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A SUBSTRATE CHANNEL IN THE NITROGENASE MoFe PROTEIN

Brett M. Barney¹, Michael G. Yurth¹, Patricia C. Dos Santos², Dennis R. Dean³, and Lance C. Seefeldt¹

¹Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322

²Department of Chemistry, Wake Forest University, Winston-Salem, North Carolina 27109

³Department of Biochemistry, Virginia Tech, Blacksburg, Virginia 24061

Abstract

Nitrogenase catalyzes the six electron/six proton reduction of N₂ to two ammonia molecules at a complex organo-metallocluster called FeMo-cofactor. This cofactor is buried within the α -subunit of the MoFe protein, with no obvious access for substrates. Examination of high-resolution X-ray crystal structures of MoFe proteins from several organisms has revealed the existence of a water-filled channel that extends from the solvent exposed surface to a specific face of FeMo-cofactor. This channel could provide a pathway for substrate and product access to the active site. In the present work, we examine this possibility by substituting four different amino acids that line the channel with other residues and analyze the impact of these substitutions on substrate reduction kinetic parameters. Each of the MoFe protein variants was purified and kinetic parameters established for the reduction of the substrates N₂, acetylene, azide and propyne. For each MoFe protein, V_{max} values for the different substrates were found to be nearly unchanged when compared to the wild-type MoFe protein, indicating that electron delivery to the active site is not compromised by the various substitutions. In contrast, the K_m values for these substrates were found to increase significantly (up to 22-fold) in some of the MoFe protein variants compared to the wild-type MoFe protein values. Given that each of the amino acids that were substituted is remote from the active site, these results are consistent with the water filled channel functioning as a substrate channel in the MoFe protein.

Nitrogenase catalyzes the reduction of N₂ to two NH₃ molecules [1] in a reaction having a minimal stoichiometry shown in equation 1.

The Mo-dependent nitrogenase, the most widely studied enzyme, has two component proteins called the Fe protein and the MoFe protein. The Fe protein delivers electrons, one at a time, from its [4Fe-4S] cluster [2] to the MoFe protein in a reaction coupled to the hydrolysis of a minimum of two MgATP molecules per electron transfer [3,4]. The MoFe protein, an $\alpha_2\beta_2$ heterotetramer, contains two unique types of metal clusters [5] called the P-cluster and the FeMo-cofactor (Figure 1). The P-cluster, an [8Fe-7S] cluster, is located between each $\alpha\beta$ subunit interface [6], where it likely functions to accept electrons from the Fe protein and to deliver electrons to the FeMo-cofactor. One FeMo-cofactor, a [7Fe-9S-Mo-X-homocitrate] cluster [7], is localized within each α -subunit and is the site where N₂ and other substrates bind and are reduced [8]. Recent studies [9] have provided evidence that a specific FeS face of

Address correspondence to: Lance C. Seefeldt, 0300 Old Main Hill, Utah State University, Logan, UT 84322. Fax: 435-797-3390; lance.seefeldt@usu.edu. Dennis R. Dean, Fralin Biotechnology Center, Virginia Tech, Blacksburg, VA 24062. Fax: 540-231-7126; deandr@vt.edu.

FeMo-cofactor (composed of Fe atoms 2, 3, 6, and 7 with the numbering from the PDB file 1M1N.pdb) is the site where N₂ and non-physiological substrates (e.g., alkynes) interact with FeMo-cofactor. This FeS face is approached by a valine side chain from the α -subunit amino acid at position 70 (α -70^{Val}), which has been shown to control the size of substrates that can gain access to the FeMo-cofactor active site [10–19].

Outside of the immediate protein environment surrounding FeMo-cofactor, little is known about how substrates gain access to and how products might exit from the active site. X-ray crystal structures of MoFe proteins from three different organisms have been reported [7,20, 21] and from all of these structures it is evident that FeMo-cofactor is buried within the MoFe protein α -subunit with no obvious way for substrates to approach from the bulk solvent. In all of these structures, a pool of water molecules surrounds the *R*-homocitrate portion of FeMo-cofactor [6,7]. Using qualitative molecular modeling, Durrant [22] proposed three potential routes for proton transfer from solvent to FeMo-cofactor. One of these routes involved a water filled channel that was suggested as a possible path for substrate and product movement from the bulk solvent to FeMo-cofactor.

Prompted by our interest in defining substrate interactions with the nitrogenase active site, coupled with a growing body of evidence that indicates well-defined channels within other gas utilizing enzymes [23–29], we examined possible substrate channels in nitrogenase that would lead from bulk solvent to FeMo-cofactor. From inspection of a high resolution X-ray structure of the *A. vinelandii* MoFe protein (1.16 Å) [7] and the channel prediction computer algorithm CAVER [30,31], we identified a possible substrate channel in the MoFe protein that roughly follows the water filled proton channel previously predicted [22]. In the present work, we have probed this putative substrate channel by substituting four different amino acids within the MoFe protein whose side chains line the channel by amino acids with side chains of different size and charge. Each of the MoFe protein variants was purified and kinetic parameters for the reduction of several substrates (protons, N₂, acetylene, azide, hydrazine, and propyne) of different size and charge were determined.

EXPERIMENTAL PROCEDURES

Materials and Protein Purification

All reagents were obtained from Sigma-Aldrich Chemicals (St. Louis, MO) and were used as provided unless otherwise specified. Wild-type and α -70^{Ala} MoFe proteins were obtained from DJ995 or DJ1310 strains of *A. vinelandii*, as already reported [10,15]. Additional amino acid substitutions in the MoFe protein included: α -94^{Ala} to Trp (strain BBA94W), α -100^{Tyr} to Phe (BBY100F), α -104^{Thr} to Ile (BBT104I), α -111^{Thr} to Val (BBT111V), α -111^{Thr} to Phe (BBT111F), the doubly modified α -70^{Val} to Ala with α -94^{Ala} to Trp (DJ1704) and α -94^{Ala} to Trp with α -111^{Thr} to Phe (BBA94W/T111F). These substitutions were constructed by gene rescue of either DJ1192 or DJ1259 using the vector pDB697 (a 1.4 kb *EcoRI* fragment of *nifD* in pUC119) containing specific codon change(s). This resulted in MoFe proteins containing the desired amino acid substitution and a seven-histidine tag near the C-terminus of the α -subunit. The tagged MoFe proteins were purified using a metal affinity chromatography procedure as previously described [32]. All proteins were greater than 95% pure as judged by SDS-PAGE analysis using Coomassie blue staining. All manipulation of proteins was done in septum-sealed serum vials under an argon atmosphere. All transfer of gases and liquids was done using gas-tight syringes.

Molybdenum Quantification

Molybdenum concentrations in MoFe proteins were determined using a colorimetric assay [33]. MoFe protein (13 mg) was placed in a ceramic crucible and dried overnight in a 70°C

oven. The sample was then heated to ash in a muffle furnace at 550°C for 60 min, and cooled to room temperature. Each sample was dissolved in 2 mL of 4 M HCl followed by addition of the following with vortexing between each addition: 200 μ L of reducing solution (300 mg of ascorbic acid and 50 mg of citric acid in 2 mL water), 600 μ L of KI solution (5 grams potassium iodide and 25 mg ascorbic acid in 5 mL water), 100 μ L of thioglycolate solution (50 mg thioglycolic acid in 1 mL water), and 200 μ L of dithiol solution (~100 mg sodium hydroxide with 8 mg toluene dithiol and 8 mg thioglycolic acid in 2 mL water). Once all of the components were added, 1.5 mL of isoamyl alcohol was added and the sample was again mixed. The green-colored complex partitioned into the isoamyl alcohol, which was removed from the aqueous solution and analyzed using a spectrometer at 678 nm. A standard curve was prepared from a Mo stock solution of sodium molybdate dihydrate (50 mg/mL).

Electron Paramagnetic Resonance Spectroscopy

MoFe proteins (75 μ M) under resting conditions were prepared in a solution containing a MgATP regeneration system (10 mM ATP, 15 mM MgCl_2 , 20 mM phosphocreatine, and 0.2 mg/mL phosphocreatine kinase) in 200 mM MOPS buffer, pH 7.0, with 50 mM sodium dithionite. All EPR samples were frozen in 4-mm calibrated quartz EPR tubes. X-band EPR spectra were recorded using a Bruker ESP-300 E spectrometer with an ER 4116 dual-mode X-band cavity equipped with an Oxford Instruments ESR-900 helium flow cryostat. Spectra were obtained at a microwave frequency of 9.65 GHz. Values of the frequency were recorded for each spectrum to determine precise g alignment. Spectra were obtained at a power setting of 1.0 mW, with a modulation frequency of 1.26 mT, and a temperature of 4.5 K as the sum of five scans. Subsequent data analysis was done using IGOR Pro (WaveMetrics, Lake Oswego, OR).

Calculations

Potential cavities and channels in the MoFe protein were examined using the computer program CAVER running as a plug-in module in the program PyMol. The protein data bank (PDB) file 1M1N.pdb was used for all calculations.

Substrate Reduction Assays

Substrate reduction rates were determined in 9 mL sealed vials with 1 mL liquid volume over 10 min at 30 °C as described earlier [15,17]. The assay liquid contained a MgATP regeneration system (5 mM ATP, 6 mM MgCl_2 , 30 mM phosphocreatine, and 0.2 mg/mL creatine phosphokinase), in a 200 mM MOPS buffer, pH 7.0 (unless stated otherwise), with 1.2 mg/mL bovine serum albumin, and 9 mM sodium dithionite. Solutions were degassed under vacuum and refilled with oxygen-free argon. MoFe protein was added (100 μ g) followed by Fe protein (500 μ g) to initiate the reaction. The reaction was quenched by the addition of 300 μ L of a 400 mM EDTA solution.

Dihydrogen was quantified in the gas phase by gas chromatography with a molecular sieve 5A column and a TCD detector.

Acetylene and propyne reduction rates were determined with different partial pressures of acetylene or propyne. Ethylene or propene was quantified by analysis of the gas phase using gas chromatography with a Porapak N column and dinitrogen as the carrier gas and a FID detector.

Dinitrogen reduction was established in assay vials containing different partial pressures of N_2 in argon. The precise partial pressure of N_2 was established for each sample by gas chromatography. Ammonia was quantified by the fluorescence method previously described with a standard curve prepared with NH_4Cl [15,17].

Azide reduction rates were established in assay vials with different quantities of azide added from a stock solution of sodium azide. The pH of the assay solution was monitored following each addition of azide, and was found not to fluctuate significantly. Ammonia was determined as described above for N₂ reduction rates.

Hydrazine was added to assay vials from a stock solution prepared as the hydrate with the pH adjusted to 7.4 using HCl or NaOH. Ammonia was quantified as described above. Since hydrazine results in a small interference with the ammonia assay, a parallel control of samples quenched first with EDTA was used to subtract the background.

Where appropriate, data were fit to the Michaelis-Menten equation using the software package Igor Pro (Wavemetrics, Lake Oswego, OR).

RESULTS

A putative substrate channel

The high resolution (1.16 Å) X-ray crystal structure of the nitrogenase MoFe protein from *A. vinelandii* (1M1N.pdb) [7] contains a number of identifiable water molecules. With the goal of identifying a potential substrate access channel, we examined this structure for chains of water molecules leading from FeMo-cofactor to the bulk solvent. A pool of water molecules surrounds the *R*-homocitrate portion of FeMo-cofactor [7]. This feature is also evident in the structures of the MoFe proteins from *Klebsiella pneumonia* [21] and *Clostridium pasteurianum* [20]. A survey of water molecules that connect the *R*-homocitrate to the protein surface suggested two possible pathways that both start at *R*-homocitrate, diverge from one another and then converge again to follow a common path to the surface.

In a complementary approach, we also used the computer program CAVER [30,31] running as a plug-in module in the program PyMol [34] to identify possible channels that lead from the protein surface to FeMo-cofactor. This program uses an analysis of molecular dynamic trajectories to identify paths leading from buried protein clefts. For the *A. vinelandii* MoFe protein structure, a number of trials were conducted starting with FeMo-cofactor as the origin and varying the grid resolution. In all cases, the program identified the same paths leading from the *R*-homocitrate to the bulk solvent at the surface that were identified by visual inspection.

The potential substrate channel (Figure 1A) consists of a ~30Å chain of water molecules that initially follows the αβ subunit interface, extends into the α-subunit and ends at the pool of water molecules that surround *R*-homocitrate (Figure 1B). This putative channel leads to an Fe-S face of FeMo-cofactor that includes Fe atoms 2, 3, 6, and 7, which has been proposed to provide the substrate-binding site and is capped by the side chain of the α-70^{Val} residue [35]. Substitution of this residue by α-70^{Ile}, having a side chain with a larger van der Waals radius, lowers the reduction rates of all substrates except protons [36]. Conversely, substitution of α-70^{Val} by either α-70^{Ala} or α-70^{Gly}, having side chains with smaller van der Waals radii, results in MoFe proteins that can reduce certain larger alkynes (e.g., propyne and butyne) at rates significantly higher than can be catalyzed by the wild-type MoFe protein [35].

Probing the predicted substrate channel by amino acid substitution

As a way to probe the possible role of the water filled channel in providing substrate access to the active site FeMo-cofactor, several amino acids whose side chains line the putative pathway were selected for substitution by amino acids having side chains of different size and polarity. The substituted MoFe proteins were purified and then examined for their capacity to reduce a range of substrates having varying size and charge. Because certain of the substituted MoFe proteins were found to have a lower content of FeMo-cofactor, as indicated by a lower total

Mo content and a lower intensity of the $S = 3/2$ EPR signal associated with FeMo-cofactor, kinetic properties for all proteins were normalized to the Mo concentration.

Four amino acid residues that define different portions of the proposed channel were selected for substitution (Figure 1). These include α -94^{Ala}, α -100^{Tyr}, α -104^{Thr}, and α -111^{Thr}. The α -100^{Tyr} residue was substituted by α -100^{Phe} in order to modify the polarity of the channel at this location. The α -94^{Ala} residue was substituted by α -94^{Trp} in an attempt to impede substrate access through the channel. The small polar threonine at positions α -111^{Thr} and α -104^{Thr} were substituted by non-polar valine, isoleucine, and phenylalanine in an attempt to block the channel at these sites.

Each of the variant proteins was expressed in *A. vinelandii* and purified to near homogeneity (>95% as judged on SDS-gels). The FeMo-cofactor content in each protein was determined by two different methods: total Mo content and from the intensity of the $S = 3/2$ EPR signal arising from the resting state of FeMo-cofactor. As can be seen from the results in Table 1, the singly substituted MoFe proteins retained greater than 91% of the FeMo-cofactor content of the wild-type MoFe protein, with good agreement between the independent Mo determination methods.

Next, each protein was examined for its ability to reduce substrates of increasing overall van der Waals radius and with different charge. The substrates used in this study are shown in Figure 2, with van der Waals surface representations shown to indicate their relative sizes. For individual substrates, the reduction rate was determined at different substrate concentrations and the results were fit to the Michaelis-Menten equation to determine the V_{max} and K_m values (Table 2). Two of the substituted MoFe proteins (α -100^{Phe} and α -111^{Val}) showed very little change in substrate reduction kinetic parameters and were not examined further.

An important control reaction to ascertain the integrity of the MoFe protein variants is the rate of proton reduction. This is the default reaction catalyzed by the enzyme in the absence of any other substrate. Protons are the smallest possible substrate for nitrogenase and are likely to gain access to the active site by multiple paths [22]. As can be seen from the results in Table 2, the three MoFe protein variants examined here (α -94^{Trp}, α -104^{Ile}, and α -111^{Phe}) retained significant proton reduction rates when compared to the wild-type MoFe protein, verifying that the amino acid substitutions had not altered the catalytic function of nitrogenase. Next, the ability of the MoFe proteins to reduce the physiological substrate N_2 and the non-physiological substrate acetylene was examined. The V_{max} for reduction of these substrates by each of the MoFe protein variants was similar (varying from no change to decreasing by 17%) to that observed for the wild-type protein. The K_m values for the α -104^{Ile} and α -111^{Phe} MoFe proteins were similar to those found for the wild-type MoFe protein. In contrast, the K_m for the reduction of N_2 and acetylene were found to increase significantly in the α -94^{Trp} MoFe protein (> 2-fold increase).

Azide is unique among known nitrogenase substrates because it is charged [1]. It is reduced by nitrogenase to ammonia and N_2 , with the possibility of N_2 being further reduced to ammonia. The size of azide is larger than N_2 or acetylene (Figure 2). The α -94^{Trp}, α -104^{Ile}, and α -111^{Phe} MoFe protein variants all maintained normal V_{max} values for azide reduction when compared to wild-type MoFe protein (100% or greater), however the K_m values increased by greater than 3-fold (Table 2).

Two residues along the proposed channel were substituted simultaneously in the same protein to maximize the effect on substrate access. The doubly substituted α -94^{Trp}/ α -111^{Phe} MoFe protein variant was purified and showed a proton reduction specific activity that was 75% of the wild-type when the activity was adjusted for FeMo-cofactor content (Table 2). The V_{max} for N_2 , acetylene, and azide reduction were near wild-type levels (86 to 100%). In contrast,

the K_m values increased by 3-fold for N_2 , 9.5-fold for acetylene, and 22-fold for azide (Table 2 and Figure 3).

The larger substrates hydrazine and propyne (Figure 2) were also used to probe the proposed substrate channel. These compounds are poor substrates for the wild-type MoFe protein [15, 19]. However, when α -70^{Val}, located in the first shell around FeMo-cofactor, is substituted by alanine, these two compounds can gain access to FeMo-cofactor and are reduced at considerable rates (Table 3). Starting from this α -70^{Ala} MoFe protein variant, α -94^{Ala} was substituted by tryptophan creating a doubly substituted MoFe protein variant (α -94^{Trp}/ α -70^{Ala}). This doubly substituted MoFe protein maintained full wild-type proton reduction activity when activity was adjusted for the FeMo-cofactor content of the protein. The V_{max} for hydrazine and propyne reduction activities were 70% of the α -70^{Ala} MoFe protein. The K_m value was 4.8-fold higher for hydrazine and 2.6-fold higher for propyne when compared to the α -70^{Ala} MoFe protein.

DISCUSSION

There is growing evidence that gas utilizing enzymes often contain specific channels for movement of a gaseous substrate or product into and away from buried active sites [24–29, 37,38]. For some of these enzymes, the channel connects one active site with another in the same protein allowing the product from one site to move as a substrate to the other site. This is the case for the carbon monoxide dehydrogenase/acetyl-CoA synthase, where CO, the product of CO_2 reduction at one active site, traverses an internal tunnel that runs the length of the protein (138 Å) to the acetyl-CoA synthesizing active site, where the CO is a substrate [26–28,38,39]. In other gas utilizing enzymes, cavities or channels connect buried active sites with the bulk solvent, providing a pathway for entrance of the substrate (e.g., H_2 , O_2) or exit of the product [23–25,37]. In the case of the H_2 oxidizing hydrogenase, Montet et al. [25] provide evidence for a hydrophobic channel that connects the Ni-Fe active site with the molecular surface. Further, it is suggested that the channel might function as a reservoir of H_2 ready for delivery to the active site.

The work presented here provides experimental evidence for a specific channel in nitrogenase that controls substrate passage from the solvent to the active site. Substituting amino acids that line the putative channel by amino acids that have larger and/or more hydrophobic side chains resulted in significant increases in K_m values for a number of nitrogenase substrates, with retention of near normal V_{max} values. These results are consistent with the amino acid substitutions altering the access of substrates to the active site, and thereby support the proposal that this channel functions as a substrate channel.

Several aspects of the present work merit further comment. The amino acids that were selected for substitution in the present study were not located near FeMo-cofactor. This is important because previous amino acid substitution studies of the MoFe protein that regulate substrate interactions were of residues located in the first shell of non-covalent side chains that interact with FeMo-cofactor [19]. Changes to amino acids in other critical locations in the MoFe protein, such as near the P-cluster [40] or at the docking interface [41], result in a general decrease in the overall activity of the enzyme either by disrupting electron transfer to FeMo-cofactor or association of the Fe protein. This general decrease in catalytic activity is clearly not occurring with the substitutions made in the present work since the catalytic function of the MoFe protein variants is near normal for proton reduction rates and largely unchanged for V_{max} values for larger substrates.

The significant increases observed here for K_m values in the channel substitution MoFe protein variants are consistent with the proposed channel functioning in substrate access. A general

trend that is observed is that the substrates having the largest van der Waals radius show the greatest increases in K_m . Further, the polarity of the substrate also appears to be significant. Azide, being an anionic substrate, showed a greater increase in K_m than was found for acetylene. Azide has an intermediate van der Waals radius among the substrates in Table 2 (dinitrogen, acetylene and azide), yet it showed the greatest increase in K_m . Azide, being charged, would favorably interact with the polar water filled channel and thus may use this channel exclusively.

Another significant observation is that the amino acid substitutions examined here do not completely eliminate substrate reduction for any substrate. One possible explanation for this could be that the MoFe protein has changed local conformation near the amino acid substitution to minimize the impact on the substrate channel. Another possibility is that the substitutions do not completely block the channel. This latter possibility is consistent with the observation that substituting two amino acids at the same time results in greater changes in the K_m for substrates, indicating an additive effect of multiple modifications to the channel. Some of the amino acid substitutions made here had no effect on substrate reduction (α -100^{Phe} and α -111^{Val}). The different side chains in these MoFe protein variants must be accommodated in such a way as to not restrict substrate access.

Finally, it is possible that there are additional substrate channels within the MoFe protein. The MoFe protein does not appear to contain any additional water filled paths from the ones identified here, but a few non-water filled (hydrophobic) paths have been identified by cavity searches using the CAVER algorithm. These other paths may provide channels for substrates to gain access to FeMo-cofactor, especially non-polar substrates. It is evident from these studies and others [22] that there must be multiple pathways for protons to gain access to FeMo-cofactor from the bulk solvent.

There is now compelling evidence that the MoFe protein restricts access of substrates to FeMo-cofactor [19]. The side chain of the α -70^{Val} residue appears to play a role in controlling substrate access. It is possible to systematically control the size of substrates that can be reduced by substituting at this position by amino acids with larger or smaller side chains [19]. Such control appears to function over a substrate size range that goes from acetylene to butynes. The work presented here indicates that a specific channel in the MoFe protein further functions to constrain access of small molecules to the active site FeMo-cofactor. Given that the FeMo-cofactor catalyzes the reduction of the N₂ triple bond, one of the most energetically demanding reactions in biology, it seems likely that if other molecules could gain access to the functioning active site (as some small non-physiological substrates do), there is sufficient reducing potential to reduce any number of compounds. From this perspective, it makes sense that nitrogenase would contain a specific substrate channel, along with gate keeper amino acids near FeMo-cofactor, like α -70^{Val}, that controls access and binding of substrates to the active site FeMo-cofactor.

Taken together, the results presented here support the identified water filled channel in the MoFe protein as functioning as at least one substrate access channel. This provides the first experimental evidence for a specific substrate channel in nitrogenase.

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Figure 1. Nitrogenase and a putative substrate channel

(panel A) Shown is the complete nitrogenase complex with MoFe protein α -subunits in cyan, β -subunits in magenta, and Fe protein subunits in green. Also shown are MgATP, the [4Fe-4S] cluster, the P-clusters and FeMo-cofactors. The putative substrate channel is shown as a tan tube leading from the surface of the MoFe protein to FeMo-cofactor at the interface between the α and β subunits. Coordinates taken from PDB file 1G21.PDB. (panel B). Close-up view of FeMo-factor with side chains of α -70^{Val}, α -94^{Ala}, α -111^{Thr}, α -100^{Tyr}, and α -104^{Thr} shown. Water molecules that define the putative substrate channel are shown as blue spheres. Colors used are carbon in black, oxygen in red, iron in green, sulfur in yellow, molybdenum in magenta, and X in orange. Coordinates are from the PDF file 1M1N.pdb with graphics done in PyMol.

$\text{N}\equiv\text{N}$
Dinitrogen

$\text{HC}\equiv\text{CH}$
Acetylene

$\text{N}=\text{N}=\text{N}^-$
Azide

$\text{H}_2\text{N}-\text{NH}_2$
Hydrazine

$\text{HC}\equiv\text{C}-\text{CH}_3$
Propyne

Figure 2. Substrates used

The substrates used in this study are shown along with van der Waals surface representations with nitrogen in blue, carbon in gray and hydrogen in white.

Figure 3. Azide reduction by MoFe protein

Plotted is the specific activity of ammonia production (nmoles ammonia/min/mg MoFe protein) against the azide concentration for the wild-type ($\blacktriangle$) and α -94^{Trp}/ α -111^{Phe} ($\diamond$) MoFe proteins. Data were fit to the Michaelis-Menten equation (solid line). See the **Materials and Methods** section for details.

Table 1

FeMo-cofactor content of MoFe proteins.

MoFe Protein	Molybdenum Content (% WT) ^a	FeMo-cofactor EPR Signal Intensity ^b (% WT)
Wild-type (α -70 ^{Val} , α -94 ^{Ala} , α -104 ^{Thr} , α -111 ^{Thr})	100	100
α -70 ^{Ala}	ND ^c	100
α -94 ^{Trp}	100	98
α -104 ^{Ile}	96	91
α -111 ^{Phe}	91	93
α -70 ^{Ala} / α -94 ^{Trp}	78	65
α -94 ^{Trp} / α -111 ^{Phe}	41	32

^a Mo content as a percentage of the wild-type (WT) MoFe protein as determined from a single measurement.^b EPR signal intensity as a percentage of the signal intensity from the wild-type MoFe protein. See **Materials and Methods** for details.^c ND; Not determined.

Table 2

Kinetic parameters for MoFe proteins.

	Substrate						
	Protons		Dinitrogen		Acetylene		Azide
	$V_{\max}$ (nmol H_2 $\text{min}^{-1} \text{mg}^{-1} a$)	K_m (atm)	$V_{\max}$ (nmol NH_3 $\text{min}^{-1} \text{mg}^{-1} a$)	K_m (atm)	$V_{\max}$ (nmol ethylene min^{-1} $\text{mg}^{-1} a$)	K_m (mM)	$V_{\max}$ (nmol NH_3 $\text{min}^{-1} \text{mg}^{-1} a$)
Wild Type (α -94 ^{Ala} , α -104 ^{Thr} , α -111 ^{Thr})	2400 $\pm$ 50	0.18 $\pm$ 0.02	650 $\pm$ 25	0.009 $\pm$ 0.001	2090 $\pm$ 63	0.93 $\pm$ 0.09	600 $\pm$ 11
α -94 ^{Trp}	2400 $\pm$ 70	0.36 $\pm$ 0.02	650 $\pm$ 20	0.020 $\pm$ 0.002	1950 $\pm$ 67	3.35 $\pm$ 0.18	650 $\pm$ 12
α -104 ^{Ile}	1900 $\pm$ 40	0.21 $\pm$ 0.02	560 $\pm$ 19	0.012 $\pm$ 0.001	1710 $\pm$ 30	3.10 $\pm$ 0.37	660 $\pm$ 25
α -111 ^{Phe}	2400 $\pm$ 50	0.25 $\pm$ 0.01	590 $\pm$ 6	0.009 $\pm$ 0.001	1530 $\pm$ 41	2.85 $\pm$ 0.21	600 $\pm$ 14
α -94 ^{Trp} / α -111 ^{Phe}	1800 $\pm$ 120	0.59 $\pm$ 0.02	560 $\pm$ 9	0.082 $\pm$ 0.004	1860 $\pm$ 45	20.5 $\pm$ 3.0	610 $\pm$ 53

^a $V_{\max}$ values are corrected to per mg of MoFe protein that contains FeMo-cofactor based on EPR and Mo quantity as described in the **Materials and Methods** section

Table 3

Larger substrates for MoFe proteins.

	Substrate			
	Protons	Hydrazine	Propyne	
	$V_{\max}$ (nmol H_2 min ⁻¹ mg ⁻¹) ^a	K_m (mM)	$V_{\max}$ (nmol NH_3 min ⁻¹ mg ⁻¹) ^a	K_m (atm) $V_{\max}$ (nmol propene min ⁻¹ mg ⁻¹) ^a
α -70 ^{Ala}	2300 ± 50	11.4 ± 0.84	1260 ± 30	0.027 ± 0.003 2600 ± 100
α -70 ^{Alu} / α -94 ^{Trp} (% α -70 ^{Ala})	2400 ± 50	55 ± 13	1130 ± 140	0.072 ± 0.006 2400 ± 100

^a $V_{\max}$ values have been corrected to per mg of MoFe protein that contains FeMo-cofactor based on EPR and Mo quantity as described in the **Materials and Methods** section.