CHAPTER 6

Early Studies on The Nitrogenase Active Site

6.1 - Introduction

Molybdenum-dependent nitrogenase is composed of the Fe protein and the MoFe protein. Together, they catalyze the reduction of nitrogen gas ($N_2$) to ammonia ($NH_3$). The Fe protein is a homodimer of 60 kDa that contains a solvent exposed [4Fe-4S] cluster. During turnover, the reduced Fe protein transfers one electron at a time to the MoFe protein in an ATP-dependent reaction. The MoFe protein is a 240 kDa $\alpha_2\beta_2$ heterotetramer with two unique types of iron-sulfur clusters. One [8Fe-7S] cluster (designated the P-cluster) is located at each $\alpha\beta$ subunit interface and seems likely to serve as an intermediary in the electron transfer process. The FeMo cofactor is a [7Fe-9S-X-Mo:homocitrate] cluster located entirely within each $\alpha$ subunit and has been identified as the substrate reduction site (Einsle et al., 2002; Hawkes et al., 1984).

In addition to its physiological substrate, nitrogenase can catalyze reduction of a variety of small triply or doubly bound substrates; among these are acetylene, azide, and nitrous oxide. In the absence of any other substrate, nitrogenase catalyzes the reduction of protons to yield hydrogen gas (Burgess, 1985). Despite years of intensive research, an understanding of the nitrogenase catalytic mechanism whereby nitrogen and other substrates are reduced at the unique FeMo cofactor has never been fully achieved. The main problem lies in the fact that substrates and inhibitors are transiently bound to the FeMo cofactor and hence are only observable spectroscopically under turnover conditions (Seefeldt et al., 2004).

Recently, we reported that the amino acid substitution $\alpha$-Gly^{69} affected enzyme specificity. This amino acid is located next to $\alpha$-Val^{70}, which caps a specific 4Fe-4S face of the FeMo cofactor (inclusive of Fe atoms: Fe 2, 3, 6 and 7 according to the X-ray structure labels; Figure 1). The importance of this amino acid was revealed during the
Figure 1- Specific [4Fe-4S] face of FeMo-cofactor and the amino acids in this molecular environment. The color scheme is as follows: sulfur = yellow, iron = green, molybdenum = magenta, nitrogen = blue, oxygen = red and carbon = gray. The amino acids detailed are Val\textsuperscript{70}, Arg\textsuperscript{96}, His\textsuperscript{195}. The PDB coordinates were extracted from the structure of *Azotobacter vinelandii* MoFe protein (PDB ID: 1M1N). The figure was constructed in Swiss PDB Viewer and edited in POV-Ray.
isolation of an acetylene-resistant MoFe protein that had \( \alpha\text{-Gly}^{69} \) substituted by Ser (Christiansen et al., 2000a; Christiansen et al., 2000b). This substitution resulted in a significant perturbation of acetylene binding and reduction. Acetylene was converted from a noncompetitive inhibitor to a competitive inhibitor of \( \text{N}_2 \) reduction. This result was interpreted as a successful elimination of one of two acetylene binding sites at the FeMo-cofactor. The low affinity acetylene binding site remained intact and unaltered, while a high affinity site was eliminated. These results suggest the nitrogen and acetylene compete for the same form of the enzyme and likely for the same substrate binding site. Next, we were able to isolate an altered MoFe protein with expanded substrate capabilities by changing the \( \alpha\text{-Val}^{70} \) to an alanine residue (Mayer et al., 2002b). This \( \alpha\text{-Ala}^{70} \) MoFe protein can effectively reduce larger alkynes such as propyne and propargyl alcohol at considerable levels.

In an attempt to further define the role of the \( \alpha\text{-Val}^{70} \) residue, we felt that additional insight could be gained by substituting the \( \alpha\text{-Val}^{70} \) by a shorter (e.g. glycine) or longer side-chain (e.g. isoleucine). By substituting the \( \alpha\text{-Val}^{70} \) residue, we established an inverse relationship between the size of the side chain at this position and the size of substrate that could be reduced. Namely, by decreasing the size of the side-chain, we were able to expand substrate size. With this work, it was determined which 4Fe-4S face of the FeMo-cofactor is involved in alkyne reduction. The results described here provided the first step towards the identification of the specific location of substrate reduction.

6.2- Experimental Procedures

Strain construction, cell growth, and purification

Strains of \( A. \) vinelandii containing amino acid substitution at the \( \alpha\text{-Val}^{70} \) position were constructed using site directed mutagenesis and gene replacement techniques previously described elsewhere (Jacobson et al., 1989; Robinson et al., 1986). These residues included: glycine (designated strain DJ1313), alanine (designated DJ1310), isoleucine (designated DJ1373) and leucine (designated DJ1312). All MoFe proteins in
this study contained a poly-histidine insertion near the carboxyl terminus of each α-subunit.

Cells were grown at 30 °C in 150 L cultures and derepressed for \textit{nif} gene expression and harvested as described previously (Christiansen et al., 1998). Crude extracts were prepared by the osmotic shock method and the MoFe protein purified by the immobilized metal-affinity chelation chromatography (IMAC) technique as previously described (Christiansen et al., 1998). The Fe protein used in all experiments was purified from wild type cells (Peters et al., 1994) and did not contain a histidine tag. All protein manipulations were kept anaerobic through the use of a Schlenk apparatus fitted with a BASF catalyst tower (Burgess et al., 1980). Protein concentration was determined by the biuret method using bovine serum albumin as the standard (Chromy et al., 1974). Protein purity was estimated by polyacrylamide gel electrophoresis (Laemmlli, 1970).

\textbf{Assays}

The overall technique and reaction mixture composition are described elsewhere (Kim et al., 1995; Peters et al., 1994). In summary, each assay contained 0.05 mg of MoFe protein and 0.45 mg of Fe protein to give a 36:1 molar ratio of Fe protein to MoFe protein. All reactions were carried out anaerobically in 9.2 ml evacuated, crimped, sealed vials containing argon. The reactions were initiated by the addition of Fe protein to the rest of the reaction components and allowed to proceed for 8 min while shaking in a 30°C water bath. The reaction was terminated by the addition of 250 \(\mu\text{L} \) 0.4 M EDTA, pH 7.4. Acetylene was freshly prepared for each experiment by the reaction of calcium carbide (Aldrich®) and water. Propyne (98% pure) was purchased from Aldrich and 1-butyne (95% pure) was purchased from Pfaltz and Bauer, Inc. Propargyl alcohol, propargyl amine, 3-butyne-2-ol, 2-butyne-1-ol, 2-butyne and 4-pentyn-1-ol were purchased from Aldrich and 1-pentyne and 3-butyne-1-ol was purchased from Fluka. \(\text{H}_2\) production was monitored by injection of 200 \(\mu\text{L}\) of the reactions’ gas phase into a Shimadzu GC-14 gas chromatograph equipped with a Supelco 80/100 molecular sieve 5A column and a thermal conductivity detector. Ethylene, propene and 1-butene productions were...
monitored using a Hewlett-Packard 5890A gas chromatograph equipped with an Al₂O₃ capillary column and a flame ionization detector. Quantification of propene and 1-butene was performed using an ethylene standard and applying a correction factor to the peak area taken from the measurement of a 2-6 carbon olefin standard (MG Scientific Gases) containing equimolar mixtures of these gases. Ammonia was quantified using the colorimetric indol-phenol method (Dilworth et al., 1992).

Hyperbolic responses to the initial velocity were seen when the partial pressure values of the substrates were increased. The Michaelis-Menten constants were derived by fitting data to the following hyperbolic equation:

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

(Eq. 1)

where \( v \) is the specific activity obtained using various substrates, \([S]\) is the concentration of the substrate, and \( V_{\text{max}} \) is the theoretical maximum specific activity. MgATP hydrolysis was determined by a colorimetric assay which measures the rate of creatine produced in the MgATP regenerating system used in the assays described above (Christiansen et al., 1998).

6.3 - RESULTS

**Short-chain alkyne substrate interactions with wild type (\(\alpha\)-Val\(^70\)), \(\alpha\)-Ala\(^70\)-, \(\alpha\)-Gly\(^70\)-, \(\alpha\)-Ile\(^70\)-, and \(\alpha\)-Leu\(^70\)- substituted MoFe proteins.**

Substitution of either an alanine or glycine at the \(\alpha\)-70 position did not significantly impair the altered MoFe protein’s enzymatic capabilities for acetylene or proton reduction (Table 1 and Figure 2A). Furthermore, no uncoupling of electron flow occurred for either enzyme as assessed by MgATP hydrolysis assays (data not shown). Results from hydrogen inhibition assays in the presence of acetylene proved to be surprising; both the \(\alpha\)-Ala\(^70\) and the \(\alpha\)-Gly\(^70\) substituted MoFe proteins showed \(\geq 95\%\) inhibition of hydrogen production in the presence of 0.1 atm of acetylene. This level of inhibition by acetylene had been predicted but never achieved, as acetylene had proved to have adverse effects on electron flux at levels greater than 0.2 atm for the wild type and other altered MoFe proteins.
Table I
Specific activities of wild-type, α-Gly\textsuperscript{70}, α-Ala\textsuperscript{70}, α-Ile\textsuperscript{70} MoFe proteins

<table>
<thead>
<tr>
<th>MoFe protein</th>
<th>Specific Activity\textsuperscript{a}</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proton</td>
<td>Nitrogen</td>
<td>Acetylene</td>
<td>Propyne</td>
</tr>
<tr>
<td>α-Ile\textsuperscript{70}</td>
<td>2300</td>
<td>170</td>
<td>130</td>
<td>nd\textsuperscript{b}</td>
</tr>
<tr>
<td>α-Val\textsuperscript{70} (wt)</td>
<td>2300</td>
<td>800</td>
<td>2000</td>
<td>44</td>
</tr>
<tr>
<td>α-Ala\textsuperscript{70}</td>
<td>1800</td>
<td>500</td>
<td>1700</td>
<td>1000</td>
</tr>
<tr>
<td>α-Gly\textsuperscript{70}</td>
<td>1500</td>
<td>nd</td>
<td>1475</td>
<td>1200</td>
</tr>
</tbody>
</table>

\textsuperscript{a} specific activities are reported in nmol of product/min/mg of MoFe protein as described in the experimental procedures section.

\textsuperscript{b} not detectable
Figure 2- Reduction of Short Chain Alkynes by α–70 MoFe Protein Variants. The plots show the specific activity (nmol/min/mg) saturation curves against the partial pressure of the substrate under Argon (atm). The inset in A and B shows the Lineweaver-Burk plot of the saturation curve. Substrates: (a) Acetylene, (B) Propyne and (C) 1-Butyne. The MoFe protein variants are shown: α–Val\(^{70}\) wild-type (-■-), α–Ala\(^{70}\) (-▲-) and α–Gly\(^{70}\) (-●-).
The substitution of the longer hydrophobic side-chains of isoleucine and leucine at the α-70 position exhibited different effects on substrate reduction than substitutions of shorter side-chains at the same position (Table I). Despite the fact that the α-Ile70 MoFe protein was as efficient in hydrogen production as the wild-type, this MoFe protein variant was very inefficient for acetylene reduction (130 nmol/min/mg vs 2000 nmol/min/mg for the wild-type MoFe protein). Substitution of a leucine at the α-70 position had prevented insertion of the FeMo-cofactor.

The interaction of short chain alkynes other than acetylene (i.e. propyne and propargyl alcohol) was shown for the wild type and α-Ala70 MoFe proteins (Mayer et al., 2002b). We, therefore, wanted to examine the α-Gly70 MoFe protein for similar interactions with propyne. We found that this altered MoFe protein is able to reduce propyne better than the α-Ala70 MoFe protein and much better than the wild type MoFe protein. Table I and Figure 2B compare wild type (α-Val70), α-Ala70 and α-Gly70 MoFe proteins for their respective propyne reduction specific activities. Based on inhibition of hydrogen formation in the presence of propyne and the lack of notable amounts of propene production, the interaction between the wild type MoFe protein and propyne was concluded to be very minimal. However, there is significant reduction of propyne in both the α-Ala70 and α-Gly70 MoFe proteins, having specific activities of 1000 and 1200 nmol/min/mg, respectively. The K_{m} and V_{max} values for propyne reduction in the α-Gly70 MoFe protein were 0.001 atm and approximately 1400 nmol/min/mg, respectively. These results suggest that propyne is able to more easily access the FeMo-cofactor in the case of the α-Gly70 MoFe protein and act as a better substrate than in the α-Ala70 MoFe protein (K_{m} of 0.02 and V_{max} of 1150 nmol/min/mg).

The next short-chain alkyne examined was 1-butyne, which is known to interact very poorly with the wild type enzyme (Burgess, 1985). Table 1 and Figure 2C show that both the α-Ala70 and α-Gly70 MoFe proteins were able to successfully reduce 1-butyne, although at much lower levels when compared to propyne reduction. These altered MoFe proteins have specific activities of approximately 175 and 350 nmol/min/mg, respectively. Obtaining a hyperbolic curve and hence accurate V_{max} and K_{m} values for
this substrate was not possible because substrate saturation measurement could not be obtained.

2-Butyne was also previously examined as a substrate for the wild type enzyme without success (Burgess, 1985). Nonetheless, in light of the reducing capabilities of the $\alpha$-Ala$^{70}$ and $\alpha$-Gly$^{70}$ MoFe proteins for 1-butyne, we thought that 2-butyne might also be reduced by these altered enzymes. However, no products could be detected and hydrogen production was not inhibited for the wild type, $\alpha$-Ala$^{70}$ or $\alpha$-Gly$^{70}$ MoFe proteins. This result might be attributed to the fact that substrates need to have an accessible terminal double or triple bond in order to bind at the FeMo-cofactor. A more likely explanation is that 2-butyne is too ‘bulky’ to gain access to the FeMo-cofactor (i.e. the substrate molecule will be linear whereas 1-butyne will have flexibility).

Similar results were also obtained when a series of alkyne alcohols were used as substrates for the $\alpha$-Gly$^{70}$ MoFe protein. The results shown in Figure 3 and Table III indicate the importance of a terminal triple bond. Twenty millimolar propargyl alcohol or 3-butyn-1-ol was able to inhibit $H_2$ production by 98% and 61% respectively. On the other hand, similar compounds that differ only in the position of the triple bond, such as 2-butyn-1-ol, were not able to inhibit $H_2$ production. Again, this observation indicates specificity for a terminal triple bond. The position of the hydroxyl group also seems to be important when alkyne alcohols are used as a substrate. When the hydroxyl group is moved closer to the triple bond, in the case of 3-butyn-2-ol, no inhibition of $H_2$ production was observed. In this experiment, the lack of inhibition can be attributed to the inability of this chemical compound to interact with the cofactor. One reasonable explanation is that the alcohol end is so bulky that this compound can not access the active site.

Finally, 1-pentyne and 4-pentyne-1-ol were analyzed as a substrate for the wild type, $\alpha$-Ala$^{70}$ and $\alpha$-Gly$^{70}$ MoFe proteins by monitoring the production of 1-pentene and the inhibition of hydrogen production. All three enzymes were unable to interact with or
Figure 3- Inhibition of Hydrogen Evolution Using Alkyne Alcohols for α–Gly70 MoFe Protein. The plot shows the inhibition of proton reduction (%) against substrate concentration (mM). Substrates: propargyl alcohol (-■-), 3-butyn-1-ol (-●-), 3-butyn-2-ol (-▲-), 2-butyn-1-ol (-◆-), and 4-pentyn-1-ol (-▼-). The table shows the values for inhibition of proton reduction using substrate concentration of 20 mM.
reduce these substrates presumably due to their sizes and their inability to access the FeMo-cofactor. This was not surprising as the previous results suggested these altered enzymes had nearly reached the size limitation of larger substrates. Nonetheless, the interdependence between side-chain size at the α-70 position and the substrate size, as seen for the substrate propyne, was unmistakably reiterated with the substrate 1-butyne and serves to further accentuate the concept that alkyne substrates are reduced at the 4Fe-4S face capped by the α-Val70 residue.

**Nitrogen interaction with wild type, α-Ala70, α-Gly70 and α-Ile70 MoFe proteins.**

Substitutions of the amino acid α-Val70 also affected the ability of nitrogenase to reduce its physiological substrate N₂. As shown in Table I, for the wild type enzyme, nitrogen is reduced at a typical rate of approximately 800 nmol/min/mg and shows 63% inhibition of hydrogen production. The α-Ala70 enzyme reduces nitrogen at a rate of approximately 500 nmol/min/mg with 37% inhibition of hydrogen production whereas, the α-Gly70 enzyme neither reduces nitrogen nor inhibits hydrogen production in the presence of N₂. Finally, the α-Ile70 enzyme reduces nitrogen at low levels (170 nmol/min/mg) and inhibits hydrogen production by only 12%. These results suggest that the α-Val70 side-chain is critically involved in nitrogen reduction. Perhaps, this amino acid residue is involved in the stabilization of an intermediate state during N₂ reduction.

**6.4 - Discussion**

The role of α-Val70 in controlling substrate reduction was defined by decreasing (α-Ala70, α-Gly70) or increasing (α-Ile70) the size of the side chain at this position. As shown in Table I, the specific activities for proton reduction were not affected by any of three substitutions (α-Gly70, α-Ala70, and α-Ile70). However, specific activity for reduction of a series of short-chain alkyne was changed dramatically for MoFe proteins having any of these substitutions. This observation indicates that substitutions at this position did not affect electron flux but rather modified substrate specificity by controlling the size of the active site.
An interpretation of these results was that either a substrate channel located very near to the FeMo-cofactor was opened or, more likely, the $\alpha$-Val$^{70}$ directly controls the size of substrates that access the active site. In the latter case, the wild type MoFe protein $\alpha$-Val$^{70}$ side chain probably places steric constrains at the substrate-binding site; so that substrates larger than acetylene can not access at the active site. Thereby, the $\alpha$-Ala$^{70}$ and $\alpha$-Gly$^{70}$ substitutions progressively relax these constrains allowing larger substrates (propyne and 1-butyne) to be reduced. The steric selectivity of wild type MoFe protein imposed by $\alpha$-Val$^{70}$ can be seen as a sophisticated mechanism of nitrogenase to ensure that resources are not wasted through reduction of non-physiological substrates.

We have demonstrated a direct correlation between the size of terminal alkyne substrates accessible and reducible at the FeMo-cofactor and the size of the $\alpha$-70 side-chain. Whether or not shortening of this side-chain opens the site of substrate binding at this particular 4Fe-4S face or whether we have simply widened the substrate channel was initially unclear. However, we favor the former idea, which is be supported by the nitrogen reducing ability of these three MoFe proteins for several reasons. First, nitrogen reduction levels were dramatically decreased in the $\alpha$-Ile$^{70}$ MoFe protein variant. Second, the $\alpha$-Gly$^{70}$ MoFe protein was not able to reduce nitrogen. This lack of reactivity towards nitrogen as substrate might be explained by the fact that, in the wild type, the $\alpha$-Val$^{70}$ side chain provides just enough room to hold and stabilize the nitrogen molecule in the correct orientation during reduction. Alternatively, this side-chain may promote stabilization of substrate intermediates. The result could also be interpreted as an inability for this altered enzyme to reach the required redox state for nitrogen reduction. This effect would not be observed during alkyne reduction because nitrogen requires a higher redox state of the enzyme to be reduced at this specific 4Fe-4S face of FeMo-cofactor.

Expanding the active site by shortening the amino acid side chain at position $\alpha$-70 is a useful tool toward understanding the nitrogenase mechanism. These MoFe proteins are now able to reduce larger substrates. This property was valuable because larger compounds that contained functional groups and/or an internal triple bound, rather than a
terminal one, could be used as substrate. Because 1-butyne was successfully reduced in both the \( \alpha \)-Ala\(^{70} \) and the \( \alpha \)-Gly\(^{70} \) MoFe proteins, we then asked if 2-butyne might not act as a substrate as well. However, none of the MoFe protein variants were able to reduce 2-butyne or 2-butyne-1-ol when used as substrates in concentrations ranging from 0-20 mM. This result was initially surprising; however, it may be explained by a number of possibilities. First, the terminal triple bond in 1-butyne and 3-butyne-1-ol (and all terminal alkynes) could mimic the acetylene triple bond and impose a similar acidic character for the molecule, which may be a factor in facilitating the initial binding at the FeMo-cofactor. Second, the initial mode of substrate binding could be end-on to a single iron atom. If this was the case, then the internal triple bond of 2-butyne would not be accessible and interaction/reduction with the FeMo-cofactor could not take place. Finally, it is possible that the strict linear character of 2-butyne imposed by the internal triple bond leaves the molecule with no flexibility preventing access to the FeMo-cofactor. In the case of 1-butyne, however, the substrate may have just enough flexibility at the 4-carbon end to maneuver its way to the FeMo-cofactor.

The use of these altered MoFe proteins for reduction of short-chain alkynes that contain functional groups was extremely useful. Functional groups could, perhaps, interact with the residues that surround the FeMo-cofactor. When used as substrates, propargyl alcohol and 3-butyne-1-ol were able to inhibit proton reduction at 98\% and 61\% respectively for the \( \alpha \)-Gly\(^{70} \) MoFe protein (Figure 3). When these substrates were used with the \( \alpha \)-Ala\(^{70} \) MoFe protein, similar results were obtained (data not shown).

The most insightful information was gained during spectroscopic analysis of the interactions between these alternative substrates and \( \alpha \)-Ala\(^{70} \) MoFe protein. When propargyl alcohol is incubated with \( \alpha \)-Ala\(^{70} \) MoFe protein, under turnover conditions, an intermediate was trapped (Benton et al., 2003). By using \( ^{13}\)C/\(^1\)H ENDOR techniques, the bound intermediate was identified as C2 and C3 of allyl alcohol bound to one iron atom forming a metallo-cyclopropane ring (Lee et al, 2004). Finally, this complex was further characterized by identifying exactly where on the FeMo-cofactor the intermediate was bound. In this proposed model, the substrate/product is bound to the Fe6 atom of the
cofactor (described in Chapter 7). This represents a snapshot of at least one of the intermediate states that occurs during alkyne reduction. The next most important question to address was whether or not both nitrogen and alkynes share the same physical binding site for substrate reduction.

More recent studies have shown that hydrazine (N$_2$H$_2$), a poor substrate for wild type MoFe protein (Davis, 1980), becomes an efficient substrate for $\alpha$-Ala$^{70}$ MoFe protein (Barney et al., 2004). Curiously, hydrazine, one of the intermediates during nitrogen reduction, is slightly larger than nitrogen. Our results lead to two important conclusions: (i) the expansion of the active site allows nitrogenase to reduce larger substrates and (ii) alkynes and nitrogen are very likely reduced at the same site.

Early studies on the $\alpha$-70 variants of MoFe protein and their ability to reduce alternative substrates provide the first set of experimental evidence for the identification of the substrate-binding site. Our results, when considered collectively (Barney et al., 2004; Benton et al., 2003; Benton et al., 2001; Igarashi et al., 2004; Lee et al., 2004; Mayer et al., 2002b), provide strong evidence that the substrate reduction site is at the specific 4Fe-4S face of the FeMo-cofactor capped by $\alpha$-Val$^{70}$. At this face, Fe6 is actually involved in at least one intermediate state of catalysis. These results, however, do not identify other intermediate steps that may occur during catalysis.
CHAPTER 7

Localization of a Catalytic Intermediate Bound to FeMo-cofactor of Nitrogenase


Robert Y. Igarashi¹, Patricia C. Dos Santos², Walter G. Niehaus², Ian G. Dance³, Dennis R. Dean², and Lance C. Seefeldt¹

This manuscript describes the identification of the location of a bound substrate-derived intermediate on FeMo-cofactor. In this work, a propargyl alcohol or propargyl amine derived intermediate is shown to hydrogen bond to the imidazole ε-NH of α-195His. Density functional- and force-field-calculation indicated that the substrate intermediate is bound to the Fe6 atom of FeMo-cofactor.

I was involved in performing experiments pertaining to bacterial strain construction, protein purification, and enzymatic assays. This chapter was written and submitted for publication with the intention for its use to satisfy a portion of the research completed for this dissertation. As co-author, I participated in writing the document at all stages of its preparation.


¹Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322; ²Department of Biochemistry, Virginia Tech, Blacksburg, Virginia 24061; ³School of Chemical Sciences, The University of New South Wales, Sydney, NSW 2052 Australia.
7.1- Introduction

Nitrogenase is comprised of two component proteins, called the Fe protein and the MoFe protein, which together catalyze the nucleotide-dependent reduction of $\text{N}_2$ to ammonia (equation 1).

$$\text{N}_2 + 8 \text{e}^- + 16 \text{MgATP} + 8 \text{H}^+ \rightarrow 2 \text{NH}_3 + \text{H}_2 + 16 \text{MgADP} + 16 \text{P}_i \quad (\text{eqn 1})$$

During catalysis, electrons are delivered one at a time from the Fe protein to the MoFe protein in a reaction coupled to the hydrolysis of two equivalents of MgATP for each equivalent of electrons transferred (Burgess & Lowe, 1996; Rees & Howard, 2000). The MoFe protein contains two metalloclusters called the P-cluster [8Fe-7S] and FeMo-cofactor [7Fe-9S-Mo-X-homocitrate], where X is proposed to be N, C, or O (Einsle et al., 2002). The P-clusters are thought to mediate electron transfer from the Fe protein to FeMo-cofactor, which in turn provides the site for substrate binding and reduction. The structure of FeMo-cofactor has been elucidated from the solution of X-ray structures of MoFe proteins (Chan et al., 1993; Einsle et al., 2002; Kim & Rees, 1992; Kim et al., 1993; Mayer et al., 1999), yet understanding where and how substrates interact with FeMo-cofactor is still unknown. Different models for where substrates bind to FeMo-cofactor have been developed, built on evidence from model compounds, theoretical calculations, and kinetic and biophysical studies on the wild-type (WT) and genetically altered MoFe proteins (Seefeldt et al., 2004). Some models propose binding and reduction of substrates at the Mo atom, whereas others suggest binding and reduction of substrates at one or more of the six Fe atoms that constitute the central portion of FeMo-cofactor. Models have also been proposed that involve substrate binding of both Mo and Fe at different steps during the reduction reaction (Demadis et al., 1996; Durrant, 2002; Malinak et al., 1997).

Recently, we have pursued genetic and biophysical approaches on nitrogenase to localize the substrate binding site on FeMo-cofactor (Benton et al., 2003; Benton et al., 2001; Christiansen et al., 2000a; Mayer et al., 2002b). It has been
demonstrated that substitution of α-70Val, a residue that approaches one Fe₄S₄ face of the FeMo-cofactor (involving Fe atoms 2, 3, 6, and 7), by amino acids with smaller side chains expands the substrate specificity to include larger alkynes (Benton et al., 2003; Christiansen et al., 2000a; Mayer et al., 2002b). For example, substitution of α-70Val by alanine has been shown to expand the substrate range of nitrogenase to include propyne (HC≡CCH₃) or propargyl alcohol (HC≡CCH₂OH, propargyl-OH) (Mayer et al., 2002b). When the α-70Ala MoFe protein is freeze-trapped during the reduction of propargyl-OH, a reduction-intermediate bound to FeMo-cofactor is captured (Benton et al., 2003). Using ¹³C- and ¹²H-labeled propargyl-OH and ENDOR spectroscopic methods, we have recently deduced that the trapped intermediate has two hydrogen atoms added (i.e., is allyl alcohol, H₂C=CHCH₂OH) and is bound to iron such that the two terminal H atoms are spectroscopically indistinguishable, suggesting the bio-organometallic complex shown in Scheme 1 (Lee et al., 2004).

In the present work, a model is developed from studies of nitrogenase for binding of a propargyl-OH reduction intermediate, as well as binding of a propargyl-NH₂ reduction intermediate, to a specific Fe atom within FeMo-cofactor.

### 7.2 - Experimental Procedures

**Protein Purification and Activity Assays**

*Azotobacter vinelandii* strains DJ1310 and DJ1316 expressing the α-70Ala and α-70Ala/α-195Gln variant MoFe proteins, respectively, were constructed using site-directed mutagenesis and gene replacement techniques as previously described (Christiansen et al., 2000a; Christiansen et al., 1998). The α-70Ala and α-70Ala/α-195Gln variant MoFe proteins were purified using a poly His-metal affinity chromatography system described
earlier (Christiansen et al., 1998). The wild-type Fe protein component of nitrogenase was purified essentially as previously described (Burgess et al., 1980). All manipulations of proteins were conducted in septum-sealed serum vials under an argon atmosphere and all anaerobic liquid and gas transfers were performed using gas-tight syringes. Acetylene reduction, H₂ evolution, and N₂ reduction activities were determined as described earlier (Seefeldt et al., 1992; Seefeldt et al., 1995). NH₃ was quantified using a liquid chromatographic-fluorescence method with o-phthalaldehyde mercaptoethanol as previously described. Thirty microliters of an assay reaction producing NH₃ was added to 1 mL of a solution containing 19 mM phthalic dicarboxyaldehyde, 3.4 mM 2-mercaptopoethanol, 5% (v/v) ethanol and 190 mM K-phosphate, pH 7.3 and allowed to react in the dark for 30 minutes. The mixture was injected and separated on C-18 Guard column and detected by fluorescence ($λ_{\text{excitation}}/λ_{\text{emission}}$ of 350/450 nm). The NH₃ quantification was standardized with NH₃Cl. The $K_i$ for propargyl-OH was estimated from the apparent affinity for acetylene ($K_{m\text{app}}$) in the presence of varying propargyl-OH concentrations and then plotting $K_{m\text{app}}$ versus propargyl-OH concentration.

**Preparation of Non-Turnover and Turnover MoFe Protein EPR Samples**

Non-turnover (resting-state) MoFe protein (100 µM) samples were made in 100 mM MOPS buffer (pH 7.0) with 30 mM sodium dithionite (Na₂S₂O₄) under 1 atm of Ar. Nitrogenase turnover samples were prepared by the addition of the MoFe protein (100 µM) to a buffer mixture (100 mM MES, 100 mM MOPS, 100 mM Tris, and 100 mM TAPS) at defined pH values and including 30 mM sodium dithionite, 10 mM ATP, 15 mM MgCl₂, 20 mM phosphocreatine, 2 mg/mL bovine serum albumin, and 0.3 mg/mL creatine phosphokinase. The reaction was initiated by the addition of Fe protein (50 µM) and was allowed to react for ca. 20 seconds at room temperature before being frozen in EPR tubes in liquid nitrogen. Proteins were added from a concentrated stock solution and did not significantly alter the reaction pH. Where appropriate, propargyl-OH (3 mM) or propargyl-NH₂ (15 mM) were included in the initial reaction mixture. Standardized 4 mm quartz EPR tubes were used for all samples.
**EPR Spectroscopy**

X-band EPR spectra were recorded on a Bruker ESP-300 E spectrometer equipped with an ER 4116 dual-mode X-band cavity including an Oxford Instruments ESR-900 helium flow cryostat. EPR spectra were recorded at a modulation frequency of 100 kHz, a modulation amplitude of 1.26 mT (12.6 Gauss), a sweep rate of 10 mT/s, and a microwave frequency of approximately 9.65 GHz (with the precise value recorded for each spectrum to ensure exact g alignment). All spectra were recorded at 8 K and a microwave power of 2.0 mW, with each trace being the sum of five scans. The program IGOR Pro (WaveMetrics, Lake Oswego, OR) was used for all subsequent manipulation of spectral data.

**Theoretical calculations.**

Spin-unrestricted all-electron density functional (DF) calculations of FeMo-cofactor with bound intermediates used the BLYP functional with numerical basis sets, as implemented in the program DMol3 [www.accelrys.com/mstudio/ms_modeling/dmol3.html] (Delley, 1990, 1995, 2000). Calculations of the FeMo-cofactor with bound intermediates within the protein involved a large protein component comprised of 1032 amino acid residues, 1332 associated water molecules, and the P-cluster, together with FeMo-cofactor. This was selected as all of chains A and B of 1M1N (Einsle et al., 2002), together with residues 494 to 523 of chain D and all water molecules within 4Å of these atoms or FeMo-cofactor. All hydrogen atoms were added, and energy-minimized by force field methods (force field cvff, program Discover [www.accelrys.com/insight/discover.html]), in order to optimize the protein and water hydrogen bonding. Structures from DF calculations of the intermediates bound to FeMo-cofactor were substituted into the protein, and investigated by further force-field optimizations. The P-cluster and the NFe$_7$MoS$_9$O$_2$ atoms of FeMo-cofactor, plus bound intermediate, were fixed during all force-field calculations.

7.3 - Results and Discussion

**Relevant features of the α-70$^{16}$-substituted MoFe protein**
Substitution of the MoFe protein $\alpha$-70$^{\text{Val}}$ residue by alanine expands the substrate range for nitrogenase to include the short chain alkynes propyne (HC CCH$_3$) and propargyl alcohol (HC CCH$_2$OH, propargyl-OH) (Mayer et al., 2002b). When propargyl-OH is used as a nitrogenase substrate and freeze-quenched under turnover conditions, a paramagnetic intermediate is observed that results from conversion of the resting state $S=3/2$ spin system (Figure 1, trace 1) to an $S=1/2$ spin system having a rhombic EPR signal with $g$ values of 2.123, 1.998, and 1.986 (Figure 1, trace 4) (Benton et al., 2003). This new EPR-active state originates from an intermediate derived from propargyl-OH that is bound to FeMo-cofactor. ENDOR studies, coupled with use of isotopically labeled propargyl-OH, have established that allyl alcohol, CH$_2$=CH-CH$_2$OH, is the probable species bound (Scheme 1) (Lee et al., 2004).

In contrast to propargyl-OH, when propyne is used as the substrate, freeze-quenching under turnover conditions does not result in formation of a trapped adduct that can be observed by EPR. An obvious explanation for this difference is that the -OH group of propargyl-OH stabilizes the bound intermediate through hydrogen bonding interactions with a functional group provided by an amino acid located within the vicinity of the substrate binding site. Identification of the proposed functional group thus provided an unprecedented opportunity to identify where and how an alkyne substrate might interact with the nitrogenase active site. Towards this end, the environment around $\alpha$-70$^{\text{Val}}$ and the FeMo-cofactor in the resting state X-ray structure of the MoFe protein (Einsle et al., 2002) was examined and the imidazole of $\alpha$-195$^{\text{His}}$ identified as the most likely candidate to provide the hydrogen-bonding interaction with propargyl-OH. This possibility suggested two predictions that could be experimentally tested. First, if a propargyl-OH reduction intermediate is stabilized by a hydrogen bond interaction with the imidazole group of $\alpha$-195$^{\text{His}}$ such stabilization should be pH-dependent, having a p$K_a$ value near that expected for a histidine residue. Second, an ability to trap the EPR-active intermediate should be dependent on having a histidine residue at the $\alpha$-195 residue position.
Figure 1. X-band EPR spectra of α-70Ala MoFe protein in the resting and turnover states with propargyl-OH at different pH values. EPR spectra of α-70Ala MoFe protein are shown for the resting state under argon (trace 1) and for the resting state in the presence of 10 mM propargyl-OH (trace 2). The lower set of traces (3, 4, and 5) are of the α-70Ala MoFe protein under turnover conditions with protons as substrate at pH 7.0 (trace 3), or with 3 mM propargyl-OH as substrate at pH 6.7 (trace 4), and pH 8.7 (trace 5). All samples contained 100 µM α-70Ala MoFe. Other conditions are presented in the Experimental Procedures section.
Appearance of the freeze-trapped propargyl-OH reduction intermediate is pH-dependent

Figure 1 illustrates the pH dependence of the intensity of the propargyl-OH derived EPR signal. The maximal EPR signal intensity, which is interpreted to indicate the maximal concentration of the trapped adduct, is observed at a pH ~ 6.7. As the pH increases from 6.7 to 8.7, the intensity of the EPR signal steadily decreases (Figure 1, traces 4 and 5). No significant changes in EPR lineshape are observed over this pH range, indicating a simple depopulation of the intermediate bound state as pH rises. A more complete data set for the intensity of the propargyl-OH dependent EPR signal versus pH is presented in Figure 2, also illustrating a depopulation of the trapped adduct as the pH increases from 6.7 to 9.0. This result is consistent with the deprotonation of a group having a pK_a ~ 7.5 and that a protonated state is required for formation of a hydrogen bond necessary to elicit the EPR signal associated with the trapped species. In proteins, histidine residues can have pK_a values ranging from 5 to 9, depending on the protein environment (Edgcomb & Murphy, 2002). The εNH of α-195His is surrounded by the hydrophobic residues α-65Ala, α-66Gly, α-70Ala, α-71Val, and α-381Phe, together with the negatively-charged sulfur atoms of FeMo-cofactor generating a net negative electrostatic potential. This environment would be predicted to increase the pK_a of the imidazole of α-195His (Chang & Swenson, 1997). Thus, one explanation of the results is that the protonated state of the εNH^+ of α-195His acts as a hydrogen-bond donor to a non-bonded electron pair of the O of bound allyl-OH (Figure 3, panel A). As the εN of α-195His becomes deprotonated (with an observed pK_a of 7.5), the hydrogen-bond with the O of allyl-OH would greatly diminish.

The pH dependence for the formation of the propargyl-OH trapped intermediate can be contrasted with the previously reported pH dependence for acetylene reduction by nitrogenase. In the latter case, maximal activity is around pH 7.3, with a decline in activity at pH values higher and lower than pH 7.3 (Pham & Burgess, 1993). The pH optimum observed here of 6.7 for the formation of the propargyl-OH trapped species is significantly lower than the pH optimum for activity of 7.3, suggesting that a specific deprotonation event is controlling the trapping of the intermediate.
Figure 2. pH dependence on formation of propargyl-OH and propargyl-NH$_2$ EPR-detected intermediates. The EPR signal ($g = 2.0$) intensity as a percentage of the maximal observed intensity is plotted against the pH. The $\alpha$-70$^{\text{Ala}}$ MoFe protein under turnover conditions with propargyl-OH at 3 mM (●) or with propargyl-NH$_2$ at 15 mM (○) is shown. Also shown is the $\alpha$-70$^{\text{Ala}}$/α-195$^{\text{Gln}}$ MoFe protein under turnover conditions with propargyl-OH (▲).
Figure 3. Proposed interactions of propargyl-OH and propargyl-NH$_2$ with $\alpha$-195$^{\text{His}}$. The proposed interaction of propargyl-OH (panel A) or propargyl-NH$_2$ (panel B) with the imidazole side chain of $\alpha$-195$^{\text{His}}$ of the MoFe protein is considered at different pH values.
Appearance of a propargyl-NH$_2$ reduction intermediate is also pH-dependent

More convincing evidence that appearance of the trapped EPR-active species is the result of specific hydrogen-bonding between a reduction intermediate and an active site residue, rather than a non-specific effect on enzyme activity, was obtained by using propargyl amine (propargyl-NH$_2$) as a substrate instead of propargyl-OH. If appearance of the trapped species derived from propargyl-OH is dependent on hydrogen-bonding provided by the protonated imidazole group of $\alpha$-195$^{\text{His}}$, it is predicted that appearance of a trapped species when propargyl-NH$_2$ is used as substrate would require a deprotonated imidazole group (Figure 3, panel B) because the pK$_a$ of propargyl-NH$_2$ is higher than that of imidazole. In control experiments, it was shown that, like propargyl-OH, propargyl-NH$_2$ is an inhibitor of acetylene reduction (50 % inhibition observed by inclusion of 20 mM propargyl-NH$_2$ in an assay with 0.003 atm acetylene at pH 8.0) and therefore interacts with the active site. When the $\alpha$-70$^{\text{Ala}}$ MoFe protein is freeze-quenched during turnover using propargyl-NH$_2$ as substrate, an EPR active species is also detected, having the same lineshape as the propargyl-OH derived species with apparent g values of 2.12, 2.00, and 2.00 (Figure 4). Given the similarity in lineshape of the EPR spectra elicited under freeze-quench conditions when either propargyl-OH or propargyl-NH$_2$ is used as substrate, it is presumed that an allyl-NH$_2$ adduct is bound to an Fe atom of FeMo-cofactor in a way similar to that proposed for propargyl-OH (Lee et al., 2004). One difference in behavior between these two substrates is the lower intensity of the propargyl-NH$_2$ (15 mM) elicited EPR signal (approximately one-fourth) when compared to propargyl-OH (3 mM) dependent EPR signal intensity. Another important difference is the pH profile for the formation of the respective intermediates (Figure 2). As can be seen, the population of the trapped propargyl-NH$_2$ reduction-intermediate species increases with rising pH, maximizing at about pH 8.2, followed by a rapid decline. The important observation is that the pH required for maximizing the propargyl-NH$_2$ elicited EPR signal is significantly shifted to a higher pH than required for maximizing the propargyl-OH elicited EPR signal. This feature is in line with a requirement for a protonated imidazole group for hydrogen bonding to propargyl-OH and a deprotonated
Figure 4. X-band EPR spectra of α-70Ala MoFe protein in the resting and turnover states with propargyl-NH2 at different pH values. EPR spectra of α-70Ala MoFe protein are shown for the resting state under argon (trace 1) and for the turnover state with protons as substrate at pH 7.0 (trace 2), or with 15 mM propargyl-NH2 as substrate at pH 6.7 (trace 3) and pH 8.0 (trace 4). All samples contained 100 µM α-70Ala MoFe protein.
imidazole group for hydrogen bonding to propargyl-NH$_2$ (Figure 3). The rapid decline in population of the trapped species when propargyl-NH$_2$ is used as substrate can be explained by the deprotonation of both the imidazole group of $\alpha$-195$^{\text{His}}$ and the amino group of propargyl-NH$_2$ (Graton et al., 1999). Taken together, the different pH-dependencies required to populate intermediate states when either propargyl-OH or propargyl-NH$_2$ is used as substrate provide a compelling case for the protonation or deprotonation of an imidazole group of $\alpha$-195$^{\text{His}}$ as controlling the stabilization of bound intermediates by hydrogen-bonding interactions.

**The imidazole group of $\alpha$-195$^{\text{His}}$ is required for intermediate stabilization**

Previous work has shown that substitution of the MoFe protein $\alpha$-195$^{\text{His}}$ residue by glutamine results in an altered MoFe protein that can bind N$_2$ as effectively as wild-type MoFe protein but is not able to effectively reduce N$_2$ (<2% N$_2$ reduction) (Dilworth et al., 1998; Fisher et al., 2000; Kim et al., 1995). In contrast, the $\alpha$-195$^{\text{Gln}}$-substituted MoFe protein is unaffected in its ability to reduce acetylene or protons (Fisher et al., 2000; Kim et al., 1995). The explanation offered for these features is that the imidazole group of $\alpha$-195$^{\text{His}}$ is specifically required to stabilize an N$_2$ reduction intermediate, or is required as a proton donor for N$_2$ reduction (Fisher et al., 2000; Kim et al., 1995). These observations, taken together, indicate that $\alpha$-195$^{\text{His}}$ is the most likely candidate for providing the ionizable group responsible for stabilizing propargyl-OH or propargyl-NH$_2$ intermediates. If $\alpha$-195$^{\text{His}}$ is responsible for hydrogen-bonding interactions necessary to stabilize a propargyl-OH reduction intermediate, such an interaction should be lost by substitution of glutamine for the MoFe protein $\alpha$-195$^{\text{His}}$ residue. This prediction was tested by construction and characterization of a doubly substituted MoFe protein where $\alpha$-195$^{\text{His}}$ is substituted by glutamine and $\alpha$-70$^{\text{Val}}$ is substituted by alanine. The $\alpha$-70$^{\text{Ala}}$/\$\alpha$-195$^{\text{Gln}}$ doubly substituted MoFe protein retains > 65% of the wild type acetylene and proton reduction activities (Table 1), indicating no major disruption in the binding or reduction of these substrates. Like the $\alpha$-195$^{\text{Gln}}$ MoFe protein, the doubly substituted MoFe protein shows very low N$_2$ reduction activity (< 2%). Further, propargyl-OH remains an effective inhibitor of acetylene reduction for the doubly substituted MoFe
Table I
Activities of wild-type, $\alpha$-70$^{\text{Ala}}$, and $\alpha$-70$^{\text{Ala}}$/\(\alpha\)-195$^{\text{Gln}}$ MoFe proteins

<table>
<thead>
<tr>
<th>MoFeP</th>
<th>C$_2$H$_4$ (0.1 atm)</th>
<th>Argon (1 atm)</th>
<th>N$_2$ (1 atm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific Activity$^a$, WT$^b$</td>
<td>Specific Activity$^a$, WT$^b$</td>
<td>Specific Activity$^a$, WT$^b$</td>
</tr>
<tr>
<td>C$_2$H$_4$</td>
<td>% atm</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1860±40 100 0.005±0.001</td>
<td>2250±70 100</td>
<td>940±60 100</td>
</tr>
<tr>
<td>$\alpha$70$^{\text{Ala}}$</td>
<td>1660±60 89 0.003±0.001</td>
<td>2200±50 98</td>
<td>790±20 84</td>
</tr>
<tr>
<td>$\alpha$70$^{\text{Ala}}$/(\alpha)-195$^{\text{Gln}}$</td>
<td>1240±60 67 0.007±0.001</td>
<td>1460±80 65</td>
<td>8±2 &lt;1</td>
</tr>
</tbody>
</table>

$^a$ Specific activities are reported in units of nmol of product/min/mg of MoFe protein and were determined with an iron protein : MoFe protein molar ratio of 40:1

$^b$ %WT were calculated by ratio of the activity of the altered MoFe protein to the activity of wild-type.
protein, with a $K_i$ of 8 mM compared to the published value of 4 mM for the $\alpha$-70$^{\text{Ala}}$ MoFe protein (Mayer et al., 2002b), clearly indicating that propargyl-OH continues to associate with the doubly substituted MoFe protein. However, no EPR detectable adduct is observed when the $\alpha$-70$^{\text{Ala}}$/$\alpha$-195$^{\text{Gln}}$ MoFe protein is freeze-quenched during turnover when either propargyl-OH or propargyl-NH$_2$ are used as substrates (data are shown in Figure 2 for propargyl-OH).

**Refined identification of the substrate binding site by density functional- and force-field-calculations**

The above results all point to the formation of a hydrogen bond between the -OH or -NH$_2$ group of the propargyl-OH or propargyl-NH$_2$ bound reduction intermediate and the imidazole of $\alpha$-195$^{\text{His}}$, thereby localizing the position of the -OH or -NH$_2$ groups to be within ~2 Å of the $\varepsilon$N of $\alpha$-195$^{\text{His}}$. Further, ENDOR spectroscopic characterization (Lee et al., 2004) has indicated that the C=C portion of allyl-OH is bound to a single Fe atom in an $\eta^2$ configuration (Scheme 1). With these constraints, the location of the bound intermediate is largely defined. To further define the likely binding site, theoretical calculations were used. The strategy employed density functional methods to elucidate the detailed geometry of bonding of the intermediate to FeMo-cofactor, and then to test the fit into the $\alpha$-70$^{\text{Ala}}$ protein using force-field methods with all hydrogen atoms explicitly included. The density functional calculations were made on a model, which includes the essential coordination features of FeMo-cofactor, namely Fe$_7$MoS$_9$N$_{\text{cen}}$(SCH$_3$)(OCH$_2$COO)(C$_3$N$_2$H$_4$), with net charge -3 corresponding to the resting state (Dance, 2003). The resulting structures with bound intermediate were then substituted (*in silico*) for FeMo-cofactor in the protein with $\alpha$-70$^{\text{Ala}}$, and relaxed to assess their ability to meet two criteria: (1) hydrogen bonding with $\varepsilon$N of $\alpha$-195$^{\text{His}}$; and (2) accommodation by the $\alpha$-70$^{\text{Ala}}$ protein but not the wild-type $\alpha$-70$^{\text{Val}}$ protein.

From these calculations, the best binding modes for allyl-OH and allyl-NH$_2$ were deduced, where the alkene portion is bound $\eta^2$ at Fe6, in a position that is closer to *exo* than *endo*. For both allyl-OH and allyl-NH$_3^+$ a good H-bond is formed with the $\varepsilon$N of $\alpha$-195$^{\text{His}}$ and with an $\mu$-S (S2B) of FeMo-cofactor (Figure 5). Normal van der Waals
contact occurs between the methyl group of $\alpha$-70$^{\text{Ala}}$ and the bound intermediates, but, as expected, there is impossible conflict with the side-chain of $\alpha$-70$^{\text{Val}}$ in the wild type MoFe protein. Binding in a similar fashion to Fe2, Fe3, and Fe7 was significantly less favorable compared to binding to Fe6, either by not satisfying the need for an H-bond between the $–\text{OH}$ or $–\text{NH}_2$ and the imidazole of $\alpha$-195, or by resulting in van der Waals collisions of the bound adduct with surrounding protein.

These results define in detail the location and geometry of the bound allyl-$\text{OH}$ and allyl-$\text{NH}_3^+$ intermediates in the substituted $\alpha$-70$^{\text{Ala}}$ MoFe protein. Calculations using the same methods and strategy as described above were made for C$_2$H$_2$ and C$_2$H$_4$ as substrate and product without the alanine substitution for the $\alpha$-70$^{\text{Val}}$ residue. These calculations reveal that the same $\eta^2$ coordination geometry of propargyl-$\text{OH}$ and propargyl-$\text{NH}_2$ is possible for C$_2$H$_2$/C$_2$H$_4$ at Fe6 in the wild-type MoFe protein.

**Mechanistic implications**

Experiments described here have established a requirement for the imidazole group of $\alpha$-195$^{\text{His}}$ to elicit a characteristic EPR spectrum under freeze-quench conditions when either propargyl-$\text{OH}$ or propargyl-$\text{NH}_2$ are used as substrates for the $\alpha$-70$^{\text{Ala}}$-substituted MoFe protein, and show that development of these respective spectra are dependent upon and differentiated by the pH of the reaction conditions. These results confine the substrate reduction site for these substrates within hydrogen-bonding distance of the $\varepsilon\text{N}$ of $\alpha$-195$^{\text{His}}$. The binding site was further refined using density functional- and force-field-calculations and consideration of the binding geometry to Fe as indicated by ENDOR experiments. Although the binding of propargyl-$\text{OH}$ and propargyl-$\text{NH}_2$ to Fe6 within FeMo-cofactor is strongly favored by the present work, they do not preclude the possibility that alkynes might also bind to other sites and in different ways. Indeed, there is abundant evidence indicating that there is more than one acetylene-binding site located within the MoFe protein (Christiansen et al., 2000b). It is therefore important to keep in
Figure 5. Proposed structure for the allyl-OH intermediates bound to FeMo-cofactor. The most favorable bound state of allyl-OH to FeMo-cofactor is shown with the C=C portion of allyl-OH bound $\eta^2$ to Fe6 and with H-bonds between the O of allyl-OH and the protonated $\varepsilon$N of $\alpha$-195$^{\text{His}}$ and between the -OH of allyl-OH and S2B of FeMo-cofactor in the $\alpha$-70$^{\text{Ala}}$ MoFe protein.
mind that the trapped species recognized by freeze-quench EPR analyses reported here and elsewhere does not necessarily represent the only binding site that is possible even for propargyl-OH and propargyl-NH$_2$. Rather, it is one binding site that can be observed and defined as a consequence of stabilization of a reduction-intermediate. The important mechanistic implication is that it provides the first experimentally supported evidence for both the location and geometry for binding of any nitrogenase substrate.

Work described here provides no direct experimental information concerning the location and geometry of alkyne binding to FeMo-cofactor in relation to the binding of the physiological substrate N$_2$. Nevertheless, it is striking that a completely independent approach was previously used to gain evidence that the MoFe protein α-195$^{\text{His}}$ residue has an important role in controlling N$_2$ reduction (Dilworth et al., 1998; Kim et al., 1995), probably through the stabilization of a reduction intermediate or as a proton donor during the catalytic process. In our view, this observation is compatible with the current work that shows α-195$^{\text{His}}$ is also required to trap an alkyne reduction intermediate that can be observed by EPR spectroscopy. Although this information can be interpreted in a variety of ways, one reasonable interpretation is that an N$_2$ reduction intermediate binds at the same place and in the same way as determined here for propargyl-OH and propargyl-NH$_2$. In this context there is ample evidence in the chemical literature for metal-hydrazine complexes having the same configuration as the metallo-cyclopropane ring structure proposed for alkyne reduction intermediates. Future work will focus on testing whether or not N$_2$ or its reduction intermediates can be trapped and characterized using the genetic, biochemical and biophysical approaches described for characterizing alkyne reduction-intermediates.