Stereospecificity of Acetylene Reduction Catalyzed by Nitrogenase

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Abstract: In addition to catalyzing the reduction of dinitrogen to ammonia, the metalloenzyme nitrogenase catalyzes the reduction of a number of alternative substrates, including acetylene (C_2H_2) to ethylene (C_2H_4) and, in certain cases, to ethane (C_2H_6). The stereochemistry of proton addition for C_2D_2 reduction to $C_2D_2H_2$ catalyzed by the Mo-dependent nitrogenase has been used to probe substrate binding and proton addition mechanisms. In the present work, the C_2D_2 reduction stereospecificity of altered MoFe proteins having amino acid substitutions within the active site FeMo-cofactor environment was examined by Fourier transform infrared (FTIR) spectroscopy. Altered MoFe proteins examined included those having the α-subunit 96^{Arg} residue substituted by Gln, Leu, or Ala, the α-subunit 69^{Gly} residue substituted by Ser, and the α-subunit 195^{His} residue substituted by Asn. The stereochemistry of proton addition to C_2D_2 does not correlate with the measured K_m values for C_2H_2 reduction, or with the ability of the enzyme to reduce C_2H_2 by four electrons to yield C_2H_6. Instead, the electron flux through nitrogenase was observed to significantly influence the ratio of *cis*- to *trans*-1,2-$C_2H_2D_2$ formed. Finally, the product distribution observed for reduction of C_2H_2 in D_2O is not consistent with an earlier proposed enzyme-bound intermediate. An alternative model that accounts for the stereochemistry of C_2H_2 reduction by nitrogenase based on a branched reaction pathway and an enzyme-bound η^2-vinyl intermediate is proposed.

Biological nitrogen fixation is catalyzed by nitrogenase, a two-component metalloenzyme responsible for the MgATP-dependent reduction of dinitrogen to ammonia.[1−3] The Mo-dependent nitrogenase is composed of an Fe protein component, which possesses the site of MgATP hydrolysis and is the electron donor to the MoFe protein component. Substrate binding and reduction occurs within the MoFe protein at a metallocluster site called FeMo-cofactor (7Fe:9S:Mo:homocitrate).[4] In addition to its physiological substrate dinitrogen (N_2), nitrogenase also catalyzes the reduction of a variety of other multiply bonded substrates,[5,6] the best known being acetylene (C_2H_2),[7] which is usually reduced by two electrons to yield ethylene (C_2H_4). Insights into the catalytic mechanism of nitrogenase have come from analysis of the stereochemistry of protonation of C_2D_2 upon its reduction.[8−11] This feature can be observed by monitoring the stereoisomers of $C_2D_2H_2$ formed when C_2D_2 is used as the substrate and reduction is catalyzed in the presence of protons. For wild-type nitrogenase, the reductive addition of protons to C_2D_2 occurs via a highly stereospecific mechanism as revealed by the production of ∼96% of the product as the cis isomer of dideuterated ethylene (*cis*-1,2-$C_2H_2D_2$), with the remaining ∼ 4% being the trans isomer.[8−12] In addition, little or no mono- or trideuterated ethylene products are detected. The stereospecificity of protonation of C_2D_2 can be perturbed in certain altered MoFe proteins that have amino acid substitutions within the first shell of noncovalent interactions with the active site FeMo-cofactor.[12] Further, in addition to C_2H_4, some of these altered proteins reduce C_2H_2 by four electrons to yield C_2H_6.[13] Thus, analysis of these substituted proteins provides a way to elucidate further details of the C_2H_2 reduction mechanism of nitrogenase.

Recently, three such altered MoFe proteins, one having the α-subunit 191^{Gln} residue substituted by Lys (α-191^{Lys}) and the others having the α-subunit 195^{His} residue substituted by either Gln(α-195^{Gln}) or Asn (α-195^{Asn}), were examined for their stereospecific protonation of C_2D_2 and production of C_2H_6.[12] From those studies it was concluded that loss of *cis* proton addition to C_2D_2 is correlated with a change in the K_m for C_2H_2 reduction and with the production of C_2H_6. A model was proposed where an increase in K_m results in a longer active site residence time of a common ethylenic intermediate, leading to the further reduction of C_2H_2 to yield C_2H_6 and the loss in stereospecificity in protonation of C_2D_2 to yield a higher fraction of *trans*-1,2-$C_2D_2H_2$.[12] In the present work the stereospecificity of C_2D_2 reduction catalyzed by other altered MoFe proteins was

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Table 1. Acetylene Reduction Properties for Wild-Type and Selected Altered MoFe Proteins

MoFe protein	1,2-$C_2H_2D_2$[a] cis (%)	1,2-$C_2H_2D_2$[a] trans (%)	C_2H_6 production[b] (%)	K_m[c] (atm)	V_{max}[c] (nmol/min/mg)[d]
wild type	96	4	0	0.006 ± 0.001	2020 ± 118
α−96^{Gln}	87	13	0	0.008 ± 0.001	1540 ± 17
α−96^{Leu}	86	14	0	0.002 ± 0.0002	303 ± 15
α−96^{Ala}	88	12	0	0.005 ± 0.0004	1710 ± 51
α−69^{Ser}	95	5	0	0.14 ± 0.01[e]	1800 ± 50[e]
α−195^{Asn}	67	33	19	0.007 ± 0.001	110 ± 2
α−69^{Ser}/α195^{Asn}	65	35	21	0.17 ± 0.08	270 ± 20

[a] Determined using a 20:1 molar ratio of Fe protein to MoFe protein. [b] Value quoted as a percent of total hydrocarbon production. Zero indicates the lower level of detection, which is <0.1%. [c] Determined using a 40:1 molar ratio of Fe protein to MoFe protein. [d] Per milligram of MoFe protein. [e] Taken from ref 22.

examined. On the basis of these results a different model is proposed.

Experimental Procedures

Nitrogenase Proteins. Wild-type Fe and MoFe proteins were expressed in *Azotobacter vinelandii* cells[14] and purified to homogeneity as previously described.[15] The specific activity for C_2H_2 reduction catalyzed by wild-type nitrogenase was >2000 nmol of product/min/mg of protein. Strains that produce altered MoFe proteins were constructed as previously described for other mutant strains of *A. vinelandii*.[16] MoFe proteins were purified by an immobilized Zn affinity liquid chromatography method using a step gradient of 250 mM imidazole for elution.[17] Protein concentrations were determined by a modified biuret method[18] with bovine serum albumin as the standard. Proteins were judged to be homogeneous on the basis of SDS-PAGE[19] with coomassie blue staining. All protein manipulations were conducted in the absence of O_2 in septum sealed vials under an argon atmosphere. Liquid and gas transfers were accomplished with gastight syringes.

C_2H_2 Reduction Assays. C_2H_2 reduction rates for the wild-type and altered MoFe proteins were measured at 30 °C with each assay containing 0.05 mg of MoFe protein together with a 40-fold molar excess of wild-type Fe protein. Each assay vial contained 20 μmol of phosphocreatine, 60 μmol of MOPS buffer, pH 7.0, 10 μmol of sodium dithionite, 6 μmol of $MgCl_2$, 3 μmol of ATP, 0.185 mg of creatine phosphokinase, and 1.2 mg of bovine serum albumin in a total volume of 1.0 mL. C_2H_2 was added as an overpressure to each assay vial containing 1.0 atm of argon, which was then vented to atmospheric pressure. The assays were initiated by addition of the wild-type Fe protein and were incubated for 8 min with gentle shaking in a water bath at 30 °C. All reactions were terminated by the addition of 250 μL of a 0.4 M EDTA, pH 7.5, solution. The production of C_2H_4 and C_2H_6 was quantified by gas chromatography on a Shimadzu GC-8A gas chromatograph with a flame ionization detector fitted with a 30 cm × 0.3 cm Porapak N column with nitrogen as the carrier gas.

Kinetic Parameters for Acetylene Reduction. To determine the kinetic parameters for C_2H_2 reduction, assays were performed as described above except the partial pressure of C_2H_2 introduced into a particular assay vial was varied. Each assay series was independently run three times with individual data points being measured in triplicate. The final quoted values for the K_m and V_{max} (Table 1) were calculated by averaging the results obtained from triplicate assays. The kinetic constants were determined by fitting the rate versus concentration data to the Michaelis−Menten equation with use of the computer program Igor Pro (Wavemetrics, Lake Oswego, OR).

Quantification of Deuterated Ethylenes by Fourier Transform Infrared (FTIR)[1] Spectroscopy. The generation of the gaseous substrate C_2D_2 was accomplished by adding an appropriate volume of D_2O to a specific quantity of CaC_2 as previously described.[12] Eighty milligrams of CaC_2 was transferred to a 25 mL vial that was subsequently capped and evacuated. D_2O (0.6 mL) was slowly added to the sample and C_2D_2 evolution was allowed to proceed for 1 h. A 5 mL aliquot of this gas was then transferred by gastight syringe to an assay flask under 1.0 atm of argon. Each 120 mL assay vial contained 200 μmol of phosphocreatine, 600 μmol of MOPS buffer, pH 7.0, 100 μmol of sodium dithionite, 60 μmol of $MgCl_2$, 30 μmol of ATP, 1.85 mg of creatine phosphokinase, and 12 mg of bovine serum albumin in a liquid volume of 10 mL. MoFe protein (5 mg) was then added and the reaction initiated by the addition of wild-type Fe protein. The assays were incubated with shaking in a water bath at 30 °C with the reaction being allowed to proceed for 15 min before termination by addition of 2.5 mL of a 0.4 M EDTA, pH 7.5, solution. The assay flask was then connected to an evacuated 100 mL volume infrared gas cell (10 cm path length) via a gastight needle and the gaseous contents equilibrated. The gaseous products were analyzed by FTIR spectroscopy by using a Mattson Galaxy Series 5000 spectrometer (Madison, WI) in conjunction with WinFirst software. All spectra were the average of 16 scans with a 1 cm^{-1} resolution. The ratio of *cis*- and *trans*-1,2-$C_2D_2H_2$ produced was established by measuring the heights of the absorption maxima in the infrared spectrum for each of the isomers. The vibrational modes monitored in the infrared spectrum for each ethylenic species were υ_7 for nondeuterated ethylene at 949 cm^{-1}, υ_8 for C_2H_3D at 943 cm^{-1}, υ_7 for *cis*-1,2-$C_2H_2D_2$ at 843 cm^{-1}, υ_4 for *trans*-1,2-$C_2H_2D_2$ at 988 cm^{-1}, and υ_8 for C_2HD_3 at 918 cm^{-1}.[8,20] Concentrations were determined from the peak height of the noted absorption band. The molar absorptivities for each species were taken as equal, with the exception of that for the *trans*-1,2-$C_2H_2D_2$ isomer, which has been reported[8] to be about half of the other values.

To assess how varying flux affects the relative formation of *cis*- and *trans*-1,2-$C_2D_2H_2$ catalyzed by wild-type and altered MoFe proteins, the procedure described above was repeated with Fe protein-to-MoFe protein molar ratios in the range between 20:1 and 1:10. This experiment was done in two different ways. In one approach, the concentration of MoFe protein was held constant and the Fe protein concentration was varied. In the second approach, the total sum of the protein concentration was held constant while the relative amounts of Fe protein and MoFe protein varied to achieve the desired molar ratio. The actual Fe protein-to-MoFe protein ratios are listed in Table 1 and the figure legends.

C_2H_2 reduction assays were also performed as described above except that either C_2H_2 or C_2D_2 was used in a reaction solution of >90% D_2O. Product distribution was determined by FTIR analysis of the gas phase as described above.

Results

Acetylene Reduction by Altered MoFe Proteins. Substitution of certain amino acids which provide the first shell of noncovalent interactions with FeMo-cofactor is known to affect

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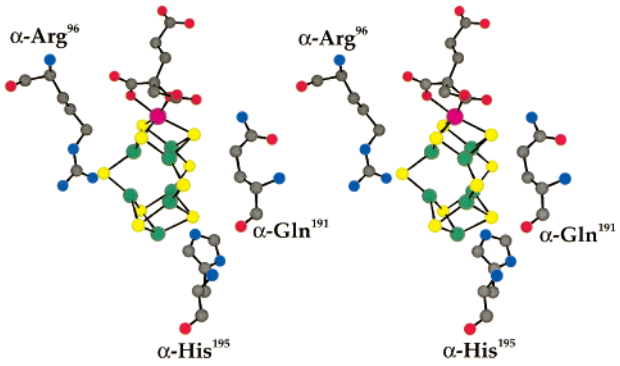

Figure 1. FeMo-cofactor protein environment. Wall-eyed stereoview of the FeMo-cofactor and α-Arg[96], α-Gln[191], and α-His[195]. The figure was generated from the MoFe protein coordinates[34] by using the programs MOLSCRIPT[35] and RASTER 3D.[36]

the reactivity of nitrogenase toward various substrates and inhibitors.[21] Figure 1 is a stereoview illustrating the FeMo-cofactor structure and the amino acids examined here. The effect of substitution of these residues on the stereochemical mechanism of proton addition during nitrogenase reduction of C_2D_2 was examined. Our rationale for these experiments was to test the hypothesis that the [4Fe-4S] face of FeMo-cofactor approached by the α-subunit 96^{Arg} residue provides the initial substrate-binding site.[21−23] It was expected that, if this face provides the substrate-binding site, substitution of amino acids approaching this face could alter steric constraints that might be manifested in a loss of stereospecificity for proton addition during C_2D_2 reduction. A list of altered MoFe proteins examined and the affects of amino acid substitutions on kinetic parameters and stereospecificity are shown in Table 1. The K_m values for C_2H_2 reduction for altered MoFe proteins range from values near the wild-type K_m of 0.0064 atm to 3-fold lower (α-96^{Leu}) and 27-fold higher (α-69^{Ser}/α-195^{Asn}) than wild type. The V_{max} values ranged from those nearly the same as the wild type (2020 nmol/min/mg) to values as low as 5% of the wild type (110 nmol/min/mg for the α-195^{Asn}-substituted protein). The α-195^{Asn}-substituted MoFe protein has been shown to contain less than the full complement of FeMo-cofactor,[12] so a true quantitative value for V_{max} is difficult to determine.

As previously reported,[7,8,10−12] the wild-type nitrogenase under high electron flux conditions is highly specific for catalyzing the reductive addition of two protons to C_2D_2, yielding ∼96% of *cis*-1,2-$C_2H_2D_2$, with the remaining ∼4% being the trans isomer. The α-69^{Ser}-substituted MoFe protein catalyzed formation of a similar product distribution of dideuterated ethylene isomers as observed for the wild-type MoFe protein. In contrast, the α-96^{Leu}-, α-96^{Gln}-, α-96^{Ala}-, α-195^{Asn}-, and α-69^{Ser}/α-195^{Asn}-substituted MoFe proteins catalyzed formation of significantly higher percentages of the trans isomer of dideuterated ethylene, ranging from 12 to 35%. The ethylene product distribution for the α-195^{Asn}-substituted MoFe protein (33% trans isomer) is similar to the value reported previously.[12] Of the altered MoFe proteins examined in the present study, only MoFe proteins having the α-195^{Asn} substitution produced detectable levels of C_2H_6 (Table 1).

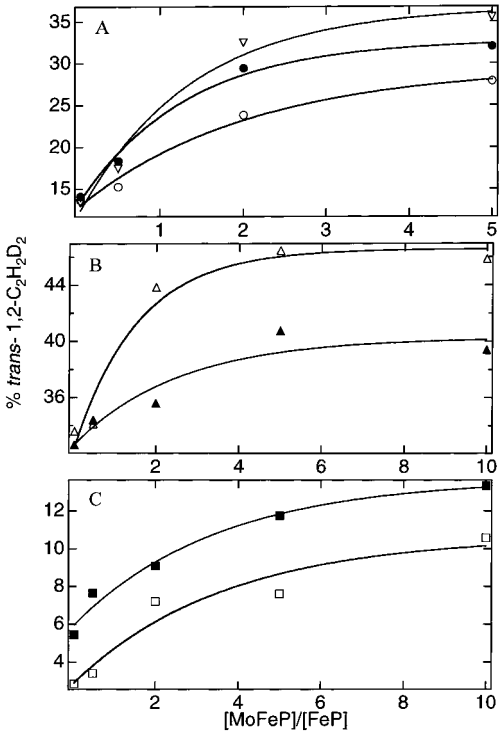

Figure 2. The effect of decreasing electron flux on the production of *trans*-1,2-$C_2H_2D_2$ for wild type and altered MoFe proteins. Assay conditions and ethylene stereoisomer quantification were conducted as described in the Experimental Procedures. The percentage of *trans*-1,2-$C_2H_2D_2$ relative to the total ethylene produced is plotted against the MoFe protein to Fe protein ratio. Panel A: MoFe proteins with α-96^{Ala} (▽), α- 96^{Gln} (●), and α- 96^{Leu} (○). Panel B: MoFe proteins with α-195^{Asn}/α-69^{Ser} (△) and α-195^{Asn} (▲). Panel C: Wild-type MoFe protein (□) and MoFe protein with α-69^{Ser} (■).

Stereospecificity of Acetylene Reduction Is Flux Dependent. For the nitrogenase system, flux is defined as the overall rate at which sufficient electrons are accumulated within the MoFe protein to achieve substrate reduction. Adjustment of electron flux through nitrogenase is known to alter the pattern of nitrogenase substrate specificity.[1] For example, under a nitrogen gas atmosphere, as the electron flux decreases, the percentage of electrons going to H^+ reduction increases while the percentage going to N_2 reduction decreases.[24] One way to regulate electron flux in vitro is by adjusting the ratio of Fe protein to MoFe protein. At a high molar ratio of Fe protein to MoFe protein (20:1), the electron flux is high, while at a low ratio (1:5), the electron flux is low. The effect of varying the MoFe protein to Fe protein ratio (i.e., electron flux) on the stereospecificity of proton addition during C_2D_2 reduction is shown in Figure 2. For all proteins examined, it was found that, as the electron flux decreased, the percentage of *trans*-1,2-$C_2H_2D_2$ formed significantly increased. For the wild-type nitrogenase (panel C), the percentage of trans isomer more than doubled, increasing from 4% to 11% as the flux was decreased. For the α-96-substituted proteins, the percentage of trans isomer increased from ∼14% to between 27 and 36%. For the α-195^{Asn} MoFe protein, the percentage increased from 33% to 40%. For the doubly substituted α-195^{Asn}/α-69^{Ser} MoFe protein, the percentage of *trans*-1,2-$C_2H_2D_2$ formed increased from 35% to a value approaching 50% as electron flux was decreased. In the above experiments, the MoFe protein concentration was held constant (5 mg in 10 mL) and the Fe protein concentration was

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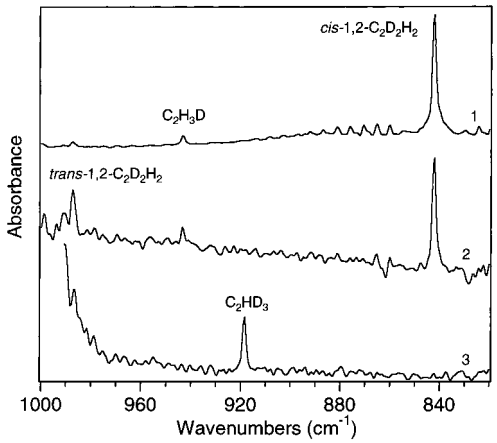


Figure 3. FTIR spectra of the gas phases for wild-type and α-195^{Asn} reductions of C_2H_2 or C_2D_2 in D_2O with some H_2O. Assay conditions and FTIR parameters were as described in the Experimental Procedures. All reactions were conducted with an Fe protein to MoFe protein molar ratio of 20:1 at 30 °C. Trace 1: Wild-type MoFe protein was allowed to react with C_2H_2 in a reaction solution of D_2O with some H_2O for 15 min. The spectrum was scaled by a factor of 0.05 for comparison to the other spectra. Trace 2: α-195^{Asn} MoFe protein was allowed to react with C_2H_2 in a reaction solution containing D_2O with some H_2O for 30 min. Trace 3: α-195^{Asn} MoFe protein was allowed to react with C_2D_2 in a reaction solution containing D_2O with some H_2O for 15 min.

varied to achieve the desired molar ratio. To rule out possible protein concentration effects, the above experiment was repeated except that the Fe protein and MoFe protein concentrations were varied to achieve the desired molar ratio while maintaining the same total protein concentration (10 mg in 10 mL). Essentially the same ratio of *cis*- and *trans*-1,2-$C_2H_2D_2$ were obtained by both experimental approaches.

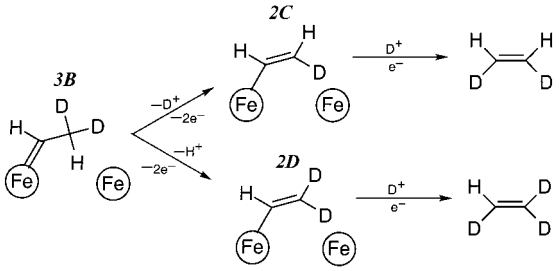
We also examined whether the K_m for C_2H_2 reduction changed as a function of electron flux under our experimental conditions. For these experiments, a series of assay vials were prepared with a fixed molar ratio of Fe protein and MoFe protein and the concentration of C_2H_2 was varied. A fit of the C_2H_2 reduction rate versus initial substrate concentration to the Michaelis−Menten equation provided the K_m values at a particular electron flux. It was observed that the K_m for C_2H_2 did not change with changing electron flux. However, when the above experiment was repeated, with the total protein concentration held constant, the K_m was found to increase 2-fold with decreasing flux over the range examined.

Reduction of C_2H_2 and C_2D_2 in D_2O. To assess the nature of intermediate states that might occur during C_2H_2 reduction, the product distribution for the wild-type and α-195^{Asn}-substituted MoFe protein catalyzed reactions was examined with C_2H_2 as the substrate in a reaction solution containing >90% D_2O, with ∼10% H_2O. As can be seen in Figure 3 (traces 1 and 2), the principal products detected for both proteins were *cis*- and *trans*-1,2-$C_2H_2D_2$, with differing percentages of the two isomers for the two proteins. A small quantity of C_2H_3D was also detected, whereas no C_2HD_3 was observed. As a control to show that formation of C_2HD_3 could be experimentally detected, the α-195^{Asn} MoFe protein was used to catalyze reduction of C_2D_2 in a D_2O/H_2O solution (trace 3).

Discussion

Fisher et al.[12] reported that certain substitutions within the MoFe protein located near the active site FeMo-cofactor can significantly change the stereochemistry of proton addition during C_2D_2 reduction. For example, it was shown that

Scheme 1



substitution of α-191^{Gln} by Lys altered proton addition resulting in ∼21% of the *trans*-dideuterated ethylene product compared to 4% for the wild-type protein. It was observed that an increase in *trans*-proton addition was correlated with both a change in the K_m for C_2H_2 and with the reduction of a small portion of C_2H_2 by four electrons to yield C_2H_6. From this correlation, a model was proposed linking the stereospecificity of proton addition with the K_m for C_2H_2 reduction and with the formation of C_2H_6.[12] In the present study, we have examined a number of altered MoFe proteins for their stereospecificity of proton addition to C_2H_2 as a way to test this model and to ask if such changes in stereospecificty could be attributed to steric constraints imposed by the active site environment. As previously reported,[12] we find that substitution of amino acid residues located within close contact to the FeMo-cofactor can result in perturbations in the stereochemistry of proton addition to C_2D_2. However, we do not find that these perturbations correlate with either the K_m for C_2H_2 reduction or the production of C_2H_6. For example, substituting α-96^{Arg} by Leu results in a MoFe protein with a 3-fold lower K_m for C_2H_2 reduction compared to the wild-type MoFe protein. This protein catalyzes 14% *trans*-ethylene formation during C_2D_2 reduction compared with 4% for the wild type. Further, no reduction of C_2H_2 to C_2H_6 could be detected for this protein. The α-96^{Ala} MoFe protein has a K_m nearly the same as that measured for wild type, and produces 12% *cis*-dideuterated ethylene. In the opposite direction, the α-69^{Ser}/α-195^{Asn} MoFe protein has a 28-fold higher K_m for acetylene and also produces significant *cis*-ethylene (35%). Thus, from these results, it must be concluded that the stereospecificity of proton addition during reduction of C_2D_2 does not correlate with either the K_m for acetylene reduction or with the formation of C_2H_6.

These new findings require reformulation of the model proposed earlier to explain the stereospecificity of proton addition during acetylene reduction. It was previously proposed that an enzyme-bound ethylenic intermediate (e.g., **3B** in Scheme 1) led to formation of C_2H_6 and loss of stereospecificity of proton addition to acetylene.[12] A longer residence time of the proposed intermediate at the active site was proposed to be responsible for the loss in stereospecificity and the increased formation of C_2H_6. It was also proposed that an increase in the population of the ethylenic intermediate was related to the K_m for acetylene. When the K_m was very low, it was postulated, acetylene would displace the intermediate, resulting in less accumulation of intermediate and less C_2H_6 formation and retention of the cis-proton addition stereochemistry. The results from the present study indicate that the K_m cannot be responsible for the increased population of the intermediate and that the stereochemistry of proton addition does not correlate with C_2H_6 formation. Further, in the case of equilibrium displacement, such as has been indicated for displacement of bound C_2H_4 from the active site by C_2H_2,[25] the binding rate for C_2H_2 is not necessarily expected to affect the off rate for C_2H_4. If free C_2H_2 does affect the residence time of the proposed ethylenic intermediate, and

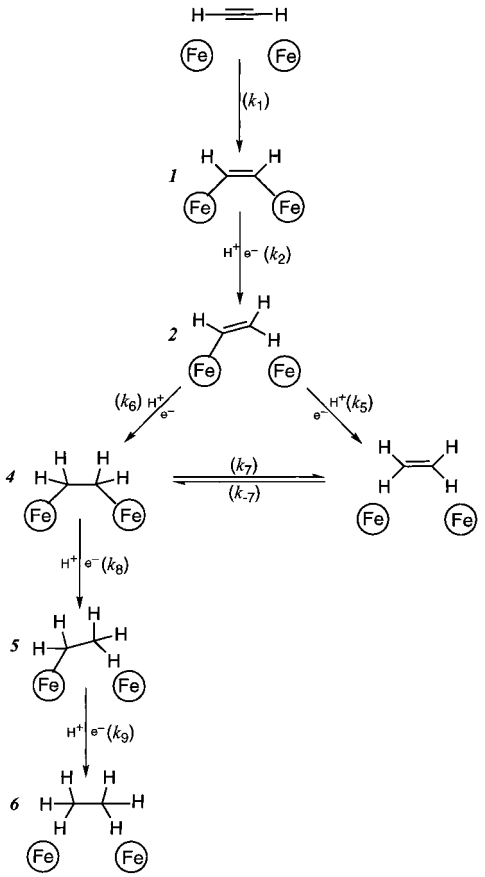


Figure 4. Proposed mechanism for nitrogenase reduction of acetylene to ethylene and ethane. The scheme depicts a model for the nitrogenase-catalyzed reduction of acetylene to both ethylene and ethane. Each intermediate is numbered and each reaction is defined by a rate constant (k) with a numerical subscript.

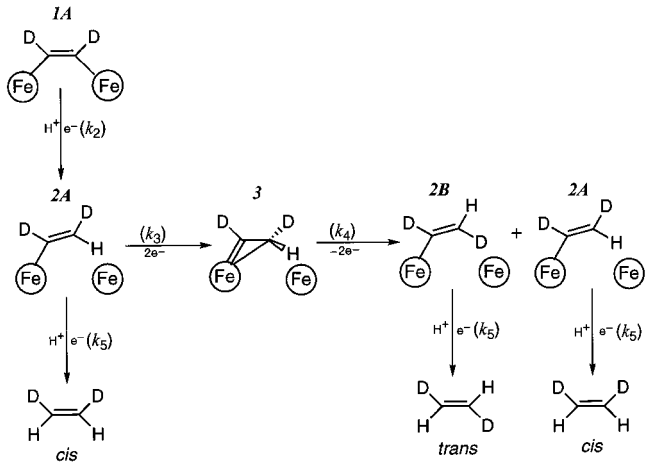


Figure 5. Proposed mechanism for nitrogenase reduction of C_2D_2. The scheme shows a model for the reduction of C_2D_2 in H_2O with the production of either *cis*- or *trans*-1,2-$C_2H_2D_2$. Each step intermediate is numbered and each reaction step is defined by a rate constant (k) with a numerical subscript. The numbering interleaves with the numbering shown in Figure 4.

C_2H_6 formation and stereospecificity are correlated to that residence time,[12] then C_2H_6 formation and stereospecificity of proton addition should be dependent on the concentration of C_2H_2, and they are not. The percentage of *cis*- and *trans*-1,2-$C_2H_2D_2$ was found not to change when the concentration of C_2D_2 was changed (data not shown).

We propose a different model based on the available information and known model chemistry (Figures 4 and 5). Based on the recent spectroscopic results from Lee et al.,[26] the initial binding of C_2H_2 to FeMo-cofactor is proposed to occur via an acetylenic bridge between two Fe atoms indicated as intermediate **1** in Figure 4. Reduction of this bound intermediate by one electron and one proton results in formation of intermediate **2** as in the model proposed by Fisher et al.[12] To explain the reduction of C_2H_2 by two or four electrons to yield either C_2H_4 or C_2H_6, two divergent reaction pathways from intermediate **2** are now proposed. For one pathway (Figure 4, left), intermediate **2** is further reduced by one electron and one proton to an enzyme-bound ethylenic intermediate such as **4**. If this intermediate remains bound to the active site further reduction by two more electrons and two protons could occur to yield C_2H_6.

The other pathway (Figure 4, right) results in the reduction of intermediate **2** to give C_2H_4, which is subsequently released. The competition between these two pathways can account for the variable levels of C_2H_6 produced by different nitrogenases. For the wild-type MoFe protein, it is expected that the reaction step characterized by the rate constant k_5 dominates over the reaction with the rate constant k_6, resulting only in C_2H_4 production. In contrast, certain alterations around FeMo-cofactor (e.g., the α-191^{Lys} substitution) could result in a decrease in the k_5/k_6 ratio resulting in a greater percentage of intermediate **2** going to C_2H_6. It has also been observed that C_2H_4 can be reduced to C_2H_6 by the V-based nitrogenase,[27] indicating the possibility for equilibrium between released and bound C_2H_4 such as described by the equilibrium constant K_7 (k_7/k_{-7}) in Figure 4. This feature can also account for the lag observed for C_2H_6 formation catalyzed by the V-based nitrogenase relative to C_2H_4 or H_2 formation during C_2H_2 reduction. Thus, by following the left-hand reaction pathway (as proposed for the MoFe protein) no lag in C_2H_6 production is expected compared to C_2H_4 production. In contrast, in the right-hand pathway (as proposed for the V-based nitrogenase) a lag could be expected for C_2H_6 production compared to C_2H_4 production since a rebinding step is required for C_2H_6 formation.

Dilworth et al. put forth two arguments against a possible C_2H_4 dissociation and association event to account for the lag in C_2H_6 formation catalyzed by the V-dependent nitrogenase.[28] First, the rate of C_2H_6 formation from added C_2H_4 is much lower than the rate of C_2H_6 formation from C_2H_2. Second, the amount of "free" C_2H_4 that could accumulate at equilibrium within the reaction vessel via C_2H_2 reduction in a typical assay would be far below the K_m for reduction of C_2H_4. Thus, our suggestion would demand a local concentration effect related to the proximity of released C_2H_4 to the active site. This possibility does not seem unreasonable considering that the active site FeMo-cofactor is buried within the polypeptide matrix and that released C_2H_4 would already be in the correct place and perhaps in the correct position for rebinding.

We also attempt to explain the observed stereochemistry of proton addition upon reduction of C_2D_2 in H_2O by the model shown in Figure 5. This model is an expanded view of steps k_2 and k_5 presented in Figure 4. Based on recent spectroscopic

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findings,[26] intermediates **1A** and **2A** for bound C_2D_2 and the one-electron reduced intermediate, respectively, seem reasonable. Proceeding from intermediate **2A**, we suggest two competing reaction pathways. Following the downward pathway in Figure 5, intermediate **2A** becomes reduced by one electron, with one proton being added to the αC on the same face as the earlier proton addition to the βC, resulting in *cis*-1,2-$C_2H_2D_2$. In the rightward pathway, intermediate **2A** would convert to an intermediate that permits rotation about the C−C bond, consequently resulting in loss of the stereochemical addition of the proton to the αC relative to the βC. An intermediate (**3B** shown in Scheme 1) was previously proposed[12] and we initially favored this model. We tested the possibility for formation of this intermediate by examining the products resulting from reduction of C_2H_2 in D_2O. Under the appropriate conditions where loss of stereospecificty is observed, accumulation of intermediate **3B** is expected. Thus, removal of a proton or deuteron from the βC of intermediate **3B** should result in formation of either intermediate **2C** or **2D**. Based on the predicted higher energy of breaking a C−D bond when compared to a C−H bond,[29] intermediate **2D** is expected to predominate. The subsequent reduction of intermediate **2D** by one electron and one deuteron would be expected to yield primarily C_2HD_3. However, this prediction is not experimentally observed. For example, in experiments with either the wild-type or the α-195^{Asn}-substituted MoFe protein for catalysis, no detectable level of C_2HD_3 is observed (Figure 3). The lack of this product clearly suggests that an intermediate like **3B** is unlikely, demanding an alternative intermediate to explain the loss of C_2D_2 reduction stereospecficity catalyzed by certain altered MoFe proteins.

Insight concerning the possible nature of the intermediate comes from well-characterized metal−alkyne complexes described in the inorganic literature.[30] Among these complexes, metal-bound η^2-vinyl complexes represent the most relevant to the present discussion.[30] On the basis of these complexes, we propose an η^2-vinyl intermediate as indicated by intermediate **3** in Figure 5. The occurrence of this intermediate is consistent with the observed product distribution of C_2D_2 reduction catalyzed by altered nitrogenases. It is known that the $\eta^1 \rightarrow \eta^2 \rightarrow \eta^1$ isomerization (**2A** → **3** → **2A** or **B**) will result in either *trans*- or *cis*-bound vinyl products (e.g., **2B** and ***2A***),[30−33] with the isomer product being controlled by the nature of ligands to the metal. In the case of nitrogenase, this isomerization could result in formation of either *cis*- or *trans*-1,2-$C_2H_2D_2$, with the protein environment around the FeMo-cofactor dictating the product distribution. Thus, it would be possible to explain the stereochemistry of proton addition observed for all nitrogenase proteins based on the protein influence controlling the transition from intermediate **3** to **2A** or **2B** without invoking the downward reaction at the far left. An intermediate such as **3** also explains why no trideuterated ethylene is observed when C_2H_2 is reduced by certain altered nitrogenases when incubated under catalytic conditions in the presence of D_2O.

The effect of electron flux on stereospecificity of C_2H_2 reduction in vitro is consistent with the sequential one-electron reduction of nitrogenase substrates. Thus, in the case of C_2D_2 reduction, a lower flux should favor a longer residence time of intermediate **2A** (shown in Figure 5) at the active site, thus giving more opportunity for the formation of intermediate **3**. It seems reasonable to expect that the formation of intermediate **3** is dependent not only on flux but also on steric constraints within the substrate-binding site. This suggestion is based on the observation that, for the wild-type protein, even under very low flux conditions, only a relatively small proportion of C_2D_2 is reduced to give the trans isomer. In contrast, lowering flux for the doubly substituted α-69^{Ser}/α-195^{Asn} MoFe protein raises the trans isomer product from 35% to nearly 50%. It seems reasonable that the range of trans product produced is a function of the additive affects of both a lower flux and the alteration of steric constraints around the FeMo-cofactor environment imposed by the protein. However, given that the specific effect of amino acid substitutions within the FeMo-cofactor environment on flux is not known, and experimentally difficult to evaluate, the interplay between these two effects remains an open question.

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