On reversible H₂ loss upon N₂ binding to FeMo-cofactor of nitrogenase

Zhi-Yong Yang*, Nimesh Khadka*, Dmitriy Lukoyanovb, Brian M. Hoffmanb,1, Dennis R. Deanc,1, and Lance C. Seefeldta,1

*Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322; bDepartments of Chemistry and Molecular Biosciences, Northwestern University, Evanston, IL 60208; and cDepartment of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

Contributed by Brian M. Hoffman, August 23, 2013 (sent for review July 15, 2013)

Nitrogenase is activated for N₂ reduction by the accumulation of four electrons/protons on its active site FeMo-cofactor, yielding a state, designated as E₄, which contains two iron-bridging hydrides [Fe–H–Fe]. A central puzzle of nitrogenase function is an apparently obligatory formation of one H₂ per N₂ reduced, which would “waste” two reducing equivalents and four ATP. We recently presented a draft mechanism for nitrogenase that provides an explanation for obligatory H₂ production. In this model, H₂ is produced by reductive elimination of the two bridging hydrides of E₄ during N₂ binding. This process releases H₂, yielding E₅ bound to FeMo-cofactor that is doubly reduced relative to the resting redox level, and thereby is activated to promptly generate bound diazene (HN=NH). This mechanism predicts that during turnover under D₂/N₂, the reverse reaction of D₂ with the N₂-bound product of FeMo-cofactor that is doubly reduced relative to the resting redox level, and thereby is activated to promptly generate bound diazene (HN=NH). This mechanism predicts that during turnover under D₂/N₂, the reverse reaction of D₂ with the N₂-bound product of reductive elimination would generate dideutero-E₄ (E₄(2D)), which can relax with loss of HD to the state designated E₅, with a single deuteride bridge [E₅(D)]. Neither of these deuteriated intermediate states could otherwise form in H₂O buffer. The predicted E₄(D) and E₅(2D) states are here established by intercepting them with the nonphysiological substrate acetylene (C₂H₃) to generate deuterated ethynes (C₂H₃D and C₂H₂D₂). The demonstration that gaseous H₂/D₂ can reduce a substrate other than H₂O to two ammonia (NH₃) molecules confirms the essential mechanistic role for H₂ formation, and hence a limiting stoichiometry for biological nitrogen fixation of eight electrons/protons, and provides direct experimental support for the reductive elimination mechanism.

Biological nitrogen fixation—the reduction of dinitrogen (N₂) to two ammonia (NH₃) molecules—is primarily catalyzed by the Mo-dependent nitrogenase. This enzyme comprises an electron-delivery Fe protein and MoFe protein, which contains the active site FeMo-cofactor (Fig. 1A) (1, 2). The nitrogenase catalyzed reaction is generally thought to have the limiting stoichiometry shown in Eq. 1 (3).

\[ N₂ + 8e^- + 16ATP + 8H^+ \rightarrow 2NH₃ + H₂ + 16ADP + 16P_i. \]  

This equation conveys one of the most puzzling aspects of nitrogenase function, for it incorporates an obligatory formation of one mole of H₂ per mole of N₂ reduced, which requires the waste of two reducing equivalents and four ATP (1, 2). A kinetic framework for nitrogenase function that incorporates the stoichiometry of Eq. 1 is provided by the Lowe–Thornley model (1, 2, 4), which describes transformations among catalytic intermediates (denoted Eₙ), where n is the number of electrons and protons (n = 0–8) delivered to MoFe protein. N₂ reduction requires activation of the MoFe protein to the E₄ state in which FeMo-cofactor has accumulated four electrons and four protons stored as two hydrides that bridge Fe atoms [Fe–H–Fe] and two protons presumably bound to sulfides of FeMo-cofactor (Fig. 1B) (5–8). The binding of N₂ to the E₄ state is coupled to the stoichiometric evolution of one H₂ per N₂ reduced.

We recently presented a draft mechanism for N₂ reduction by nitrogenase that incorporates a mechanistic explanation for obligatory, reversible H₂ loss upon N₂ binding (5). We considered two models for this process, both built on our characterization of the key E₅ state, and tested them against the numerous constraints imposed by turnover under N₂ plus D₂ or T₂ (5, 9–16). In particular, these constraints include the key findings that during catalytic reduction of N₂ (see Scheme S1), a molecule of D₂ or T₂ will reduce two protons to form two HD or HT without D²/T² exchange with solvent, even though neither D₂ nor T₂ by themselves react with nitrogenase during turnover under Ar (5). One model, involving protonation of one of the hydrides to form H₂, and its replacement by N₂, was shown to violate the turnover constraints, and was therefore rejected.

In the second model, H₂ is produced by reductive elimination (re) (17–20) of the two bridging hydrides of E₄ during N₂ binding (Fig. 1B and Scheme S1). This yields N₂ bound to FeMo-cofactor that has been doubly reduced relative to the resting redox level, and thereby is activated to promptly deliver two electrons plus the two protons to N₂, generating diazene (HN=NH) bound to FeMo-cofactor. The re activation of FeMo-cofactor for N₂ binding and reduction involving obligatory H₂ formation (i) provides a compelling rationale for the apparently wasteful evolution of H₂ and (ii) satisfies the constraints provided by the interplay of N₂ and H₂ binding. In particular, as schematized in Fig. 1C and Fig. S1, according to the re mechanism, D₂ or T₂ can reverse the N₂ binding equilibrium (1, 21), displace N₂ generate an E₄ intermediate with two [Fe–D/T–Fe], which do not exchange with solvent (5, 11). This intermediate in turn can relax in two steps to form two HD or HT, thereby satisfying these two key constraints. As a result, the re mechanism formed the keystone of our draft mechanism.

Subsequently, we recognized that this re mechanism for H₂ release upon N₂ binding (Fig. 1B) makes testable predictions. As
N2 binding, and thus of the eight-electron stoichiometry for nitrogenase under a mixture of D2/N2, but not under D2 alone (9, 12, 16, 23), can be explained by an re mechanism in which N2 is displaced from the E4[N2/N2H2] state by D2 through oxidative addition to form the E4(2D) state containing two Fe-bridging deuterides [Fe–D–Fe] that are not exchangeable with solvent (5, 6). In the absence of reactions with other substrates, this intermediate relaxes to the resting E0 level in a two-step process: (i) release of HD to form E0(D) followed by (ii) release of a second HD to form E0. The formation of the E2(2D) and E2(D) deuterated intermediates of Fig. 1 is unique to the re mechanism (5). In an attempt to intercept these intermediates, and thereby reveal their presence, turnover conditions were created that favor HD formation (0.25 atm N2 and 0.7 atm D2) (12), but included a small amount of 13C-acetylene (typically 0.02 atm). As indicated in Fig. 1 and in Discussion, reduction of C2H2 by E3(2D) is predicted to yield C2H2D2 and possibly C2H2D, whereas reduction by E2(D) would yield C2H2D. These studies used 13C-H2 to avoid confusion from the small but significant natural abundance of 13C (1.07%) (25) in acetylene.

Most of the ethylene formed in either the presence or absence of N2 is 13C3H4, which is detected by mass spectrometry (MS) as a peak with mass/charge (m/z) of 30, following separation from other gases by gas chromatography (GC). The 13C3H4 clearly forms by the normal acetylene reduction process, with the two protons added to C2H2 coming from solvent without intervention of D2 or N2. However, deuterated ethylenes were also formed with the full 13C3H2D2/N2 reaction mixture, and as expected from prior work, they were not formed when N2 was not included (or the reactions were conducted with H2 instead of D2), Fig. 2. A substantial amount of monodeuterated ethylene (13C2H2D; m/z = 32) was detected (Fig. 1), its rate of formation was found to be ∼27 nmol C2H2D/nmol MoFe protein over 20 min (Fig. 2), equivalent to 2% of the amount of 13C3H4 generated. Most significantly, a product with m/z of 32, corresponding to 13C2H2D2

![Figure 1](image1.png)

**Fig. 1.** FeMo-cofactor and key mechanistic steps. (A) FeMo-cofactor inorganic core with Mo in magenta, Fe in rust, S in yellow, and C in gray. The structure and numbering of Fe atoms are based on the protein database file Protein Data Bank ID code 1M1N. The face composed of Fe atoms 2, 3, 6, and 7 is noted with a red circle. (B) Proposed reductive elimination (re) mechanism for N2 binding at E4 with re of H2 and hydrogenation of N2 to generate a metal bound diazene intermediate. Colors are N in blue and H in green. Fe0 in red indicates the Fe6 valence state. (C) Predicted steps for the N2-dependent formation of 2HD (right to left) during relaxation of E4(2D) formed by D2 displacement of N2. The possible interception of the key E2(2D) and E4(D) intermediates by acetylene to produce C2H2D and C2H2D2 is shown. In the absence of information about the structural rearrangements that are likely to occur during formation of intermediates, these visualizations are based on the resting FeMo-cofactor structure.

**Results**

The earlier finding that HD is formed during turnover of nitrogenase under a mixture of D2/N2, but not under D2 alone (9, 12-16, 23), can be explained by an re mechanism in which N2 is displaced from the E4[N2/N2H2] state by D2 through oxidative addition to form the E4(2D) state containing two Fe-bridging deuterides [Fe–D–Fe] that are not exchangeable with solvent (5, 6). In the absence of reactions with other substrates, this intermediate relaxes to the resting E0 level in a two-step process: (i) release of HD to form E0(D) followed by (ii) release of a second HD to form E0. The formation of the E2(2D) and E2(D) deuterated intermediates of Fig. 1 is unique to the re mechanism (5). In an attempt to intercept these intermediates, and thereby reveal their presence, turnover conditions were created that favor HD formation (0.25 atm N2 and 0.7 atm D2) (12), but included a small amount of 13C-acetylene (typically 0.02 atm). As indicated in Fig. 1 and in Discussion, reduction of C2H2 by E3(2D) is predicted to yield C2H2D2 and possibly C2H2D, whereas reduction by E2(D) would yield C2H2D. These studies used 13C-H2 to avoid confusion from the small but significant natural abundance of 13C (1.07%) (25) in acetylene.

Most of the ethylene formed in either the presence or absence of N2 is 13C3H4, which is detected by mass spectrometry (MS) as a peak with mass/charge (m/z) of 30, following separation from other gases by gas chromatography (GC). The 13C3H4 clearly forms by the normal acetylene reduction process, with the two protons added to C2H2 coming from solvent without intervention of D2 or N2. However, deuterated ethylenes were also formed with the full 13C3H2D2/N2 reaction mixture, and as expected from prior work, they were not formed when N2 was not included (or the reactions were conducted with H2 instead of D2), Fig. 2. A substantial amount of monodeuterated ethylene (13C2H2D; m/z = 32) was detected (Fig. 1), its rate of formation was found to be ∼27 nmol C2H2D/nmol MoFe protein over 20 min (Fig. 2), equivalent to 2% of the amount of 13C3H4 generated. Most significantly, a product with m/z of 32, corresponding to 13C2H2D2

![Figure 2](image2.png)

**Fig. 2.** Time-dependent formation of mono- and dideutero 13C-ethylene catalyzed by nitrogenase reduction of 13C2H2. Shown is the formation of monodeutero-13C-ethylene (13C2H2D) as a function of time determined by GC/MS monitoring of m/z = 31 for a reaction mixture containing 13C2H2 and including D2 and N2 (●), just D2 (□), or H2 and N2 (◇). The slight increase in m/z = 31 for the +D2 and the +H2/N2 controls can be attributed to incorporation of natural-abundance D into nominally 13C3H2 reduction product; the difference between the two controls is a consequence of either or both, reaction of the N2 substrate in the +H2/N2 control and/or a consequence of a trace amount of N2 contamination in the D2 control. (Inset) Formation of dideutero-13C-ethylene (13C2H2D2; m/z = 32) monitored at m/z = 22 starting with 13C2H2D2/N2 (●), 13C2H2D/T (●), or 13C2H2D/H2 (○). The amounts of 13C2H2D2 are over 20 times higher than would arise from natural abundance D (∼0.0115%) incorporated in nominally 13C3H2. The assays were conducted with partial pressures of 0.02 atm 13C2H2, 0.25 atm N2, and 0.7 atm H2/D2, where present. The molar ratio between Fe protein and MoFe protein was 2:1. All assays were incubated at 30 °C.
was cleanly detected (Fig. 2, Inset), with a lower, although still significant, production rate of \( \sim 1.9 \text{ nmol } ^{13}\text{C}_2\text{H}_2\text{D}_2/\text{nmol MoFe protein} \) over 20 min. This species can only have been produced by \( ^{13}\text{C}_2\text{H}_2 \) intercepting \( \text{E}_4(2\text{D}) \) (Fig. 1), the intermediate formed through \( \text{N}_2 \) replacement by \( \text{D}_2 \). As indicated in Fig. 1 and Fig. S1, formation of \( ^{13}\text{C}_2\text{H}_3\text{D} \) is likely to occur primarily through \( ^{13}\text{C}_2\text{H}_2 \) intercepting \( \text{E}_2(\text{D}) \), but could also occur by an alternative channel for the reaction of \( ^{13}\text{C}_2\text{H}_2 \) with \( \text{E}_4(2\text{D}) \).

Reduction of acetylene and \( \text{N}_2 \) are mutually exclusive, with complicated inhibition kinetics between these two substrates (26). Therefore, it was of interest to determine the effect of varying the \( \text{C}_2\text{H}_2 \) partial pressure on the formation of \( ^{13}\text{C}_2\text{H}_3\text{D} \) and \( ^{13}\text{C}_2\text{H}_2\text{D}_2 \) at a fixed \( \text{N}_2 \) and \( \text{D}_2 \) concentration. As the partial pressure of \( \text{C}_2\text{H}_2 \) is increased, the amounts of \( ^{13}\text{C}_2\text{H}_3\text{D} \) and \( ^{13}\text{C}_2\text{H}_2\text{D}_2 \) expressed as fractions of total ethylene formed decrease with increasing acetylene concentration, with the strongest decrease in the fraction of \( ^{13}\text{C}_2\text{H}_3\text{D} \) (Fig. 3). The overall suppression of deuterated ethylene products can be understood as the result of increased capture by \( \text{C}_2\text{H}_2 \) of states less reduced than \( \text{E}_4[\text{N}_2/\text{N}_2\text{H}_2] \), thereby preventing the formation of this state and its reaction with \( \text{D}_2 \) which is required for the creation of deuterated ethylenes. It is also possible to explain the differential influence of increasing \( \text{C}_2\text{H}_2 \) concentration on the fraction of \( ^{13}\text{C}_2\text{H}_3\text{D} \) and \( ^{13}\text{C}_2\text{H}_2\text{D}_2 \) formed. Increasing the \( \text{C}_2\text{H}_2 \) concentration would enhance the tendency of any \( \text{E}_4(2\text{D}) \) that does form to react with the \( \text{C}_2\text{H}_2 \) to form \( ^{13}\text{C}_2\text{H}_3\text{D}_2 \), somewhat relieving the suppression of \( ^{13}\text{C}_2\text{H}_3\text{D} \) formation. This would further suppress \( ^{13}\text{C}_2\text{H}_3\text{D} \) production, as enhanced reaction of \( \text{E}_4(2\text{D}) \) would compete with HD release by \( \text{E}_4(2\text{D}) \) to form the \( \text{E}_2(\text{D}) \) state, whose reaction with \( \text{C}_2\text{H}_2 \) generates \( ^{13}\text{C}_2\text{H}_3\text{D} \).

Davis et al. showed that when both \( \text{N}_2 \) and \( \text{C}_2\text{H}_2 \) are included in a nitrogenase assay, increasing the partial pressure of \( \text{H}_2 \) increasingly diverts nitrogenase from reduction of \( \text{N}_2 \) to reduction of \( \text{C}_2\text{H}_2 \) (27), an observation interpreted as resulting from increased reaction of \( \text{H}_2 \) with \( \text{E}_4[\text{N}_2/\text{N}_2\text{H}_2] \), displacing \( \text{N}_2 \) and suppressing the formation of \( \text{NH}_3 \). The \( \text{re} \) mechanism further predicts that in the presence of \( \text{D}_2 \), the displacement of \( \text{N}_2 \) generates \( \text{E}_4(2\text{D}) \), which can release HD to form \( \text{E}_2(\text{D}) \) (Fig. 1); reaction of \( \text{C}_2\text{H}_2 \) with those deuterated \( \text{E} \) states would yield \( ^{13}\text{C}_2\text{H}_3\text{D}_2 \) and \( ^{13}\text{C}_2\text{H}_2\text{D}_2 \), respectively, as described in the previous paragraph. Correspondingly, as shown in Fig. 4, the formation of both \( ^{13}\text{C}_2\text{H}_3\text{D} \) and \( ^{13}\text{C}_2\text{H}_2\text{D}_2 \) increases in parallel with increasing \( \text{D}_2 \) partial pressure, as expected from this analysis.

The yield of \( ^{13}\text{C}_2\text{H}_3\text{D} \) and \( ^{13}\text{C}_2\text{H}_2\text{D}_2 \) similarly increases in parallel with increasing partial pressure of \( \text{N}_2 \) (Fig. 5). This can be explained by enhanced formation of \( \text{E}_4[\text{N}_2/\text{N}_2\text{H}_2] \) by reaction of \( \text{N}_2 \) with \( \text{E}_4 \). Increased formation of \( \text{E}_4[\text{N}_2/\text{N}_2\text{H}_2] \) in turn would enhance reaction with \( \text{D}_2 \) to form \( \text{E}_4(2\text{D}) \), which can be intercepted by acetylene to form deuterated ethylenes (Fig. 1).
The semilogarithmic plot of amounts of C$_2$H$_3$D and C$_2$H$_2$D$_2$ formed as a function of the electron flux, as characterized by the ratio of Fe protein to MoFe protein. The partial pressure of C$_2$H$_2$ was 0.02 atm, D$_2$ was 0.68 atm, and N$_2$ was 0.3 atm. The molar ratio of Fe protein to MoFe protein varied from 2:1 to 32:1. Assay conditions as in Fig. 3.

**Discussion**

**Predictions of the re Mechanism.** Subsequent to formulation of the re mechanism for the activation of FeMo-cofactor to reduce N$_2$ (Fig. 1) (5), we noted that addition of C$_2$H$_2$ to a N$_2$/D$_2$ reaction mixture should offer a rigorous test of the mechanism. The test is founded on a defining characteristic of nitrogenase catalysis, an exact distinction between hydrons (H/D/T) associated with the gaseous diatomics, H$_2$/D$_2$/T$_2$, and those derived from solvent water. Thus, when nitrogenase in protic buffer is turned over under N$_2$/D$_2$, gaseous D$_2$ can displace N$_2$ from the E$_4$(N$_2$/N$_2$H$_2$) state (Fig. 1), stoichiometrically yielding two HD (11). This and other observations clearly show that diatomic H$_2$/D$_2$ is not used to reduce N$_2$ during turnover under N$_2$/H$_2$/D$_2$/T$_2$ (in particular, T incorporated into the ammonia product of N$_2$ fixation would exchange with solvent) (1). Likewise, as demonstrated here (Fig. 2), when C$_2$H$_2$ is reduced in the presence of D$_2$, no deuterated ethylenes are generated.

With this foundation, we recognized that the re mechanism predicts that turnover under C$_2$H$_2$/D$_2$/N$_2$ should not only incorporate H from solvent to generate C$_2$H$_4$ by the normal reduction process, but through the agency of the added N$_2$ also should breach the separation of gaseous D$_2$ from solvent protons by generating both C$_2$H$_3$D and C$_2$H$_2$D$_2$. According to the re mechanism (Fig. 1), when turnover is carried out under N$_2$/D$_2$, D$_2$ can react with E$_4$(N$_2$/N$_2$H$_2$), replacing the N$_2$ and undergoing oxidative addition to generate E$_2$(2D). We recognized that this state in fact can be expected to react with C$_2$H$_2$ to form C$_2$H$_3$D$_2$, with the idealized mechanism (Fig. S1) involving terminalization of an [Fe–D–Fe] bridge of E$_4$ and migratory insertion of bound C$_2$H$_2$ into the Fe–D bond to form an Fe–alkenyl intermediate, followed by reductive elimination of C$_2$H$_2$D$_2$ (17, 28). Previous studies (1, 4) could not distinguish reaction at the E$_4$ state from reaction at the E$_2$ state when C$_2$H$_2$ is reduced in the absence of N$_2$, as N$_2$ is required to enable gaseous D$_2$ to enter the nitrogenase catalytic process. The possibility that acetylene can access different nitrogenase redox states, however, has been suggested on the basis of experiments using a nitrogenase variant that exhibits N$_2$ reduction that is resistant to inhibition by acetylene (29).

The E$_0$(2D) state would also relax through the loss of HD to form E$_2$(D), an E$_2$ state whose unique isotopic composition can be generated in no other way. Interception of the E$_2$(D) state by C$_2$H$_2$ would then generate C$_2$H$_3$D$_2$, with Fig. S1 presenting a plausible mechanism: hydride terminalization and insertion, followed by alkényl protonolysis (17, 28); this reaction also might occur through an alternative reaction channel of E$_4$(2D), as noted in Fig. 1 and Fig. S1.

**Testing the Predictions.** In this study, we have tested the predictions of an involvement of gaseous D$_2$ in substrate reduction by using C$_2$H$_2$ reduction under N$_2$/D$_2$/C$_2$H$_2$ gas mixtures to intercept the E$_0$(2D) and E$_2$(D) states. As expected, the control reaction of turnover under D$_2$/C$_2$H$_2$ generates only C$_2$H$_4$, without incorporation of D from gaseous D$_2$ to generate either C$_2$H$_3$D$_2$ or C$_2$H$_2$D$_2$ (Fig. 2). In dramatic contrast, C$_2$H$_2$ reduction by nitrogenase under a N$_2$/D$_2$/C$_2$H$_2$ gas mixture in fact produces readily measured amounts of C$_2$H$_3$D$_2$ and even greater amounts of C$_2$H$_2$D$_2$ (Fig. 2). The lower yield of C$_2$H$_3$D$_2$ likely indicates that the probability for binding and reduction of C$_2$H$_2$ by E$_4$(2D) is substantially lower than that for the relaxation to E$_2$(D) through loss of HD, and the reduction of C$_2$H$_2$ by E$_2$(D) (Fig. 1). These observations are enriched by the considerations of the dependence of the yields of C$_2$H$_3$D$_2$ and C$_2$H$_2$D$_2$ on the partial pressures of C$_2$H$_2$, D$_2$, N$_2$, and electron flux, all of which are understandable in terms of the production of the E$_0$(2D) and E$_2$(D) states under these turnover conditions, as predicted by the re mechanism for FeMo-cofactor activation for N$_2$ binding and reduction.

Note that the reduction of C$_2$H$_2$ to C$_2$H$_3$D by reaction with E$_4$(D) formally corresponds to the reduction of C$_2$H$_2$ by the HD that otherwise would form during relaxation of E$_2$(D) to E$_0$(D) as N$_2$ is required to enable gaseous D$_2$ to enter the nitrogenase catalytic process. The possibility that acetylene can access different nitrogenase redox states, however, has been suggested on the basis of experiments using a nitrogenase variant that exhibits N$_2$ reduction that is resistant to inhibition by acetylene (29).

The E$_2$(2D) state would also relax through the loss of HD to form E$_2$(D), an E$_2$ state whose unique isotopic composition can be generated in no other way. Interception of the E$_2$(D) state by C$_2$H$_2$ would then generate C$_2$H$_3$D$_2$, with Fig. S1 presenting a plausible mechanism: hydride terminalization and insertion, followed by alkényl protonolysis (17, 28); this reaction also might occur through an alternative reaction channel of E$_4$(2D), as noted in Fig. 1 and Fig. S1.

**Testing the Predictions.** In this study, we have tested the predictions of an involvement of gaseous D$_2$ in substrate reduction by using C$_2$H$_2$ reduction under N$_2$/D$_2$/C$_2$H$_2$ gas mixtures to intercept the E$_0$(2D) and E$_2$(D) states. As expected, the control reaction of turnover under D$_2$/C$_2$H$_2$ generates only C$_2$H$_4$, without incorporation of D from gaseous D$_2$ to generate either C$_2$H$_3$D$_2$ or C$_2$H$_2$D$_2$ (Fig. 2). In dramatic contrast, C$_2$H$_2$ reduction by nitrogenase under a N$_2$/D$_2$/C$_2$H$_2$ gas mixture in fact produces readily measured amounts of C$_2$H$_3$D$_2$ and even greater amounts of C$_2$H$_2$D$_2$ (Fig. 2). The lower yield of C$_2$H$_3$D$_2$ likely indicates that the probability for binding and reduction of C$_2$H$_2$ by E$_4$(2D) is substantially lower than that for the relaxation to E$_2$(D) through loss of HD, and the reduction of C$_2$H$_2$ by E$_2$(D) (Fig. 1). These observations are enriched by the considerations of the dependence of the yields of C$_2$H$_3$D$_2$ and C$_2$H$_2$D$_2$ on the partial pressures of C$_2$H$_2$, D$_2$, N$_2$, and electron flux, all of which are understandable in terms of the production of the E$_0$(2D) and E$_2$(D) states under these turnover conditions, as predicted by the re mechanism for FeMo-cofactor activation for N$_2$ binding and reduction.

Note that the reduction of C$_2$H$_2$ to C$_2$H$_3$D by reaction with E$_4$(D) formally corresponds to the reduction of C$_2$H$_2$ by the HD that otherwise would form during relaxation of E$_2$(D) to E$_0$(D) as N$_2$ is required to enable gaseous D$_2$ to enter the nitrogenase catalytic process. The possibility that acetylene can access different nitrogenase redox states, however, has been suggested on the basis of experiments using a nitrogenase variant that exhibits N$_2$ reduction that is resistant to inhibition by acetylene (29).

The E$_2$(2D) state would also relax through the loss of HD to form E$_2$(D), an E$_2$ state whose unique isotopic composition can be generated in no other way. Interception of the E$_2$(D) state by C$_2$H$_2$ would then generate C$_2$H$_3$D$_2$, with Fig. S1 presenting a plausible mechanism: hydride terminalization and insertion, followed by alkényl protonolysis (17, 28); this reaction also might occur through an alternative reaction channel of E$_4$(2D), as noted in Fig. 1 and Fig. S1.
Materials and Methods

Chemical Reagents and Protein Purification. Gases were purchased from Air Liquide. Ultrapure helium was purchased from Airgas. Ethylene (99.9% vol/vol) was obtained from Praxair Inc. 13C-acetylene (99 atom % 13C) and D2 (99.8 atom % D) were purchased from Sigma-Aldrich and Isotec, respectively. All other reagents were obtained from Sigma-Aldrich or Fisher Scientific and were used without further purification, unless specified otherwise. Azotobacter vinelandii strains DJ1260 (hydrogenase genes removed) and DJ884 (R1871) were grown as previously described. 

Assay Methods. All assays were conducted in 9.4-mL serum vials with gases at 25°C. All gases and liquid transfers used gas-tight syringes. Assays were done in triplicate. Data were expressed as pmol of product formed/min.

Conclusions

The incorporation of 30 atm of D2 into the nitrogenase reduction products C2H2D2 and C2H2D4 during turnover under C3H2D2N2 in H2O demonstrates the presence of the E2(2D) and E2(D) states under these conditions. This incorporation provides a very clear demonstration of the essential mechanistic role for obligatory, reversible loss of H2 upon N2 binding and thus of the eight-electron stoichiometry for nitrogen fixation by nitrogenase embodied in Eq. 1. Until now, the data indicating that some H2 must be evolved during N2 reduction have been viewed as being much more compelling than the data indicating an obligatory evolution of one H2 for every N2 reduced, leading to the stoichiometry of Eq. 1 (1).

The formation of E2(2D) and E2(D) during turnover under D2/N2 in H2O is predicted by the r mechanism for the activation of FeMo-cofactor for reduction of N2 (Fig. 1), and the interception of these intermediates by C3H2 thus provides direct experimental evidence in support of this mechanism.

The known reduction of protons by D2 to form 2HD during turnover under D2/N2 in H2O and the reductions of C2D2 by D2/N2 reported here should be viewed as being catalyzed by nitrogenase with N2 as cocatalyst. The purities of these proteins were estimated to be 95% based on denaturing polyacrylamide gel separation with Coomassie blue staining. Manipulation of proteins, solutions, and buffers was done in separate rooms in a clean area. Intensities for all ethylene peaks were established by subtraction of the background signal.

GC-MS Measurement of Products. Ethylene was quantified by analysis of the headspace gas with a Shimadzu GC-2010 gas chromatograph equipped with a programmed temperature vaporization (PTV) injector and a Shimadzu GCMS-QP2010S mass spectrometer in selected ion monitoring mode using electron ionization as the ion source. Separation of ethylenes from other gases was achieved with an Rt-Alumina Bond/PCI column (30 m, 0.32-mm i.d., and 5.0-μm film thickness) (Restek). The injector and column temperatures were set to 35°C. Ultrapure helium was used as the carrier gas set at a linear velocity of 60 cm/s. For each injection, 100 or 150 μL of headspace gas was directly injected into the PTV injector. When 13C-acetylene (C2H2) was the substrate, ethylene (C2H4, mass 28) was monitored at m/z = 28 (m), with minor species being 29 (m-1) and 30 (m+2) resulting from natural abundance 13C (D) and 13C in the acetylene. For samples using 13C2H2, ethylene (13C2H4, mass 30) was monitored as m/z = 30 (m) with minor products detected at 31 (m-1) and 32 (m+2) from natural abundance 13C. When the reaction was run with D2 in place of H2, the ethylene products CD2H2 (mass 29) and CD2D2 (mass 30) or 13C2DH2 (mass 31) and 13C2D2H2 (mass 32) were detected. Ethylene was quantified from the peak area using a standard curve generated with known amounts of ethylene in argon. It was taken that all isopropomers of ethylene had the same retention time and that the ionization and fragmentation efficiency of all isopropomers was the same. Intensities for all ethylene peaks were established by subtraction of the signal background at that retention time.

Acknowledgments. We thank Dr. Bradley D. Wahlen for his kind help with GC-MS experiments. We thank Prof. Michael Hausinger, Patrick Hufnagel, and Jonas Peters for insightful comments. This work was supported by the National Institutes of Health GM59087 (to L.C.S. and D.R.D.) and HL 13531 (to B.M.H.).


Supporting Information
Yang et al. 10.1073/pnas.1315852110

Fig. S1. Formation of deuterated acetylenes according to re mechanism. As noted in Fig. 2 legend, cartoons depict the Fe2,3,6,7 face of FeMo co as in the resting structure, and make no attempt to incorporate likely structural modifications in various states depicted. (A) Extended version of Fig. 1C, showing the reverse of re mechanism caused by displacement of N2 by D2, along with potential reaction channels for capture of E4(2D) and E4(D) intermediates with C2H2.

(B) Schematic mechanism for reaction of C2H2 with E4(2D) to form C2H2D2 following scheme 15.20 of Hartwig (1); mi = migratory insertion; re = reductive elimination. In braces: possible alternative reaction channel that leads to formation of C2H3D: ap = alkenyl protonolysis (1). (C) Schematic mechanism for formation of C2H3D from reaction of C2H2 with E4(D) (1).

Scheme S1. For convenience we include the full formulation of the two alternative models for activation of FeMo-co for N₂ reduction through H₂ release upon N₂ binding (1). (Upper) Reductive elimination (re) model, which we have adopted. (Lower) Hydride protonolysis (hp) model that we considered and rejected (1).