Differentiation of Acetylene-Reduction Sites by Stereoselective Proton Addition during *Azotobacter vinelandii* Nitrogenase-Catalyzed C_2D_2 Reduction†

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ABSTRACT: The interactions of acetylene with its binding site(s) on the FeMo cofactor of the MoFe protein of *Azotobacter vinelandii* nitrogenase were probed using C_2D_2. Specifically, the effects of changing the C_2D_2 concentration, electron flux, pH, or the individual presence of N_2, ethylene, or CO on the formation of both *cis*- and *trans*-1,2-ethylene-d_2 from C_2D_2 were measured. A hypothesis, involving two acetylene-reduction sites, was developed to explain the changes observed in the stereoselective protonation during both substrate-concentration-dependent and electron-flux-dependent C_2D_2 reduction. One of these sites is a higher-affinity acetylene-binding site that produces only *cis*-1,2-ethylene-d_2 from C_2D_2. The other is a lower-affinity acetylene-binding site, which produces both *cis*- and *trans*-1,2-ethylene-d_2. Added N_2 specifically inhibited the production of *cis*-1,2-ethylene-d_2 from C_2D_2, which indicates that N_2 binds to (and is reduced at) the higher-affinity acetylene-binding site. High concentrations of added ethylene behaved like very high concentrations of acetylene and inhibited both the electron flux flowing through the enzyme and cis-isomer formation. Added CO, at very low concentrations, did not affect the relative distribution of cis- and trans-isomers, indicating a separate CO-binding site. The results of pH-dependence experiments showed that substrate inhibition at high C_2D_2 concentrations is enhanced under acidic conditions but is absent under basic conditions and suggest that a low proton flux has a similar impact to that of a low electron flux; both inhibit *cis*-1,2-ethylene-d_2 formation selectively. Apparently, the factors affecting stereoselective protonation during C_2D_2 reduction could be the same as those that perturb protonation of the FeMo cofactor when acetylene is reduced. The observed nitrogenase-catalyzed production of ethylene-d_1 from C_2D_2 implicates a reversible protonation step in the mechanistic pathway.

Nitrogenases are enzymes that fix N_2 and produce ammonia (*1*) by a reaction mechanism that is yet to be elucidated. Molybdenum−nitrogenase (Mo-nitrogenase) is a two-component system, consisting of the MoFe protein[1] and the Fe protein, and the catalyzed substrate-reduction reaction is known to occur on the MoFe protein. The MoFe protein contains two different metal-cluster centers, the FeMo cofactor and the P cluster. The reduction of many alternative substrates, in addition to N_2, is catalyzed by nitrogenase. All are suggested to interact with the FeMo cofactor (Figure 1), which has the composition of *R*-homocitrate-$MoFe_7S_9X$ (X = unknown atom) (*2*). There is no clear consensus as to whether different substrates are reduced at different redox states of one primary site or at physically different sites on the FeMo cofactor (*1*, *3*).

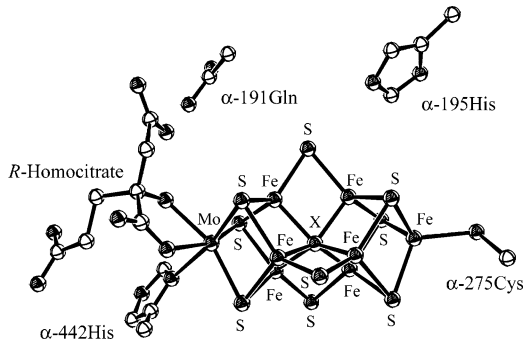

FIGURE 1: Structure of the FeMo cofactor (*R*-homocitrate-$MoFe_7S_9X$, where X is an unknown atom, likely C, N, or O) and some neighboring amino acid residues considered important in the reduction of nitrogenase substrates. Only α-275^{Cys} and α-442^{His} directly bond to metal ions in FeMo cofactor, to Fe and Mo, respectively. *R*-Homocitrate binds to Mo by its 2-hydroxy and 2-carboxy groups and is also the acceptor in a >NH→O− hydrogen bond with the amide function of α-191^{Gln}. The ϵ-NH function of the imidazole group of the α-195^{His} residue is the donor in a >NH→S− hydrogen bond with a central sulfide of FeMo cofactor.

The catalyzed reduction of acetylene (and C_2D_2) has been especially useful both for measuring nitrogenase activity (*4*, *5*) and for characterizing aspects of the nitrogenase reaction mechanism (*6*−*9*). Current understanding of the Mo-nitrogenase−acetylene interaction includes: (a) acetylene is reduced exclusively to ethylene (a two-electron reduction product), but some genetically altered MoFe proteins and

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[1] Abbreviations: Fe protein, iron protein of nitrogenase; MoFe protein, molybdenum−iron protein of nitrogenase; FeMo cofactor, the 7Fe-Mo-9S-homocitrate-X-containing prosthetic group in the MoFe protein; P-cluster, the 8Fe-7S-containing prosthetic group in the MoFe protein; Na_2-EDTA, the disodium salt of ethylenediamine-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; FT-IR, Fourier transform infrared; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure.

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V-nitrogenase produce ethane (a four-electron reduction product) as well (*10*−*12*); (b) acetylene is an inhibitor of proton reduction (*3*), and, at high concentrations, it inhibits its own reduction (*3*, *13*, *14*) by slowing electron flux through the enzyme most likely due to the tighter binding of the oxidized Fe protein to the MoFe protein (*15*, *16*); (c) acetylene and N_2 are likely reduced at different redox states of the MoFe protein because acetylene is a noncompetitive inhibitor of N_2 reduction, whereas N_2 is a competitive inhibitor of acetylene reduction (*17*, *18*); (d) there are at least two acetylene-reduction sites (*18*−*20*); (e) at least one of these reduction sites is suggested to be on an Fe_4S_4 face of the FeMo cofactor (*19*, *21*, *22*); and (f) one of the acetylene binding and reduction sites is likely involved with N_2 reduction (*22*, *23*).

When C_2D_2 is used as a substrate, Mo-nitrogenase produces *cis*-1,2-ethylene-d_2 mainly with only 4% of *trans*-1,2-ethylene-d_2 formed under an atmosphere of 4−5% C_2D_2 in argon. Using some altered *Azotobacter vinelandii* MoFe proteins, two papers (*24*, *25*) have recently provided insight into the factors controlling the stereoselectivity of C_2D_2 reduction by Mo-nitrogenase. First, the substitution of amino acid residues in the local environment of the FeMo cofactor perturbed the stereoselectivity of proton addition during C_2D_2 reduction and the relative K_m values for both ethylene production from acetylene and ethane production from ethylene by the various MoFe proteins were proposed to be important in determining both the cis/trans ratio of the 1,2-ethylene-d_2 produced and whether the production of ethane from acetylene might occur (*24*). Second, using the same experimental protocol but with different altered MoFe proteins, the electron flux through the Mo-nitrogenase rather than the relative K_m values was suggested to be the important factor in determining the stereoselectivity of C_2D_2 reduction (*25*). It is clear, however, that the K_m value for substrate reduction depends on the electron flux through nitrogenase (*14*, *26*).

We have studied C_2D_2 reduction by wild-type Mo-nitrogenase from *A. vinelandii* to examine how the stereoselectivity of catalyzed C_2D_2 reduction is affected by various reaction conditions, such as substrate concentration, electron flux, and pH. The effect of adding other nitrogenase substrates and inhibitors, namely, N_2, ethylene, and CO, was also monitored. Identification of the factors that influence the stereoselectivity of C_2D_2 reduction by wild-type Mo-nitrogenase could provide more precise information on the acetylene-reduction mechanism and the interactions between the acetylene-binding site(s) on the FeMo cofactor with other substrates and inhibitors.

EXPERIMENTAL PROCEDURES

The MoFe-protein and Fe-protein components of nitrogenase were isolated from wild-type *A. vinelandii* cells, which were diazotrophically grown in a 24-L fermentor at 30 °C according to the published method (*24*). The specific activities of the purified MoFe protein and the Fe protein were about 3000 nmol H_2/(min·mg) and 1600 nmol H_2/(min·mg) at 30 °C, respectively. The Mo content of the MoFe protein was 1.97 Mo atoms per molecule.

Unless stated otherwise, all specific activities are reported on a MoFe-protein basis as nmol of product formed per min·mg; total nitrogenase specific activity is the sum of the specific activities for both H_2 and ethylene production; total ethylene specific activity is the sum of the specific activities of all ethylenic species as quantified by gas chromatography; and all assays were carried out according to the published method in regular 9.25-mL assay vials with 70 μg of MoFe protein at a 20:1 molar ratio of Fe protein-to-MoFe protein at 30 °C with an appropriate amount of C_2D_2 (*24*). Except for when the three-buffer system was used for the pH-dependence experiments (*27*), all experiments were carried out in 25 mM HEPES buffer at pH 7.4. After all gaseous components were added, the assay vials were preequilibrated for 3 min at 30 °C before venting to atmospheric pressure, and then the reaction was started by adding the enzyme. A time-course experiment under 10% C_2D_2 was conducted for 1 h to monitor changes both in the specific activity of total ethylene production and in the cis/trans-isomer ratio. Typically, the reaction time was set for 10 min. The acetylene-reduction assay was stopped by addition of 0.25 mL of 0.5 M Na_2-EDTA pH 7.5, and gas chromatography was used to quantify both H_2 and hydrocarbons as described previously (*24*).

Headspace gas (4 mL) from the assay vial was also sampled with a gastight syringe and injected into a N_2-filled 100-mm path length IR gas cell. A Bruker IFS66v/S FT-IR spectrometer with a liquid nitrogen-cooled detector was used to collect IR spectra. All spectra were collected as 1000 scans at 4 cm^{-1} resolution, and the baseline was corrected using the blank obtained from an assay vial that contained every component of the C_2D_2-reduction experiment except the Fe protein. The peaks between 800 and 1100 cm^{-1} were identified by the comparison with the published data (*4*, *28*−*30*). Peak height was used for the calculation of the percentage of both the trans-isomer and the mono-deuterated product, C_2DH_3, in the deuterated ethylene products. The molar absorptivities for both the trans-characteristic peak at 988 cm^{-1} and the C_2DH_3-characteristic peak at 943 cm^{-1} are similar and about half that of the cis-characteristic peak at 843 cm^{-1} (*30*). Therefore, the peak heights at 943 and 988 cm^{-1} were doubled in our calculations as has been done for other published data. In these experiments, ca. 8% *trans*-1,2-ethylene-d_2 was typically observed at 4−5% C_2D_2 in argon compared to ca. 4% reported previously (*23*, *24*). We believe this difference is likely due to the lower specific-activity Fe protein used.

C_2D_2-concentration-dependent experiments were carried out similarly but with different amounts of C_2D_2 present in the reaction vials. Flux-dependent experiments were carried out with a constant amount (70 μg) of MoFe protein with the amount of Fe protein varied to achieve Fe protein-to-MoFe protein molar ratios from 2.5:1 to 38:1. For lower flux conditions, i.e., from 0.125:1 to 1:1 molar ratios of Fe protein-to-MoFe protein, the total protein amount was kept as 0.5 mg to prevent inhibition by high protein concentration. Each set of flux-dependent experiments was carried out over a concentration range (0.5−100%) of C_2D_2 in argon.

C_2D_2 reduction was also carried out in the presence of N_2 and the amount of *trans*-1,2-ethylene-d_2 formed was compared with that under argon. Because N_2 is an effective inhibitor of acetylene reduction only at low concentrations of acetylene due to the partial nature of its inhibition (*17*, *18*, *20*, *31*), the first experiment to test the effect of N_2 on

C_2D_2 reduction had a minimal amount (0.5%) of C_2D_2 present under atmospheres of 0, 20, 40, 60, 80, or 100% N_2 with argon as the balance gas. For this specific experiment, the reactions were carried out for 20 min instead of 10 min to ensure sufficient product was available for measurement. A second experimental series had a constant 80% N_2 present in all assay vials plus increasing amounts of C_2D_2 (0−20%) with argon as the balance gas.

The effect of ethylene on the stereoselectivity of C_2D_2 reduction was also monitored. A constant amount of ethylene (27%) was present in assay vials containing various amounts (from 0.5 to 11%) of C_2D_2 with argon as the balance gas. The vials were equilibrated at 30 °C for 3 min before venting to atmospheric pressure and initiating the reaction with the enzyme. To check the influence of CO concentration on the stereoselectivity of C_2D_2 reduction, small amounts of both CO (0−0.1%) and C_2D_2 (5%) were used to produce sufficient ethylene for IR measurement without introducing substrate inhibition by high concentration of C_2D_2.

The effect of pH on the stereoselectivity of C_2D_2 reduction was examined in the three-buffer system (*27*). The final pH was attained by adding either 6 M NaOH or 6 M HCl solution prior to degassing the reaction solution. The pH values of the reaction vials, which were measured before addition of EDTA solution, were reported as the pH values for the data sets. The experiments were restricted to the pH 6−8 region because the MoFe protein is denatured at pH values below 6 and above 8.2 (*27*). Because acetylene is a weak acid (*4*), the rate of formation of C_2DH from nonenzymatic $C_2D_2-H_2O$ exchange at pH 7 and 8 was checked by monitoring the increase in the mass27/mass28 ratio in the gas phase over a 2-mL standard three-buffer reaction mixture plus dithionite in a 15-mL round-bottomed flask shaken at 30 °C. Exchange was initiated by the injection of 50% (v/v) C_2D_2 in argon to give a final concentration of 10% C_2D_2 in the gas phase. At times up to 60 min, flasks were frozen in dry ice/ethanol and mass spectra were recorded. After 60-min incubation, 5.6% of the total acetylene was present as C_2DH.

RESULTS

Because the stereoselectivity of C_2D_2 reduction catalyzed by both wild-type and altered nitrogenases has been investigated under a variety of reaction conditions (*8*, *9*, *24*, *25*), we initiated a systematic investigation with wild-type Mo-nitrogenase. An initial time-course experiment under 10% C_2D_2 at a 20:1 molar ratio of Fe protein-to-MoFe protein showed that both total ethylene and *trans*-1,2-ethylene-d_2 formation remained linear with time for 25 min and the cis/trans isomer ratio was constant at ca. 7:1. Even when the reaction was run until the MgATP supply was exhausted (up to 1 h at pH 7.4), there was no change in the ratio of *cis*- to *trans*-1,2-ethylene-d_2 in the total ethylene produced, indicating that nonenzymatic reactions were not involved in ethylene formation.

trans-1,2-Ethylene-d_2 Production as a Function of C_2D_2 Concentration. No differences in terms of kinetic parameters and interactions with other substrate and inhibitors (compared to those observed using C_2H_2) were observed when deuterated acetylene was used as the substrate for wild-type nitrogenase. Using a 20:1 molar ratio of Fe protein-to-MoFe protein to provide a constant electron flux, the percentage of 1,2-*trans*-ethylene-d_2 in the reduction product was found to increase with increasing C_2D_2 concentration (see Figure 2). These percentages were then used to calculate the specific activity for 1,2-*trans*-ethylene-d_2 formation. When the specific activity of both total ethylene (cis- plus trans-) formation and 1,2-*trans*-ethylene-d_2 formation were plotted as a function of C_2D_2 concentration (see Figure 2), two different patterns of response at C_2D_2 concentrations greater than 20% were observed. The specific activity of total ethylene (cis- plus trans-) formation decreased markedly with increasing C_2D_2 concentration, whereas the specific activity of trans-isomer production was hardly affected. These different responses at the higher C_2D_2 concentrations resulted from a significant increase in trans-isomer percentage, indicating that cis-isomer production was selectively inhibited. The rate of cis-isomer production can be calculated by subtracting the rate of trans-isomer formation from the total ethylene-formation rate. Similar inhibition of total ethylene production by high concentrations of C_2D_2 was observed under a variety of electron-flux conditions, which were generated by varying the molar ratio of Fe protein-to-MoFe protein from 2.5:1 to 38:1 (data in Supporting Information, Figure S-1). The rate of trans-isomer production in these experiments, when plotted as a function of C_2D_2 concentration (see Supporting Information, Figure S-2), was not inhibited by high concentrations of C_2D_2. These data confirm the previously reported substrate inhibition of nitrogenase-catalyzed acetylene reduction at high acetylene concentrations (*3*, *18*, *20*) but, in addition, indicate that it affects cis-isomer production specifically.

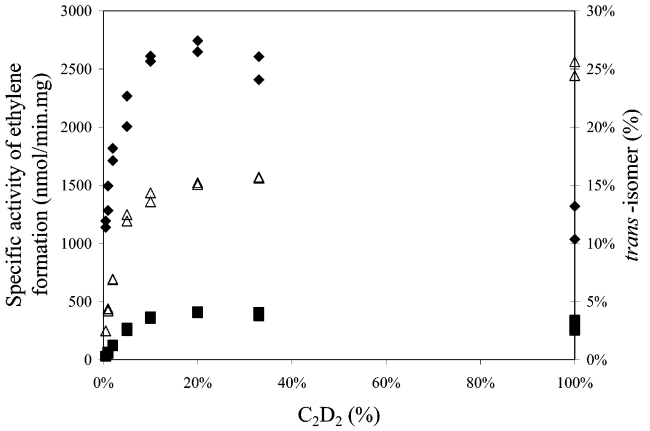

FIGURE 2: The effect of increasing C_2D_2 concentration on the production of *trans*-1,2-ethylene-d_2. Assay conditions and quantification of ethylene stereoisomers were as described under Experimental Procedures, using a 20:1 Fe protein-to-MoFe protein molar ratio. The specific activities of both total (cis- plus trans-) ethylene formation (◆) and *trans*-1,2-ethylene-d_2 production (■), which was obtained by multiplying the trans-isomer percentage (△) by the specific activity of total ethylene production, are plotted as a function of C_2D_2 concentration,

Effect of Changing Electron Flux on the Stereoselectivity of C_2D_2 Reduction. The electron flux, as defined by the molar ratio of Fe protein-to-MoFe protein, is known to be a major factor controlling the electron allocation to reducible substrates during nitrogenase catalysis (*14*−*16*, *26*). Because the rates of total ethylene formation and 1,2-*trans*-ethylene-d_2 formation responded differently to increasing C_2D_2 (see above), the stereoselectivity of protonation was examined under various electron-flux conditions to determine how each

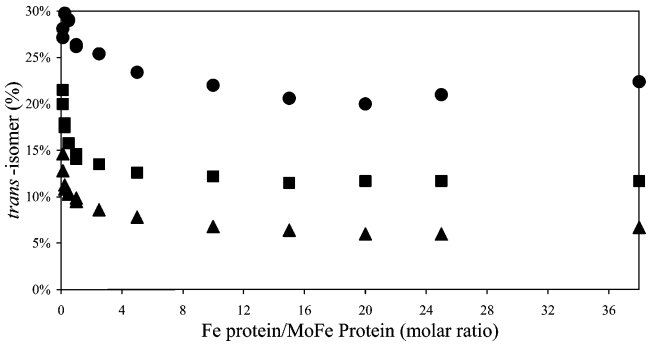

FIGURE 3: The effect of increasing electron flux on the production of *trans*-1,2-ethylene-d_2 at selected C_2D_2 concentrations. Assay conditions and quantification of ethylene stereoisomers were as described under Experimental Procedures. The percentage of *trans*-1,2-ethylene-d_2 relative to the total ethylene produced is plotted as a function of electron flux, as produced by varying the Fe protein-to-MoFe protein molar ratio, at different C_2D_2 concentrations (●, 100% C_2D_2; ■, 10% C_2D_2; ▲, 2% C_2D_2).

of these reactions would respond. Each set of flux-dependent experiments (from 0.125:1 to 38:1 molar ratio of Fe protein-to-MoFe protein) was carried out under 2, 10, or 100% C_2D_2. Figure 3 shows that the percentage of trans-isomer increased as the Fe protein-to-MoFe protein molar ratio decreased, but only below a ratio of ca. 10:1 as previously reported (*25*), under all three C_2D_2 concentrations. Under 100% C_2D_2 (which corresponds to a substrate-inhibition condition under higher-flux conditions), the percentage of trans-isomer increased from ca. 20% to ca. 30% as the electron flux decreased from ca. 15:1 to 0.125:1. Even under 2% C_2D_2 (where there is no substrate inhibition under higher-flux conditions), the percentage of trans-isomer increased from ca. 6% to ca. 14% over the same electron-flux range.

A replot (see Supporting Information, Figure S-3) of these data showed substrate inhibition by high C_2D_2 concentrations over a wide range of electron-flux conditions, but trans-isomer production was not similarly inhibited. Even in the lowest electron-flux regime (a molar ratio of Fe protein-to-MoFe protein of 1:1 and less), the rate of total ethylene formation was significantly inhibited by 100% C_2D_2 (the open circles were always lowest), whereas the rate of *trans*-1,2-ethylene-d_2 production was not inhibited.

Effect of N_2 on Stereoselectivity during C_2D_2 Reduction. To determine if the presence of N_2 selectively inhibits protonation of C_2D_2 during its catalyzed reduction, the rates of both *trans*-1,2-ethylene-d_2 and total ethylene production were monitored at a constant electron flux (maintained by a 20:1 Fe protein-to-MoFe protein molar ratio) under different concentrations of N_2 in argon to compare with data from a similar experiment under argon alone. Because N_2 is a partial inhibitor (*31*) of acetylene reduction, only 0.5% C_2D_2 was used initially. As the concentration of N_2 increased from 0 to 100%, the percentage of trans-isomer in the deuterated ethylene product increased linearly from ca. 2.5% to ca. 5%. Simultaneously, the specific activity of *trans*-1,2-ethylene-d_2 production decreased linearly from 22 to 16 nmol/(min·mg of MoFe protein) (data not shown).

In a second experiment, the substrate-concentration (from 0 to 20% C_2D_2) dependence of the stereoselectivity of C_2D_2 protonation was monitored (at a constant electron flux) under a constant 80% N_2 with argon as the balance gas to compare with data from similar experiments under argon alone as the

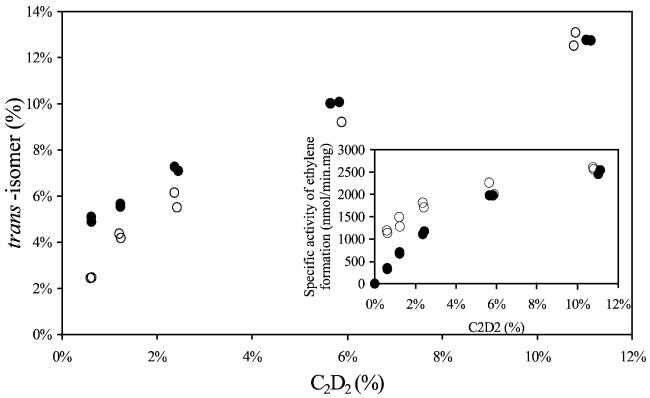

FIGURE 4: The effect of added N_2 on the production of *trans*-1,2-ethylene-d_2 as a function of C_2D_2 concentration. Assay conditions and quantification of ethylene stereoisomers were as described under Experimental Procedures, using a 20:1 Fe protein-to-MoFe protein molar ratio. The percentage of *trans*-1,2-ethylene-d_2 relative to the total ethylene produced is plotted as a function of C_2D_2 concentration under either 80% N_2 plus argon (●) or argon alone (○). The inset shows the effect of 80% N_2 in argon (●) versus argon alone (○) on the specific activity of total (cis- plus trans-) ethylene formation as a function of C_2D_2 concentration.

balance gas. The rate of total ethylene production from C_2D_2 reduction was the same under either argon or 80% N_2 at all C_2D_2 concentrations greater than ca. 6% (see Figure 4). At lower C_2D_2 concentrations, a significant loss in ethylene-production activity was observed in the reaction under 80% N_2, but this activity loss was compensated by N_2 reduction to ammonia (data not shown). The percentage of trans-isomer produced under 80% N_2, just like in the experiment under argon alone, increased as the concentration of C_2D_2 increased. But a plot of the trans-isomer percentage versus C_2D_2 concentration showed that the increase under 80% N_2 did not parallel that under argon alone (see Figure 4); a greater percentage of trans-isomer was produced under 80% N_2 at concentrations of less than 6% C_2D_2. The largest difference in the trans-isomer percentages was at 0.5% of C_2D_2, which was the lowest experimental C_2D_2 concentration used.

Effect of Ethylene on Stereoselectivity during C_2D_2 Reduction. Added ethylene affects acetylene reduction similarly to high concentrations of acetylene by slowing the overall nitrogenase catalytic cycle and lowering the effective electron flux (*24*, *32*). To check the effect of added ethylene on the stereoselectivity of C_2D_2 protonation, the concentration of C_2D_2 was increased from 0.5 to 12% in reaction vials containing 27% ethylene with argon as the balance gas. Assuming that total nitrogenase specific activity under 27% ethylene remains constant regardless of how much C_2D_2 is in the reaction vial, the rate of ethylene production from acetylene at each C_2D_2 concentration can be calculated by subtracting the rate of H_2 evolution in the presence of C_2D_2 from the H_2-evolution rate in the absence C_2D_2. No C_2H_6 was formed under these conditions. Figure 5 shows the calculated rates of both total ethylene and *trans*-1,2-ethylene-d_2 production under 27% ethylene as a function of C_2D_2 concentration together with both rates in the absence of ethylene. The calculated rate of total ethylene formation under 27% ethylene was always lower than the rate observed under argon alone. Furthermore, the total nitrogenase specific activity (which includes H_2-evolution activity) under 27% ethylene was ca. 80% of that obtained under argon alone (data not shown). In contrast, the percentage of trans-isomer

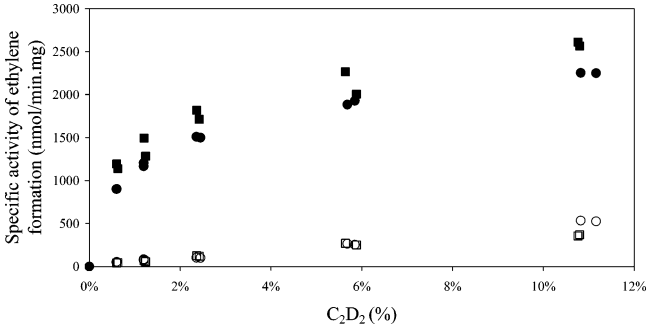

FIGURE 5: The specific activity of both total (cis- plus trans-) ethylene formation and *trans*-1,2-ethylene-d_2 production as a function of C_2D_2 concentration under either argon alone (■, □) or 27% C_2H_4 plus argon as the balance gas (●, ○). Assay conditions were as described under Experimental Procedures, using a 20:1 Fe protein-to-MoFe protein molar ratio. Filled symbols (■, ●) represent the specific activity of total ethylene formation, and open symbols (□, ○) represent the specific activity of *trans*-1,2-ethylene-d_2 production.

formed from C_2D_2 was always higher under an atmosphere containing 27% ethylene than under argon alone (data not shown). When combined, these two effects resulted in the same rate of trans-isomer production under both atmospheres (see Figure 5).

C_2D_2 Reduction in the Presence of CO. A noninhibitory concentration of C_2D_2 (5%) was used at a 20:1 molar ratio of Fe protein-to-MoFe protein in the presence of varying CO concentrations to determine if CO perturbs the stereoselectivity of C_2D_2 protonation. The specific activity of both total ethylene formation and trans-isomer production decreased linearly by ca. 60% as the concentration of CO increased from 0% to ca. 0.10%. These parallel decreases were the result of the percentage of trans-isomer relative to the total ethylene content remaining constant at ca. 11% (see Supporting Information, Figure S-4). Thus, both *cis*- and *trans*-1,2-ethylene-d_2 production decreased proportionally as the concentration of CO increased and diverted electron flux from acetylene reduction to H_2 evolution.

Effect of pH Changes on Stereoselectivity during C_2D_2 Reduction. The formation of both *trans*-1,2-ethylene-d_2 and ethylene-d_1 (C_2DH_3) was monitored as a function of pH at values between pH 6.0 and pH 8.0 under 10% C_2D_2 at an Fe protein-to-MoFe protein molar ratio of 20:1. The percentage of both ethylenic species increased with increasing pH but only at the higher pH values (see Figure 6). The percentage of trans-isomer increased from ca. 12% at pH values below 7 to ca. 20% at pH 7.9 and the percentage of ethylene-d_1 increased from zero at pH values below pH 7 to ca. 2% at pH 7 and ca. 18% at pH 7.9. Although an increase in the percentage of trans-isomer was observed at pH values below 6 (data not shown), the MoFe protein is being denatured here as also occurs at pH values above 8.2 (*27*). Control reactions (see Experimental Procedures) demonstrated that at most 5% (but more likely 3%) C_2DH_3 might be formed from the catalyzed reduction of contaminating C_2DH produced by nonenzymatic C_2D_2/H_2O exchange in the assay vials. Sufficient contaminating C_2DH might, therefore, be present to account for all C_2DH_3 produced at pH 7, but it is clearly insufficient to account for the amount of C_2DH_3 produced at pH 8.

The data derived from C_2D_2-concentration-dependent experiments at pH values of 6.2, 7.2, and 8.2 (using the three-

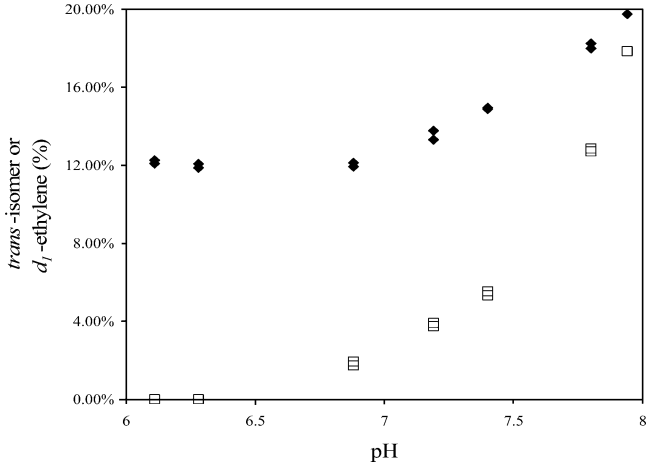

FIGURE 6: The effect of pH on the production of both *trans*-1,2-ethylene-d_2 and ethylene-d_1. Assay conditions and quantification of ethylene stereoisomers were as described under Experimental Procedures, except that the three-buffer system was used together with a 20:1 Fe protein-to-MoFe protein molar ratio under 10% C_2D_2. The percentages of *trans*-1,2-ethylene-d_2 (◆) and ethylene-d_1 (□) relative to the total ethylene produced are plotted as a function of pH.

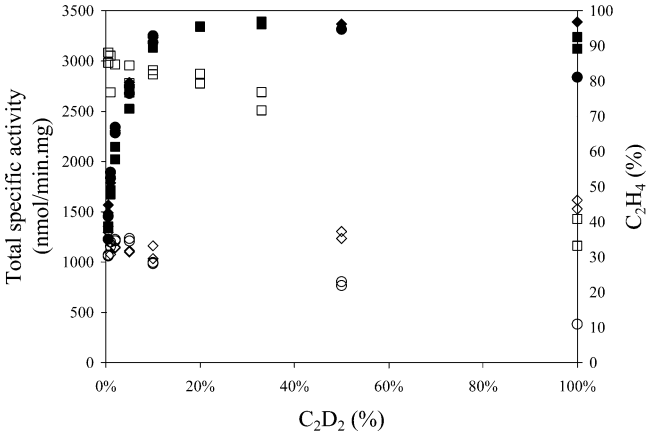

FIGURE 7: The effect of pH both on the total nitrogenase specific activity, which is the sum of the specific activities for all ethylenic species plus that of H_2 evolution, and on the distribution of electrons to products as a function of C_2D_2 concentration. Assay conditions and product quantification were as described under Experimental Procedures, except that the three-buffer system was used together with a 20:1 Fe protein-to-MoFe protein molar ratio. The total nitrogenase specific activity is plotted as a function of C_2D_2 concentration at the pH values of 6.2 (○), 7.2 (□), and 8.2 (◇). The percentage of ethylene produced as a function of C_2D_2 concentration is also plotted for the pH values of 6.2 (●), 7.2 (■), and 8.2 (◆).

buffer system with a 20:1 Fe protein-to-MoFe protein molar ratio) showed that total nitrogenase (ethylene plus H_2) specific activity was clearly impacted by the pH of the assay (see Figure 7). At pH 7.2, total nitrogenase specific activity remained ca. 3000 nmol/(min·mg of MoFe protein) up to 20% C_2D_2 and then showed the typical substrate-inhibition decrease (by ca. 55% at 100% C_2D_2) as observed in HEPES buffer at pH 7.4 (see above). Under acidic conditions (at pH 6.2), total nitrogenase specific activity (ca. 1200 nmol H_2/(min·mg) under 100% argon) showed even greater substrate inhibition; it was now observable at 10% C_2D_2 and resulted in an ca. 65% loss of specific activity at 100% C_2D_2. In contrast, at the basic pH of 8.2, total nitrogenase specific activity (ca. 1400 nmol H_2/(min·mg) under 100% argon) was

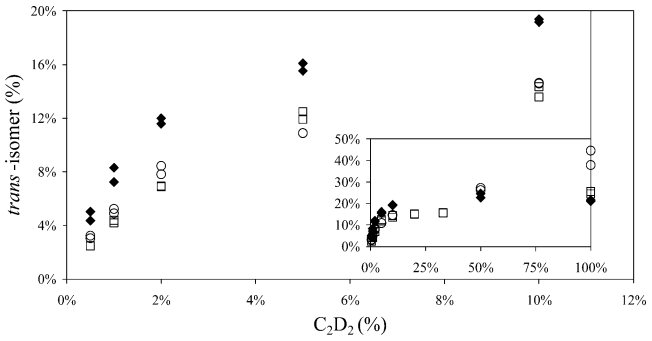

FIGURE 8: The effect of pH on the production of *trans*-1,2-ethylene-d_2 as a function of C_2D_2 concentration. Assay conditions and quantification of ethylene stereoisomers were as described under Experimental Procedures, except that the three-buffer system was used together with a 20:1 Fe protein-to-MoFe protein molar ratio. The main figure is a plot of the percentage of *trans*-1,2-ethylene-d_2 relative to the total ethylene produced as a function of C_2D_2 concentration (from 0 to 10% in argon) at the pH values of 6.2 (○), 7.2 (□), and 8.2 (◆). The inset, using the same units, shows the data set over the complete 0−100% C_2D_2-concentration range.

not inhibited by increasing C_2D_2 concentrations, not even at 100% C_2D_2. In fact, an activity enhancement occurred at the higher C_2D_2 concentrations at pH 8.2.

A plot of subsets of these data (see Figure 7) showed that the percentage of electron flux used for total ethylene production was similar for all three pH values up to 50% C_2D_2, maximizing at ca. 95%. At 100% C_2D_2, however, some differences were apparent, especially at pH 6.2, where the ethylene percentage was somewhat lower (ca. 80%) than at the other two pH values.

Double-reciprocal plots of these same data (see Supporting Information, Figure S-5) showed several different pH effects. The plot of the pH-7.2 data was curvilinear for total ethylene production over the 0.5−100% range of C_2D_2 concentration. This curve is quite similar to a previously published curve (see Figure 1a of ref *20*) and had three apparently distinct sections. In the 0.5−2% C_2D_2-concentration section, the curve has a shallow slope, which (staying mindful of the minimal number of data points) suggests a low K_m value; in the 2−20% concentration range, a more steeply downward-sloping portion of the curve indicates a higher K_m value; and, above 20% C_2D_2, the substrate-inhibition phenomenon becomes observable as the curve breaks sharply and steeply upward (with the data points now above any extrapolated line).

The pH-6.2 double-reciprocal plot did not show the initial shallow-sloped section, but instead had a linear section extending from 0.5 to 5% C_2D_2 that indicated a K_m value of ca. 1%. The impact of substrate inhibition was apparent at 10% C_2D_2 and above. The plot of the pH-8.2 data was different again. Now, both the shallow-sloped (indicative of the low K_m value) and the more steeply sloped (compatible with the higher K_m value) sections, as observed in the pH-7.2 plot, were visible again in the curvilinear plot, but substrate inhibition was completely lacking. Instead of curving sharply upward, the last several points of the plot curved downward, indicating a possible rate enhancement at high C_2D_2 concentrations (see Supporting Information, Figure S-5 and ref *20*).

The percentage of trans-isomer formed responded differently to changes in C_2D_2 concentration depending on the pH conditions (see Figure 8). Up to 10% C_2D_2 (see the main part of Figure 8), the percentage of trans-isomer in the total ethylene product increased similarly (to ca. 14%) at both pH 6.2 and pH 7.2 but a significantly greater percentage (to ca. 20%) of trans-isomer was always formed at pH 8.2. The percentage of trans-isomer at pH 8.2 did not increase further as the C_2D_2 concentration was increased from 10 to 100%. However, the trans-isomer percentage did continue to increase at both pH 7.2 (to ca. 20% at 100% C_2D_2) and pH 6.2 (to ca. 40% at 100% C_2D_2) due to substrate inhibition and its selective inhibition of cis-isomer production (see inset to Figure 8).

The formation of ethylene-d_1 was also monitored in these experiments. As expected from the results of the initial pH study, no ethylene-d_1 was formed at pH 6.2 at any C_2D_2 concentration. At pH 7.2, the ethylene-d_1 content relative to total ethylene increased from ca. 3% at 0.5% C_2D_2 to only ca. 4% at 10% C_2D_2. At pH 8.2, the percentage of ethylene-d_1 was already ca. 11% of the total ethylene produced under 0.5% C_2D_2 and increased to ca. 15% at 10% C_2D_2.

DISCUSSION

Stereoselective Protonation and trans-1,2-Ethylene-d_2 Production. The results presented here clearly show that the stereoselectivity of proton addition, which occurs during wild-type Mo-nitrogenase-catalyzed C_2D_2 reduction, is affected by (i) acetylene concentration; (ii) electron flux, i.e., the Fe protein-to-MoFe protein molar ratio; (iii) the presence of other substrates or inhibitors; and (iv) the pH of the assay. Inhibition of acetylene reduction catalyzed by wild-type Mo-nitrogenase by high concentrations of acetylene, a process known as substrate inhibition (*33*; see Figure 3), has been recognized for a long time (*3*, *13*, *14*, *20*). However, our results clearly show that acetylene substrate inhibition selectively affects *cis*-1,2-ethylene-d_2 production from C_2D_2. A corollary of this result is that the percentage of trans-isomer formed relative to total ethylene production is dependent on the C_2D_2 concentration (see Figure 2), a result that contrasts with a previous report (*25*), although that report did not indicate the range of C_2D_2 concentrations studied.

Similar levels of substrate inhibition by high C_2D_2 concentrations occur over a wide range of electron-flux conditions and, in no case, is there significant inhibition of trans-isomer production (see Supporting Information, Figures S-1, S-2, and S-3). An enhancement in trans-isomer formation relative to total ethylene formation might be expected under low electron-flux conditions, if its formation is dependent (at least partly) on the residence time of an intermediate at the binding site as previously suggested (*24*). This suggestion is also supported by the direct effect of C_2D_2 concentration on the percentage of trans-isomer formed (see above), which is also expected, if the residence time of the putative intermediate is affected by the free acetylene concentration.

Added substrates and inhibitors also affected the stereoselectivity of protonation. N_2 is a partial inhibitor of catalyzed acetylene reduction (*18*, *31*), which means operationally that its effect is observable only at a high N_2 concentration and a low C_2D_2 concentration. Under such conditions (80% N_2 with 6% or less C_2D_2), the percentage of trans-isomer formed was always greater than under argon alone, even though the

rate of total ethylene formation was lower (see Figure 4). Thus, N_2 binding to the enzyme selectively inhibited cis-isomer formation. A similar result was observed when 27% ethylene was present in the assays. Ethylene is both a substrate and an electron-flux inhibitor of nitrogenase. Ethylene is such a poor substrate for wild-type Mo-nitrogenase that it is not reduced at all when acetylene is present (*24*, *32*). Its inhibitory effect on nitrogenase activity has been likened to that of high concentrations of acetylene (*6*, *24*). Ethylene can be a good diagnostic tool because its inhibition of electron flux can be observed in the presence of small amounts of C_2D_2. Although the calculated rate of total C_2D_2 reduction was decreased by ca. 20% in the presence of 27% ethylene, the calculated rate of *trans*-1,2-ethylene-d_2 production was unaffected (see Figure 5). Thus, ethylene not only acts as an electron-flux inhibitor but selectively inhibits the production of *cis*-1,2-ethylene-d_2 similarly to substrate inhibition. One caveat for this conclusion is that the rate of total ethylene production was calculated and may be an overestimation.

The effect of added CO was different to those of N_2 and C_2H_4. Although CO is isoelectronic with N_2, it is not a substrate. Rather, it is an inhibitor of all Mo-nitrogenase-catalyzed substrate reductions, except H^+ reduction. In contrast to the high concentrations of acetylene needed to elicit substrate inhibition, CO effectively inhibits acetylene reduction at very low concentrations. Moreover, it does so noncompetitively and without affecting the electron flux through nitrogenase. CO simply diverts all electron flux to H_2 evolution (*13*, *20*). CO cannot completely reverse the electron-flux inhibition caused by higher concentrations of either acetylene or ethylene (*15*, *16*, *24*, *32*). Under conditions that both circumvented acetylene substrate inhibition and allowed the formation of sufficient ethylene for detection, CO inhibited the rate of total ethylene production as expected, but did not change the percentage of trans-isomer in the total ethylene produced. This result was in marked contrast to the response under either N_2 or C_2H_4 and indicated that CO inhibition did not distinguish among cis- and trans-isomer production.

Suggested Scheme for Stereoselective Protonation. The differential effects on cis- and trans-isomer production, which are described above and occurred on changing substrate concentration, electron flux, or adding other nitrogenase substrates/inhibitors, cannot be explained by the presence of a single substrate-binding/reduction site on the enzyme. Instead, we have used a two-site model (see Figure 9) in which site 1 produces both *cis*- and *trans*-1,2-ethylene-d_2, whereas site 2 produces only *cis*-1,2-ethylene-d_2 from C_2D_2 reduction. We propose that substrate inhibition at high C_2D_2 concentrations occurs specifically for site 2, so inhibiting only *cis*-1,2-ethylene-d_2 production, and is due to the binding of an additional acetylene molecule (to site C in Figure 9) that somehow interrupts either productive binding at or internal electron transfer to site 2. This suggestion is consistent with previous EPR observations under turnover conditions (*6*). Moreover, the selective inhibition of cis-isomer production by added N_2 suggests that N_2 competitively interacts with (and likely is reduced at) site 2 of our two-site model. This conclusion suggests that the reaction mechanism for N_2 reduction could share common characteristics with acetylene reduction at this site.

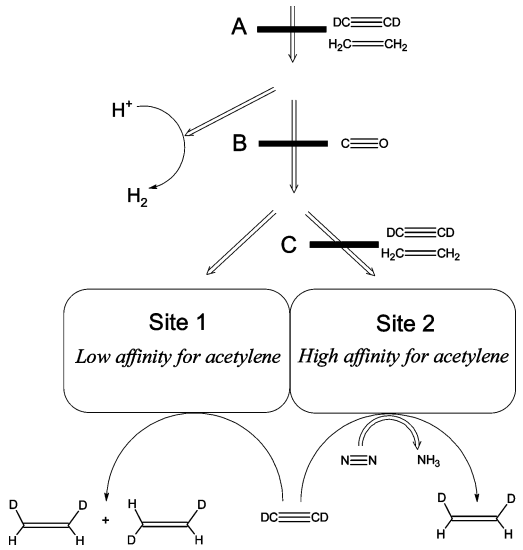

FIGURE 9: A proposed arrangement of substrate- and inhibitor-binding sites along the electron-transfer pathway. There is evidence to support the location of many of these sites on the FeMo cofactor of the nitrogenase MoFe protein (*10*, *19*, *21*, *23*, *24*, *36*). The two major acetylene-binding and reduction sites are labeled as site 1 and site 2. Site 1 is proposed to have a K_m of ca. 1.5%, a presumed lower affinity for acetylene, and produces both *cis*- and *trans*-1,2-ethylene-d_2, whereas site 2 is proposed to have a K_m of ca. 0.1%, a presumed higher affinity for acetylene, produces only *cis*-1,2-ethylene-d_2, and interacts with N_2. The nonproductive (inhibitory) sites are labeled as A, B, and C. Electrons enter the pathway from the Fe protein at the top of the figure, where they may encounter any of several situations. If either ethylene or acetylene (at high concentrations) is bound at site A, electron flux decreases. Because this site is not susceptible to the effect of added CO, it may be located elsewhere than on the FeMo cofactor. When CO is bound at site B, it prevents electron flux from reaching both site 1 and site 2 and diverts all electron flux to H_2 evolution. Site C is a substrate-inhibition site, which (when occupied) selectively inhibits *cis*-1,2-ethylene-d_2 production at site 2.

Added ethylene, although not being catalytically reduced to ethane, also inhibits the production of *cis*-1,2-ethylene-d_2 selectively and may be mimicking the effect of acetylene substrate inhibition by binding to the third substrate-inhibition site (site C in Figure 9). This effect would be in addition to its role as an overall electron-flux inhibitor (by binding at site A in Figure 9) as suggested previously (*24*). The effect of the other inhibitor used, CO, contrasts with that of added ethylene because CO is completely unselective in its inhibition of C_2D_2 reduction. This observation indicates that, when CO is bound to Mo-nitrogenase, the enzyme is totally inactive toward acetylene reduction and catalyzes only proton reduction. Thus, CO binds to the enzyme at a site (site B in Figure 9) quite distinct from all other binding sites.

Can we find any support for these several putative acetylene-binding sites through further analysis of both our and previously published data? A double-reciprocal plot of specific activity of total ethylene production as a function of C_2D_2 concentration at pH 7.2 (see Supporting Information, Figure S-5) indicated that there could be as many as three acetylene-binding sites: the substrate-inhibition site plus two reduction sites. Analysis of a similar curvilinear plot (see Figure 1a of ref *20*) had given the two K_m values of ca. 0.1% and ca. 1% for ethylene production. We can correlate our cis-isomer-producing site (site 2) with the lower-K_m (ca. 0.1%) and presumably higher-affinity acetylene-binding site

and our trans-isomer-producing site (site 1) with the higher-K_m (ca. 1%) and presumably lower-affinity site for the following reasons. The rate of total ethylene production, under low electron-flux conditions, was essentially the same at 2 and 10% C_2D_2 (see inset of Supporting Information, Figure S-3), and yet the rate of trans-isomer formation increased with increasing C_2D_2 concentrations (see Figure 3 and Supporting Information, Figure S-3). These results indicate that the cis-isomer-producing site was already saturated at 2% C_2D_2, which is consistent with a lower K_m value for site 2. In contrast, the trans-isomer-producing site was not similarly saturated, indicating a higher K_m value for site 1. Further, our preliminary simulations (data not shown), which are complicated by the substrate-inhibition phenomenon, support this conclusion because similar patterns of response result only when the trans-isomer is assumed to be formed at the putative lower-affinity site 1.

Effects of pH on Stereoselective C_2D_2 Reduction. Both total nitrogenase (H_2 plus ethylene) and total ethylene-production specific activities are influenced by the pH of the reaction (*14*, *27*, *34*, *35*). Total nitrogenase specific activity versus pH plots are distorted bell-shaped curves with a maximum activity at pH ca. 7.2 and pK_a (as 50% of maximum activity) values of ca. 6.2 and ca. 8.2. When the curve produced under 100% argon is compared to the curve for 10% acetylene/90% argon under high electron-flux conditions, they differ only on the basic side (>pH 7.0), where the pK_a value shifts in the acidic direction by ca. 0.4 pH unit when acetylene is present (*35*). This result contrasts with a previous report (*27*), which indicated that the whole specific activity versus pH plot was shifted in the acid direction in the presence of acetylene. This acetylene-induced pK_a shift suggests that the protonation process varies with the substrate being reduced.

Similarly, the stereoselectivity of protonation under 10% C_2D_2 at high electron flux is also affected at pH values greater than ca. 7, with the trans-isomer percentage relative to the total ethylene produced increasing with increasing pH (see Figure 6). Consistent with this observation, the trans-isomer percentage increased more rapidly with C_2D_2 concentration at pH 8.2 than at either pH 7.2 or 6.2 (see Figure 8). It also maximized earlier (at 10% C_2D_2) with ca. 20% trans-isomer present, which was the same maximum found for the trans-isomer percentage at pH 7.2, but here 100% C_2D_2 was required (see inset to Figure 8). A similar enhancement of trans-isomer formation relative to total ethylene formation was observed when electron flux was low (see above). Because all nitrogenase substrates require a proton for every electron accepted, it may not be surprising that trans-isomer formation is also enhanced when the flux of protons is low (as would be the case at higher pH). In contrast to the constant 20% of trans-isomer observed at both pH 7.2 and 8.2 under 50% (and more) C_2D_2, the trans-isomer percentage at pH 6.2 continued to increase dramatically until it accounted for ca. 40% of total ethylene production at 100% C_2D_2, thus emphasizing the impact of the increased substrate inhibition on trans-isomer formation.

The rate of total ethylene production under 100% C_2D_2 was the same at both pH 7.2 and pH 8.2 (see Figure 7). However, this rate was achieved at 10% C_2D_2 at pH 8.2 rather than at the 100% C_2D_2 required at pH 7.2. It appears then that the effect of higher pH and substrate inhibition offset one another and result in the same rate.

Effects of pH on Substrate Inhibition. The impact of changing pH provided insight into the phenomenon of substrate inhibition. Apparently, basic conditions offset substrate inhibition, whereas acidic conditions exacerbate it, suggesting that protonation state is an important factor. Substrate inhibition, in addition to inhibiting cis-isomer formation (see above), also decreased total electron flux but without affecting its distribution between ethylene formation and H_2 evolution at all three pH values at least up to 50% C_2D_2 (see Figure 7). However, under 100% C_2D_2, ethylene formation appears to suffer a small selective increase in inhibition at pH 6.2 and 7.2, but not at pH 8.2. This last difference may be a consequence of the increase in activity that occurs under higher C_2D_2 concentrations at pH 8.2.

Neither *Klebsiella pneumoniae* (*18*) nor *Clostridium pasteurianum* (*18*, *20*) nitrogenase exhibits substrate inhibition when assayed for catalyzed acetylene reduction at pH ca. 7 under higher flux conditions. The *A. vinelandii* nitrogenase is unique in this respect. However, when assayed at pH 8.2, *A. vinelandii* nitrogenase-catalyzed acetylene reduction loses this uniqueness and becomes quite similar to that of *C. pasteurianum* (*18*, *20*) nitrogenase, including a stimulation of activity at high acetylene concentrations that suggests the presence of a very low-affinity binding site. Lineweaver−Burk plots for both nitrogenases are quite similar (see Supporting Information, Figure S-5 and Figure 1a in ref *20*), and a K_m value of ca. 20% has been measured for the very low-affinity *C. pasteurianum* site. If substrate inhibition is due to the nonproductive binding of a second acetylene molecule to the *A. vinelandii* enzyme (at site C in Figure 9), this additional acetylene-binding (and normally electron-flux-inhibiting) site must be compromised at basic pH (most likely by one or more acid−base groups becoming deprotonated). Our pH-8.2 data set suggests that this substrate-inhibition site may have been either replaced by or converted into a productive, but very low-affinity, site and indicates a K_m value of at least 10% in line with the *C. pasteurianum* value. Under normal assay conditions, an acetylene-reduction site such as this would at best be only marginally effective at pH ca. 7 with the wild-type enzyme.

A body of inferential evidence has accumulated to implicate the FeMo cofactor prosthetic group of the MoFe protein as providing the substrate/inhibitor-binding sites (see, e.g., *10*, *36*−*37*). The enhancement of substrate inhibition under acidic conditions and its absence at basic pH may be related to the chemical stability of the unproductive FeMo cofactor-(acetylene)$_2$ complex that is likely responsible for this phenomenon. Acetylene is known to form vinyl-sulfide complexes readily and does so when it reacts with either dinuclear (*38*) or trinuclear (*39*) molybdenum- (or tungsten-) chalcogenide clusters, many of which have a M_3S_3 moiety that is structurally similar to those found in the FeMo cofactor. Furthermore, the formation of a vinyl-sulfide complex is often reversible and sometimes can result in ethylene formation (*40*). Formation of such a vinyl-sulfide complex may be responsible for substrate inhibition with nitrogenase because vinyl-sulfide complex formation is expedited by acidic pH which is consistent with the increased substrate inhibition observed at pH 6.2. Moreover, substrate inhibition on wild-type nitrogenase is also reversible and, at pH 8.2, might even become productive.

Comparison of our Model with Other Proposals for Acetylene Reduction. Previous studies (*18*−*20*, *22*−*23*) with nitrogenase have implicated two major acetylene-binding/reduction sites. The results from two of these studies (*18*, *20*) were quite similar to ours and provide the K_m values that we have assigned to site 1 and site 2 in our model (see above and Figure 9), but neither of those reports included much mechanistic insight. Results from two other studies, using variant MoFe proteins, were used to assign K_m values and to distribute substrates/inhibitors quite differently to the two sites (*22*−*23*). A higher-affinity C_2H_2-binding site (with a K_m of ca. 0.7% C_2H_2) was proposed as the site that is normally assayed under 10% acetylene and may also bind CO, whereas a lower-affinity site (with a K_m of ca. 14% C_2H_2) was deemed responsible for the binding/reduction of N_2, and some other substrates and may also bind CO.

This last proposal (*22*−*23*) has three obvious differences when compared to our model. The first is the magnitudes of the kinetic constant, K_m. Although these differences may simply be a direct result of their use of variant MoFe proteins, their higher-affinity site may actually reflect an average of the individual K_m values of our sites 1 and 2, a phenomenon that has been encountered before (*18*). Furthermore, their lower-affinity site could be the same as the "third" site that becomes observable (and productive, with a K_m of ca. 10% C_2H_2) at pH 8.2 in our data and that might be formed from the original substrate-inhibition site. If so, this site would be invisible with wild-type *A. vinelandii* nitrogenase at neutral pH because of acetylene substrate inhibition. The second difference involves the binding of N_2. Our results favor N_2 binding at the higher-affinity C_2H_2-binding site, whereas theirs favor binding at the lower-affinity site. It would be difficult to explain the partial (and competitive) inhibition by N_2 of acetylene reduction if N_2 binds only at the lower-affinity C_2H_2-binding site. For example, N_2 only inhibits acetylene reduction at high N_2 and low C_2H_2 concentrations; in our studies, 80% N_2 was an effective inhibitor only at acetylene concentrations of less than 6%. Of course, N_2 might bind competitively at both sites, but binding at their lower-affinity (with a K_m of ca. 14%) site would be undetectable in wild type due to the onset of substrate inhibition. The third difference involves CO binding. Our model proposes a CO-binding site that is quite distinct from the acetylene-binding sites (sites 1 and 2) and so is consistent with the known noncompetitive pattern of CO inhibition with wild-type nitrogenase. Their proposal implicates CO binding at both of their acetylene-binding sites based on the competitive pattern of inhibition observed with their variant MoFe proteins.

The problem of how the cis- and trans-isomers of deuterated ethylene are produced has also been addressed using model compounds. The results led to the suggestion that their formation during nitrogenase-catalyzed C_2D_2 reduction is due to the uncontrolled protonation of the FeMo cofactor (*41*). However, our observation that the stereoselectivity of protonation during C_2D_2 reduction is pH-dependent makes this suggestion unlikely. Our results are, however, consistent with protonation/deprotonation of the FeMo cofactor as detected by the pH-dependent change in the $S = 3/2$ EPR signal of the MoFe protein (*42*) and during catalytic turnover (*43*). Therefore, the factors that affect stereoselective C_2D_2 reduction could be the same as those that perturb protonation/deprotonation of the FeMo cofactor when it reduces C_2D_2. This pH dependence implies that the protonation status of acid−base group(s) close to substrate-binding sites is an important factor for catalysis in general (*27*, *35*, *44*).

In this regard, the observation that ethylene-d_1 (C_2DH_3) is formed in a pH-dependent reaction during C_2D_2 reduction in H_2O is an important one. Formation of small amounts of C_2DH_3 has been observed at pH ca. 7 before (*24*, *25*) but is usually ascribed to the reduction of presumed contaminating C_2DH in the substrate C_2D_2. This assumption is no longer tenable because no C_2DH_3 is observed in the infrared spectrum of reactions run at pH values below ca. 6.7, even though acetylene reduction is occurring. C_2DH_3 production is only observed at the higher pH values, where the supply of protons to accompany the electrons used to reduce substrate could become limited. Thus, C_2DH_3 formed as a product of catalyzed C_2D_2 reduction, especially at higher pH values, strongly implicates a reversible protonation step as an integral part of the mechanism of stereoselective protonation during acetylene reduction.

We previously suggested a scheme, which included a reversible protonation step on a key σ-alkenyl intermediate (*24*; step III in Scheme 2), that attempted to rationalize both the mix of stereoisomers produced and whether ethane is formed during catalyzed C_2D_2 reduction. A more recent scheme (*25*; Figure 5), which does not include reversible protonation and so is incompatible with catalyzed ethylene-d_1 formation, discarded this intermediate in favor of a η^2-vinyl intermediate based on (i) no C_2D_3H being observed from the catalytic reduction of C_2H_2 in D_2O at pH 7, even though some C_2DH_3 was produced from C_2H_2 in D_2O at pH 7; and (ii) the cis-/trans-isomer product ratio being independent of substrate concentration. The data presented herein are at odds with both of these observations, suggesting that the σ-alkenyl intermediate should be reconsidered.

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SUPPORTING INFORMATION AVAILABLE

Figure S-1 is a plot of the specific activity of total ethylene production as a function of C_2D_2 concentration over a wide range of electron-flux conditions. Figure S-2 is a plot of specific activity of *trans*-1,2-ethylene-d_2 formation as a function of C_2D_2 concentration over a limited range of electron-flux conditions at pH 7.4. Figure S-3 shows plots of the specific activity of both total ethylene production and *trans*-1,2-ethylene-d_2 formation as a function of increasing electron flux (generated by Fe protein-to-MoFe protein molar ratios from 0.1:1 to 1:1) under 2, 10, and 100% C_2D_2 at pH 7.4. Figure S-4 shows plots of both normalized total ethylene specific activity and normalized *trans*-1,2-ethylene-d_1 specific activity as a function of CO concentration at pH 7.4 using a 20:1 molar ratio of Fe protein-to-MoFe protein. Figure S-5

shows double-reciprocal plots of the specific activity of total ethylene production as a function of C_2D_2 concentration at the pH values of 6.2, 7.2, and 8.2 using a 20:1 molar ratio of Fe protein-to-MoFe protein. This material is available free of charge via the Internet at http://pubs.acs.org.

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