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The Critical E_4 State of Nitrogenase Catalysis

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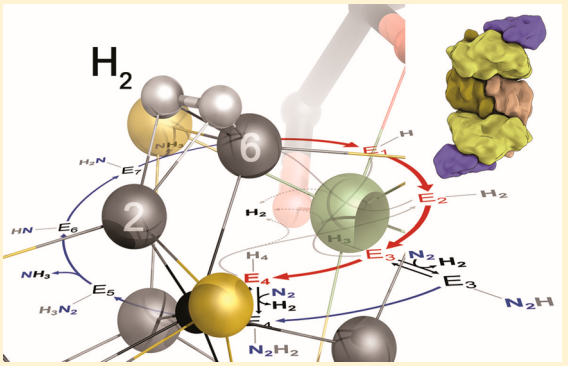
ABSTRACT: The reaction catalyzed by the nitrogenase enzyme involves breaking the stable triple bond of the dinitrogen molecule and is consequently considered among the most challenging reactions in biology. While many aspects regarding its atomic mechanism remain to be elucidated, a kinetic scheme established by David Lowe and Roger Thorneley has remained a gold standard for functional studies of the enzyme for more than 30 years. Recent three-dimensional structures of ligand-bound states of molybdenum- and vanadium-dependent nitrogenases have revealed the actual site of substrate binding on the large active site cofactors of this class of enzymes. The binding mode of an inhibitor and a reaction intermediate further substantiate a hypothesis by Seefeldt, Hoffman, and Dean that the activation of N_2 is made possible by a reductive elimination of H_2 that leaves the cofactor in a super-reduced state that can bind and reduce the inert N_2 molecule. Here we discuss the immediate implications of the structurally observed mode of binding of small molecules to the enzyme with respect to the early stages of the Thorneley−Lowe mechanism of nitrogenase. Four consecutive single-electron reductions give rise to two bridging hydrides at the cluster surface that can recombine to eliminate H_2 and enable the reduced cluster to bind its substrate in a bridging mode.



BIOLOGICAL NITROGEN FIXATION

The complete reduction of atmospheric N_2 gas to two molecules of ammonium, NH_4^+, is an exergonic process under ambient conditions but is kinetically disfavored by the high energy barrier for activating the inert N_2 triple bond.[1] As a consequence, the vast majority of all nitrogen in Earth's biosphere exists as N_2, making the element quite frequently the limiting factor for organismic growth in a given habitat.[2,3] The technological solution to dinitrogen fixation is the well-established Haber−Bosch process, where high temperature and pressure sustain the reaction of N_2 with H_2 on an iron catalyst.[4] Biological nitrogen fixation, on the other hand, is restricted to ambient conditions and the use of a biological energy source, ATP.[1] In nature, this has been exclusively achieved by the enzyme nitrogenase, a two-component metalloenzyme system that is thought to derive evolutionarily from bacterial protochlorophyllide reductases,[5] indicating that the growth limitation by nitrogen deprivation may have become relevant only after the advent of photosynthesis provided the organismic world with virtually unlimited energy supply for growth. In essence, nitrogenases employ a heterotetrameric protein of approximately 240 kDa as a scaffold for a unique, iron−sulfur-based cofactor that is assembled *ex situ* and inserted only as the final step of protein maturation.[6] Because of its intricate chemistry and its obvious significance for the living world, nitrogenase has been the subject of intense studies for decades, starting out with the discovery of the process by Hellriegel and Willfarth in 1888[7] and eventually with the discovery of the enzyme itself.[8] A subsequent, fundamentally important finding was that biological nitrogen fixation is accompanied by the release of H_2 in at least stoichiometric amounts.[9,10] Early on, nitrogenase was characterized as requiring molybdenum,[11] although it lacked any homology to known Mo-dependent enzymes, and we now know that the most common class of nitrogenase indeed contains a Mo^{3+} ion as part of a [Mo:7Fe:9S:C]:homocitrate cluster, the FeMo cofactor.[12] During the late 1970s, nitrogen fixation under Mo-depleted conditions was reported,[13,14] and vanadium was implicated as an alternative heterometal; however, only after 1980 was a V-dependent alternative nitrogenase eventually isolated.[15−17] Interestingly, a third class of nitrogenases even functions without either Mo or V, producing a structurally similar active site cofactor that contains only iron.[18] This Fe-dependent nitrogenase exhibits a N_2-reducing activity lower than that of the vanadium-containing enzyme, which in turn is less active than Mo-nitrogenase, suggesting that in particular those organisms that are equipped with all three systems resort to a preferential expression that depends on heterometal bioavailability. Both Mo and V are far less abundant than iron, but while Mo predominates over V in marine environments, the situation is reversed in soil.[18] Over time, a somewhat more complex picture of alternative nitrogen fixation has emerged, as V-nitrogenase that was already known to reduce CO to hydrocarbons in analogy to Fischer−Tropsch chemistry *in vitro*[19] also performs this reaction under physiological

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conditions,[20] while Mo-nitrogenase is strongly inhibited by CO. Fe-nitrogenase, on the other hand, additionally catalyzes reduction of CO_2 to methane,[21] a remarkable reaction that does not take place in either of the other classes, except in a particular variant protein.[22] In spite of such differences in reactivity, however, it is generally assumed that all types of nitrogenases employ the same mechanism of reduction, at least for their most challenging substrate, N_2. There is broad agreement that the respective cofactors, FeMoco and the accordingly labeled FeVco and FeFeco, are the sites of catalysis and bind, activate, and reduce dinitrogen in largely the same way. With initial structural models for the two component proteins of nitrogenase obtained by Rees and co-workers as the starting point (Figure 1),[23,24] high-resolution crystal structures were highly instrumental in understanding the architecture of nitrogenases[25−28] and were ideally complemented with an impressive body of biochemical, spectroscopic, and theoretical work that starts to integrate into a functional understanding of this outstanding enzyme in molecular and even electronic detail. However, a major obstacle in understanding nitrogenase catalysis to date has been the lack of detailed information regarding the actual position and mode of coordination of N_2 to the complex metal sites. All metal centers in all versions of the nitrogenase cofactor are coordinatively saturated, so that a binding site for the substrate is not readily apparent.[12]

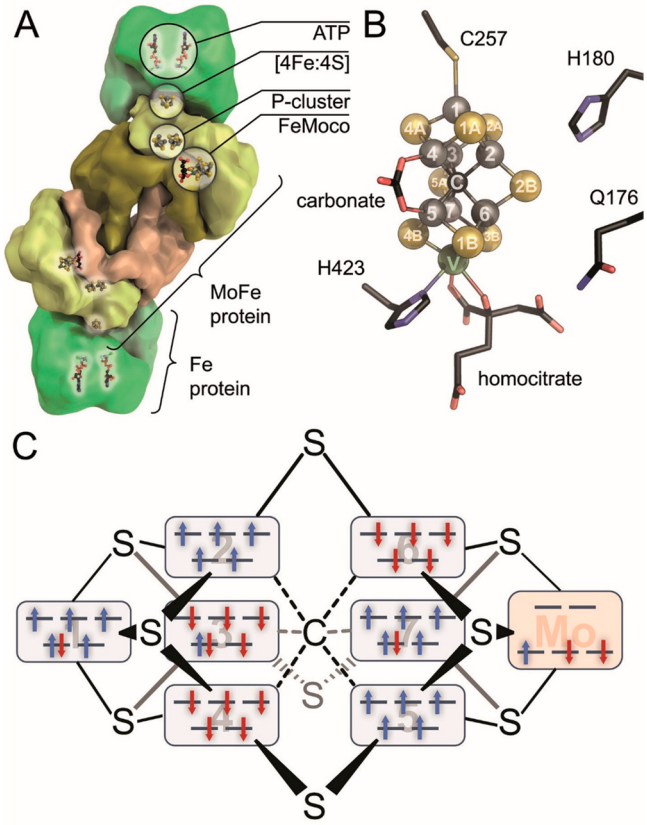

Figure 1. Structural features of nitrogenase. (A) The molybdenum nitrogenase of *Azotobacter vinelandii* is a complex formed by the catalytic MoFe protein (brown) and two copies of the electron-donating Fe protein (green). Two sets of metal sites allow for electron transfer, triggered by ATP hydrolysis, from a [4Fe:4S] cluster in Fe protein via the [8Fe:7S] P-cluster to the active site FeMo cofactor. Figure generated from Protein Data Bank (PDB) entry 1N2C. (B) FeVco of vanadium nitrogenase from *A. vinelandii* as an example for nitrogenase cofactors. V is in an apical position, and this cluster includes two organic ligands, CO_3^{2-} and homocitrate. Cluster atoms are labeled according to the standard nomenclature. Figure generated from PDB entry 5N6Y. (C) Current model for the electronic structure of the FeMo cofactor. The antiferromagnetically coupled iron sites are in a high-spin configuration with three additional electrons residing on Fe1, Fe3, and Fe7. With Mo^{3+} in a non-Hund configuration that originates from coupling to the adjacent iron sites, the total spin of the systems adds to $S = {}^3/_2$. Note that the depicted electron configurations assume that all S atoms in the cluster are sulfides (S^{2-}) and the central C atom is a carbide (C^{4-}).

ELECTRONIC STRUCTURE OF NITROGENASE COFACTORS

Nitrogenase cofactors are arguably the largest and most complex metal cofactors synthesized in nature to sustain enzymatic catalysis. They are assembled *ex situ* by a multistep maturation machinery and subsequently inserted into apo-nitrogenases.[29] The well-characterized MoFe protein of molybdenum nitrogenase is isolated in a stable resting state, in which a highly rhombic $S = {}^3/_2$ configuration of the cofactor yields a characteristic, broad EPR spectrum (note that nitrogenase contains a second, large iron−sulfur cluster, P-cluster, which is all-ferrous and thus EPR-silent in the as isolated state). Understanding the electronic structure underlying this site is a prerequisite for unraveling its functionality, but in a system of this complexity, multiple hypotheses have been put forward over time. Basically, the seven Fe ions of the FeMo cofactor reside in a tetrahedral ligand field and will thus be in a high-spin configuration. The sites will be electronically coupled in either a ferromagnetic (aligned spins) or antiferromagnetic (opposing spins) manner, giving rise to different coupling patterns that were explored in a broken-symmetry DFT study by Case and Noodleman.[30] Shortly thereafter, the cofactor was found to contain an interstitial light atom[26] that was later identified as a carbide (C^{4-}) by a combination of different approaches.[12,31,32] The nine sulfur atoms in this cluster are at the sulfide level (S^{2-}), and the remaining component, molybdenum, was originally assigned as Mo^{4+}, the canonical reduced state of the element in proteins.[33]

In this configuration, either two ($2Fe^{2+}$:$5Fe^{3+}$) or four (4 Fe^{2+}:$3Fe^{3+}$) additional electrons on the iron ions could give rise to an $S = {}^3/_2$ system. To clarify this question, we studied the electron distribution in the FeMo cofactor by spatially refined anomalous scattering (SpReAD), a method that combines the three-dimensional resolution of X-ray diffraction with an analysis of X-ray absorption properties.[34] The analysis of MoFe protein indicated a ($3Fe^{2+}$:$4Fe^{3+}$) configuration for the FeMo cofactor, which would not combine to a total spin of $S = {}^3/_2$ with Mo^{4+} and its d^2 configuration.[35] This apparent discrepancy was resolved by an XES study by DeBeer and co-workers, who identified the Mo ion as a unique Mo^{3+} with a non-Hund ground state resulting in a total spin of $S = {}^1/_2$.[36] Together with the BS7 coupling scheme proposed by Noodleman, this then resulted in a complete electronic model consistent with quantum mechanics/molecular mechanics calculations for the FeMo cofactor (Figure 1C).[37] Several aspects of this model have direct implications for understanding the reactivity of the cofactor, in particular that iron sites Fe2 and Fe6 appear to be the most highly oxidized sites in the cluster, and that they are antiferromagentically coupled to all their surrounding metal sites, with the exception of the apical Fe1 and Mo.[38]

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MECHANISM OF DINITROGEN REDUCTION

A true milestone for understanding nitrogenase catalysis was a series of meticulous kinetic studies by Thorneley and Lowe that cast the eight-electron reduction of N_2 to two NH_3 molecules under generation of H_2 into a concise reaction scheme comprising eight individual states, E_0–E_7 (Figure 2).[39]

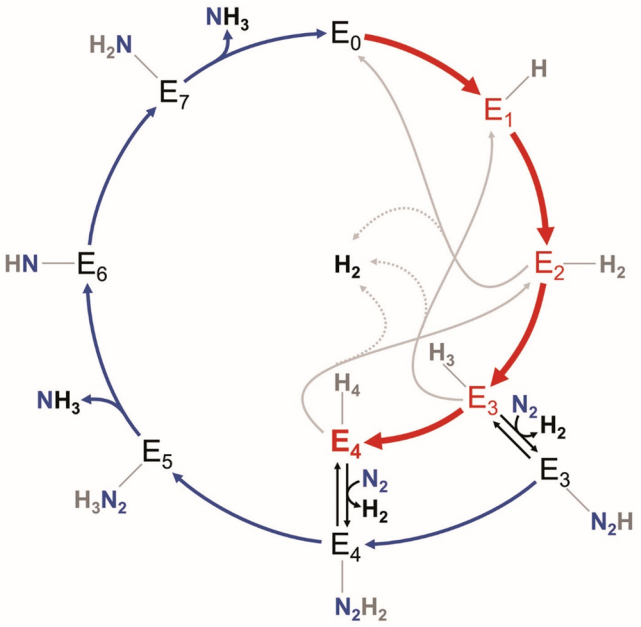

Figure 2. Eight-electron reduction scheme for nitrogenase catalysis according to Thorneley and Lowe. At a limiting stoichiometry, the enzyme releases at least one H_2 for each N_2 molecule that is reduced to two NH_3 molecules. The substrate is only bound in exchange for H_2 at E_3, where the association is reversible, or at E_4, from where the reaction was found to proceed to completion. The accumulation of four electrons prior to substrate binding was rationalized by the formation of a surface hydride in the active site cofactor, which can result in unproductive release of H_2 upon spontaneous protonation.

Thorneley and Lowe identified these states and assigned rate constants to the majority of transitions linking them. Among their most significant findings was that the resting state (E_0), in which the enzyme is typically isolated, must be reduced by at least three, better four, electrons before N_2 is bound. Under typical reaction conditions, a significant proportion of the electron flux through nitrogenase is diverted toward the reduction of protons to yield H_2. This was initially considered an unwanted side reaction, but in 1984, Simpson and Burris showed that even at 50 atm of H_2 the generation of hydrogen gas does not cede but persists at least in a 1:1 stoichiometry with N_2 reduction.[10] Thorneley and Lowe integrated this in the form of a required exchange of N_2 for H_2 upon binding. This can already occur in state E_3, but at this point, N_2 is only reversibly bound to the enzyme and a further reduction step is required to initiate its reduction. What remained unclear was whether there was any mechanistic rationale for H_2 generation by nitrogenase or whether this seemingly unproductive diversion of electrons was an inevitable price to pay to generate the massive reducing power required to break the N_2 triple bond. Electrons are delivered individually from Fe protein, the reductase component of the system that utilizes the energy released by the hydrolysis of two molecules of ATP to transfer a single low-potential electron to the active site cofactor. The mechanism for this transfer is highly orchestrated and involves the unique properties of the P-cluster,[40] but the key features relevant to the present discussion are that (i) each electron is delivered at the same redox potential and (ii) the time between two electron transfer events is undetermined and possibly long, so that all intermediates must be sufficiently stable to persist. This raises the difficulty of adding four consecutive electrons to a metal cofactor that already in its resting state E_0 is highly reduced. Even if the four remaining formal Fe^{3+} sites were to accept one electron each, this should have a profound effect on the structural integrity and stability of the cofactor (and increasingly so), while Fe protein will always donate one electron at a constant potential. To address this problem, Seefeldt, Hoffman, and Dean have recently suggested that the increasing charge is essentially not stored in the cofactor at all but that each pair of electrons can combine with a proton to form a metal hydride (H^-) at the cluster surface.[41−43] In this scheme, only a single electron would ever be required to reside within the cofactor itself, representing the odd-numbered E states of the early stages of the Thorneley–Lowe scheme (Figure 2). A first surface hydride will be generated in state E_2, followed by a second in E_4, so that when the enzyme is sufficiently reduced for N_2 binding, the four acquired electrons are all bound in superficial hydrides. Cryo-annealing protocols were established to trap intermediates of these reaction steps in molybdenum nitrogenase, and a very convincing point was made in that turnover of the enzyme in the presence of D_2 leads to HD formation.[44,45] This can be rationalized if D_2 is bound by the activated enzymes and split into two deuterides that can subsequently be protonated to release HD (*vide infra*). Most importantly, however, in the E_4 state, the recombination of the two surface-bound hydrides into a single H_2 molecule that immediately departs from the cluster can leave the active site in a high-energy state, super-reduced by two electrons in a way that could not be achieved by electron transfer from Fe protein alone. It is this active state, designated E_4* for the following discussion, that nitrogenase is uniquely able to bind to activate the substrate N_2, breaking the stable triple bond and initiating the biological fixation of nitrogen.

ACTIVE STATE E_4

In an eight-electron reduction of N_2 that yields two molecules of ammonia together with H_2, the E_4 state is reached halfway through the entire reaction cycle. Binding of the substrate N_2 to this state occurs concomitant with the release of H_2, and from here on, the reaction cycle is committed to proceed to completion. With the functional and spectroscopic studies performed on Mo-nitrogenase, it was postulated that the key for breaking N_2 by this enzyme is to reach the E_4 state to reductively eliminate H_2.[41] For nitrogenases, H_2 release is not a particular feat, and indeed, any intermediate state from E_2 on will be able to revert backward on the cycle and release hydrogen. A major strength of the Seefeldt–Hoffman–Dean proposal of surface hydrides is that the reason for this involuntary (and obviously futile) H_2 evolution becomes apparent when assuming that the enzyme must try to support a surface hydride for an extended period of time (until the next electron is delivered by Fe protein) in an active site that is optimized to deliver protons for N_2 reduction. It requires only the direct protonation of a hydride to form H_2 and make the enzyme lose two electrons it had already acquired at the cost of four molecules of ATP. In this picture, the observed H_2

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evolution in nitrogenase marks failed attempts by the enzyme to reach the E_4 state required for N_2 binding and thus exclusively constitutes an unwanted side reaction. Note that this type of H_2 evolution may well occur also in E_4, so that even then an unproductive reversion to E_2 is possible.

The E_4 state of nitrogenase thus has not one but two possible reaction pathways to follow. One is the unproductive protonation of a hydride, leading back to E_2 with loss of two electrons. The other, however, proceeds to also release H_2, but in a different, productive way that leads to further progress of the reaction. The "Janus intermediate" was the name given to this point in the reaction that can be seen as facing forward and backward at the same time, defining the further fate of the reaction not by whether H_2 is evolved but rather by how it is generated.[46] Only if two hydrides are available, in state E_4, do the two hydrides have the possibility of eliminating H_2 without involving further protonation. This process will leave two electrons on a now super-reduced cofactor that could not be generated by successive reduction from Fe protein alone. The reductive elimination thus couples H_2 evolution to the activation of the enzyme for substrate reduction, rationalizing why stoichiometric H_2 evolution persists even under a high overpressure of the gas.[10] Through the first stages of the catalytic cycle, the nitrogenase cofactor in this model alternates exclusively between an oxidized $S = {}^3/_2$ state and a one-electron-reduced integer-spin state, while every second electron leads to hydride formation. As it takes two such hydrides to undergo reductive elimination of hydrogen, it is only in state E_4 that the cofactor can be doubly reduced through H_2 elimination, rendering it able to bind N_2.

The concept of reductive elimination elegantly rationalizes key aspects of the Thorneley–Lowe model, and it allows us to ask very specific questions regarding the further progress and mechanism of dinitrogen reduction. Three points that are left open, however, are the actual nature of the activated state, its molecular structure, and also the electronic state that describes E_4* after H_2 release. The two-electron reduction of the cluster could lead either to a single, super-reduced site that is formally Fe(I) or even Fe(0) or to the symmetric reduction of two iron ions. Also, the nitrogenase cofactor does not show obvious coordination sites for any ligand, so the positioning of surface hydrides as well as their nature, terminal versus bridging, remained speculative.

LIGAND BINDING TO THE NITROGENASE COFACTOR

The active sites of metal-based enzymes typically feature open coordination sites that straightforwardly suggest the position and mode of substrate binding, providing a very good starting point for understanding the respective reaction pathways. With the structural analysis of Mo-nitrogenase, Rees and co-workers pointed out that for the nitrogenase cofactor this is not the case. In particular, after the discovery of a central ligand, later identified as a carbide, all metal ions presented a complete coordination sphere, with tetrahedral geometry for all irons and octahedral for molybdenum.[12] From the study of point variants of the enzyme, it was known that the replacement of the conserved Val70 with Ile would hamper substrate access. This was interpreted to indicate that substrate binding occurs on the cluster face situated below this residue, in particular at Fe6, with a possible end-on binding of N_2 to this site.[41] As a result, the Fe6–C bond should elongate, resulting in a trigonal bipyramidal geometry that inspired extensive synthetic work by Peters and co-workers, who showed that such complexes would not only bind but also activate and catalytically reduce N_2.[47−49] Similarly, Seefeldt, Hoffman, and Dean suggested that the formation of hydrides would occur on the same cluster face in the form of bridging hydrides that are typically less likely to be lost through protonation than their terminal counterparts.[50,51] The precise placement of such hydrides remained an open question, as much as the more fundamental point as to which exact features of the cluster determine this reactivity.

For more than 25 years, structural biology has made crucial contributions to our understanding of nitrogenase catalysis and has provided a picture of the enzyme's cofactor in very great detail. At no point, however, was it possible to depict a ligand-bound form that could finally address the questions raised above. This changed only in 2014, when further advances in protein handling and crystallization protocols resulted in a structure for a CO-inhibited state of MoFe protein that had been generated under turnover conditions following previously established protocols.[52] In the presence of ATP and reducing equivalents, CO bound to the cofactor as a metal carbonyl bridging irons Fe2 and Fe6. The remarkable aspect of this mode of binding was that it involved the replacement of one of the characteristic μ_2-bridging sulfides at this position, S2B, and thus created a chemically reasonable binding site for the inhibitor CO. Moreover, continued turnover of nitrogenase in the absence of CO would lead back to the resting state, reinstating S2B to its original place, although in the CO-bound form the only hint toward the fate of this sulfide was a possible storage site ∼22 Å from the cluster. With CO being a noncompetitive inhibitor of Mo-nitrogenase for all substrates other than protons, but (almost) not a substrate, the significance of this observation remained under debate. Most recently, however, further progress was made through the characterization of the VFe protein, the catalytic component of the alternative, vanadium-dependent nitrogenase enzyme (Figure 3A).[53] Its active site is located on the FeV cofactor that retains the features of the FeMo cofactor, with the exception of one bridging sulfide being replaced by carbonate and, obviously, the exchange of Mo for V. The FeV cofactor also features an $S = {}^3/_2$ resting state with a V^{3+} ion (d^2 configuration), implying that one of the iron sites must be more highly reduced than in FeMoco.[54] Nevertheless, the VFe protein was initially observed in a resting state (E_0), with sulfide S2B in place and an $S = {}^3/_2$ spin state. In contrast to the MoFe protein, this E_0 did not remain the only form in which the protein was found, and when the amount of chemical reductant present during protein isolation and crystallization was decreased, the first structure of a nitrogenase with a bound reaction intermediate was obtained.[38] Its defining feature was once more the displacement of sulfide S2B that created a binding site for a light atom that we interpreted as an NH unit representing state E_6 of the catalytic cycle.[38] Most notably, the replacement of the bridging ligand also led to a conformational rearrangement of a nearby glutamine residue, Q176, which by rotating toward the bound ligand exposed a binding pocket where the released S2B was identified (Figure 3B). While on the basis of the structural data alone a final distinction between an OH and an NH ligand is not possible and it may be that in the VFe protein the association of OH^- was what made this state detectable, this finding nevertheless corroborated the implication of the Fe2–Fe6 edge of the cofactor in substrate binding that arose from the CO complex of the MoFe protein,[52] and while the structure depicted what is assumed to

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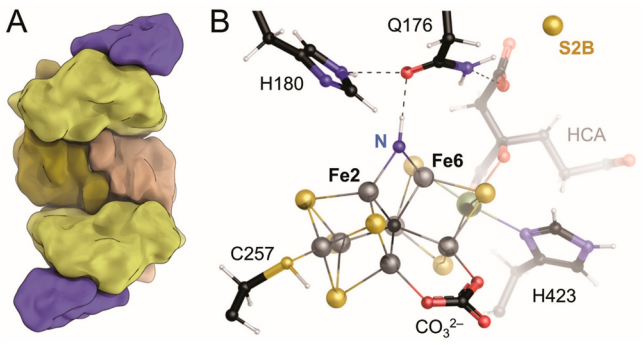

Figure 3. Vanadium nitrogenase reveals a ligand coordination site on the nitrogenase cofactor. (A) Similar to the more extensively characterized MoFe protein of molybdenum nitrogenase, the core of VFe protein from *A. vinelandii* forms an $\alpha_2\beta_2$ heterotetramer ($VnfD_2K_2$, with VnfD colored yellow and VnfK colored olive and wheat). In addition, the VFe protein contains a third subunit, VnfG (blue), that exclusively contacts VnfD. (B) A recent structural analysis of an active state of the VFe protein revealed that belt sulfide S2B is reversibly displaced from the cluster to a holding site 7 Å away. A bound intermediate can be an NH or OH and is tentatively labeled N. While this awaits clarification, either ligand quite likely reveals the binding mode of a monatomic, protonated species, in analogy to state E_6 of the Thorneley–Lowe scheme, in that it highlights that ligands to the nitrogenase cofactors are bound in a μ-bridging fashion at Fe2 and Fe6. Figure generated from PDB entry 6FES.

be a late stage of the Thorneley–Lowe cycle, it also holds strong implications for earlier stages, further supporting the concept of reductive elimination.

ANATOMY OF THE E_4 STATE

In the transition from resting state E_0 to E_4, four electrons that accumulate in nitrogenase recombine at the cofactor surface with two protons to form two bridging hydrides. Assuming a structurally intact cofactor, the model of reductive elimination implied that the formation of these hydrides was most likely to occur on one of the cluster surfaces comprising four Fe ions in a square arrangement, with preference given to the Fe2–3–6–7 face located beneath residue $V70^{NifD}$ of the MoFe protein, as its alteration had been shown to influence substrate binding and reduction (Figure 4A).[41] Seefeldt, Hoffman, and Dean have discussed the possibility of an E_4 state that includes two bridging hydrides on this face, either sharing one iron site or forming two parallel, bridging sites.[55] Should two hydrides sharing one Fe site combine to release H_2, the two electrons remaining on the cluster can then lead to a single, super-reduced site [formally Fe(I), d^7] that binds and activates N_2. However, one consequence of the current electronic structure proposal for the cofactor (Figure 1C) is that neither the formation of two hydrides sharing one Fe site (Figure 4B) nor the juxtaposition of the hydrides across the cluster face (Figure 4C) could generate a situation whereupon reductive elimination of H_2 the two remaining electrons are located at a single Fe site. This is due to the requirement for the two electrons to be eliminated as H_2 to have opposing spin states. The same is then true for the remaining electrons, and there is no single Fe site in the cluster that would be able to accept a spin $+^1/_2$ and spin $-^1/_2$ electron simultaneously.

The ligand-bound structures of MoFe and VFe proteins now highlight a dinuclear binding site comprising Fe2 and Fe6 that could not have been anticipated from resting state structures, and the coordination sites generated on both iron centers by

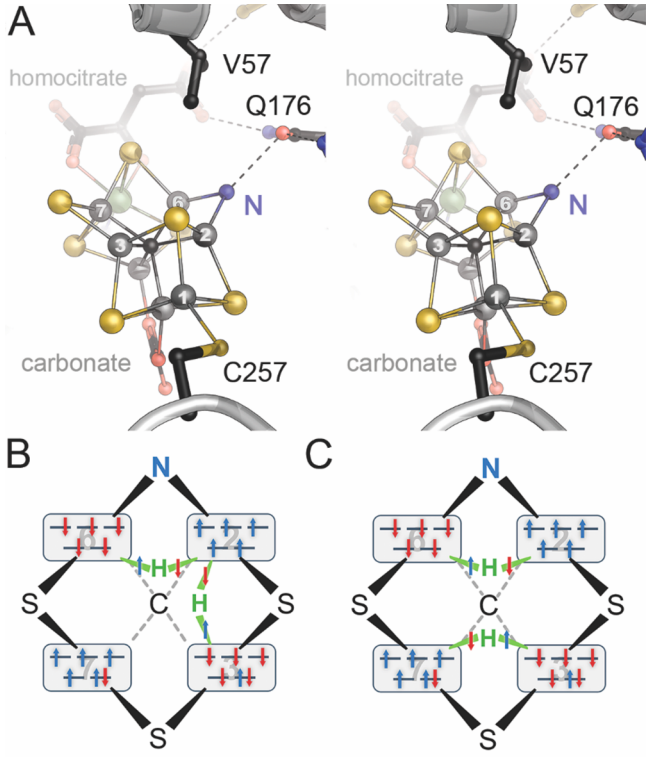

Figure 4. Proposed substrate binding site on the cofactor. (A) Stereo representation of the Fe2–3–6–7 face of the FeV cofactor. Residue V57 is analogous to V70 of the *A. vinelandii* MoFe protein. An altered substrate specificity after mutagenesis of this residue was taken as an indication that substrates bind here. Note, however, the proximity of V57 to the bridging ligand bound at Fe2 and Fe6 [tentatively labeled N (see Figure 3)]. Figure generated from PDB entry 6FES. (B) Proposed binding mode for two surface hydrides on the Fe2–3–6–7 face, with the hydrides sharing one Fe site with arrows indicating electron spins according to Figure 1C ($+^1/_2$ colored blue, $-^1/_2$ colored red). (C) Analogous binding proposal for a juxtaposition of two hydrides. In both cases, the reductive elimination of H_2 will remove one electron of either spin, and consequently, the two remaining electrons will be of opposing spin, leaving no straightforward way for both to generate a single, super-reduced iron.

the temporary removal of sulfide S2B allow for new considerations regarding the formation and reactivity of surface hydrides in the early E states of the Thorneley–Lowe cycle. With the higher stability of a bridging hydride with respect to a terminal one, a μ_2-bridging binding mode between Fe2 and Fe6 is highly likely for state E_2. A second hydride will be formed in state E_4, and the concept of ligand binding at Fe2 and Fe6 after removal of S2B accommodates the formation of this second hydride at the same position, resulting in two adjacent hydrides.

No structural data are presently available for hydride formation on a nitrogenase cofactor, but a hypothetical E_4 state with this configuration can be modeled and reveals that the structure is indeed suited to accommodate bridging H^-, with an Fe–H distance of 1.65 Å (Figure 5A), as observed in model compounds and also in a structure of the Ni-R state of Ni,Fe-hydrogenase refined to 0.89 Å resolution.[56] With all possible binding modes for hydride anions on the complex and structure surface of a nitrogenase cofactor, this suggestion remains a working hypothesis, but the degree to which it integrates known data on the E_4 state is noteworthy. First, the

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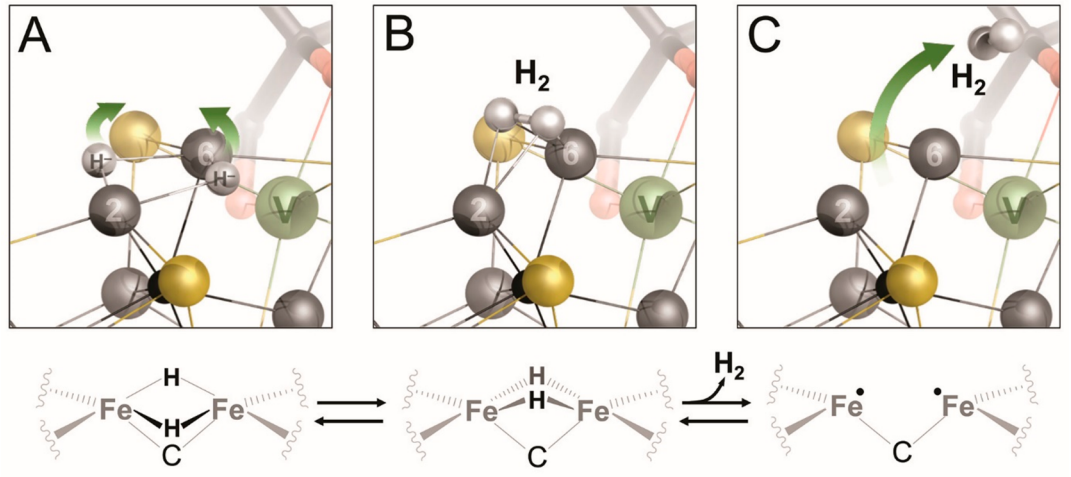

Figure 5. Hypothetical model for the E_4 state of the Thorneley–Lowe scheme and reductive elimination of H_2, depicted in modeled structures (above) and as schemes (below). (A) Successive reduction and protonation of the cofactor first lead to a single bridging hydride in state E_2 (likely concomitant with S2B removal and subsequently to a dihydride form in E_4 that is reminiscent of known model compounds). (B) The transition to a μ:η^2-η^2 binding mode supports the formation of H_2 that is subsequently eliminated from the cluster, under effective reduction of Fe2 and Fe6. (C) This results in an open, dinuclear binding site for substrates that is more reduced by two electrons than a comparable state that could be generated from E_2 after removal of S2B. However, only the super-reduced open site is capable of binding and activating the physiological substrate N_2, leading to an immediate cleavage of the stable triple bond. Importantly, studies of HD formation (through D_2 association and protonation of the resulting deuterides) in MoFe and FeFe proteins underline the reversibility of these steps, indicating that all three forms are energetically nearly equivalent.

recombination of two bridging hydrides in this state merely requires an approach of both toward the Fe2–Fe6 edge, leading to a decreasing H–H distance (Figure 5B) and, eventually, the formation and dissociation of H_2 (Figure 5C). Importantly, all these steps should be fully reversible. Upon dissociation of H_2, the cluster remains in a super-reduced state that allows it to bind inert substrates such as N_2 and to rebind the H_2 molecule that can subsequently be split again into two distinct, bridging hydrides. This is in line with the reported feature of nitrogenase to react with D_2 under turnover conditions (i.e., in the presence of a reductant, ATP, and Fe protein) and form HD.[44,45]

In the activated E_4 state, the antiferromagnetic coupling of Fe2 and Fe6 invariably implies that each metal retains one electron rather than generating a single super-reduced site, and thus, a bridging binding mode for any ligand should be strongly preferred (Figure 6). For a diatomic ligand, this either can result in a μ_2 configuration as observed for binding of CO to the FeMo cofactor[52] or may lead to a side-on bridging (μ:η^2-η^2) configuration as we suggest for H_2. None of these possibilities can be ruled out at this point, and in fact, both have been extensively discussed in the literature, even though

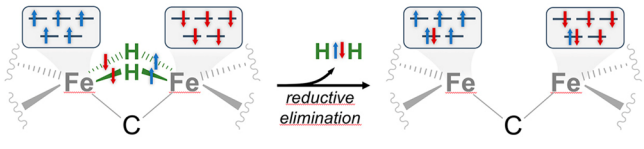

Figure 6. Bridging hydrides imply bridging binding for N_2 and other substrates. According to the electronic structure proposal for the nitrogenase cofactor (Figure 1C), substrate binding iron sites Fe2 and Fe6 are antiferromagnetically coupled. In this case, the model of an E_4 state containing two hydrides bridging both sites implies that upon reductive elimination of H_2 the two electrons remaining on the cofactor will have opposing spin states and will thus be localized to different irons rather than at a single super-reduced site.

no molecular structure of this state was known. A μ_2 binding mode at Fe2 and Fe6 implies a preferred protonation of the distal nitrogen and consequently its release early in the reaction cycle. This has been described as a distal mechanism. In contrast, the side-on binding would leave both nitrogen atoms in an equivalent position, facilitating an alternating transfer of electrons and protons that would lead to a successive reduction of the N–N bond order and a final cleavage of this bond only very late in the cycle.[51] The vanadium-containing variant of nitrogenase now presents a unique opportunity to directly address those questions, and the generation of further reaction intermediates may be the best chance yet to unravel the molecular details of the mechanism of biological nitrogen fixation.

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■ ABBREVIATIONS

EPR, electron paramagnetic resonance; DFT, density functional theory; SpReAD, spatially resolved anomalous dispersion.

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