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Nitrogenase: A Draft Mechanism

Brian M. Hoffman^{†,*}, Dmitriy Lukoyanov[†], Dennis R. Dean^{#,*}, and Lance C. Seefeldt^{§,*}

[†]Department of Chemistry, Northwestern University, Evanston, Illinois 60208

[§]Department of Chemistry and Biochemistry, Utah State University, Logan Utah 84322

[#]Department of Biochemistry, Virginia Tech University, Blacksburg, Virginia 24061.

Conspectus

Biological nitrogen fixation — the reduction of N₂ to two NH₃ molecules — supports more than half the human population. This reaction is catalyzed by the enzyme nitrogenase, whose predominant form, discussed here, comprises an electron-delivery Fe protein and a catalytic MoFe protein. Nitrogenase has been studied extensively but the catalytic mechanism has remained unknown. At 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. Nitrogenase kinetics have been described by the Lowe-Thorneley (LT) model, which provides rate constants for transformations among intermediates, denoted E_n, indexed by the number of electrons (and protons), *n*, that have been accumulated within the MoFe protein. However, until recently, research on purified nitrogenase had not resulted in characterization of any E_n state beyond E₀.

In this article we summarize the recent characterization of three freeze-trapped intermediate states formed during nitrogenase catalysis, and their placement within the LT kinetic scheme. First we discuss the key E₄ state, which is primed for N₂ binding and reduction and which we refer to as the “Janus intermediate”. This state contains the active-site iron-molybdenum cofactor ([7Fe-9S-Mo-C-homocitrate]; FeMo-co) at its resting oxidation level, its four accumulated reducing equivalents being stored as two [Fe-H-Fe] bridging hydrides. The other two trapped intermediates contain reduced forms of N₂. One, intermediate **I**, has *S* = 1/2 FeMo-co. ENDOR/HYSCORE measurements indicate that **I**, is the final catalytic state, E₈, having NH₃ product bound to FeMo-co at its resting redox level. The other characterized intermediate, designated **H**, has integer-spin FeMo-co (Non-Kramers; *S* = 2). ESEEM measurements indicate that **H** binds the [–NH₂] fragment and therefore corresponds to E₇. These assignments, plus consideration of previous studies, imply a pathway in which (i) N₂ binds at E₄ with liberation of H₂, (ii) N₂ is promptly reduced to N₂H₂, (iii) the two N’s are hydrogenated alternately to form hydrazine-bound FeMo-co, and (iv) two NH₃ 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, it does not incorporate one of the most puzzling aspects of nitrogenase catalysis: obligatory generation of H₂ upon N₂ binding that apparently ‘wastes’ two reducing equivalents and thus 25% of the total energy supplied by the hydrolysis of ATP. The finding that E₄ stores its four accumulated reducing equivalents as two bridging hydrides, considered in the context of the organometallic chemistries of hydrides and dihydrogen, leads us to propose an answer to this puzzle. Namely, that H₂ release upon N₂ binding involves reductive elimination of two hydrides to yield N₂ bound to doubly reduced Fe. Coupled delivery of the two available electrons and two activating protons yields cofactor-bound diazene, in keeping with the **P-A** scheme. This keystone completes a draft mechanism for nitrogenase that

*Corresponding Authors: bmh@northwestern.edu, phone 847-491-3104, (B.M.H.); lance.seefeldt@usu.edu, phone 435-797-3964, (L.C.S.); deandr@vt.edu, phone 540-231-5895, (D.R.D).

organizes the vast body of data upon which it is formulated, and is intended to serve as a basis for future experiments.

Introduction

Biological nitrogen fixation — the reduction of N₂ to two NH₃ molecules — supports more than one-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 N₂ binding/reduction active site called the iron-molybdenum cofactor ([7Fe-9S-Mo-C-homocitrate]; FeMo-co, Fig 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 endings with release of the second NH₃ and return to the resting state; (ii) an understanding of the kinetics/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 Fig 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

One of the most puzzling aspects of nitrogenase function embodied in the LT scheme (Fig 2) is the generation of H₂ upon N₂ binding. In the absence of other substrates, MoFe protein reduces protons to form H₂ by a cycle of electron/proton accumulation and relaxation back towards resting state, as illustrated in Fig 2. With increasing partial pressure of N₂, the reducing equivalents supplied to MoFe protein are increasingly used to produce NH₃, but it was surprisingly shown that the limiting stoichiometry for 'enzymological' nitrogen fixation requires eight electrons/protons to reduce each N₂ to two NH₃,^{3,7} with two electrons/protons being lost through obligatory H₂ evolution during the process.⁹ Thus, there is a fundamental 'mismatch' between the chemical stoichiometry of six electron and protons per N₂ 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 Fig 2 to take the values, *n* = 0 (resting) ↔ 8, *not* 0 ↔ 6. The delivery of each electron requires the hydrolysis of two MgATP, and so the optimum enzymological stoichiometry becomes,

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 N₂ binding and H₂ loss, which occurs only after the MoFe protein has been activated by the accumulation of three or four electrons and protons (E₃ or E₄) (Fig 2).

The first forty years of study of purified nitrogenase¹⁰ did not identify any E_n catalytic intermediates beyond E₀,^{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 (Fig 1).¹³ Substitution for one or both of 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 report begins with a summary of progress since our last Account.⁵ The characterization by 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 N₂ fixation by nitrogenase. It then offers an explanation of why Nature generates H₂ upon N₂ 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

E₄: the ‘Janus Intermediate’

Early in the search for intermediates,^{14,15} the α 70^{Val→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 ⁹⁵Mo ENDOR measurements established that $M = M = Fe$: FeMo-co in this state contains two Fe-H-Fe fragments (Fig 3).¹⁸ To complete the characterization of the metal-ion core of FeMo-co in this intermediate, we recently characterized its ⁵⁷Fe atoms of the E₄ 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 H₂ molecules, thus revealing that the trapped intermediate is the E₄ state, which has accumulated $n = 4$ electrons and four protons.²⁰ Examination of a simplified version of the LT scheme of Fig 2 reveals that E₄ is a key 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 E₄ to the steps by which it is formed, E₄ is the culmination of one-half of the electron/proton deliveries during N₂ fixation: four of the eight reducing equivalents are accumulated in E₄, before N₂ even becomes involved. Looking ‘forward’, towards NH₃ formation, E₄ is the state at which N₂ hydrogenation begins, and it is involved in one of the biggest puzzles in N₂ fixation, ‘why’ and ‘how’ is H₂ lost upon N₂ binding.

Storage of the reducing equivalents accumulated in the E₄ state as bridging hydrides has major consequences. In this binding mode a hydride is less susceptible to protonation, and the tendency to lose H₂ (Fig 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 towards substrate hydrogenation, relative to that of terminal hydrides.^{21,22} As a result, substrate hydrogenation most probably incorporates the conversion of hydrides from bridging to terminal binding modes.²³ We next discuss how the structure found for E₄ guides our assignment of structures for the E₁-E₃

states. Subsequently, we show how the E_4 structure defined 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 reside not 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 Fig 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 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), Fig 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, Fig 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 respectively might well contain one and two bridging hydrides bound to a formally *oxidized* metal-ion core (Fig 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 Fig 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).

‘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 complexes²⁸ 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

NH₃. 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 NH₃ only liberated during the fifth step (Scheme 1). Simple arguments can be made for both pathways.³⁻⁵³¹

As shown by Scheme 1, characterization of catalytic intermediates formed during the reduction of N₂ could distinguish between the **D** and **A** pathways. However, such intermediates had long eluded capture until four intermediates associated with N₂ 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 N₂ or semi-reduced forms of N₂ or their analogs: N₂; NH=NH; NH=N-CH₃; H₂N-NH₂.^{3,4}

Intermediate I—A combination of X/Q-band EPR and ¹⁵N, ^{1,2}H ENDOR measurements on the intermediates formed with the three semi-reduced substrates during turnover of the α-70^{Val→Ala}, α6195^{His→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 normal N₂-reduction pathway (Scheme 1), and the diazene reduction must have ‘caught up’ with the ‘later’ hydrazine reaction.

^{1,2}H and ¹⁵N 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 CH₃-¹⁵N=NH, ¹³CH₃-N=NH, or C²H₃-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 released³¹ 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 N₂.³¹ As one example, to explain how nitrogenase could reduce each of the substrates, N₂, N₂H₂ and N₂H₄, to two NH₃ 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 nitrogenase² reduce N₂ by the same pathway. As V-nitrogenase produces traces of N₂H₄ while reducing N₂ to NH₃,³² 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; $S = 1/2, 3/2, \dots$; E_n , $n = \text{even}$),^{4,5} plus 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 , $n = \text{odd}$ ($n = 2m + 1$, $m = 0-3$) intermediates in the LT scheme. Such states may contain diamagnetic FeMo-co, or FeMo-co in integer-spin ($S = 1, 2, \dots$), ‘non-Kramers (NK)’ states,²⁴³³ 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 $\alpha\text{-70}^{\text{Val}\rightarrow\text{Ala}}$, $\alpha\text{-195}^{\text{His}\rightarrow\text{Gln}}$ MoFe protein; $\text{H-}^{14}\text{N}=\text{N-}^{14}\text{N-CH}_3$, $\text{H-}^{15}\text{N}=\text{N-}^{14}\text{N-CH}_3$, and $\text{H-}^{14}\text{N}=\text{N-}^{14}\text{N-CD}_3$. The measurements indicate that the NK-EPR signals arises from FeMo-co in an integer-spin state, $S = 2$, 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 it is not NH_3 , and that **H** binds as $[-\text{NH}_2]$.

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 , $5 \leq n \leq 8$. 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, Fig 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) As 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 $n = \text{odd}$. As **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, for it comes *before* hydrazine appears (Fig 6). (iii) Thus, **H** corresponds to the $[\text{NH}_2]^-$ -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 (Fig 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 $\alpha\text{-70}^{\text{Val}\rightarrow\text{Ala}}$ MoFe protein with the alkyne, propargyl alcohol; this intermediate was shown to be the allyl alcohol alkene product of reduction³⁶. With assignments, of E_4 , E_7 , and E_8 , then filling in the LT ‘boxes’ for E_5 , E_6 , is straightforward, Scheme 1, thus unifying the reaction pathway for N_2 reduction with the LT scheme.

The obligatory evolution of H₂ in nitrogen fixation

Although the E_n assignments of Fig 4 (or Fig 5) plus those of Fig 6, give proposed structures to all E_n states of the LT kinetic scheme (Fig 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 E₄ Janus intermediate, Fig 3: (i) the 4-electron/proton activation of FeMo-co to generate E₄; (ii) the reduction of bound N₂ 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 H₂ molecule upon N₂ binding (eq 1).⁹ Why nitrogenase should 'waste' fully 25% of the ATP required for nitrogen fixation through H₂ generation (eq 1) has remained a mystery, and indeed is not even accepted uniformly.³⁷³⁸

Consideration of the finding that E₄ stores its four reducing equivalents as two bridging hydrides (Fig 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 Fig 7, by which this state binds and activates N₂ with release of H₂, along with the prompt formation of FeMo-co-bound diazene (N₂H₂) featured in the *P-A* reaction pathway, Fig 6.⁴¹

The *re* mechanism for N₂ binding and diazene formation

The *re* mechanism for H₂ loss upon N₂ binding begins with transient terminalization of *both* E₄ hydrides, Fig 7, followed by reductive elimination of H₂ as N₂ binds, steps with strong precedence.^{21,3940} Of key importance, the departing H₂ carries away only two of the four reducing equivalents stored in E₄, while the Fe that binds N₂ becomes highly activated through formal reduction by two equivalents, for example from formal redox states of Fe(II) to Fe(0). Such a highly reduced Fe is poised to deliver the two activating electrons to N₂, 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)) would not only 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 H₂ formation during N₂ reduction: the transient formation of a state in which an electrostatically activated N₂ is bound to a highly activated, doubly-reduced site, thereby generating a state optimally activated to carry out the initial hydrogenations of N₂, the most difficult process in N₂ fixation.

Strong support for the *re* mechanisms is obtained from tests against the numerous constraints provided by the interplay of N₂ and H₂ 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* N₂ and D₂, then two HD are formed through D₂ 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 N₂, and not during reduction of H⁺ or *any* other substrate.

The second key constraint was revealed by Burgess and coworkers thirty years ago; the absence of exchange into solvent of D⁺/T⁺ derived from D₂/T₂ gas, Chart 1, (ii).⁴³ When nitrogenase turns over under a mixture of N₂ and T₂, 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 N₂/D₂ requires that the enzyme be at least at the E₄ redox level, with a FeMo-co-bound N-N species at the reduction

level of N₂H₂ or beyond, corresponding to the third constraint, Chart 1, (iii).⁴⁴ Constraint (iii), plus the stoichiometry of HD formation according to (i) implies a process described as,

and thus that N₂H₂ formation is reversible, as shown in Fig 7.

In the reverse of the *re* mechanism, shown in Fig 8, D₂ binding and N₂ release would generate E₄ in which *both* atoms of D₂ exist as deuteride bridges. One possible fate of this state would be relaxation to E₂ with loss of HD, then to E₀ with loss of the second HD, thus satisfying the stoichiometry of eq 2. If the reaction were carried out under T₂, essentially no T⁺ would be lost to solvent because the bridging deuterides 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 E₄ formed by D₂ replacement of N₂, it could rebind an N₂, which would merely release the D₂ that had started the reverse process, creating a cycle invisible to detection. As a second alternative, the mono-deutero E₂ state could acquire two additional electrons/protons to achieve the mono-deutero E₄ state. As shown in Fig 8 if this state then bound N₂ it would release the second HD, again without solvent exchange. Thus, the *re* mechanism for N₂ binding and H₂ 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 D₂ binds to and reacts with N₂-bound FeMo-co not by the reverse of the enzymatic reaction pathway (Scheme 2), but by a '*branch* pathway' (Scheme 3) that leads back towards the resting state by a sequence of steps different from that which generated the N₂-bound state? As discussed in SI, 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 D₂ binding and HD formation (See SI),^{3,42} and our analysis indicates that the reverse-*re* pathway for HD formation satisfies these, as well.

In summary, the *re* mechanism, Fig 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 D₂ 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 D₂ 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 H₂ when N₂ binds?" The answer: reductive elimination of H₂ upon binding of N₂ to FeMo-co of the E₄ state generates a state in which highly reduced FeMo-co binds N₂, 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, Fig 7, in keeping with the *P*-*A* scheme of Fig 6. It appears that only through this activation is the enzyme able to hydrogenate N₂.

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 Fig 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, Fig 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biographical Information

Dennis R. Dean received a B.A. from Wabash College and a Ph.D. from Purdue University. He is currently a University Distinguished Professor at Virginia Tech where he also serves as the Director of the Fralin Life Science Institute and the Virginia Bioinformatics Institute.

Brian M. Hoffman was an undergraduate at the University of Chicago, received his Ph.D. from Caltech, and spent a postdoctoral year at MIT. From there he went to Northwestern University, where he is the Charles E. and Emma H. Morrison Professor in the Departments of Chemistry and of Molecular Biosciences.

Dmitriy Lukoyanov received a M.S. degree and a Ph.D. from Kazan State University. He is a postdoctoral fellow at Northwestern University.

Lance C. Seefeldt received a B.S. degree from the University of Redlands and a Ph.D. from the University of California at Riverside. He was a postdoctoral fellow in the Center for Metalloenzyme Studies at the University of Georgia and is now professor of Chemistry and Biochemistry at Utah State University, where he recently received the D. Wynne Thorne Researcher of the Year Award.

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Fig 1.
FeMo-co and key residues

Fig 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 are shown; (iii) N_2 binding and H_2 release at either the E_3 or E_4 levels, with the pathway through E_3 deemphasized. LT also denote the protons added to FeMo-co (eg. E_1H_1); for clarity we have omitted this.

Fig 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 checkxstarinfinity.blogspot.com/

Fig 4.

Formulation of E₁-E₃ derived from consideration of E₄. **Note** M denotes FeMo-co inorganic core in its entirety; the superscript (+/-) represents difference between core charge and that of core in the resting state.

Fig 5.

Alternative formulation of E_1 - E_4 under assumption that hydride formation occurs at each stage. As in Fig 4, **Note:** **M** denotes FeMo-co inorganic core and superscript (+/-) represents difference between core charge and that of core in the resting state.

Fig 6.

Integration of LT kinetic scheme with Alternating (A) pathways for N_2 reduction. 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 hydride 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

Fig 7.

Visualization of *re* mechanism for H₂ release upon N₂ (blue) binding to E₄. 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 N₂ 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 E₄ (green) are positioned as suggested by *re* mechanism of H₂ release upon N₂ binding. Alternative binding modes for N₂-derived species can be envisaged.

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

Scheme 1.

Schme 2.

Schme 3.

Chart 1: Key Constraints on HD Formation under N₂/D₂:

- (i) *Stoichiometry*: $\text{M-N}_2 + \text{D}_2 + 2\text{H}^+ + 2\text{e}^- \Rightarrow 2\text{HD} + \text{M} + \text{N}_2$
 (ii) *Scrambling*: 'No' T^+ released to solvent under T_2
 (iii) *Reduction Stage*: D_2 reacts at 'N₂H₂' level

Chart 1.Key Constraints on HD Formation under N₂/D₂