CHAPTER 3

Substrate Interactions with the Nitrogenase Active Site

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This manuscript describes the background and recent literature concerning identification of the substrate-binding site within the nitrogenase FeMo-cofactor. Emphasis is placed on recent advances on this topic that have emerged from a collaborative project among the Dean, Seefeldt and Hoffman laboratories. Much of the reviewed work represents publications for which I have been a co-author and these primary publications are included or are discussed within this dissertation. This chapter was written and submitted for publication with the intention for its use to satisfy a portion of the literature survey for this dissertation. As first author, my responsibility was to gather and interpret recently published work, participate in writing the document at all stages of its preparation, and to prepare the figures that are included in the review.

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3.1 - Nitrogenase Catalyzes Biological Nitrogen Fixation

The agronomic and economic significance of nitrogen fixation – reduction of N_2 to yield 2 NH_3 - can be appreciated through the perspective of an estimate that suggests more than a third of today's human population would not exist without application of the synthetic Haber-Bosch process (Smil, 2000). Biological nitrogen fixation, which utilizes energy in the form of MgATP, is catalyzed by a complex metalloenzyme called nitrogenase (see Christiansen et al., 2001 for a recent review) .

$$N_2 + 8e^- + 8H^+ + 16MgATP \Rightarrow 2NH_3 + H_2 + 16MgADP + 16\ Pi$$

Whether or not the biological process can be more effectively exploited for agronomic benefit remains an open question. Nevertheless, nitrogen fixation is necessary to sustain life on earth, and how organisms manage to activate and cleave N_2 at ambient temperature and pressure remains a fascinating and unsolved chemical problem. There are three different types of nitrogenases (Eady, 1996), distinguished by the metal composition of their respective active site metalloclusters, but all of them share common structural and mechanistic features. The best studied of these is the Mo-dependent enzyme that contains an active-site organometallic cluster called FeMo-cofactor (Burgess & Lowe, 1996). All nitrogenases comprise two catalytic components and, in the case of the Mo-dependent enzyme, these are designated as the Fe protein and the MoFe protein. The Fe protein is a specific, [4Fe-4S] cluster-containing, nucleotide-dependent, reductant of the MoFe protein, which contains the active site FeMo-cofactor. Complex formation and electron transfer between the Fe protein and MoFe protein is controlled by MgATP binding and hydrolysis (Howard & Rees, 1994). The interaction of the Fe protein and MoFe protein can be considered to involve two coupled cycles. During catalysis, the Fe protein [4Fe-4S] cluster cycles between 1^+ and 2^+ states as single electrons are delivered to the MoFe protein, whereas the MoFe protein cycle involves the accumulation of multiple electrons necessary for substrate binding and reduction. Because multiple electrons are required for substrate reduction, multiple Fe protein cycles are required to complete a single MoFe protein cycle.

Nitrogenase also catalyzes the reduction of a variety of triply bonded substrates other than N_2, the most familiar being acetylene (Dilworth, 1966). In its resting state the MoFe protein is not capable of reducing or even covalently binding any substrate. Rather, substrate interaction requires the prior activation of the MoFe protein by accumulation of electrons donated from the Fe protein (Lowe & Thorneley, 1984). In the absence of other substrates, the activated MoFe protein reduces protons to yield H_2, thereby continuously cycling the protein back to its resting state. These properties, and the fact that accumulation of a different number of electrons is required to make the MoFe protein competent to bind different substrates, has enormously complicated analysis of nitrogenase catalysis. Perhaps the most important feature that has frustrated study of the chemical mechanism is an inability to capture a homogenous form of the enzyme having substrate bound at high occupancy. Consequently there has been no direct information concerning where and how substrates interact with the active site. This article describes a combination of genetic, biochemical and biophysical strategies that were used to overcome this problem.

3.2 - A Genetic Approach

The FeMo-cofactor metal-sulfur core is constructed from Fe_4S_3 and Fe_4S_3Mo sub-clusters (Figure 1) joined by a shared central ligand, suspected to be a nitride, and linked by three bridging S^{2-} (Dos Santos et al., 2004a; Einsle et al., 2002). An organic constituent, homocitrate, is attached to the Mo atom through its 2-hydroxyl and 2-carboxy groups and the cofactor is covalently anchored to the MoFe protein through an N-ligand provided by a histidine imidazole to the Mo atom and a cysteine thiolate-ligand to an Fe atom at the opposite end. This unusual structure has invited considerable theoretical speculation concerning the identity of the substrate-binding site, description of possible mechanisms for substrate activation, and has also inspired a variety of model chemistries as a venue to understand N_2 activation (Seefeldt et al., 2004).

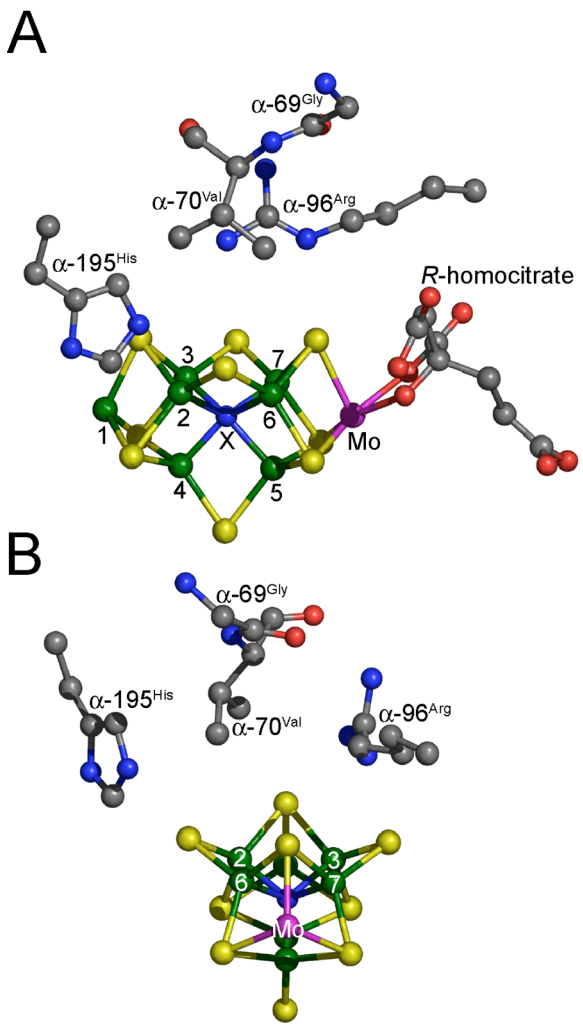

Figure 1. Two views of FeMo-cofactor and selected residues that provide the first shell of non-covalent interactions. Side-on view (panel A) and a Mo end-on view (with homocitrate removed) (panel B). FeMo-cofactor is a [7Fe-8S-Mo-X-homocitrate] cluster, where X is an atom of unknown identity but is believed to be a nitride. The figure was generated from PDB file 1M1N using the program PyMOL. The color scheme is Fe in green, S in yellow, C in gray, N in blue, O in red, and Mo in magenta.

An initial approach to resolve the question, where do substrates interact with the active site, involved evaluation of the catalytic and biophysical consequences arising from substitution of the residues that provide the first shell of non-covalent interactions with FeMo-cofactor (Scott et al., 1990; Kim et al., 1995; Benton et al., 2001). It soon became apparent, however, that the complexity of the system denied unequivocal interpretation of most information gained in this way. For example, for most substitutions it was not possible to assign catalytic defects as specifically arising from electron transfer, chemical reactivity of the cofactor, or substrate access to the cofactor.

Because N_2 and acetylene compete for the available reducing equivalents during nitrogenase catalysis, acetylene is a growth inhibitor under conditions where N_2 fixation is required. We therefore decided to use a genetic strategy to circumvent the above problems by isolation of mutant strains resistant to the inhibitory effects of acetylene (Christiansen et al., 2000a; Christiansen et al., 2000b). This approach was valuable for two important reasons. First, the method cannot be biased by an investigator's preference for the location of the substrate-binding site. Second, because N_2 reduction is thermodynamically more demanding and requires more electrons than acetylene reduction, a mutant strain impaired in acetylene reduction, but not N_2 reduction, cannot arise from defects in either electron transfer or in active site reactivity. Rather, this feature can only be assigned to an inability of acetylene to effectively access the active site.

MoFe protein purified from an acetylene resistant strain exhibited a substantially lower affinity for acetylene binding/reduction, whereas N_2 reduction parameters remained unaltered. Nucleotide sequence analysis of the genes encoding the MoFe protein from this strain showed that the MoFe protein α- 69^{Gly} residue was substituted by serine and inspection of the MoFe protein crystal structure revealed that α-69^{Gly} is located on a short helix that skirts one of three Fe-S "faces" that compose the central portion of FeMo-cofactor (Figure 1). Although the basis for acetylene resistance could be interpreted in a variety of different ways, we proposed a model where this substitution alters local

conformational flexibility so that the side-chain of the adjacent α-70^{Val} residue is locked into a conformation that impairs active site access by acetylene, but does not affect access by the slightly smaller N_2 molecule. If this model is correct, we reasoned that substitution of α-70^{Val} by residues having smaller side chains would expand the size of substrates that can access the substrate reduction site. This possibility was easily tested because previous studies had established that short-chain alkynes other than acetylene are not effectively reduced by nitrogenase, presumably because they are denied access to the activation site due to the larger size (Hardy & Jackson, 1967; McKenna et al., 1979). Indeed, shortening the side-chain at the α-70^{Val} residue position, by substitution with either alanine or glycine, progressively expands the size of short-chain alkyne substrates, for example, propyne and 1-butyne, which can be accommodated at the nitrogenase active site (Mayer et al., 2002b).

3.3 - Biophysical Analysis of a Trapped Substrate Reduction Intermediate

The as-isolated form of the MoFe protein exhibits a characteristic S = 3/2 EPR (Electron Paramagnetic Resonance) spectrum that can be uniquely assigned to a semi-reduced state of FeMo-cofactor. When the MoFe protein is freeze-trapped under turnover conditions, where electrons are being delivered from the Fe protein to the MoFe protein, a more reduced form of FeMo-cofactor is accessed, which exists predominantly in an EPR-silent state (Figure 2). Attempts to freeze-quench the nitrogenase system in the presence of various substrates as a way to trap an EPR-active substrate/intermediate bound species has proven to be problematic. In particular, this approach has resulted in trapping bound species at only very low occupancy and often in multiple forms. For the inhibitor CO, two different CO bound states have been trapped at relatively high occupancy: one at lower CO concentrations (called lo-CO) and one at higher CO concentrations (called hi-CO). Characterization of these states by various spectroscopic methods has resulted in models where one or two CO molecules are bound to Fe atoms (Lee et al., 1997a; Lee et al., 1997b). Very low occupancy intermediates have been trapped when nitrogenase is freeze-quenched during reduction of the substrates carbon disulfide and acetylene (Lee et al., 2000; Ryle et al., 2000). The low occupancy of these

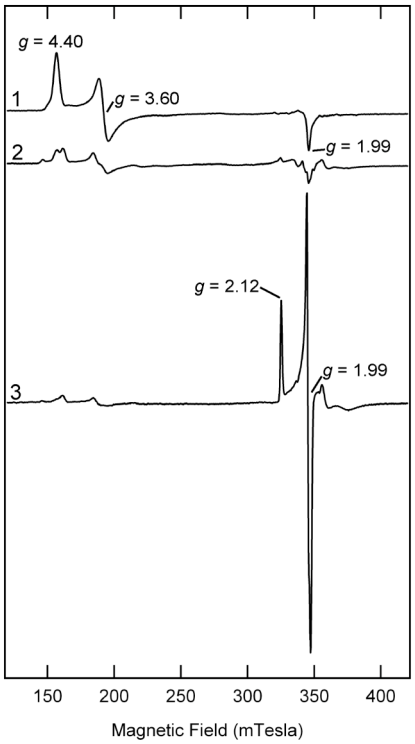

Figure 2. X-band EPR spectra of the MoFe protein. EPR spectra of the α-70^{Ala} MoFe protein are shown for the resting state under argon (trace 1), the turnover state under argon (trace 2), and the turnover state in the presence 3 mM propargyl-OH at pH 6.7 (trace 3). The *g*-values for select inflections are shown.

intermediates limited the information that could be deduced, although it was concluded that both substrates are likely bound to one or more Fe atoms.

Expansion of the active site of the MoFe protein so that larger substrates can be accommodated permitted the use of substrates that contain functional groups as a new strategy to trap a substrate reduction intermediate in an EPR-active form and at concentrations amenable to spectroscopic analyses (Benton et al., 2003). During the course of evaluating various functionalized substrates, we discovered that when either propargyl alcohol ($HC\equiv C\text{-}H_2OH$) or propargyl amine ($HC\equiv C\text{-}CH_2NH_2$) is used as a substrate for the substituted α-70^{Ala} MoFe protein, the resting state S = 3/2 EPR spectrum is converted to an intense S = 1/2 signal (Figure 2). In the context of earlier work identifying the S =1/2 intermediate formed during turnover in the presence of the inhibitor CO, the properties of the propargyl alcohol derived signal indicated that a single species was trapped in high occupancy.

These features provided an unprecedented opportunity to explore the properties of a bound nitrogenase substrate-reduction intermediate by EPR and ENDOR (Electron Nuclear Double Resonance) spectroscopies. By using uniformly ^{13}C-labeled propargyl alcohol and ^{13}C ENDOR spectroscopy, the coupling of three unique C atoms to FeMo-cofactor could be observed (Benton et al., 2003). This result confirmed that the substrate-derived intermediate was covalently bound to FeMo-cofactor, and further indicated an asymmetric spin coupling of the propargyl alcohol carbon atoms (C3 > C2 > C1) to FeMo-cofactor. The nature of the bound intermediate was revealed by examination of the ENDOR parameters when either H- or D-labeled propargyl alcohol (PA-H; PA-D) was used as substrate with turnover occurring in either H_2O or ^2H_2O (Lee et al., 2004). Key was the combined use of a new quantitative ^1H ENDOR technique, Stochastic Field-Modulated (SF) ENDOR, plus Mims pulsed ^2H ENDOR, to study strongly coupled protons (H^a) observed in the PA-H/H_2O spectrum (hyperfine coupling of $A(^1H^a) \sim 20$ MHz). This signal appears with half intensity in the spectra of the PA-H/D_2O and PA-D/H_2O samples, and is lost with the 'doubly-deuterated' PA-D/D_2O sample (Figure 3). These observations imply that the H^a doublet in the PA-H/H_2O

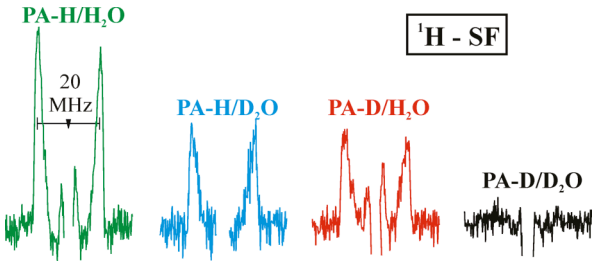

Figure 3. Quantitative Stochastic Field modulated-ENDOR spectra (^{1}H-SF) of the α-70^{Ala} MoFe protein incubated with propargyl alcohol under turnover conditions. The deuteration patterns are indicated; spectra are centered at the ^{1}H frequency, and split by the hyperfine coupling. The important observation from these spectra is that the intensity for the non-deuterated sample (green) is halved when either D_2O is used as solvent (blue) or the substrate is deuterated (red), and eliminated when deuterated substrate is used in D_2O (black). These results show that the bound intermediate contains two strongly coupled, magnetically identical protons. One of these protons must be from the substrate whereas the other originates from the solvent. A third, weakly coupled proton is seen in the red and green spectra originating from the solvent. The structural interpretation of these data is shown in Figure 4.

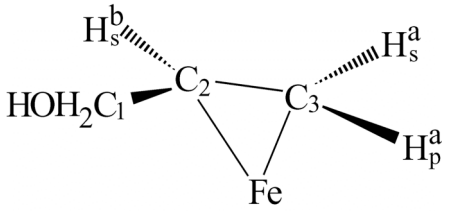

Figure 4. Proposed structure of the trapped propargyl alcohol reduction intermediate. This structural model is based on the results of ENDOR parameters and consideration of model compounds. The figure indicates two magnetically identical protons (H^a) bound to C3, one derived from the solvent (H_s) and one derived from the substrate (H_p). The weakly coupled proton (H^b) derived from the solvent is assigned to that bound to C2. Asymmetric binding of the propargyl alcohol C3 and C2 atoms (C3 coupling > C2) as revealed by ^{13}C ENDOR is indicated by a longer Fe-C2 bond than the Fe-C3 bond.

spectrum is the superposition of doublets from two magnetically identical, and hence symmetry-equivalent protons, one derived from propargyl alcohol (H_p) and the other acquired from solvent (H_s) during reduction. In addition, the experiments disclosed one weakly coupled proton (H^b) derived from solvent. Inspection of inorganic model compounds having similar compositions suggested two models that could explain the ENDOR observations, in particular, the two equivalent protons having different chemical origins. Both involve a three-membered ring that includes the propargyl alcohol C3 and C2 atoms and a single Fe atom. One of these is a one proton added, semi-reduced (ferracyclopropene) intermediate, and the other is a two proton added, further reduced (ferracylopropane) adduct of the allyl alcohol product. Between these we favor the latter (Figure 4), largely because it gives a more 'natural' explanation for the presence of the solvent-derived H^b. DFT (Density Functional Theory) calculations on FeMo-cofactor subsequently showed that the proposed model is an energetically favorable one (Dance, 2004).

3.4 - Elucidating the Location of the Bound Propargyl Alcohol Adduct

Genetics established the general location for binding of alkyne substrates and advanced spectroscopic techniques provided insight on how the reduction intermediate is bound but neither approach pinpointed which of the Fe atoms at the Fe 2, 3, 6, and 7 face is involved. To address this question we sought to determine why a propargyl alcohol reduction intermediate becomes trapped at the active site. The important observation was that although a propargyl alcohol intermediate becomes trapped at the active site, a propyne reduction intermediate does not, even though both molecules are substrates. This suggested that the propargyl alcohol -OH group is likely to interact with a functional group provided by the protein to stabilize a reduction intermediate. Among the candidate amino acids that could provide this function, the α-195^{His} residue was the most attractive because it is appropriately positioned within the identified binding region (Figure 1) and because previous work indicated a possible role for α-195^{His} as an obligate proton donor during N_2 reduction. The possibility that α-195^{His} stabilizes a propargyl alcohol

reduction-intermediate was tested and confirmed in two different ways (Igarashi et al., 2004). First, it was shown that the appearance of the S = 1/2 EPR signal under freeze quench conditions when propargyl alcohol is used as substrate disappears when the α-195^{His} residue is substituted by glutamine, even though propargyl alcohol retains an ability to interact with the active site of the substituted MoFe protein. Second, appearance of the characteristic EPR signal is pH dependent when either propargyl alcohol or propargyl amine is used as substrate. The respective pH-dependence profiles are different and show that optimum population of the trapped propargyl alcohol intermediate occurs when the α-195^{His} imidazole is protonated (pH 6.7, Figure 5A) whereas optimum population of the propargyl amine intermediate occurs when the imidazole group is deprotonated and propargyl amine is protonated (pH 8.0, Figure 5B).

The above results indicated formation of a hydrogen bond between the –OH group of a propargyl alcohol reduction intermediate and the imidazole of a-195^{His} thereby localizing the position of the –OH group within ~2 Å of the εN of a-195^{His}. Furthermore the previously described ENDOR studies showed that the bound adduct is allyl alcohol, which is proposed to interact with a single Fe atom in an η^2 configuration. Within these constraints the location of the bound intermediate is largely defined. To further refine the likely binding site DFT computational methods were used to suggest a detailed bonding geometry of the cofactor-reduction intermediate adduct, which was then fit into the α-Ala^{70} MoFe protein using force field methods to give the model shown in Figure 6.

3.5 - Relevance of the Identified Alkyne Binding Site to N_2 Binding

The results of experiments described so far provide compelling evidence that alkyne substrates can bind and be reduced at a specific FeS face of FeMo-cofactor, and, perhaps at a single Fe atom within that face. A remaining question is whether or not N_2 is also activated and reduced at this same face. This question is an important one because acetylene is a non-competitive inhibitor of N_2 reduction whereas N_2 is a weak

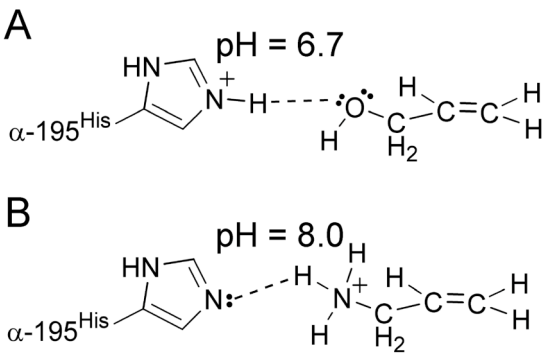

Figure 5. Proposed H-bonding of the trapped intermediates. The proposed H-bonding (dashed lines) is shown between the protonated α-195^{His} and propargyl alcohol (panel A) and between the deprotonated α-195^{His} and protonated propargyl amine (panel B).

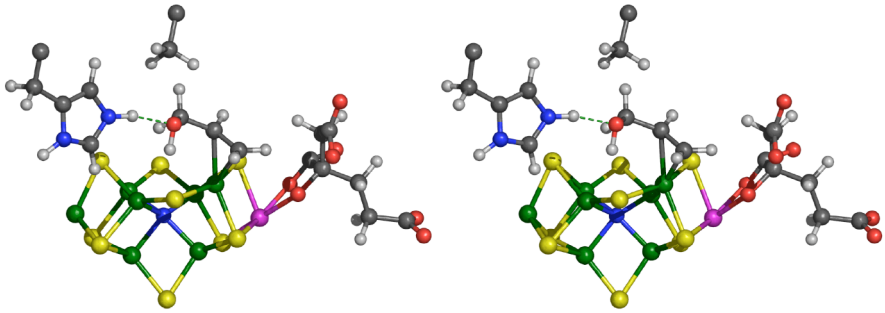

Figure 6. Stereo-view of the proposed structure for the trapped propargyl alcohol reduction intermediate bound to FeMo-cofactor. The most favorable state of the propargyl alcohol reduction intermediate bound to FeMo-cofactor in the α-70^{Ala} MoFe protein is shown. The alkane unit of allyl alcohol is bound to Fe6 of the FeMo-cofactor. A H-bond is shown (dashed line) between the hydroxyl O of allyl alcohol and the εN of α-195^{His} in the α-70^{Ala} MoFe protein. This model was deduced from experimental evidence for hydrogen bonding between the –OH group of the reduction intermediate and the α-195^{His} side-chain imidazole group and a combination of density functional theory and molecular mechanics computational methods.

competitive inhibitor of acetylene reduction (Seefeldt et al., 2004). Also, there is abundant evidence that acetylene can bind to the MoFe protein with both high and low affinity (Han & Newton, 2004). A reasonable interpretation of these observations is that either there are multiple and separate binding sites or that a single site can be accessed at different redox states. Differentiation between these possibilities, which are not mutually exclusive, is experimentally challenging because the MoFe protein pool is populated by a variety of different redox states under turnover conditions.

Although not yet answered unequivocally, there are recent results that bear on the question of whether or not acetylene and N_2 share the same binding site. In experiments that have already been described it was possible to expand the substrate reduction site by substitution of the α-70^{Val} residue by amino acids having smaller side-chains. We therefore reasoned that substitution of α-70^{Val} by amino acids having larger side-chains should compromise the reduction of all substrates that must access this face. To test this possibility the α-70^{Val} residue was substituted by isoleucine and the impact on acetylene and N_2 reduction evaluated (Barney et al., 2004). The results of these experiments showed that the substituted α-70^{Ile} MoFe protein is severely compromised for both acetylene reduction (>120 fold increase in K_m) and N_2 reduction (>15 fold increase in K_m) with no affect on proton reduction. The observations that proton reduction is not affected by the substitution and that acetylene and N_2 reduction can still be detected, although at very low levels, are consistent with an interpretation that neither electron delivery to the active site nor the ability of the enzyme to access the redox state required for substrate binding have been affected. Although an effect of the substitution on N_2 activation has not been ruled out, a logical interpretation of the results is that the α-70^{Ile} substitution denies access of both N_2 and acetylene to the same or overlapping binding sites. Whatever the relationship between N_2 binding and acetylene binding, the effect of the α-70^{Ile} substitution indicates that all acetylene reduction reactions must occur at the same face for several reasons. First, both acetylene and proton reduction involve the same redox states of the MoFe protein, both requiring two electrons. Second, proton reduction is unaffected by the α-70^{Ile} substitution, which means that the capacity for the altered MoFe protein to access the redox states required for acetylene reduction is not

affected by the substitution. Third, the ability of the substituted protein to catalyze acetylene reduction at substrate concentrations used to saturate the normal enzyme is virtually eliminated. Of course, the possibility that multiple substrate sites for acetylene binding exist within the same FeS face has not been ruled out.

A second line of experiments that indicates N_2 and acetylene share the same or overlapping binding sites involves analysis of the semi-reduced form of N_2, hydrazine, as a substrate (Barney et al., 2004). It was previously shown that hydrazine is a very poor substrate for nitrogenase (Davis, 1980). Further, acid quenching of nitrogenase under turnover conditions results in the release of small amounts of hydrazine when N_2 is used as substrate (Thorneley et al., 1978). Hydrazine is also a minor reduction product when N_2 is used as a substrate for the vanadium-dependent nitrogenase (Dilworth & Eady, 1991). In aggregate these observations provide compelling evidence that a bound form of hydrazine is an intermediate during nitrogenase-catalyzed N_2 reduction. One possible explanation for the slow rate of hydrazine substrate reduction is that its relatively larger size when compared to N_2 denies facile access to the active site. If this explanation is correct and N_2 is reduced at the same FeMo-cofactor face as acetylene reduction, then expansion of the active site by the α-Ala^{70} substitution should increase the capacity for hydrazine reduction. This prediction was verified experimentally where it was shown that substitution of the α-70^{Val} residue by alanine dramatically increases the capacity for hydrazine reduction (8 fold decrease in K_m) (Barney et al., 2004).

3.6 - The Riddle of N_2 Activation is Not Solved

In spite of nearly 40 years of intense investigation, the chemical mechanism of biological nitrogen reduction has remained enigmatic. In this account we have described a comprehensive approach that has been used to gain evidence that nitrogenase substrates bind and are reduced at a specific FeS face of FeMo-cofactor. Work described here cannot be considered to have eliminated Mo as the activation site for N_2 reduction but none of our results are compatible with that model. Perhaps the most important

contribution of the current work is that it has provided a framework for future theoretical models and proposed chemical mechanisms that can be tested experimentally.

From the experimental perspective there are two immediate goals of our research efforts. First, although there is now strong evidence that hydrazine, a likely active site bound N_2 reduction intermediate, is localized to the same general site as identified for propargyl alcohol reduction, it is not known exactly where and how this intermediate is bound. Towards this end, we are attempting to trap an EPR-active N_2 reduction intermediate in a highly populated state, using the same general approach described for detection and characterization of the alkyne reduction intermediates. Our second goal is related to the activation of N_2. Although nitrogenase is able to reduce a variety of substrates, N_2 reduction is differentiated from other substrates in the following ways: (*i*) a more reduced state of the MoFe protein is required for N_2 binding, (*ii*) N_2 binding/reduction is associated with the obligate evolution of H_2, (*iii*) H_2 is a competitive inhibitor of N_2 reduction and (*iv*) in the presence of N_2 and D_2 nitrogenase catalyzes HD formation (Burgess & Lowe, 1996). These observations suggest that the N_2 activation, but not activation of other substrates, could involve the obligate displacement of hydrides bound to FeMo-cofactor. Validation of this model requires elucidation of whether or not hydrides are bound to FeMo-cofactor in the activated state and, if so, determination of where they are bound. Again we believe that a combined genetic and biochemical approach is a way to trap a highly populated, EPR-active state so that advanced spectroscopies can be applied, and provides the best opportunity for success. These experiments are in progress.