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Nitrogenase: A Draft Mechanism

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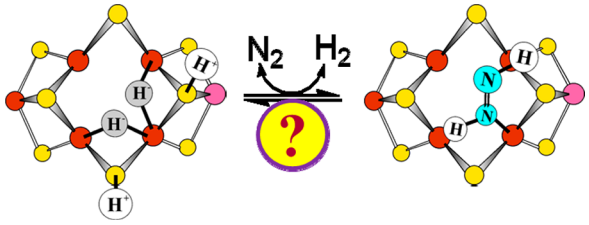

Biological nitrogen fixation, the reduction of N_2 to two NH_3 molecules, supports more than half the human population. The predominant form of the enzyme nitrogenase, which catalyzes this reaction, comprises an electron-delivery Fe protein and a catalytic MoFe protein. Although nitrogenase has been studied extensively, the catalytic mechanism has remained unknown. At a minimum, a mechanism must identify and characterize each intermediate formed during catalysis and embed these intermediates within a kinetic framework that explains their dynamic interconversion. The Lowe–Thorneley (LT) model describes nitrogenase kinetics and provides rate constants for transformations among intermediates (denoted E_n, where n is the number of electrons (and protons), that have accumulated within the MoFe protein). Until recently, however, research on purified nitrogenase had not characterized any E_n state beyond E_0.

In this Account, we summarize the recent characterization of three freeze-trapped intermediate states formed during nitrogenase catalysis and place them within the LT kinetic scheme. First we discuss the key E_4 state, which is primed for N_2 binding and reduction and which we refer to as the "Janus intermediate" because it lies halfway through the reaction cycle. This state has accumulated four reducing equivalents stored as two [Fe–H–Fe] bridging hydrides bound to the active-site iron–molybdenum cofactor ([7Fe–9S–Mo–C–homocitrate]; FeMo-co) at its resting oxidation level. The other two trapped intermediates contain reduced forms of N_2. One, intermediate, designated *I*, has $S = 1/2$ FeMo-co. Electron nuclear double resonance/hyperfine sublevel correlation (ENDOR/HYSCORE) measurements indicate that *I* is the final catalytic state, E_8, with NH_3 product bound to FeMo-co at its resting redox level. The other characterized intermediate, designated *H*, has integer-spin FeMo-co (non-Kramers; $S \geq 2$). Electron spin echo envelope modulation (ESEEM) measurements indicate that *H* contains the [$-NH_2$] fragment bound to FeMo-co and therefore corresponds to E_7.

These assignments in the context of previous studies imply a pathway in which (i) N_2 binds at E_4 with liberation of H_2, (ii) N_2 is promptly reduced to N_2H_2, (iii) the two N's are reduced in two steps to form hydrazine-bound FeMo-co, and (iv) two NH_3 are liberated in two further steps of reduction. This proposal identifies nitrogenase as following a "prompt-alternating (*P-A*)" reaction pathway and unifies the catalytic pathway with the LT kinetic framework. However, the proposal does not incorporate one of the most puzzling aspects of nitrogenase catalysis: obligatory generation of H_2 upon N_2 binding that apparently "wastes" two reducing equivalents and thus 25% of the total energy supplied by the hydrolysis of ATP. Because E_4 stores its four accumulated reducing equivalents as two bridging hydrides, we propose an answer to this puzzle based on the organometallic chemistry of hydrides and dihydrogen. We propose that H_2 release upon N_2 binding involves reductive elimination of two hydrides to yield N_2 bound to doubly reduced FeMo-co. Delivery of the two available electrons and two activating protons yields cofactor-bound diazene, in agreement with the *P-A* scheme. This keystone completes a draft mechanism for nitrogenase that both organizes the vast body of data on which it is founded and serves as a basis for future experiments.

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Introduction

Biological nitrogen fixation, the reduction of N_2 to two NH_3 molecules, supports more than half of today's human population.[1] This process is catalyzed by the nitrogenase enzymes,[2] with the best-characterized and most prevalent being the Mo-dependent enzyme discussed here.[3−6] It consists of two components, the electron-delivery Fe protein and the catalytic MoFe protein. The latter contains two remarkable metal clusters, the N_2 binding/reduction active site called the iron−molybdenum cofactor ([7Fe−9S−Mo−C−homocitrate]; FeMo-co, Figure 1), and the [8Fe−7S] P cluster, which is involved in electron transfer to FeMo-co.[3,4]

We seek to uncover the nitrogenase mechanism, which at minimum incorporates (i) a reaction pathway that identifies each intermediate that forms, beginning with resting-state nitrogenase and ending with release of the second NH_3 and return to the resting state and (ii) an understanding of the kinetics and dynamics through which these intermediate states are formed. Our efforts build on the "kinetic" foundation provided by the Lowe−Thorneley (LT) kinetic model for nitrogenase function.[3,7,8] The LT scheme, shown in a highly simplified form in Figure 2, is formulated in terms of states, denoted E_n, indexed by the number of electrons (and protons), n, that have been accumulated within the MoFe protein; it is characterized by the rate constants for transformations among those states. Single-electron transfer from Fe protein to MoFe protein is driven by the binding and hydrolysis of two MgATP within the Fe protein; the *release* of the Fe protein after delivery of its electron is the rate-limiting step of catalysis.[7]

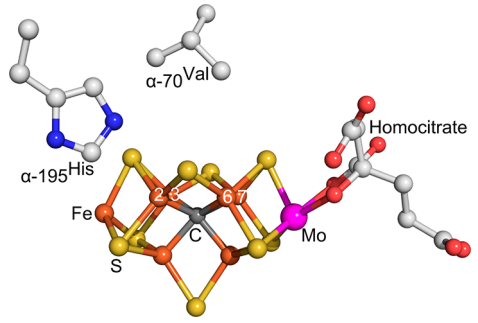


FIGURE 1. FeMo-co and key residues.

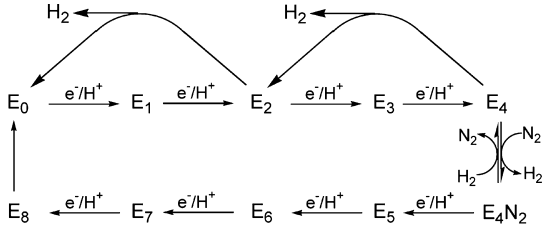


FIGURE 2. Highly simplified LT kinetic scheme, highlighting (i) correlated electron/proton delivery in eight steps, (ii) some of the possible pathways for decay by H_2 release, and (iii) N_2 binding and H_2 release at the E_4 level. LT also denote the protons added to FeMo-co (e.g., E_1H_1); for clarity, we have omitted this.

One of the most puzzling aspects of nitrogenase function embodied in the LT scheme (Figure 2) is the generation of H_2 upon N_2 binding. In the absence of other substrates, MoFe protein reduces protons to form H_2 by a cycle of electron/proton accumulation and relaxation back toward resting state, as illustrated in Figure 2. With increasing partial pressure of N_2, the reducing equivalents supplied to MoFe protein are increasingly used to produce NH_3, but it was surprisingly shown that the limiting stoichiometry for "enzymological" nitrogen fixation requires eight electrons/protons to reduce each N_2 to two NH_3,[3,7] with two electrons/protons being lost through obligatory H_2 evolution during the process.[9] Thus, there is a fundamental "mismatch" between the chemical stoichiometry of six electrons and protons per N_2 reduced and the "enzymological" stoichiometry, in which eight electrons and protons are required. This mismatch causes the index for the LT E_n intermediates in Figure 2 to take the values, $n = 0$ (resting) to 8, *not* 0 to 6. The delivery of each electron requires the hydrolysis of two MgATP, and so the optimum enzymological stoichiometry becomes

$$N_2 + 8e^- + 16MgATP + 8H^+ \Longrightarrow 2NH_3 + H_2 + 16MgADP + 16P_i \quad (1)$$

As a result, this mismatch introduces the apparent "waste" of 25% of the total energy supplied by the hydrolysis of ATP.[7] In the LT scheme, the chemical/enzymological mismatch is introduced by a reversible coupling between N_2 binding and H_2 loss, which occurs only after the MoFe protein has been activated by the accumulation of three or four electrons and protons (E_3 or E_4) (Figure 2).

The first 40 years of study of purified nitrogenase[10] did not identify any E_n catalytic intermediates beyond E_0,[11,3,12] leaving the identity of the reaction pathway unresolved.[3] The way forward was provided by microbiological experiments that showed the α-70^{Val} acts as a "gatekeeper" that sterically controls the access of substrate to the Fe6 ion at the waist of FeMo-co,[4] thereby also implicating Fe as the site of substrate binding, while α-195^{His} was inferred to be involved in proton delivery (Figure 1).[13] Substitution for one or both of

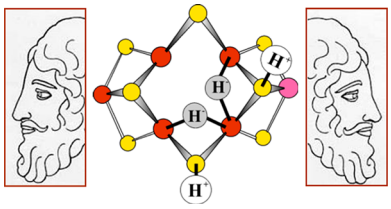


FIGURE 3. ENDOR-derived description of E_4 as containing two Fe−H−Fe moieties, emphasizing our view of the essential role of this key "Janus intermediate", which comes at the halfway point in the LT scheme and whose properties have implications for the first and second halves of the scheme. Janus image adapted from Janus12.jpg checkx-starinfinity.blogspot.com/.

these residues allowed us to freeze-quench trap a number of nitrogenase turnover intermediates, each of which shows an EPR signal arising from an $S = 1/2$ state of FeMo-co, rather than the $S = 3/2$ state of resting-state FeMo-co; this was accompanied by the trapping of an analogous state formed with the WT enzyme.[4]

This Account begins with a summary of progress since our last Account.[5] The characterization by electron nuclear double resonance/electron spin echo envelope modulation/hyperfine sublevel correlation (ENDOR/ESEEM/HYSCORE) of three freeze-trapped nitrogen fixation intermediates has enabled us to propose identities for all the LT E_n nitrogen-fixation intermediates, thereby identifying the reaction pathway and unifying it with the kinetic scheme for N_2 fixation by nitrogenase. It then offers an explanation of why Nature generates H_2 upon N_2 binding to nitrogenase. Together, these two components lead to a draft mechanism for nitrogen fixation by nitrogenase that organizes the vast efforts, by many investigators,[3,7] on which it builds, and provides a framework for future efforts.

Progress

E_4: The "Janus Intermediate". Early in the search for intermediates,[14,15] the $\alpha 70^{Val\rightarrow Ile}$ substitution in the MoFe protein was shown to deny access of all substrates to the active site, except protons.[16] Samples of this substituted MoFe protein freeze-quenched during turnover under Ar exhibited a new $S = 1/2$ EPR signal, and $^{1,2}H$ ENDOR spectroscopic analysis of this trapped state,[16] which also is observed at lower concentrations during turnover of WT enzyme under Ar,[17] revealed the presence of two metal-bridging hydrides M−H−M′. Recent ^{95}Mo ENDOR measurements established that M = M′ = Fe: FeMo-co in this state contains two Fe−H−Fe fragments (Figure 3).[18] To complete the characterization of the metal-ion core of FeMo-co in this intermediate, we recently characterized its ^{57}Fe atoms of the E_4 FeMo-co through use of a suite of advanced ENDOR methods.[19]

Annealing this intermediate in the frozen state, which prevents further delivery of electrons, showed that it relaxes to the resting state by the successive loss of two H_2 molecules, thus revealing that the trapped intermediate is the E_4 state, which has accumulated $n = 4$ electrons and four protons.[20] Examination of a simplified version of the LT scheme of Figure 2 reveals that E_4 is a key midpoint stage in the process of nitrogen fixation. Indeed, we have come to view it as the "Janus" intermediate, referring to the Roman God of transitions who is represented with two faces, one looking to the past and one looking to the future. On the one hand, looking "back" from E_4 to the steps by which it is formed, E_4 is the culmination of one-half of the electron/proton deliveries during N_2 fixation: four of the eight reducing equivalents are accumulated in E_4, before N_2 even becomes involved. Looking "forward", toward NH_3 formation, E_4 is the state at which N_2 reduction begins, and it is involved in one of the biggest puzzles in N_2 fixation, why and how is H_2 lost upon N_2 binding.

Storage of the reducing equivalents accumulated in the E_4 state as bridging hydrides has major consequences. In this binding mode, a hydride is less susceptible to protonation, and the tendency to lose H_2 (Figure 2) is thereby diminished, favoring the accumulation of reducing equivalents. This mode also lowers the ability of the hydrides to undergo exchange with protons in the environment, a characteristic that is shown to be of central importance below. However, the bridging mode also lowers hydride reactivity toward substrate reduction, relative to that of terminal hydrides.[21,22] As a result, substrate reduction most probably incorporates the conversion of hydrides from bridging to terminal binding modes.[23] We next discuss how the structure found for E_4 guides our assignment of structures for the E_1−E_3 states. Subsequently, we show how the E_4 structure defines possible mechanisms for coupling H_2 loss to N_2 binding.

E_1−E_3 and "Why Such a Big Catalytic Cluster?". Given our finding that the four accumulated electrons of E_4 do not reside on the metal ions but, instead, are formally assigned to two Fe-bridging hydrides, what then are the proper descriptions of E_1−E_3? The addition of one electron/proton to the MoFe protein results in the E_1 state. When experimentally observed in Mossbauer experiments,[24] it was presumed to contain the reduced metal-ion core of FeMo-co, denoted M^- in Figure 4, with the proton bound to sulfur. Given the presence in E_4 of *two* bridging hydrides/two protons, it is an obvious extension to propose that upon

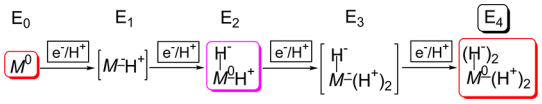


FIGURE 4. Formulation of E_1–E_3 derived from consideration of E_4. Note: M denotes FeMo-co inorganic core in its entirety; the superscript (±) represents the difference between core charge and that of core in the resting state.

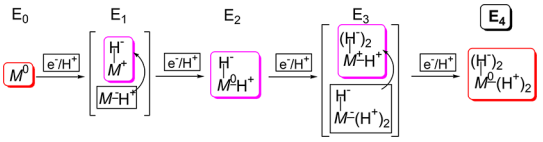


FIGURE 5. Alternative formulation of E_1–E_3 under the assumption that hydride formation occurs at each stage. As in Figure 4, M denotes FeMo-co inorganic core and superscript (±) represents difference between core charge and that of core in the resting state.

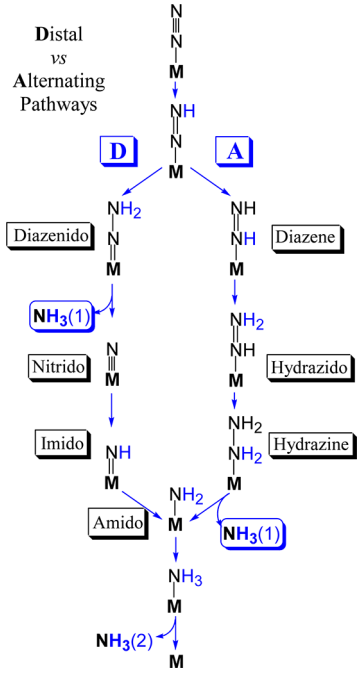
delivery of the second electron/proton to form E_2 the metal–sulfur core of the FeMo-cofactor "shuttles" both electrons onto one proton to form an Fe–H–Fe hydride, leaving the second proton bound to sulfur and the core at the resting-state, M^0, redox level (also commonly referred to as M^N), Figure 4. A subsequent, analogous, two-stage process would then yield the E_4 state, with its two Fe–H–Fe hydrides, two sulfur-bound protons, and the core at the resting-state, M^0, redox level.

Such a process of acquiring the four reducing equivalents of E_4 involves only a single redox couple connecting two formal redox levels of the FeMo-co *core* of eight metal ions, M^0 the resting state and M^- the one-electron reduced state of the core, Figure 4.[19] Indeed, comparisons of the ^{57}Fe ENDOR results for the E_4 intermediate with earlier ^{57}Fe ENDOR studies and "electron inventory analyses" of nitrogenase intermediates led us to the remarkable conclusion that throughout the nitrogenase catalytic cycle the FeMo-cofactor might cycle through only two formal redox levels of the metal-ion core. On reflection, it seems obvious that only by "storing" the equivalents as hydrides is it possible to accumulate so much reducing power at the constant potential of the Fe protein. We further proposed that such "simple" redox behavior of a complex metal center might apply to other FeS enzymes carrying out substrate reductions.[19]

Considering the critical role of hydrides in storing reducing equivalents, we also suggested that the E_1 and E_3 states might well contain one and two bridging hydrides, respectively, bound to a formally *oxidized* metal-ion core (Figure 5).[6]

If the FeMo-cofactor does not utilize more than one redox couple during catalysis, then why is it constructed from so many metal ions? As discussed below, the hydrides of E_4 bind to no fewer than three Fe atoms of a 4-Fe face of FeMo-co, as shown in Figure 3. It is further possible that catalysis is modulated by the linkage of Fe ion(s) to the anionic atom C that is centrally located within the metal–sulfur core of the FeMo-cofactor.[25,26] Formation of such a 4Fe face and the incorporation of C is not likely with less than a trigonal prism of six Fe ions linked by sulfides to generate these structural features. In this view, the trigonal prismatic FeMo-cofactor core of six Fe ions plus C generates the catalytically active 4Fe face. This prism is capped, and its properties are likely "tuned", by two "anchor" ions; one Fe plus a Mo (or a V or Fe in the alternative nitrogenases).

SCHEME 1



"Dueling" N_2 Reduction Pathways and Intermediates of N_2 Reduction. Researchers have long considered two competing proposals for the second half of the LT kinetic scheme, the reaction pathway for N_2 reduction that begins with the Janus E_4 state.[3,5,27] These invoke distinctly different intermediates, Scheme 1, and computations suggest they likely involve different metal-ion sites on FeMo-co.[27] In the "distal" (***D***) pathway, utilized by N_2-fixing inorganic Mo complexes[28] and suggested to apply in reaction at Mo of FeMo-co,[29] a single N of N_2 is hydrogenated in three steps until the first NH_3 is liberated, then the remaining nitrido-N is hydrogenated three more times to yield the second NH_3. In the "alternating" (***A***) pathway that has been suggested to apply to reaction at Fe of FeMo-co,[30] the two N's instead are hydrogenated alternately, with a hydrazine-bound state generated upon four steps of hydrogenation and the first

NH_3 only liberated during the fifth step (Scheme 1). Simple arguments can be made for both pathways.[3−5,31]

As shown by Scheme 1, characterization of catalytic intermediates formed during the reduction of N_2 could distinguish between the ***D*** and ***A*** pathways. However, such intermediates had long eluded capture until four intermediates associated with N_2 fixation were freeze-trapped for ENDOR spectroscopic studies.[4,5] These four were generated under the hypothesis that intermediates associated with different reduction stages could be trapped using N_2 or semireduced forms of N_2 or their analogs: N_2; NH═NH; NH═N−CH_3; H_2N-NH_2.[3,4]

Intermediate *I*. A combination of X/Q-band EPR and ^{15}N,$^{1,2}H$ ENDOR measurements on the intermediates formed with the three semireduced substrates during turnover of the α-$70^{Val\rightarrow Ala}$, α-$195^{His\rightarrow Gln}$ MoFe protein subsequently showed that in fact they all correspond to a common intermediate (here denoted ***I***) in which FeMo-co binds a substrate-derived $[N_xH_y]$ moiety.[4,5] Thus, both the diazenes and hydrazine enter and "flow through" the N_2-reduction pathway (Scheme 1), and the diazene reduction must have "caught up" with the "later" hydrazine reaction.

$^{1,2}H$ and ^{15}N 35 GHz CW and pulsed ENDOR measurements next showed that ***I*** exists in two conformers, each with metal ion(s) in FeMo-co having bound a single nitrogen from a substrate-derived $[N_xH_y]$ fragment.[4,5] Subsequent high-resolution 35 GHz pulsed ENDOR spectra and X-band HYSCORE measurements showed *no* response from a second nitrogen atom, and when ***I*** was trapped during turnover with the selectively labeled $CH_3-{}^{15}N$═NH, $^{13}CH_3-N$═NH, or C^2H_3-N═NH, no signal was seen from the isotopic labels.[31] From these results, we concluded the N−N linkage had been cleaved in forming ***I***, which thus represents a late stage of nitrogen fixation, after the first ammonia molecule already has been released[31] and only a $[NH_x]$ ($x = 2$ or 3) fragment of substrate is bound to FeMo-co.

The Nitrogenase Reaction Pathway: *D* vs *A*. Given that states that could correspond to ***I*** are reached by both ***A*** and ***D*** pathways (Scheme 1), the *identity* of this $[NH_x]$ moiety need not in itself distinguish between pathways. However, the spectroscopic findings about ***I***, in conjunction with a variety of additional considerations, led us to propose that nitrogenase functions via the ***A*** reaction pathway of Scheme 1 for reduction of N_2.[31] As one example, to explain how nitrogenase could reduce each of the substrates, N_2, N_2H_2, and N_2H_4, to two NH_3 molecules via a common ***A*** reaction pathway, one need only postulate that each substrate "joins" the pathway at the appropriate stage of reduction, binding to FeMo-co that has been "activated" by accumulation of a sufficient number of electrons (possibly with FeMo-co reorganization) and then proceeds along that pathway. Energetic considerations,[27] in combination with the strong influence of α-70^{Val} substitutions of MoFe protein *without* modification of FeMo-co reactivity, then implicate Fe, rather than Mo, as the site of binding and reactivity.[16]

As further support of this conclusion, it is most economical to suggest that both the Mo-dependent nitrogenase studied here and the V-dependent nitrogenase[2] reduce N_2 by the same pathway. Because V-nitrogenase produces traces of N_2H_4 while reducing N_2 to NH_3,[32] then according to Scheme 1 this enzyme clearly functions via the ***A*** pathway, implying the same is true for Mo-nitrogenase.

Intermediate *H*. When nitrogenase is freeze-quenched during turnover, the EPR signals from trapped intermediates in odd-electron FeMo-co states (Kramers states; $S = 1/2$, 3/2,...; E_n, $n =$ even),[4,5] and the signals from residual resting-state FeMo-co never quantitate to the total FeMo-co present, indicating that EPR-silent states of FeMo-co must also exist. These silent MoFe protein states must contain FeMo-co with an even number of electrons, and thus correspond to E_n, $n =$ odd ($n = 2m + 1$, $m = 0-3$) intermediates in the LT scheme. Such states may contain diamagnetic FeMo-co or FeMo-co in integer-spin ($S = 1$, 2, ...) "non-Kramers (NK)" states,[24,33] but no EPR signal from an integer-spin form of FeMo-co had been detected. However, careful examination of samples that contain intermediate ***I***[4,5] now have revealed a broad EPR signal at low field in Q-band spectra that arises from an integer-spin system with a ground-state non-Kramers doublet.[34]

In earlier work, we showed how to characterize a non-Kramers doublet with ESEEM spectroscopy (NK-ESEEM),[35] so NK-ESEEM time-waves were collected for the NK intermediates trapped during turnover with ^{14}N and ^{15}N isotopologs of N_2H_2 and N_2H_4 substrates; [95] Mo-enriched α-$70^{Val\rightarrow Ala}$, α-$195^{His\rightarrow Gln}$ MoFe protein; $H-{}^{14}N={}^{14}N-CH_3$, $H-{}^{15}N={}^{14}N-CH_3$, and $H-{}^{14}N={}^{14}N-CD_3$. The measurements indicate that the NK-EPR signals arise from FeMo-co in an integer-spin state, $S \geq 2$, that this state corresponds to a common intermediate, ***H***, formed with all of these substrates, and that ***H*** binds an NH_x fragment formed after cleavage of the N−N bond of substrate bound to FeMo-co and loss of NH_3. Quadrupole coupling parameters for the NH_x fragment indicate that it is not NH_3 and that ***H*** binds $[-NH_2]$.

Assignment of the Nitrogenase Reaction Pathway and Unification with the LT Kinetic Scheme. The ***H*** and ***I*** intermediates provide "anchor points" that allow assignment of the complete set of E_n intermediates that follow E_4, $5 \leq n \leq 8$.

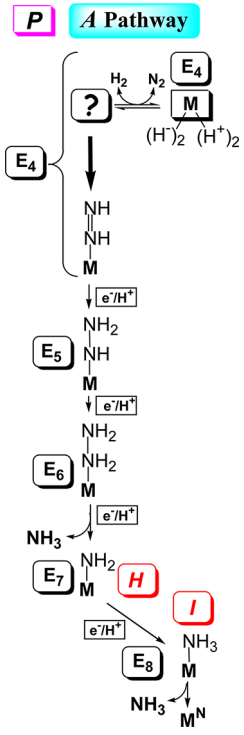


FIGURE 6. Integration of LT kinetic scheme with alternating (***A***) pathways for N_2 reduction. M again denotes FeMo-co core, and substrate-derived species are drawn to indicate stoichiometry only, not mode of substrate binding. Bold arrow indicates transfer to substrate of two reducing equivalents remaining after N_2 binding in E_4; ***P*** represents "prompt" transfer. E_n states, n = even, are Kramers states; n = odd are non-Kramers. M^N denotes resting-state FeMo-co. Adapted from ref 29.

The loss of two reducing equivalents and two protons as H_2 (eq 1) upon N_2 binding to the FeMo-co of E_4 leaves FeMo-co activated by two reducing equivalents and two protons. We argued that the enzyme could only be following a "prompt" (***P***)-alternating pathway: when N_2 binds to FeMo-co, it is "nailed down" by prompt hydrogenation, Figure 6, with N_2 binding, H_2 loss, and reduction to diazene all occurring at the E_4 kinetic stage of the LT scheme.[31] The identification of ***H*** with its E_n stage is achieved as follows. (i) Because the same intermediate ***H*** is formed during turnover with the two diazenes and with hydrazine, the diazenes must have catalytically "caught up" to hydrazine, and ***H*** must occur at or after the appearance of a hydrazine-bound intermediate. (ii) As noted above, ***H*** contains FeMo-co in an integer-spin (NK) state and thus corresponds to an E_n state with n = odd. Because ***H*** is a common intermediate that contains a bound fragment of substrate, it must, therefore, correspond to E_5 or E_7. But ***H*** cannot be E_5 on the ***P*** pathway, because it comes *before* hydrazine appears (Figure 6). (iii) Thus, ***H*** corresponds to the $[NH_2]^-$-bound intermediate formed by N−N bond cleavage at the E_7 stage.

By parallel arguments, the only possible assignment for the $S = 1/2$ state ***I***, which we showed earlier to occur after N−N bond cleavage,[31] is as E_8: ***I*** must correspond to the final state in the catalytic process (Figure 6), in which the NH_3 product is bound to FeMo-co at its resting redox state, prior to release and regeneration of the resting-state form of the cofactor. The trapping of a product-bound intermediate ***I*** is analogous to the trapping of a bio-organometallic intermediate during turnover of the α-$70^{Val\rightarrow Ala}$ MoFe protein with the alkyne, propargyl alcohol; this intermediate was shown to contain the FeMo-co-bound allyl alcohol alkene product of reduction.[36] With assignments of E_4, E_7, and E_8, then filling in the LT "boxes" for E_5 and E_6 is straightforward, Scheme 1, thus unifying the reaction pathway for N_2 reduction with the LT scheme.

The Obligatory Evolution of H_2 in Nitrogen Fixation. Although the E_n assignments of Figure 4 (or Figure 5) and those of Figure 6 give proposed structures to all E_n states of the LT kinetic scheme (Figure 2), they have been developed through independent analyses of the two four-electron halves of the eight-electron catalytic cycle (eq 1) as linked by the E_4 Janus intermediate, Figure 3: (i) the 4-electron/proton activation of FeMo-co to generate E_4; (ii) the reduction of bound N_2 by two of those electrons plus an additional four electrons. However, the analysis so far is silent about the mechanism that connects these two halves: the obligatory production of an H_2 molecule upon N_2 binding (eq 1).[9] Why nitrogenase should "waste" fully 25% of the ATP required for nitrogen fixation through H_2 generation (eq 1) has remained a mystery and indeed is not even accepted uniformly.[37,38]

Consideration of the finding that E_4 stores its four reducing equivalents as two bridging hydrides (Figure 3) within the context of the well-known organometallic chemistries of hydrides[21,39] and dihydrogen[40] leads us to propose a "reductive elimination (*re*)" mechanism,[22] Figure 7, by which this state binds and activates N_2 with release of H_2, along with the prompt formation of FeMo-co-bound diazene (N_2H_2) featured in the ***P-A*** reaction pathway, Figure 6.[41]

The *re* Mechanism for N_2 Binding and Diazene Formation. The *re* mechanism for H_2 loss upon N_2 binding begins with transient terminalization of both E_4 hydrides, Figure 7, followed by reductive elimination of H_2 as N_2 binds, steps with strong precedence.[21,39,40] Of key importance, the departing H_2 carries away only two of the four reducing equivalents stored in E_4, while the Fe that binds N_2 becomes highly activated through formal reduction by two equivalents, for example, from formal redox states of Fe(II) to Fe(0).

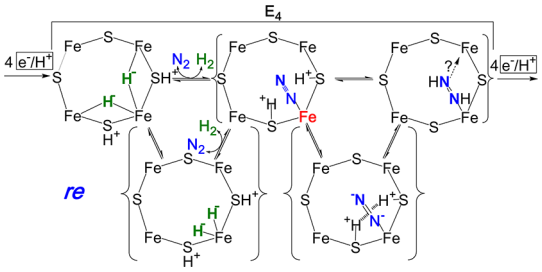


FIGURE 7. Visualization of *re* mechanism for H_2 release upon N_2 (blue) binding to E_4. Shown, the Fe-2,3,6,7 face of resting FeMo-co; the structure of FeMo-co may vary in different stages of catalysis. The Fe shown binding N_2 is presumed to be Fe6, as indicated by studies of α-70^{Val} variants; when bold, red, formally Fe(0) (see text). The two bridging hydrides of E_4 (green) are positioned as suggested by *re* mechanism of H_2 release upon N_2 binding. Alternative binding modes for N_2-derived species can be envisaged.

CHART 1. Key Constraints on HD Formation under N_2/D_2

(i) Stoichiometry: $\mathbf{M\text{-}N_2 + D_2 + 2H^+ + 2e \Rightarrow 2HD + M + N_2}$
(ii) Scrambling: **'No' T^+ released to solvent under T_2**
(iii) Reduction Stage: **D_2 reacts at 'N_2H_2' level**

Such a highly reduced Fe is poised to deliver the two activating electrons to N_2, whose π acidity could be further enhanced by electrostatic interactions with the two sulfur-bound protons; coupled delivery of the two activating protons would yield cofactor-bound diazene. The diminished electron donation to Fe by protonated sulfides (possibly even with breakage of Fe−S bond(s)) not only would facilitate reductive elimination but also would act to localize the added electrons on the Fe involved, instead of delocalizing the charge over the rest of the cofactor. This mechanism provides a compelling rationale for obligatory H_2 formation during N_2 reduction: the transient formation of a state in which an electrostatically activated N_2 is bound to a doubly reduced site, thereby generating a state optimally activated to carry out the initial hydrogenations of N_2, the most difficult process in N_2 fixation.

Strong support for the *re* mechanisms is obtained from tests against the numerous constraints provided by the interplay of N_2 and H_2 binding, the three principal ones being listed in Chart 1. The first test of a mechanism is that it accommodate the finding that when nitrogenase turns over in the presence of *both* N_2 and D_2, then two HD are formed through D_2 cleavage and solvent-proton reduction, with the stoichiometry summarized as constraint (i) of Chart 1.[3,42] Such HD formation *only* occurs in the presence of N_2 and not during reduction of H^+ or *any* other substrate.

The second key constraint was revealed by Burgess and co-workers 30 years ago; the absence of exchange into

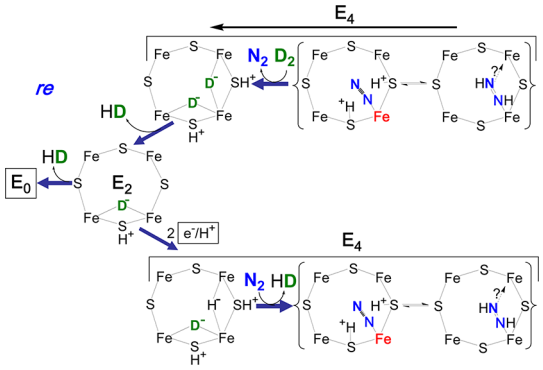


FIGURE 8. Reversal of *re* mechanism upon D_2 binding. Details as in Figure 7.

solvent of D^+/T^+ derived from D_2/T_2 gas, Chart 1, (ii).[43] When nitrogenase turns over under a mixture of N_2 and T_2, HT is formed with stoichiometry corresponding to Chart 1, (i), but during this process only a negligible amount of T^+ is released to solvent (~2%). A later study of α-195^{His}- and α-191^{Gln}-substituted MoFe proteins provided persuasive evidence that HD formation under N_2/D_2 requires that the enzyme be at least at the E_4 redox level, with a FeMo-co-bound N−N species at the reduction level of N_2H_2 or beyond, corresponding to the third constraint, Chart 1, (iii).[44] Constraint (iii) and the stoichiometry of HD formation according to (i) implies a process described as

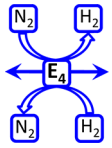
$$N_2H_2 + D_2 \rightarrow N_2 + 2HD \qquad (2)$$

and thus that N_2H_2 formation is reversible, as shown in Figure 7.

In the reverse of the *re* mechanism, shown in Figure 8, D_2 binding and N_2 release would generate E_4 in which *both* atoms of D_2 exist as deuteride bridges. One possible fate of this state would be relaxation to E_2 with loss of HD, then to E_0 with loss of the second HD, thus satisfying the stoichiometry of eq 2. If the reaction were carried out under T_2, essentially no T^+ would be lost to solvent because the bridging tritides are deactivated for exchange with the protein environment and solvent, thus satisfying the "T^+ exchange" constraint, Chart 1.

As one alternative fate of the doubly deuterated E_4 formed by D_2 replacement of N_2, it could rebind a N_2, which would merely release the D_2 that had started the reverse process, creating a cycle invisible to detection. As a second alternative, the monodeutero E_2 state could acquire two additional electrons/protons to achieve the monodeutero E_4 state. As shown in Figure 8, if this state then bound N_2 it would

SCHEME 2



release the second HD, again without solvent exchange. Thus, the *re* mechanism for N_2 binding and H_2 release not only has the compelling chemical rationale discussed above but also satisfies the three critical HD constraints for the various alternatives that arise when it is run in reverse, Chart 1.

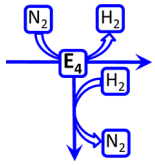
But what if the exogenous D_2 binds to and reacts with N_2-bound FeMo-co not by the reverse of the enzymatic reaction pathway (Scheme 2), but by a "*branch* pathway" (Scheme 3) that leads back toward the resting state by a sequence of steps different from that which generated the N_2-bound state? As discussed in Supporting Information, such a branch to the *re* mechanism also would satisfy the HD constraints.

Finally, other constraints on the nitrogenase mechanism have been generated, most especially those associated with D_2 binding and HD formation (see Supporting Information),[3,42] and our analysis indicates that the reverse-*re* pathway for HD formation satisfies these, as well.

In summary, the *re* mechanism, Figure 7, satisfies both the stoichiometric constraint of HD formation (Chart 1, (i)) and the "T^+" constraint against exchange of gas-derived hydrons with solvent (Chart 1, (ii)). The *re* mechanism further involves D_2 binding to a state at the "diazene level" of reduction, as required by the constraint of eq 2 and Chart 1, (iii). Finally, to the best of our knowledge, all other constraints on the mechanism, most of which are not directly tied to D_2 binding, are satisfied as well.

This mechanism answers the long-standing and oft-repeated question, "Why does Nature "waste" four ATP/two reducing equivalents through an obligatory loss of H_2 when N_2 binds?" The answer: *reductive elimination of H_2 upon binding of N_2 to FeMo-co of the E_4 state generates a state in which highly reduced FeMo-co binds N_2, which likely is activated for reduction through electrostatic interactions with the remaining two sulfur-bound protons*. Transfer of the two reducing equivalents generated by the reductive elimination, combined with transfer of the two activating protons, then forms diazene, Figure 7, in keeping with the ***P-A*** scheme of Figure 6. It appears that only through this activation is the enzyme able to hydrogenate N_2.

SCHEME 3



Conclusions

The trapping and characterization of three of the eight electron-reduction stages involved in nitrogen fixation by nitrogenase (eq 1), including the "Janus intermediate", E_4, and the nitrogenous intermediate states ***H*** and ***I*** have identified the "prompt-alternating (***P-A***)" pathway of Figure 6 as most likely operative for nitrogenase and led to the first unification of the nitrogenase reaction pathway and the LT kinetic scheme. This unification is completed, thereby generating a first-draft mechanism for biological nitrogen fixation, with the proposed *re* mechanism for H_2 release upon N_2 reduction by nitrogenase, Figure 7, described in this Account for the first time. This mechanism will serve as a basis for future experiments to test, refine, correct as needed, and extend it.

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Supporting Information. Representative electron/proton distribution for E_1-E_4 and a discussion of the alternative *hp* mechanism. This material is available free of charge via the Internet at http://pubs.acs.org.

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FOOTNOTES

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The authors declare no competing financial interest.

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