated cycles of degassing and flushing with argon gas. When the air was removed, 100 μL of a 0.1 M solution of Na₂S₂O₄ was added to each reaction vial. The reaction vials were vented with a gas bubbler previous to injecting any substrate or inhibitor gas to the vial head-space. After the injection of the gas, the vial head-space was vented again. The assays were initiated by addition of wild-type Fe protein and were run for 10 min (unless otherwise stated). All reactions were terminated by the addition of 300 μL of a EDTA 0.4 M (pH 8) solution. The production of C₂H₄ and C₂H₆ was quantified by gas chromatography on a Shimadzu GC-8A gas chromatograph with a flame ionization detector fitted with a 30 cm × 0.3 cm Porapak N column using nitrogen as the carrier gas. H₂ evolution was also quantified by gas chromatography using a molecular sieve 5A column installed on a Shimadzu GC-8A gas chromatograph fitted with a thermo-conductivity detector. A fluorescence assay was used to determine ammonia in the post-reaction solutions. A 25-μL aliquot of post-reaction solution was added to 1 mL of detection buffer containing 20 mM o-phthalic dicarboxaldehyde, 3.5 mM 2-mercaptoethanol, 5% (v/v) ethanol, and 200 mM potassium phosphate (pH 7.3) and allowed to react in the dark for 30 min. A standard curve was prepared with 25-μL aliquots of NH₄Cl solutions dissolved in 1 mL of detection buffer. The ammonia presented in the post-reaction solution was detected by fluorescence
Gas-tight syringes were used to transfer all the liquids and gases.

**Metal Analysis.** Molybdenum and vanadium analysis were performed in the Utah Veterinary Diagnostic Laboratory (Logan, UT). In brief, the protein samples were digested (1:1) in trace mineral grade nitric acid under heat. The digests were then diluted with ultra-pure water to a final nitric acid content of 5 %, which provided a matrix match for the analytical standards. The prepared samples were analyzed by ICP-MS and assessed against concentration curves of known mineral standards. The detection limit for both molybdenum and vanadium was 0.001 ppm. The concentration of ferrous iron in the protein samples was determined using the 2,2’ dipyridyl method 31 after the protein samples had been resuspended in 400 µL of distilled water and digested with HCl 1 % at 80 ºC for 10 min.

**Treatment of Kinetic Data.** All the collected data were fitted into the Michaelis-Menten equation 32,

\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} \]  

(Eq. 2)

where \( v \) is the rate of product formation, \( V_{\text{max}} \) is the theoretical maximum rate, \( [S] \) is the concentration of substrate, and \( K_m \) is the Michaelis constant. Eq. 2 was edited into the computer program Igor Pro and used to calculate the \( V_{\text{max}} \) and \( K_m \) values from the average of three independent data sets for each of the substrates examined. Inhibition patterns of various substrates and inhibitor combinations were determined by visual examination of the double-reciprocal (Lineweaver-
Burk) plots of the rate of enzymatic reaction vs different substrate concentrations in the presence of different fixed inhibitor concentrations. The inhibition constant $K_i$ for competitive inhibitors was derived by plotting the slope of the double-reciprocal plots vs the different fixed inhibitor concentrations, which gives a straight line with a slope of $K_m/V_{max}K_i$ and an intercept on the $y$ axis of $K_m/V_{max}$. When $y = 0$, the intercept on the $x$ axis gives $-K_i^{32}$.

RESULTS

Protein Purification. A histidine-tagged V-nitrogenase was constructed to circumvent the problems associated with protein instability which has been widely recognized to plague V-nitrogenase preparations $^{1,33}$. The same strategy has been successfully applied to the Mo-nitrogenase, as a result of which large amounts of highly pure and active enzyme can be obtained following a simple purification protocol $^{28}$. In the V-nitrogenase, the histidine-tag is located at the N-terminus of the VFe protein $\beta$ subunit (VnfK). The purification protocol, which uses a Zn$^{2+}$-loaded IMAC resin, readily purifies the VFe protein $\alpha$ and $\beta$ subunits (VnfDK) (Figure 4-1S). The identities of these proteins were confirmed by N-terminal sequence analysis (Table 4-1S). Unexpectedly, the iron (Fe$^{2+}$) content and the C$_2$H$_2$ and H$^+$ reduction activities (Table 4-1) are lower than what has been reported in the literature $^{34-36}$. 
Table 4-1. Specific activities of the VFe protein (VnfDK) purified on a Zn$^{2+}$-loaded IMAC resin and moles of Iron associated to the protein preparation as determined by the 2,2’ dipyridyl method.

<table>
<thead>
<tr>
<th></th>
<th>C$_2$H$_2$ (0.11 atm)</th>
<th>Ar (1 atm)</th>
<th>Fe$^{2+}$ mol of metal/mol of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>VFe protein</td>
<td>VFe protein</td>
<td>Ar</td>
<td>Fe$^{2+}$</td>
</tr>
<tr>
<td></td>
<td>C$_2$H$_4$ H$_2$</td>
<td>H$_2$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>23</td>
<td>39</td>
</tr>
</tbody>
</table>

Specific activity values are expressed in nmol.min$^{-1}$.mg$^{-1}$ of protein. VnfDK to NifH molar ratio was 1:20

A striking observation is that the δ subunit marginally co-purifies with the rest of the complex (Figure 4-1S). Importantly, the lack of a functional δ subunit has been documented to affect the catalytic capabilities of the V-nitrogenase 14. Therefore, a plausible hypothesis to explain the low iron content and poor rates of enzymatic reduction of substrates by the VFe protein herein presented is that the δ subunit stabilizes the VFe protein quaternary structure and, in its absence, the protein is unable to attain the high reduction potential needed for substrate reduction. Thus, adding purified δ subunit to the DJ1258 cell supernatant, which contains histidine-tagged VFe protein, could stabilize the protein quaternary structure during the purification of the protein, yielding a protein preparation with improved enzymatic activity and higher metal content. In order to test this hypothesis and in an attempt to obtain histidine-tagged VFe protein with improved enzymatic activity and in high yields, a molecular biology strategy was designed to clone vnfG, to insert six histidine-coding codons on its 3’ prime end, and to overexpress the resulting gene in a heterologous expression system so as to obtain recombinant, pure histidine-tagged δ subunit in large amounts. Thus: i)
vnfG was genetically modified and inserted into *Escherichia coli* strain BL21; ii) The modified vnfG gene was overexpressed and its product purified to homogeneity (Figure 4-1S), and iii) Purified histidine-tagged δ subunit was added to a supernatant containing histidine-tagged VFe protein. To further stabilize the interactions among the VFe subunits, all the buffers used throughout the purification procedure contained 15 % glycerol. The resulting VFe protein complex, which was purified with a Ni\(^{2+}\)-loaded IMAC resin, showed the following characteristics: i) It co-purified with both wild type and histidine-tagged δ subunit (Figure 4-1S); ii) It contained ~ 1.3 atoms of vanadium and ~ 22 atoms of iron per molecule of protein (Table 4-2); iii) Its C\(_2\)H\(_2\) and H\(^+\) reduction activities were improved by a factor of ~ 10 (Table 4-3); and iv) It produces C\(_2\)H\(_6\) upon C\(_2\)H\(_2\) reduction, which is a hallmark of the V-nitrogenase. Metal analysis detected almost no molybdenum in the protein preparation. This, combined with the N-terminus sequence analysis results and the fact that C\(_2\)H\(_6\) is a product of C\(_2\)H\(_2\) reduction, confirms that the examined protein is the VFe protein.

Unfortunately, only ~ 2/3 of the protein molecules are loaded with V atoms, in spite of several modifications to the optimized protein purification protocol developed herein to isolate the histidine-tagged version of the VFe protein.

**Table 4-2. Metal content of the nitrogenases (MoFe and VnfDK+VnfG) examined in this study.**

<table>
<thead>
<tr>
<th></th>
<th>Fe(^{2+})</th>
<th>V</th>
<th>Mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>MoFeprotein</td>
<td>23</td>
<td>0.001</td>
<td>1.81</td>
</tr>
<tr>
<td>VFe protein</td>
<td>22</td>
<td>1.28</td>
<td>0.001</td>
</tr>
</tbody>
</table>

All the values are expressed in mol of metal per mol of protein. Mo and V were quantified by ICP-MS and Fe was quantified using the 2,2’-dipyridyl method.
Table 4-3. Specific activities of VFe protein (VnflDK+VnflG) purified on a Ni^{2+}-loaded IMAC resin.

<table>
<thead>
<tr>
<th></th>
<th>C(_2)H(_2) (0.11 atm)</th>
<th>Ar (1 atm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C(_2)H(_4)</td>
<td>H(_2)</td>
</tr>
<tr>
<td>VFe protein</td>
<td>130</td>
<td>290</td>
</tr>
</tbody>
</table>

All values are expressed in nmol.min\(^{-1}\).mg\(^{-1}\) of protein. The VFe to NifH molar ratio used in this study was 1:20.

Since the V content is less than the ideal 2 atoms per protein molecule and the ratio of V:Fe was 1:17 (the expected value is 1:15), it is to be concluded that there are three different protein populations: one containing holo-VFe protein, another one containing Fe but not V, and a third one containing neither metal.

*Activity Profile, Electron Allocation, and Michaelis-Menten Constants.*

Table 4-4 groups the specific activity values, the total electrons shuttled, and the percentage of electrons going to each of the products of the reduction of H\(^+\), C\(_2\)H\(_2\), N\(_2\), N\(_2\)H\(_4\), and N\(_3\)^\(^{-}\), as catalyzed by the histidine-tagged VFe protein and the MoFe protein. In an Ar atmosphere and under optimal turn-over conditions (30 °C, pH 7, VFe to Fe protein molar ratio of 1:35), the enzyme delivers electrons to H\(^+\) at a rate of ~ 1000 nmol.min\(^{-1}\)(mg\(^{-1}\) of protein)(~ 520 nmol of H\(_2\) produced min\(^{-1}\).mg\(^{-1}\) of protein). This is the maximum flux of electrons that this protein could achieve under the experimental conditions herein presented, which contrasts with the specific activity value for H\(_2\) evolution (1400 nmol of H\(_2\) produced min\(^{-1}\).mg\(^{-1}\) of protein) previously reported for the wild-type VFe protein from *A. vinelandii*\(^{36}\). For substrates other than protons, the flux of electrons to the different substrates tested in this work decreased according to the following series: C\(_2\)H\(_2\) > N\(_2\) > N\(_3\)^\(^{-}\) > N\(_2\)H\(_4\). A similar trend is observed for the MoFe protein,
although this protein seems to deliver approximately the same number of
electrons to C2H2 and N2. For the MoFe protein, the electron flux decreases
according to the following series: C2H2 ≈ N2 > N3− > N2H4.

As previously reported, the enzyme catalyzes the 4H+/4e− reduction of
C2H2 to form a small but readily detectable amount of C2H6 (~ 2 % of the total
electron flux is diverted to this product), whereas ~ 60 % of the total electron flux
goes to the 2H+/2e− reduction of C2H2 to form C2H4. The remainder of the
electron pool (~ 40 %) reduces protons from solution. The electron distribution is
accomplished differently in the wild-type MoFe protein: ~ 90 % of the electron
flux is diverted to the 2H+/2e− reduction of C2H2 to form C2H4; the residual
electrons reduce protons from solution. In contrast with the VFe protein, wild-
type MoFe protein does not support the 4H+/4e− reduction of C2H2 [an exception
being a MoFe protein which α-195His residue has been substituted by an
asparagine. The altered MoFe protein deviates ~ 20 % of the electron flux to
C2H2 to form C2H6 and ~ 30 % to protons to form H220].

The histidine-tagged VFe protein also supports the reduction of N2,
although at a specific activity lower than previously reported34-36 even though the
vanadium content of the protein preparation presented herein (~ 1.3
atoms/protein molecule) is higher than that of the enzyme studied in36 (0.7 ± 0.3
atoms/protein molecule). In any case, the enzyme preparation herein presented
distributes the total electron pool to the substrates N2, N2H4, and N3− in a similar
manner: ~ 20 % of the total electron flux goes to each individual substrate to form
NH3 and the remaining 80 % reduce protons to hydrogen gas. On the other hand,
when the Mo-nitrogenase turns over $N_2$ (under 0.4 atm of $N_2$), it allocates 60% of the total electron flux to this substrate. Interestingly, the Mo-nitrogenase allocates the electron flux to the substrates $N_2H_4$ and $N_3^-$ in a manner that resembles that seen for the V-nitrogenase when it is turning over the same substrates (~20% to $N_2H_4$ and $N_3^-$ and ~80% to $H^+$). Therefore, the Mo and V-based active sites direct the electron flux to $N_2H_4$ and $N_3^-$ similarly, the difference being the substrate $N_2$ which is preferentially reduced by the Mo-based active site but not by the V counterpart.

The fact that both enzymes direct similarly the electron flux to $N_2H_4$ and $N_3^-$ can be explained in terms of the Michaelis constant $K_m$ for $N_2H_4$ and $N_3^-$: both Mo- and V-nitrogenases depict very similar $K_m$ values for the mentioned substrates (Table 4-5). However, for $N_2$ reduction, the same rational cannot be applied since the Mo- and V-nitrogenases have very similar $K_m$ values for $N_2$ (Table 4-5).

*Inhibition Patterns: The Nonreciprocal Response of $N_2$ and $C_2H_2*.

During the 1970s it was observed that the reduction of $N_2$ by the Mo-nitrogenase was inhibited by $C_2H_2$ in a non-competitive manner and that the reduction of $C_2H_2$ was inhibited by $N_2$ in a competitive manner. At the time, the nonreciprocal response of $N_2$ and $C_2H_2$ laid a scenario that could not be explained by any scheme that had been proposed for the mechanism of substrate reduction. Rivera-Ortiz *et al.* interpreted this results as arising from the competition of both $C_2H_2$ and $N_2$ for the same electron sink.
Table 4-4. Specific activities of the VFe and MoFe proteins for the reduction of different substrates.

<table>
<thead>
<tr>
<th></th>
<th>Ar (1 atm)</th>
<th>C₂H₂ (0.11 atm)</th>
<th>N₂ (0.4 atm)</th>
<th>N₂H₄ (100 mM)</th>
<th>NaN₃ (10 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H₂</td>
<td>H₂</td>
<td>C₂H₄</td>
<td>C₂H₆</td>
<td>H₂</td>
</tr>
<tr>
<td>VFe protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Product (nmol.min⁻¹.mg⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total e⁻ (nmol.min⁻¹.mg⁻¹)</td>
<td>510</td>
<td>150</td>
<td>260</td>
<td>5</td>
<td>320</td>
</tr>
<tr>
<td>Total e⁻ (%)</td>
<td>100</td>
<td>36</td>
<td>62</td>
<td>2</td>
<td>81</td>
</tr>
<tr>
<td>MoFe protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Product (nmol.min⁻¹.mg⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total e⁻ (nmol.min⁻¹.mg⁻¹)</td>
<td>2050</td>
<td>250</td>
<td>1970</td>
<td>----</td>
<td>860</td>
</tr>
<tr>
<td>Total e⁻ (%)</td>
<td>100</td>
<td>11</td>
<td>89</td>
<td>----</td>
<td>40</td>
</tr>
</tbody>
</table>

All the enzymatic assays were carried out at pH 7, 30 °C, and a VFe protein to Fe protein molar ratio of 1:35.
Table 4-5. Michaelis-Menten constants for the MoFe and VFe protein.

<table>
<thead>
<tr>
<th></th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N$_2$ (atm)</td>
</tr>
<tr>
<td>MoFe protein</td>
<td>0.04</td>
</tr>
<tr>
<td>VFe protein</td>
<td>0.05</td>
</tr>
</tbody>
</table>

All the enzymatic assays were carried out at pH 7, 30 ºC, and a VFe protein to Fe protein molar ration of 1:35.

Table 4-6. Inhibition constants for the MoFe and VFe protein.

<table>
<thead>
<tr>
<th></th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N$_2$ (atm)</td>
</tr>
<tr>
<td></td>
<td>inhibits</td>
</tr>
<tr>
<td>C$_2$H$_2$</td>
<td>N$_2$</td>
</tr>
<tr>
<td>MoFe protein</td>
<td>0.250 C</td>
</tr>
<tr>
<td>VFe protein</td>
<td>0.045 C</td>
</tr>
</tbody>
</table>

All the enzymatic assays were carried out at pH 7, 30 ºC, and a VFe protein to Fe protein molar ration of 1:35.
Symbols: C, competitive inhibition; NC, non-competitive inhibition; M, mixed inhibition; UC, uncompetitive inhibition.
The authors reasoned that in order to reduce N\textsubscript{2}, the electron sink must accumulate six electrons, but only two electrons to reduce C\textsubscript{2}H\textsubscript{2}. Thus, high C\textsubscript{2}H\textsubscript{2} partial pressures would keep the sink depleted, so that it will never contain more than two electrons (making the sink incapable of reducing N\textsubscript{2}), whereas high N\textsubscript{2} partial pressures will never prevent the access of C\textsubscript{2}H\textsubscript{2} to the sink, resulting in the non-competitive inhibition trend seen when C\textsubscript{2}H\textsubscript{2} is the substrate. Figure 4-1 shows that the same rational can be applied to the V-nitrogenase, since it also depicts the nonreciprocal response of N\textsubscript{2} and C\textsubscript{2}H\textsubscript{2} (Panels C and D). The substitution of Mo for V in the active site of the enzyme does not alter the nonreciprocal response of N\textsubscript{2} and C\textsubscript{2}H\textsubscript{2}. However, the V-nitrogenase shows a different degree of inhibition by N\textsubscript{2} (when C\textsubscript{2}H\textsubscript{2} is the substrate) and by C\textsubscript{2}H\textsubscript{2} (when N\textsubscript{2} is the substrate) when compared with the Mo-nitrogenase. The values for the inhibition constant $K_i$ (Table 4-6) for N\textsubscript{2} and C\textsubscript{2}H\textsubscript{2} reveal that, for the Mo-nitrogenase, N\textsubscript{2} is a poor inhibitor of the reduction of C\textsubscript{2}H\textsubscript{2}. On the other hand, N\textsubscript{2}, with a $K_i$ five times smaller than that seen for the Mo-nitrogenase is a better inhibitor of the reduction of C\textsubscript{2}H\textsubscript{2} when the reaction is catalyzed by the V-nitrogenase. As for C\textsubscript{2}H\textsubscript{2}, it is a better inhibitor of the reduction of N\textsubscript{2} when the enzyme assayed is the Mo-nitrogenase which, with a $K_i$ of 0.009 atm, is approximately seven times smaller than the corresponding $K_i$ for C\textsubscript{2}H\textsubscript{2} for the inhibition of N\textsubscript{2} reduction as catalyzed by the V-nitrogenase.
Inhibition Patterns: $H_2$ Inhibits $N_2$ but not $C_2H_2$, $N_2H_4$, or $N_3^-$ Reduction.

Dihydrogen gas has long been recognized to inhibit the Mo-dependent reduction of $N_2$. The nature of the inhibition was determined to be competitive, with a $K_i$ of ~ 0.1 atm. Hwang et al. reported that $H_2$ had no effect on the reduction of $C_2H_2$, $N_3^-$, HCN, or methylisocyanide, thus suggesting a common binding site for $H_2$ and $N_2$ and a common (or multiple) binding site(s) for $C_2H_2$, $N_3^-$, HCN, and methylisocyanide that cannot be accessed by $H_2$. It was of interest to investigate
the effect H₂ has on the reduction of C₂H₂, N₂, N₂H₄, and N₃⁻ when the reaction is catalyzed by the V-nitrogenase so as to establish whether the substitution of a metal and/or variations on the amino acid residues delineating the active site of these enzymes would yield a different H₂ inhibition pattern.

**Figure 4-2. Double-reciprocal plots for the reduction of different partial pressures of N₂ in the absence and in the presence of the inhibitor H₂.** Panel A correspond to the reaction catalyzed by the Mo-nitrogenase whereas panel B is for the reaction catalyzed by the V-nitrogenase. Panel A: ■) H₂, 0 atm; □) H₂, 0.4 atm. Panel B: ●) H₂, 0 atm; ○) H₂, 0.4 atm.

Figure 4-2 (panel B) shows that H₂ indeed inhibits N₂ reduction, although in an uncompetitive manner. The inhibition constant Kᵢ for H₂ was determined to be 0.7 atm, which is seven times larger that that determined for the inhibition of N₂ reduction by H₂ as catalyzed by the Mo-nitrogenase.²⁵ Dihydrogen gas showed no effect on the reduction of C₂H₂, N₂H₄, and N₃⁻ (not shown). Therefore, H₂ as an inhibitor of the reduction of N₂, C₂H₂, N₂H₄, and N₃⁻ behaves similarly on the V- and Mo-nitrogenases in that it only inhibits the reduction of N₂ without affecting the reduction of any other substrate. However, the degree by which H₂ inhibits N₂ reduction differs for both enzymes: H₂ seems to be a stronger inhibitor of the Mo-based catalyzed reaction, as judged by the lower Kᵢ
value it shows for the inhibition of N$_2$ reduction when compared with that of the V-based catalyzed reaction (Table 4-6). This, and the differences in $K_i$ values for N$_2$ and C$_2$H$_2$ as inhibitors clearly reflects that the effect of substituting Mo for V in the active site of these enzymes goes beyond those differences seem in terms of electron allocation and specificities for the substrates N$_2$ and C$_2$H$_2$.

*Inhibition Patterns: N$_2$H$_4$ Inhibits the Reduction of C$_2$H$_2$.* Davis$^{24}$ determined that, for the MoFe protein, N$_2$H$_4$ was a weak, noncompetitive (mixed) inhibitor of the reduction of C$_2$H$_2$. Figure 4-3 (panel A) shows a double-reciprocal of the reduction of C$_2$H$_2$ as catalyzed by the Mo-nitrogenase in the presence of 80 mM N$_2$H$_4$. This plot reproduces the results reported by Davis: when the enzyme is turning over C$_2$H$_2$ in the presence of hydrazine, the latter seems to bind to the free enzyme and to the enzyme-substrate complex, which suggests that hydrazine can bind an enzymatic state with a less reduced character that that needed for C$_2$H$_2$ binding. In contrast, the double-reciprocal plot for the reduction of C$_2$H$_2$ as catalyzed by the V-nitrogenase in the presence of N$_2$H$_4$ shows a different inhibition trend: hydrazine acts as an uncompetitive inhibitor of the reduction of C$_2$H$_2$. Only after C$_2$H$_2$ binds on the active site can hydrazine exerts its inhibitory effect. It is unknown what type of chemical changes are promoted upon C$_2$H$_2$ binding on the active site of the V-nitrogenase that unexpectedly permit hydrazine to exert its inhibitory effect on C$_2$H$_2$ reduction. In any case, the interactions between these chemical species occurring at the active site of the V-nitrogenase profoundly affect the distribution of protons and electrons among the molecules being reduced. This is described below.
Figure 4-3. Double-reciprocal plots for the reduction of different partial pressures of C\textsubscript{2}H\textsubscript{2} in the absence and in the presence of the inhibitor N\textsubscript{2}H\textsubscript{4}. Panel A correspond to the reaction catalyzed by the Mo-nitrogenase whereas panel B is for the reaction catalyzed by the V-nitrogenase. Panel A: ■ N\textsubscript{2}H\textsubscript{4}, 0 atm; □ N\textsubscript{2}H\textsubscript{4}, 80 mM. Panel B: ● N\textsubscript{2}H\textsubscript{4}, 0 atm; ○ N\textsubscript{2}H\textsubscript{4}, 80 mM.

The Effect of H\textsubscript{2}, N\textsubscript{2}, N\textsubscript{2}H\textsubscript{4}, and N\textsubscript{3}\textsuperscript{-} on the Electron Distribution to the Products of C\textsubscript{2}H\textsubscript{2} Reduction. Wild-type Mo-nitrogenase catalyzes the two electron reduction of C\textsubscript{2}H\textsubscript{2} to form C\textsubscript{2}H\textsubscript{4}. Only at high temperatures \textsuperscript{37}, or when the reaction is catalyzed by altered enzymes \textsuperscript{20}, has the Mo-nitrogenase been reported to achieve the four electron reduction of C\textsubscript{2}H\textsubscript{2} to yield C\textsubscript{2}H\textsubscript{6}. In contrast, the V-nitrogenase reduces C\textsubscript{2}H\textsubscript{2} to yield a mixture of C\textsubscript{2}H\textsubscript{4} and C\textsubscript{2}H\textsubscript{6} under normal conditions (pH 7, 30 °C, Fe protein:VFe protein molar ratio 1:35). As with Mo-nitrogenase, CO inhibits the reduction of C\textsubscript{2}H\textsubscript{2} by the V-nitrogenase, although at partial pressures as low as < 0.02 atm CO surprisingly enhances the formation of both C\textsubscript{2}H\textsubscript{4} and C\textsubscript{2}H\textsubscript{6} in equimolar amounts \textsuperscript{38}. Figure 4-4 shows that, for the V-nitrogenase, the electron flux to C\textsubscript{2}H\textsubscript{2} is not only altered by low partial pressures of CO to produce more C\textsubscript{2}H\textsubscript{4} and C\textsubscript{2}H\textsubscript{6} as reported in \textsuperscript{38}; the electron flux can also be diverted to the reduction of C\textsubscript{2}H\textsubscript{2} to specifically increase the formation of C\textsubscript{2}H\textsubscript{6} when the reaction is catalyzed in the presence of N\textsubscript{2}H\textsubscript{4} (80 mM) and N\textsubscript{3}\textsuperscript{-} (10 mM).
The effect was not provoked by the gaseous substrate \( \text{N}_2 \) (0.4 atm) or the inhibitor \( \text{H}_2 \) (0.95 atm), indicating that only upon \( \text{N}_2\text{H}_4 \) or \( \text{N}_3^- \) binding on a specific location within the enzyme (a binding site common to \( \text{N}_2\text{H}_4 \) and \( \text{N}_3^- \) but not to \( \text{H}_2 \) or \( \text{N}_2 \)) can the effect be triggered.

![Figure 4-4](image)

**Figure 4-4.** The effect of \( \text{H}_2 \), \( \text{N}_2 \), \( \text{N}_2\text{H}_4 \), and \( \text{N}_3^- \) on the distribution of electrons to the products of the reduction of \( \text{C}_2\text{H}_2 \). All the reactions were carried out under an atmosphere consisting of \( \text{Ar} \) and the appropriate amount of the gaseous substrate(s). The final pressure inside the reaction vessels was 1 atm.

A 10-fold increase on the electron flux to \( \text{C}_2\text{H}_2 \) to form \( \text{C}_2\text{H}_6 \) is observed when the V-nitrogenase is turning over substrates in a reaction mixture containing both \( \text{C}_2\text{H}_2 \) and \( \text{N}_2\text{H}_4 \) (or \( \text{N}_3^- \)) (Table 4-7). The effect could not be recreated when the substrate was \( \text{C}_2\text{H}_4 \), indicating that an intermediate of the reduction of \( \text{C}_2\text{H}_2 \)
(and not C₂H₄) is involved in redirecting the electron flux to increase the formation of C₂H₆. In any case, the six electron product of the C₂H₂ reduction, CH₄, could not be detected under any of the conditions tested on this work.

Table 4-7. Electron distribution to H⁺ and to the products of the reduction of C₂H₂ in the presence of the inhibitor H₂ and the substrates N₂, N₂H₄, and N₃⁻.

<table>
<thead>
<tr>
<th></th>
<th>H₂</th>
<th>C₂H₄</th>
<th>C₂H₆</th>
<th>NH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar + C₂H₂</td>
<td>74</td>
<td>25</td>
<td>1</td>
<td>----</td>
</tr>
<tr>
<td>Ar + C₂H₂ + H₂.</td>
<td>74</td>
<td>24</td>
<td>2</td>
<td>----</td>
</tr>
<tr>
<td>Ar + C₂H₂ + N₂</td>
<td>62</td>
<td>27</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Ar + C₂H₂ + N₂H₄</td>
<td>57</td>
<td>17</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>Ar + C₂H₂ + N₃⁻</td>
<td>64</td>
<td>14</td>
<td>9</td>
<td>13</td>
</tr>
</tbody>
</table>

"The direct determination of the total electron flux was not possible for experiment since the hydrogen evolved as a product of the reaction was masked by the hydrogen added as part of the experimental design. However, since hydrogen does not inhibit neither the reduction of C₂H₂ nor the reduction of H⁺ from solution, in this case the total electron flux was assumed to be that for the reduction of C₂H₂ under an Ar atmosphere.

As previously mentioned, altered Mo-nitrogenases, but not wild-type Mo-nitrogenase, can support the 4[H⁺/e⁻] reduction of C₂H₂ at 30 ºC to form C₂H₆. In one of the altered MoFe proteins, α-191Gln has been replaced by a lysine (α-191Gln→Lys); the second altered MoFe protein possesses a mutation in which α-195His has been replaced by an asparagine (α-195His→Asn). MoFe proteins α-191Gln→Lys and α-195His→Asn deviated to the product C₂H₆ 13 % and 23 % of the total electron flux, respectively. It seems that a requisite for re-routing the electron flux to the formation of C₂H₆ is the presence of an amine or amine-like group in the vicinity of the C₂H₂ binding site. A third altered MoFe protein (α-195His→Gln) did not catalyzed the 4[H⁺/e⁻] reduction of C₂H₂, which suggests that
the proximity of the N-H species to the C₂H₂ adduct is important for the stimulation of the effect. Interestingly, neither of these mutant enzymes reduces N₂, whereas the VFe protein used in this study is fully capable of reducing N₂. In light of these results, it was of interest to test whether a diversion of the electron flux could be provoked when the wild-type Mo-nitrogenase turns over C₂H₂ in the presence of either N₂H₄ or N₃⁻. When under reduction, these two species could mimic the N-H group that seems to be needed to divert the electron flux to form C₂H₆. Under the experimental conditions herein tested (Fe protein:MoFe protein molar ratio of 35:1, pH 7, and at 30 ºC), the results showed that neither N₂H₄ (120 mM) nor N₃⁻ (12 mM) were able to divert the electron flux to the formation of C₂H₆ when the substrate was C₂H₂.

DISCUSSION

Knowledge regarding biological nitrogen fixation has been derived from studies mainly conducted on the Mo-nitrogenase. Research on biological nitrogen fixation has been biased towards the Mo-dependent system because it is preferentially expressed in bacteria in the presence of Mo and because the Mo-nitrogenase displays the highest rates for N₂ reduction of the three phylogenetically-related nitrogenases known to date. It is worth noting that the progress achieved within the last 10 years in understanding the molecular basis of Mo-dependent biological nitrogen fixation has been due in part to the fact that the wild-type MoFe protein component has been genetically modified into a MoFe protein variant that can be readily purified in a reproducible manner, yielding
increased amounts of highly active protein preparations. As good for the field as this might be, it is in detriment to the understanding of biological nitrogen fixation accomplished by systems whose activities depend on transition metals such as V or exclusively dependent on Fe.

In an attempt to shorten the knowledge gap existing between Mo- and V-dependent biological nitrogen fixation, an effort was undertaken to genetically modify the *A. vinelandii* VFe protein following the same genetic strategy designed for the MoFe protein as described in \(^{28}\). Importantly, it is of interest to compare the kinetic properties of isoenzymes that contain either Mo or V and that are slightly different in terms of the amino acid environment that compose their active sites. To this end, a histidine tagged VFe protein was constructed and purified; unfortunately, the as-purified protein displayed poor enzymatic activities towards the reduction of H\(^+\) and C\(_2\)H\(_2\). It was noticed that the stoichiometry of the histidine-tagged protein did not correspond to the \(\alpha_2\beta_2\delta_2\) hexamer; the protein species purified as a tetramer of composition \(\alpha_2\beta_2\). Therefore, it was decided to clone, express, and purify a recombinant \(\delta\) subunit in order to add it to the cell supernatant containing the histidine-tagged VFe protein in an attempt to increase the stability of the \(\alpha_2\beta_2\delta_2\) hexamer as it is loaded and eluted from the metal affinity column. This strategy proved to be partially successful since the proton reduction activity, from being \(~ 3\%\) of the best reported activity for the wild-type enzyme, was improved more than 10-fold to finally yield an enzyme which reduces protons with a specific activity that is \(~ 40\%\) of that of the wild-type enzyme. This illustrates what has been insinuated in the literature regarding the
weak interactions that take place among the VFe protein components. This work
demonstrate for the first time how these interactions can be strengthen upon
addition of an excess amount of $\delta$ subunit, which results on a VFe protein with
improved enzymatic activity and with higher metal content. However, the final
protein preparation was a mixture of holo- and apo-enzyme: The V content was
estimated to be $\sim 1.3$ mol of V per mol of protein (an insignificant amount of Mo
was detected in the preparation). The N-terminal sequence of the two major
colpeptides that eluted from the metal affinity chromatography, combined with
the metal analysis of the protein preparation, confirmed that the purified protein
was indeed the VFe protein. Further confirmation came from the fact that the
protein showed the hallmark of the alternative nitrogenases, the reduction of $C_2H_2$
to $C_2H_6$ at 30 ºC.

This work confirms the work of others $^{35,36,39}$: The V-based reduction of
nitrogen is less efficient than the reduction of nitrogen as catalyzed by the Mo-
nitrogenase in that more hydrogen gas evolves per mol of $N_2$ fixed when the
reaction is catalyzed by the V-nitrogenase. The same is true when $C_2H_2$ is the
substrate of the reaction: whereas hydrogen evolution is almost suppressed when
the Mo-nitrogenase turns over $C_2H_2$, the V-nitrogenase allocates $\sim 60$ % of the
total electron flux to $C_2H_2$ and the remaining to $H^+$ reduction. That $C_2H_2$ is less
efficiently reduced by the V-nitrogenase can be explained considering its $K_m$
value (0.04 atm), which is ten times higher as compared to that for the Mo-nitrogenase
(0.0045 atm). However, the same rational cannot be applied to explain why $N_2$ is
reduced less efficiently by the V-nitrogenase as compared with the Mo-
nitrogenase since, as pointed out in \(^1\), the \(K_m\) for \(N_2\) as a substrate is similar for both Mo- (0.04 atm) and V-nitrogenase (0.05 atm). Kästner et al. \(^{40}\) points out that the most difficult step of the reaction from \(N_2\) to \(NH_3\) is the first protonation of dinitrogen. The authors state that, in the gas phase, the reaction step \(N_2 + H^+ + e^- \rightarrow N_2H\) is endothermic (164 kJ/mol). However, at the FeMo-cofactor, the reaction was calculated to be 47 kJ/mol, which indicates that the main goal of nitrogenase is to reduce the energy barrier necessary to achieve the first protonation of \(N_2\). Therefore, it seems reasonable to believe that the energy barrier for the addition of the first (or subsequent) proton(s) onto \(N_2\) is higher when the reaction is carried out on a V-containing active site (and also on an active site composed by a different amino acid population). As a consequence of this, more ATP molecules are hydrolyzed and higher amounts of \(H_2\) evolves per mol of \(N_2\) fixed even though the latter interacts similarly with the Mo and V active sites, as judged by the similarities in \(K_m\) value for \(N_2\) for both systems.

But in spite of the differences regarding \(N_2\) and \(C_2H_2\) reduction efficiencies, the V-nitrogenase: i) reduces \(N_2H_4\) and \(N_3^-\) with \(K_m\) values that resemble those for the Mo-nitrogenase; ii) depicts the same nonreciprocal response of \(N_2\) and \(C_2H_2\); and iii) its \(N_2\) reduction activity is inhibited by \(H_2\) but not its \(C_2H_2, N_2H_4,\) and \(N_3^-\) reduction activity. That the enzyme depicts the same nonreciprocal response of \(N_2\) and \(C_2H_2\) suggests that, as it has been interpreted for the Mo-nitrogenase \(^{23}\), \(C_2H_2\) and \(N_2\) are not equivalent substrates: \(C_2H_2\) binds on a less reduced whereas \(N_2\) binds on a more reduced V active site. Mo and V, in conjunction with their respective atomic environments, show the same reactivity
when simultaneously facing N₂ and C₂H₂. However, the degree of inhibition varies for N₂ (when C₂H₂ is the substrate) and for C₂H₂ (when N₂ is the substrate) when compared with those for the Mo-nitrogenase. Dihydrogen gas is also an inhibitor of the V-catalyzed reduction of N₂ although it exerts its inhibitory effect in an uncompetitive manner whereas H₂ is a competitive inhibitor of the reduction of N₂ as catalyzed by a Mo-containing active site. That H₂ is an uncompetitive inhibitor of the reduction of N₂ suggests that it becomes an inhibitor of N₂ reduction only after the substrate binds on the V-nitrogenase active site. In the Thorneley-Lowe scheme for the sequential reduction of the MoFe protein (Figure 4-1), N₂ and H₂ compete for the same active sites which apparently possess similar reduction states (E₃, E₄). The results presented in this work suggest that, for the VFe protein, N₂ must first bind to the V-containing active site for H₂ to exert its inhibitory effect. Apparently, the site of N₂ binding is not the site for attachment of H₂. This observation must be taken into account if the mechanism for N₂ reduction, as summarized in the Thorneley-Lowe cycle for the reduction of the MoFe protein, were to be extrapolated to the sequential reduction of the VFe protein. A question that arises after examining the previous results regarding N₂ and H₂ interactions at the V-containing active site is whether this enzyme is able to activate the exchange of D₂ with protons from solution to form HD in the presence of N₂ or any other substrate.

As previously pointed out, the VFe protein is not inhibited by H₂ when reducing C₂H₂, N₂H₄, and N₃⁻, indicating that these substrates and H₂ do not share binding sites or possible bind on an active site which is less reduced than that
needed for H$_2$ or N$_2$ binding. Here both enzymes coincide in terms of the lack of effect by H$_2$ toward the reduction of these “semi-reduced” substrates.

The “oxidized” species H$_2$ (and N$_2$) is also ineffective at re-routing electrons to the products of the reduction of C$_2$H$_2$, effect that is readily provoked by the “semi-reduced” hydrazine and azide when both species are in the reaction mixture as the V-nitrogenase turns over C$_2$H$_2$. For the first time, chemical species extraneous to the nitrogenase protein folds are observed to dramatically direct the flux of electrons to augment the formation of C$_2$H$_6$. This intriguing observation has so far been reported for altered MoFe proteins\textsuperscript{19,20} which amino acid substitutions have added amine-like (N-H) species in close proximity to what seems to be the C$_2$H$_2$ binding site\textsuperscript{41}. This species is already an integral part of N$_2$H$_4$ and it can be envisioned to arise as N$_3^-$ is reduced at the V active site, thus providing the functional group that would stabilize a C$_2$H$_2$ adduct such that it can undergo a 4[H$^+$/e$^-$] reduction. An important observation is that the effect is not stimulated when C$_2$H$_4$ is used as the substrate of the reaction, indicating that only a M-C$_2$H$_2$ adduct (in which M is the active site) can provoke the redistribution of the electron flux to form more C$_2$H$_6$ (scheme 4-1S). It is unclear whether this is a kinetic effect, in which the N-H species would prolong the C$_2$H$_2$-active site interaction such that more C$_2$H$_6$ is formed, or whether the effect is the product of decreasing the energy barrier for the 4[H$^+$/e$^-$] reduction of C$_2$H$_2$.

In summary, this work demonstrate that: i) the VFe protein quaternary structure can be stabilized by adding excess δ subunit to a supernatant containing histidine-tagged VFe protein; ii) the histidine-tagged VFe protein reduces N$_2$H$_4$
and N$_3^-$ with $K_m$ values that resemble those for the Mo-nitrogenase; iii) the nonreciprocal response of N$_2$ and C$_2$H$_2$ seen for the Mo-nitrogenase is also a pattern seen for the V-nitrogenase; iii) as with the Mo-nitrogenase, H$_2$ inhibits N$_2$ reduction but not C$_2$H$_2$, N$_2$H$_4$, and N$_3^-$ reduction; and iv) chemical species that possible form amine-like adducts at the enzyme active site divert the electron to the formation of the 4[H$^+/e^-$] reduction C$_2$H$_6$ when the VFe protein is turning over C$_2$H$_2$. This work seeks to contribute with experimental evidence with which to delineate a mechanism for the reduction of N$_2$ on a metal active site containing V, a transition metal exotic to biological systems.

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CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

The biological reduction of molecular nitrogen has been the subject of intensive study for over 40 years because: i) It is central to maintain the biogeochemical nitrogen cycle on earth, on which life in the biosphere depends; ii) It is an oxido-reduction reaction that requires a high amount of free energy – supplied by the hydrolysis of at least 16 phosphohodiester bonds – to deliver electrons to the active site so as to cleave an otherwise inert triple-bonded N₂ molecule \(^1\); iii) It is accomplished by mutually-dependent, two-component proteins which possess unusual metal assemblies composed of a mixture of transition metals and inorganic sulfur ligands \(^2\); and, iv) As mentioned in Chapter 1 in this thesis, it is the source for inspiration to those who are in the quest of small molecular-weight chemical catalysts capable of achieving the high yields of nitrogen fixation obtained by the Haber-Bosch industrial nitrogen fixation process but under the mild conditions in which the nitrogenase systems operate.

Studies on the paradigm of the field, the Mo-nitrogenase, has provided most of the insight into how the metal centers, in conjunction with their protein environments, provide the reactivity necessary to promote substrate binding and to funnel electrons through metal clusters and along distances larger than 20 Å \(^3\) until they reach the active site of the enzyme. The Mo-nitrogenase has received much attention due in part to the fact that it is preferentially expressed when there are no metal limitations where the bacteria thrive; because it is the most efficient
at reducing $N_2$ of the three phylogenetically-related nitrogenases; and because it is the most stable nitrogenase when subjected to purification procedures. Due to these, the field of biological nitrogen fixation has been profoundly biased toward the study of a biochemical reaction that it is not only carried out on a Mo:Fe:S metal center but on active sites which are also composed of V:Fe:S or only Fe:S. In order to shorten the knowledge gap existing between the Mo- and V-based nitrogen fixation systems, this thesis presents a comparative study of the structural features and enzymatic properties of the MoFe and VFe proteins from *Azotobacter vinelandii*.

**The FeFe and VFe Proteins from *Azotobacter vinelandii*: Structural Features Revealed by Homology Modeling.** The structure of the VFe and FeFe protein metal active sites, the FeV- and FeFe-cofactors, respectively, has been inferred with the aid of spectroscopic techniques such as V and Fe K-edge EXAFS, DFT and BS calculations $^{4-8}$, and the chemical structure of the FeMo-cofactor determined from the crystal structure of the MoFe protein $^{9,10}$. However, before this thesis was written, there was no information regarding the structural nature of the VFe and FeFe protein folds, thus hampering the efforts to establish a link between the structure of the alternative nitrogenases and their function as biochemical catalysts.

The models that represent the protein folds of both nitrogenases provide for the first time a view of the spatial location of the chemical groups that could potentially be involved in many aspect related to protein function. Amino acid residues that line the putative active site cavity have been identified by visual
inspection of the area that is 5 Å away from the surface of the putative metal cluster active site. A total of 60 % of the surveyed amino acids have been conserved during the evolution of the active site protein component. However, the remaining 40 % represents distinct functional groups that, in conjunction with the different nature of the transition metal atoms (Mo, V, or Fe-only) present in such protein environment, are most likely responsible for the differences in reactivity and selectivity depicted by the nitrogenases. These could be listed as: i) The VFe protein releases four-electron-products upon reducing N₂ and C₂H₂; ii) Even though the \( K_m \) for the substrate N₂ is similar for both MoFe and VFe proteins, the MoFe protein is better at preferentially reducing this substrate; iii) C₂H₂ is a poorer substrate for the VFe protein and its \( K_m \) value is significantly higher to that reported for the MoFe protein; iv) H₂ is an uncompetitive inhibitor (and a poorer inhibitor, as judged by its \( K_i \)) of the reduction of N₂ as catalyzed by the VFe protein whereas it is a competitive inhibitor when the reaction is catalyzed by the MoFe protein; v) N₂ does not inhibit the reduction of C₂H₂ when the VFe protein catalyses the reaction as strongly as if the reaction were carried out by the MoFe protein; on the other hand, for the VFe protein, C₂H₂ is a poorer inhibitor of the reduction of N₂ when compared to the MoFe protein-catalyzed reaction; lastly vi) semi-reduced nitrogenous species, such as N₂H₄ and N₃⁻, which might resemble amine groups when in the active site of the VFe protein, redirect the electron flux to increase the formation of the product C₂H₆ when the enzyme is turning over C₂H₂.
Such effect has only been documented on an altered MoFe protein whose side chain $\alpha$-191$^{\text{Gin}}$ has been replaced by a lysine. A second altered nitrogenase depicting the same trend has had $\alpha$-195$^{\text{His}}$ replaced by an asparagine; a third altered MoFe protein, whose side chain $\alpha$-195$^{\text{His}}$ was replaced by a glutamine, did not catalyzed the 4$[^{\text{H}+/e^-}]$ reduction of $\text{C}_2\text{H}_2^{11,12}$, thus indicating that there could be two requisites for re-routing the electron flux to increase the formation of $\text{C}_2\text{H}_6$ upon $\text{C}_2\text{H}_2$ reduction: i) The presence of an N-H species in the vicinity of the $\text{C}_2\text{H}_2$ binding site, and ii) the proximity of the N-H species to the $\text{C}_2\text{H}_2$ adduct. Interestingly, the mentioned amino acid side chains are directed toward a unique FeMo-cofactor Fe-S face composed of iron atoms 2, 3, 6, and 7 (Figure 1-1). Inspection of the VFe protein active site models reveals that $\alpha$-82$^{\text{Lys}}$ could provide the amine group that seems to be the chemical group responsible for re-routing the electron flux. According to the model, the side chain of this residue points directly toward where the iron atoms 2, 3, 6, and 7 of the FeV-cofactor could be located. This hypothesis could only have been proposed with the aid of a model for the protein fold for the VFe protein $\alpha$ subunit. However, in silico experiments in which models of the FeV-cofactor are docked onto the putative active site cavity of the VFe protein fold are needed in order to completely construct the VFe protein active site in the absence of X-ray diffraction data [Lovell et al. 13 constructed models for the FeV- and FeFe-cofactors using DFT and BS calculations. These metal cluster models could be used to perform the proposed docking experiments].
As for the P-cluster, its putative protein cavity is composed of amino acid residues that have almost all been conserved through the evolution of the nitrogenases. Approximately 80% of the amino acids that were found to delineate the putative cavity are identical to their MoFe protein counterparts. This trend, in which there is high structural identity among the modeled alternative nitrogenase folds their MoFe protein counterpart, is repeated on structural features such as the putative proton-transfer pathway and the Fe protein docking surface, all of which are crucial for protein function. Therefore, even though there is a rather low sequence homology among the primary structure of the nitrogenases α and β subunits, there is very high structural correspondence on the areas that are likely involved in the correct functioning of the protein. Nature, through a natural selection process, has obviously conserved those pivotal structural features that are localized within certain regions of the proteins.

*The vnfG Gene from Azotobacter vinelandii: Cloning, Expression, Purification, and Crystallization of its Product, the VFe Protein δ Subunit.* The vnfG gene, and its expression product, the δ subunit, has been demonstrated to be essential for the correct functioning of the VFe protein as a reductant of N₂. Interestingly, in the absence of the δ subunit, the VFe protein can still reach the reduction potential required for the reduction of H⁺ from solution and the non-physiological substrate C₂H₂.

The δ subunit has been hypothesized to act as a molecular chaperon for the insertion and stabilization of FeV-cofactor into the VFe protein active site. However, there is yet to be presented structural evidence supporting these
propositions. For this reason, Chapter 3 in this thesis describes the cloning and the expression of the \textit{vnfG} gene, and the purification and crystallization trials conducted with the \( \delta \) subunit.

The recombinant \( \delta \) subunit is readily expressed by \textit{E. coli} strain BL21-pCRVFe19 in the form of a soluble protein which interacts specifically with a \( \text{Ni}^{2+} \)-loaded IMAC column. Only one chromatographic step is needed to obtain a protein preparation which, as judged by SDS-PAGE, is 100\% pure. Approximately 30 mg of recombinant \( \delta \) subunit can be purified from 1 L of induced \textit{E. coli} strain BL21-pCRVFe19. This protein retains its capacity to improve the catalytic activity of the VFe protein: When a supernatant containing histidine-tagged VFe protein is mixed with a 3-molar excess of recombinant \( \delta \) subunit, the resulting histidine-tagged VFe protein quaternary structure is stabilized such that upon it being loaded on a \( \text{Ni}^{2+} \)-IMAC column and eluted from it its catalytic activity is ten times improved, as compared with the histidine-tagged VFe protein subjected to the same procedure but with no added recombinant \( \delta \) subunit.

The \( \delta \) subunit was subjected to crystallization trials (using the sitting drop vapor diffusion method) with the aid of an automated crystallization facility at the Hill Laboratories (UofU). Two crystallization cocktails promoted crystal growth after a 60-day incubation period: i) Bis-Tris 0.1 M pH 6.5, PEG 3350 25\% w/v, and ii) HEPES 0.1 M pH 7.5, PEG 3350 25\% w/v. The crystallization of the \( \delta \) subunit at USU has only been tested with cocktail (i). The crystallization experiments have so far yielded no crystals as of the time of writing this thesis.
As mentioned in Chapter 3, the second crystallization cocktail remains to be tested. However, before any other attempt to obtain crystals is pursued, it is highly advisable to find the conditions in which a single oligomeric species of the δ subunit is favored in solution. Figure 2-4 shows that, at pH 7, the δ subunit elutes from a gel filtration column mostly as an hexamer, although a very small percentage of the loaded protein eluted at a volume that corresponds to the approximate molecular weight of a monomer. Thus, it is recommended that the buffer in which the protein is purified (Tris-HCl 50 mM pH 8, NaCl 0.2 M) is exchanged by MOPS 0.1 M pH 7 (or HEPES 0.1 M pH 7), with NaCl 0.5 M. After the buffer is exchanged, the protein should exist in solution as a single oligomeric species that could crystallize easier (as compare to multiple oligomeric species in solution) when exposed to the precipitant PEG 3350. In any case, the ideal condition for crystallization could be that in which the protein concentration is 5-10 mg/mL, the pH of the buffer is in the range of 6.5-7.5, and the precipitant is PEG 3350. A necessary step for quick and reproducible crystallization could involve removing NaCl before setting up the crystallization experiments. This could be accomplished by dialyzing the protein (using Snake Skin Pleated Dialysis Tubing, as mentioned in the Expression and Purification of Histidine-tagged VnfG, Chapter 3) against buffer MOPS 0.1 M pH 7 (or HEPES 0.1 M pH 7), with no added NaCl or by means of a de-salting column equilibrated on the previously mentioned buffers. The resin Sephadex G-25 (GE Healthcare) could be the ideal media to perform the buffer exchange and de-salting procedure. However, it must be taken into account that the oligomeric state of the protein
could change upon decreasing the ionic strength of the solution in which it is suspended.

It is of interest to solve the 3D structure of the recombinant \( \delta \) subunit because: i) The \( \delta \) subunit is a novel polypeptide whose primary structure has apparently been conserved in Nature for the solely purpose of supporting V- and Fe-only dependent reduction of molecular nitrogen; ii) It could provide the phase information needed to solve the structure of the \( \delta \) subunit when in complex with the VFe protein \( \alpha \) and \( \beta \) subunit; iii) It will provide insight as to what region of the protein interacts with the VFe protein \( \alpha/\beta \) fold and; iv) With the aid of the models for the VFe and FeFe protein \( \alpha/\beta \) folds shown in Chapter 2, the 3D structure of the recombinant \( \delta \) subunit could be used to construct the totality of the VFe and FeFe protein \( \alpha_2\beta_2\delta_2 \) quaternary structure.

Solving the 3D structure of the recombinant \( \delta \) subunit would definitively contribute with the understanding of V-dependent reduction of nitrogen. As previously suggested, it seems that the \( \delta \) subunit allows the FeV-cofactor (and the FeFe-cofactor) to reach the redox potential to bind and reduce \( \text{N}_2 \) by a still unknown mechanism. It might be that the \( \delta \) subunit, when interacting with the rest of the VFe protein complex, promotes conformational changes within the protein scaffold that favors the correct FeV-cofactor geometry/isomer for \( \text{N}_2 \) reduction. Or it might be that the \( \delta \) subunit reconFigures the VFe protein quaternary structure such that the electron transfer path (or the proton-transfer path, or both) is correctly formed within the protein fold. However, since the enzyme still reduces \( \text{H}^+ \) and \( \text{C}_2\text{H}_2 \) even in the absence of the \( \delta \) subunit, the latter
hypothesis cannot be totally accepted, unless there are proton and electron transfer paths that are exclusive for the reduction of N₂ and only arise when the VFe protein is in the α₂β₂δ₂ oligomeric state.

Another approach that could be employed to understand the role the primary structure of the δ subunit plays in the V-based reduction of nitrogen would involve mutating conserved amino acids existing in both VnfG and AnfG (AnfG is the δ subunit of the FeFe protein) to then use the altered subunits in assays measuring the degree by which the histidine-tagged VFe protein is stabilized during the purification procedure presented in Chapter 4 (i.e. amount of Fe²⁺ and V associated to the purified histidine-tagged VFe protein and specific activity values for the reduction of H⁺, C₂H₂, and N₂). A series of altered δ subunit can be readily constructed since the vnfG gene is already cloned in both cloning and expression vectors. The strategy designed to construct the current vnfG can also be used to clone anfG into an expression vector [anfDGK was amplified and cloned into a cloning vector using a similar strategy to that designed to clone vnfDGK. This is briefly described in Chapter 3 under the vnfG Cloning and Insertion of Poly-Histidine Tag section]. Truncated versions of the proteins could also be constructed and their ability to stabilize the histidine-tagged VFe protein tested. None of the suggested work has been done and if it were, it could eventually provide clues as to the elements within the δ subunit required exclusively for the reduction of H⁺ and C₂H₂ and for the reduction of N₂.

Problems Associated with the Correct Functioning of the VFe Protein.

The histidine-tagged VFe protein preparation initially isolated to conduct research
on V-based nitrogen reduction had very low Fe$^{2+}$ content and poor substrate reduction activities. The purified protein had an $\alpha_2\beta_2$ composition with no $\delta$ subunit. It was logical to hypothesize that the added histidine residues (located at the N-terminus of the VFe protein $\beta$ subunit) could be interfering with the proper $\delta$ subunit-$\alpha\beta$ dimer interactions needed for maintaining a functional enzyme. Therefore, it was decided to perform the *de novo* construction of a histidine-tagged VFe protein which histidine tag would be located at the C-terminus of its $\alpha$ subunit. It is at this position where a histidine tag was added to the $\alpha$ subunit of a MoFe protein which can be isolated using a Zn$^{2+}$-loaded IMAC column. The purification protocol yields high amounts of fully active MoFe protein. Having this in mind, the construction of the VFe protein was started and eventually it was obtained a plasmid containing a 800 bp-long segment of *vnfD* with histidine-coding codons on its 3’ end, the entirety of *vnfG*, and a 800 bp-long segment of *vnfK*. This plasmid, the so-called rescue plasmid (pPCRVFe28-5), was used to transform a Nif$^-$ *A. vinelandii* strain (DJ33strep/kan/gen resistant and incapable of fixing nitrogen) which *nifDK* genes, *vnfDGK* genes, *anfDGK* genes, and *modGEABC* genes (involved in Mo homeostasis), had been disrupted for the purpose of this work. Colonies which displayed nitrogen fixing phenotype (Nif$^+$), called *A. vinelandii* DJ33VFe28, were isolated to conduct genomic DNA analysis to confirm that they indeed had acquired the rescued plasmid (Figure 5-1).
Figure 5-1. PCR and restriction enzyme analysis of genomic DNA extracted from *A. vinelandii* strain Trans (wild-type) and DJ33VFe28 colony 1. A PCR experiment was designed to amplify, using primers BBP465 and BBP520, a 1558 bp genomic DNA region encompassing a segment of *vnfD* and a segment of *vnfG*. The PCR product corresponding to strain Trans does not possess a *Hind*III restriction site sequence whereas its counterpart in DJ33VFe28 possesses a single *Hind*III restriction, which was incorporated as a result of the DNA manipulations carried out to construct plasmid pPCRVFe28-5. The restriction enzyme *Hind*III does not digest the PCR product from *A. vinelandii* strain Trans (lane 1). However, *Hind*III does digest the PCR product from strain DJ33VFe28 (lane 2). The sizes of the digestion products match well with the theoretical expected sizes of the fragments (1350 bp and 208 bp). The restriction analysis of the PCR product from DJ33VFe28 indicates that plasmid pPCRVFe28-5 was incorporated into the genome of Nif− *A. vinelandii* strain DJ33strep/kan/gen.

The PCR experiments conducted with isolated genomic DNA from the selected colonies, in conjunction with restriction analysis of the PCR products, confirmed that each of the colonies possessed the DNA material that could only
have come from recombination events between the rescue plasmid and the cell’s genomic DNA. Thus it was obtained phenotypic and genotypic evidence indicating that the cells have the genetic information to manufacture a VFe protein with a histidine tag attached on the C-terminus of its \( \alpha \) subunit.

Unfortunately, the only component that could be purified was the histidine-tagged \( \alpha \) subunit (58.9 kDa) (Figure 5-2). The \( \beta \) and \( \delta \) subunits (52.8 kDa and 13.4 kDa, respectively) did not co-purified with the histidine-tagged \( \alpha \) subunit.

![Figure 5-2. 18 % SDS-PAGE showing the histidine-tagged VFe protein \( \alpha \) subunit purified from \textit{A. vinelandii} DJ33VFe28. Lane 1, MoFe protein \( \alpha \) subunit (56.4 kDa) and \( \beta \) subunit (59.3 kDa); lane 2, VFe protein \( \delta \) subunit; lane 3, VFe protein constructed in the laboratories of Dr. Dennis Dean; lane 4, VFe protein \( \alpha \) subunit purified from \textit{A. vinelandii} DJ33VFe28.]

This negative result should be taken into account when deciding whether to re-construct a histidine-tagged VFe protein. In the future, one histidine tag could be added to the N-terminus of the \( \alpha \) subunit and a second histidine tag to the C-terminus (or N-terminus) of the \( \delta \) subunit, thus creating a double-histidine-
tagged VFe protein. The construction of the plasmid with the genes coding for these modified proteins should not be time-demanding due to the fact that the parent plasmid needed to undertake this project has already been built. Likewise, the parent strain with the correct genotypic background for incorporation of the proposed plasmid has also been constructed. Thus, the construction and expression of a VFe protein with histidine tags on one or more of its subunits can be accomplish without requiring a lengthy investment of time.

A plausible approach that could be employed to obtain a VFe protein to conduct studies on V-dependent nitrogen fixation would involve cloning the $vnfDGK$ operon from the cyanobacterium *Anabaena variabilis* $^{18}$, adding histidine-coding codons to any of its structural genes, and then inserting the modified genes into the previously mentioned *A. vinelandii* strain. According to Thiel, the $vnfDGK$ genes of *A. variabilis* are organized much like those of *Azotobacter* pp. and show strong similarity to those alternative nitrogenase genes. However, in *A. variabilis*, but not in *Azotobacter* pp., the gene for the $\delta$ subunit of the VFe protein is fused to the $vnfD$ gene, yielding a single open reading frame which it was called $vnfDG$. This poses an interesting scenario in which a VFe protein could be composed of two subunits: an $\alpha\delta$ and a $\beta$ subunit. In theory, such VFe protein would not display the problems largely recognized as the culprit for obtaining a mixture of protein with various peptide stoichiometries, namely poor protein-protein interactions among the different VFe protein subunits. As commented in $^{18}$, *A. variabilis* and *A. vinelandii* possess many of the $nif$ genes
required for the maturation of the MoFe and VFe proteins, indicating that it could be possible to obtain mature, active *A. variabilis* VFe protein in *A. vinelandii*.

**Electron Allocation and Substrate-Inhibitor Interactions at the**

Azotobacter vinelandii VFe Protein Active Site. The recombinant δ subunit was a factor that definitively contributed with the isolation of a histidine-tagged VFe protein with higher Fe²⁺ content and improved substrate reduction activity. This version of the wild-type VFe protein was examined in terms of its capability to reduce H⁺, C₂H₂, N₂, N₂H₄, and N₃⁻, in the absence and in the presence of inhibitors. Likewise, its kinetic properties were determined (for the different substrates and inhibitor examined) using the classic Michaelis-Menten approach for unireactant enzymes and the results were compared to those available for the MoFe protein-catalyzed reactions.

The work conducted determined that the transition metal V and a slightly different amino acid environment (as revealed by the homology modeling study and compared to that of the MoFe protein active site) yield: i) an active site that reduces N₂, N₂H₄ and N₃⁻ with *Kₘ* values that resemble those for the Mo-nitrogenase; iii) the nonreciprocal response of N₂ and C₂H₂ seen for the Mo-nitrogenase; and iii) an active site that is incapable of reducing N₂ but capable of reducing C₂H₂, N₂H₄, and N₃⁻ in the presence of H₂. These are catalytic patterns that seem to be shared between the Mo and the V-containing active sites. However, the work presented in Chapter 4 also uncovered a number of catalytic dissimilarities existing between the Mo- and the V-nitrogenases. It has been previously reported that the V-nitrogenase is a better hydrogenase and a worse
nitrogenase and “acetylenease” than the Mo-nitrogenase. Chapter 4 reports that for the V-nitrogenase: i) $N_2$ is a better inhibitor of the reduction of $C_2H_2$; ii) $C_2H_2$ is a worse inhibitor for the reduction of $N_2$; iii) $H_2$ inhibits the reduction of $N_2$ in an uncompetitive manner (for the Mo-nitrogenase, $H_2$ is a competitive inhibitor of the reduction of $N_2$); iii) $N_2H_4$ inhibits the reduction of $C_2H_2$ also in an uncompetitive manner (for the Mo-nitrogenase, $N_2H_4$ is a competitive inhibitor of the reduction of $C_2H_4$); and iv) semi-reduced nitrogenous species such as $N_2H_4$ and $N_3^-$, but not $N_2$ or $H_2$, causes the electron flux to be re-directed so as to increment the formation of $C_2H_6$ when the enzyme turns over $C_2H_2$. Although the VFe protein readily reduces $C_2H_2$ with $4[H^+/e^-]$ to yield a small amount of $C_2H_6$, the presence of $N_2H_4$ and $N_3^-$ increases 10-fold the formation of $C_2H_6$. The effect can only be observed when the enzyme reduces $C_2H_2$ but not $C_2H_4$ (which is also a substrate for the reaction), clearly indicating that a $C_2H_2$ reduction adduct and not a $C_2H_4$ adduct is required for the effect to take place. This also suggest that, at least for the VFe protein, the reduction of $C_2H_2$ and $C_2H_4$ are probably accomplished by means of different routes, one to which $N_2H_4$ and $N_3^-$ have access and another which is not accessible to the mentioned species.

ENDOR measurements on the resting state of the histidine tagged protein and when it is turning over various substrates could disclose some of the chemistry taking place at the V-containing active site of the VFe protein. The natural abundance of the stable vanadium isotope $^{51}V$ is over 99%. This isotope possesses a highly anisotropic character (electron spin $S = \frac{1}{2}$ and nuclear spin of $I = \frac{7}{2}$) which allows for single-crystal-like spectra ENDOR spectra to be obtained.
at the $M_I = \pm \frac{7}{2}$ parallel turning points of the EPR spectrum. The ENDOR technique could be used to measure the $^{51}$V nuclear quadrupole coupling constant associated with the VFe protein when in its ground state (which is paramagnetic) and exited state (i.e. resting state and under turning over conditions). Changes in the quadrupole coupling constant could reveal the dynamics within the active site from the vanadium atom stand point. A series of oxovanadium complexes have been examined using $^{51}$V ESE-ENDOR $^{19}$. This work determined that the quadrupole coupling constants calculated for $^{51}$V in the complexes $^{51}$VO(oep), $^{51}$VO(acac)$_2$, and $^{51}$VO(salen), with axial ligands such as $n$-butylamine and imidazole, change on the basis of the type and location of the ligand. Such measurements may be useful for identifying the types and locations of $^{51}$V ligands (specifically regarding N/O ligands) as the enzyme catalyses substrate reduction. These types of experiments are unprecedented in the field of V-based biological nitrogen fixation. The same approach could be used to describe the types of interactions occurring among V and its sulphide ligands in the resting state or during enzymatic turn-over if there were $^{51}$V ENDOR measurements of vanadium complexes in complex with different types of thio-functional ligands.

The VFe Protein as a Halocarbon Reductase: The fact that the VFe protein accomplishes the $4[H^+/e^-]$ reduction of alkynes such as C$_2$H$_2$ and the $2[H^+/e^-]$ reduction of alkenes such as C$_2$H$_4$ makes this enzyme an attractive catalyst for reducing small, unsaturated aliphatic halocarbons such as vinyl chloride and trichloroethylene, both of which have been identified as highly toxic and carcinogenic compounds that can be readily detected in drinking-water
aquifers. There are a variety of monooxigenases that can catalyze the oxygenation of the mentioned compounds, thus priming them for further degradation which results in complete mineralization of the halocarbons. However, the product of the oxygenation, a reactive epoxide, can itself be a xenobiotic agent that could ultimately kill the organisms that carry out the mineralization. Therefore, the reduction of these compounds by a route that do not require their prior oxidation would circumvent the toxicity associated with epoxide rings. Such route could involve the participation of an enzyme such as the VFe protein since it is able to reduce small, unsaturated aliphatic compound homologous to both vinyl chloride and trichloroethylene. Therefore, it would be interesting to assess whether the histidine-tagged VFe protein presented in this thesis is capable of reducing such compounds.

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APPENDIXES
**Figure 2-1S. Amino acid sequence alignments of the nitrogenase α-subunit found in eight different bacteria.**

The amino acid sequences used in this study come from: *Azotobacter vinelandii* Mo-containing nitrogenase (AvMo), *A. vinelandii* Fe-only nitrogenase (AvFe), *A. vinelandii* V-containing nitrogenase (AvV), *Klebsiella pneumoniae* Mo-containing nitrogenase (Kp), *Rhizobium* sp. Mo-containing nitrogenase (Rhiz), *Bradyrhizobium japonicum* Mo-containing nitrogenase (Bj), *Rhodobacter capsulatus* Mo-containing nitrogenase (Rc) and *Clostridium pasteurianum* Mo-containing nitrogenase (Cp). The letter color code indicates, in red, identical amino acids; in blue, similar amino acids and, in green, amino acids that are almost conserved in all the aligned sequences.
**Table 2-1S.** List of amino acid residues that delineate the FeMo-cofactor and the FeV- and FeFe-cofactor cavities in the MoFe and predicted VFe and FeFe protein α subunits. The amino acid numbering follows that of the *A. vinelandii* α subunit.

<table>
<thead>
<tr>
<th>Identical</th>
<th>MoFe protein</th>
<th>VFe protein</th>
<th>FeFe protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-229Tyr</td>
<td>α-279Ser</td>
<td>α-259Ser</td>
<td>α-210Tyr</td>
</tr>
<tr>
<td>α-275Cys</td>
<td>α-195His</td>
<td>α-256Cys</td>
<td>α-256Cys</td>
</tr>
<tr>
<td>α-191Gln</td>
<td>α-258Arg</td>
<td>α-179His</td>
<td>α-179His</td>
</tr>
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<td>α-381Phe</td>
<td>α-357Gly</td>
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<td>α-336Gly</td>
</tr>
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<td>α-361Phe</td>
<td>α-361Phe</td>
</tr>
<tr>
<td>α-231Ile</td>
<td>α-422His</td>
<td>α-179Gln</td>
<td>α-422His</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Similar</th>
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<th>VFe protein</th>
<th>FeFe protein</th>
</tr>
</thead>
<tbody>
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<td>α-360Lys</td>
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<tr>
<td>α-277Arg</td>
<td>α-380Glu</td>
<td>α-337Pro</td>
<td>α-337Ser</td>
</tr>
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<td>α-65Ala</td>
<td>α-279Ser</td>
<td>α-360Lys</td>
<td>α-360Lys</td>
</tr>
<tr>
<td>α-95Gly</td>
<td>α-65Ala</td>
<td>α-51Cys</td>
<td>α-51Cys</td>
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<th>VFe protein</th>
<th>FeFe protein</th>
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<td>α-51Cys</td>
<td>α-81Thr</td>
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<tr>
<td>--------------------</td>
<td>--------------</td>
<td>-------------</td>
<td>--------------</td>
</tr>
<tr>
<td>α-212Ile</td>
<td>α-212Ile</td>
<td>α-81Thr</td>
<td></td>
</tr>
</tbody>
</table>
Table 2-2S. List of amino acid residues that delineate the P-cluster cavity in the MoFe and predicted VFe and FeFe protein α and β subunit interface. The amino acid numbering follows that of the *A. vinelandii* α and β subunit.

<table>
<thead>
<tr>
<th></th>
<th>α subunit</th>
<th>β subunit</th>
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<tr>
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<td>FeFe</td>
</tr>
<tr>
<td>Identical</td>
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</tr>
<tr>
<td>62 Cys</td>
<td>48 Cys</td>
<td>48 Cys</td>
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<tr>
<td>88 Cys</td>
<td>74 Cys</td>
<td>74 Cys</td>
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<tr>
<td>154 Cys</td>
<td>137 Cys</td>
<td>137 Cys</td>
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<tr>
<td>185 Gly</td>
<td>169 Gly</td>
<td>169 Gly</td>
</tr>
<tr>
<td>64 Tyr</td>
<td>50 Tyr</td>
<td>-----</td>
</tr>
<tr>
<td>85 Pro</td>
<td>71 Pro</td>
<td>71 Pro</td>
</tr>
<tr>
<td>87 Gly</td>
<td>73 Gly</td>
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<td>Similar</td>
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<td></td>
</tr>
<tr>
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<td>77 Asp</td>
</tr>
<tr>
<td>153 Gln</td>
<td>135 Thr</td>
<td>135 Thr</td>
</tr>
<tr>
<td>64 Tyr</td>
<td>50 Phe</td>
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</table>
Table 2-3S. List of the amino acid residues that delineate the proposed proton-transfer pathway within the MoFe protein $\alpha\beta$ interstice and the modeled VFe and FeFe proteins. The selected amino acids are those located 6 Å away from the hypothetical path.

<table>
<thead>
<tr>
<th></th>
<th>MoFe</th>
<th>FeFe</th>
<th>VFe</th>
<th></th>
<th>MoFe</th>
<th>FeFe</th>
<th>VFe</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ subunit</td>
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<td></td>
<td></td>
<td>$\beta$ subunit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----</td>
<td>55$^{\text{His}}$</td>
<td>55$^{\text{Leu}}$</td>
<td></td>
<td>105$^{\text{Arg}}$</td>
<td>55$^{\text{Gln}}$</td>
<td>66$^{\text{Gln}}$</td>
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</tr>
<tr>
<td>70$^{\text{Val}}$</td>
<td>56$^{\text{Val}}$</td>
<td>56$^{\text{Val}}$</td>
<td></td>
<td>449$^{\text{Lys}}$</td>
<td>399$^{\text{Arg}}$</td>
<td>413$^{\text{Arg}}$</td>
<td></td>
</tr>
<tr>
<td>94$^{\text{Ala}}$</td>
<td>80$^{\text{Gln}}$</td>
<td>80$^{\text{His}}$</td>
<td></td>
<td>472$^{\text{Phe}}$</td>
<td>417$^{\text{Tyr}}$</td>
<td>431$^{\text{Phe}}$</td>
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</tr>
<tr>
<td>95$^{\text{Gly}}$</td>
<td>81$^{\text{Thr}}$</td>
<td>81$^{\text{Thr}}$</td>
<td></td>
<td>473$^{\text{Asp}}$</td>
<td>418$^{\text{Asp}}$</td>
<td>432$^{\text{Asp}}$</td>
<td></td>
</tr>
<tr>
<td>96$^{\text{Arg}}$</td>
<td>82$^{\text{Lys}}$</td>
<td></td>
<td>----</td>
<td>55$^{\text{His}}$</td>
<td>56$^{\text{Val}}$</td>
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<tr>
<td>97$^{\text{Arg}}$</td>
<td>83$^{\text{Arg}}$</td>
<td></td>
<td>----</td>
<td>105$^{\text{Arg}}$</td>
<td>55$^{\text{Gln}}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>99$^{\text{Tyr}}$</td>
<td></td>
<td>----</td>
<td>----</td>
<td>449$^{\text{Lys}}$</td>
<td>399$^{\text{Arg}}$</td>
<td>66$^{\text{Gln}}$</td>
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<tr>
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<td>175$^{\text{Gln}}$</td>
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<td>355$^{\text{Ile}}$</td>
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<td>212$^{\text{Ile}}$</td>
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<td>231$^{\text{Ile}}$</td>
<td>212$^{\text{Ile}}$</td>
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<td>----</td>
<td>213$^{\text{Gln}}$</td>
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</tr>
<tr>
<td>355$^{\text{Gly}}$</td>
<td>335$^{\text{Gly}}$</td>
<td>335$^{\text{Gly}}$</td>
<td></td>
<td>338$^{\text{Arg}}$</td>
<td>338$^{\text{Arg}}$</td>
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</tr>
<tr>
<td>359$^{\text{Arg}}$</td>
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<td>338$^{\text{Arg}}$</td>
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<td>361$^{\text{Phe}}$</td>
<td>361$^{\text{Phe}}$</td>
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<tr>
<td>381$^{\text{Phe}}$</td>
<td>361$^{\text{Phe}}$</td>
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<td>----</td>
<td>440$^{\text{Glu}}$</td>
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<td>442$^{\text{His}}$</td>
<td>422$^{\text{His}}$</td>
<td>422$^{\text{His}}$</td>
<td></td>
<td>443$^{\text{Ser}}$</td>
<td>423$^{\text{Ala}}$</td>
<td>423$^{\text{Gly}}$</td>
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<tr>
<td>444$^{\text{Trp}}$</td>
<td>424$^{\text{Tyr}}$</td>
<td>424$^{\text{Tyr}}$</td>
<td></td>
<td>446$^{\text{Tyr}}$</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>446$^{\text{Tyr}}$</td>
<td></td>
<td>----</td>
<td>----</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The amino acids placed on the same row are spatially equivalent among the MoFe protein and the modeled VFe and FeFe protein folds.
SUPPLEMENTARY MATERIAL FOR CHAPTER 4
Table 4-1S. Identification of the VFe protein by N-terminal sequencing.

<table>
<thead>
<tr>
<th>VFe protein subunit</th>
<th>Theoretic N-terminal sequence*</th>
<th>Determined N-terminal sequence**</th>
</tr>
</thead>
<tbody>
<tr>
<td>VnfD</td>
<td>MPMVLLECDKD…</td>
<td>PMVLLE(s?)DKD</td>
</tr>
<tr>
<td>VnfK</td>
<td>MSNCELTV…</td>
<td>SNLNHHH</td>
</tr>
</tbody>
</table>

*NCBI protein entry code M32371.
**This position was assigned to either a serine or a cysteine.
**N-terminal sequences were determined at the ASU Proteomic and Protein Chemistry Laboratory using the Edman degradation method.
Figure 4-1S. 18 % SDS-PAGE loaded with recombinant VnfG (lane 1), VFe protein purified from *A. vinelandii* DJ1258 (lane 2), and VFe protein purified from the supernatant of *A. vinelandii* DJ1258 to which excess, recombinant VnfG had been added before it was loaded onto a N$_{2}^{2+}$-IMAC column (lane 3).
Scheme 4-1S. Representation of the routes for C$_2$H$_2$ reduction as catalyzed by the V-nitrogenase. In route A C$_2$H$_2$ binds to the metal active site (M) which then reduces the substrate to yield a mixture of C$_2$H$_4$ and C$_2$H$_6$. When the enzyme turns over C$_2$H$_2$ in the presence of N$_2$H$_4$ or N$_3^-$ (but not H$_2$ or N$_2$), an active site species M$^*$ - C$_2$H$_2$ is formed which augments the flux of electrons to the product C$_2$H$_6$. In route B, the enzyme turns over C$_2$H$_4$ in the presence of N$_2$H$_4$ or N$_3^-$, forming the species M - C$_2$H$_4$. The latter is incapable of redirecting the electron flux to increase the formation of C$_2$H$_6$. 

\[ \text{N}_2\text{H}_4/\text{N}_3^-, \text{C}_2\text{H}_2 \rightarrow M \rightarrow M^* - \text{C}_2\text{H}_2 \rightarrow M + \text{C}_2\text{H}_4 \rightarrow M + \text{C}_2\text{H}_6 \]

\[ \text{C}_2\text{H}_4, \text{N}_2\text{H}_4/\text{N}_3^- \rightarrow M \rightarrow M^* - \text{C}_2\text{H}_4 \rightarrow M + \text{C}_2\text{H}_6 \]

\[ \text{A} \rightarrow \text{B} \]