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Nirogenase

SPECIAL ISSUE

Nitrogenase Cofactor: Inspiration for Model Chemistry

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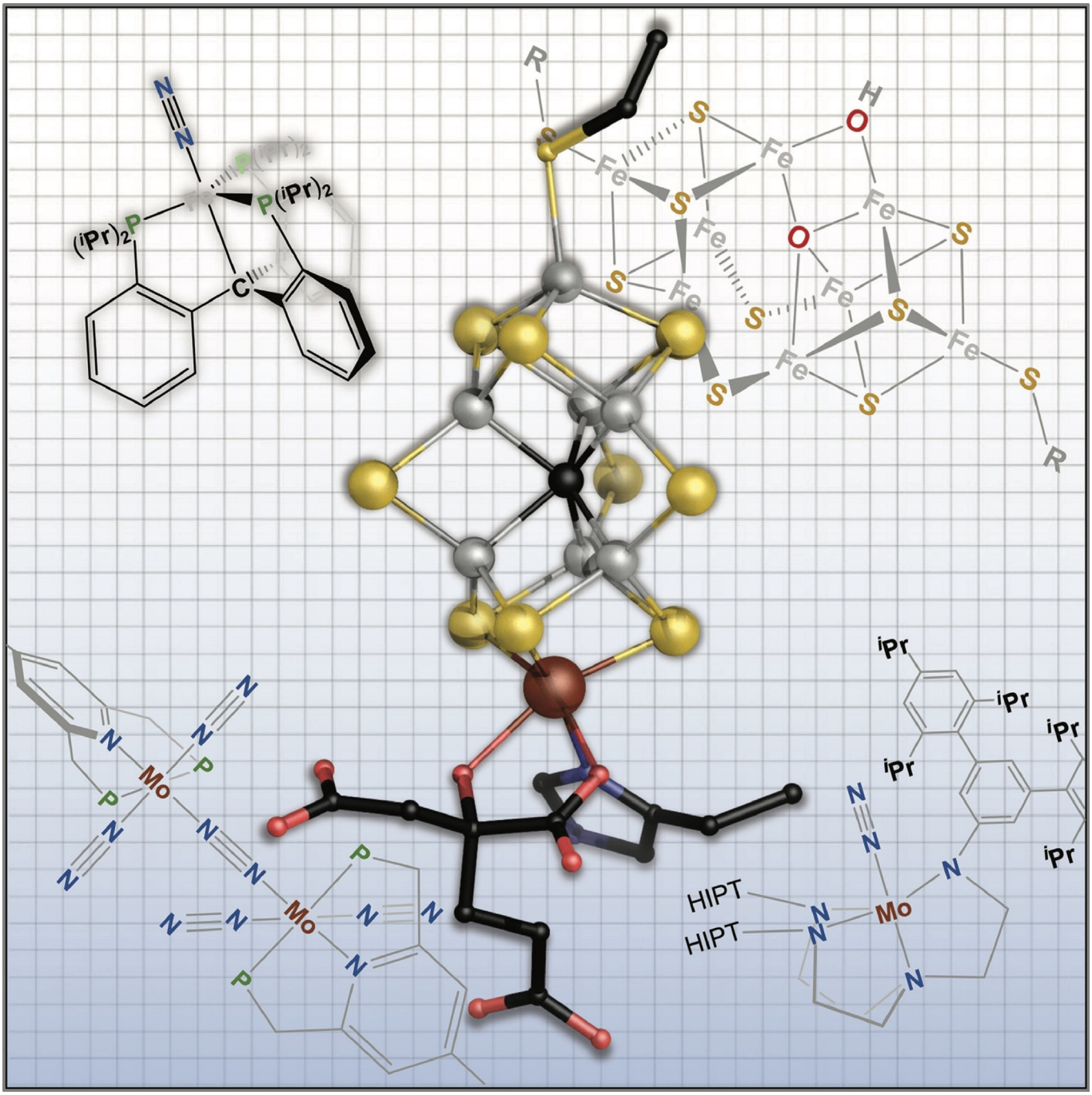
Dedicated to Professor Kazuyuki Tatsumi



Abstract: The cofactor of nitrogenase is the largest and most intricate metal cluster known in nature. Its reactivity, mode of action and even the precise binding site of substrate remain a matter of debate. For decades, synthetic chemists have taken inspiration from the exceptional structural, electronic and catalytic features of the cofactor and have tried to either mimic the unique topology of the entire site, or to extract its functional principles and build them into novel catalysts that achieve the same—or very similar—astounding transformations. We review some of the available model chemistry as it represents the various approaches that have been taken from studying the cofactor, to eventually summarize the current state of knowledge on catalysis by nitrogenase and highlight the mutually beneficial role of model chemistry and enzymology in bioinorganic chemistry.

1. Nitrogen Fixation

1.1. Ways and Means of Breaking the N_2 Triple Bond

Atmospheric dinitrogen (N_2) is the primary global sink for nitrogen, an element that is essential for all living organisms. However, due to its extraordinary stability, the reduction of its triple bond is only achieved along few known routes. Besides a minor contribution to nitroxide generation through lightning, all living organisms rely on the ability of a group of microorganisms, the diazotrophs, to fix nitrogen. A single class of enzymes, the nitrogenases, catalyzes this reduction of N_2 to ammonia [NH_3, Eq. (1)]. Frequently, organismic growth is limited by the availability of reduced nitrogen, and for large-scale crop production this was only overcome with the introduction of the Haber–Bosch process of industrial ammonia production in 1906. Here, N_2 and H_2 are reacted at high temperature (400–500 °C) and high pressure (15–25 MPa) on the surface of an iron catalyst, with particularly high activity at the 111-face of face-centered cubic (α-)iron [Eq. (2)].

$$N_2 + 8\,e^- + 16\,ATP + 8\,H^+ \rightarrow NH_3 + H_2 + 16\,ADP + 16\,P_i \quad (1)$$

$$N_2 + 3\,H_2 \rightarrow 2\,NH_3 \quad (2)$$

Nitrogenase consists of two enzymatic components: a reductase (the Fe-protein) containing a [Fe_4S_4] cluster, and a catalytic moiety with an [Fe_8S_7], electron-transferring P-cluster and a [heterometal-Fe_7S_9C]:homocitrate moiety known as *the cofactor*, where catalysis occurs.[1] Three types of nitrogenases are known, differing by the apical heterometal in the cofactor: molybdenum (in FeMo-cofactor), vanadium (FeV-co), or iron (FeFe-co).[2] Although all these sites likely retain similar structures, V- and Fe-nitrogenases reduce N_2 less efficiently than Mo-nitrogenase that also is the most thoroughly studied enzyme by far.

FeMo-co (Figure 1) can be described as a carbon-ligated dicubane, as it consists of a [Fe_4S_3] and a [$MoFe_3S_3$] unit connected via a central μ_6-carbide and additionally bridged by three μ_2-sulfides.[3] The apical molybdenum ion is coordinated by homocitrate, and the entire cofactor is linked only to two amino acid residues: by H442$^\alpha$ to Mo, and by C275$^\alpha$ to iron Fe1 at the opposing end. Two other residues in proximity to the active site were suggested to be involved in the reaction: H195$^\alpha$ and V70$^\alpha$, above the [Fe2,6,3,7] face of the cofactor.[4] Mutagenesis of either residue led to a substantial loss of catalytic activity, and as the imidazole side chain of H195$^\alpha$ is found within hydrogen-bonding distance of S2B, the μ_2-sulfide bridging Fe2 and Fe6, this residue may operate in proton delivery to the cofactor. On the other hand, V70$^\alpha$ was suggested to act as a gate controlling substrate access to FeMo-co.[5] For the $S = 3/2$ resting state, the electron configuration of FeMo-co was found to be $3Fe^{2+}$:$4Fe^{3+}$:Mo^{3+},[6] and the consistent Fe-S bond lengths of 2.2–2.3 Å suggest that none of the 9 S is protonated.[7]

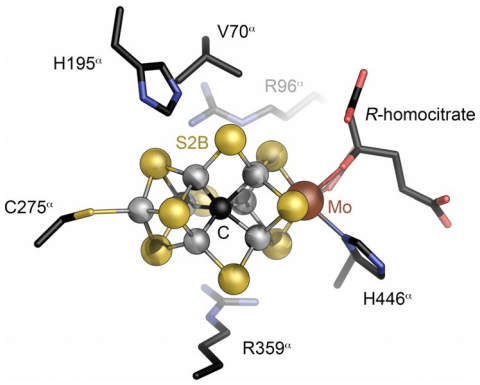


Figure 1. The FeMo-cofactor of molybdenum nitrogenase. The [Mo:7Fe:9-S:C]:homocitrate moiety is coordinated to the enzyme exclusively through residues C275$^\alpha$ and H442$^\alpha$, and only two further amino acid residues are known to be relevant for catalysis, H195$^\alpha$ and V70$^\alpha$, while two positively charged residues, R96$^\alpha$ and R395$^\alpha$, contribute to provide a defined electrostatic environment for the cluster. Figure generated from PDB entry 3U7Q.[9]

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1.2. The Mechanism of Biological Nitrogen Fixation

Numerous efforts have been undertaken on Mo-nitrogenase to shed light on the intricate multi-electron multi-proton transfer reaction required for N_2 reduction. Electrons are delivered exclusively by Fe-protein, in single-e^- transfer steps concomitant with the hydrolysis of two molecules of ATP. Each transfer requires the transient complex formation of the two proteins and at least eight such encounters are required per N_2 molecule, as H_2 is generated as a by-product. This outlines a basic kinetic Scheme comprising eight states that were characterized by Thorneley and Lowe to yield a model for catalysis that is still valid today (Scheme 1 A).[8]

Most notably, while nitrogenase is isolated in a resting state E_0, binding of the substrate N_2 requires reduction to at least the E_3, possibly the E_4 state (Scheme 1 A). The E_4 state is thus considered critical for catalysis, and the use of a V70I$^{\alpha}$ variant allowed Hoffman and Seefeldt to trap this state and characterize it by ENDOR spectroscopy.[10] They suggested the electrons to be stored as metal hydrides at the cluster surface, and proposed the release of H_2 to be the result of a reductive elimination from two adjacent hydrides (Figure 4 D).[11] This mechanism allows for the accumulation of up to four electrons at isopotential, driven by ATP hydrolysis, and may yet create a reducing power that otherwise is not reachable in an aqueous milieu. The subsequent reduction of N_2 can then follow a *distal* or an *alternating* pathway (Scheme 1 B), and although diazene and hydrazine are known substrates for nitrogenase, the question of which reaction pathway is preferred remains under debate.

Deciphering the mechanism of biological nitrogen fixation represents a key challenge for chemistry, not only due to the enigmatic nature of the nitrogenase cofactor and the challenging reaction it catalyzes, but also because the ability to harness this reactivity holds the promise to develop new chemical and biotechnological applications of the process in agriculture and industry that may help to alleviate the growing problem of nitrogen pollution of the environment. With its ability to break the most stable bond known in biological systems, it comes as no surprise that nitrogenase is also able to reduce other stable molecules, including acetylene (C_2H_2), hydroxylamine (NH_2OH), nitrite (NO_2^-), and carbon dioxide (CO_2). The interaction of nitrogenase with carbon monoxide (CO) is of even higher relevance, as CO is a substrate for V-nitrogenase but an inhibitor of Mo-nitrogenase. This non-competitive, reversible inhibition of the reduction of all substrates except protons is partially abolished in V70A$^{\alpha}$ and V70G$^{\alpha}$ variants.[12] The structure of CO-bound Mo-nitrogenase was reported recently and revealed CO to bridge Fe2 and Fe6, reversibly displacing sulfide S2B.[13] Other hints on N_2 binding modalities on FeMo-co had been previously reported from spectroscopy experiments and studies of artificial complexes, either structurally analogous to FeMo-co or demonstrating nitrogenase-like catalytic activities. We review here the recent advances in the elucidation of the mechanism of nitrogenase brought by studies on various synthetic compounds.

2. Chemical Inspiration from FeMo Cofactor

2.1. Structural Analogs of Nitrogenase Cofactors

The complexity of FeMo-co and its unique properties, in particular its μ_6-carbide, present a substantial challenge for synthetic chemistry. The core structure itself is not without precedent among metallocarbonyls, as an octahedral Fe_6 cluster with an interstitial carbide, $[Fe_6C(CO)_{16}]^{2-}$, was synthesized and crystallized in 1971,[14] and spectroscopically characterized forty years later via iron valence-to-core Fe Kβ *x*-ray emission spectroscopy (V2C XES).[15] Even earlier, a Co_6 carbidocarbonyl compound was made that closely resembled the—then unknown—prismatic arrangement of the nitrogenase cofactor core.[16] However, none of these structural elements were connected to the enzyme before the discovery of the interstitial carbide in the X-ray structure of Mo nitrogenase.[3b,7,17]

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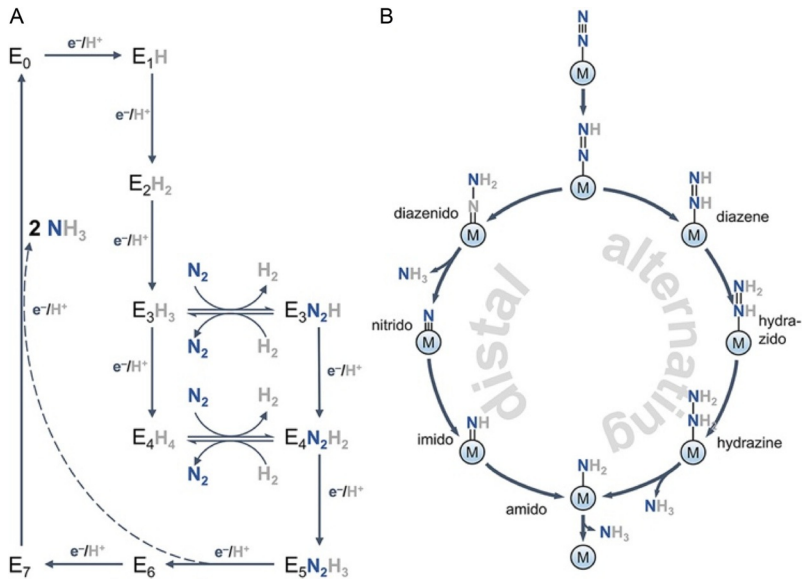

Scheme 1. Mechanistic models for nitrogenase catalysis. A) Simplified kinetic Scheme for nitrogenase catalysis after Thorneley and Lowe. N_2 is bound upon release of H_2 only after reduction to the E_3 or E_4 state. Each reduction step is likely coupled to protonation for charge compensation. B) Binding of N_2 to a (structurally undefined) binding site on the cofactor (M) will be followed by a first protonation/reduction that represents the most challenging step as it involves breaking the triple bond. Subsequently, electron transfer can occur either only to the distal nitrogen (left branch), releasing the first NH_3 after the third electron is transferred, yielding a bound nitride species. The finding that diazene and hydrazine are alternative substrates of nitrogenase led to postulating an alternating mechanism of reduction (right branch), where the N–N bond is only broken in the penultimate reduction step.

After the first three-dimensional structures of the cofactor became available in 1992,[18] the laboratories of Coucouvanis, Holm, Tatsumi and others have invested strongly into synthesizing structural analogs of FeMo-co, notably a $[(Tp)_2(Mo)_2Fe_6(S_9(SH)_2]_2$ cluster that—besides the apical Mo—is a complete isotopolog of P-cluster, highlighting its relationship (and possibly common ancestry) with the cofactor (Figure 2 A)[19] and an $[Fe_8S_7O_2]$ cluster (Figure 2 B),[20] as well as a $[Fe_6S_9]$ cluster, Fe_6^{RHH} ($[Et_4N]_4[Fe_6S_9(SEt)_2]$), with an overall similar shape as the enzyme clusters (Figure 2 C).[21] More recently, a $[Cp^*MoFe_5S_9(SH)]_3^-$ cluster (Figure 2 D) could be assembled that represents an interesting functional analog to the cofactor. In the presence of Et_3N-buffered $HNEt_3[BF_4]$ and upon addition of SmI_2 in THF, this cluster catalyzed the reduction of C_1 compounds with turnover numbers (TON) close to those of isolated FeMo-co in solution.[22] However, until now no dinitrogen reduction activity could be detected within such organometallic analogs of FeMo-co.

Nevertheless, valuable clues regarding the mechanism of dinitrogen reduction on FeMo-co were gathered from compounds that are structurally different, but share architectural and electronic features with the cofactor. In recent years, substantial progress was made in the design and synthesis of novel compounds based on molybdenum or iron that cleave N_2 and yield NH_3, and in the following a selection of key achievements is presented and discussed in brief.

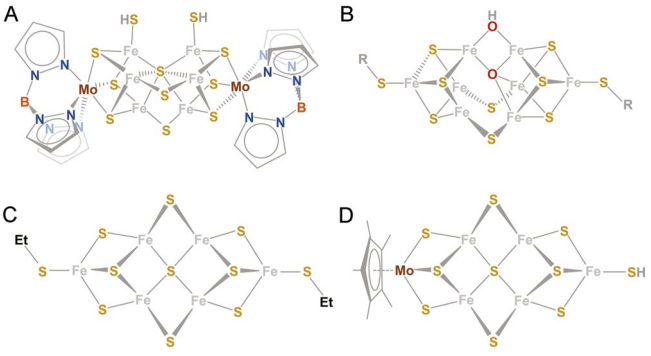

Figure 2. Structural mimics for nitrogenase cofactors. **A**) In spite of its terminal Mo ions, the symmetric $[(Tp)_2(Mo)_2Fe_6(S_9(SH)_2]_2$ cluster by Holm and coworkers underlines the topological analogy of cofactor and P-cluster. **B**) The remarkable $[Fe_8S_7O_2]$ cluster from the Tatsumi group includes two oxygen light atoms. **C**) The Fe_6^{RHH} cluster. **D**) The $[Cp^*MoFe_5S_9(SH)]_3^-$ cluster catalyzes the reduction of C_1 compounds in vitro. Counterions and protons are omitted for clarity.

2.2. Molybdenum-based Compounds

The insertion of molybdenum into FeMo-co represents a key maturation step of the cofactor in vivo and is complex: A topologically complete, *D*32 symmetric $[Fe_8S_9C]$ cluster precursor (termed L-cluster or NifB-co) is transferred to a most remarkable scaffold protein complex, NifEN, where its maturation to FeMo-co (M-cluster) is completed.[23] This requires the action of the Fe-protein, likely as a reductase, and the delivery of molybdenum by NifQ, possibly on a $[Fe_3S_4Mo]$ cluster.[24] *R*-homocitrate, synthesized by NifV,[25] completes the maturation of the cofactor and may ligate the metal even before its insertion into the precursor. Only then the mature cofactor is transferred to apo-nitrogenase.

The requirement of several specific cellular factors for the maturation of FeMo-co with molybdenum, in addition to the superior nitrogen fixation activity of Mo-nitrogenase compared

to the alternative, V- and Fe-dependent enzymes, suggests that molybdenum has a major influence on activity. Quite evidently, molybdenum could facilitate binding or protonation of the substrate by altering the redox potential of FeMo-co or its electronic structure. Also, although recent publications hint to Fe6 as the site for N_2 fixation and cleavage, molybdenum still has not been formally excluded. Furthermore, molybdenum was suggested as the site of one of the latest steps of the alternating mechanism of ammonia formation, that is, the reduction of hydrazine, after substrate migration.[26] Several organometallic compounds containing molybdenum are reported to catalyze the reduction of N_2 or nitrogen derivatives, and the first of these was synthesized in 2003 by Schrock and co-workers.[27] They obtained 8 equivalents of ammonia using catalysts based on tetradentate $[HIPTN_3N]^{3-}$ triamidoamine ligands such as $[HIPTN_3N]Mo(N_2)$ (Figure 3A) (where $[HIPTN_3N]^{3-}$ is $[\{3,5\text{-}(2,4,6\text{-}i\text{Pr}_3C_6H_2)_2C_6H_3NCH_2CH_2\}_3N]^{3-}$), using CrCp2* as a reducing reagent and 2,6-lutidinium *tetrakis*[3,5-*bis*-(trifluoromethyl)phenylborate] ([LutH]$BAr^F{}_4$; Ar^F = 3,5-*bis*(trifluoromethyl)phenyl)) as a proton source.

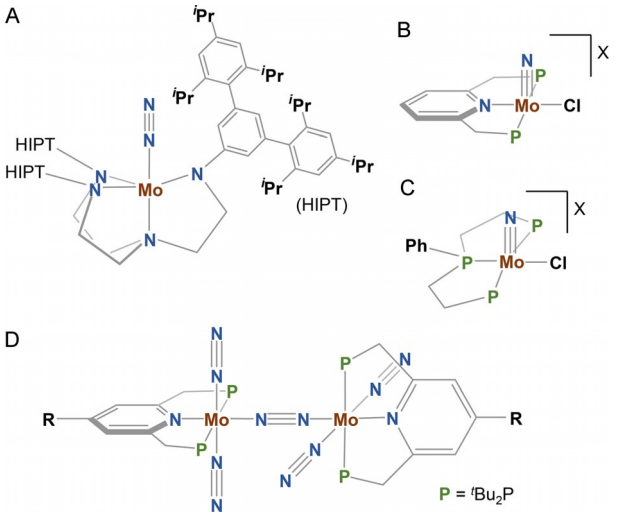

Figure 3. Mo-based catalytic complexes for N_2 activation. **A**) The Mo-HIPT-triamidoamine complex presented by Yandulov and Schrock was the first synthetic catalyst for N_2 reduction, yielding 8 equiv of NH_3. **B**) The mononuclear Mo complexes with PNP-type pincer ligands by Nishibayashi and co-workers. **C**) The switch to triphosphine ligands led to a substantial increase in catalytic activity. **D**) The N_2-bridged binuclear Mo complex yielded 63 equiv NH_3, the highest turnover achieved to date.

Extensive theoretical studies together with the characterization of intermediates yielded a consistent reaction cycle (Schrock cycle) that closely resembles the classic Scheme of Chatt.[28] After end-on binding of N_2 at molybdenum, notably in the oxidation state Mo^{III}, the transfer of two protons and three electrons leads to reduction to Mo^{V} and the threefold protonation of the distal nitrogen atom. A subsequent electron transfer causes the release of the first NH_3 molecule from Mo^{VI}. The following transfer of three protons and three electrons yields the ammonia complex of Mo^{III}, from which the second NH_3 is released by competition with a new N_2 molecule. This cycle is reminiscent of the distal mechanism proposed for the reduction of N_2 on FeMo-co (Scheme 1B). Only minor amounts of the hydrazine complex were detected, further deemphasizing the alternating reduction pathway.[29] Recent work performed by the group of Nishibayashi has yielded the highest TON for molybdenum-based NH_3 production from N_2 so far, with up to 63 equivalents of NH_3. These results were achieved with dinitrogen-bridged dimolybdenum complexes bearing PNP-type pincer ligands (PNP = 2,6-*bis*(di-tert-butylphosphinomethyl)pyridine). Up to 23 equivalents of ammonia were produced with $[Mo(N_2)_2(PNP)]_2(\mu\text{-}N_2)_4$ (Figure 3D) using $CoCp_2$ as a reducing reagent and 2,6-lutidinium trifluoromethanesulfonate ([LutH]OTf) as a proton source.[30] The formation of the hydrazido complex in absence of [LutH]OTf pointed to a distal mechanism, similar to the Schrock cycle.

To study the putative intermediates of this reaction, mononuclear molybdenum-nitrido complexes bearing the PNP-type pincer ligand were prepared. Two of these, the neutral molybdenum(IV) nitrido complex [(MoN)Cl(PNP)] (Figure 3B) and the cationic molybdenum(V) nitrido complex [(MoN)Cl(PPP)]X (X = OTf and $BAr^F{}_4$), had similar activities than $[Mo(N_2)_2(PNP)]_2(\mu\text{-}N_2)_4$. These findings, in addition to DFT calculations, indicated that the reaction on the dimolybdenum complex would not involve protonation-induced dissociation of the two molybdenum atoms, but rather the additional binding of N_2 on each molybdenum. The reduction of dinitrogen, following a distal mechanism, would be triggered by the transfer of an electron from one Mo to the other. Supporting this proposal, variants of these dimolybdenum complexes with ligands substituted with electron-donating or redox active moieties were shown to have an increased activity. In particular, the ferrocene-substituted compound yielded 45 equiv ammonia,[31] revealing initial electron transfer to the molybdenum as a critical step of the reaction.

Finally, research on triphosphine systems identified the most efficient catalysts as molybdenum-nitrido complexes bearing [Mo(N)-Cl(PPP)] (8a) and [Mo(N)-Cl(PPP)]$BAr^F{}_4$ (Figure 3C) ligands (PPP = *bis*(di-tert-butylphosphinoethyl)phenylphosphine).[32] However, the mechanism of this reaction has not yet been clarified, and its relation to the proposed reduction of N_2 by dimolybdenum-dinitrogen complexes remains uncertain.

Although the catalytic performance of these molybdenum-based catalysts proves that molybdenum still needs to be considered as a possible binding and cleavage site for N_2 reduction, their molecular composition and electronic structure remains too remote from FeMo-co to allow definitive conclusions about the reaction taking place on the cofactor. Cubane models have formerly given rise to the hypothesis that the molybdenum ion of FeMo-co would be the site for the last steps of ammonia production, that is, the reduction of hydrazine. Studies on $[MoFe_3S_4]^{3+}$ cubanes, using $Co(Cp)_2$ as electron donor and 2,6-lutidinium (Lut-HCl) as proton source, showed they catalyzed the $2e^-$ reduction of hydrazine to ammonia.[33] Interestingly, a similar activity was observed with synthetic compounds featuring a $[VFe_3S_4]^{2+}$ core.[34] These findings suggest that the first steps of N_2 reduction would occur on another site of FeMo-co—such as Fe6, as discussed below; followed

by the migration of a reaction intermediate on the molybdenum atom, where the final steps would take place.

2.3. Iron-based Compounds

A further point that questions the role of Mo as the catalytically relevant component of FeMo-co is the mere existence of the alternative, Mo-free nitrogenases that differ in the heterometal, but that likely still contain the [Fe_7S_9C] core of the cluster. Consequently, the Fe sites have long been discussed as possible substrate-binding sites, and more recently a series of successful syntheses have introduced iron-based catalysts as models for the reduction of N_2 by nitrogenase.

With the discovery of the central atom,[3b] all iron sites in FeMo-co were coordinatively saturated, making the actual coordination site for N_2 a matter of extensive debate, flanked by a plethora of theoretical calculations. A frequently raised and chemically intuitive suggestion are the six core irons (Fe2-Fe7) surrounding the carbide.[3a] Their ligand geometry is distorted tetrahedral, with the metal ions shifted towards the plane of the three sulfur ligands and the carbon attaining a more axial character. N_2 was suggested to coordinate to iron such that it completes a trigonal bipyramidal ligation, and can then be further reduced to ammonia via nitrogenous (N_xH_y) ligands.[35] Some research groups reported diazene (N_2H_2), hydrazine (N_2H_4) and ammonia complexes of iron as intermediates in the *alternating* pathway.[36] In the *alternating* pathway, contrary to the *distal* pathway (Scheme 1 B), both N of N_2 are alternatively hydrogenated at each protonation step. A hydrazine-bound intermediate is formed after four proton-couple electron transfer steps, and the first NH_3 is released from the E_5 state.

The loss of N_2 reductase activity, but not of proton reduction activity, by nitrogenase V70I$^{\alpha}$ variants was taken as an indication that N_2 binds to the [Fe2,6,3,7] face of FeMo-co, and more specifically to Fe6 that is positioned most closely to the altered amino acid side chain. Binding of substrate would occur either in *exo* position (Figure 4 A), leading to an elongation or even dissociation of the Fe–C bond, or to the breaking of a Fe–S bound, or in *endo* coordination, where N_2 would be juxtaposed to a sulfur atom and inward from three iron atoms. Fe–C bond elongation has been indicated by extended X-ray absorption fine structure (EXAFS) and nuclear resonance vibrational spectroscopy (NRVS).[37] On the other hand, the experimentally demonstrated replacement of the μ_2-sulfide S2B sulfide replacement by CO or selenocyanate established that a reversible Fe–S bond cleavage is possible (Figure 4 B),[13,38] as did the observation of Fe–S dissociation in smaller Fe–S clusters.[39] In contrast, the *endo* coordination (Figure 4 C) was supported by electron nuclear double resonance (ENDOR) experiments.[40]

In 2015, Holland and co-workers introduced a new iron-sulfur-carbon compound as a model for N_2 binding to a site with the properties of a core iron of FeMo-co.[41] Notably, this system included soft sulfido ligands into N_2-reducing compounds for the first time, providing a closer analogy to the biological metal site. Structural analysis of the ligated compound showed that upon reduction by potassium graphite (KC_8) at −70 °C, N_2 binds to an iron atom coordinated by two thiolate ligands and an arene ring. This coordination is retained using either an iron *bis*(thiolate) (Figure 5 A) or *tris*(thiolate) complex (Figure 5 B), revealing the dissociation of an Fe-S bond upon binding of N_2. However, no dinitrogen reduction activity has been assayed using this compound, and N_2 dissociated from the complex upon incubation at room temperature.

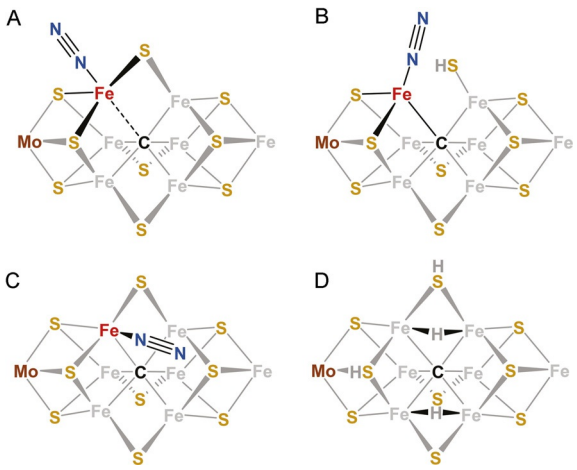

Figure 4. Hypotheses for N_2 binding to FeMo cofactor. **A**) With Fe as potential binding site suggested by the V70I$^{\alpha}$ variant of MoFe protein, binding of N_2 can occur in an *exo* position, leading to an elongation of the FeC bond. **B**) This mode of binding might also afford the cleavage of an Fe-S bond, rationalizing the CO-inhibited complex that was characterized by X-ray crystallography. **C**) Binding of N_2 in an *endo* position would located the substrate above a cluster face. **D**) The catalytically relevant E_4H_4 state of the cofactor was supposed to contain surface hydrides that can eliminate H_2, leaving the cluster in a supperreduced and highly reactive state.

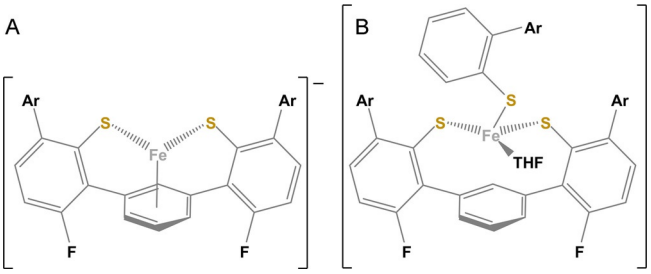

Figure 5. Thiolate-containing iron complexes by the Holland group. **A**) The Fe-*bis*(thionate) complex. **B**) in the Fe *tris*(thiolate) compound, a sulfide was eliminated upon N_2 binding.

Several models for the binding and the functionalization of N_2 at a single iron site were generated by Peters and co-workers, based on tetradentate ligands related to *tris*(phosphine) complexes.[42] In particular, elongation of the Fe-C bound has been modeled using four- and five-coordinate Fe complexes featuring an axial tri(silyl)methyl ligand,[43] or an anionic *tris*(phosphinoalkyl)FeN_2^- complex (Figure 6 A, X = C).[44] This $(CP^iPr_3)FeN_2^-$ catalyzed the formation of 4.6 equiv of NH_3 upon addition of KC_8 and $[H(Et_2O)_2]BAr^F_4$. The reaction proceeded under 1 atm N_2 at −78 °C to avoid the formation of dihydrogen as a result of reaction between the strong reducing agent and strong acid.

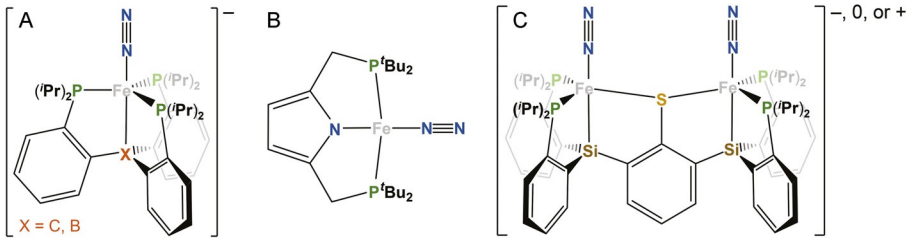

Figure 6. Catalytically active Fe compounds for N_2 activation. **A**) The tetradentate triphosphine ligand from the Peters group was inspired by the possible N_2 binding in *exo* position on a core iron of nitrogenase cofactor. **B**) The PNP-type complex of Nishibayashi and co-workers effectively catalyzed N_2 reduction with KC_8 as a reductant and yielded hydrazine as a by-product. **C**) Introducing a sulfido ligand, this dinuclear compound from the Peters group retained N_2 across at least three redox states.

Under identical conditions, up to 7 equiv of NH_3 were obtained with the related triphosphine compound $[(TPB)Fe(N_2)][Na(12\text{-crown-}4)_2]$ (TPB = *tri*(phosphine)borane) (Figure 6A, X = B) upon addition of excess $[H(Et_2O)_2]BAr^F{}_4$,[45] underlining the importance of the nature of the *trans* ligand of Fe. Yields were 10-fold higher when increased amounts of $HBAr^F{}_4$ and KC_8 were used.[46] Importantly, an iron-hydrazido complex, characterized as $[(TPB)(FeN)\text{-}NH_2][BAr^F{}_4]$, has been characterized as a reactive intermediate of this reaction.[47] This intermediate points to a distal mechanism, or a hybrid distal-to-alternating mechanism, for N_2 reduction. Contrasting results have been obtained by the research group of Nishibayashi, who reported effective catalysis of N_2 reduction to NH_3 using an iron-dinitrogen complex bearing an anionic PNP-pincer ligand (PNP = 2,5-*bis*(di-tert-butylphosphinomethyl)pyrrolide) with KC_8 as a reductant and $[H(OEt_2)_2]BAr^F{}_4$ as proton donor, at −78 °C (Figure 6B).[48] No activity was observed with $CoCp_2$ and [LutH]OTf, the electron and proton donors previously used by this group for their studies on PNP-pincer molybdenum compounds. Hydrazine was a side-product when the reaction was performed in Et_2O, and the N_2H_4/NH_3 ratio increased when THF was used as solvent, yielding nearly as much hydrazine as ammonia. The production of hydrazine points to an *alternating* pathway and seems to be at variance with the results of Peters, who reported no hydrazine production from their iron-based compounds.

More recently, Creutz and Peters also addressed the introduction of sulfide ligands into their systems in order to more closely approach the architecture of FeMo-co. They reported a dinuclear iron system featuring a thiolate donor, (N_2-Fe(μ-SAr)Fe-N_2) that produced NH_3. Interestingly, the N_2 ligands are retained through at least three redox states ($Fe^{II}Fe^{II}$, $Fe^{II}Fe^{I}$, $Fe^{I}Fe^{I}$) upon treatment with $FcPF_6$ and $FcBAr^F{}_4$, which is interpreted as a good representation of the thiolate-ligated, low-valent iron sites of FeMo-co (Figure 6C).[49]

3. Reuniting Biology and Chemistry

Starting from attempts to predict—and later mimic—the structural features of the unique catalytic cofactor of nitrogenases, synthetic organometallic chemistry has come a long way to generate functional, rationally designed catalysts for the reduction of the most inert substrate known to the chemistry of living organisms. In parallel, molecular biology has progressed and our understanding of the reaction mechanism of the enzyme nitrogenase has advanced considerably. Recent efforts have thus aimed at making the enzyme and its complex biogenesis cascade available to other, non-diazotrophic organisms, as well as at combining the biological protein matrix with some of the model compounds described above to explore the possibility of biohybrid solutions to nitrogen fixation.

3.1. Refactored Nitrogenase and Nitrogenase Biohybrids

The complexity of nitrogenase cofactors and their acute sensitivity to oxygen represent the main obstacles on the path towards the generation of new diazotrophs. While the construction of eukaryotic (yeast or plant) cells producing a functional nitrogenase is still under way, a few successful attempts have been reported in microbes.[50] Searching for a minimal nitrogenase gene cluster seems to be a rewarding approach, and investigations on the biosynthesis of Fe-nitrogenase, which requires less factors for its maturation, look promising. Nitrogen fixation has been demonstrated via $^{15}N_2$ incorporation assays for some strains, and a further biochemical characterization of such recombinant nitrogenases would be highly interesting.

FeMo-co, extracted from nitrogenase into organic solvents, can catalyze the reduction of several substrates such as protons, CN^-, or CO.[51] However, it so far cannot be used for the reduction of N_2, highlighting the crucial influence of the protein matrix on its reactivity. Very recently, a synthetic $[Et_4N]_4[Fe_6S_9(SEt)_2]$ cluster (termed the $Fe_6{}^{RHH}$-cluster) (Figure 2), vaguely reminiscent of the structure of the $[Fe_8S_9C]$ precursor (L-cluster) of FeMo-co, but even more so of P-cluster, catalyzed similar reactions when using SmI_2 as a reductant.[52] Most interestingly, an artificial nitrogenase with FeMo-co replaced by the $Fe_6{}^{RHH}$-cluster catalyzed the reduction of acetylene to ethylene in assays comprising the reductase moiety (NifH), ATP, and dithionite as a reductant.[22] The reported activity reached 2% of the one of holo-nitrogenase. When Eu^{II}DTPA (DTPA = diethylene triamine pentaacetic acid) was used instead of the reductase component, the biohybrid nitrogenase with $Fe_6{}^{RHH}$ had twice the activity of holo-nitrogenase, while $Fe_6{}^{RHH}$ alone in solution was inactive. No N_2 reduction activity was observed, which could be due to compositional differences between the clusters, such as the lack of the central μ_6-carbide or of one of the Fe faces of FeMo-co. Furthermore, these results underline the importance of interactions between the cofactor and its protein shell. Structural or spectroscopic studies would be of

great interest to assess how Fe_6^{RHH} is inserted in nitrogenase and whether it is coordinated by similar protein residues than FeMo-co.

3.2. New Mechanistic Insights from Nitrogenase

While the basic structure and atomic composition of FeMo-co was eventually fully understood in 2011,[7] further advances were made since, in particular towards elucidating its electronic structure and possible reactivity. This included the orientation of the magnetic tensor of the cluster by single-crystal EPR,[9] as well as the individual assignment of Mo[53] and Fe oxidation states by SpReAD analysis,[54] and a re-evaluation of Mössbauer data available in literature.[55] This level of chemical understanding now provides a sound basis for a new generation of theoretical studies that may help to finally provide an answer to how this enzyme achieves its unique catalytic properties. Here, the insights gained from the CO-inhibited and Se-ligated structures of molybdenum nitrogenase may well be crucial.[13,38] The binding of CO provided direct evidence for the lability of the μ_2-coordinated belt sulfides, and generated a coordination site for the exogenous ligand that is chemically reasonable and matches most spectroscopic observations. Possibly the most striking aspect of this binding mode, however, was the straightforward reconstitution of the sulfide ligand upon removal of CO. This has been taken up in a mechanistic proposal by the Nørskov group,[56] where the rebinding of S^{2-} was suggested to be essential for the release of the second NH_3 molecule. In the three-dimensional structure, however, the displaced sulfide was not found in proximity to the cofactor, although a storage position in reasonable vicinity of the active site should be a prerequisite for efficient catalysis if this was indeed part of the reduction mechanism. The questions regarding the fate of this sulfide and the binding mode of N_2 with respect to CO will therefore be most urgent for nitrogenase research in the near future.

3.3. Conclusions

The enzyme nitrogenase has retained its enigmatic character to date, and only slowly we are beginning to understand the possibilities for substrate coordination at its unique cofactors. At the same time, model chemistry has seen fantastic advances and has come a long way from compounds that only coordinated N_2 or mediated stoichiometric cleavage through structural mimics of the entire cofactor to the functional catalysts we have today. Notably, however, these systems do not necessarily reproduce the chemistry of the enzyme. Compounds based on either Mo or Fe are *bioinspired* rather than *biomimetic*, and while they do draw their inspiration from the solutions that nature has found, synthetic chemistry is not limited by the availability of metals in the environment or the fixed set of proteinogenic amino acids to serve as ligands. Enzymes, on the other hand, are far more than complex metal clusters locked in an inert protein matrix. The combination of both aspects indeed creates emergent properties whose intricacy is way beyond the narrow concepts of homogeneous and heterogeneous catalysis, and the de novo generation of true biomimetics or biohybrids will remain a challenge for chemists and molecular biologists for many years to come.

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