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Structural Enzymology of Nitrogenase Enzymes

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Abstract

The reduction of dinitrogen to ammonia by nitrogenase reflects a complex choreography involving two component proteins, MgATP, and reductant. At center stage of this process resides the active site cofactor, a complex metallocluster organized around a trigonal prismatic arrangement of iron sites surrounding an interstitial carbon. As a consequence of the choreography, electrons and protons are delivered to the active site for transfer to the bound N₂. While the detailed mechanism for the substrate reduction remains enigmatic, recent developments highlight the role of hydrides and the privileged role for two irons of the trigonal prism in the binding of exogenous ligands. Outstanding questions concern the precise nature of the intermediates between N₂ and NH₃, and whether the cofactor undergoes significant rearrangement during turnover; resolution of these issues will require the convergence of biochemistry, structure, spectroscopy, computation and model chemistry.

Graphical Abstract

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1 INTRODUCTION

Nitrogenase, the enzyme solely responsible for replenishing the nitrogen cycle from atmospheric dinitrogen, catalyzes the ATP-dependent reduction of N_2 to ammonia. The optimal turnover frequency of nitrogenase is approximately one N_2 per second, with an effective second order rate constant $k_{\text{cat}}/K_{\text{m}} \sim 10^4 \text{ M}^{-1} \text{ s}^{-1}$.¹ While this is several orders of magnitude slower than diffusion limited enzymatic reactions,² given the stability of the $\text{N}\equiv\text{N}$ triple bond and the immeasurably slow rate of the uncatalyzed reaction, this is a remarkable achievement. Since the physiological substrate of nitrogenase is readily available and since nitrogenase proceeds at a rather leisurely pace relative to faster and more complicated enzyme systems (including such massive assemblies as the ribosome or polymerases that are approximately 10–100 times faster adding monomers),^{3,4} one could reasonably expect that just about everything to know about nitrogenase would be known by now. Ironically, the small size of the substrate and the complexity of the active site clusters has made it challenging to define the N_2 reduction mechanism in atomic detail. Recently, however, there have been exciting advances based on structural, spectroscopic and biochemical probes of the mechanism that have established important details of how substrates interact with the active site under turnover conditions. This review discusses the current state of understanding of the nitrogenase mechanism and highlights some of the outstanding issues.

Nitrogenase consists of two component metalloproteins, the iron (Fe) protein and the molybdenum-iron (MoFe) protein [or the homologous alternative vanadium (VFe) and iron-only (FeFe) nitrogenases].⁵ The MoFe protein is organized as an $\alpha_2\beta_2$ tetramer NifD₂K₂, of molecular weight approximately 230 kDa (Fig. 1), where the α and β subunits NifD and NifK have similar folds, pointing towards an evolutionary relationship (see 2.2.1). Coordinated to the MoFe protein are two copies each of two extraordinary metalloclusters designated the FeMo cofactor and the P-cluster. Functionally, the FeMo cofactor represents the site of substrate reduction, while the P-cluster serves as the initial acceptor of electrons from the Fe protein. Overall, the MoFe protein tetramer may be considered to be composed of a pair of $\alpha\beta$ subunits that coordinate one FeMo cofactor (located in a cleft between the three domains of the α subunit) and one P-cluster buried at the interface between the α and β subunits (Fig. 1). The Fe protein, NifH₂, is a dimer of identical subunits (total molecular weight ~60 kDa) that symmetrically coordinate one [4Fe:4S] cluster, with the ATP binding sites at the dimer interface (Fig. 2).

While we are focused in this review on the molecular mechanism of nitrogenase, the cumulative effect of the fixation of N₂ by microorganisms is to drive the global nitrogen cycle, and it is instructive to consider the scale of N₂ fixed annually by nitrogenase. Although the overall flux is not straightforward to measure,⁶ an order of magnitude estimate is $\sim 10^{14}$ g [N] fixed y⁻¹, representing less than 10^{-7} of the total atmospheric N₂. At the maximum velocity, nitrogenase reduces about one N₂ per active site, producing four NH₃ per MoFe protein per second at 30 °C. Working through the conversion factors, $3 \cdot 10^{10}$ g MoFe-protein operating at maximal velocity will generate 10^{14} g of fixed [N] in one year. Of course, this amount of MoFe-protein represents a lower boundary, since under physiological conditions of temperature and the general metabolic state with sub-maximal growth rates, the rate of N₂ fixed by nitrogenase will undoubtedly be less than 1 s^{-1} . An analysis of the global mass of Rubisco, the enzyme responsible for CO₂ fixation, suggested that the average flux through this pathway for terrestrial organisms was ~1% of the maximal rate.⁷ A similar effect for nitrogenase would imply that the global mass of nitrogenase is $\sim 10^{12}$ g, which can serve as an upper limit. For reference, the global mass of Rubisco is estimated as 10^{15} g, with an estimated 10^{17} g [C] fixed yr⁻¹.⁷ The volume associated with 10^{12} g MoFe protein would be roughly 10^{12} cm^3 or $(100 \text{ m})^3$. Of this, ~1% is occupied by the active site cofactor. Until the advent during the past century of the large-scale industrial Haber Bosch process, this scale of biological nitrogen fixation was required to sustain life on Earth.

Understanding how the component proteins of nitrogenase work together to reduce N₂ has required a multidisciplinary effort involving biochemistry, molecular biology, spectroscopy, crystallography, inorganic chemistry and computational chemistry. Each approach has its own strengths – and limitations – for these studies. The critical advantage of a structural biology approach to the study of nitrogenase is the ability to define this system in three-dimensional, atomic detail. As described in the following sections, one of the rewarding aspects of the structural characterization of the nitrogenase has been the opportunity to establish the unprecedented structures of the associated metalloclusters and how ligands bind to the active site.

2 MOLYBDENUM NITROGENASE

2.1 Crystallization of Nitrogenase Components

The propensity of the MoFe protein to crystallize was recognized several years after the first purifications of the component proteins were reported.^{8,9} The crystallizability of the MoFe protein was integrated into early purification protocols^{10,11} until they were replaced by improved chromatography methods.^{12,13} The promise of high resolution structural information on nitrogenase was fueled by the 1982 report from the Mortenson group describing crystals of the *Clostridium pasteurianum* MoFe protein diffracting to a resolution of 2.4 Å.¹⁴ This was subsequently followed by an analysis with Rossmann of the molecular symmetry demonstrating the homology between the α - and β -subunits.¹⁵ Crystals of the Fe protein were published in 1983.¹⁶ Despite the presence of metalloclusters that would make the structure determination from these crystals straightforward at a present-day synchrotron source, solutions of the phase problems for these two proteins took a decade to achieve. During this period, an important insight was the finding by Bolin¹⁷ that the *C. pasteurianum* MoFe protein contained two copies each of the FeMo cofactor and P-cluster, arranged such that the two FeMo cofactors were separated by ~ 70 Å and hence could not simultaneously interact with the same substrate. This observation contrasted with the findings of the first published report of a MoFe protein crystal structure determination (at 8 Å resolution, phased by direct methods) that indicated the two FeMo cofactors were sufficiently close to be bridged by N₂.¹⁸

The first crystal structures for the nitrogenase proteins were published in 1992 for the *Azotobacter vinelandii* MoFe protein^{19,20} and Fe protein,²¹ followed shortly by structures for the *C. pasteurianum* MoFe protein.^{22,23} The resolutions of these structures were insufficient to unambiguously define the metallocluster architecture, and so the initial models integrated elemental analysis on the MoFe protein and isolated FeMo cofactor, stereochemical information from small molecule iron-sulfur cluster structures, and spectroscopic inferences. As the resolution of the crystal structures increased due to improvements in crystal quality, synchrotron beamlines, X-ray detectors, refinement algorithms and computational resources, errors in the original models were corrected. These changes included the existence of an interstitial ligand in the center of the FeMo cofactor (see 3.1)²⁴ and the presence of 7, not 8, sulfurs in the P-cluster (*vide infra*).²⁵ The original indication that one of the FeMo cofactor belt positions, initially identified as “Y” and now assigned as S5A, was lighter than sulfur did not survive scrutiny at high resolution. Ironically, this is the one belt sulfur position that has not subsequently been observed to be stoichiometrically substituted with lighter ligands in certain forms of either the MoFe protein or the VFe protein (see 3.3, 4.4).

2.2 MoFe Protein – The Dinitrogenase

2.2.1 Structure of MoFe Protein—In spite of the use of distinct structural genes clustered separately in the genome of *A. vinelandii*, all three nitrogenase systems are remarkably similar in architecture. The core of each of the three catalytic components is formed by a NifD₂K₂ heterotetramer of approximately 230–240 kDa (Fig. 1), arranged around a twofold symmetry axis (Fig. 3A). The two subunits are the 55 kDa NifD and

the 53 kDa NifK. Interestingly, the *nifD* and *nifK* genes themselves show significant sequence homology, hinting at an early duplication of a common precursor gene. The D and K subunits are further structured into three globular domains with a canonical $\beta\alpha\beta$ -fold (Rossmann fold), where a four- or five-stranded parallel β -sheet is flanked by connecting α -helices (Fig. 3B).¹⁹ In each of these domains, the C-terminal loops emanating from the β -strands interact with the two metal clusters of the protein, as is typical for this fold (Fig. 3C). The Rossmann-domains of the nitrogenase proteins are themselves phylogenetically related, suggesting that the complex nitrogen-fixing systems known today can be traced back to an original single-domain protein, possibly already functioning as ligand for an iron-sulfur cluster. This primordial ferredoxin then extended its possibilities through a gene triplication, and the dimerization of two such units further generated the possibility to bind cofactors at the dimer interface. This presumed history of nitrogenase is supported by recent studies on structurally related enzyme systems that are considered to be ancestral to the nitrogen-fixing machinery. A pair of genes that was originally described to be ‘Nif-like’ (*nif*) was recently identified to play a role in the biogenesis of the tetrapyrrole cofactor F₄₃₀, the unique Ni-porphyrin that forms the active site of methyl-coenzyme M reductase, a key enzyme in archaeal methanogenesis.^{26,27} In the Nif-like enzyme CfbDC, the CfbD proteins forms a dimeric reductase with a bridging [4Fe:4S] cluster and strong homologies to the Fe protein of nitrogenases and its relatives.²⁶ CfbD interacts with CfbC that shows the very same three-domain architecture observed in NifD and NifK, but then forms a simple homodimer as the catalytically active component.

CfbDC thus has already evolved cofactor binding in a three-domain protein, but has not yet differentiated into two distinct subunits for the catalytic part. This differentiation is not unique to nitrogen fixation, however. Two further remarkable homologs of nitrogenases are found in the biosynthesis of bacteriochlorophyll, another tetrapyrrole cofactor that predates (and that eventually heralded) the aerobic era in the evolution of life. Here, the dark-operative protochlorophyllide reductase (DPOR, Fig. 4) and the downstream-acting chlorophyllide oxidoreductase (COR) form homologous $\alpha_2\beta_2$ heterotetramers that each interact with a designated, dimeric reductase. A recent structural analysis of DPOR revealed that the enzyme contains a regular [4Fe:4S] cluster at the interface of its two subunits.²⁸ It lacks the two unique iron-sulfur sites of nitrogenases (*vide infra*) and binds its substrate, a bulky tetrapyrrole, at a position close to the binding site of the catalytic cofactor in nitrogenases. The origin of nitrogenases can thus be traced back to tetrapyrrole-modifying systems from the anaerobic world (Fig. 4).²⁹ These were then repurposed as a scaffold to accommodate a large and intricate iron-sulfur moiety, the catalytic cofactor that mediates the cleavage of the N₂ triple bond.

Mo-nitrogenase – like the other variants – thus contains two types of large iron-sulfur clusters in each DK dimer, forming two presumably independent, functional units in the heterotetrameric assembly. The position of the [4Fe:4S] cluster in DPOR and COR is occupied by the P-cluster, a unique [8Fe:7S] moiety that is formed through the fusion of two canonical [4Fe:4S] centers during the maturation of the enzyme.^{30,31} It functions as an electron relay between the reductase, Fe protein, and the second of the metal clusters of nitrogenase where reductive catalysis takes place. All three known variants of nitrogenase contain this P-cluster, and at least in the two structurally characterized proteins utilizing

molybdenum and vanadium, respectively, its architecture is highly conserved (see 2.2.2, Figs. 5, 13C). In contrast, the catalytic cofactor, an even larger, iron-sulfur-based metal cluster, differs among the classes of nitrogenases. Historically, Mo-nitrogenase is by far the most thoroughly characterized enzyme. It contains the FeMo cofactor at its active center, a [Mo:7Fe:9S:C] cluster of high symmetry complemented with an organic *R*-homocitrate ligand (see 2.2.3). As a key difference, V-nitrogenases replace the molybdenum ion with vanadium, while in Fe-nitrogenases, iron is employed exclusively. Recent years have seen substantial progress in understanding the atomic and electronic structure of FeMo cofactor, forming the basis for most current hypotheses regarding the functionality of nitrogenases.

2.2.2 The Role of the [8Fe:7S] P-cluster—Among the peculiarities of P-cluster, a site that according to current knowledge is entirely unique to the three classes of nitrogenase enzymes, is that it is typically isolated in an all-ferrous state, i.e. formally with 8 Fe²⁺, conveying a total charge of +2 (Fig. 5A). All-ferrous iron-sulfur clusters are rare, but interestingly, a second occurrence of such a highly reduced site is found in the corresponding reductase, Fe protein (see 2.3.1). Fe protein serves as the electron donor to MoFe protein, with the P-cluster acting as a relay for a single electron moving from the [4Fe:4S] site in Fe protein to the active site, FeMo cofactor. However, with P-cluster in an all-ferrous state, the addition of another electron from Fe protein is far from trivial. According to the current state of knowledge, this problem is solved through an intricate sequence of events triggered by the formation of a complex between ATP-bound Fe protein and the catalytic MoFe protein that starts with a single-electron transfer from P-cluster to the cofactor that only then is followed by the oxidation of the [4Fe:4S] cluster of Fe protein.³² This has been designated a ‘deficit-spending’ mechanism, and it implies that upon docking to MoFe protein, Fe protein has a means of lowering the reduction potential of P-cluster such that it can reduce the cofactor.^{33,34} Whether complex formation in itself is sufficient to achieve this or ATP hydrolysis is already required at this point remains a matter of debate. A kinetic study by Seefeldt and co-workers concluded that the hydrolysis of ATP would only occur after the oxidation of Fe protein and that the subsequent release of inorganic phosphate from hydrolyzed ATP constitutes the rate-limiting step of the process,³⁵ although this model is challenged by the finding that the known structures of complexes of MoFe and Fe proteins do not show any structural changes within the catalytic component that were triggered by complex formation alone.

A further significant feature of the P-cluster was found through structural analysis, showing that the cluster as isolated (i.e. reduced with sodium dithionite that is used throughout the purification procedure, designated P^N) shows a different conformation than a P^{Ox}-form that is 2-electron oxidized, either by chemical oxidants such as phenosafranine, indigo carmine or ascorbate or by short air exposure. The P^N state of P-cluster is highly symmetric, with the central sulfide S1 placed almost precisely on the pseudo-twofold symmetry axis relating the D and K subunits of the MoFe protein, reflecting their common origin. Upon oxidation to the P^{Ox} state, however, this symmetric arrangement is broken by two of the Fe ions, Fe5 and Fe6, which shift position to release their coordination by S1 and approach new ligands. In the case of Fe6 this is a largely conserved serine residue, Ser 188^K in the β -subunit (NifK) of the *A. vinelandii* MoFe protein, while in another group of nitrogenases

this oxygen-based ligand is instead provided by a tyrosine, as in the case of Tyr 98^K of the enzyme from *Gluconacetobacter diazotrophics*, where this leads to a repositioning of Fe8 rather than Fe6.³⁶ In addition, Fe5 in both groups moves to interact with the backbone amide of residue Cys 88^D in the α -subunit (NifD, *A. vinelandii* numbering), notably requiring deprotonation of the amide nitrogen atom (Fig. 5C), while a recent computational study has found that in *A. vinelandii* MoFe protein, Ser 188^K is also deprotonated in the P^{Ox} state.³⁷ Various structural analyses have shown that this conformational change is reversible and that it is found in V-nitrogenase as well as in the Mo-dependent variant.^{38,39} The transition from P^N to P^{Ox} represents a 2-electron oxidation, while the mechanism of N₂ reduction by the enzyme is commonly discussed in single-electron steps. A one-electron oxidized P¹⁺ state of P-cluster has been identified spectroscopically, but has long remained elusive, until a recent structural analysis of redox-poised crystals of MoFe protein by Peters and co-workers revealed that in this state only Fe6 shows the previously defined ligand exchange for Ser 188^K, while Fe5 retains the position observed in the P^N state (Fig. 5B). This finding was corroborated by the first crystal structure of a vanadium nitrogenase, where an otherwise largely identical P-cluster was isolated in a mixed state between P^N and a P¹⁺ with a shift only of Fe6.³⁸ The unusual conformational rearrangement of P-cluster is further reminiscent of an observation made in the oxygen-tolerant hydrogenase form *Cupriavidus necator* (previously *Ralstonia eutropha*), where a single Fe site of a unique [4Fe:3S] cluster undergoes a movement that is highly analogous to what is found for P-cluster (Fig. 5D).^{40,41} Such structural flexibility in a metal site is likely to be of functional significance, and in nitrogenase it seems safe to assume that this feature is mechanistically linked to the 'deficit-spending' electron release from the P^N state that only occurs after the docking of a reduced Fe protein dimer. A conformational change in P-cluster not only alters its midpoint potential for re-reduction drastically, but also relays information about its oxidation state to affect the affinity of both protein components and trigger ATP hydrolysis.

The fact that both the [4Fe:4S] cluster of Fe protein and P-cluster were found to accept and release one or two electrons has led to considering the possibility that such 2-electron transfer events might occur under physiological conditions.^{25,42–44} This would be of major mechanistic significance, as the interaction of Fe protein with the dinitrogenase is linked to the hydrolysis of two ATP molecules, so that a concerted transfer of two electrons would effectively reduce the ATP requirement of the enzyme by 50 percent. While no instance of physiological two-electron transfer has yet been found in any functional assay or *in vivo*, the known flexibility of the nitrogenase system does not allow to generally rule out this type of mechanism.

Surprisingly, mutagenesis studies of residues coordinating the P-cluster have established that at least certain substitutions, or even deletions, can be tolerated, while maintaining the ability to fix nitrogen at high enough levels so that the cells can grow diazotrophically.^{36,45–49} Structural studies from the Tezcan group have further demonstrated the compositional lability of the P-cluster upon mutagenesis of surrounding residues, where despite the loss of one or two Fe, nitrogen reduction activity is retained.^{36,49} One of the challenges in understanding the role of the P-cluster in nitrogen fixation is how to reconcile the evident sensitivity of this metallocluster to structural perturbations, yet at the same time, the modified nitrogenases can still fix dinitrogen at levels sufficient to support cell growth.

2.2.3 The Catalytic Cofactor of Nitrogenase, FeMoco—The second cluster of the nitrogenases constitutes the active site for the reduction of a variety of small-molecule substrates. Like the P-cluster it is a variation on the theme of iron-sulfur clusters, but differs starkly from all other metal sites within this family. Its structure has been of long-standing interest (see 3), and is now recognized to have the composition [Mo:7Fe:9S:C]:homocitrate in the case of Mo-nitrogenase, where it is designated ‘FeMo cofactor’ (Fig. 6).⁵⁰ This cluster complements 7 iron ions with a single molybdenum, digresses from regular iron-sulfur clusters by containing one more acid-labile sulfide than it has metal ions and features a μ_6 -carbide that is unprecedented in biology. It also incorporates an organic homocitrate molecule that serves as a ligand to molybdenum and is synthesized by a homocitrate synthase, the NifV protein, from acetyl-coenzyme A and 2-oxoglutarate.^{51,52} In the FeMo cofactor, the carbide holds a central position, fusing two cubane-like half clusters that are additionally connected by three μ^2 -bridging sulfides. The entire cluster is D_3 -pseudosymmetric, with only the apical Mo ion breaking the point group symmetry.

More importantly, with a coordination to the protein *via* a cysteine residue to Fe1 and a histidine to molybdenum, all seven iron ions are tetrahedrally four-coordinate, while molybdenum is octahedrally coordinated through three sulfide ligands from the cluster, homocitrate and the aforementioned histidine. At first glance this implies that the entire cluster does not contain any free coordination site for a ligand, indicating that some kind of structural change or rearrangement should occur during catalysis. Since the first structural models for FeMo cofactor became available in 1992,²⁰ a multitude of proposals based on spectroscopy, theory or model chemistry were put forward to address the questions regarding substrate binding and mechanism. The present review focuses on the implications of these models and on more recent findings that have led to substantial progress in understanding the chemical properties and functional features of this cofactor.

2.3 Fe Protein / Nitrogenase Reductase is a P-loop NTPase

2.3.1 Structure of Fe protein and Functional Implications—The Fe protein consists of a dimer of two highly conserved subunits that symmetrically coordinate a [4Fe:4S] cluster (Fig. 2A). As sequence information became available, regions and residues could be identified that were important for the function of the Fe protein in coupling ATP hydrolysis to electron transfer to the MoFe protein. Anticipating that the cluster ligands were plausibly cysteines, the coordinating residues were identified as Cys 97 and Cys 132 (residue numbering of the *A. vinelandii* protein sequence) through sequence conservation and protein chemistry,⁵³ and subsequently confirmed by site directed mutagenesis.⁵⁴ It was further evident that regions of the Fe protein exhibited significant sequence similarities to other proteins known to interact with nucleotides.⁵⁵ Of particular note was the region near the N-terminus now recognized as the P-loop or Walker motif A with a characteristic sequence motif GKGGXGKS that interacts with the terminal phosphates of ATP of the non-hydrolyzable analog AMPPCP (Fig. 7A) and ADP (Fig. 7B). The Fe protein was subsequently found to be a member of a distinct branch of dimeric P-loop containing ATPases and GTPases including proteins mediating protein targeting, DNA replication and transport that are characterized by a Walker A motif with the two conserved lysines.^{56–58} A related family of P-loop-containing GTPases includes Ras and other G-proteins; both

families are characterized by the P-loop and switch I and II regions that couple the protein conformation and the nucleotide state.

The crystal structure of the *A. vinelandii* Fe protein confirmed these predictions and revealed the detailed molecular architecture.²¹ The Fe protein dimer exhibits approximately two-fold molecular symmetry, with the rotation axis passing through the [4Fe:4S] cluster at one end of the dimer surface. The two chemically identical subunits fold as a single α/β domain of a type frequently observed in nucleotide binding proteins with a predominantly parallel β -sheet flanked by α -helices. Two residues (Cys 97 and 132) from each subunit coordinate the [4Fe:4S] cluster (Fig. 2B). Significantly, conserved nucleotide binding motifs from each subunit are present at the dimer interface, immediately suggesting that the Fe protein conformation would be intimately coupled to the nucleotide state (Fig. 7C). These features are conserved in the structurally characterized NifH Fe proteins from *C. pasteurianum*⁵⁹ and *Methanosarcina acetivorans*,⁶⁰ and the VnfH protein from *A. vinelandii* (see 4.3).⁶¹

The [4Fe:4S] cluster is solvent exposed at the surface that is coordinated by Cys 97. Remarkably for protein bound [4Fe:4S] clusters, the Fe protein can exist in three distinct oxidation states, that include the oxidized [4Fe:4S]²⁺ and dithionite-reduced [4Fe:4S]¹⁺ forms identified in early work on nitrogenase, as well as the all-ferrous [4Fe:4S]⁰ form that can be obtained after incubation with Ti(III),^{62,63} but also with the physiological electron donor, the flavodoxin NifF.⁶⁴ The electron transfer reaction between the Fe protein and MoFe protein is generally envisioned as involving the 2+ to 1+ couple, although a role for the all-ferrous form has been proposed, implying two-electron transfer and thus an ATP/e⁻ ratio of 1:1 under certain conditions.^{62,64} The Fe in [4Fe:4S] clusters may be assigned as either valence-localized sites (Fe²⁺ or Fe³⁺) or as valence delocalized Fe^{2.5+}:Fe^{2.5+} pairs from the isomer shifts observed in Mössbauer spectra.^{65,66} The Fe protein in the S=1/2 spin state of the [4Fe:4S]¹⁺ form is composed of a delocalized Fe^{2.5+} pair and a pair of Fe²⁺. A recent analysis of the site-specific oxidation state assignments in the Fe protein using spatially refined anomalous dispersion (SpReAD, see 3.2.2) identified the delocalized Fe^{2.5+} pair at the solvent exposed face of the cluster, coordinated by Cys97, while the buried Fe coordinated to Cys132 are ferrous. As the form of the Fe protein competent to transfer electrons to the MoFe protein might be expected to have the reduced Fe nearer to the surface, the role of MgATP could be to promote an internal redox rearrangement such that the positions of the Fe²⁺ and Fe^{2.5+} pairs are switched. This may be promoted by the dipole moment of a long α -helix pointed directly at the cluster in both monomers (Fig. 2A).⁶⁷

2.4 Nitrogenase Complexes

Complex formation between the MoFe protein and Fe protein plays a critical role in the substrate reduction mechanism as this is the species in which intermolecular electron transfer is coupled to ATP hydrolysis. Complexes between the two component nitrogenase proteins from *A. vinelandii* have been characterized crystallographically in the presence and absence of various nucleotides. The initial structure determination was of the ADP·AlF₄⁻-stabilized complex with two Fe protein dimers bound to one MoFe protein tetramer (Fig. 8A).^{68,69} The molecular twofold axis of the Fe protein coincided with the pseudo-two fold

axis relating the α - and β -subunits of the MoFe protein, such that the [4Fe:4S] cluster was positioned near and aligned with the P-cluster. Consequently, the relative positions of the metalloclusters indicated that electron transfer from the Fe protein to the FeMo cofactor proceeds through the P-cluster. Two ADP·AlF₄[−] complexes per Fe protein dimer are bound at the interface between subunits. The nucleotide binding interactions are similar to those observed in other nucleotide switch proteins, with the phosphates bound to the P-loops and the Mg²⁺ coordinated by residues in the switch I and switch II regions. While the MoFe protein conformation is essentially unchanged from the uncomplexed structure, significant structural changes occur in the Fe protein that may be described as a ~13° rotation of each subunit towards the dimer interface in the complex. The [4Fe:4S] cluster is not a hinge point of this motion, but rather the cluster shifts ~4 Å towards the MoFe protein as the switch II region undergoes extensive rearrangements between the free and complexed forms of the Fe protein.

The transition between different conformational states of nucleotide switch proteins can be controlled by the rate of nucleotide hydrolysis serving as a timing mechanism. Ultimately, the rate of nucleotide hydrolysis reflects the ability of the protein to create the appropriate catalytic site.⁷⁰ Efficient hydrolysis of ATP requires the appropriate positioning of two catalytic residues, a general base in the switch II region near the γ -phosphate and a positively charged residue near the β -phosphate. One of the fascinating aspects of these systems is that the rate of nucleotide hydrolysis can be controlled by the interaction of various external factors; for G-proteins, these include GTPase Activating Proteins (GAPs) that provide the positively charged arginine finger, and Regulators of G-protein Signaling, or RGS proteins that stabilize the conformation that is catalytically competent for nucleotide hydrolysis.^{71,72} In the ADP·AlF₄[−]-stabilized nitrogenase complex, the catalytically critical residues are Asp 129 and Lys 10 of NifH, which unexpectedly interact with the nucleotide predominantly bound to the other subunit. Hence, each Fe protein subunit serves as the GAP (or more appropriately AAP) for the other subunit, while the MoFe protein stabilizes the catalytically competent form and hence is functionally equivalent to the RGS protein. The lack of significant ATP hydrolysis by the isolated Fe protein likely reflects the requirement of binding to MoFe protein to stabilize the catalytically competent conformation.

By cocrystallization using near-physiological MoFe protein and Fe protein concentrations and ionic strengths, nitrogenase complexes were prepared and structurally characterized in the nucleotide-free state, in the presence of the non-hydrolyzable ATP analog MgAMPPCP, and in the presence of the MgADP product (Fig. 8B).⁷³ These structures showed the Fe protein occupying distinct, but mutually exclusive and overlapping docking modes showing a variation in distance between the [4Fe:4S] cluster and P-cluster. By co-crystallization in the presence of both MgADP and MgAMPPCP, a structure with two nucleotides asymmetrically bound to the Fe protein was prepared, suggesting that ATP hydrolysis and phosphate release may proceed by a stepwise mechanism.⁷⁴ The conformational richness of the Fe protein underlies the coupling of protein structure and nucleotide state central to the orchestration of the sequence of electron transfer reaction. The ability in turn largely reflects the quaternary architecture of the Fe protein with the two subunits linked through the [4Fe:4S] cluster and the nucleotide binding sites at the subunit-subunit interface, so that changes in nucleotide state can be linked to intermolecular electron transfer processes.

2.5 Mo-Nitrogenases from Other Species

While the majority of the structural studies of the MoFe-protein have utilized the protein isolated from *A. vinelandii*, structural studies have also been reported for the orthologs from *C. pasteurianum*,^{23,75} *Klebsiella pneumoniae*^{76,77} and *G. diazotrophicus*.³⁶ Highlights of these studies include how the *C. pasteurianum* MoFe-protein accommodates a ~50-residue insertion in the α subunit positioned over the FeMo-cofactor, along with an ~50-residue deletion in the β subunit. This reflects the reported subclassification of nitrogenase genes into four different groups (excluding the Vnf and Anf systems), as defined in 2013, where *A. vinelandii*, *K. pneumoniae* and *G. diazotrophicus* belong to group I, while the 50-residue insertion is a hallmark of group II enzymes such as the one from *C. pasteurianum*.⁷⁸ Different changes at low pH in the active sites of the *A. vinelandii* and *C. pasteurianum* MoFe-proteins have been reported⁷⁹ that presumably reflect their variations in the FeMo-cofactor environment. The structure of the NifV⁻ variant of the *K. pneumoniae* MoFe protein directly established that citrate coordinates the Mo in place of homocitrate and also served as the first nitrogenase mutant to be structurally characterized. An unusual feature of the *G. diazotrophicus* MoFe-protein is that the residue corresponding to Ser^{188K} in the *A. vinelandii* MoFe-protein is an alanine; as a compensating change in the P^{Ox} state, the side chain of a non-equivalent Tyr residue, Tyr 99^K, coordinates Fe8. These two changes covaried in about 20 sequences within the 2013 study and are consequently likely to represent a widespread variation.⁷⁸

3. UNDERSTANDING FEMOCO

When isolated MoFe protein became available and various spectroscopic techniques were applied to generate an increasing amount of data,⁸⁰ the uniqueness of the metal cofactors nitrogenase became apparent early on. In the absence of a three-dimensional structure, synthetic chemistry made many attempts to obtain small-molecule models that reproduce all or some of the observable features of the enzyme. Based on elemental analysis, a molybdenum site within (or directly coupled to) an iron-sulfur-based scaffold was generally assumed.^{81–83} For the P-cluster, such analyses postulated a pair of [4Fe:4S] clusters, coming very close to the actual structure.⁸⁴ However, the highly unusual FeMo cofactor could not be convincingly modelled, so that understanding its structural features required the determination of a crystal structure at high resolution.

3.1. Completing the Atomic Structure of FeMoco

When the first crystal structure of Mo-nitrogenase was reported in 1992, the structure of FeMo cofactor defied expectations in many aspects. The initial structural model of *A. vinelandii* NifDK at a resolution of 2.7 Å consisted of two incomplete cubane-type subclusters, a [4Fe:3S] and a [Mo:3Fe:3S] unit, μ_2 -bridged by three non-protein ligands.^{19,20} Two of these were modelled as sulfides, while the third was designated 'Y'.¹⁹ The structure also revealed that the entire cluster was only liganded by the protein through its apical atoms, coordinated to two amino acid side chains, Cys 275^D to Fe1 and His 442^D to Mo. The previously identified homocitrate molecule exclusively coordinated the molybdenum ion.²⁰ In its center, the novel metal site contained a surprisingly large, internal cavity with a diameter of approximately 4 Å, surrounded by six central and coordinatively undersaturated

Fe ions. While possibly quite small for accommodating N₂, this unusual position presented itself as a prime candidate for a substrate binding site,⁸⁵ but at the same time it raised the question how this cluster was able to retain its structural integrity. FeMo cofactor can be extracted from the protein into *N*-methylformamide, following denaturation under acidic conditions and used to reconstitute active nitrogenase by adding it to separately purified apoprotein.^{86–93} Also, the resting state that was represented in the crystal structure is unable to bind N₂ prior to activation by a 3- or 4-electron reduction (see 5.4).⁹⁴ At first glance, the structure could not readily explain the stability and relative chemical inertness of the cofactor. Regarding structure, subsequent computational studies tended to include metal-metal bonding interactions that helped to reproduce the atomic structure observed in the crystal.⁹⁵ The central cavity of the cofactor thus did not seem to be a high-affinity substrate binding site, and the function of the cluster – for the time being – remained largely unknown.

3.1.1. Identification of a Central Ligand in FeMoco—In the following years, *A. vinelandii* Mo-nitrogenase continued to be the most highly resolved structure, but underwent successive improvements from the initial analysis at 2.7 Å,^{19,20} a further refinement to 2.2 Å,⁸⁵ and eventually a model at 2.0 Å.²⁵ For the understanding of the accessory P-cluster this progress was crucial, allowing to correct the original assignment of a [8Fe:8S] moiety to [8Fe:7S] and revealing the structural changes it undergoes upon change of redox state (see 2.2.2, Fig. 5). For FeMo cofactor, however, the structural model remained unchanged with the exception of identifying the bridging ‘Y’ as a third μ²-sulfide, S5A. A more highly resolved 1.6 Å structure for the enzyme from *K. pneumoniae* showed an identical FeMo cofactor.⁷⁷ In the 2F_o–F_c difference electron density maps of all these analyses, the central cavity of the cluster was well-defined and quite obviously empty, both in the presence and absence of substrates.

In 2002, the change of crystallization protocols from batch to vapor diffusion then produced single crystals of unprecedented quality, allowing for a re-determination of the structure of *A. vinelandii* MoFe protein at a truly atomic resolution of 1.16 Å that radically changed the picture of the cofactor.²⁴ At this resolution, the central cavity of the cofactor was found to contain a previously unseen, well-defined electron density maximum. It was modeled as a μ₆-coordinated light atom that according to its intensity could represent either a carbide (C^{4–}), nitride (N^{3–}) or oxide (O^{2–}). The sudden appearance of this atom raised the question whether the same species had been present, but overlooked, in earlier analyses, considering that the best available structures of MoFe proteins from *A. vinelandii* (2.0 Å) and *K. pneumoniae* (1.6 Å) were highly resolved already and depicted the very same, dithionite-reduced resting state. It was then recognized that the unusual structure of the cofactor itself led to the obfuscation of the central atom in a unique, resolution-dependent manner. The theoretical basis for this, in brief, is as follows: Each atom in FeMo cofactor individually scatters the incident X-rays in a diffraction experiment, and the diffracted photons interfere to generate an observable diffraction pattern that can be converted into a real-space electron distribution function by means of a Fourier transform. The diffraction data provides structure factors, i.e. the Fourier coefficients, and in theory, the synthesis of an infinite number of structure factors will yield a perfect representation of the electron distribution in real

space. In practice, of course, any Fourier synthesis is carried out with a finite number of coefficients, leading to a discrepancy between the experimental map and the real structure. More precisely, a limited number of structure factors leads to Fourier series termination artifacts that manifest as periodic noise (known as ‘ripples’). In a typical electron density map of a protein molecule that overwhelmingly consists of light atoms from H to C, N or O, such ripples are small and tend to cancel each other out. The noticeable exception are individual heavy atoms in a structure, such as metal ions or – more severely – large metal clusters.

FeMo cofactor is the largest biological metal cluster known to date, and in addition it features an unmatched structural symmetry that is focused exactly on the central position. It is surrounded by the six iron ions Fe2-Fe7, arranged as a trigonal prism, and in addition it is equidistant to all nine sulfide ions present in the cluster. As a consequence, the minor, individual ripple effects of six iron and nine sulfides add up to a severe and resolution-dependent distortion of the electron density *only* at this very position. This leads to a defined profile of electron density vs. resolution that actually represents an artifact attributable to the unique cofactor geometry. Unexpectedly, the result of this additive ripple effect at the center of the cofactor was found to be a *negative* electron density feature. It occurs in a resolution range between 2.2 Å and 1.55 Å and is of a magnitude that is sufficient to mask the presence of a light atom at this point (Fig. 9A). The calculated limit of 1.55 Å was precisely reflected in the fact that this atom was not yet visible in the structure of *K. pneumoniae* MoFe protein determined to 1.6 Å resolution, but was clearly defined at 1.16 Å in *A. vinelandii* MoFe protein (Fig. 9D).²⁴ It has since been established that the central atom is an integral structural component of nitrogenase cofactors, although for a long time after the discovery of its presence, its chemical nature and function remained under debate.⁵⁰

After its discovery, the central ligand of FeMo cofactor was designated as a ‘light atom X’ (with X being either C, N, or O).²⁴ In the initial characterization at 1.16 Å resolution, a careful quantification of the observed electron density feature was interpreted as N being the most likely candidate for the μ_6 -ligated interstitial atom, but even at this resolution, a definitive answer could not be given. Considering the physiological reactivity of the enzyme, it was obviously tempting to speculate whether an interstitial nitrogen atom might originate from the cleavage of N₂, in line with the earlier concept of the central position representing a substrate binding site. However, during the following years, spectroscopic studies using ENDOR and ESEEM by Hoffman and co-workers first could not detect evidence for an exchangeable N atom,⁹⁶ and subsequently pointed out that in any case the presence of nitrogen should have been recorded in this approach, so that the central atom should be of a different nature.⁹⁷ In parallel, density functional calculations of the cofactor had been established through the work of Noodleman and co-workers^{98,99} that subsequently investigated the possible nature of a central ligand, concluding that neither O²⁻ or C⁴⁻ were likely candidates for the central atom,¹⁰⁰ in line with the Hoffman group that also could not conclusively assign the central ligand X.¹⁰¹ In the following years, a series of attempts were undertaken to clarify this questions, and nearly a decade later it was the combination of a broad range of methodologies that laid the issue to rest. DeBeer and co-workers presented a study using Fe K β X-ray emission spectroscopy, where they showed that an experimental valence-to-core X-ray emission difference spectrum of intact MoFe protein

and a cofactor-free variant (*nifB* MoFe) was in far better agreement with the calculated signature of an interstitial C than with those of an N or an O.¹⁰² Similarly, the same authors also found that this atom is already present in the NifEN-bound precursor of the cofactor.¹⁰³ This work was complemented with a new crystallographic analysis of MoFe protein at 1.0 Å resolution (Fig. 9B) and with ESEEM studies of MoFe protein labelled with either ¹⁵N or ¹³C (Fig. 9C). All these techniques provided consistent evidence for an interstitial C⁴⁻.¹⁰⁴ Important information regarding the origin of the central carbide then came through work of Ribbe and Hu, who confirmed S-adenosylmethionine as the carbon donor during cofactor biogenesis, mediated by the radical/SAM enzyme NifB.¹⁰⁵ However, even with the complete atomic structure of the cofactor finally established, little was revealed about the mechanism and location of substrate binding and activation. As a carbon, the central atom clearly was not an intermediate of N₂ reduction, and a series of theoretical studies by several groups that ensued from the new data also came to widely diverging conclusions regarding the its role for the reactivity of the cluster.^{106–109}

3.1.2. Implications for Structure and Function—The discovery of the central carbide in nitrogenase FeMo cofactor provided an explanation for the relative stability and chemical inertness of the cluster in its resting (as isolated) state. Without the requirement to explain the presence of a large, open central cavity, it was easier to conceive that the entire cluster could be extracted from the protein after acid denaturation (pH 2) into *N*-methylformamide and retain sufficient structural integrity to be reconstituted into apo-nitrogenase, yielding active protein.¹¹⁰ At the same time, the completed structure now left every single metal ion in the cluster in a coordinatively saturated state, indicating that a conformational rearrangement or even a ligand exchange was required prior to the binding of N₂. Multiple suggestions were put forward in the following years, covering all bases from a substantial enhancement of cluster stability by the central ligand to a flexibilization that would allow for major rearrangements and an opening of the core to bind substrate.^{111–115} Unfortunately, the different theoretical approaches towards FeMo cofactor did not converge to a generally accepted model, and if anything, the confusion in the field increased. Not in the least this was due to the problem that the theoretical description, in particular of the electronic structure of the cofactor, was itself far from settled. In the absence of clarifying experimental data, several laboratories therefore set out to accumulate more information on the enzyme and its metal clusters, although for the time being only the resting state was accessible.

3.2. Electronic Structure of FeMo Cofactor

Early on, spectroscopic studies indicated that FeMo cofactor is a highly reduced site. EPR spectroscopy showed that in its resting state, E₀, the cluster is an *S* = 3/2 system. Iron ions most likely are ferric (Fe³⁺) or ferrous (Fe²⁺), containing five or six *d*-electrons, respectively. As is the case for other iron-sulfur clusters, in a tetrahedral environment with weak-field sulfide ligands all seven Fe ions will be in *high-spin* configuration, but may couple ferro- or antiferromagnetically. This leads to a large number of combinatorial possibilities for the electron arrangement in the cluster, as well as for the distribution of the additional electrons for ferrous sites. Finally, the oxidation state of molybdenum in biological systems in most cases is Mo⁴⁺ or Mo⁶⁺, adding up to a total cluster charge that is compensated by the

9 formal sulfides (S^{2-} , total charge contribution -18) and the carbide (C^{4-}). Independent of the actual charge distribution in detail, all electrons in question have to add up to the observed $S = 3/2$ system, while leaving the cofactor with a total charge that is chemically reasonable.

3.2.1. Broken-Symmetry Approaches toward Coupling Interactions in FeMo Cofactor

—Much spectroscopic data probing the electronic properties of FeMo cofactor were compiled over decades, but a comprehensive description of the electronic structure of the site was crucially dependent on the development of appropriate theoretical methods. Noodleman and co-workers made a highly significant contribution by introducing spin-polarized broken-symmetry density functional theory (BS-DFT) to interpret the possible combinations of relatively localized electronic configurations. Among a series of chemically reasonable solutions, they favored a coupling scheme termed BS7 that has been widely used since and is currently the basis for most proposed electronic models.⁹⁹ In the BS7 scheme, four Fe sites in spin-up configuration couple to three Fe sites in spin-down, maximizing the amount of antiferromagnetic coupling of each site with its neighbors. The molybdenum ion was investigated by ^{95}Mo ENDOR. It was found to be highly reduced and was consequently assigned as a Mo^{4+} , in line with other Mo sites in biological systems.¹¹⁶ Within these boundaries, the question how many ferrous sites then are required to result in a total spin of $3/2$ is a matter of combinatorics: Based on ^{57}Fe Mössbauer spectroscopy – but prior to the discovery of the central carbide – Burgess and Münck proposed a configuration of 4Fe^{2+} and 3Fe^{3+} ,¹¹⁷ but combinations of $2\text{Fe}^{2+} : 5\text{Fe}^{3+} : \text{Mo}^{4+}$ and $6\text{Fe}^{2+} : 1\text{Fe}^{3+} : \text{Mo}^{4+}$ would yield the correct spin state as well. The resulting ambiguity regarding the charge assignments within the cofactor prevented any model from gaining general acceptance, and furthermore contributed to the divergence in the results of theoretical studies due to the lack of a guiding electronic description.

3.2.2. SpReAD Assignment of Individual Oxidation States to the Cluster

Metals—The element-specific absorption of X-rays by core electrons of any given element provides an elegant approach for the assignment of charges in metal clusters. In addition, the element iron is also amenable to Mössbauer spectroscopy, where individual iron sites manifest characteristic quadrupole splitting and isomer shifts that reveal details about their oxidation state and chemical environment. Of the two types of metal ions present in FeMo cofactor, molybdenum was the more straightforward to address, as it is present in only a single copy. In biology, the element molybdenum is most commonly used to support two-electron transfer reactions or oxygen atom transfers, as in the reactions of formate dehydrogenase or nitrate reductase.^{118,119} The element is found in two stable redox states, Mo^{4+} ($4d^2$) and Mo^{6+} ($4d^0$), both of which are diamagnetic. The original assignment of the Mo site in FeMo cofactor as Mo^{4+} thus was canonical and in line with expectations. It would also determine the arithmetic for the charge distribution of the iron sites, as with two unpaired electrons on Mo, the observed $S = 3/2$ system of the resting state of the enzyme then implied either 2, 4 or 6 Fe^{2+} sites ($3d^6$). The complication in charge assignment to the Fe sites is in their number: Nitrogenase MoFe protein contains 7 iron ions in the cofactor, and another 8 in P-cluster, and both X-ray absorption and Mössbauer spectroscopies invariably only detect the cumulated spectroscopic signature of all these sites.

Münck and co-workers had published an extensive Mössbauer analysis of nitrogenase, but were left with an ambiguity in their assignment,¹¹⁷ and X-ray absorption spectra are not sufficiently feature-rich to deconvolute 15 individual sites. For molybdenum, however, the technique was ideally suited. DeBeer and co-workers investigated both the enzyme and several model compounds of known electronic state by high energy resolution fluorescence-detected (HERFD) X-ray absorption spectroscopy (XAS) at the K-edge of Mo, and found that the pre-edge and also the rising edge features of the enzyme were not consistent with an Mo⁴⁺ assignment. An analogous finding was made for an important model, a (Et₄N) [(Tp)MoFe₃S₄Cl₃] complex originally synthesized by Holm.¹²⁰ In both cases the lower energy of the edge position indicated a more reduced species, and in the case of the smaller model compound, DFT calculations supported an assignment as Mo³⁺ (4d³). Interestingly, the coupling of the spin system of Mo with the partially delocalized Fe sites in the complex led to a highly unusual, non-Hund $\alpha\alpha\beta$ ground state.¹²¹

A Mo³⁺ site in a biological system is unprecedented, which further highlighted the requirement to understand the charge distribution among the Fe sites within the cluster. The solution here was to exploit the effect that X-rays are diffracted by the electron shell of an atom. When in close proximity to an absorption edge, X-rays are absorbed by the core electrons of an atom (the basis for XAS), which will have a profound effect on the diffraction properties of this particular element. The interaction of an ejected core electron with the remaining particles in the shell of the atom breaks the internal centrosymmetry of the diffraction process that is described by Friedel's law.¹²² It states that the intensity of a reciprocal lattice point at a position (h, k, l) is identical to one at position ($-h, -k, -l$), albeit with opposite phase angles. In the proximity of an absorption edge this is no longer true, giving rise to an *anomalous difference* D_{ano} in intensity. This is directly proportional to the absorption coefficient for X-rays, so that it provides an information equivalent to an X-ray absorption spectrum. The advantage of reading this information out of a diffraction process, however, is that a three-dimensional reciprocal lattice is recorded that preserves spatial resolution in addition to the absorbance measurement.¹²³ The contribution of each individual absorbing site to the magnitude of D_{ano} can be determined during the structure refinement. If a series of data sets is collected along the absorption edge, the refined values of D_{ano} thus approximate the individual absorption edges of single atoms in space. This *spatially refined anomalous dispersion* (SpReAD) was first applied to the [2Fe:2S] ferredoxin Fd4 from *A. aeolicus* that contains localized, antiferromagnetically coupled Fe(II) and Fe(III) sites.¹²³ In the SpReAD analysis, the individual edge positions for Fe(III) were higher in energy by approximately 2 eV with respect to those for Fe(II), in line with expectations from XAS and theory.

This simple test case contained a dimer of the single-cluster protein Fd4 in the asymmetric unit, with a total of four individual Fe sites to be refined. Applying the SpReAD method to nitrogenase involved at least a complete NifD₂K₂ heterotetramer with two copies each of the P-cluster and FeMo cofactor, amounting to 30 Fe sites. The SpReAD analysis of the resting state of FeMo cofactor showed that Fe1, Fe3 and Fe7 were more reduced (lower energy edges), while Fe2, Fe4, Fe5 and Fe6 were more oxidized (Fig. 10A). In addition, the presence of P-cluster in the all-ferrous P^N state provided a precise internal reference for Fe²⁺, aligning fully with the profiles of Fe1, Fe3 and Fe7. If, accordingly, the remaining four

Fe ions are assumed to be ferric the system indeed added up to the observed total spin of $S = 3/2$,¹²⁴ as FeMo cofactor is a $S = 3/2$ state as isolated, and the BS7 coupling scheme established by Noodleman and co-workers⁹⁸ in conjunction with a spin-coupled Mo(III) site in a non-Hund ground state¹²¹ implies a formal distribution of 4 Fe(III) and 3 Fe(II). The data was in agreement with the valence electrons of the system being largely localized. This could be rationalized through the maximized antiferromagnetic coupling implied in the BS7 scheme, although model complexes show that in canonical iron-sulfur clusters the delocalization of spins across ferromagnetically coupled metal centers should be the rule.¹²¹ Based on the re-assignment of Mo as Mo^{3+} and the SpReAD results with respect to the Fe ion, Björnsson and DeBeer re-evaluated Münck's Mössbauer and largely confirmed the new interpretation. In the accompanying DFT calculations, although the respective ferromagnetic coupling of Fe3/Fe4 and Fe5/Fe7 resulted in spin delocalization that was not reproduced in the SpReAD data.¹²⁵ Importantly, in both approaches Fe2 and Fe6 were identified as the most oxidized sites in the E_0 state of FeMo cofactor (Fig. 10B).¹²⁶

3.2.3. Spatial Analysis of the Magnetic Properties of FeMo cofactor—In its as isolated state, Mo nitrogenase shows a surprisingly simple spectral signature in continuous-wave electron paramagnetic resonance spectroscopy (EPR). With P-cluster in the P^{N} state, FeMo cofactor exhibits an $S = 3/2$ signal with a rhombic g tensor with apparent principal components $g_x = 2.01$, $g_y = 3.65$ and $g_z = 4.31$. This feature is not straightforward to reconcile with the structure of the cofactor and its D_3 pseudosymmetry. An alignment of the magnetic tensor and the crystal structure was made by X-band EPR spectroscopy of single crystals, where only the respective cross-section of the g tensor ellipsoid with the magnetic field of the spectrometer absorbs microwaves, so that the rotation of the crystal in the magnet allows for scanning the tensor orientation that can subsequently be correlated to the crystal orientation that is obtained by X-ray diffraction on the same crystal.¹²⁷ As expected, the longest apparent principal component, g_z , oriented along the threefold cluster axis. Of the other principal axes, $g_x = 2.01$ was in the plane formed by Fe1, Fe2, Fe6 and Mo in FeMo cofactor, providing another indication that the three-fold symmetry of the cluster is not reflected in its electronic properties and that Fe2 and Fe6, together with the apical metals of this moiety, play a prominent role (Fig. 10B, 11A).

3.2.4. An Electronic Structure for the Resting State—Through the combination of several data sources regarding the structural, spectroscopic and thus electronic features of FeMo cofactor as discussed in the previous sections, a comprehensive model for the resting state E_0 of the enzyme has emerged and gained increasing acceptance. Interestingly, the three-fold symmetry that gives the cofactor its unique appearance is not reflected in its internal electronic properties. Instead, two adjacent Fe positions, Fe2 and Fe6, are distinguished as the most highly oxidized sites in the resting state. A possible reason for finding these sites so clearly localized is in the complex electrostatic environment provided by the protein matrix. It embeds the cofactor in its entirety, limiting access of solvent molecules to defined pathways and providing positively charged amino acid side chains that stabilize more reduced Fe sites opposing the Fe2-Fe6 edge. This distribution furthermore depends on the complex antiferromagnetic coupling scheme revealed by the broken-symmetry model BS7.⁹⁸ In order to maintain the asymmetric electron distribution in

the cofactor, this mechanism effectively counteracts charge delocalization within the cluster. While the analysis of FeMo cofactor thus had not revealed an obvious binding site for substrate molecules, it provided a convincing rationale for an internal asymmetry of the cluster that already hinted at very specific binding events rather than a catalysis based on the extensive metal-sulfur surface of FeMo cofactor. However, these considerations did not yet address the pressing issue that in a cofactor structure with a central carbide all individual metal seems seem to be entirely coordinatively saturated, so that a binding position for any ligand – inhibitor or substrate – is not readily apparent.

3.3. Ligand Binding to FeMoco

Even before a three-dimensional structure of the enzyme had become available in 1992, inorganic model complexes had provided multiple suggestions regarding the nature of the metal clusters in nitrogenase. Holm and co-workers produced large iron-sulfur systems with obvious similarities to the metal sites found in the first crystal structures,^{19,20,128} and Tatsumi and co-workers assembled a topological equivalent of P-cluster with apical, symmetric Mo sites in a single step from [2Fe:2S] precursors.¹²⁹ Early on, the six central iron atoms of the cluster were considered as the most probable binding sites for a ligand.¹³⁰ These positions show a complete tetrahedral ligand environment, but the Fe ion is closer to the plane formed by its three sulfide ligands than to the geometric center of the tetrahedron, so that an external ligand could be envisioned to distort the site towards a pentacoordinate trigonal bipyramid. This idea was the basis for the successful series of model complexes by Peters and co-workers that eventually yielded compounds competent in the catalytic reduction of N₂ at a single, reduced iron ion. The Peters compounds used three coplanar phosphine ligands that in later studies were replaced with thiolates and featured axial Si, B or C ligands, resulting in some of the most efficient small-molecule catalysts for activation of N₂ under mild conditions.¹³¹ They require a highly reduced Fe site as well as low-potential electrons, and while such conditions are not readily compatible with an aqueous environment they emphasize the primary boundary conditions for N₂ activation that any catalytic system must address (*vide infra*). Interestingly, the discovery of a Mo³⁺ site in FeMo cofactor raised new considerations regarding a catalytic role of the heterometal that had been proposed early on to be the actual catalytic site of the enzyme by Coucouvanis and others.¹³² Indeed, molybdenum was the metal used in the first actual small-molecule catalysts for dinitrogen reduction by Schrock¹³³ and Nishibayashi.¹³⁴ These compounds exploited the wide range of oxidation states that the heterometal is able to attain. A reduced Mo³⁺ species is the site for N₂ binding, while the stabilization of a Mo⁶⁺ nitride is required after N-N bond cleavage.^{135,136} At first glance, both types of complexes feature a free coordination site on the metal cation that is obviously not present in the octahedrally coordinated Mo³⁺ ion of the FeMo cofactor. However, considering that two of the six coordination positions are occupied by the organic homocitrate ligand that is located in a solvent-filled cavity within the protein matrix, it cannot be excluded that a structural rearrangement of the ligand exposes a coordination site on Mo. From a chemist's perspective, both Mo and Fe thus are suitable metals for the task, but the coordination of a ligand invariably requires an accessible coordination site, so that a distortion of the cluster from its resting state conformation, or even the release or exchange of a ligand are necessary before N₂ can bind. For long, none of the available crystal structures of

nitrogenases provided any indication as to what kind of change might occur and what actual sites and modes of binding ligands are possible for this cofactor.

3.3.1. Ligand Binding: CO and Selenide—In enzymology, a frequently used strategy to evaluate ligand binding modes in enzymes that rapidly turn over their actual substrates is to generate stable complexes with inhibitors instead and investigate the possible mechanistic relevance of their observable binding mode. As nitrogenase is a reductase of extraordinary strength, only few molecules are known to inhibit the enzyme, the most relevant being carbon monoxide (CO) that blocks the reduction of any other known substrate, with the notable exception of protons.¹³⁷ CO binds to a range of metal cations as a terminal or μ -bridging strong-field ligand, and in nitrogenase the inhibition of N_2 reduction by CO was found to be non-competitive.¹³⁸ Mechanistically, this type of inhibition implies that the inhibitor binds to a site where substrate cannot bind. This may be a physically different site, making the inhibition allosteric, or it might be a different state, as is the case here. As a critical part of the catalytic mechanism, the binding of N_2 to nitrogenase requires reduction of the enzyme by four electrons, while CO binds after a two-electron reduction of the cofactor.¹³⁹ Substrate and inhibitor therefore do not compete for binding, even if their binding sites were identical. In addition, EPR and infrared spectroscopy have differentiated two separate CO binding events that are commonly designated the low-CO and high-CO states and are assumed to have one or two molecules of CO bound to the cofactor, respectively.^{138,140–146} Part of the challenge in interpreting the inhibitory mechanism of CO is that while the K_i is $\sim 1\text{--}10\cdot 10^{-4}$ atm,¹³⁷ the concentrations used in biophysical studies are typically 1000-fold higher (0.1–1 atm). Furthermore, the time scale for development of the characteristic EPR signals following CO addition is several orders of magnitude longer than for CO inhibition of dinitrogen reduction.¹⁴¹ Obtaining such structures is severely complicated by the inability of Mo-nitrogenase to bind CO in its resting state. The relevant two-electron-reduced state E_2 is only reached under turnover conditions, and the dynamic formation of the electron transfer complex with Fe protein makes this virtually impossible to achieve in a crystal.

The solution to this problem was found by inhibiting the enzyme under turnover and subsequently isolating MoFe protein, growing crystals and measuring diffraction data, leading to a 1.5 Å resolution crystal structure of the MoFe protein of nitrogenase inhibited by a single molecule of CO, i.e. the low-CO state of the enzyme.¹⁴⁷ In this structure, the CO ligand could be unambiguously assigned, but its binding position was entirely unexpected. Replacing the μ_2 -bridging sulfide S2B at Fe2 and Fe6 of the cofactor, the CO ligand caused a structural change of the cluster and formed a bridging carbonyl that is chemically reasonable, but would have been impossible to predict correctly in the absence of three-dimensional structural information (Fig. 12A). When CO was removed under continued turnover, a resulting crystal structure showed that sulfide S2B had returned to its position, with the resulting structure being indistinguishable from the known resting state.¹⁴⁷ In spite of an anomalous electron density maximum detected approximately 20 Å away from the cluster, the fate of S2B in the CO complex remained unknown, but the binding position of CO at the cluster inspired further hypotheses on mechanistic relevance. Importantly, Holland and co-workers reported a small-molecule Fe:S-based compound that

bound N₂ under dissociation of an Fe-S bond, emphasizing that a connection between Fe-S bond cleavage and substrate binding may well be chemically reasonable.¹⁴⁸

The observed replacement of a μ_2 -bridging sulfide with CO and the reinstatement of the original ligand upon removal of the inhibitor then prompted experiments aimed at replacing atom S2B with a spectroscopically and structurally more easily detectable selenide anion, Se²⁻. While eventually successful, this could not be achieved by simple addition of selenide under turnover conditions, but rather required the alternative substrate SeCN⁻, whose reduction yields the products methane and ammonium, leaving the enzyme in a formal resting state, but with the exchangeable ligand as a Se2B bridging Fe2 and Fe6 (Fig. 12B).¹⁴⁹ Under turnover in the presence of acetylene, the selenide initially bound at the 2B position was additionally found to migrate through the remaining μ_2 -sulfides S3A and S5A. Remarkably, although the belt sulfur positions are separated by 5.7 Å in the resting state of the cofactor, these sites can all interchange during acetylene reduction. After several thousand catalytic turnovers, the total Se was lost and apparently replaced by S, leading to a recovery of the original FeMo cofactor. Neither the source of this S, nor the pathway(s) by which Se exits the MoFe protein, were identified. The observation that Se2B can migrate to S5A and S3A and is ultimately chased from the cofactor provided clear evidence that structural flexibility is an intrinsic feature of the nitrogenase cofactor. The coordination of the cofactor through only two protein ligands may facilitate the underlying metallocluster rearrangements.

3.3.2. CO as a Substrate for Nitrogenases—The strong-field ligand CO forms stable complexes with a range of synthetic compounds and metalloenzyme active sites. Its binding to FeMo cofactor highlights the flexibility of the complex cluster, but at second glance the fact that an enzyme able to activate and reduce N₂ is inhibited by CO is not immediately obvious. Accordingly, Ribbe, Hu and co-workers found that the alternative vanadium nitrogenase from *A. vinelandii* did indeed reduce CO, albeit at lower rates.¹⁵⁰ The product was not primarily methane, but instead the reduction overwhelmingly involved a C-C bond formation leading to 93% of the carbon to be found as ethylene, C₂H₄.¹⁵¹ In order to achieve this, the enzyme most likely has to bind two molecules of CO, conveying potential mechanistic significance to the high-CO state mentioned previously.¹⁵² Methane was found at yields below 1% in these reactions, even less than the further products ethane (3%) and propane (2.5%). The formation of hydrocarbons from CO is obviously reminiscent of the well-established Fischer-Tropsch process, but there the main product is the comparatively low-value methane that requires further, energy-intensive conversion to more chemically versatile products, while the enzyme's standard mode of operation already includes the formation of carbon chains. It is not surprising that this ability of vanadium nitrogenase has raised immediate interest regarding the potential for biofuel production from CO, and subsequent studies showed that this reactivity can be driven in an ATP-independent fashion, in contrast to N₂ fixation. Using isolated cofactor and chemical reductants, the product chain length could be extended up to C₇, although ethylene mostly remained the primary product.¹⁵³ Using highly sensitive analytical methods, even Mo-nitrogenase was shown to reduce CO, but the rates observed here were more than 700-fold lower than for the V-dependent enzyme.¹⁵⁴ CO is therefore correctly described as an inhibitor of substrate

reduction by nitrogenase (with the important exception of proton reduction to form H₂). The fact that MoFe and VFe proteins exhibit such striking differences in their ability to reduce this alternative substrate is an important reminder that the two variants of nitrogenase differ in key aspects of their functionality.

4. VANADIUM NITROGENASE

4.1. Isolation of native VFe Protein

The observation that *A. vinelandii* retains the ability to fix N₂ in the absence of available molybdate goes back to 1971,¹⁵⁵ but was initially suspected to be based on traces of molybdenum in the growth media or the used vanadium salts.¹⁵⁶ Bishop and co-workers then presented evidence for a second enzymatic system for nitrogen fixation that was based on vanadium in 1980,¹⁵⁷ and its presence was subsequently confirmed through the construction of a *nifDK* strain that was still able to fix N₂ in the presence of vanadium, although it lacked the structural genes for molybdenum nitrogenase.^{158,159} Such deletion strains allowed for the isolation of the component proteins of vanadium nitrogenase from *A. vinelandii* and the closely related *Azotobacter chroococcum*.^{160,161} More recently, Hu and Ribbe reported the *A. vinelandii* variant YM68A that contained a hexahistidine-tagged VnfK in a *nifDK* background, allowing for straightforward isolation of the protein,¹⁶² but still retaining the non-natural situation of a *nif*-deletion strain. The early reports on vanadium nitrogenase indicated that the removal of molybdenum was non-trivial to start with, as *A. vinelandii* contains a Mo-storage protein to counteract phase of metal depletion. In order to counteract this effect in the *A. vinelandii* type strain,¹⁶³ molybdate was omitted from all growth and selection media for at least five complete cycles of cell growth and individualization on agar plates. Eventually, diazotrophically growing were obtained cells that almost exclusively produced the vanadium-based nitrogen fixation system.¹⁶⁴ Both the catalytic VFe protein and its cognate Fe protein VnfH were isolated by anion exchange and size exclusion chromatography, forming the basis for the determination of the three-dimensional structures described in the following sections.

4.2. Structure of VFe Protein

Based on the optimized isolation protocols for unmodified VFe protein from its native *A. vinelandii*, well-diffracting single crystals of the enzyme were obtained that allowed for the determination of a three-dimensional structure to a resolution of 1.35 Å. As expected, the protein was based on a heterotetrameric D₂K₂ assembly that underlined its close relationship with MoFe protein (Fig. 13A, B).³⁸ As in the latter, vanadium dinitrogenase contained an [8Fe:7S] P-cluster at each DK-interface, and contrary to reports suggesting a different architecture for this cluster, the observed dithionite-reduced state had the exact same arrangement of cubane-type subclusters sharing a μ₆-sulfide as found in MoFe protein (Fig. 13C). The active site FeV cofactor was also cradled in the three Rossmann-subdomains of VnfD, as is the case with FeMo cofactor in MoFe protein. With this high overall similarity, the major structural difference between the two nitrogenases was an N-terminal extension of NifK that wraps around the surface of the adjacent NifD in three extended α-helices (Fig. 13A). In VnfK this extension is missing, and is replaced by a β-strand that adds to the central β-sheet of the third Rossmann-domain of VnfD. This visible difference to the

outward appearance of both proteins actually obfuscates the high degree of conservation of the relevant functional features of both enzymes. The K-subunits of the VFe protein are bridged by two symmetrically bridging metal cations. While in the MoFe protein the analogous sites were modelled as Ca^{2+} ,^{19,24} but later shown to be at least partly replaced by a 16th Fe,¹⁶⁵ the 1.35 Å resolution structure of the VFe protein strongly indicated that in this case the ion is an octahedral Mg^{2+} .³⁸ In both enzymes these sites are presumed to play a structural role in stabilizing the heterotetramer, but beyond this, a function related to the observed half-side reactivity of the enzyme cannot be ruled out,^{166,167} implying some degree of conformational communication between the two DK-heterodimers.

Beyond these strong similarities, VFe protein differs from its Mo-containing counterpart through the presence of an additional subunit, encoded by the *vnfG* gene that is located between the structural genes *vnfD* and *vnfK* in the genome of *A. vinelandii*.¹⁶⁸ Before the availability of a crystal structure, only little was known about structure, role, and even stoichiometry of this additional subunit.³⁸ The 113 aa VnfG forms a globular four α -helix-bundle (Fig. 13D) that occurs in two copies exclusively contacting with VnfD on opposing sides of the resulting 240 kDa $\text{VnfD}_2\text{K}_2\text{G}_2$ heterohexamer (Fig. 13B). VnfG contains no cofactor and is located sufficiently remote from the putative docking site for the Fe protein VnfH to not interfere in the formation of an electron transfer complex during substrate reduction at VFe protein.³⁸

4.2.1. Properties of FeV cofactor—As the cofactors of all three known variants of the nitrogenase enzyme derive from a common precursor, the ‘NifB cofactor’ or ‘L-cluster’ produced by the radical/SAM enzyme NifB, the three-dimensional structure of VFe protein fulfilled expectations in that FeV cofactor indeed retained the overall bridged double-cubane structure of its Mo-containing analog, of course with the apical heterometal molybdenum replaced by vanadium (Fig. 14). The biogenesis pathways of the cofactors branch after the action of NifB, and the insertion of the heterometal then occurs on the EN complex, a scaffold protein that is a structural homolog of the enzymatic component itself,¹⁶⁹ and that in the *nif* cluster comprises a NifEN machinery, while in the *vnf* cluster a separate VnfEN system is used.¹⁶⁸ Consequently, FeV cofactor is a heterometal cluster with a central, interstitial carbide, as predicted by XAS,¹⁷⁰ that contains a single vanadium ion taking the position of Mo in the FeMo cofactor. V is coordinated by a single histidine residue from the VnfD subunit and the organic ligand *R*-homocitrate, the product of the enzyme NifV. In spite of their different sizes, the Mo ion in FeMo cofactor and the vanadium ion in the cluster of VFe protein show almost identical bond distances to their respective ligands, and both clusters are structurally very similar.³⁸ One difference between the two moieties was, however, entirely unexpected. Rather than sharing the three μ_2 -sulfides of FeMo cofactor, S3A, S5A and S2B that form the characteristic edges of the cluster,²⁴ FeV cofactor retained only S2B and S5A, while the S3A sulfide at Fe4 and Fe5 was replaced by a 1,3-bridging tetraatomic ligand (Fig. 14A).³⁸ According to the 1.35 Å resolution electron density map this ligand was assigned as carbonate, CO_3^{2-} , with identical bond distances and a hydrogen-bonding pattern that excluded protonation of its oxygen atoms. At this stage the presence of a nitrate anion, NO_3^- in place of CO_3^{2-} could not be fully excluded based on the crystallographic analysis alone, but strong points speaking for carbonate were its

availability as the hydrated product of aerobic glucose oxidation that drives diazotrophy in *A. vinelandii*, as well as the known immediate ‘switch-off’ of nitrogenase activity in the presence of nitrate in the cell, a signal that sufficient bioavailable nitrogen is present. More recently, computational studies have also supported the assignment as carbonate.¹⁷¹

4.2.2. The Role of a Carbonate Ligand—The larger ligand and its binding mode increase the Fe4 - Fe5 distance to 2.76 Å,³⁸ compared to 2.61 Å in FeMo cofactor,¹⁰⁴ leading to the most significant distortion in metal-metal distances in the two clusters, but the functional role of this ligand change remains unclear. Carbonate is firmly embedded in a loop of the VnfD subunit (residues 335–340 in *A. vinelandii*) that varies from the corresponding loop in NifD by having the positions of a proline and a leucine residue swapped (Fig. 14B). This generates a cavity in VnfD that accommodates carbonate, but is blocked by the proline in NifD. As the active site cofactor of nitrogenase is formed *ex situ* and inserted into apo-nitrogenase only as the final step of its biosynthesis, the S3A ligand that is present in the L-cluster precursor of all cofactors must be exchanged for carbonate prior to this insertion. The protein will then bind the cluster tightly, precluding any further dissociation of the ligand. The carbonate ligand of the FeV cofactor does not seem to be directly involved in catalysis in VFe protein, including in particular the reduction of CO that occurs here.^{39,172} It is not currently known what factors are involved in the removal of sulfide and its replacement with carbonate. The process likely takes place on VnfEN, but may well require further maturation factors, and the effect of carbonate on the catalytic properties of FeV cofactor remains to be elucidated.

4.2.3. Biochemical and Biophysical Distinction from MoFe Protein—In the physiologically relevant reduction of dinitrogen to ammonia, V-nitrogenase only shows approximately one third of the catalytic activity of Mo-nitrogenase. One may therefore speculate that the insertion of carbonate is a posterior optimization, without which the alternative system might be performing even more poorly. On the other hand, the insertion of a carbonate might even be required to affect the electronic structure of FeV cofactor such that it becomes functional in N₂ reduction in the first place. A recent XAS study revealed vanadium in FeV cofactor to reside in the 3+ oxidation state in its resting state, as is the case for Mo in FeMo cofactor.¹⁷¹ However, V³⁺ has a 3d² configuration, while Mo in FeMo cofactor is 4d³, in an unusual, non-Hund ααβ configuration.¹²¹ Both clusters are commonly considered to have a *S* = 3/2 multiplet ground state, which then implies that one Fe site in FeV cofactor must be more highly reduced than in FeMo cofactor. A carbonate ligand may fundamentally influence the spin distribution within the system and thus its reactivity towards substrates, but further studies will be required to assess this effect.

4.3. VnfH, the Fe Protein of Vanadium Nitrogenase

The three nitrogenase variants of *A. vinelandii* use active site cofactors that differ in their namesake heterometal, but are otherwise very similar in their core structure as they are derived from a common precursor, the NifB cofactor or ‘L-cluster’.¹⁷³ In contrast, the P-clusters present in all three systems seem to be very similar in all aspects. The same is true for the basic functionality of receiving electrons from the ATP-hydrolyzing Fe protein (see 2.3). Nevertheless, the distinct gene loci of the three nitrogenase variants all contain

a distinct ortholog of the NifH protein of Mo-nitrogenase that is designated VnfH in the V-dependent system and AnfH in iron-only nitrogenases. The expression of these H-genes is coregulated with that of the structural genes for the cognate dinitrogenase, and yet the reductase components share the same, conserved features of a homodimeric P-loop NTPase with a bridging [4Fe:4S] cluster that obtains two cysteine ligands from each monomer. In *A. vinelandii*, NifH and VnfH share 91 % sequence identity, a degree of similarity that is also reflected in the recently determined structure of the ADP-bound form of VnfH (Fig. 15A).⁶¹ The two proteins are exchangeable and work with either dinitrogenase without any discernible functional difference. Moreover, the ADP-bound states of both reductases are near identical in their quaternary structure, so that this conformation – ready for nucleotide exchange – is also a defined state rather than a flexible one (Fig. 15B). From this state, Fe protein obtains an electron from its redox partner and exchanges ADP for ATP to be ready for the next round of interaction with the dinitrogenase.

4.4. Ligand Binding to FeVco

Although the exchange of molybdenum for vanadium and the insertion of an additional carbonate ligand seem to infer substantial differences between FeMo and FeV cofactors, most structural features are conserved, and the clusters follow the same mechanistic principles for the reduction of N₂.¹⁷⁴ This extends to the organic homocitrate ligand, as well as to the attachment of the clusters via an apical cysteine to Fe1 and a histidine to the heterometal. The carbonate ligand that is present in FeV cofactor likely only finetunes the electronic properties of the cluster, but not to a degree that would affect its basic functionality. This is also reflected in the fact that the immediate surrounding of both cofactors remains nearly identical. The bridging sulfide S2B that was already highlighted above is in close proximity to two conserved residues, His180 and Gln176 in VFe protein and His195 and Gln191 in MoFe protein, respectively. The exchange of either residue in MoFe protein had a substantial effect on N₂ reduction activities,^{175,176} and the histidine forms a direct hydrogen bond to S2B, connecting it to a conserved hydrogen-bonding network within the protein that extends to the nearby protein surface, suggesting a role in proton transfer to the cluster during substrate reduction.³⁹ Note that based on theoretical approaches other pathways have also been proposed.¹⁷⁷ In contrast, the side chain of glutamine, the other conserved residue, points away from the cofactor in all known structures, making its role less clear. Sulfide S2B is the ligand that is reversibly exchanged for CO and also for selenide in MoFe protein,^{147,149} and in the first structure available for VFe protein the ligand was fully in place.³⁸ This resting state structure of the enzyme also showed the typical EPR features reported previously by others and the enzymatic activity of the protein was well within the expected range, amounting to approximately 1/3 of the activity of MoFe protein.¹⁶⁴

When isolation protocols were refined further, however, a second, distinct species became apparent, whose EPR signature showed the same apparent *g* values as the resting state, but was characterized by distinct changes in the relative intensities of the signals.¹⁶⁴ The EPR spectrum of VFe protein indeed differs significantly from the straightforward, rhombic *S* = 3/2 signal of the resting state (*E*₀) of MoFe protein with its apparent *g* values of 2.01, 3.65, and 4.31 (see 3.2.3, Fig. 16A).¹²⁷ The alignment of this *g* tensor with the cluster

structure is compatible with the evaluation of its electronic structure by XAS and SpReAD (see 3.2.2).¹²⁴ At the same time, the FeMo and FeV cofactors are very similar in structure and are suggested to follow the same principles for substrate reduction. The EPR signal of resting state VFe protein is similarly broad as for MoFe protein, nearly 3000 G, and shows signals at apparent g values similar to the ones for FeMo cofactor. In addition, however, an axial signal at 3470 G as well as a band at 1300 G are visible, indicating a mixture of several spin states in the resting state preparations (Fig. 16B).¹⁶⁴ Münck and co-workers had previously suggested an explanation of the spectral differences based on Mössbauer and EPR studies. They described the $S = 3/2$ system of FeMo cofactor with a slightly axial effective g value of $g_x = g_y = 2.003$ and $g_z = 2.03$, and used this model to rationalize the observed differences between FeMo and FeV cofactor by variations of the systems' rhombicity parameter E/D representing the lower Kramer's doublet of the $S = 3/2$ system (Fig. 16C).¹⁷⁸ Here, an E/D of 0.05 would model the apparent g values of FeMo cofactor, while the resting state spectrum of FeV cofactor would result from an E/D of 0.30 (Fig. 16D). Alternatively, the EPR spectrum of resting state VFe protein was frequently described as a mixture of an $S = 1/2$ signal at a field of 3470 Gauss, the $S = 3/2$ signal known from FeMo cofactor and a further contribution at 1300 Gauss that was attributed to an $S = 5/2$ signal. The changes observed during the optimization of the isolation procedure can be explained as a change in the population of these signals, but not their positions.³⁹

The structural analysis of this state at 1.2 Å resolution then revealed an unexpected change at the FeV cofactor, in that sulfide S2B was removed from the metal cluster, as previously observed in the CO and Se adducts of the analogous FeMo cofactor (Fig. 17). The site was instead occupied with a protonated light atom, NH or OH, which in turn allowed for further rearrangements to occur in the vicinity of the Fe2-Fe6 edge of the cluster. Bound to either Fe ion at a bond distance of only 2.0 Å compared to the 2.3 Å of a sulfide or selenide, the ligand created the space for the side chain of the adjacent Q176 to rotate by 130° towards the cluster and form a short (2.8 Å) hydrogen bond with the imidazole side chain of H180. In turn, this rearrangement then opened a binding pocket that was previously occupied by the amide moiety of Q176 and that now provided a binding site for sulfide S2B, at a distance of only 7 Å from its former position in the cluster. At the same time, the carbonate ligand and the remaining μ_2 -sulfide S5A were retained. The presence of S2B in this binding pocket was confirmed by analysis of the anomalous scattering signal of sulfide, and the charge distribution in the surrounding protein matrix indicated that the ion was present as a hydrosulfide anion, HS⁻.³⁹ The positive electrostatic potential contribution required to accommodate the anion in this pocket was provided by backbone amides of the protein chain of the D subunit, reminiscent of the arrangement of an oxyanion hole in serine proteinases that is essential for stabilizing the oxyanion in the tetrahedral transition state of peptide bond hydrolysis in these enzymes.¹⁷⁹

Importantly, a rearrangement of the residue corresponding to Q176 was not observed in the CO-bound structure of MoFe protein.¹⁴⁷ Compared to the structure of the turnover state of VFe protein, it is the larger size of the CO ligand, whose oxygen atoms forms a stable 2.8 Å hydrogen bond with H195 (corresponding to H180 in VFe protein) that would block the rotation of the glutamine (Fig. 12A). The ligand bound to FeV cofactor in its turnover state, however, is at 2.55 Å distance from the amide oxygen of Q176 in its

inward-facing position. This implies protonation of the ligand, as is also supported by an analysis of the experimental electron density maps,³⁹ yielding a very short hydrogen bond. The nature of the ligand atom itself remains under debate, as both nitrogen and oxygen would satisfy the observed electron density maximum. As μ -bridging ligands, however, both a hydroxide, HO^- , and an imide, HN^{2-} , would represent the fully reduced forms of the respective heteroatom, and the release as either H_2O or $\text{NH}_3/\text{NH}_4^+$ should merely require protonation, but no further electron transfer. Given the timescale of crystallization and structure determination, the observation of this adduct in vanadium nitrogenase is quite remarkable in itself. A hydroxo ligand would imply the binding of water to the enzyme, suggesting that water would indeed compete for this binding site on the cofactor with the substrates of reduction by nitrogenase. With several lines of evidence pointing at this binding side to be relevant for substrate turnover, it seems unproductive that VFe protein should suffer from water as a competitive inhibitor, while MoFe protein does not. Moreover, binding to Fe2 and Fe6 requires dissociation of sulfide S2B, which in turn only occurs in more highly reduced states than the resting state E_0 , likely from E_2 onwards (see 5.3). Water and ammonia are already fully reduced modifications of O and N, respectively, and would require deprotonation immediately upon binding. These protons would then have to be removed and the ligand would have to be protected from reprotonation to allow for its experimental observation. Also, a strong binding of $\text{NH}_3/\text{NH}_4^+$ in vanadium nitrogenase should manifest as substantial product inhibition in the kinetic profile of the reaction, which has not been reported (see 5.3).

A possible rationalization for observing either adduct was brought forth, arguing that while the observed ligand may represent a reduced HO^- or HN^- , its stable association was due to the cofactor itself being in a more oxidized state than the resting state E_0 .³⁹ In this model, the cluster would donate two electrons to the ligand, allowing for a substantial stabilization through backbonding interactions with the cluster. This would place an O or N ligand formally at the oxido (–II) or nitrido (–III) level, but would have the cluster in a two-electron oxidized state, corresponding to E_6 in the catalytic cycle of the enzyme rather than E_0 . Interestingly, this proposal would apply to either O or N as a ligand with analogous mechanistic implications, but while a protonated nitrogen species could originate from reduction of N_2 , the presence of O_2 can be safely excluded, as the high sensitivity of VFe protein and its reductase VnfH would lead to immediate inactivation of the enzyme at the stoichiometric amounts of O_2 required to generate a hydroxo adduct. For these reasons, HN^- was suggested the most suitable candidate for the observed ligand, but a final confirmation through corroborating analytical methods will be required to provide a definitive answer. In either case, the key finding from this work is the establishment of a dinuclear substrate binding site at Fe2 and Fe6 that was already suggested by earlier observations of CO and Se^{2-} binding. With respect to understanding nitrogenase mechanism, it provides a structural framework essential for integrating existing data, in particular from extensive kinetic studies, spanning the work of multiple laboratories over several decades.

5. KINETIC ANALYSIS OF THE NITROGENASE REACTION

5.1 Reaction Components

The first step in establishing a reaction mechanism is to identify all the necessary components (reactants, products and catalytic species). For biological nitrogen fixation, the reactants are unquestionably N_2 , a source of reducing equivalents, and protons. Although the products of nitrogen fixation could be either more oxidized or more reduced than elemental N_2 , in pioneering studies, Wilson and Burris established that the key intermediate is ammonia.¹⁸⁰ It is likely that there is also 1 H_2 is produced per N_2 reduced.¹⁸¹ There does not appear to be a unique electron donor to nitrogenase *in vivo*, and various ferredoxins¹⁸² or flavodoxins can serve this role,¹⁸³ while *in vitro* studies almost exclusively employ sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$).¹⁸⁴ Finally, nitrogenase requires MgATP ,¹⁸² while MgADP is a potent inhibitor.¹⁸⁵ Nitrogenase is not uniquely specific for N_2 and other substrates can be reduced, including protons to H_2 (so nitrogenase is also a hydrogenase) and acetylene to ethylene (the vanadium nitrogenase⁵ as well as some variants of the MoFe protein¹⁸⁶ can also produce ethane). Because of the relative ease of monitoring hydrogen and ethylene by gas chromatography, these two reactions are commonly used to assay enzyme activity rather than the physiologically relevant production of NH_3 . Other small substrates with unsaturated groups may be reduced, including acetylene, HCN , and N_3^- . Nitrogenase can also catalyze C-C bond formation, which was first observed during the reaction with CH_3NC ^{187,188} and more recently with CO .¹⁵⁴

The working model for the overall stoichiometry of N_2 reduction by nitrogenase may be summarized as

It should be emphasized that under most conditions, however, the indicated ratios of $\text{H}_2/\text{N}_2 = 1$ and $\text{MgATP} / 2 \text{e}^- = 4$ are not observed. The reasons for this range from relatively trivial (reaction conditions have not been “optimized”, with uncoupled ATPase activity) to having profound implications for our mechanistic understanding of nitrogenase, e.g. how do we know that one H_2 is produced per N_2 reduced? The ubiquitous presence of protons means that nitrogenase cannot be studied in the absence of reducible substrates. A further implication is that H_2/N_2 ratios > 1 are nearly always observed and there is no easy way to tell if H_2 production is an obligatory consequence of N_2 reduction or the consequence of a parallel process of H^+ reduction. $\text{MgATP} / 2 \text{e}^- \sim 5$ are typically observed under optimal reaction conditions with dithionite as the electron donor, which likely reflects the contribution of some uncoupled ATPase activity to the overall stoichiometry. With other reductants such as Ti(III)citrate and flavodoxin,^{43,64} $\text{MgATP} / 2 \text{e}^-$ stoichiometries ~ 2 have been reported, although a recent evaluation of flavodoxin as the electron donor found $\text{MgATP} / 2 \text{e}^- \sim 4$.³⁵ There is also an intriguing report from Mortenson that $\text{MgATP} / 2 \text{e}^- \sim 2$ is observed for $(\text{ADP})/(\text{ATP})$ ratios characteristic of nitrogen-fixing cells.¹⁸⁹

5.2 Challenges to Studying Nitrogenase Kinetics

The maximal specific activity reported for N₂ reduction by the MoFe protein is ~ 600 nmol N₂ min⁻¹ mg⁻¹ MoFe protein,¹⁹⁰ which is equivalent to ~1 dinitrogen reduced per second per active site. To put this rate in perspective, it would take one active site over 20 min to reduce sufficient N₂ to produce the amount of NH₃ equivalent to that present in the constituent amino acids of one MoFe protein tetramer. Clearly, femtosecond spectroscopy is not essential to follow this reaction – so what is the problem in studying nitrogenase kinetics? There is no one dominant issue, but rather a combination of factors that contribute to these challenges. Among these are:

Oxygen sensitivity: The nitrogenase proteins exhibit significant oxygen sensitivity and must therefore be manipulated using anaerobic techniques.¹⁹¹ The mechanism of inactivation is not well understood, but presumably is a consequence of oxidative damage or the formation of reactive oxygen species that can degrade the metalloclusters.

Sample heterogeneity: The biosynthesis of nitrogenase is a complex process involving over a dozen proteins to generate the mature metalloclusters.¹⁹² As this is an ongoing process in growing cells fixing nitrogen, it is possible that partially assembled clusters are present, generating a heterogeneous mixture of proteins in the population. Oxidatively damaged clusters constitute another potential source of heterogeneity. For example, the *K. pneumoniae* nitrogenase samples used in the development of the Thorneley-Lowe kinetic model were reported¹⁹³ to consist of 70 % active MoFe protein (on the basis of Mo content) and 45% active Fe protein (on the basis of specific activity considerations (see below)).

Multiple intermediates during substrate reduction: The nitrogenase mechanism involves multiple electrons (up to 8 for nitrogen reduction with obligatory hydrogen evolution) transferred from the external reductant to the active site by multiple cycles of ATP-dependent interaction between the two component proteins. Mechanistic studies must take into account the dynamic nature of the nitrogenase system, requiring multiple association and dissociation events between the two component proteins, as well as the ubiquitous presence of protons that are reduced to dihydrogen even in competition with other substrates. The resulting distribution of intermediates under turnover conditions significantly complicates the structural and spectroscopic investigation of substrate interactions. The contrast to photosystem II is striking, where electron transfer processes are triggered photochemically rather than by diffusion, and it is possible to prepare well-defined intermediate states of the system.¹⁹⁴ Photoinitiated electron transfer systems for delivering electrons to the nitrogenase have been reported; the system developed by Syrtsova and coworkers used eosin to reduce the Fe-protein,¹⁹⁵ while more recently, Tezcan's group engineered covalently linked a Ru-photochemical donor to the MoFe-protein and demonstrated that substrate reduction could occur in the absence of Fe-protein and ATP.^{196,197} Although the reported quantum yield was low, this approach has great potential for dissecting the nitrogenase mechanism at the level of individual electron transfer steps.

Spectroscopic challenges: Iron sulfur clusters generally have similar optical absorption spectra with broad features at ~400 nm and comparable extinction coefficients on a per Fe

basis for a given average oxidation state.¹⁹⁸ For example, the extinction coefficients at 410 nm for the as-isolated forms of the Fe protein and MoFe protein are $\sim 10 \text{ mM}^{-1} \text{ cm}^{-1}$ and $76 \text{ mM}^{-1} \text{ cm}^{-1}$ with 4 and 30 Fe, respectively. Cluster oxidation is typically accompanied by an increased absorption; upon oxidation of the Fe protein [4Fe:4S] cluster from the +1 to +2 state, the absorption at 430 nm increases by approximately $+7 \text{ mM}^{-1} \text{ cm}^{-1}$.¹⁹⁹ More importantly, it is not possible to easily use optical spectroscopy to monitor redox changes at specific clusters since they all have similar optical properties. Iron sulfur clusters exist in multiple states that are often paramagnetic, making them accessible to study by EPR and related spectroscopies.²⁰⁰ While these techniques are exquisitely sensitive to changes (in appropriately paramagnetic states) and have been effectively used to monitor populations of various nitrogenase intermediates,²⁰¹ the underlying chemical interpretations (structures of clusters and intermediates) can be challenging to decipher.

Determining the time course of product formation: Standard assays for nitrogenase activity (reduction of C_2H_2 , H^+ and N_2) are typically not monitored in real time, but rather by collecting samples at discrete time points for product quantitation by gas chromatography (C_2H_4 and H_2) or colorimetrically (NH_3). As a result, continuous time courses for product formation are not measured, and even if they were measured in the gas phase, the contribution of physical diffusion from solution to gas would need to be taken into account in interpreting the results. A notable exception is Burris' use of a hydrogen electrode to monitor formation of H_2 in solution.²⁰² There are some studies, such as the use of stopped-flow IR spectroscopy to monitor the binding of certain substrates and inhibitors²⁰³ or monitoring the consumption of dithionite²⁰⁴ that can be done in real time; advances in this area would be of great significance. Insights into intermediates has been achieved using pulsed EPR methods, principally by Hoffman and collaborators,²⁰⁵ but due to the relaxation properties, samples must be trapped at low temperature and the assignments require heroic efforts to establish the structures of intermediates.

Dithionite: Since the initial report by Bulen,²⁰⁶ dithionite has been widely used as the electron donor for nitrogenase assays, as well as a general additive to maintain oxygen-free solutions by reducing residual oxygen. It does have some potentially significant drawbacks; with nitrogenase, dithionite works as a one-electron reductant through dissociation to the radical SO_2^- species^{193,204} and does not generate the all-ferrous form of the Fe protein;⁴⁴ accumulation of the oxidation products sulfite (SO_3^{2-}) and bisulfite (HSO_3^-) can significantly influence the reduction potentials of dithionite solutions;²⁰⁷ dithionite decomposes during storage and stock supplies can contain significant levels of impurities, particularly sulfite;²⁰⁸ and dithionite provides a source of adventitious sulfur that can complicate analyses of this crucial element of metallocluster function. It is worth keeping in mind that the apparent first order rate constant for the reduction of Fe protein by 10 mM dithionite is $\sim 10 \text{ s}^{-1}$,^{193,209} which is comparable to the turnover rate; given the significant problem of dithionite decomposition, this rate may be quite sensitive to the condition of the dithionite used in a particular experiment which constitutes a source of potentially uncontrolled variability.²⁰⁸ It is also worth noting that sulfite and potentially other dithionite-related compounds can serve as sulfur donors in cluster biosynthetic reactions.²¹⁰

5.3 General Features of Nitrogenase Kinetics

A key component of the kinetic analysis of a reaction mechanism is determining the dependence of the rate of product formation on the concentrations of the various reactants. Given all the components, a comprehensive analysis of the complete time and concentration dependence of the nitrogenase reaction for multiple substrates is not feasible. Here we highlight key features of the kinetics of nitrogenase that have been established over the past half century of research.

5.3.1. The Nitrogenase Proteins Form a Dissociable Complex: The existence of a dissociable complex formed between the component proteins was established from the concentration dependence of nitrogenase activity. For a fixed component ratio (CR; given by the molar ratio of [Fe protein] to [MoFe protein] active site), the specific activity of the individual proteins decreases non-linearly as the total concentration of proteins decreases.²¹¹ This so-called “dilution effect” was interpreted by Silverstein and Bulen²¹¹ as indicating that nitrogenase can be considered a complex of “dissociable dissimilar subunits, neither being catalytic in the absence of the other”. Typical values of the dissociation constant estimated from activity measurements are $\sim 10^{-7}$ M to 10^{-6} M.^{94,212–214}

5.3.2. The Nitrogenase Proteins Dissociate After Each Cycle of Electron Transfer—Burris and Hageman²¹³ made the key observation that component protein dissociation after each cycle of electron transfer is an obligatory feature of the nitrogenase mechanism. By using an H_2 electrode to monitor proton reduction, they observed a lag period for H_2 production (but not ATP hydrolysis) under conditions of very slow electron transfer by having the MoFe protein in large excess over the Fe protein. Significantly, the lag time was proportional to the time required produce H_2 at the MoFe protein active site (i.e. – the turnover time of the MoFe protein) for a given set of conditions, but not the turnover time of the Fe protein in electron transfer. The latter relationship would be expected if the catalytically competent Fe protein:MoFe protein complex remained intact during turnover. These observations are consistent with a ping-pong mechanism of the type

where E_0 and E_1 represent consecutively reduced states of the MoFe protein, and the rate constants k_n denote complex formation with, and electron transfer from, the Fe protein. That each step is associated with electron transfer to the MoFe protein was demonstrated by following the EPR signal from the FeMo cofactor during the lag period.²⁰¹ Since electron transfer and ATP hydrolysis (but not H_2 evolution) occur during the lag phase, this indicates that ATP hydrolysis is coupled to inter-protein electron transfer, but not directly to substrate reduction.

As with any kinetic study, alternative interpretations are not precluded; the requirement for cycles of dissociation/association may reflect the protein concentrations (0.01 to 3×10^{-6} M, relative to a dissociation constant of $\sim 10^{-7}$ M) used in this study, so that the complex is likely to dissociate and the proteins must then re-associate for electron transfer. Whether obligatory cycles of association and dissociation are required at the concentrations found in

nitrogen fixing cells (~ 25 to 45×10^{-6} M)²¹⁵ is not clear. It is also possible that the initial electron transfers are required to activate an inactive (or dormant) form of the nitrogenase proteins^{216,217} and that the lag phase reflects this activation event rather than turnover. An example of this type of behavior is provided by cytochrome *c* oxidase, where the fully oxidized form in the as-isolated resting state is distinct from the fully oxidized form produced under turnover conditions.^{218,219}

5.3.3. Flux Through Nitrogenase Is Independent of Substrates Being

Reduced—A striking feature of the reaction catalyzed by nitrogenase is that the electron flux through the system is largely independent of the substrate that is being reduced.^{211,220,221} Under a given set of conditions, the number of electrons transferred to substrate per active site per unit time is (nearly) the same for the reduction of dinitrogen to ammonia (the physiological reaction), the reduction of acetylene to ethylene (commonly used to assay nitrogenase activity), or the reduction of protons to dihydrogen, which occurs in the absence (or sufficiently low concentrations) of other reducible substrates. This implies that all nitrogenase substrates effectively compete for the same pool of electrons that are pumped into the MoFe protein by the Fe protein at a constant rate and with a constant rate of ATP consumption, independent of the substrate being reduced.

5.3.4. Component Protein Dependence—An important type of mechanistic analysis is provided by titration curves, where the reaction velocity is measured for a fixed concentration of one component while the second component is varied. This is typically expressed in terms of the component ratio (CR), defined as the ratio of Fe protein to MoFe protein; an important point is whether this is defined in terms of molar ratio of proteins or molar ratio of active sites. Experimentally, when the MoFe protein is titrated with Fe protein (see ²²²), the velocity increases with increasing CR until saturation is achieved at a MoFe protein specific activity of ~ 2500 nmoles $\text{C}_2\text{H}_4 \text{ min}^{-1} \text{ mg}^{-1}$. This titration curve typically can be fit by a hyperbolic (Michaelis-Menten) type curve, although sigmoidal kinetics have been reported.^{202,223–225} The reverse titration of a fixed amount of Fe protein with increasing amounts of MoFe protein also shows apparent hyperbolic behavior with activity increases with increasing MoFe protein up to a maximal value of ~ 1500 – 2000 nmol $\text{H}_2 \text{ min}^{-1} \text{ mg}^{-1}$ (although there appears to be considerable variability in the reported Fe protein specific activities). Interestingly, as [MoFe protein] increases beyond these levels promoting maximal activity, it has been observed that the Fe protein specific activity decreases; i.e. the MoFe protein is inhibitory towards Fe protein at high concentrations.

The results of the previous two sections effectively imply that nitrogenase cycles through a series of increasingly reduced states driven by association and dissociation of nitrogenase proteins at a rate independent of the substrate. After sufficient electrons have been transferred, the product is released and nitrogenase returns to the resting state. For hydrogen evolution, the simplest form of this mechanism may be described

where E and F designate MoFe protein and Fe protein, respectively, which cycle through different redox states. This mechanism makes several simplifying assumptions, such as neglecting the binding of F_{ox} to MoFe protein and that the exchange of ATP for ADP on the Fe protein is fast. When k_R , the rate of F_{ox} reduction (and exchange of ATP for ADP) is fast relative to k , then the titration curve equations will be symmetrical with respect to interchange of the MoFe protein and the Fe protein concentrations. For the case where either component protein is in sufficient excess over the other, then the steady-state rate equation is hyperbolic in that component. When the concentrations of the components are comparable, the effect of complex formation on the concentration of the free component proteins may be significant, although the shapes of the titration curves remain approximately hyperbolic (i.e., they are not sigmoidal).

It is clear that a mechanism of the type described by eq 3 does not qualitatively fit the titration curve data, especially for the Fe protein titration. An important indication of this comes from comparison of the maximal specific activities of the MoFe protein and Fe protein. It may be shown that the specific activity for either component protein for this mechanism is proportional to $k/2$, which based on the MoFe protein specific activity = 2500 nmol H_2 min⁻¹ mg⁻¹ yields $k \sim 10$ s⁻¹. This value for k then predicts that the Fe protein specific activity should be ~ 4700 nmol H_2 min⁻¹ mg⁻¹, which is over twice that typically observed. Various reasons for this significant discrepancy have been proposed, including that some other step becomes rate limiting under conditions of high Fe protein turnover (such as maintaining the Fe protein in the reduced, ATP-bound form (i.e., k_R is not $\gg k$); the Fe protein is only ~ 45 % active (see 5.4); that there are problems with the experimental design, such as non-constant ionic strength, that compromise the activity measurement; or that eq 3 (and the underlying assumptions) is fundamentally flawed.

An example of the last possibility is provided by the observation of sigmoidal kinetics, which have been interpreted from some of the earliest kinetic studies of nitrogenase,²²³ and by subsequent studies^{212,226} to indicate that a complex with 2 Fe protein and 1 MoFe protein (i.e. a 2:1 complex), but not a 1:1 complex, is the active species for substrate reduction. In a detailed kinetic analysis of this point, Hageman and Burris concluded that while both 2:1 and 1:1 complexes are possible, the 1:1 complex was most consistent with their observations.²⁰² Crystal structures of nitrogenase complexes have identified multiple discrete, but overlapping Fe protein binding sites on the MoFe protein (see 2.4, Fig. 8B),⁷³ and, at least for the sites that have been characterized, it would not be possible for two Fe proteins to simultaneously bind adjacent to one MoFe protein active site. The observation of sigmoidal kinetics does not, of course, require that there be a 2:1 complex involved in the mechanism, but it could have distinct mechanistic origins. While the two active sites of the MoFe protein tetramer are generally assumed to be independent, sigmoidal kinetics could indicate cooperativity in Fe protein binding between the two MoFe protein active sites in a

tetramer.^{167,227} The simplest kinetic models assume that the rate of electron transfer from the Fe protein to the MoFe protein is independent of how many electrons have already been transferred, but if that is not the case, sigmoidal kinetics could also be observed.²²⁶

5.3.5. Substrates and Inhibitors Bind to Different E_n States—The competition between substrates for reducing equivalents is not simply based on the relative concentrations of the different forms and their apparent affinities (K_m s), but also depends on the details of the experiment, including the flux of electrons through the system, which is set by the concentration of component proteins. If the reduction of substrate S₁ is preferred at low electron flux, while reduction of substrate S₂ becomes favored as the electron flux increases, this is indicative that the two substrates preferentially bind to different E_n states, with S₁ favoring less reduced and S₂ more highly reduced E_n states. Studies of this type indicate that N₂ binds to the E₃ and E₄ states,^{193,228} while C₂H₂ binds to E₁ and E₂ states,²²⁹ and CN[−], CH₃CN and HN₃ may bind to an even more oxidized state, likely E₀.²³⁰ The inhibitor/poor substrate CO binds after a two-electron reduction of the cofactor.¹³⁹ An interesting consequence of this type of behavior is that the apparent K_m values of substrates will depend on the electron flux through the system. An important consequence of the binding of N₂ to higher E_i states is that ammonia production is favored over proton reduction (in the absence of any other reducible substrates) under conditions of high electron flux. This constraint coupled to the relatively low turnover rate of nitrogenase requires high concentrations of the nitrogenase proteins, but not too high if substrate binding and product dissociation require free MoFe protein (*vide infra*). The cellular concentrations of the *A. vinelandii* MoFe protein and Fe protein have been estimated by EPR measurements to be $\sim 28 \times 10^{-6}$ M (6.0 mg ml^{−1}) and 45×10^{-6} M (2.8 mg ml^{−1}), respectively, for CR = 45/(2×28) = 0.8 on the basis of MoFe protein active sites.²¹⁵ This corresponds to $\sim 6\%$ of the total cellular protein in *Azotobacter*, and as discussed in that reference, even higher levels ($\sim 10\%$) have been reported.

Enzyme inhibitors can provide important insights into the nature and number of ligand binding sites on an enzyme. In principle, this information can be used to establish whether substrates bind at the same site (competitive) or distinct sites (non-competitive). However, for complex enzymes such as nitrogenase with multiple oxidation states and potential substrate binding modes, this distinction is not required.¹⁴¹ One of the most important inhibitors is carbon monoxide. CO is isoelectronic to the physiological substrate, only binds to partially reduced MoFe protein generated under turnover conditions, and has been found to be a non-competitive inhibitor for all substrates except protons.^{137,138} Intriguingly, CO can partially block proton reduction in certain MoFe protein mutants,²³¹ as well as in the NifV[−] nitrogenase system,²³² where the homocitrate is replaced by citrate. These cases are among the few examples of an inhibitor that reduces electron flux through nitrogenase. For the inhibition of C₂H₂ reduction by CO, evidence has been presented that both molecules can bind simultaneously,²²⁹ although multiple binding modes have been identified for both species, so perhaps it is to be expected that a subset of these be occupied together.

The effect of one substrate on the reduction of another substrate can be analyzed for inhibitory modes, which can illustrate the complexities of nitrogenase kinetics. As an example, C₂H₂ is a non-competitive inhibitor of N₂ reduction, but N₂ is a competitive

inhibitor of C₂H₂ reduction.²³³ This is a consequence of the observation noted above that N₂ binds to a more reduced form of nitrogenase than C₂H₂. A substrate binding to a more reduced state appears as a competitive inhibitor towards one binding to a less reduced (i.e. more oxidized) state; conversely, the second substrate appears as a mixed-type/non-competitive inhibitor of the first. On the basis of these patterns of inhibitions, attempts have been made to assign substrates and inhibitors to different binding sites (ranging from two to five different sites).^{137,234} Not surprisingly, a self-consistent pattern has not identified, undoubtedly due to the challenges arising from different binding modes, binding to different E_n states and the possibility of multiple types of binding modes for a given substrate.

Mechanism-based inhibition can be effectively used to investigate the catalytic mechanism and would provide a potent new direction for probing substrate reduction, particularly for the more highly reduced states of the MoFe protein that bind substrates. One solution would be to work at higher pH to minimize the rate of proton reduction, thereby increasing the concentrations of more highly reduced states. While it has been reported that the MoFe protein is denatured by incubation above pH 8.65,²³⁵ a subsequent analysis demonstrated instead that the MoFe protein undergoes a turnover-dependent inactivation reaction at pH 9.5, implying this is a mechanism-based inactivation process that likely involves higher reduction states.²³⁶ While the inactivated MoFe protein has distinct properties from wild type MoFe protein (including an expanded hydrodynamic radius), it retains a full complement of metals and can still interact with Fe protein and supports ATP hydrolysis, but not substrate reduction. The underlying inactivation mechanism is unknown, but perhaps reflects a rearrangement of a more reduced, deprotonated form of the FeMo cofactor.

5.3.6. N₂ Reduction, H₂ Evolution and HD Formation—There is clearly a special relationship between H₂ and N₂, although the exact relationship is complex since H⁺ is a ubiquitous substrate. Key observations include that N₂ reduction cannot (apparently) eliminate H₂ production,²³⁷ with extrapolation of limiting H₂ yields under high N₂ pressures^{181,238} suggesting that a residual 1 H₂ is produced per N₂ reduced for the enzyme from *A. vinelandii*. H₂ is a competitive inhibitor of the reduction of N₂, but remarkably does not affect the reduction of any other substrate, including H⁺ reduction. In one of the most characteristic, but enigmatic processes, N₂ is required for the nitrogenase-catalyzed exchange reaction between D₂ and H⁺ to yield HD²³⁹

This reaction was interpreted by Chatt²⁴⁰ as reflecting the displacement of hydride ligands as H₂ upon binding of N₂ as observed for small molecule complexes. In the case of nitrogenase, N₂-dependent HD exchange may reflect D₂ displacement of bound N₂ to form deuterides at the catalytic center that are subsequently protonated to form 2HD;^{241,242} if N₂ displaces H₂ upon initial binding this overall process requires 2 electrons or 1 electron per HD, as observed.²³⁹ More detailed studies suggest that HD exchange requires not just bound N₂, but a reduced form of dinitrogen, perhaps at the diimide (N₂H₂) level.^{176,239}

5.4 A Kinetic Mechanism for Nitrogenase: the Thorneley-Lowe Model

The conceptual framework at the foundation for most discussions of the nitrogenase mechanism derives from two key insights of Joseph Chatt:^{240,243}

- i. Binding of N_2 to a metal center can proceed through displacement of H_2 generated by the 2-electron reduction of protons.
- ii. Metal bound N_2 was proposed to be reduced to NH_3 through a 6-electron process involving an alternating sequence of proton and electron transfers.

According to this framework, the reduction of N_2 to NH_3 by nitrogenase is an 8-electron process with obligatory H_2 evolution, represented in the overall stoichiometry of Eq. 1.

The Thorneley-Lowe kinetic scheme for nitrogenase^{94,244} reflects this view and builds on the kinetic foundations discussed above that the nitrogenase component proteins undergo obligatory cycles of ATP-dependent complex formation as the MoFe protein cycles through states E_0 to E_7 . The Thorneley-Lowe model is organized around two interconnected cycles (the Fe protein and MoFe protein cycles) rooted in observations of Hageman and Burris that the nitrogenase proteins dissociate after each electron transfer:^{202,245,246}

Fe protein cycle, where electrons are transferred from the Fe protein to the MoFe protein in an ATP dependent process. For each electron transferred, the Fe protein must undergo a cycle of nucleotide exchange and reduction by either flavodoxin or ferredoxin; this likely takes place following dissociation of the Fe protein – MoFe protein complex (Fig. 18A).

MoFe protein cycle, where the MoFe protein goes through a sequence of increasingly reduced states, starting with the resting (“as-isolated”) state (E_0), with the subsequent states designated E_i to reflect the cumulative delivery of i electrons from the Fe protein (Fig. 18B).

Because the reduction of all known substrates by nitrogenase entails two or more electrons, the complex of the two nitrogenase proteins must turnover multiple times. Even for a simple substrate such as H^+ , it can be appreciated that there are a significant number of distinct states that need to be incorporated in a realistic kinetic model. Key features of the nitrogenase mechanism integrated into the Thorneley-Lowe model to model substrate interactions and the transitions between these states include:

- i. Substrates do not bind to the resting E_0 state, but rather the MoFe protein must be reduced by ~2–4 electrons to the E_2 to E_4 states before they can bind, with the possible exception of acetylene binding to E_1 .
- ii. The flux of electrons through the MoFe protein is independent of the substrate being reduced, or the number of electrons already transferred. In the absence of other reducible substrates (typically C_2H_2 or N_2), the electron flux through nitrogenase continues at the same rate to reduce protons to H_2 .
- iii. Only free MoFe protein (i.e. MoFe protein not in complex with the Fe protein) can bind substrates or release products.
- iv. The MoFe protein active sites are independent.

The full form of the kinetic scheme involves 15 rate constants whose values were determined using a series of pre-steady state stop flow and steady-state kinetic experiments on the *K. pneumoniae* molybdenum nitrogenase at 23 °C, pH 7.4. The rate-determining step under optimized conditions in these studies was described as dissociation of the Fe protein – MoFe protein complex, with $k \sim 6.4 \text{ s}^{-1}$ at 23 °C, corresponding to maximal specific activities of MoFe and Fe protein of 1650 and 3000 $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, respectively.¹⁹³

While the Thorneley-Lowe model is widely cited in connection with the nitrogenase mechanism, it is rarely used to simulate time courses for intermediate and product formation. Undoubtedly one important factor is that the kinetic model was explicitly evaluated for *K. pneumoniae* nitrogenase at 23 °C, while most contemporary nitrogenase studies use the *A. vinelandii* nitrogenase at 30 °C. There may be deeper reasons for this lack of quantitative application of the Thorneley-Lowe models to nitrogenase kinetics and it is worth noting two major reservations:

- Independent efforts to reproduce kinetic fits using the published data were unsuccessful²⁴⁷ and the rate constants needed to be reparametrized to obtain responsible fits. It is unclear why the numerical simulations could not be reproduced, but given the number of rate constants and the extent of the data, computational instabilities in correlated parameters would seem one possibility. As a related issue, rapid freeze-quench EPR studies of an intermediate described as E₃ based on the kinetics of appearance²⁴⁸ was reassigned to E₂ based on the EPR properties; the discrepancy was ascribed to “uncertainties in the rate constants used in the kinetics analysis”.^{205,249} This reassignment suggests that the kinetic description is already unreliable early in the MoFe protein cycle.
- The Thorneley-Lowe model requires the Fe protein to be only ~ 45 % active, based on the observed specific activity measurements. This inactive protein is assumed to be able to form complexes with the MoFe protein with the same kinetic parameters as native Fe protein. In contrast, the 30 % inactive MoFe protein is assumed to be unable to participate in complex formation with the Fe protein. It is hard to understand the molecular basis of these properties given that inactive Fe protein has never been directly characterized, but only postulated to make the specific activity measurements fit an assumed kinetic model.

One resolution to this conundrum would be the presence of Fe protein in non-reactive redox states. As a perhaps hypothetical illustration of this behavior, Watt has measured the equilibrium constant = 7.4 for the disproportionation of ATP-bound Fe protein between the oxidized (2+), reduced (1+) and all-ferrous (0) oxidation states:⁴⁴

A Fe protein solution originally in the +1 state will equilibrate to a distribution of 0.42, 0.16, 0.42 between the 2+, 1+ and 0 states; assuming for the sake of this discussion that the all-ferrous form was the only form of the Fe protein active for electron transfer to the MoFe protein, then 42 % of the Fe protein would be in this form at equilibrium. The role, if any, of the all-ferrous form remains controversial, however.³⁵

6. MECHANISTIC PRINCIPLES OF BIOLOGICAL NITROGEN FIXATION

6.1 General Mechanistic Framework for N₂ Reduction

While we know now that nitrogenase produces ammonia, as a general chemical problem, the transformation of atmospheric N₂ to a “fixed” form could yield a variety of different products, ranging from fully reduced ammonia (NH₃) to fully oxidized nitrate (NO₃[−]), as well as nitrite (NO₂[−]), hydroxylamine (NH₂OH), cyanic acid (HCN), urea (H₂N-CO-NH₂) and undoubtedly other species. A perceptive analysis in 1927 by Burk concluded that the overall free energy change for all these reactions would be generally favorable.²⁵⁰ “Fixation of nitrogen even with liberation of energy or free energy, will take place if either oxygen gas or hydrogen gas, or other substances, especially gases, whose standard free energies are close to zero, are involved to form either nitrates, ammonia, or cyanide, not to speak of still other compounds.” Hence, thermodynamics does not give us any direct guidance in terms of identifying a specific product of nitrogen fixation that would be uniquely thermodynamically favorable under ambient conditions. Although ammonia was early on implicated as the product of biological nitrogen fixation, it was not until the introduction of isotopically labeled ¹⁵N that Wilson and Burris firmly established that the key intermediate is indeed ammonia.¹⁸⁰

The mechanistic challenge in the reduction of N₂ to NH₃ is that while the overall reaction is thermodynamically favored (when H₂ or a low potential ferredoxin or flavodoxin are used as the reductant), the kinetics of the uncatalyzed reaction are highly unfavorable under ambient conditions. This behavior is due to a high activation energy for the initial reduction of the N≡N triple bond to form diimide (N₂H₂), which is also reflected in the highly unfavorable thermodynamics for this reaction. In the gas phase, the free energy change for the reduction of N₂ to N₂H₂ is estimated as +210 kJ·mol^{−1}, significantly higher than the corresponding reductions of the triple bonded compounds C₂H₂ to yield C₂H₄, or CO to H₂CO (−140 kJ·mol^{−1} and +25 kJ·mol^{−1}, respectively). The free energy of formation of N₂H₂ was estimated in an analysis by Stiefel,²⁵¹ and it is likely the value he used for the enthalpy of formation of N₂H₂ was underestimated by ~50 kJ·mol^{−1} relative to contemporary values,²⁵² making this reaction even more unfavorable. It is worth noting that this situation does not mirror the relative bond strengths of the triple bonds in these compounds, since the N₂ triple bond energy (941 kJ·mol^{−1}) is comparable to the triple bond energies in C₂H₂ and CO (962 and 1070 kJ·mol^{−1}, respectively).²⁵³ An important distinction between N₂ relative to C₂H₂ or CO is the significant additional stabilization in the N₂ triple bond compared to the single and double bond forms, so that the initial reduction of N₂ is significantly more unfavorable than for these other compounds.

Two general mechanistic frameworks have been considered for the reduction of N₂ to NH₃:
253–255

1. a sequence of proton-coupled electron transfers to N₂, resulting in the formation of intermediates successively reduced by 1 [H] (N₂H, N₂H₂, ..., 2NH₃).
Protonation of N₂ lowers the energetic barrier to reduction; as an example, the enthalpy change associated with N₂H⁺ reduction in the gas phase is nearly 1000 kJ·mol^{−1} lower than for the reduction of N₂.²⁵² (Equivalently, it is easier to

protonate N_2^- than N_2 by the same amount). The binding of N_2 to transition metals significantly lowers the barrier to reduction through back-bonding effects, and an approach to the catalytic conversion of transition metal coordinated N_2 to ammonia through a sequence of proton-coupled electron transfers was pioneered by Chatt.²⁴⁰ The experimental realization of this general scheme at a single transition metal was subsequently achieved by Schrock with Mo,²⁵⁶ and by Peters with Fe.²⁵⁷

2. a multi-electron transfer dissociative process where the $\text{N}\equiv\text{N}$ triple bond is split in the initial step. In the extreme case, this would involve initial reduction to form two nitrides (N^{3-}), but it is also possible that this could involve reduction to the hydrazido level (N_2^{4-}). Following transfer of the requisite number of protons and electrons, NH_3 would be produced. To facilitate the multi-electron natures of these scenarios, catalysts are typically multi-metallic, such as the homogenous di-molybdenum system of Nishibayashi and coworkers,²⁵⁸ or the heterogenous system of Shilov.²⁵⁹ In essence, the catalyst serves as a capacitor that is charged up during an activation phase of the reaction before subsequently discharging multiple electrons to the substrate to form more highly reduced nitrogen species. The Haber-Bosch process^{260,261} provides an important example where N_2 dissociates into N atoms on the surface of a Fe catalyst, subsequent to reacting with dissociated H atoms to form NH_3 .

It should be noted that other mechanistic approaches to nitrogen fixation are possible. For example, the Frank-Caro cyanamide process was an early large-scale method for fixing nitrogen that involved the reaction of calcium carbide with N_2 at high temperature to form the calcium salt of the cyanamide anion (NCN^{2-}) and graphite.²⁶² As with the Haber-Bosch process, the reactants and reaction conditions are irrelevant to nitrogenase, but the fact that there is an interstitial carbide at the heart of the FeMo cofactor suggests that an intermediate involving the reaction of the carbide with N_2 to form perhaps a diazomethane or diazirine that is subsequently reduced to NH_3 should not be dismissed immediately.

6.2 The Mechanism of N_2 Reduction by Nitrogenase

The chemical reduction mechanism of N_2 by nitrogenase is generally viewed through the mechanistic framework introduced by Chatt, integrated with the Thorneley-Lowe kinetic model. In this model, dinitrogen reduction proceeds through a sequence of single proton-coupled electron transfer steps leading from N_2 to N_2H to N_2H_2 ... to 2NH_3 . Indirect experimental evidence supporting this model includes:

1. hydrazine can be detected upon acid or alkali quenching of actively fixing nitrogenase.²⁶³ This behavior strictly demonstrates that an intermediate containing N_2 and hydrogens at the proper reduction level for hydrazine appears during quenching,²⁴³ but not necessarily that hydrazine is an obligatory intermediate during N_2 reduction. Indeed, hydrazine can be generated during acid quenching of well-characterized N_2 complexes.²⁶⁴
2. hydrazine can be detected as an intermediate in the reduction of N_2 by the VFe protein.²⁶⁵ This observation suggests that NH_3 is not formed directly from N_2

(without intermediates), but it has not been demonstrated that hydrazine is an on-path intermediate.

3. nitrogenase can reduce N_2 to NH_3 under conditions where the Fe protein transfers single electrons to the MoFe protein using dithionite as a reductant, supporting the concept of a mechanistic scheme composed of single-electron transfer events. There are, however, reports that nitrogenase functions more efficiently with the all-ferrous Fe protein²⁶⁶ or using reduced flavodoxin as the electron donor²⁰² that could serve as two electron donors. To be clear, it is not understood how the electron transfer properties of the Fe protein are related to the N_2 reduction process.
4. Mössbauer spectroscopy studies of nitrogenase indicate that the average isomer shift of irons in the FeMo cofactor and P-cluster change little under turnover conditions in the presence of N_2 .¹⁷⁸ From the correlation between average isomer shift and iron oxidation state in FeS clusters, the change is estimated to correspond to a net reduction of less than one iron per FeMo cofactor.¹¹⁷ A dissociative type of process with a transfer of six electrons from the metalloclusters to N_2 could be expected to result in a significant net oxidation of irons in the metalloclusters during turnover conditions. That this is not observed could indicate that nitrogenase does not utilize a dissociative mechanism with N_2 reduced to the nitride level in one step; it could also mean that under the experimental conditions, the enzyme was not efficiently reducing N_2 . NH_3 formation was not reported in the experimental protocols¹¹⁷ and at the low component ratio utilized in this study (~ 1), significant H^+ reduction was likely, so the system may more reflect low E_n states rather than the complete nitrogen fixing cycle.

The most detailed version of this model has been developed by Hoffman, Seefeldt and Dean, who have used sophisticated pulsed EPR methodologies with various substrates and nitrogenase mutants to trap and characterize intermediates.²⁰⁵ These intermediates have been assigned to specific states in a modified Thorneley-Lowe mechanism (Fig. 18B) that has N_2 binding to E_4 (the binding of N_2 to E_3 is “deemphasized” in the Hoffman *et al.* model)²⁶⁷ and includes an E_8 state. To date, 3 intermediates have been characterized with bound nitrogen(s) – a species with two nitrogens assigned to E_4 and two species containing single nitrogens assigned to E_7 and E_8 in the Hoffman mechanism.²⁰⁵ The E_4 intermediate has been identified as existing in two forms,²⁶⁸ with either 2 bridging hydrides or with bound N_2 following displacement of bound H_2 generated by hydride protonation. The replacement of two hydride ligands as H_2 by N_2 is well preceded in transition metal complexes.^{243,269} This reductive elimination reaction on nitrogenase has been shown to be reversible²⁷⁰ and has been observed in all three types of nitrogenase.¹⁷⁴ Whether or not this process is a true reductive elimination, resulting in significant Fe reduction upon H_2 dissociation has been challenged.²⁷¹ E_4 has been termed the “Janus” intermediate, since it faces both forward and backward, either advancing to NH_3 formation upon binding of N_2 or returning to the resting state with loss of H_2 .

Despite this significant progress in identifying intermediates in the nitrogenase N_2 reduction pathway, as of yet, there is still no definitive characterization of an intermediate with a reduced $N\equiv N$ triple bond. Only one intermediate containing two nitrogens has been observed, assigned to the E_4 state. As noted in the initial characterization of this species,²⁶⁸ this is “a state in which FeMo-co binds the components of diazene (an N–N moiety, perhaps N_2 and two $[e^-/H^+]$ or diazene itself)”, i.e. it was not possible to distinguish whether this species has a reduced N–N bond or not. In addition to E_4 , this mechanism predicts that two additional intermediates (E_5 , E_6) with a reduced N–N bond should be present that have not yet been observed. Since the intermediates that have been observed to date are either not diagnostic for any specific type of reduction pathway (the E_7 and E_8 intermediates with a single N) or the identity of the N–N species has not been conclusively demonstrated (E_4), key aspects of the chemical mechanism of N_2 reduction by nitrogenase remain open.

7. FROM STRUCTURAL TO MECHANISTIC UNDERSTANDING

Structures of the resting state of the MoFe and VFe proteins highlight conserved features of the active site cofactor relevant to the substrate reduction mechanism (Figs. 6, 14).

1. the cofactors are coordinated to the protein through only two sidechains, a cysteine bound to the apical Fe (Fe1) and a histidine coordinated to the heterometal.
2. the coordination sphere of the heterometal is completed through bidentate ligation by *R*-homocitrate.
3. the six Fe sites without a protein ligand (Fe2–Fe7) form a trigonal pyramid encapsulating a biologically unprecedented interstitial carbide ligand. The two triangular faces are bridged by three non-protein ligands – either three μ_2 “belt sulfurs” in the FeMo cofactor or two μ_2 belt sulfurs and a planar species, likely carbonate, in the FeV cofactor.

With the orchestrated addition of electrons, protons and substrate, the cofactor becomes activated to the state catalytically competent for substrate reduction and product formation. Key features of the integration of structure and mechanism have been detailed in Rohde *et al.*,¹⁷² and the steps in this process may be summarized:

electron transfer:

ATP-coupled electron transfer from the Fe protein results in sequential reduction of the MoFe protein (or the alternative nitrogenases), likely by single-electron transfer events, but a role for two-electron transfer processes cannot be ruled out. Although the Fe protein reduction potential is comparable to that of ferredoxins and flavodoxins (the natural reductants of Fe protein), these low potential electron transfer proteins cannot replace the Fe protein as the electron donor to the MoFe protein. Likewise, low-potential, small molecule reductants have not been identified that can replace Fe protein for the efficient reduction of N_2 . This suggests that the ATP requirement for substrate reduction reflects the importance of the proper timing of the electron transfer events more than the thermodynamic driving force. Indeed, the flux of electrons through the system and ATP/ e^- ratio is independent of

the substrate reduced, indicative of an intermolecular electron transfer process where the electrons are delivered at constant potential from the Fe protein.

Mössbauer studies detailed above indicate that there is little change during turnover in the average oxidation state of MoFe protein irons, relative to the resting state. This indicates that there is neither a significant accumulation of electrons on the nitrogenase metallocusters during turnover, nor is the substrate reduced by a significant number of electrons in one step. The resolution of this paradox of the transfer of electrons without the resulting accumulation of electrons on the metallocusters was the key finding by Hoffman, Seefeldt and Dean that the electrons are used to form metal hydrides on the clusters.^{249,268,272–274} In this fashion, reducing equivalents could be accumulated without a build of charge that would thermodynamically disfavor successive electron transfers from the Fe protein.

proton transfer:

Including obligatory H₂ production and protonation to form NH₄⁺, the reduction of dinitrogen by nitrogenase is associated with the transfer of 10 protons to the buried active site. In addition to the *R*-homocitrate, the other potentially ionizable groups on the cofactor include the cluster sulfurs (based on the pH titration properties of synthetic and protein-based clusters)^{275–278} and the carbonate ligand in the VFe protein. Structural changes observed in the crystallographic analysis of MoFe proteins at low pH suggest that the belt sulfurs S3A and S5A are potential protonation sites.⁷⁹ In the immediate protein environment, the sole group with an expected pK_a near neutrality is His 195. Other hydrogen bonding interactions between the protein and cofactor belt sulfurs involve main chain NH groups and the guanidinium groups of nearby arginine residues; while these groups are not appreciably ionized at neutral pH, they could participate in proton transfer processes. No water molecules are within hydrogen bonding distance of the cluster sulfurs, although the long arm of homocitrate (i.e. the carboxylate arm in homocitrate with the additional methylene group relative to citrate) is surrounded by an extensive network of waters. Proton transfer pathways involving water channels have been identified.^{177,279,280} The solvation of homocitrate by this buried water pool is suggestive of a role for homocitrate in proton transfer. Still unclear is why homocitrate is essential for N₂ fixation relative to citrate, as the “long” arm of the homocitrate points away from the cofactor, precluding any direct interaction between this feature and the cofactor in the absence of any rearrangements.

substrate binding:

Multiple potential access pathways have been identified for substrates to reach the active site, making it unlikely there is a unique path for this process.^{177,279–284} Since nitrogenase has a relatively leisurely turnover rate of about 1 N₂ s^{−1} per active site, migration through the protein scaffold in the absence of permanent pathways should not be rate limiting, given the nanosecond-scale structural fluctuations that facilitate gas diffusion through proteins.²⁸⁵ Substrate access to the active site is insufficient for binding, however, as the cofactor needs to be reduced to the appropriate level. A routine demonstration of this requirement is that despite immersing crystals of the resting state forms of the MoFe protein or VFe protein in liquid substrate (liquid nitrogen) prior to data collection, binding of N₂ to the active site (or indeed anywhere in the protein) has not been observed. Also, the observed removal of

sulfide S2B in all ligand-bound structures obtained to date not only describes a possible binding site for N₂, but the observed geometry is also very well suited to accommodate a bridging hydride at the very same position when modeled according to the bond distances and geometries observed in other metal-bridging hydride structures (Fig. 19). Importantly, in this conformation the inward-facing side chain of residue Gln 176 in the VFe protein structure from *A. vinelandii* forms a short hydrogen bond with the N_ε nitrogen of His 180, stabilizing the protonated form of the imidazole moiety and thus preventing proton transfer from this like entry point for H⁺ during substrate reduction. In addition to the direct, stabilizing effect of the amide oxygen interaction with the positively polarized side of the hydride, this protective effect would extend the lifetime of the E₂ state – at least under conditions of high electron flow – to allow for the next reduction steps to occur before H₂ is lost due to unwanted protonation.

Furthermore, substrate binding to Fe2 and/or Fe6 is likely associated with the reduction of these sites, plausibly either by direct reduction following electron transfer from the Fe protein, or by the reductive elimination process described above. Protonation of the S2B sulfide will facilitate dissociation from the reduced Fe, thereby opening up these sites for substrate binding. The lack of reactivity of the FeMo cofactor in the resting state is likely due to the incorrect redox state of the cofactor, together with the belt sulfurs effectively serving as protecting groups that reversibly block substrate-binding sites. Given the rearrangements observed in the selenated FeMo cofactor under turnover conditions (Fig. 12B),¹⁴⁹ it is possible that additional rearrangements may occur such that other iron sites participate in ligand binding,²¹⁷ but there is as yet no direct experimental evidence for this.

The sequence of events following N₂ binding have yet to be uniquely defined in terms of the specifics of the electron and proton transfers, including whether the N-N bond is reduced in 1-electron steps or possibly 2- or more electron steps, and identification of the specific sites of protonation of bound N₂ that distinguish different reduction mechanisms, such as the distal *vs* alternating *vs* hybrid reduction mechanisms.^{253–255} Related questions are whether the same (or at least similar) mechanisms are utilized by nitrogenase during the reduction of N₂ and such alternative substrates as C₂H₂ or substrates that undergo CC coupling reactions such as CO and CH₃NC.

Experimental elucidation the atomic mechanism of N₂ reduction will be ultimately limited by the transient nature of catalytic intermediates and the challenges in unambiguously establishing the temporal sequence of intermediate structures in a reaction mechanism. Computation is essential for the integration of structure, energetics and dynamics and studies to date have been valuable for exploring possible reaction pathways. At some future point, the nitrogenase mechanism will be amenable to *ab initio* studies, but in the meantime the integration of structure and experiment is essential for moving the problem forward.

8. CONCLUSIONS AND OUTLOOK

The past 5 years have seen major advances in our understanding of the mechanism of substrate reduction by nitrogenase, particularly in the identification of hydride and nitrogen containing intermediates by pulsed EPR methods²⁰⁵ and in the crystallographic

characterization of forms of nitrogenase establishing reversible displacement of a belt sulfur in the active site cofactor by exogenous ligands.^{39,147,149} Keeping in mind Halpern's tenet "if you can identify a compound from a catalytic system, it is probably not the catalyst",²⁸⁶ the challenge is to relate these observations to the nitrogenase catalytic mechanism. A related view on the mechanistic analysis of nitrogen fixing reactions was summarized by Chatt: "Unfortunately, it is not in general possible to isolate intermediates from these systems, and the proposed mechanisms are based on kinetic measurements, overall stoichiometry, and, not least, chemical intuition".²⁴⁰ The latter quality has been the most elusive, but fortunately, advances in small molecule catalysts and computational chemistry are complementing multidisciplinary approaches on the enzymatic system to provide an increasingly detailed view inside the nitrogenase black box.

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Biography

Oliver Einsle obtained a diploma in Biology from Konstanz University, Germany, and a doctorate of natural sciences under the supervision of Peter Kroneck and Robert Huber at the Max-Planck-Institute for Biochemistry, Martinsried, Germany. He joined the group of Doug Rees at Caltech as a postdoctoral fellow in 2001 and was appointed junior professor for protein crystallography at the University of Göttingen, Germany, in 2003. Since 2008 he is the chair of Biochemistry at the Faculty for Chemistry and Pharmacy at the University of Freiburg, Germany, where he studies the structural and functional properties of metalloproteins and integral membrane proteins. He is currently the director of the Institute for Biochemistry and Dean of the Faculty of Chemistry and Pharmacy.

Douglas C. Rees received his BS in Molecular Biophysics and Biochemistry from Yale College and his PhD in Biophysics working with William Lipscomb at Harvard University. As a graduate student, Rees was introduced to nitrogenase through James B. Howard, at that time a sabbatical visitor with Lipscomb. Following postdoctoral work with Howard at the University of Minnesota, Rees joined the UCLA faculty in the Department of Chemistry and Biochemistry in 1982. He moved to the California Institute of Technology in 1989, where his group studies the structures and functions of metalloproteins and integral membrane proteins. Rees is an investigator of the Howard Hughes Medical Institute and is currently Dean of Graduate Studies at Caltech.

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Figure 1: Architecture of molybdenum nitrogenase MoFe protein NifDK (PDB 3U7Q). The dinitrogenase component is a NifD₂K₂ heterotetramer that contains a set of two complex iron-sulfur clusters in each $\alpha\beta$ dimer. While the electron-transferring [8Fe:7S] P-cluster is located at the interface of NifD and NifK, the active site FeMo cofactor, a unique [Mo:7Fe:9S:C]:homocitrate moiety, is buried within NifD.

Figure 2:

The Fe protein of molybdenum nitrogenase. **A)** The NifH protein forms a homodimer, with each 30 kDa monomer binding ATP or ADP (in stick representation). A conformational change is related to the different ligand-bound states, as Fe protein is a P-loop NTPase. It requires docking to MoFe protein to trigger ATP hydrolysis and electron transfer from its metal site to P-cluster. **B)** The [4Fe:4S] cluster of Fe protein is coordinated symmetrically at the dimer interface, coordinated by two cysteines from each monomer. Figure generated from PDB 6N4L.

Figure 3:

The structure of MoFe protein. **A)** MoFe protein forms an $\alpha_2\beta_2$ heterotetramer in which two $\alpha\beta$ units (NifDK) are connected solely via the NifK peptides. Each $\alpha\beta$ unit holds a FeMo cofactor and a P-cluster (PDB 3U7Q). **B)** NifD (above) and NifK (below) are structurally and evolutionarily related and consist of three consecutive Rossmann-fold domains. The domains are highlighted in red, green and blue according to their occurrence in the chain. In NifD, all three domains cradle the FeMo cofactor in their center, while the P-cluster is symmetrically coordinated between the third domains of each chain. **C)** As typical for this fold, the P-cluster and FeMo cofactor are coordinated at the loop regions at the C-terminus of the parallel β -sheet of a Rossmann domain.

Figure 4:

A structural comparison of Mo- and V-nitrogenases and their orthologs NifEN and the protochlorophyllide reductase BchNB (DPOR). **A)** Subunit arrangement of the four orthologs. Coloring scheme as in Fig.1. **B)** Top view of the complexes, with corresponding coloring of homologous subunits. **C)** The respective α -subunits of each enzyme in cartoon representation, colored from blue at the N-terminus to red at the C-terminus. Each chain contains three consecutive Rossmann-fold domains (blue, green, red). **D)** The respective β -subunits in the same orientation as in C). The figure was generated from the PDB entries 3U7Q (NifDK), 5N6Y (VnfDKG), 3PDI (NifEN) and 3AEK (BchNB).

Figure 5:

Redox-dependent conformational states of P-cluster. **A)** In the P^N state obtained upon reduction with sodium dithionite, the [8Fe:7S]²⁺ cluster is all-ferrous and symmetrically arranged around a central, six-coordinate sulfide. **B)** The one-electron-oxidized P¹⁺ state shows a rearrangement of Fe6 only, releasing its interaction with the central sulfide and orienting towards a nearby serine, the largely conserved Ser 188^K. **C)** Two-electron oxidation of the P^N state leads to P^{Ox}, a [8Fe:7S]⁴⁺ state in which Fe5, in addition to Fe6, re-orient away from the central sulfide, this time towards a backbone amide. Notably, all observed changes are reversible. **D)** Redox-dependent structural changes in the [4Fe:3S] cluster of *O*₂-tolerant hydrogenase of *C. necator*. The shift of Fe4 towards a backbone amide is highly reminiscent of the shift of Fe6 upon oxidation of P-cluster. Panels A)-C) generated from PDB entries 1M1N and 3U7Q, panel D) generated from PDB entry 4IUD.

Figure 6:

The catalytic FeMo cofactor of Mo nitrogenase. This complex iron-sulfur cluster prominently contains molybdenum at an apical position as a heterometal, bidentally coordinated by a homocitrate molecule. It obtains its highly symmetric structure through the insertion of a central carbide (formally C^{4-}) that originates from S-adenosyl methionine. The $[Mo:7Fe:9S:C]:homocitrate$ cluster is coordinated only by two residues of the NifD subunit, Cys 275 and His 442, and all eight metal ions are coordinatively saturated. Figure made from PDB entry 3U7Q.

Figure 7:
Nucleotide binding and conformational changes in the Fe protein NifH from *A. vinelandii*.
A) The three phosphate groups of the ATP analog AMPPCP are cradled in the P-loop (green) of the protein, with a bound Mg^{2+} cation liganded by the β - and γ -phosphate and residues from the switch I (blue) and switch II (red) loops (PDB 4WZB). **B)** In the ADP-bound state after dissociation of the γ -phosphate, residue K41 from the switch I region (blue) becomes a ligand to Mg^{2+} , leading to a major conformational change (PDB 6N4L). **C)** In an overlay of the NifH monomers in the AMPPCP- and ADP-bound states, the effect of the conformational change of the switch I (blue) and switch II (red) regions is seen as a displacement of the $[4Fe:4S]$ cluster at the dimer interface (arrow).

Figure 8:

Nitrogenase complexes. Electron transfer from Fe protein to the catalytic MoFe protein requires the formation of a stoichiometric complex of both components (PDB 1N2C). **A)** With bound Mg-ADP·AlF₄⁻, the [4Fe:4S] of Fe protein is positioned at a distance from P-cluster that is suitable for electron transfer. **B)** Different nucleotide-bound or -free states of Fe protein bind in different orientations on MoFe protein, but without nucleotide (PDB 2AFH) or with ADP (PDB 2AFI), the distance between the [4Fe:4S] cluster and P-cluster is increased with respect to the form with bound ATP analogs AMPPCP (PDB 4WZB) or ADP·AlF₄⁻.

Figure 9:

Discovery and Identification of the central carbide in FeMo cofactor. **A)** Calculated, resolution-dependent electron density profiles of the central position of FeMo cofactor, highlighting that the different surrounding atoms (Fe1, Mo, all nine S and the six remaining Fe) have varying effects that sum up to a profile (below) that highlights a negative electron density artifact in the resolution range between 2.2 and 1.55 Å. **B)** At 1.0 Å resolution, a statistical evaluation of the diffraction behavior of all carbon (black), nitrogen (blue) and oxygen (red) atoms of the structure (PDB 3U7Q), the central light atoms of the two cofactors of the MoFe protein clearly lined up with carbon. **C)** The result from (B) was corroborated by ESEEM spectroscopy, where only ¹³C, but not ¹⁵N-labelling generated a new signal at the correct Larmor frequency ν . **D)** F_{calc} electron density maps calculated to the stated limiting resolutions underline that the maximum indicating the central carbide indeed disappears at resolutions lower than 1.55 Å, as a direct consequence of the distorting ripple effects of the surrounding atoms as dissected in (A).

Figure 10:

Analysis of the E_0 state of FeMo cofactor by spatially resolved anomalous dispersion (SpReAD). **A)** In an experiment involving 17 data sets along the K-edge of iron, SpReAD refinement of the 7 individual iron sites of FeMo cofactor revealed the existence of a set of more oxidized Fe sites (Fe2, 4, 5, 6, red average) and a more reduced set (Fe1, 3, 7, green average). The edge position for the latter was very well in line with the all-ferrous P-cluster (blue average). As complementary information, the cofactor structures below show anomalous difference electron density maps for the data sets recorded at the indicated positions along the edge. **B)** Structure of FeMo cofactor with the more reduced Fe sites in green and the more oxidized ones in red. The axes indicate the principal components of the oriented magnetic g tensor (see Fig. 11A).

Figure 11:

The electronic structure of the resting state E_0 of FeMo cofactor. **A)** Orientation of the $S = 3/2$ g tensor of resting-state MoFe protein with respect to the cluster structure from single-crystal EPR analysis. The longest principal component, $g_z = 4.31$, orients along the pseudo-threefold axis of the cofactor. Notably, the $g_x = 2.01$ component is directed towards the Fe2-Fe6 edge that later also proved to be the site of ligand binding. **B)** Spin-localized model for the $S = 3/2$ E_0 state of MoFe protein. The maximized antiferromagnetic coupling of this model leads to largely localized charges, leaving Fe2 and Fe6 as the most oxidized metal sites in the cluster.

Figure 12:

Ligand complexes of FeMo cofactor. **A)** CO complex of *A. vinelandii* MoFe protein obtained under turnover conditions (PDB 4TKV). The ligand replaced bridging sulfide S2B, but induced no other discernible changes at the active site. **B)** Under turnover with SeCN^- , selenide also replaced sulfide S2B (PDB 5BVG). Under continuous turnover, replacement of the other bridging sulfides was also observed, albeit to a lesser degree.

Figure 13:

The vanadium-iron protein from *A. vinelandii*. **A)** Similar to the $NifD_2K_2$ heterotetramer (left), the core of VFe protein is a $VnfD_2K_2$ assembly with high structural homology to MoFe protein. It additionally contains a further subunit, VnfG, which is in exclusive contact with VnfD. **B)** Cartoon representation of *A. vinelandii* VFe protein (PDB 5N6Y). Green spheres denote the bridging Mg^{2+} ions. **C)** The P-cluster of VFe protein bridges the D- and K-subunits. In its reduced state it is nearly identical to its MoFe protein counterpart. **D)** The VnfG subunit forms a four-helix bundle and does not contain additional cofactors. Every tenth residue of VnfG is denoted by a small sphere.

Figure 14:

The active site FeV cofactor of vanadium nitrogenase. **A)** FeV cofactor is a $[\text{V}:7\text{Fe}:8\text{S}:\text{C}:\text{CO}_3^{2-}]:\text{homocitrate}$ cluster with high overall similarity to FeMo cofactor (Fig. 5). A V^{3+} cation replaces Mo^{3+} , but retains its binding geometry, as well as the organic *R*-homocitrate ligand (PDB 5N6Y). In addition, FeV cofactor has one μ_2 -bridging sulfide, S3A, replaced with a μ -1,3-bridging carbonate. **B)** The carbonate ligand is tightly bound within a loop region of VnfD. In MoFe protein (PDB 3U7Q), the corresponding loop has a single leucine-proline swap due to which the carbonate ligand cannot be accommodated.

Figure 15:

The Fe protein of V-nitrogenase, VnfH. **A)** Superposition of NifH (black) and VnfH (red) from *A. vinelandii*. The two reductases are 91% identical in sequence and can fully complement each other in function. **B)** The ADP-bound state of VnfH with an octahedrally coordinated Mg^{2+} cation shows the close spatial proximity of the nucleotide-binding P-loop, the conformationally flexible switch I and II regions and the bridging [4Fe:4S] cluster. Figure made from PDB entry 6Q93.

Figure 16:

Continuous-wave EPR spectra of nitrogenases. **A)** X-band spectrum of *A. vinelandii* MoFe protein in the resting state E_0 . **B)** X-band spectrum of the resting state of *A. vinelandii* VFe protein. **C)** In the $S=3/2$ system of the cofactor, the lower Kramer's doublet dominates the EPR signal. **D)** In a rhombogram for the lower doublet, both nitrogenases can be described by an axial g -tensor, but differing in rhombicity, with $E/D = 0.05$ for MoFe and 0.30 for VFe protein.

Figure 17:

A turnover state structure for *A. vinelandii* VFe protein. Sulfide S2B that in the resting state E_0 bridges Fe ions 2 and 6 relocated by 7 Å to a binding pocket that was created by an inward rearrangement of the sidechain of residue Q176^D. It was replaced by a light atom, N or O that must be protonated according to the interatomic distances observed in the 1.2 Å resolution crystal structure. Figure generated from PDB entry 6FEA.

Figure 18:

The Thorneley-Lowe model for nitrogenase. **A**) In a Fe protein cycle, the transfer of a single electron obtained from central metabolism *via* a ferredoxin or flavodoxin requires transient complex formation of the reduced Fe protein with the catalytic component (MFe protein), concomitant with the hydrolysis of 2 ATP/e[−]. As a result, the enzyme is advanced by one E-state in the catalytic cycle. **B**) The cycle of the MFe protein describes an 8-electron process and consequently cycles through 8 distinct 1-electron steps (E₀–E₇). An alternating transfer of electrons and protons (or *vice versa*) is assumed, and notably the binding of the substrate N₂ requires the enzyme to be at least in state E₃ or E₄. From states E₁–E₄, unproductive H₂ evolution is observed, while the exchange of N₂ for H₂ upon substrate binding is presumed to be a mechanistic requirement.

Figure 19:

A model for hydride binding to Fe2 and Fe6 in FeV cofactor. Based on the turnover state structure of VFe protein from *A. vinelandii*, a bridging hydride can be modeled in place of the NH (or OH) ligand observed in the electron density map. Here, the positioning of residue Gln 176 between the likely proton source His 180 and the substrate binding site may be instrumental for the stabilization of the bridging hydride.