

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/8881091>

Substrate Interactions with Nitrogenase: Fe versus Mo

ARTICLE *in* BIOCHEMISTRY · MARCH 2004

Impact Factor: 3.02 · DOI: 10.1021/bi036038g · Source: PubMed

CITATIONS

149

READS

65

3 AUTHORS, INCLUDING:



Lance Seefeldt

Utah State University

136 PUBLICATIONS 5,394 CITATIONS

SEE PROFILE



Ian Dance

University of New South Wales

312 PUBLICATIONS 8,436 CITATIONS

SEE PROFILE

Current Topics

Substrate Interactions with Nitrogenase: Fe versus Mo[†]

Lance C. Seefeldt,^{*,‡} Ian G. Dance,[§] and Dennis R. Dean^{*,||}

Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84332, School of Chemical Sciences, The University of New South Wales, Sydney, NSW 2052, Australia, and Department of Biochemistry, Virginia Polytechnic and State University, Blacksburg, Virginia 24061

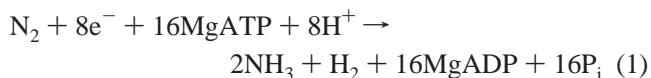
Received November 13, 2003; Revised Manuscript Received December 14, 2003

ABSTRACT: Biological nitrogen reduction is catalyzed by a complex two-component metalloenzyme called nitrogenase. For the Mo-dependent enzyme, the site of substrate reduction is provided by a [7Fe-9S-Mo-X-homocitrate] metallocluster, where X is proposed to be an N atom. Recent progress with organometallic model compounds, theoretical calculations, and biochemical, kinetic, and biophysical studies on nitrogenase has led to the formulation of two opposing models of where N₂ or alternative substrates might bind during catalysis. One model involves substrate binding to the Mo atom, whereas the other model involves the participation of one or more Fe atoms located in the central region of the metallocluster. Recently gathered evidence that has provided the basis for both models is summarized, and a perspective on future research in resolving this fundamental mechanistic question is presented.

Conversion of N₂ to NH₃ ("nitrogen fixation") is an essential step in the global nitrogen cycle, and this reaction is necessary to sustain life on earth (1). Biological nitrogen fixation is the process by which a major portion of the metabolically accessible nitrogen enters the biosphere, and enzymes responsible for this activity are designated nitrogenases. Nitrogenases are found exclusively in microbes, where three major families have been identified. These families are mechanistically related; however, their catalytic components are genetically distinct, and they are differenti-

ated by the composition of their respective active site metalloclusters. Among these is the Mo-dependent nitrogenase, which is the most extensively studied (2, 3) and is the topic of this perspective.

Mo-dependent nitrogenases require the participation of two separately purified protein partners designated the Fe protein¹ (or nitrogenase reductase) and the MoFe protein (or dinitrogenase) (Figure 1), which under ideal conditions catalyze the reaction



The Fe protein is a homodimer that contains a single, bridging [4Fe-4S]^{2+/1+} cluster and two MgATP binding sites (Figure 1) (4). During turnover, the reduced, nucleotide-

[†] This work was supported by National Institutes of Health Grant GM59087 (L.C.S. and D.R.D.), the Australian Research Council, the Australian Centre for Advanced Computing and Communications, and The University of New South Wales (I.G.D.).

^{*} To whom correspondence should be addressed. L.C.S.: phone, (435) 797-3964; fax, (435) 797-3390; e-mail, seefeldt@cc.usu.edu. D.R.D.: phone, (540) 231-5895; fax, (540) 231-7126; e-mail, deandr@vt.edu.

[‡] Utah State University.

[§] The University of New South Wales.

^{||} Virginia Polytechnic and State University.

¹ Abbreviations: Fe protein, iron protein; MoFe protein, molybdenum-iron protein; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance.

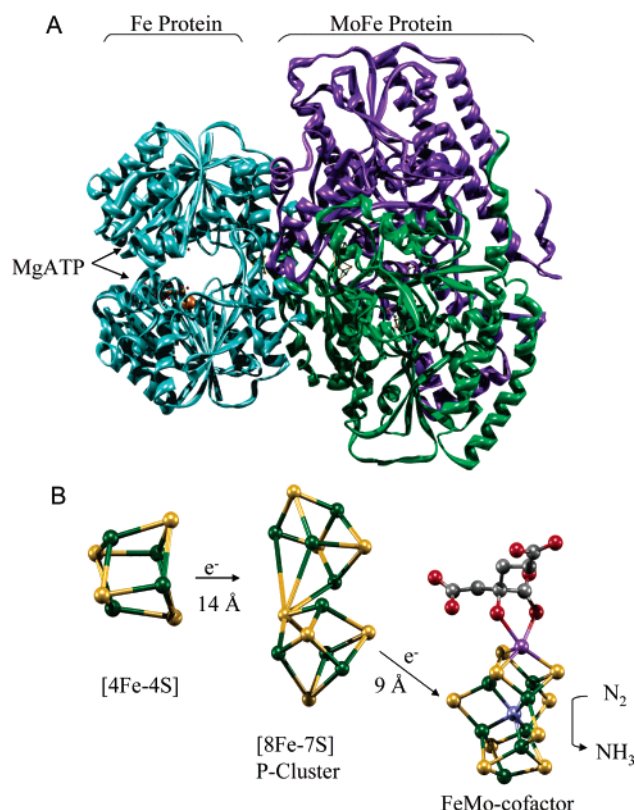


FIGURE 1: Nitrogenase Fe protein and one $\alpha\beta$ unit of the MoFe protein. (A) The Fe protein (light blue) is shown docked to one-half ($\alpha\beta$ unit) of the MoFe protein (β -subunit in purple and α -subunit in green) with the location of the bound MgATP noted (PDB entry 1G21). (B) The flow of electrons from the Fe protein [4Fe-4S] cluster to the MoFe protein [8Fe-7S] cluster (or P-cluster) and to the FeMo cofactor is shown with approximate edge-to-edge distances. Atom colors are as follows: Fe in green, S in yellow, Mo in purple, O in red, C in gray, and X in blue (PDB entry 1M1N). This figure was generated using the computer programs Discovery Viewer Pro 5.0 (Accelrys Inc.) and POV-Ray.

bound Fe protein docks with the MoFe protein, which triggers MgATP hydrolysis in a reaction coupled to one-electron transfer and subsequent dissociation of the two component proteins (5, 6). Because multiple electrons are required for substrate reduction, this situation demands multiple rounds of Fe protein–MoFe protein association and dissociation during catalysis, and that MoFe protein must be able to sustain a number of levels of reduction. The MoFe protein is a heterotetramer ($\alpha_2\beta_2$), with each $\alpha\beta$ unit functioning as a catalytic unit and containing two novel metalloclusters involved in electron transfer and substrate binding (Figure 1) (7). One of these is an [8Fe-7S] cluster, termed the P-cluster, which is located near an $\alpha\beta$ unit pseudosymmetric interface that provides the Fe protein docking surface (8, 9). The P-cluster initially receives the electron delivered from the Fe protein, which is subsequently transferred to the other metallocluster, called FeMo cofactor [7Fe-9S-Mo-X-homocitrate], where X is proposed to be an N atom (10–14). A variety of complementary genetic (15) and biochemical studies (16) have established that the FeMo cofactor provides the substrate binding/reduction site.

Fundamental aspects of the nitrogenase mechanism have been examined in recent years, including how MgATP binding and hydrolysis are coupled to intercomponent electron transfer (5, 6). The ability to address these issues

has been significantly advanced by various high-resolution structures of the Fe protein (4, 17), the MoFe protein (7, 10, 18), and the Fe protein–MoFe protein complex (8, 9). Nevertheless, one of the most important aspects of nitrogenase catalysis, exactly where and how substrates are bound to the FeMo cofactor during catalysis, has remained enigmatic.

Ideally, what is needed to fully understand how N_2 interacts with the nitrogenase active site is to have N_2 or some partially reduced form of N_2 trapped at the active site FeMo cofactor at a sufficient concentration and in a state that is amenable to structural or biophysical examination. However, several complicating features of nitrogenase have denied the successful application of this approach. For example, the resting state of the MoFe protein does not bind N_2 . Rather, the MoFe protein is only able to bind N_2 under “turnover” conditions that require the Fe protein, an electron donor, and MgATP, and binding only occurs after three or more electrons have accumulated within the MoFe protein (19). Consequently, all efforts to characterize N_2 - or intermediate-bound states have utilized freeze or chemical quenching of nitrogenase during turnover. A general problem with this approach has been the relatively low concentration of trapped states that can be achieved because of the many possible states that are in equilibrium. Thus, formulation of substrate binding models has relied on a combination of indirect approaches, including (1) examination of the interaction of alternative substrates and inhibitors with the FeMo cofactor using kinetic and spectroscopic methods, (2) examination of the effect on catalytic activity elicited by amino acid substitution or by modification of the composition of the FeMo cofactor, (3) development of theoretical models based on the structure of the FeMo cofactor, and (4) synthesis and reactivity of FeMo cofactor model compounds. These approaches have led to the development of two different views of substrate binding and the catalytic mechanism of nitrogenase: one involving the direct participation of Mo and (R)-homocitrate and the other involving binding at a [4Fe-4S] face of the FeMo cofactor.

General Features of the FeMo Cofactor

The structure of the nitrogenase active site, FeMo cofactor, was resolved by the solution of the X-ray structure of the MoFe protein from *Azotobacter vinelandii* by the Rees group in 1992 (20). Subsequent X-ray structures of MoFe proteins, including structures of MoFe proteins from other organisms (18, 21), have confirmed the general structural features (Figure 2A). The cluster is composed of two metal–sulfur substructures, [Mo-3Fe-3S] and [4Fe-3S], which are bridged by three μ_2 -S $^{2-}$ ligands. (R)-Homocitrate is an organic constituent that provides two O ligands to the Mo. Recently, a high-resolution X-ray structure (1.16 Å) of the MoFe protein has identified an atom (10), most probably nonexchangeable N (11–14), at the center of the FeMo cofactor. Important aspects of the structure that are relevant to formulation of substrate binding models include (1) six-coordinate Mo, with three ligands provided by three μ_3 -S $^{2-}$ ions from one cuboidal unit, two ligands from O atoms of (R)-homocitrate, and an N ligand from the MoFe protein (α -442^{His}); (2) six Fe atoms in the central portion of the FeMo cofactor, arranged as a trigonal prism, and each four-

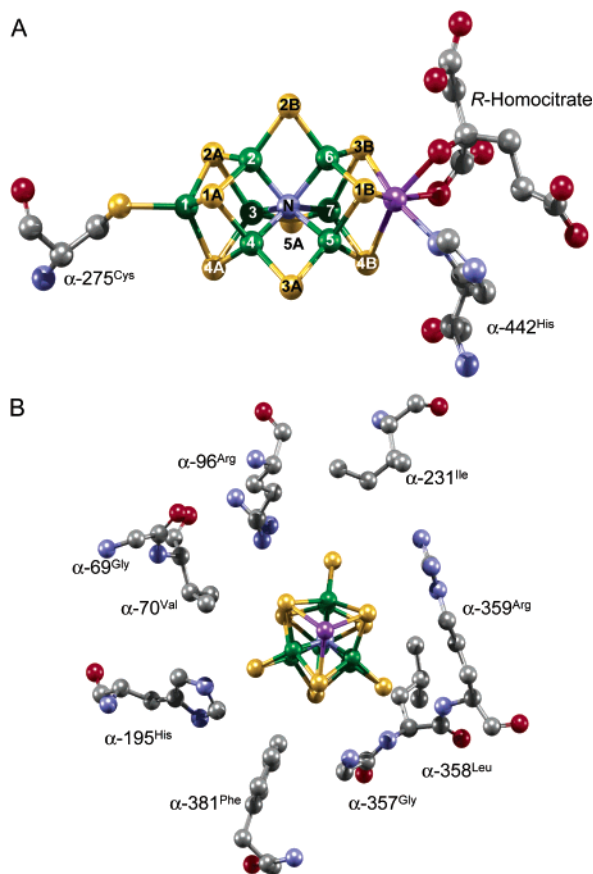


FIGURE 2: FeMo cofactor and parts of its protein environment. (A) The FeMo cofactor is shown with MoFe protein ligands. The atom numbering in the FeMo cofactor is from ref 10. (B) View down the Mo end of the FeMo cofactor (homocitrate removed) with MoFe protein amino acids in the first shell of interaction shown (PDB entry 1M1N). The central atom X is shown as N in this review, although its identity has not been definitively established. This figure was generated using the computer programs Discovery Viewer Pro 5.0 (Accelrys Inc.) and POV-Ray.

coordinate; and (3) a four-coordinate Fe (designated Fe1) with ligands from α -275^{Cys} and three μ_3 -S²⁻ ligands.

The FeMo cofactor can undergo at least two redox changes, with three oxidation states that are well-described (eq 2).



When bound in the MoFe protein, the as-isolated, dithionite-reduced state of the FeMo cofactor is termed the M^N state (22). This is an $S = 3/2$ spin state and therefore is EPR active with a strong signal having g values of 4.3, 3.7, and 2.0 at low temperatures (<12 K). The M^N state can be oxidized by the addition of dye mediators, accessing a state termed the M^{OX} state (23, 24). This state is diamagnetic and is therefore EPR silent. There is no experimental evidence that this latter oxidation state is accessed during nitrogenase catalysis, but with an E_m value of -40 mV, the involvement of this oxidation state cannot be ruled out (25, 26).

Further reduction of the M^N state has not been achieved by the addition of small molecule reductants. However, when the MoFe protein is freeze-quenched under turnover conditions (i.e., with the Fe protein, MgATP, and dithionite), the FeMo cofactor is trapped in an EPR silent state that is termed the M^R state (27, 28). The E_m for this redox couple has not

been measured directly, but has been estimated to be more negative than -465 mV (29). While the M^R state is known to be more reduced than the M^N state, it almost certainly does not represent a single oxidation state of the FeMo cofactor. There is ample kinetic evidence that from one to seven electrons per $\alpha\beta$ dimer can accumulate in the MoFe protein depending on the substrate that is present (termed E_0 , E_1 , etc., in the Thorneley and Lowe cycle) (19). While it is not clear how many of these electrons accumulate on the FeMo cofactor versus the P-cluster at any particular stage of catalysis, it seems reasonable to conclude that multiple electrons accumulate on the FeMo cofactor in the form of partially reduced substrates. Given that the FeMo cofactor remains in an EPR silent state (M^R), it would appear that the redox state of the core cluster is constant, implicating localization of the added electrons on bound substrates (e.g., hydrogen atoms).

The fact that at least one redox state of the FeMo cofactor is EPR active has been a valuable tool in investigations of binding of substrates and inhibitors to the FeMo cofactor. Importantly, this feature provides a way of monitoring the electronic properties of the FeMo cofactor exclusive of all other components in the protein. Furthermore, the paramagnetic state allows the application of more sophisticated spectroscopic methods, such as ENDOR (30) and Mössbauer (28) spectroscopies, which provide information about ligands bound to the FeMo cofactor and the valence states of the Fe and Mo atoms. While the paramagnetic nature of the M^N state has been instrumental in examining the mechanism of nitrogenase, the spectroscopic silence of the M^R state has been a limitation.

The FeMo cofactor can also be extracted from the MoFe protein using chaotropic organic solvents, such as *N*-methylformamide (NMF) (16). In this case, NMF appears to replace the protein ligands (α -275^{Cys} and α -442^{His}), thus stabilizing the cofactor. Two features of the organic solvent-extracted cofactor are important for the current discussion. First, the resting state of the isolated cofactor is in the same oxidation state as the M^N state, giving rise to a similar EPR spectrum. Second, while the isolated FeMo cofactor has been shown to have limited reactivity (31), it does not have the range of activities found when bound in the protein, clearly pointing to the importance of the protein in controlling reactivity that is relevant to catalysis.

Nitrogenase Enzymology Is Complicated

As shown in eq 1, the reduction of N_2 requires the obligate evolution of one H_2 for each N_2 that is reduced (32). Whether such H_2 evolution represents an activation step required for N_2 binding is not yet known, but this feature highlights a major complicating aspect of nitrogenase enzymology. In the absence of N_2 or other substrates, all electron flow is directed toward proton reduction. Because the availability of protons cannot be restricted, the enzyme is constantly returned to the resting state, and for this reason, it is not possible to obtain a highly populated form of the semireduced enzyme that is amenable to biophysical or structural analysis. One feature that has proven to be useful with respect to the development of kinetic and substrate binding models is that, under turnover conditions, nitrogenase is able to bind a variety of different substrates and inhibitors, including

acetylene (substrate) (33) and CO (inhibitor) (34). However, the relationship among the binding of N_2 , alternative substrates, and inhibitors has also turned out to be very complicated. One such complication is highlighted by the peculiar nature of the kinetic relationship between acetylene reduction, which is a *noncompetitive* inhibitor of N_2 reduction, and N_2 reduction, which is a *competitive* inhibitor of acetylene reduction (35). This nonreciprocity in substrate inhibition patterns can be explained if it is considered that a more reduced form of the MoFe protein is required for N_2 binding than for acetylene binding (19, 36). That is, the MoFe protein must receive more electrons from the Fe protein for N_2 binding than are required for acetylene binding. Thus, one simple explanation for the respective inhibition patterns for N_2 and acetylene reduction that emerges is that these substrates are able to access the same site or overlapping sites, but at different redox states. However, it has not yet been established whether acetylene or other substrates and inhibitors do, in fact, share the same or an overlapping binding site accessed by N_2 . Features that contribute to uncertainty about this issue include the following. Although H_2 evolution appears to be obligately associated with N_2 binding and/or reduction, it is not required for the binding of other substrates and inhibitors (32). As already mentioned, this situation could mean that H_2 evolution represents an activation step that is uniquely required for N_2 binding. Related to this question is the fact that H_2 is a competitive inhibitor of N_2 binding, and in the presence of D_2 and N_2 , the enzyme is able to evolve HD (37, 38). Neither of these properties is associated with the binding of any other substrate or inhibitor. Further, there is abundant evidence for multiple sites for the binding of substrates and inhibitors. One good example of this feature is that CO is able to induce cooperativity in acetylene reduction catalyzed by an altered MoFe protein produced by amino acid substitution (39).

In contrast to the above complicating issues, there is also evidence that at least one acetylene-binding site represents the same site, or an overlapping site, that is accessed by N_2 . For example, a MoFe protein was produced by amino acid substitution that is able to reduce acetylene at the normal rate but can reduce N_2 only very poorly (<2% of the normal rate) (40, 41). Despite a very low N_2 reduction rate for this protein, N_2 is still able to inhibit acetylene reduction as effectively as for the normal protein. Because N_2 cannot be effectively reduced by the altered MoFe protein, it must be that N_2 binding, rather than competition with acetylene for reducing equivalents, is responsible for inhibition of acetylene reduction. In another case, an altered MoFe protein was produced by amino acid substitution for which acetylene becomes a *competitive* rather than a *noncompetitive* inhibitor of N_2 reduction (42). The explanation offered for this result is that an acetylene-binding site or state, not accessed by N_2 , has been eliminated by the substitution, but a binding site or state that can be accessed by both N_2 and acetylene is left intact.

Our interpretation of all of these observations is that while alternative substrates and inhibitors are likely to be useful probes for identification of the N_2 binding site, there are two important caveats to this approach. First, because there are multiple binding sites, any site identified by using alternative substrates and inhibitors must also be shown to be the same binding site accessed by N_2 . Second, although N_2 , alternative

substrates, and inhibitors might access the same or overlapping sites, the mechanism for N_2 activation appears to be fundamentally different from that required for activation of other substrates and inhibitors, indicating a probable distinction between *where* and *how* substrates might bind to the active site.

Mo as the Substrate-Binding Site

Interest in the participation of Mo in nitrogenase catalysis arose early, well before there was definitive information about the enzyme structure, from knowledge that the enzyme contained Mo, and from strong and continuing development of Mo coordination chemistry of N_2 and its reduced forms, including diazene (N_2H_2) and hydrazine (N_2H_4) (43, 44). On the basis of Mo-phosphine complexes with trapped nitrogen and its hydrides, Chatt and co-workers developed a Mo-based mechanism for the reduction of N_2 to ammonia that has since been made more elaborate and is now often called the "Chatt cycle" (45). More recently, support for such a mechanism has come from the observation of reactivities of some Mo model compounds toward N_2 or its reduction intermediates. For example, Coucouvanis and co-workers reported the catalytic reduction of hydrazine to ammonia with [Mo-3Fe-4S-polycarboxylate] clusters, with reduction occurring on the Mo (46, 47). This reaction could be analogous to the final steps in nitrogenase-catalyzed N_2 reduction. More recently, Schrock and co-workers have reported a remarkable catalytic reduction of N_2 all the way to ammonia at a single Mo center of a model compound (48, 49). Their proposed mechanism, derived from some trapped intermediates, and considering the Chatt cycle, involves binding of N_2 and its reduction intermediates at a Mo site. It needs to be recognized, however, that these complexes use nonbiological ligands, and the chemical conditions and reagents in N_2 reduction cycles involving model compounds are quite different from those of active nitrogenase.

For Mo in the FeMo cofactor to serve as the substrate-binding site, one or more of its ligands needs to be dissociated to provide an open coordination site. One possibility would be for the carboxylate O of (*R*)-homocitrate to be displaced by the substrate. Support for this model has come from examination of the effects of changes in (*R*)-homocitrate on the catalytic properties of the MoFe protein and on the reactivity of the isolated FeMo cofactor (15, 50–52). For example, when the gene encoding homocitrate synthase is inactivated, an altered FeMo cofactor is produced having (*R*)-homocitrate substituted with citrate (53). The resulting MoFe protein is observed to possess altered catalytic properties, exhibiting a substantial level of proton and acetylene reduction, but only a low level of N_2 reduction, when compared to that of the normal enzyme. Isolated FeMo cofactors that contain either citrate or (*R*)-homocitrate also exhibit differential reactivities toward binding of thiophenolate when imidazole is a ligand to the Mo (54). These results were interpreted to indicate hydrogen bonding between a carboxylate of (*R*)-homocitrate and an NH group of the imidazole. Modeling was used to suggest that this hydrogen bonding could involve the longer arm carboxylate ligand of (*R*)-homocitrate, with dissociation from Mo of the shorter arm carboxylate (Figure 3). In a series of studies where different organic acids were substituted for (*R*)-homocitrate in the FeMo cofactor, Ludden and co-workers

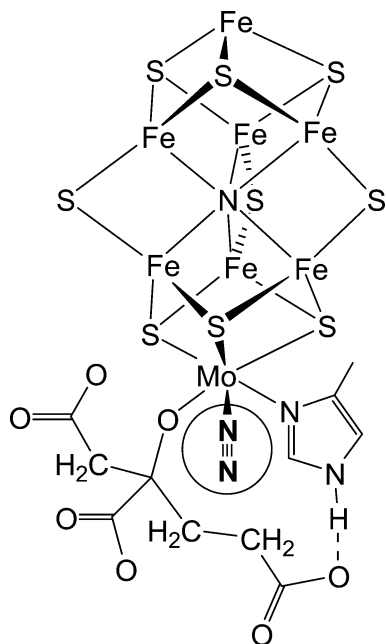


FIGURE 3: Possible binding of N_2 to Mo of the FeMo cofactor. A proposed model for creation of a binding site for N_2 at Mo by dissociation of the carboxylate ligand of (*R*)-homocitrate, and hydrogen bonding of the longer arm of (*R*)-homocitrate to α -442^{His}.

(55) deduced some minimum features of the bound organic acid that are necessary to support N_2 reduction activity: (1) 1- and 2-carboxyl groups, (2) a hydroxyl group, (3) an *R* configuration, and (4) a carbon chain length of four to six atoms. All of these studies, as well as some amino acid substitution studies (56), show that alterations near the Mo have a significant impact on the reactivity of the FeMo cofactor toward N_2 binding and/or reduction.

Two other classes of nitrogenase are known, with V or Fe in place of Mo in the cofactor (57, 58). The current thinking is that these active site clusters are identical except for the identity of Mo, V, or Fe at the end metal position of the heterometal-containing subcluster (Figure 2). The catalytic efficiency toward N_2 reduction is much greater for the Mo-containing enzyme than for those containing V or Fe. This activity variation could be interpreted in terms of Mo, V, or Fe being an important site in the mechanism, with Mo optimal. Alternatively, these data could be interpreted as reflecting a secondary influence of the heterometal end, with the expectation that a site common to all three of the FeMo cofactor homologues (the central Fe atoms) is the most reasonable candidate.

Theoretical Models Invoking Substrate Binding to Mo

Durrant has used density functional theory (DFT) to investigate possible stages in the nitrogenase catalytic cycle with Mo as the main binding site (59, 60). This model requires the dissociation of one O ligand from the Mo, as already described, permitting participation of the Mo in N_2 binding and reduction. Durrant's schemes attempt to explain a number of experimental observations, including N_2 -dependent HD formation (61). Nevertheless, a limitation of this calculated mechanism is that its truncated chemical system is a poor representation of the FeMo cofactor. Other

detailed theoretical investigations of Mo-only models of the Mo site have been reported (62).

Evidence for Central Fe Atom(s) as the Substrate-Binding Site

The case for the central Fe region as the site for substrate binding in nitrogenase is based on experimental studies of model compounds, theoretical modeling, and more recently a series of studies with residue-modified nitrogenases that clearly point to binding of several different substrates and one inhibitor at one or more Fe atoms in the central section of the FeMo cofactor.

A number of well-defined model compounds have N_2 or its partially hydrogenated intermediates, and/or H and H_2 , bound to Fe (63–70). Certain other observations also support the possible involvement of Fe in N_2 binding. For example, the industrial Haber–Bosch process, which accounts for a large fraction of N_2 fixation in the global nitrogen cycle, is a catalytic reaction that uses an Fe-based catalyst. Several studies support an end-on binding of N_2 to one or more Fe atoms during this reaction (71). N_2 reduction to ammonia in a low yield in the presence of hydrogen sulfide and iron sulfide has also been reported (72).

The most compelling experimental evidence that favors Fe as the substrate-binding site in nitrogenase comes from studies with substrates or inhibitors other than N_2 . In early work it was recognized that when nitrogenase is freeze-trapped during turnover in the presence of the inhibitor CO, a new EPR signal is observed (34, 73). Subsequent studies, using ^{13}CO - and ^{57}Fe -labeled FeMo cofactor, in conjunction with ENDOR spectroscopy, established that CO is bound through C to one or more of the Fe atoms of the FeMo cofactor (74, 75). These data were interpreted to indicate that the lo-CO state (observed at low CO concentrations) results from a single CO bridged between two Fe atoms, probably in the central portion of the FeMo cofactor. The hi-CO state (observed at higher CO concentrations) was concluded to result from two molecules of CO bound end-on to two Fe atoms of the FeMo cofactor. Similar EPR and ENDOR studies with freeze-trapped states of MoFe proteins under turnover conditions with C_2H_2 (76, 77) or CS_2 (78) as substrates also indicate they are probably bound through C to one or more Fe atoms within the FeMo cofactor.

While these studies identify Fe atom(s) as the site for binding of these substrates or inhibitor, they do not specify which of the six central Fe atoms of the FeMo cofactor provide the binding site. The central portion of the FeMo cofactor is symmetrical, with the six Fe atoms being arranged as three geometrically equivalent [4Fe-4S] faces (Figure 2A). Nevertheless, these faces are differentiated within the protein matrix by the amino acid side chains that approach each of them (Figure 2B). Therefore, the experimental strategy was to make amino acid substitutions at these residues that provide the first shell of noncovalent interactions with the FeMo cofactor (42, 79, 80). The logic of this approach was based on the knowledge that nitrogenase is able to effectively reduce acetylene but is unable to accommodate reduction of longer chain alkynes such as propyne and butyne. In these experiments, it was found that substitution of the MoFe protein α -subunit 70^{Val} residue (Figure 2B) with alanine expands the substrate range to include larger alkynes such

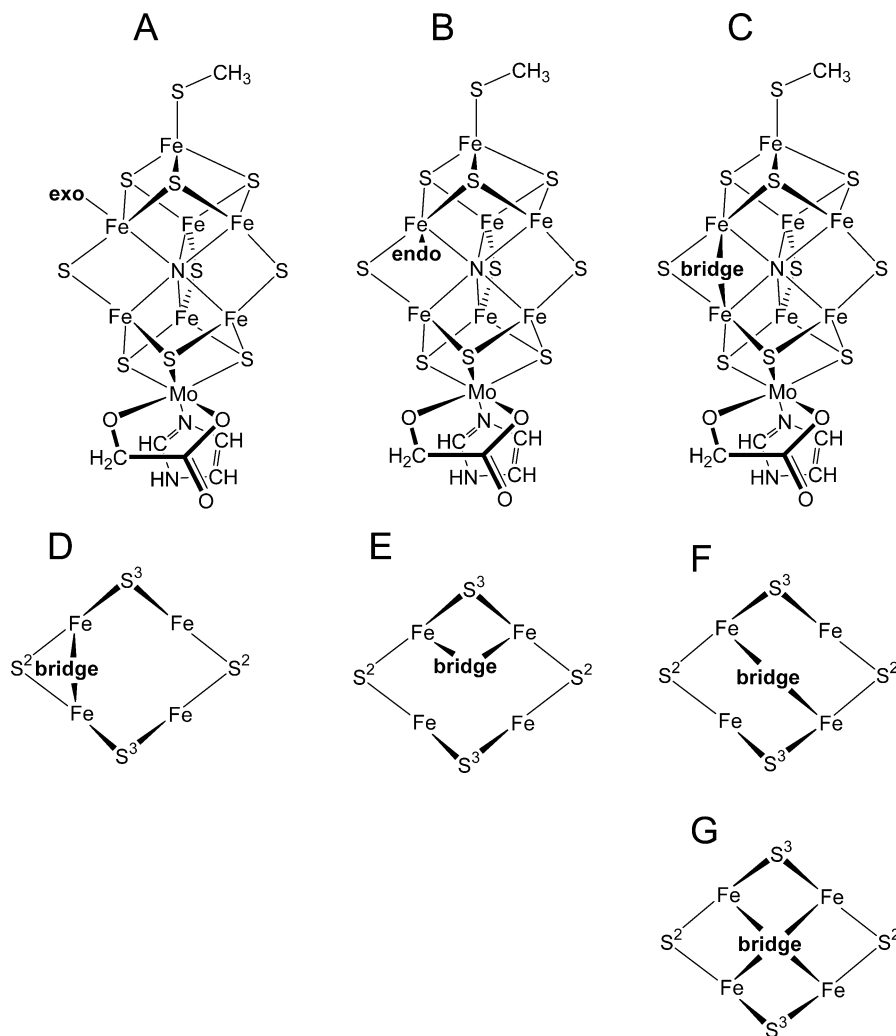


FIGURE 4: Binding of substrate to Fe of the FeMo cofactor. DFT-calculated models (A–C) for substrate binding positions at the FeMo cofactor and four different geometries (D–G) for substrate bridging on the FeS face.

as propyne, butyne, and propargyl alcohol ($\text{HC}\equiv\text{CCH}_2\text{OH}$) (80).

The α -70^{Ala} substituted MoFe protein has proven to be especially useful for spectroscopic studies where propargyl alcohol is used as the substrate. When the α -70^{Ala} substituted MoFe protein is trapped during turnover with this substrate, a new rhombic EPR signal is observed in the $g \sim 2$ region (81). Analysis of this signal indicated a high concentration of an intermediate bound to the FeMo cofactor, and subsequent ENDOR studies using ^{13}C -labeled propargyl alcohol were used to establish that the EPR signal arises from a derivative of propargyl alcohol bound to the FeMo cofactor. The importance of these results is that they clearly indicate binding of an alkyne substrate or its partially reduced intermediate to a specific location on the FeMo cofactor that involves one face of the FeMo cofactor defined by Fe atoms 2, 3, 6, and 7 (Figure 2A). Thus, there is a clear indication that substrates and inhibitors can access a binding site involving one or more Fe atoms, which now raises the important question of whether these results can be extended to N_2 binding.

Theoretical Models for Binding of N_2 to Fe

A number of authors have investigated theoretically the binding of N_2 , N_2H_2 , and N_2H_4 to single-Fe model com-

plexes, or small Fe cluster fragments (82–84). Density functional theory results have been reported for a symmetrized Fe_8S_9 cluster (Fe replacing Mo), but with an excessive enlargement of the central prism when two μ_2 -S–H bonds are introduced (83). The binding of N_2 , H, N_2H species, and CO at Fe sites of a periodic $\text{MoS}_3\text{Fe}_3\text{S}_3\text{Fe}_3\text{S}_3$ -Mo model, together with H binding at S, has been investigated by density functional methods, and energy changes for steps in a possible mechanistic cycle were estimated (85).

Recent density functional calculations have used more complete and realistic FeMo cofactor models, including the central N atom, $^-\text{SCH}_3$ for α -275^{Cys}, imidazole for α -442^{His}, and $^-\text{OCH}_2\text{COO}^-$ for (*R*)-homocitrate (11). Favorable possibilities for the binding of substrates and intermediates to the central Fe atoms have been characterized. At each of these Fe atoms there is an exo coordination position (Figure 4A) located essentially along the extension of the $\text{N}^{\text{central}}-\text{Fe}$ vector, and an endo coordination position (Figure 4B), which is located near an extension of a μ_3 -S–Fe bond. The endo coordination position requires that the adjacent μ_2 -S atom be folded away, which involves only bond bending and has been calculated (11) to be a low-energy adjustment, compensated by the substrate binding energy. The exo binding mode creates an Fe that is approximately trigonal bipyramidal, whereas the endo binding mode results in an

Fe atom that has square pyramidal geometry. It is possible for both exo and endo coordination to occur at the same Fe atom, which would then result in approximately octahedral geometry. A third ligation mode is bridging between central Fe atoms (Figure 4C), which can occur in four quite different ways (Figure 4D–G), suggesting a variety of mechanistic possibilities involving the central Fe atoms (I. G. Dance, unpublished work). An attractive aspect of the proposed participation of the central Fe atoms is the suggestion of a mechanistic role for the central N atom in modulating the coordination at different central Fe atoms during catalysis. In the resting state, the central N atom is overcoordinated with six N–Fe bonds, and when substrates and intermediates bind to Fe, one N–Fe interaction can be substantially elongated and two can be moderately elongated. From this, we envision a “coordinative allosteric” mechanism where substrate (or inhibitor) interaction at one Fe influences, via the central N atom, the coordinative abilities and geometries of the other Fe atoms.

Perspective

From the discussion given above, it is apparent that a consensus view has yet to emerge on whether Fe or Mo is the key player in substrate binding during nitrogenase catalysis. Arguments for both possibilities have been developed from consideration of the catalytic features of different forms of the FeMo cofactor (or alterations in its polypeptide environment), from chemical considerations based on Fe- or Mo-based model compounds, and from theoretical calculations. Each of these approaches has inherent limitations that, so far, have denied any opportunity to support one model while eliminating the alternative possibility. For example, changes in the FeMo cofactor or its polypeptide environment could elicit indirect effects on substrate binding as a result of modifications in electron transfer. A genetic strategy has been described as a way of circumventing this issue (79), and this approach has clearly indicated that alkyne substrates can be reduced at a specific Fe–S face of the FeMo cofactor. Nevertheless, it has not yet been rigorously established that alkyne substrates and N₂ bind at the same site. The elegant system developed by Schrock (48) for the catalytic reduction of N₂ using a Mo model compound also does not provide a clear answer to the question, because there are alternative nitrogenases that have the Mo position of the FeMo cofactor substituted with either V or Fe, and it is difficult to envision that Fe, in particular, would be capable of the same chemistry described in the Schrock scheme. Also, if Fe is capable of such chemistry, then there is no *a priori* reason one or more of the Fe atoms common to the structures of all nitrogenase cofactors would not provide the substrate-binding site. Finally, theoretical calculations use incomplete and sometimes inadequate chemical models and can be biased by assumptions about electronic structure and have accuracy limitations. In this regard, it is emphasized that, although there is general agreement that the atom located within the center of the FeMo cofactor is probably an N atom, this has not been experimentally proven.

How then might this question of Mo versus Fe be resolved? In our view, a satisfactory answer will require a more robust characterization of nitrogenase with substrate-derived intermediates bound to the FeMo cofactor in forms that are tractable to biophysical characterization. Trapping

a substrate-derived catalytic intermediate on nitrogenase is likely to be difficult but probably not intractable. Recent results indicate that it is already possible to detect acetylene (77) and propargyl alcohol-derived (81) intermediates that form a paramagnetic complex with the FeMo cofactor. Paramagnetic intermediates have also been detected during reduction of N₂ (86), although these species have as yet been at very low concentrations. Thus, a major challenge will involve the advancement of ways to prepare nitrogenase samples having an enriched population of the substrate-derived intermediate bound to the FeMo cofactor, perhaps coupled with the use of isotopes (e.g., ^{1/2}H or ^{14/15}N) and advanced spectroscopic methods (e.g., ENDOR) to characterize the bound species. We are particularly encouraged by recent successes in trapping alkyne intermediates under turnover conditions in forms amenable to careful spectroscopic analyses (81). In principle, the same approach involving stabilization of semireduced N₂ intermediates should be possible by amino acid substitution coupled with freeze trapping. In this respect, the development of new relevant model compounds, and calculations that include realistic models for the FeMo cofactor and take into account the surrounding protein, will be essential in understanding the bound intermediates characterized on the FeMo cofactor. The prospects of solving the problem of understanding where and how substrates interact with the nitrogenase active site, while not yet in hand, look promising over the near term.

REFERENCES

1. Smil, V. (2001) *Enriching the Earth: Fritz Haber, Carl Bosch, and the Transformation of World Food Production*, MIT Press, Cambridge, MA.
2. Burgess, B. K., and Lowe, D. J. (1996) The mechanism of molybdenum nitrogenase, *Chem. Rev.* 96, 2983–3011.
3. Rees, D. C., and Howard, J. B. (2000) Nitrogenase: standing at the crossroads, *Curr. Opin. Chem. Biol.* 4, 559–566.
4. Georgiadis, M. M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J. J., and Rees, D. C. (1992) Crystallographic structure of the nitrogenase iron protein from *Azotobacter vinelandii*, *Science* 257, 1653–1659.
5. Seefeldt, L. C., and Dean, D. R. (1997) Role of nucleotides in nitrogenase catalysis, *Acc. Chem. Res.* 30, 260–266.
6. Howard, J. B., and Rees, D. C. (1994) Nitrogenase: A nucleotide-dependent molecular switch, *Annu. Rev. Biochem.* 63, 235–264.
7. Kim, J., and Rees, D. C. (1992) Crystallographic structure and functional implications of the nitrogenase molybdenum iron protein from *Azotobacter vinelandii*, *Nature* 360, 553–560.
8. Schindelin, H., Kisker, C., Schlessman, J. L., Howard, J. B., and Rees, D. C. (1997) Structure of ADP-AlF₄-stabilized nitrogenase complex and its implications for signal transduction, *Nature* 387, 370–376.
9. Chiu, H.-J., Peters, J. W., Lanzilotta, W. N., Ryle, M. J., Seefeldt, L. C., Howard, J. B., and Rees, D. C. (2001) MgATP-bound and nucleotide-free structures of a nitrogenase protein complex between the Leu 127-Delta-Fe-protein and the MoFe-protein, *Biochemistry* 40, 641–650.
10. Einsle, O., Tezcan, F. A., Andrade, S. L. A., Schmid, B., Yoshida, M., Howard, J. B., and Rees, D. C. (2002) Nitrogenase MoFe-protein at 1.16 angstrom resolution: A central ligand in the FeMo-cofactor, *Science* 297, 1696–1700.
11. Dance, I. (2003) The consequences of an interstitial N atom in the FeMo cofactor of nitrogenase, *Chem. Commun.*, 324–325.
12. Lee, H. I., Benton, P. M., Laryukhin, M., Igarashi, R. Y., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2003) The interstitial atom of the nitrogenase FeMo-cofactor: ENDOR and ESEEM show it is not an exchangeable nitrogen, *J. Am. Chem. Soc.* 125, 5604–5605.
13. Lovell, T., Liu, T., Case, D. A., and Noodleman, L. (2003) Structural, spectroscopic, and redox consequences of a central

- ligand in the FeMoco of nitrogenase: A density functional theoretical study, *J. Am. Chem. Soc.* 125, 8377–8383.
14. Hinemann, B., and Norskov, J. K. (2003) Modeling a central ligand in the nitrogenase FeMo cofactor, *J. Am. Chem. Soc.* 125, 1466–1467.
 15. McLean, P. A., and Dixon, R. A. (1981) Requirement of *nifV* gene for production of wild-type nitrogenase enzyme in *Klebsiella pneumoniae*, *Nature* 292, 655–656.
 16. Shah, V. K., and Brill, W. J. (1977) Isolation of an iron–molybdenum cofactor from nitrogenase, *Proc. Natl. Acad. Sci. U.S.A.* 74, 3249–3253.
 17. Jang, S. B., Seefeldt, L. C., and Peters, J. W. (2000) Insights into nucleotide signal transduction in nitrogenase: Structure of an iron protein with MgADP bound, *Biochemistry* 39, 14745–14752.
 18. Mayer, S. M., Lawson, D. M., Gormal, C. A., Roe, S. M., and Smith, B. E. (1999) New insights into structure–function relationships in nitrogenase: A 1.6 Å resolution X-ray crystallographic study of *Klebsiella pneumoniae* MoFe-protein, *J. Mol. Biol.* 292, 871–891.
 19. Thorneley, R. N. F., and Lowe, D. J. (1985) Kinetics and mechanisms of the nitrogenase enzyme system, in *Molybdenum Enzymes* (Spiro, T. G., Ed.) pp 221–284, Wiley, New York.
 20. Kim, J., and Rees, D. C. (1992) Structural models for the metal centers in the nitrogenase molybdenum–iron protein, *Science* 257, 1677–1682.
 21. Kim, J., Woo, D., and Rees, D. C. (1993) X-ray crystal structure of the nitrogenase molybdenum–iron protein from *Clostridium pasteurianum* at 3.0-Å resolution, *Biochemistry* 32, 7104–7115.
 22. Orme-Johnson, W. H., Hamilton, W. D., Jones, T. L., Tso, M. Y. W., Burris, R. H., Shah, V. K., and Brill, W. J. (1972) Electron paramagnetic resonance of nitrogenase and nitrogenase components from *Clostridium pasteurianum* W5 and *Azotobacter vinelandii* OP, *Proc. Natl. Acad. Sci. U.S.A.* 69, 3142–3145.
 23. Lindahl, P. A., Papaefthymiou, V., Orme-Johnson, W. H., and Münck, E. (1988) Mössbauer studies of solid thionin-oxidized MoFe protein of nitrogenase, *J. Biol. Chem.* 263, 19412–19418.
 24. Zimmermann, R., Münck, E., Brill, W. J., Shah, V. K., Henzl, M. T., Rawlings, J., and Orme-Johnson, W. H. (1978) Nitrogenase X: Mössbauer and EPR studies on reversibly oxidized MoFe protein from *Azotobacter vinelandii* OP: Nature of the iron centers, *Biochim. Biophys. Acta* 537, 185–207.
 25. Schultz, F. A., Gheller, S. F., and Newton, W. E. (1988) Iron molybdenum cofactor of nitrogenase: electrochemical determination of the electron stoichiometry of the oxidized semi-reduced couple, *Biochem. Biophys. Res. Commun.* 152, 629–635.
 26. O'Donnell, M. J., and Smith, B. E. (1978) Electron paramagnetic resonance studies on the redox properties of the molybdenum iron protein of nitrogenase between +50 and –450 mV, *Biochem. J.* 173, 831–839.
 27. Huynh, B. H., Henzl, M. T., Christner, J. A., Zimmermann, R., Orme-Johnson, W. H., and Münck, E. (1980) Nitrogenase XII: Mössbauer studies of the MoFe protein from *Clostridium pasteurianum* W5, *Biochim. Biophys. Acta* 623, 124–138.
 28. Yoo, S. J., Angove, H. C., Papaefthymiou, V., Burgess, B. K., and Münck, E. (2000) Mössbauer study of the MoFe protein of nitrogenase from *Azotobacter vinelandii* using selective ⁵⁷Fe enrichment of the M-centers, *J. Am. Chem. Soc.* 122, 4926–4936.
 29. Watt, G. D., Burns, A., Lough, S., and Tennent, D. L. (1980) Redox and spectroscopic properties of oxidized MoFe protein from *Azotobacter vinelandii*, *Biochemistry* 19, 4926–4932.
 30. DeRose, V. J., Kim, C. H., Newton, W. E., Dean, D. R., and Hoffman, B. M. (1995) Electron-spin–echo envelope modulation spectroscopic analysis of altered nitrogenase MoFe proteins from *Azotobacter vinelandii*, *Biochemistry* 34, 2809–2814.
 31. Smith, B. E., Durrant, M. C., Fairhurst, S. A., Gormal, C. A., Gronberg, K. L. C., Henderson, R. A., Ibrahim, S. K., Le Gall, T., and Pickett, C. J. (1999) Exploring the reactivity of the isolated iron–molybdenum cofactor of nitrogenase, *Coord. Chem. Rev.* 185–186, 669–687.
 32. Simpson, F. B., and Burris, R. H. (1984) A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase, *Science* 224, 1095–1097.
 33. Dilworth, M. J. (1966) Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasteurianum*, *Biochim. Biophys. Acta* 127, 285–294.
 34. Davis, L. C., Henzl, M. T., Burris, R. H., and Orme-Johnson, W. H. (1979) Iron–sulfur clusters in the molybdenum–iron protein component of nitrogenase. Electron paramagnetic resonance of the carbon monoxide inhibited state, *Biochemistry* 18, 4860–4869.
 35. Rivera-Ortiz, J. M., and Burris, R. H. (1975) Interactions among substrates and inhibitors of nitrogenase, *J. Bacteriol.* 123, 537–545.
 36. Davis, L. C., Shah, V. K., and Brill, W. J. (1975) Nitrogenase: Effect of component ratio, ATP and H₂ on the distribution of electrons to alternative substrates, *Biochim. Biophys. Acta* 403, 67–78.
 37. Li, J., and Burris, R. H. (1983) Influence of pN₂ and pD₂ on HD formation by various nitrogenases, *Biochemistry* 22, 4472–4480.
 38. Guth, J. H., and Burris, R. H. (1983) Inhibition of nitrogenase catalyzed NH₃ formation by H₂, *Biochemistry* 22, 5111–5122.
 39. Shen, J., Dean, D. R., and Newton, W. E. (1997) Evidence for multiple substrate-reduction sites and distinct inhibitor-binding sites from an altered *Azotobacter vinelandii* nitrogenase MoFe protein, *Biochemistry* 36, 4884–4894.
 40. Kim, C. H., Newton, W. E., and Dean, D. R. (1995) Role of the MoFe protein alpha-subunit histidine-195 residue in FeMo-cofactor binding and nitrogenase catalysis, *Biochemistry* 34, 2798–2808.
 41. Fisher, K., Dilworth, M. J., Kim, C. H., and Newton, W. E. (2000) *Azotobacter vinelandii* nitrogenases containing altered MoFe proteins with substitutions in the FeMo-cofactor environment: effects on the catalyzed reduction of acetylene and ethylene, *Biochemistry* 39, 2970–2979.
 42. Christiansen, J., Seefeldt, L. C., and Dean, D. R. (2000) Competitive substrate and inhibitor interactions at the physiologically relevant active site of nitrogenase, *J. Biol. Chem.* 275, 36104–36107.
 43. Chatt, J., Dilworth, J. R., and Richards, R. L. (1978) Recent advances in the chemistry of nitrogen fixation, *Chem. Rev.* 78, 589–625.
 44. Barriere, F. (2003) Modeling of the molybdenum center in the nitrogenase FeMo-cofactor, *Coord. Chem. Rev.* 236, 71–89.
 45. Pickett, C. J. (1996) The Chatt cycle and the mechanism of enzymic reduction of molecular nitrogen, *J. Biol. Inorg. Chem.* 1, 601–606.
 46. Demadis, K. D., Malinak, S. M., and Coucouvanis, D. (1996) Catalytic reduction of hydrazine to ammonia with MoFe₃S₄-polycarboxylate clusters. Possible relevance regarding the function of the molybdenum-coordinated homocitrate in nitrogenase, *Inorg. Chem.* 35, 4038–4046.
 47. Malinak, S. M., and Coucouvanis, D. (2001) The chemistry of synthetic Fe–Mo–S clusters and their relevance to the structure and function of the Fe–Mo–S center in nitrogenase, *Prog. Inorg. Chem.* 49, 599–662.
 48. Yandulov, D. V., and Schrock, R. R. (2003) Catalytic reduction of dinitrogen to ammonia at a single molybdenum center, *Science* 301, 76–78.
 49. Schrock, R. R. (2003) Catalytic reduction of dinitrogen under mild conditions, *Chem. Commun.*, 2389–2391.
 50. Hoover, T. R., Imperial, J., Ludden, P. W., and Shah, V. K. (1988) Dinitrogenase with altered substrate specificity results from the use of homocitrate analogues for *in vitro* synthesis of the iron–molybdenum cofactor, *Biochemistry* 27, 3647–3652.
 51. Madden, M. S., Paustian, T. D., Ludden, P. W., and Shah, V. K. (1991) Effects of homocitrate, homocitrate lactone, and fluorochomocitrate on nitrogenase in *nifV*- mutants of *Azotobacter vinelandii*, *J. Bacteriol.* 173, 5403–5405.
 52. Hawkes, T. R., McLean, P. A., and Smith, B. E. (1984) Nitrogenase from *nifV* mutants of *Klebsiella pneumoniae* contains an altered form of the iron–molybdenum cofactor, *Biochem. J.* 217, 317–321.
 53. Mayer, S. M., Gormal, C. A., Smith, B. E., and Lawson, D. M. (2002) Crystallographic analysis of the MoFe protein of nitrogenase from a *nifV* mutant of *Klebsiella pneumoniae* identifies citrate as a ligand to the molybdenum of iron molybdenum cofactor (FeMoco), *J. Biol. Chem.* 277, 35263–35266.
 54. Gronberg, K. L. C., Gormal, C. A., Durrant, M. C., Smith, B. E., and Henderson, R. A. (1998) Why R-homocitrate is essential to the reactivity of FeMo-cofactor of nitrogenase: Studies on NifV(–)-extracted FeMo-cofactor, *J. Am. Chem. Soc.* 120, 10613–10621.
 55. Imperial, J., Hoover, T. R., Madden, M. S., Ludden, P. W., and Shah, V. K. (1989) Substrate reduction properties of dinitrogenase activated *in vitro* are dependent upon the presence of homocitrate or its analogues during iron–molybdenum cofactor synthesis, *Biochemistry* 28, 7796–7799.
 56. Scott, D. J., Dean, D. R., and Newton, W. E. (1992) Nitrogenase-catalyzed ethane production and CO-sensitive hydrogen evolution

- from MoFe proteins having amino acid substitutions in an α -subunit FeMo cofactor-binding domain, *J. Biol. Chem.* 267, 20002–20010.
57. Eady, R. R., Robson, R. L., and Smith, B. E. (1988) Alternative and conventional nitrogenases, in *The Nitrogen and Sulfur Cycles* (Cole, J. A., and Ferguson, S., Eds.) pp 363–382, University Press, Cambridge, MA.
58. Eady, R. R. (1996) Structure–function relationships of alternative nitrogenases, *Chem. Rev.* 96, 3013–3030.
59. Durrant, M. C. (2001) A molybdenum-centered model for nitrogenase catalysis, *Inorg. Chem. Commun.* 4, 60–62.
60. Durrant, M. C. (2002) An atomic-level mechanism for molybdenum nitrogenase. Part 1. Reduction of dinitrogen, *Biochemistry* 41, 13934–13945.
61. Durrant, M. C. (2002) An atomic-level mechanism for molybdenum nitrogenase. Part 2. Proton reduction, inhibition of dinitrogen reduction by dihydrogen, and the HD formation reaction, *Biochemistry* 41, 13946–13955.
62. Szilagyi, R. K., Musaev, D. G., and Morokuma, K. (2001) Theoretical studies of biological nitrogen fixation. I. Density functional modeling of the Mo-site of the FeMo-cofactor, *Inorg. Chem.* 40, 766–775.
63. Van der Sluys, L. S., Eckert, J., Eisenstein, O., Hall, J. H., Huffman, J. C., Jackson, S. A., Koetzle, T. F., Kubas, G. J., Vergamini, P. J., and Caulton, K. G. (1990) An attractive cis-effect of hydride on neighbor ligands: experimental and theoretical studies on the structure and intramolecular rearrangements of $\text{Fe}(\text{H})_2(\text{H}_2)(\text{PEtPh}_2)_3$, *J. Am. Chem. Soc.* 112, 4831–4841.
64. Sellmann, D., Soglowek, W., Knoch, F., Ritter, G., and Dengler, J. (1992) Transition-metal complexes with sulfur ligands. 88. Dependence of spin state, structure, and reactivity of $[\text{FeII}(\text{L})-(\text{N}_\text{H})\text{S}_4']$ complexes on the coligand L (L = CO, N_2H_2 , N_2H_4 , NH_3 , pyridine, NHCH_3NH_2 , CH_3OH , THF, $\text{P}(\text{OCH}_3)_3$, $\text{P}(\text{OPh})_3$): model complexes for iron nitrogenases ($\text{NHS}_4'^2$ -dianion of 2,2'-bis[(2-mercaptophenyl)thio]diethylamine), *Inorg. Chem.* 31, 3711–3717.
65. Buys, I. E., Field, L. D., Hambley, T. W., and McQueen, A. E. D. (1993) Structure of bis[1,2-bis(diethylphosphino)ethane-P,P']-hydrido(dinitrogen-N)iron(II) tetraphenylborate, *Acta Crystallogr. C* 49, 1056–1059.
66. Albertin, G., Antoniutti, S., Bordignon, E., and Pattaro, S. (1997) Synthesis, characterization and reactivity of hydrazine complexes of iron(II), *J. Chem. Soc., Dalton Trans.*, 4445–4454.
67. Franke, O., Wiesler, B. E., Lehnert, N., Naether, C., Ksenofontov, V., Neuhausen, J., and Tuczek, F. (2002) Five-coordinate complexes $[\text{FeX}(\text{depe})_2]\text{BPh}_4$, X = Cl, Br: Electronic structure and spin-forbidden reaction with N_2 , *Inorg. Chem.* 41, 3491–3499.
68. Betley, T. A., and Peters, J. C. (2003) Dinitrogen chemistry from trigonally coordinated iron and cobalt platforms, *J. Am. Chem. Soc.* 125, 10782–10783.
69. Lehnert, N., Wiesler, B. E., Tuczek, F., Hennige, A., and Sellmann, D. (1997) Activation of diazene and the nitrogenase problem: an investigation of diazene-bridged Fe(II) centers with sulfur ligand sphere. 1. Electronic structure, *J. Am. Chem. Soc.* 119, 8869–8878.
70. Sellmann, D., Fursattel, A., and Sutter, J. (2000) The nitrogenase catalyzed N_2 dependent HD formation: a model reaction and its significance for the FeMoco function, *Coord. Chem. Rev.* 200–202, 545–561.
71. Bozso, F., Ertl, G., and Weiss, M. (1977) Interaction of nitrogen with iron surfaces, *J. Catal.* 50, 519–529.
72. Dorr, M., Kassbohrer, J., Grunert, R., Kreisel, G., Brand, W. A., Werner, R. A., Geilmann, H., Apfel, C., Robl, C., and Weigand, W. (2003) A possible prebiotic formation of ammonia from dinitrogen on iron sulfide surfaces, *Angew. Chem., Int. Ed., Engl.* 42, 1540–1543.
73. Hwang, J. C., Chen, C. H., and Burris, R. H. (1973) Inhibition of nitrogenase-catalyzed reductions, *Biochim. Biophys. Acta* 292, 256–270.
74. Lee, H. I., Cameron, L. M., Hales, B. J., and Hoffman, B. M. (1997) CO binding to the FeMo cofactor of CO-inhibited nitrogenase: ^{13}C and ^1H Q-band ENDOR investigation, *J. Am. Chem. Soc.* 119, 10121–10126.
75. Christie, P. D., Lee, H. I., Cameron, L. M., Hales, B. J., Orme-Johnson, W. H., and Hoffman, B. M. (1996) Identification of the CO-binding cluster in nitrogenase MoFe protein by ENDOR of ^{57}Fe isotopomers, *J. Am. Chem. Soc.* 118, 8707–8709.
76. Lee, H. I., Sorlie, M., Christiansen, J., Song, R., Dean, D. R., Hales, B. J., and Hoffman, B. M. (2000) Characterization of an intermediate in the reduction of acetylene by nitrogenase α -Gln195 MoFe protein by Q-band EPR and ^{13}C , ^1H ENDOR, *J. Am. Chem. Soc.* 122, 5582–5587.
77. Sørlie, M., Christiansen, J., Dean, D. R., and Hales, B. J. (1999) Detection of a new radical and FeMo-cofactor EPR signal during acetylene reduction by the α -H195Q mutant of nitrogenase, *J. Am. Chem. Soc.* 121, 9457–9458.
78. Ryle, M. J., Lee, H. I., Seefeldt, L. C., and Hoffman, B. M. (2000) Nitrogenase reduction of carbon disulfide: freeze-quench EPR and ENDOR evidence for three sequential intermediates with cluster-bound carbon moieties, *Biochemistry* 39, 1114–1119.
79. Christiansen, J., Cash, V. L., Seefeldt, L. C., and Dean, D. R. (2000) Isolation and characterization of an acetylene-resistant nitrogenase, *J. Biol. Chem.* 275, 11459–11464.
80. Mayer, S. M., Niehaus, W. G., and Dean, D. R. (2002) Reduction of short chain alkynes by a nitrogenase α -70Ala-substituted MoFe protein, *J. Chem. Soc., Dalton Trans.* 5, 802–807.
81. Benton, P. M. C., Laryukhin, M., Mayer, S. M., Hoffman, B. M., Dean, D. R., and Seefeldt, L. C. (2003) Localization of a substrate binding site on FeMo-cofactor in nitrogenase: trapping propargyl alcohol with an α -70-substituted MoFe protein, *Biochemistry* 42, 9102–9109.
82. Deeth, R. J., and Field, C. N. (1994) A computational study of metal-dinitrogen co-ordination, *J. Chem. Soc., Dalton Trans.*, 1943–1948.
83. Siegbahn, P. E. M., Westerberg, J., Svensson, M., and Crabtree, R. H. (1998) Nitrogen fixation by nitrogenases: A quantum chemical study, *J. Phys. Chem. B* 102, 1615–1623.
84. Reiher, M., and Hess, B. A. (2002) A quantum-chemical study of dinitrogen reduction at mononuclear iron–sulfur complexes with hints to the mechanism of nitrogenase, *Chem. Eur. J.* 8, 5332–5339.
85. Rod, T. H., and Norskov, J. K. (2000) Modeling the nitrogenase FeMo cofactor, *J. Am. Chem. Soc.* 122, 12751–12763.
86. Fisher, K., Newton, W. E., and Lowe, D. J. (2001) Electron paramagnetic resonance analysis of different *Azotobacter vinelandii* nitrogenase MoFe-protein conformations generated during enzyme turnover: Evidence for $S = 3/2$ spin states from reduced MoFe-protein intermediates, *Biochemistry* 40, 3333–3339.