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Climbing Nitrogenase: Toward a Mechanism of Enzymatic Nitrogen Fixation

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Conspectus

"Nitrogen fixation"—the reduction of dinitrogen (N_2) to two ammonia (NH_3) molecules—by the Mo-dependent nitrogenase is essential for all life. Despite four decades of research, a daunting number of unanswered questions about the mechanism of nitrogenase make it the 'Everest of enzymes'. This *Account* describes our efforts to climb one "face" of this mountain by meeting two interdependent challenges central to determining the mechanism of biological N_2 reduction. The first challenge is to determine the reaction pathway: the composition and structure of each of the substrate-derived moieties bound to the catalytic FeMocofactor (FeMo-co) of the molybdenum-iron (MoFe) protein of nitrogenase. To overcome this challenge, we need to discriminate between the two classes of potential reaction pathways: 1) a "*distal*" *(D)* pathway, in which H atoms add sequentially at a *single* N or 2) an "*alternating*" *(A)* pathway, in which H atoms add alternately to the *two* N atoms of N_2. Secondly, we need to characterize the dynamics of conversion among intermediates within the accepted Lowe-Thorneley kinetic scheme for N_2 reduction. That goal requires us to experimentally determine both the number of electrons/protons delivered to the MoFe protein and their "inventory"—a partition into those residing on each of the reaction components and released as H_2 or NH_3.

The principal obstacle to this "climb" has been the inability to generate N_2 reduction intermediates for characterization. A combination of genetic, biochemical, and spectroscopic approaches recently overcame this obstacle. These experiments identified one of the four-iron Fe-S faces of the active-site FeMo-cofactor as the specific site of reactivity, indicated that the sidechain of residue α70V controls access to this face, and supported the involvement of the sidechain of residue α195H in proton delivery. We can now freeze-quench trap N_2 reduction pathway intermediates and use ENDOR/ESEEM spectroscopies to characterize them.

However, even successful trapping of a N_2 reduction intermediate occurs without synchronous electron delivery to the MoFe protein. As a result, the number of electrons and protons, *n*, delivered to MoFe during its formation is unknown. To determine *n* and the electron inventory, we initially employed ENDOR spectroscopy to analyze the substrate moiety bound to the FeMo-co and ^{57}Fe within the cofactor. Difficulties in using that approach led us to devise a robust kinetic protocol for determining *n* of a trapped intermediate.

This Account describes strategies that we have formulated to bring this "face" of the nitrogenase mechanism into view *and* afford approaches to its climb. Although the summit remains distant, we look forward to continued progress in the ascent.

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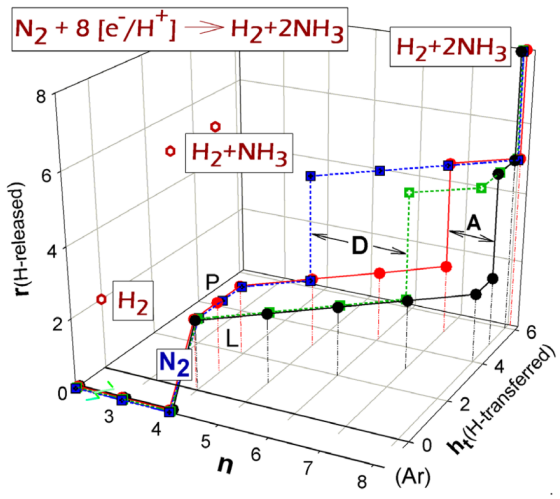

Introduction

'Nitrogen fixation' – the reduction of dinitrogen (N_2) to two ammonia (NH_3) molecules – is essential to all life, as it is the exclusive pathway for the input of nitrogen from N_2 into the global biogeochemical N-cycle. The majority of N_2 fixation occurs biologically, by the action of the microbial enzyme, nitrogenase. There are four known types of nitrogenase, primarily distinguished by the metal composition of their active-site metalloclusters.[1] The most important is the Mo-dependent enzyme[2] on which this *Account* focuses, henceforth denoted, 'nitrogenase'. It comprises two components, denoted the MoFe and Fe proteins. The former contains the active-site, multimetallic iron-molybdenum cofactor (FeMo-co) cluster as well as an auxiliary 'P' cluster, presumed to mediate electron transfer from Fe protein to FeMo-co. The Fe protein contains a [4Fe-4S] cluster and delivers electrons one-at-a-time to the MoFe protein.

Nitrogenase has been subjected to four decades of intensive kinetic[3,4] and spectroscopic studies,[2] and structures have been determined for MoFe and Fe proteins and for multiple forms of their complex.[5] Given the remarkable insights these studies provide into nitrogenase structure and function, one might expect that we would be well on the way to understanding the full mechanism of biological N_2 fixation. However, as outlined by Howard and Rees[6] and Schrock,[7] a daunting number of mechanistic questions remain unanswered, in our view, making nitrogenase the 'Everest of enzymes'. This *Account* describes our efforts to climb one 'face' of this mountain by meeting two interdependent challenges that are central to determining the mechanism of biological N_2 reduction. The first is to determine the reaction pathway for N_2 reduction. The second challenge is to characterize the dynamics of conversion among intermediates by incorporating them into a 'multidimensional' formulation of the accepted Lowe-Thorneley (LT) kinetic scheme for N_2 reduction.[2–4] This task in turn requires an experimental determination for each intermediate of *n*, the number of electrons/protons delivered to MoFe protein, as well as the 'electron/proton inventory route' for catalysis, a partition of each intermediate's *n* electrons/protons into those residing on FeMo-co, those residing on the substrate-derived moiety bound to it, and those released as H_2 or NH_3. These efforts seek some of the enzyme's most closely held secrets. This *Account* both describes the barriers to ascent and summarizes recent progress[8–10] in experiment and concept that are slowly advancing us towards the summit.

The first decades

NH_3 formation by nitrogenase proceeds through a series of intermediates in which N_2 and its reduced forms bind to FeMo-co.[2,3] However, during the first four decades of studying purified nitrogenase, no reduction intermediate was trapped for characterization, although intermediates that accumulate during CO inhibition were trapped early on.[11,12] Instead, the 1970s and 1980s witnessed the formulation of a kinetic scheme for substrate reduction, with some insights into the nature of intermediates being provided by studies of pre-steady-state kinetics. These efforts culminated in the LT kinetic scheme,[2–4] which has proven to satisfactorily describe the kinetics of nitrogenase catalysis in full.

Such efforts surprisingly showed that nitrogen fixation under optimized conditions exhibits the reaction stoichiometry,

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

Thus, the enzymatic reaction is both expensive, requiring the hydrolysis of two MgATP/reducing equivalent, and curious, in that eight reducing equivalents, not six, are needed to reduce each N_2 to two NH_3, with two equivalents being 'wasted' through the evolution of H_2.[2,3]

The LT scheme, shown *in part* in Fig 1, is formulated in terms of states, denoted E_n, that are indexed by the number of electrons and protons, *n*, that have been delivered to the MoFe protein during their formation: $n = 0$ (resting) ↔ 8 (as required by eq 1); it is characterized by the rate constants for transformations among those states. Particular features of note include the following: during catalysis the Fe protein delivers one electron at a time to the MoFe protein as its 4Fe-4S cluster cycles between (1+) and (2+) oxidation states; protein complex formation and 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.[3] N_2 reduction involves eight of these cycles to deliver the eight electrons necessary for substrate binding and reduction, eq 1.

There is little evidence for binding of any substrate to the resting state of the MoFe protein, and N_2 does not appear to bind until the MoFe protein has been activated by the accumulation of three or four electrons and protons (E_3 or E_4).[3] In the absence of other substrates, activation of MoFe protein leads to the reduction of protons to form H_2, with the protein thereby cycling back to its resting state. In the presence of N_2, this hydrogenase capability leads to a competition between H^+ and N_2 reduction. As a result, even the optimum stoichiometry of one H_2 formed per N_2 reduced (eq 1) represents only a lower limit.

The X-ray structure of MoFe protein was first determined sixteen years ago.[5] It revealed the active-site FeMo-cofactor to be an unprecedented [Fe_7S_9Mo; homocitrate] cluster, Fig 2, with roughly trigonal symmetry along the terminal Fe-Mo axis. Subsequently, refinement showed the presence in FeMo-co of an interstitial first-row atom, X = C, N, or O, making its final composition, [Fe_7S_9MoX; homocitrate],[13] and structures were obtained for multiple [Fe; MoFe] protein complexes.[5]

The structure of FeMo-co (Fig 2) was a major surprise, and its determination provides the foundation for any discussion of mechanism. But in some ways it deepened the mystery. Firstly, examination of the resting-state structure does not reveal the site of N_2 binding/reduction! Mo is an obvious candidate for the active site, as it is incorporated in the only known inorganic metal complexes that catalytically reduce N_2.[14] However, Fe is an equally attractive candidate, given that it is the catalytic metal in the commercial Haber-Bosch process for NH_3 formation

and that there are V and Fe nitrogenases that reduce N_2 but do not have Mo.[15] Then, if Fe is involved, the structure does not indicate which one of the three 4Fe-4S 'faces' of the central [Fe_6] iron prismane is active, much less identify the active Fe(s). In addition, there are such questions as: why homocitrate;[16] and what is X?[17]

Thus, at the beginning of this millennium we knew the structure of nitrogenase in its resting state, but not where catalysis occurred within that structure. Kinetics measurements culminating in the LT scheme provided an excellent understanding of how nitrogenase converts among the E_n intermediates during N_2 reduction, but we had absolutely no experimental evidence as to the identity and structure of *any* of these intermediates.

What's the problem?

The LT scheme reveals that the major obstacle to generating an E_n intermediate in sufficient amounts for characterization, and thus perhpas *the* reason for such an unsatisfying state of affairs, is that the MoFe protein acquires electrons one-at-a-time from its partner Fe protein and this process involves the nucleotide-dependent association *and* dissociation of the two proteins.[2] As a consequence, multiple electron additions to MoFe protein cannot be synchronized, for complex *dissociation* represents the rate-limiting step of substrate reduction,[2,3] while electron accumulation is opposed by the competing loss of H_2. EPR has been the primary tool in monitoring nitrogenase under turnover, and efforts to trap turnover intermediates for study indeed showed loss of the resting-state FeMo-co EPR signal under turnover, indicating that it has converted to intermediate E_n states. However, no signals from such states appeared in its place, indicating that the system distributes itself among multiple intermediates and/or that the preponderant states are EPR-silent. This barrier to study contrasts, for example, with the ready use of flash photolysis in photosynthesis to synchronously generate a particular desired intermediate for study.[18]

Beginning an Ascent

A way up this face was revealed[9,10] in a series of studies that combined genetic, biochemical, and spectroscopic approaches that: *(i)* identified the specific site of reactivity as the 4Fe-4S face of FeMo-cofactor defined by Fe atoms 2, 3, 6, and 7 (numbering based on pdb 1M1N); *(ii)* demonstrated that the sidechain of residue α70V acts as a 'gatekeeper' that controls access to the FeMo-co active site; and *(iii)* supported the idea that proton delivery involves the sidechain of residue α195H.[10,19,20] These structural features all are shown in Fig 2.

These conclusions stem from the observation that decreasing the size of the α70V residue by substitution with alanine allows the enzymatic reduction of molecules excluded from the wild type (WT) active site, such as propyne, propargyl alcohol, and 1-butyne, while increasing its size by substitution with isoleucine represses reaction of all WT substrates except H^+.[9,10] In both variants, the change leaves the reactivity of FeMo-co unaltered. These observations were accompanied by the discovery that reduction intermediates could be trapped in MoFe variants with mutations at α70V and/or α195H, and subsequently in WT enzyme itself. [10] This discovery open a trail to the summit.

Intermediates

Beginning in 2004, a series of reports described the first freeze-quench trapping and characterization of enzymatic intermediates,[8,9] initially ones that form during the reduction of alkyne substrates,[21,22] then one formed during the reduction of H^+ under Ar,[23] then four intermediates associated with N_2 reduction.[24–27] In all but one of these cases a key was the use of MoFe proteins that contain the amino acid substitutions, separately or in combination, that inhibit proton delivery to substrates (α-195^{Gln}) and modulate substrate accessibility to the reduction site (α-70^{Ala}). The trapping of an intermediate during N_2 reduction by WT

nitrogenase supports the view that these intermediates reflect normal nitrogenase function. They are being characterized biochemically, and as is now illustrated, their structures are being determined by the spectroscopic methods of choice, electron-nuclear double resonance (ENDOR) and electron spin-echo envelope modulation (ESEEM).[28,29]

CO; alkyne; hydride

The first use of ENDOR to study any nitrogenase intermediate employed ^{57}Fe to prove that the EPR signals from the two CO-inhibited forms of the MoFe protein arise from the FeMo-cofactor, not the P cluster,[30] used ^{13}C ENDOR to demonstrate that one CO binds to the 'lo-CO' state formed under low CO pressure while two bind to 'hi-CO', and offered proposals for the CO binding modes.[31]

The first determination of the structure of a nitrogenase catalytic intermediate combined ^{13}C and ^{1,2}H Mims 35 GHz ENDOR techniques to reveal the structure of the substrate-derived moiety bound to FeMo-co in the intermediate formed during reduction of the alkynes propargyl alcohol (*PA*; HC≡CHCH$_2$OH)[21] and HC≡CH[22] by MoFe variants. This study provides the 'template' for the ongoing applications of ENDOR/ESEEM spectroscopies to determine structure in such a complicated system. These techniques in general do not provide sufficient information with which to directly deduce the nature and binding geometry of the substratederived moiety, and quantum mechanical computations on FeMo-co are not advanced enough to unambiguously interpret the hyperfine/quadrupole couplings that are measured. Instead, the measurements supply constraints against which possible models can be tested and the correct model selected.

In the case of the *PA* intermediate,[21] ^{13}C ENDOR showed that the *PA* backbone remains intact and is covalently bonded to FeMo-co, and that no two of the backbone ^{13}C are equivalent. Thus, there are only three possible species that might be bound to FeMo-co: *PA* itself; singlyreduced *PA*; the alkene product (allyl alcohol), Eq 2.

$$HO-C1H_2-C2\equiv C3-H\,[PA]\xrightarrow{H^+/e^-}HO-CH_2-C{=}CHH\xrightarrow{H^+/e^-}HO-CH_2-CH{=}CHH \qquad (2)$$

^{1,2}H ENDOR further disclosed that the terminal carbon (C3) of *PA* is bonded to a non-exchangeable H of the reactant and a solvent-derived H, and that the two are symmetry equivalent through a reflection in a g-tensor plane; in addition, there is another, more weakly-coupled, solvent-derived H, indicated in red. Upon testing these constraints against every relevant structure in the Cambridge Structural Database, we reached the remarkable conclusion that the intermediate contains a bio-organometallic complex – the alkene reduction product, allyl alcohol, bound 'side-on' to one Fe of FeMo-co (Fig 3) – and a similar conclusion was shown to apply to the corresponding intermediate trapped during C_2H_2 reduction.[22] Subsequent experiments and DFT computations supported this conclusion.[9]

PA is not a substrate of WT nitrogenase, but becomes one when substrate access is expanded by reducing the size of the α-V70 side-chain through the α-V70A amino acid substitution. If the size of this side-chain is instead increased through the α-V70I substitution the result is a MoFe protein variant effective only in the reduction of $2H^+$ to H_2. During turnover under Ar this variant accumulates a novel intermediate that was shown by ^{1,2}H-ENDOR to have two chemically equivalent hydrides bound to metal ions of FeMo-co;[23] the central role of this intermediate in N_2 reduction is discussed below.

N_2 Intermediates and the Reaction Pathway

A molecular mechanism of N_2 reduction must include three constituents: *(i)* the 'Reaction Pathway' – an enumeration of the states that arise during the conversion of reactants to products, *(ii)* the molecular structures of these intermediates; and *(iii)* a kinetic scheme, in this case the LT scheme, that incorporates information about the kinetics/dynamics of conversion among these intermediates. Considering constituent *(i)*, we recently formalized the division of N_2 reduction pathways into two classes. In a *'distal' (D)* pathway, H-atoms add sequentially to a *single* N prior to N-N bond cleavage after the third hydrogenation, Fig 4. Catalytic NH_3 formation at mononuclear Mo metal complexes follows such a pathway, as shown by the early work of Chatt and coworkers[32] and recently by Schrock and coworkers.[14] This contrasts with an 'alternating' (*A*) pathway in which H-atoms add alternately to the *two* N atoms of N_2 before N-N bond cleavage at the fifth hydrogenation, Fig 4, often favored in computational studies, for example [33]. Interestingly, current discussions of possible reduction mechanisms have tended to support one or the other class without explicitly noting that there are two classes, although this was well understood in early days.[34]

Until recently, the limited information about the nitrogenase pathway was predominanatly derived from kinetics studies, and these were interpreted as supporting the *D* version.[3] As the substrate-derived species bound to FeMo-co along the alternative pathways of N_2 reduction are different at many stages of N_2 fixation, Fig 4, the enzyme's pathway would be revealed if these species could be characterized by ENDOR/ESEEM. Thus it was of pivotal importance when in 2004 we first reported the successful trapping of intermediates associated with N_2 reduction.[25] To date, four such S = ½ states have been trapped:[9,10,24–27] *e*(N_2), an early stage in reduction obtained from wild type MoFe protein with N_2 as substrate;[25] *m*(NH=N-CH_3), from α195Gln MoFe protein with CH_3-N=NH as substrate;[25,26] *m*(N_2H_2) prepared[27] by freeze-quenching the MoFe protein α70Ala/α195Gln variant during steady-state turnover with *in situ* generated diazene; and *l*(N_2H_4), from α70Ala/α195Gln MoFe protein with H_2N-NH_2 as substrate.[24,25]

A combination of ^{15}N and ^{1}H ENDOR measurements (Fig 5) showed that three of the intermediates *l*(N_2H_4), *m*(N_2H_2), and *m*(NH=N-CH_3) contain a substrate-derived [$-NH_x$] moiety bound to the cofactor.[24–27] In contrast, FeMo-co of *e*(N_2) appears to bind a non-protonated N, which would indicate that *e*(N_2) represents an earlier step in the catalytic pathway.[25] Recent $^{14,15}N$ and ^{1}H ENDOR measurements showed that the substrate-derived moiety in turnover states trapped with diazene and hydrazine substrates have extremely similar characteristics.[27] Hydrazine is well known to be a *bona fide* nitrogenase substrate[35] and biochemical experiments showed that the same holds for diazene.[27] Together, the above observations indicate that diazene enters the normal N_2 reduction reaction pathway (Fig 4) and further suggest that during turnover and freeze-quench, diazene is reduced by the enzyme until it 'catches up' to the species trapped during turnover with hydrazine, with the two substrates thus generating a common intermediate that falls along the normal pathway of N_2 reduction by nitrogenase. If this speculation proves to be correct, considerations of Fig 4 show that this interpretation favors an *A* mechanism.

Full characterization of these, possibly common, intermediates and of the others by ENDOR and by relaxation methods discussed below would definitively test this suggestion. However, the use of $^{1,2}H$ and $^{14,15}N$ ENDOR/ESEEM to determine the structures of the trapped nitrogenous reduction intermediates is a much more formidable task than was the determination of the structure of the alkyne reduction intermediates. Firstly, there are six stages of N_2 reduction, not two (Eq 1). Secondly, when alternate *A/D* pathways are taken into account (Fig 4), there are numerous possible [N_xH_y] species to be considered at every stage of N_2 reduction; this is illustrated by Chart 1 for later stages. Thirdly, one has less control over the placement of nuclear-spin probes. As the N_2H_m (*m* = 0, 2, 4) substrates used to generate intermediates

are symmetric, unlike *PA*, selective labeling of the N's thus is impossible—except with the unnatural substrate NH=N-CH_3;[26] all the N-H are solvent exchangeable, so selective deuteration as with *PA* is impossible. On the other hand, just a very few ENDOR-derived *constraints* can act as powerful means of narrowing the list of candidates. To characterize a [N_yH_x] cofactor-bound moiety one must answer the questions: *(i)* is the N-N bond broken (*y =1* or *2*), and if not, are the two N's equivalent; *(ii)* what is the reduction level of the bound fragment (what is *x*); *(iii)* what is the binding mode if *y* = 2? Some of these constraints will come directly from ENDOR results for the intermediates, as complemented and strengthened by comparisons[36] with newly emerging biomimetic complexes,[37,38] but some will come from a relaxation-kinetics protocol to be described below.

Connecting Intermediates and Kinetics.[22,39]

The nitrogenase reaction pathway is specified by determination of the composition and structure of the intermediates of N_2 reduction, Fig 4. However, even when a N_2 reduction intermediate has been trapped, this occurs without synchronous electron delivery to the MoFe protein. As a result, the number of electrons and protons, *n*, delivered to MoFe during its formation is unknown and the intermediate is untethered from the LT kinetic scheme for substrate reduction. Thus, *n* for the intermediate must be determined, but beyond this, one must determine how those electrons/protons are partitioned: are they on FeMo-co, P cluster, or substrate? One may loosely define an 'N_2 reduction mechanism' as comprising complete answers to three conjoined questions for all intermediates: composition/structure; the value of *n*; and the partition of *n*. The next section discusses efforts to address the second and third of these.

Electron Inventory

The first effort to determine *n* for an intermediate focused on those generated during alkyne reduction. It introduced the concept of a MoFe protein 'electron inventory',[22] seeking to determine the number (*n*) of electrons and protons that have been delivered to the MoFe protein to form an E_n state by partitioning *n* into distinct categories and determining the number in each: those that reside on the substrate-derived moiety bound to FeMo-co (*s*); those that remain on FeMo-co (*m*); the number of electrons/protons that have been 'released' (*r*) through liberation of H_2 and reduced substrate (*eg*., NH_3); further allowing for the possibility that P cluster has donated (*p*) electrons to FeMo-co. The index, *n*, thus can be written as the sum over categories,[22]

$$n=m+s+r-p \tag{3}$$

For the alkyne intermediates, $r = p = 0$, so $n = m + s$. The idea was to use ENDOR of nuclei associated with the substrate-derived moiety bound to FeMo-co to determine *s* and ^{57}Fe ENDOR of FeMo-co to determine *m*.

The inventory concept implicitly recognizes that the single index, *n*, is inadequate to specify the properties of an intermediate, and that additional indices are needed. For example, as in other formal valency schemes, the inventory depends on the nature of the binding of substrate-derived fragments to FeMo-co. Consider the E_n intermediate that contains the ethylene product of acetylene reduction bound to FeMo-co. If ethylene acts as a dative π-donor (π-C_2H_4; Fig 6), this binding does not alter the formal reduction level of the FeMo-co (*m*) or the alkene (*s* = 2), and the complex has an electron inventory, $n = m + s = m + 2$. However, one might instead imagine that C_2H_4 actually binds through C-Fe σ-bonds as the ferracyclopropane (σ-C_2H_4; Fig 6). Such a structure corresponds to oxidative addition of C_2H_4 to the FeMo-co, and in this case the alkene must be described formally as having accepted two additional electrons

from FeMo-co, making a total of $s = 4$ electrons transferred to the initial C_2H_2 substrate. As a result, $n = m + 4$. Analysis of ^{57}Fe ENDOR measurements of the acetylene-reduction intermediate, in combination with a variety of other considerations, led us to prefer $n = 4$ for this intermediate, compatible with the finding of Lowe *et al*. that C_2H_4 is released from the E_3 and E_4 states during C_2H_2 reduction by WT enzyme.[40] Thus, depending upon the binding mode, the E_4 MoFe protein would have accumulated four electrons, but FeMo-co of this intermediate could formally retain $m = 2$ electrons (π-C_2H_4; Fig 6), or none, $m = 0$ (σ-C_2H_4). This proposed n will be tested with a kinetic procedure described below.

Inventory Routes and the LT Scheme in 3D

Once one introduces the concepts of electron and proton inventories for intermediates, it becomes clear that there are not just two alternative pathways for the sequential addition of electrons/protons to substrate, *D* and *A*, (Fig 4), but each represents a multiplicity. For example, according to the LT scheme (Fig 1), N_2 binding to the E_4 state of nitrogenase occurs with loss of two electrons/protons as H_2, leaving N_2-bound FeMo-co reductively activated by the remaining $m = 2$ electrons. But these electrons can in principle be delivered to substrate at any subsequent stage in the reaction, and thus the *A* and *D* pathways in Fig 4 each really represents many possible 'electron inventory routes' of reduction. As one limiting route, transfer of the $m = 2$ electrons and protons to N_2 could occur *P*romptly (*P*), to form bound diazene; in the opposite limit, these electrons might remain on FeMo-co until the final, *L*ate (*L*), stages of N_2 reduction; and of course there are intermediate cases. To describe the alternative intermediate states embodied in these multiple pathways/routes requires other 'quantum numbers' in addition to *n*. In this light, the LT scheme as typically drawn (*eg*., Fig 1) is best viewed as a 1D projection of a 'multi-dimensional' kinetic scheme.

One way to represent such a scheme is to extend the notation for an E_n intermediate formed during N_2 reduction so as to include indices that specify the electron (*e*) inventory (eq 3): $E_n[m, s, r, p]^e$. However, as indicated above, an *electron* inventory in general is as much a formal construct, analogous to formal atomic valencies in a molecule, as an experimental one. Instead, it seems more instructive to denote an E_n intermediate by its 'proton (*H*) inventory'. As each electron delivered to MoFe protein is accompanied by a proton, $n = n(e^-) = n(H^+)$, we may denote the multiple possible intermediates at the E_n stage of reduction by specifying h_m, the number of protons that remain bound to cofactor as protonated sulfide or hydride, h_s, the number that are associated with the substrate-derived moiety bound to FeMo-co, and r, the number of protons/electrons that have been released as H_2 or reduced substrate/fragment. The proton inventory analogue to eq3 then equates sum of these protons to n,

$$\begin{aligned} n &= h_m + h_s + r \\ &\equiv h_m + h_t \end{aligned} \qquad (4)$$

where we further define the total number of protons that have been transferred to substrate, regardless of their fate: $h_t = [h_s + r]$. For clarity we will sometimes denote an intermediate as, $E_n[h_m, h_s, r]^H$, but in fact, for a given n the alternate intermediates can be specified with only two 'quantum number': $[h_m, h_s]$, $[h_m, r]$, or $[h_t, r]$; the remaining index is fixed by Eq 4. For completeness, one should further incorporate into the notation the substrate-derived species (*S*) bound to FeMo-co, leading to a notation in which the alternate intermediates associated with a given value of n may be written in terms of the three inventory indices, $E_n[h_m, h_s, r; S]^H$, or more economically in terms of two, as $E_n[h_t, r; S]^H$, etc. An advantage of this approach is that it is not merely formal: a proton on FeMo-co is a 'classical' particle; it really is bound either to an atom of substrate or one of FeMo-co.

Fig 7 presents a 3D proton inventory plot that that displays the four *limiting* alternate routes of N_2 reduction that involve N_2 binding to E_4 with release of H_2: *D* vs *A* pathways; each with either *P*rompt (*P*) or *L*ate (*L*) transfer to substrate of the $m = 2$ electrons/protons that remained on MoFe after N_2 binding. The plot omits the corresponding routes associated with N_2 binding at E_3 because their inclusion would make it unreadable. In this plot, the alternate proton inventories available for E_n are denoted, $E_n[h_t, r; S]^H$, with a particular inventory route represented by a series of points for which the abscissa is *n*, the ordinate is h_t, and the z coordinate is *r*, the number of protons (and electrons) released as H_2 and NH_3 during catalysis.

The routes displayed begin with the accumulation of $n = 4$ electrons/proton to generate the 'hydride' intermediate activated for N_2 binding, E_4 $[h_t = 0, r = 0]^H$, and hence $m = 4$ from Eq 4. Next, N_2 binding and the accompanying release of H_2 ($r = 2$) generates E_4 $[0, 2]^H$, with $m = 2$. The *P/L* routes then diverge, each subsequently splitting into *D/A* branches. Release of the first NH_3 ($r = 5$) occurs at a different state on each of the four branches; the four finally converge to release the second NH_3 ($r = 8$). The volume enclosed by these limiting paths contains the points for states that would arise in a 'mixed' pathway.

This approach also helps in thinking about such enduring mysteries as: why is release of H_2 an obligatory feature of N_2 binding (eq 1); why must N_2 bind to MoFe protein that has acquired $n = 4$ electrons/protons (Fig 4), written most descriptively with the three inventory indices as state $E_4[h_m, h_s, r; -]^H = E_4[4, 0, 0; -]^H$, when the accompanying loss of H_2 yields an N_2-bound state in which MoFe protein retains only two equivalents, $E_4[2, 0, 2; N_2]^H$, and analogously for binding to $n = 3$? Consider the 'overpotential' requirement, that N_2 bind to a state with $n > 2$. *If* it were to be shown that catalysis follows a *P* route in which, for example, $E_4[4, 0, 0; -]^H$ binds N_2 and loses H_2 to form $E_4[2, 0, 2; N_2]^H$, with prompt followup reduction of N_2 to generate $E_4[0, 2, 2; N_2H_2]^H$, then one would interpret this overpotential as reflecting a need to reductively stabilize bound N_2 On the other hand, *if* an *L* route is operative, for example with the $e(N_2)$ intermediate corresponding to $E_4[2, 0, 2; N_2]^H$, then one might speculate that the required high level of reduction ($n = 4$ or 3) is accompanied by a structural rearrangement of the FeMo-cofactor that is required for N_2 binding.

Relaxation Protocol

The use of an 'electron inventory' to determine *n* for intermediates other than those trapped during alkyne reduction was blocked by several difficulties, one being that the effects of slow ^{57}Fe nuclear relaxation made it difficult to analyze ^{57}Fe ENDOR spectra. This led us to devise a robust new kinetic protocol for determining the E_n state of a trapped intermediate.[39] It is founded on the recognition that no matter how an intermediate state of the MoFe protein has been trapped, *it has* accumulated a specific number of electrons, *n*, and *it is* in a specific E_n state. Thus, *it acts* as a 'synchronously prepared' initial state. In particular, we hypothesized that *n* for an intermediate state early in the kinetic scheme (Fig 1) could be revealed by following its 'synchronous' relaxation back to the resting state (E_0) through the loss of one or more equivalents of H_2, and 'simply' counting the number of such $\Delta n = 2$ steps.

This approach, of course, requires that no additional electrons transfer from Fe protein to MoFe protein during the relaxation, which raises the question: How can one 'turn off' intercomponent electron transfer without perturbing the MoFe protein in a sample that retains both the reducing equivalents and ATP required for this electron transfer? The answer rests in the fact that each ET from the Fe protein to the MoFe protein *absolutely* requires dissociation/association of the Fe-MoFe protein complex.[2,3] We recognized that this electron transfer would be abolished if Fe-MoFe association/dissociation were prevented by keeping the sample frozen, while we knew from our studies of heme monooxygenases[41] that reactions *within* the MoFe protein can proceed in the frozen state. In the resulting relaxation protocol, a 77 K freeze-trapped intermediate relaxes in the solid (typically at 253K) without additional intercomponent electron

transfer, while reaction progress is monitored periodically by cooling the sample to 2K for EPR measurements of the EPR-active species, at which temperature no reactions occur.

With this protocol we showed that the hydride intermediate (**A**) relaxes to the resting state (**C**) in two steps, **A** ➡ **B** ➡ **C**. Both steps show appreciable solvent kinetic isotope effects, *KIE* ~ 3–4 (85% D_2O) (Fig 8), assigned to the generation of H_2 during relaxation. According to the LT scheme, Fig 1, this intermediate must then be the catalytically central E_4 state that has been activated for N_2 binding and reduction by the accumulation of four electrons. Its relaxation by loss of H_2 generates the state **B** = E_2, and this relaxes with loss of H_2 to the resting state, **C** = E_0.

We propose that the combination of this relaxation protocol with ENDOR/ESEEM studies of structure offers a real prospect of connecting trapped E_n intermediates to mechanism. Beyond that, it will help to characterize individual intermediates and enable us to distinguish among the multiplicity of reaction pathways that are revealed when considering alternate 'inventory routes', as in Fig 7. For example, if the same intermediate is trapped during turnover with diazene and hydrazine, then the two should show the same relaxation behavior.

The many faces of nitrogenase

This *Account* has discussed progress and prospects for determination of an 'N_2 reduction mechanism' defined as comprising: the reaction pathway detailing the composition and structure of intermediates; the substrate reduction 'route' that links those intermediates to kinetics through the determination of *n* and the electron inventory for each. As noted above, the Perspectives of Schrock[7] and of Howard and Rees[6] provide a context for this work and discuss challenges not addressed in this *Account* -- the other unclimbed 'faces of the nitrogenase mountain'. Three issues noted there are of particular relevance to the efforts described here. *(i)* Do the bound intermediates 'migrate' over FeMo-co during catalysis and is Mo directly involved at some stage?[7,9] *(ii)* What is **X** (Fig 1)?[17] *(iii)* How does the P cluster participate?[42] The first two of these can be addressed by ENDOR studies of trapped intermediates, the third by relaxation measurements. But such challenges will require much 'climbing' by us and others before they can be discussed in some future *Account*.

The Climb

At the start of this milennium the 'face' of nitrogenase discussed here was shrouded in clouds. This discussion has argued that strategies have been formulated that bring this face into view *and* afford approaches to its climb. This does *not* mean that this summit will soon be reached, but rather that vigorous climbing will be rewarded.

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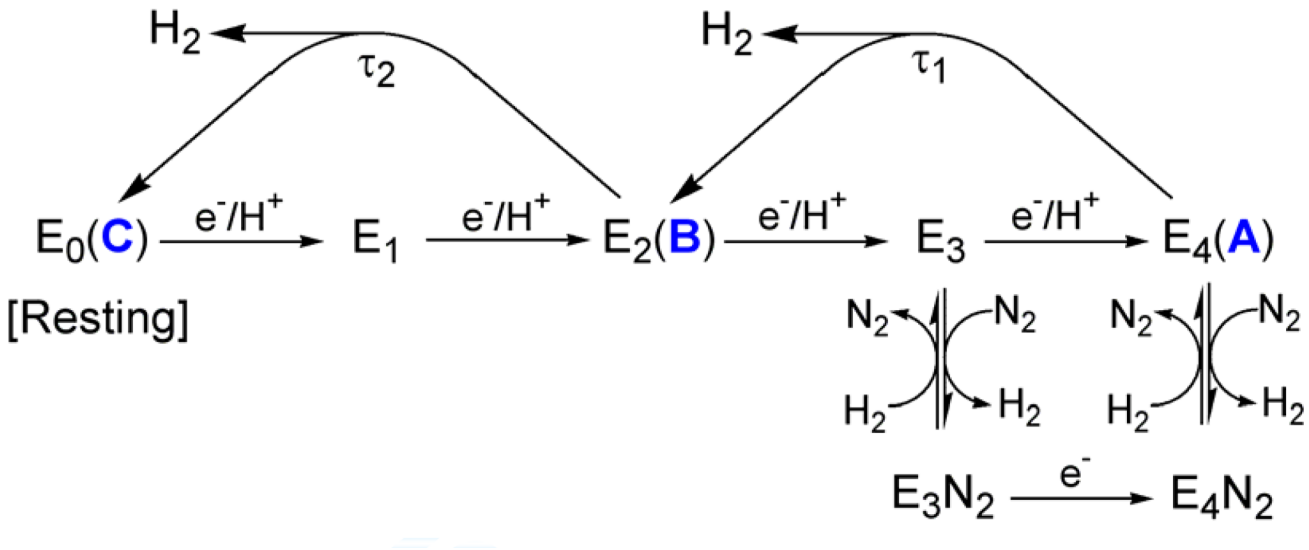

Fig. 1.

Early stages of Lowe-Thorneley (LT) kinetic scheme for N_2 reduction, the portion that connects states E_0 through E_4; for a full scheme, see references [2–4]. As discussed in conjunction with Fig 8, **A** is the MoFe protein 'hydride' intermediate; **B** forms during its relaxation to resting state, **C**.

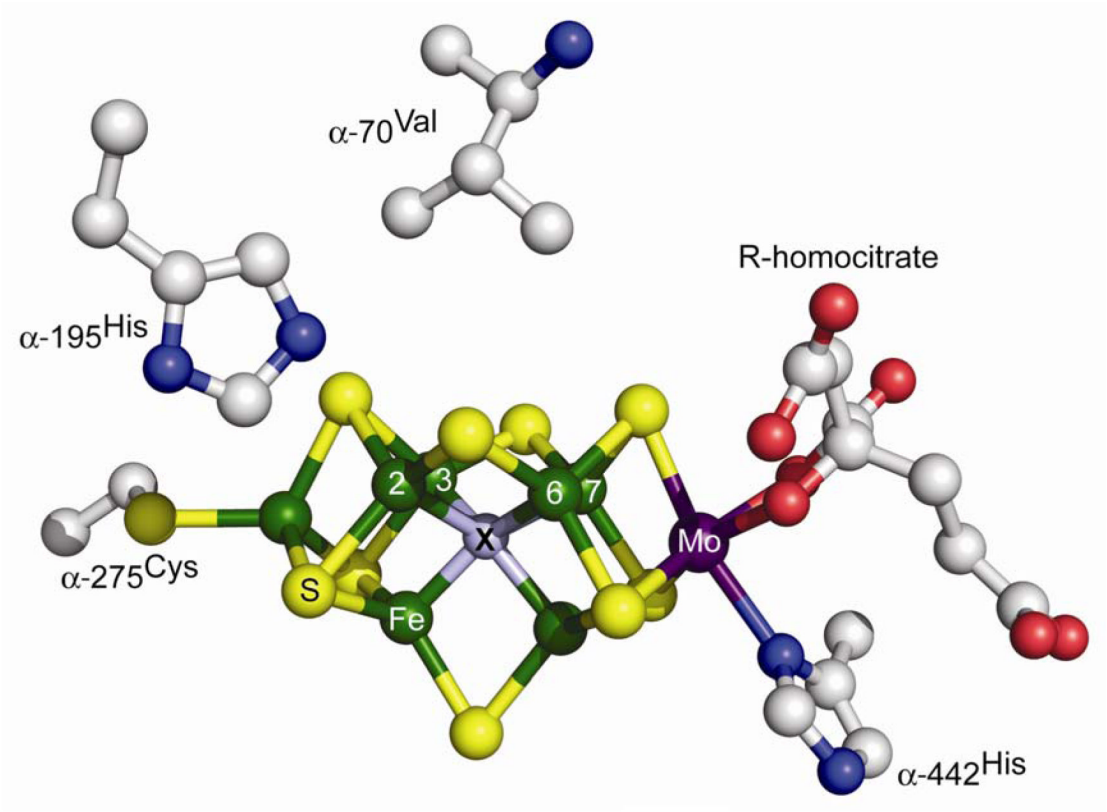

Fig 2.

Structure of FeMo-co, including the two residues that covalently link it to the apo-protein and the two implicated in function, α-V70 as substrate 'gatekeeper' and α-H195 as agent for proton delivery.

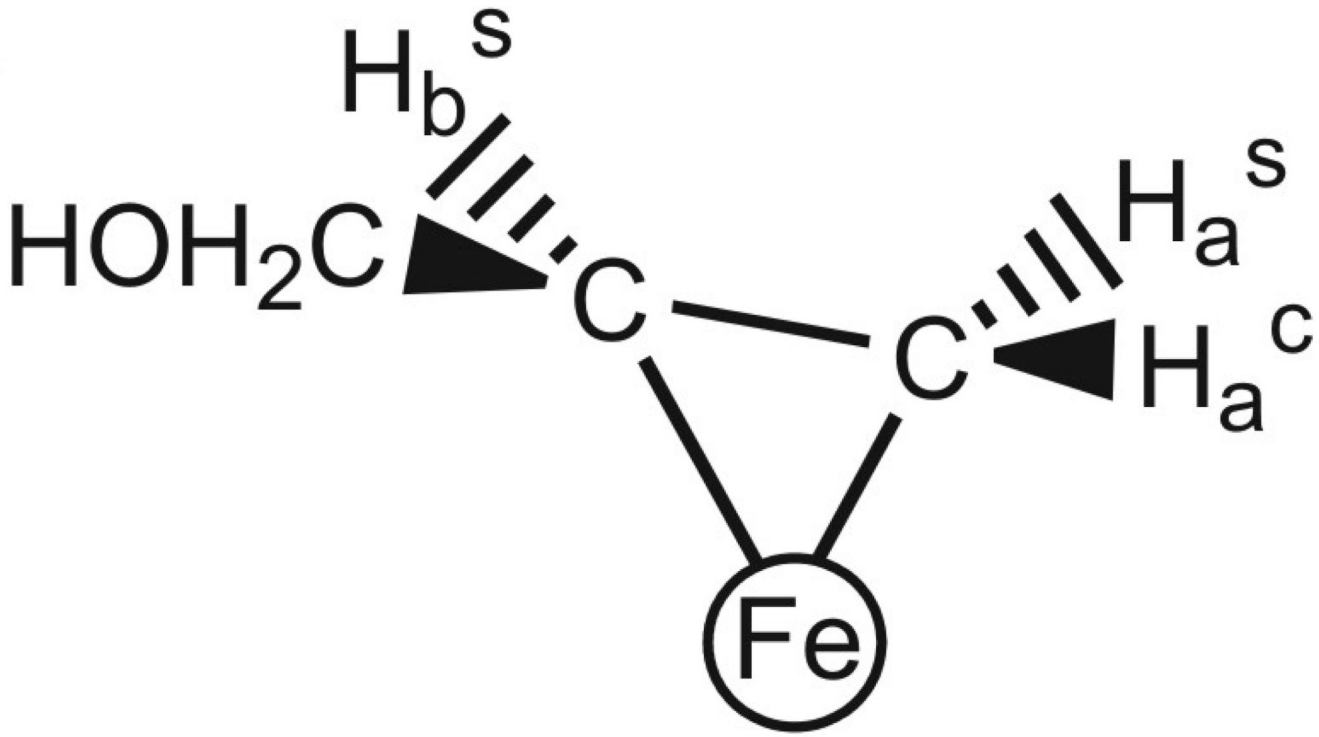

Fig 3.
Proposed structure for the intermediate trapped during propargyl alcohol reduction – the product alkene bound side on to a single Fe ion.

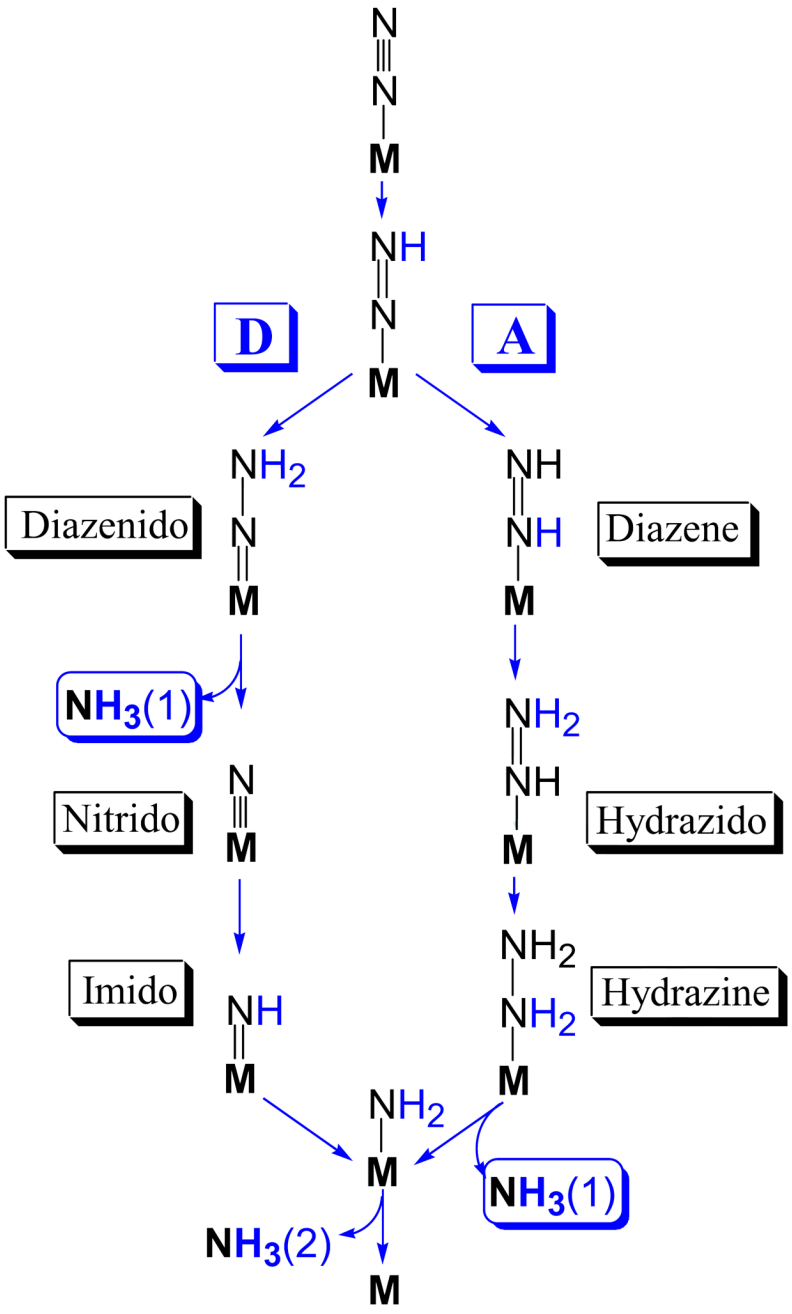

Fig 4.

'Alternating' (*A*) and 'Distal' (*D*) N_2 reduction pathways. **M** represents FeMo-co without specifying metal ion(s) involved. Likewise, the representations of binding are meant to emphasize the distinction between pathways and do not imply specific binding modes. Small straight arrows represent addition of H^+/e^- to substrate.

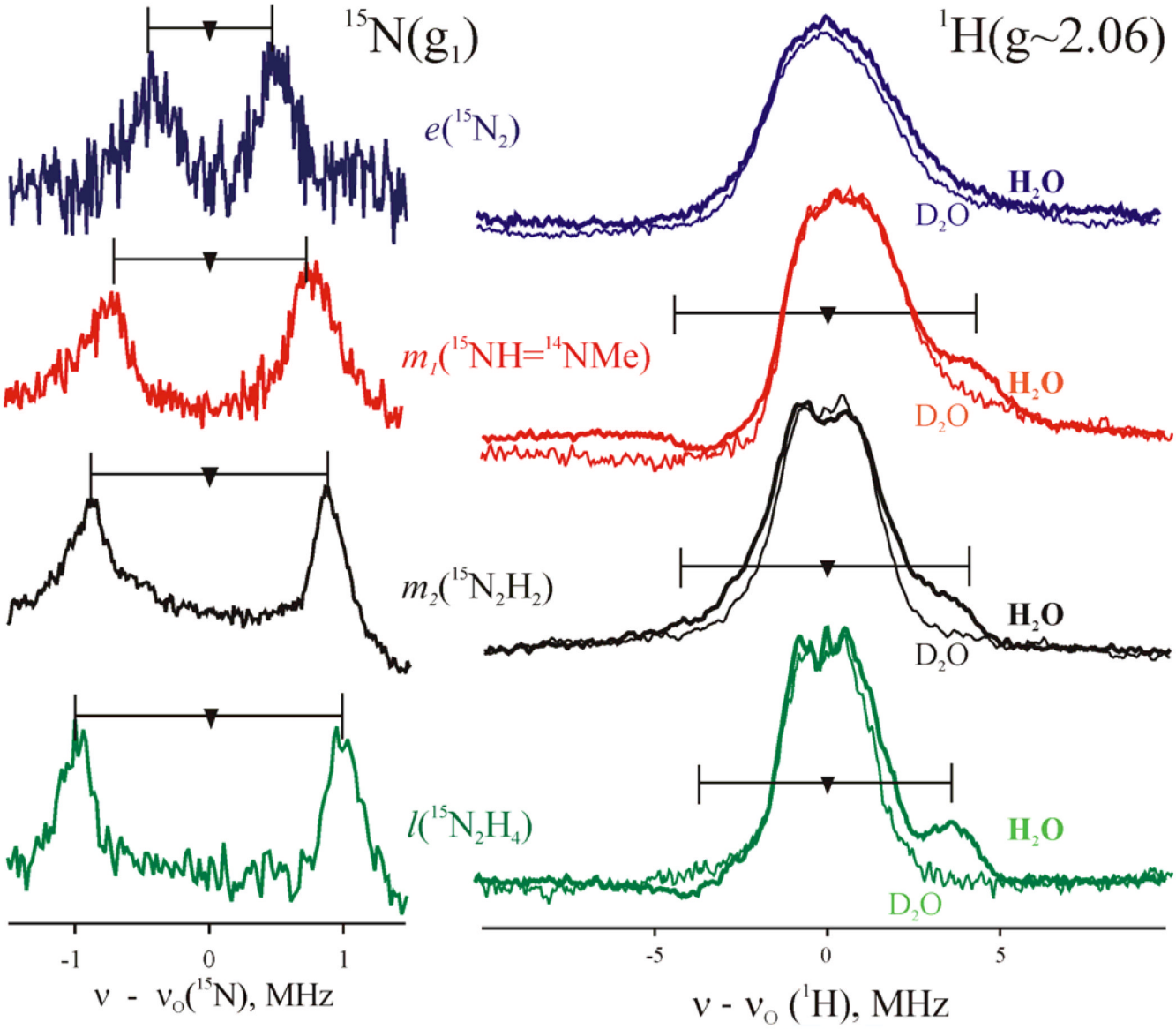

Fig 5.

(**Left**) Q-band Mims (*e* and *m*) and Re-Mims (*l*) ^{15}N-ENDOR spectra (g_1) of nitrogenous trapped intermediates, labeled with ^{15}N as indicated. *Conditions*: microwave frequency = 34.808–34.819 GHz; $\pi/2$ = 52 ns (*e*, *m*) and 32 ns (*l*); RF = 20 ~ 30 μs; τ = 500 ns (*e*), 300 ns (*m*), and 200 ns (*l*); sampling = ~ 1000 transients/point; repetition rate = 100 Hz (*e* and *m*), 50 Hz (*l*); 2 K. (**Right**) CW ^{1}H-ENDOR of ^{14}N intermediates in H_2O and D_2O. *Conditions*: microwave frequency, 35.057–35.171 GHz; modulation amplitude = 4 G; RF sweep speed = 1 MHz/s; bandwidth of RF broadened to 100 kHz; 2K.

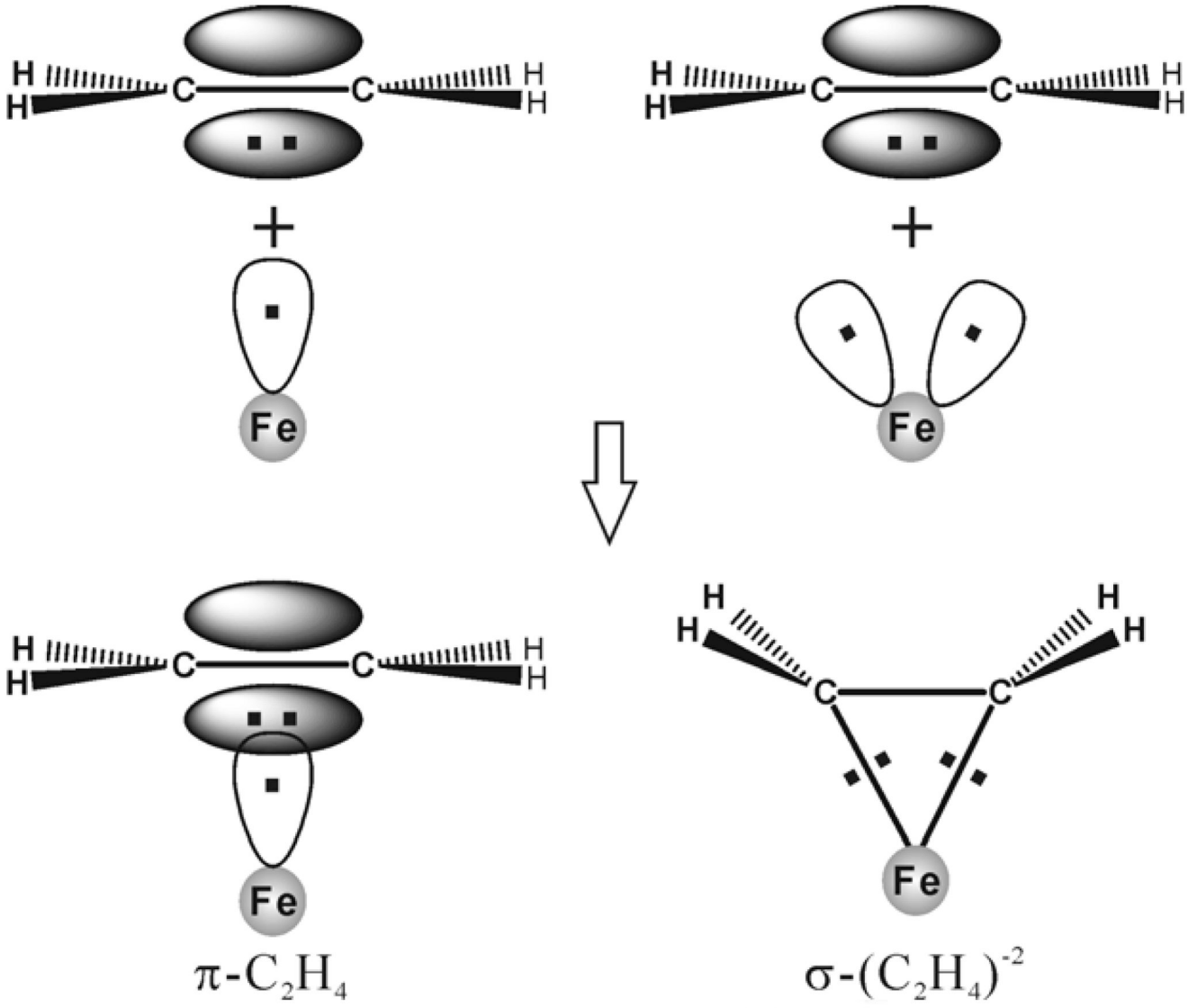

Fig 6.
Cartoon representation of alternate schemes for binding C_2H_4 to a cofactor metal ion. *Left:* dative π bonding. *Right:* oxidative addition to form Fe-C σ bonds.

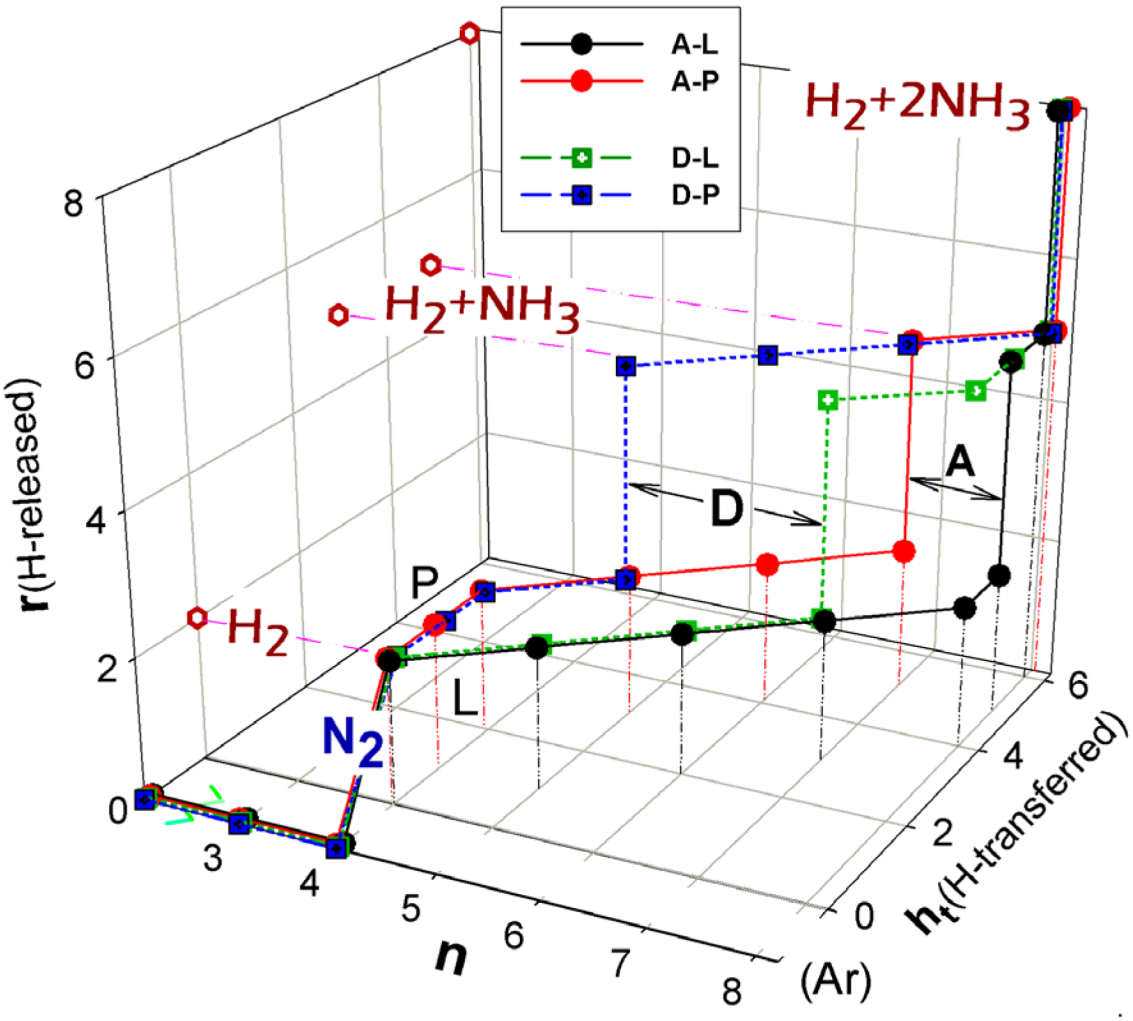

Fig 7.

3D representation of LT scheme plotted in terms of the proton inventory (eq 4), showing alternate limiting reaction pathways for N_2 reduction by nitrogenase following N_2 binding to intermediate E_4 of Fig 1. Indices of intermediate states are: *n* (*abscissa*); h_s (*ordinate*) = number of protons delivered to substrate; r (*z axis*) = number of electrons/protons release during catalysis.

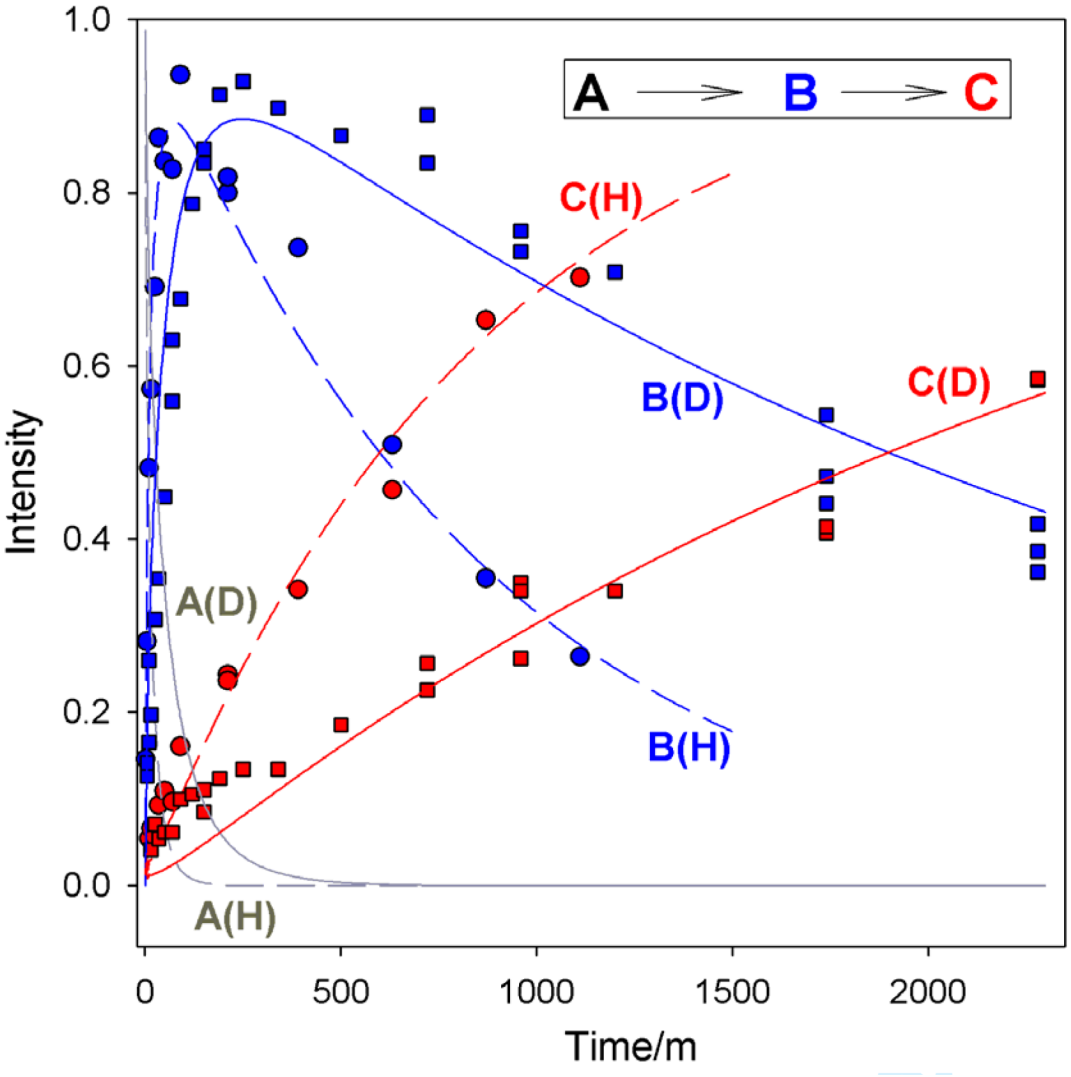

Fig 8.

Step-annealing curves (253K) of hydride intermediate (**A**), leading to formation of **B** intermediate and ground state **C**, in H_2O/D_2O buffers. Fits to distributed (stretched exponential) two-step relaxation: $t_1(H_2O)$ = 13 min, $t_1(D_2O)$ = 49 min; $t_2(H_2O)$ = 870 min, $t_2(D_2O)$ = 2700 min. For clarity, data for **A(H)** and **A(D)** are omitted, and only the fits are presented.

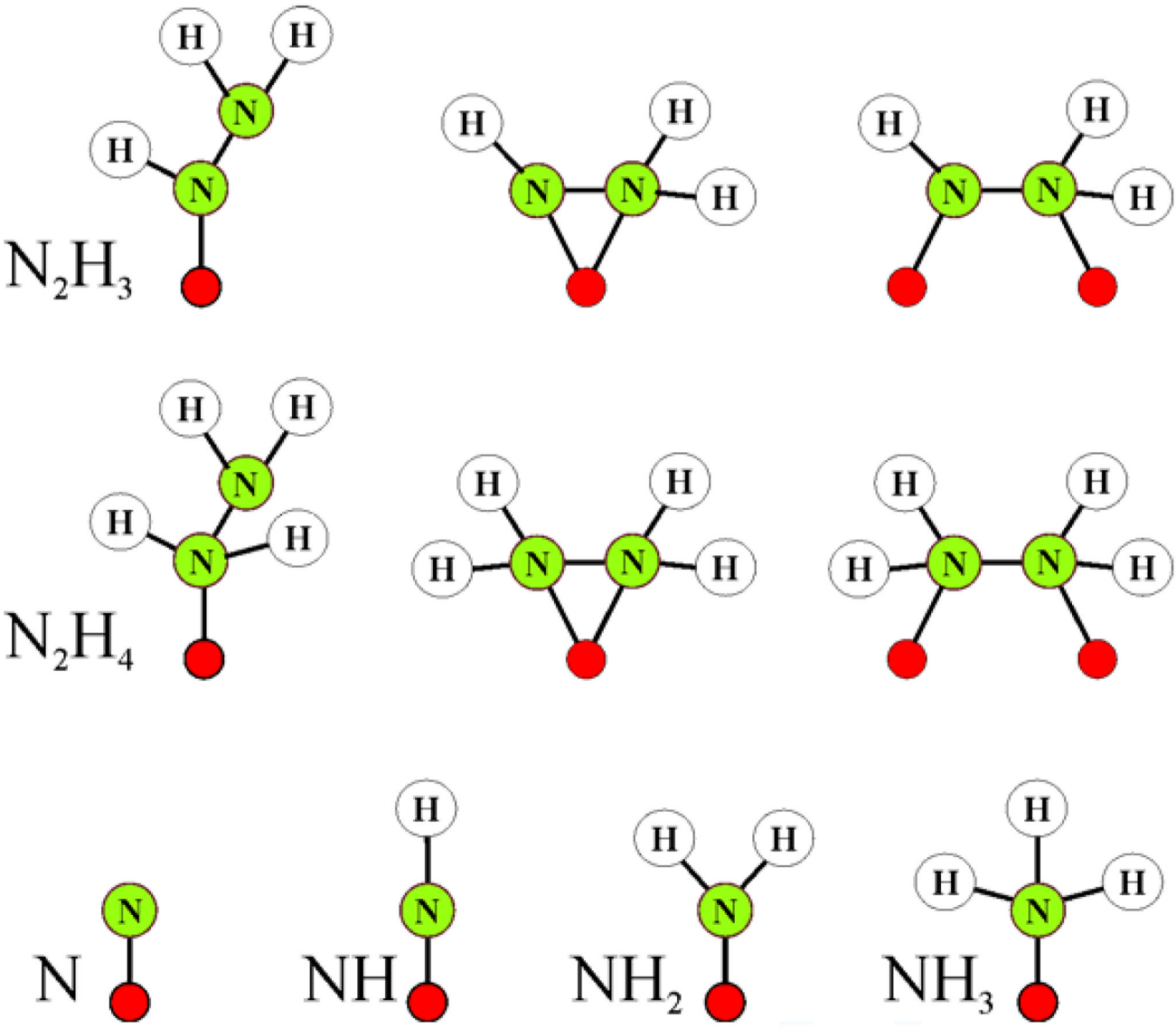

Chart 1.
Substrate-derived species bound to FeMo-co that might form in late stages of N_2 reduction.