Dalton Perspectives

Metals in the Nitrogenases

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In the 1960s and 1970s there was an explosion of chemical and biochemical work on nitrogen fixation.[1] This period saw the first reproducible preparation of cell-free extracts of nitrogen-fixing organisms which were active in catalysing the reduction of dinitrogen,[2] and also the characterization of the first transition-metal dinitrogen complexes.[3] There was great intellectual excitement and the feeling in some quarters, essentially a misunderstanding however, that a source of cheap fertiliser was within our grasp. Now, in the decade of the 1990s, we know an enormous amount about chemical and biological fixation, but we don't yet know how biological fixation works, and we certainly don't have cheap fertiliser. Consequently, interest has to some degree faded, and this is sad because the reality is that chemical and biological nitrogen fixation remain as puzzling, challenging, and intellectually demanding as ever they were, and, some might maintain, even more so.

Nitrogen fixation has been a challenge because it was difficult to explain how microorganisms could convert dinitrogen to ammonia, whereas humans had to invent high-pressure and high-temperature chemical engineering to do it with any facility. Indeed, the great Justus von Liebig[4] proved empirically and apparently conclusively in the 1840s that it was not necessary to posit biological nitrogen fixation in order to explain the accumulation of fixed nitrogen in plants. In this, as in several other matters, he was wrong. The destroyer of the vital force theory of organic compounds could not accept the possibility of biological nitrogen fixation.

Liebig notwithstanding, the reality of biological nitrogen fixation was firmly established empirically by 1888;[5] chemical nitrogen fixation essentially in its current industrial form was developed by Haber and Bosch in the early years of the twentieth century; and in the period 1930–1936 the microbiologist Bortels[6] described how the elements molybdenum, and to a lesser extent vanadium, were necessary to stimulate growth and nitrogen fixation by the soil bacterium *Azotobacter vinelandii*. The role of vanadium was generally ignored, although subsequent physiological studies in the period up to 1950 confirmed Bortels' original observations.[7]

Two discoveries which stimulated intense activity in the chemical and biochemical fields, respectively, were the preparation of the first dinitrogen complex by Allen and Senoff[3] in 1965, and the preparation of cell-free extracts of nitrogenase from the anaerobe *Clostridium pasteurianum* by Carnahan *et al.*[2] in 1960. Subsequent work rapidly revealed that, irrespective of the type of nitrogen-fixing organism from which they are isolated, nitrogenases are two-protein component enzymes which all have similar properties and requirements for activity. Among these are the absence of dioxygen, a source of low-potential reducing equivalents (with a mid-point potential of *ca.* −450 mV), and MgATP (ATP = adenosine 5'-triphosphate).[8]

In the 1970s the genetics of nitrogen fixation were established[9] and spectacular progress was made in characterizing the genome responsible for diazotrophy, initially in *Klebsiella pneumoniae* and later in other organisms. *K. pneumoniae* was a fortunate choice, because the *ca.* 20 *nif** genes are all clustered together on the chromosome and, although not recognized at the time, this organism only has a Mo-containing nitrogenase. These factors facilitated analysis and enabled rapid identification of the genes which encode the polypeptides of nitrogenase. It was quickly established that nitrogenase is a highly conserved enzyme among a wide range of nitrogen-fixing organisms. Subsequently, it was shown[9] that the organization of *nif* genes is more complex in organisms other than *K. pneumoniae*, and some were discovered to have additional genes which enable them to synthesize nitrogenases which, as discussed below, do not use molybdenum.

Biochemical studies up to 1985 had shown all Mo-nitrogenases to be two-component enzymes, consisting of a Fe protein which functioned as an ATP-dependent electron donor to a MoFe protein, which contained an Fe- and Mo-containing cofactor centre (FeMoco), the probable site of substrate binding and reduction. Studies of the genes required for FeMoco biosynthesis and insertion into the MoFe protein[8,9] revealed that they are part of the *nif* gene cluster, and that a surprising number of them are involved (*nif H*, *B*, *E*, *N*, *V* and *Q*).† The juxtaposition of these genes with the structural genes for the enzyme, and the fact that all the nitrogenases which had been characterized contained molybdenum in a FeMoco centre, led to the dogmatic view that molybdenum had a unique and essential role in nitrogenase function. This proposal fitted well with the parallel developments which had occurred in the characterization of chemical systems capable of reducing dinitrogen.[10]

It was a 1980 study of the genetics and physiology of diazotrophic growth of *A. vinelandii* (the organism studied by Bortels some 50 years earlier) which changed this view of the unique role of molybdenum.[11] The experimental observations were simple—some mutant strains which had lost the ability to use dinitrogen as a source of nitrogen for growth surprisingly regained this ability when cultured on media lacking molybdenum. It was not until 1986, when the powerful techniques of molecular biology were applied to remove the structural genes encoding Mo-nitrogenase (*nif HDK*), that the reality of a Mo-independent route for nitrogen fixation was established beyond question.[12] This relied on vanadium rather than molybdenum. Subsequently, V-nitrogenases were purified from *A. vinelandii*[13] and from a strain of the closely related organism, *Azotobacter chroococcum*, lacking the structural genes of Mo-nitrogenase.[14,15] An even more surprising development in 1988 was when a third nitrogen-fixing system which apparently lacks molybdenum or vanadium was purified from *A. vinelandii*.[16] It should be emphasized that the three systems, although related, are genetically distinct, and do not arise simply by the substitution of different metals into the same apoenzyme. The

* *nif* genes are the genes responsible for Mo-dependent nitrogen fixation.
† These letters are used internationally as a trivial nomenclature for specific genes involved in Mo-nitrogenase.

VFe and FeFe proteins, analogues of the MoFe protein, are distinct but similar.

These were shattering developments. They defined a new enzymic role for vanadium, which previously had only been shown to be involved in a single class of enzyme, the bromoperoxidases of some sea algae.[17] They also demolished the dogma of the essential role for molybdenum in biological nitrogen fixation.

Chemical research was stimulated by these findings and responded rapidly, extending the plausible chemistry which had been developed for nitrogen fixation on molybdenum, first to vanadium,[18,19] and more recently iron.[20]

Finally at the end of 1992 the long-awaited detailed X-ray crystal structures of the Fe and MoFe proteins of *A. vinelandii* were published by Kim and Rees.[21] These revealed the structure of the polypeptide chains of both proteins, and the locations of their redox centres, and also generated models of these centres consistent with other spectroscopic and structural data.

Instead of clarifying the nature of the active site, as had generally been expected it would, the model proposed for the substrate-binding site (FeMoco), raised a whole host of chemical and biochemical questions, but in particular, the identity of the atoms at which substrate binding and reduction occurs. The 'active site' has faded like a mirage the closer we appear to be approaching it.

The aim of this Perspective is to explain how and why this has happened, with particular emphasis on the identification and function of the active sites of the different nitrogenases. In order to restrict the large numbers of references which this would otherwise entail, the reader is guided to the many excellent review articles, as appropriate, and only the most recent work is referred to as primary references.

The Structure and Function of Nitrogenases

Molybdenum-containing nitrogenases which have been studied in most detail are the enzymes from *K. pneumoniae*, *Clostridium pasteurianum* and *A. vinelandii* a facultative anaerobe, an obligate anaerobe, and an obligate aerobe, respectively. (For reviews of this area see refs. 8 and 22–24.)

The Iron Proteins of Nitrogenase.—Each of the nitrogenase systems discussed in this Perspective has a specific genetically distinct Fe protein associated with it. These proteins function as ATP-dependent electron donors to MoFe-, VFe- or FeFe-proteins.

The DNA sequences of the genes of all known Fe proteins are highly conserved and so the physicochemical properties of these proteins should be similar. In so far as biochemical data are available, this expectation is confirmed to the extent that in most instances the Fe protein from one organism (or type of nitrogenase) will form a functional hybrid nitrogenase when combined with MoFe or VFe protein. This being so, and since most information is available for the Fe proteins associated with Mo-nitrogenase, the properties of a typical Fe protein associated with Mo-nitrogenase will be considered here.

Iron proteins are dimers (M_r *ca.* 64 000) of two identical subunits and are extremely sensitive to irreversible inactivation by dioxygen. They bind two molecules of ATP or ADP (adenosine 5′-diphosphate) and contain a single 4Fe4S centre. Chemical modification experiments suggested the FeS centre to be bound in the interface between the subunits, cysteine residues Cys-97 and Cys-132 from each subunit ligating the cluster. The cluster functions as a one-electron donor operating between the 1+ and 2+ cluster oxidation levels, typically with a mid-point potential of −300 mV. The binding of MgADP or MgATP results in a conformational change of the protein and a decrease in the mid-point potential of the cluster by *ca.* 100 mV. The recent 2.9 Å resolution X-ray crystal structure of the Fe protein from *A. vinelandii*[25] showed each of the subunits to be folded in a single αβ domain and confirmed the location of the cluster at the subunit interface. It also showed that the cluster is symmetrically co-ordinated by two cysteines from each subunit and that it is located at one end of the molecule. The cluster is exposed to the solvent to a much greater extent than is found in the ferredoxins, and this may account for the extreme sensitivity of the Fe proteins to inactivation by dioxygen. The crystal showed partial occupancy of the two likely ATP/ADP binding sites by ADP in the cleft formed between the two subunits, some 20 Å from the 4Fe4S centre. Such a separation implies that the decrease in redox potential of the cluster consequent on ATP or ADP binding is mediated by a conformational change in the protein. This suggestion is futher supported by the finding that the secondary structure around the nucleotide binding site shows strong homology with a human protein designated $p21^{ras}$, which couples the hydrolysis of the nucleotide guanosine triphosphate to drive a reversible switch between two conformational states of this protein. It has been proposed that both nucleotide-binding proteins utilize a common signal-transducing system triggered by nucleotide binding and hydrolysis.

Molybdenum–Iron Protein Structure and Function.—The MoFe proteins are complex proteins that are tetramers ($\alpha_2\beta_2$) which contain (in the preparations of highest activity) 2 Mo, 30–34 Fe atoms and 30 acid-labile S atoms. The arrangement of these atoms and the types of redox centre present in MoFe proteins were subject to extensive spectroscopic and chemical studies during the 1970s and 1980s (see ref. 22). Such studies showed that these proteins contain two types of metal–sulfur cluster which are unique to nitrogenase, the P clusters which are FeS clusters with unusual spectroscopic properties, and FeMoco, an Fe- and Mo-containing cofactor.

Four years ago Bolin *et al.*[26] reported the first low-resolution X-ray crystallographic data for any MoFe protein which showed that in Cp1* the P clusters and the FeMoco centres were some 19 Å apart and that the two cofactor centres themselves are 70 Å apart. This ruled out a bridging of N_2 between two Mo atoms during dinitrogen reduction. The P clusters are also too large to be isolated 4Fe4S clusters as was then the consensus (see ref. 22). A subsequent 2.7 Å resolution X-ray crystal structure of Av1 confirmed this separation of the redox centres and allowed structural models of P clusters and FeMoco to be developed.[21] Since at this resolution individual atoms in a crystal-structure determination are often not resolved, the identities were inferred from the wealth of available spectroscopic data, also utilizing information derived from directed mutagenesis to identify amino acid residues implicated in binding the redox centres in MoFe proteins. The structure of P clusters and the FeMoco centres in Av1 and Cp1 have now been refined to 2.2 Å, a resolution which allows a definitive assignment to most of the atoms in these centres.[27,28]

P Clusters.—The P clusters can be extruded from the MoFe protein as 4Fe4S clusters by treatment with thiophenol under denaturing conditions, as has been demonstrated for a number of simple FeS proteins, but the spectroscopic properties of these clusters in the MoFe protein have no counterpart in other FeS proteins studied. Directed mutagenesis studies of Kp1 and Av1 implicated a number of conserved cysteine residues in the binding of these clusters. For many years the consensus was that the MoFe protein contained four unusual 4Fe4S clusters. However, in 1987 EPR signals arising from P centres of dye-oxidized MoFe protein were detected, their redox properties being consistent with them arising from larger centres containing 6–8 Fe atoms.[29]

* Nitrogenase proteins are represented by abbreviations indicating the organism from which they are isolated (Kp1, Cp1, Rc1, Ac1 and Av1 represent the MoFe proteins from *Klebsiella pneumoniae*, *Clostridium pasteurianum Rhodobacter capsulatus*, *Azotobacter chroococcum and Azotobacter vinelandii*, respectively).

The X-ray crystal structure of these MoFe proteins shows that the P clusters are located at the α/β subunit interface and ligated by cysteines from both subunits, a structure not known in other enzymes (see Fig. 1). The P clusters are two 4Fe4S clusters linked by bridging cysteine thiolate groups and may possess an S–S link or a common S atom. In the structure of Cp1 at 3 Å resolution,[30] the metal clusters appear to be identical to those of Av1. However, at 2.2 Å resolution,[29] only the two FeMoco structures are similar, whereas the P clusters are not. It has been suggested that the bridging S–S bond in the P clusters observed in a 2.2 Å resolution structure of Av1, may be a single shared S atom in these clusters in Cp1. What these differences may signify remains to be resolved.

The bridged double-cubane structure of the P clusters, and the possible structural variant of a shared S atom, readily account for their unusual spectral and redox properties. These centres have recently been shown to undergo oxidation during the reduction of dinitrogen, providing experimental support[31] for their long-suggested role as electron capacitors.

FeMoco.—As isolated in the dithionite-reduced state, MoFe proteins are characterized by an EPR signal arising from an $S = \frac{3}{2}$ spin system. The effect of ^{57}Fe and ^{95}Mo on the broadening of the EPR signal indicates that the unpaired electron does not reside on the Mo atom for a significant time. This EPR signal, with g values close to 4.3, 3.7 and 2.01, is a sensitive spectroscopic fingerprint of FeMoco. Mössbauer spectroscopy indicated that approximately half the 30–34 Fe atoms present in the MoFe protein are associated with this centre and the remainder are associated with the P clusters.

Mössbauer and EPR studies of the enzyme during enzyme turnover have shown that the FeMoco centre becomes further reduced following the ATP-dependent electron transfer from the Fe protein.

In 1977 a seminal advance was made[32] with the extraction from the MoFe protein of the molybdenum and approximately half the iron into *N*-methylformamide (nmf). The nmf extract was capable of activating the cofactor-free MoFe protein synthesized by mutants of *K. pneumoniae* and *A. vinelandii* which were themselves unable to synthesize FeMoco and produce the complete protein. Extracted FeMoco, which contains Fe, S^{2-} and Mo with a general composition of Mo: Fe_{6-8}: S_{4-10}, is extremely sensitive to inactivation by dioxygen.

The expectation at this time was that the chemical synthesis of FeMoco and the determination of its structure separate from the protein would be rapidly achieved. Despite considerable efforts worldwide, 16 years later neither of these objectives has been met.

During this period it was established that FeMoco has an organic constituent, homocitrate. In addition, changes in the structure of FeMoco by mutation of *nif V*, which results in citrate rather than homocitrate [(*R*)-2-hydroxybutane-1,2,4-tricarboxylic acid] being incorporated into FeMoco, alter the substrate specificity of the mutated or reconstituted MoFe protein. It also became clear that the ability to reduce dinitrogen is lost on mutation of amino acids located close to FeMoco, whereas C_2H_2 and protons remained effective substrates (see ref. 22).

The chemical environment of molybdenum both in the intact MoFe protein and in extracted FeMoco studied by Mo EXAFS (extended X-ray absorption fine structure) spectroscopy is consistent with an environment of Fe, S and C (or N) which is essentially unchanged by extraction into nmf. When FeMoco from a mutant strain with an altered substrate specificity was extracted and recombined with cofactor-free MoFe protein the altered specificity was transferred with the cofactor (see ref. 22). These data strongly imply that FeMoco is the active site of nitrogenase, and, reassuringly, that this site contains molybdenum.

The X-ray crystal structures of the MoFe proteins show that the two FeMoco centres are located within the α subunits of the $\alpha_2\beta_2$ tetramer some 20 Å from the P clusters. At 2.2 Å resolution, the Av1 and Cp1 structures[27,28] refine and largely confirm the model of Kim and Rees[21] that FeMoco consists of two clusters of stoichiometry Fe_4S_3 and $MoFe_3S_3$ that are bridged by three sulfides, one of which was not assigned in the earlier model[22] (Fig. 2). The Fe–Fe distances between bridged iron sites average *ca.* 2.5 Å, close enough to co-ordinate *via* metal–metal bonds to these otherwise trigonal, and therefore unusual, Fe atoms. The Mo atom is at one end of the cluster and is co-ordinated to homocitrate by hydroxide and carboxylate oxygen atoms. Two protein amino acid residues are bound to the FeMoco centre, His-442 to Mo and Cys-275 to Fe(1), at the other end of the cluster.

Despite the compelling evidence that FeMoco forms the active site of Mo-nitrogenase, Mo EXAFS studies on the protein during enzyme turnover in the steady-state indicated little or no change in the co-ordination of the Mo atom and certainly provided no evidence for the binding of N_2 to molybdenum (see refs. 8 and 10). Similar EXAFS studies on V-nitrogenase also showed little change in co-ordination of the V atom during turnover. The isolation of the third nitrogenase which may contain only iron in the cofactor raises the possibility that N_2 binding and reduction may occur on an Fe atom in all three nitrogenases. Possible modes of binding of N_2 to FeMoco are discussed below.

Biochemistry of FeMoco Synthesis.—Little is known about the details of the biosynthesis of the two types of redox centre that the MoFe proteins contain. The involvement of *nif S*, which has very recently (see ref. 23) been shown to encode a cysteine sulfur transferase, appears to be essential for the synthesis of the 4Fe4S centre of the Fe protein, and probably for the P centres of the MoFe protein. Although intuitively one might expect FeS clusters to assemble spontaneously, in biological systems this process, and their insertion into proteins, appears to be controlled enzymically.

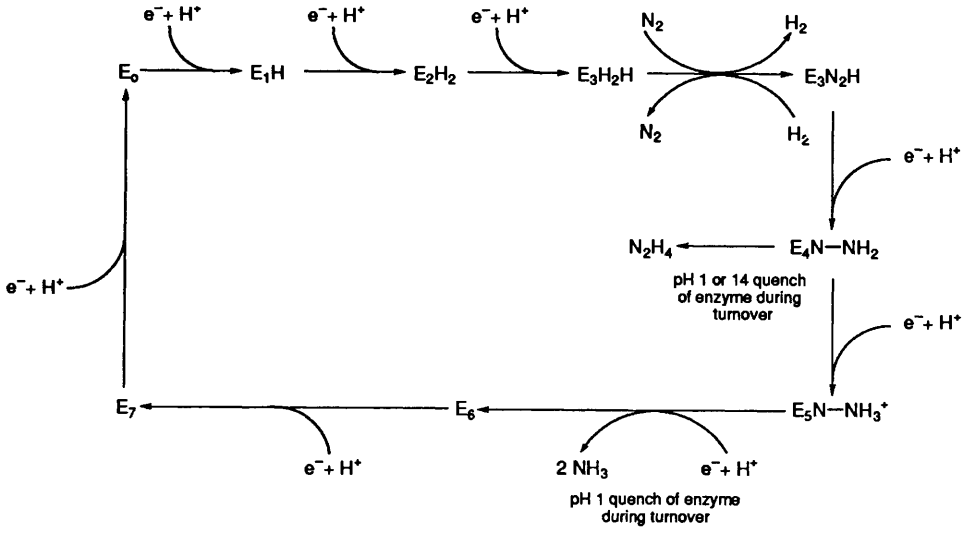
A number of *nif* genes (*nif Q, B, E, N, V* and *H*) have been shown to be essential for FeMoco synthesis and insertion into the cofactor-free MoFe protein, although the details of their involvement are not understood. It is clear that cofactor-free MoFe protein is not the template on which FeMoco is synthesized since some mutants lacking the structural genes of MoFe protein can nevertheless synthesize FeMoco. It had been suggested on the basis of the strong sequence homology of *nif EN* with *nif DK* that the template is the $\alpha_2\beta_2$ tetrameric protein formed by the products of the *nif EN* genes. The role of *nif V* in the nitrogen-fixation process is the clearest since it probably encodes a homocitrate synthase, and homocitrate is now known to be a ligand to the Mo atom of FeMoco and is extracted with it into nmf. It appears that *nif Q* is involved in the uptake or processing of molybdenum.

The ATP-dependent incorporation of molybdenum into FeMoco in crude extra-cellular extracts has allowed the effect of carboxylic acids other than homocitrate or its isomers on the nitrogen-fixing ability of the reconstituted MoFe protein to be tested (see ref. 33). These studies showed that the specific stereochemistry around C(3) of homocitrate is essential for the reduction of N_2, and that though other carboxylic acids may be effective partially in supporting nitrogen-fixing activity, none is as effective as homocitrate.

Substrate Reduction by Molybdenum-nitrogenase.—Under optimal conditions, the reaction (1) in which Pi indicates inorganic phosphate is that catalysed by Mo-nitrogenase.

$$N_2 + 8\,e + 8\,H^+ + 16\,ATP \longrightarrow 16\,ADP + 16\,Pi + 2\,NH_3 + H_2 \quad (1)$$

During the reduction of N_2, a dinitrogen–hydride intermediate is believed to be generated, which produces N_2H_4

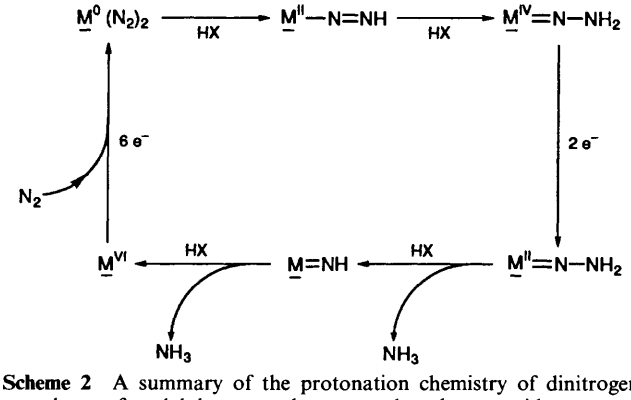


Scheme 1 The catalytic cycle for the reduction of N_2 by the Mo-nitrogenase of *K. pneumoniae*. The resting state of the enzyme as isolated is represented by E_0; the later subscripts indicate the number of times the MoFe protein has been reduced by the Fe protein in an ATP-dependent reaction and the arrows between each state represent complex formation, electron transfer and protonation, followed by protein dissociation—the rate-limiting step in the overall reaction. The side-reaction at E_4 and E_5 show where enzyme-bound intermediates give rise to the products shown when the reaction is quenched at the pH values indicated. Dinitrogen binds to the species E_3 accounting for the stoichiometry of equation (1). The designation of structures such as E_3H_3 does not imply the existence of metal–hydride species as intermediates or the precise level of protonation of a metal site (see ref. 34 for a full description of this scheme)

upon quenching the reaction mixture in acid or alkali. In the most successful kinetic model for the eight-electron reduction of equation (1), there is a successive MgATP-dependent transfer of eight electrons from the Fe protein to the MoFe protein, in an electron-transfer complex of the two proteins.[34] After each electron transfer the two proteins dissociate, in what is the rate-limiting step in the overall reaction. The free Fe protein–MgADP species is subsequently reduced, MgADP dissociates and is replaced by MgATP, and the Fe protein–MgATP species reassociates with the MoFe protein for the next electron transfer. In the absence of an added reducible substrate, as when the enzyme functions under argon, protons are reduced to dihydrogen. A number of small molecules with multiple bonds are also reducible, the most important being C_2H_2 which is reduced to C_2H_4.

An extensive kinetic analysis of the pre-steady-state rates of electron transfer, intermediate generation and product formation has led to the development of the Lowe–Thorneley scheme for nitrogenase function (Scheme 1).[34] The protonation of N_2 in several molybdenum and tungsten dinitrogen complexes has been shown to yield ammonia in a series of well defined steps which fit exceedingly well with this kinetic model (Scheme 2). In this scheme the dinitrogen is assumed to bind end-on at one end only to the metal atom at the active site. The protonation then proceeds with electron flow from the metal to the dinitrogen as protons are delivered from solution. The Lowe–Thorneley scheme is the most comprehensive and predictive yet proposed. The stoichiometry of reaction (1), which involves eight rather than the expected six electrons, is accounted for by the initial formation of a hydride species at the substrate-binding site of FeMoco from which dihydrogen is displaced when dinitrogen binds. This mode of binding provides a rationale for the specific competitive nature of the inhibition of N_2 reduction by H_2, whereas the reduction of other substrates by nitrogenase is not inhibited by H_2.

Hydride ligands at the active site have also been invoked to explain dihydrogen evolution by nitrogenase and also the formation of HD when N_2 is reduced in the presence of some D_2. Although the presence of enzyme-bound hydride intermediates is consistent with the Lowe–Thorneley scheme, physical evidence for them is lacking. However, very recent work has demonstrated that a carboxylate can function as a



Scheme 2 A summary of the protonation chemistry of dinitrogen complexes of molybdenum and tungsten based on a wide range of chemical, electrochemical and kinetic researches. Species characteristic of all these stages except M^{VI} have been isolated and characterized. In a typical case $\underline{M}$ = $M(Ph_2PCH_2CH_2PPh_2)_2$, X = Cl and M = Mo or W. The stoichiometric displacement of H_2 by N_2 is not specifically indicated here, but there are several examples known

leaving group on reduction of the molybdenum(IV) dihydride centre in $[MoH_2(O_2CMe)(dppe)_2]^+$ [dppe = 1,2-bis(diphenylphosphino)ethane], exposing a site at which N_2 (and CO or C_2H_2) can bind.[35] Under dinitrogen, $[MoH_2(N_2)(dppe)_2]$ is formed, and this, in turn, slowly loses H_2. Thus binding of N_2 precedes the loss of H_2, rather than being a consequence, as usually assumed. These are exciting developments. The authors emphasize the clear analogy with the homocitrate in FeMoco, which is a tethered carboxylate that may function as a leaving group from the Mo atom during biological nitrogen fixation.

Regulation of Expression of the Molybdenum-independent Nitrogenase Systems by Molybdenum and Vanadium.—Studies in *Azotobacter* have shown that low levels of molybdenum (2 nmol dm^{-3}) in the growth medium repress V- and Fe-nitrogenase by preventing the transcription of their structural genes. Consequently, in the presence of sufficient molybdenum, only the Mo-nitrogenase is synthesized. The discovery of Mo-independent nitrogenases was delayed many years by this control mechanism. Molybdenum had been routinely included

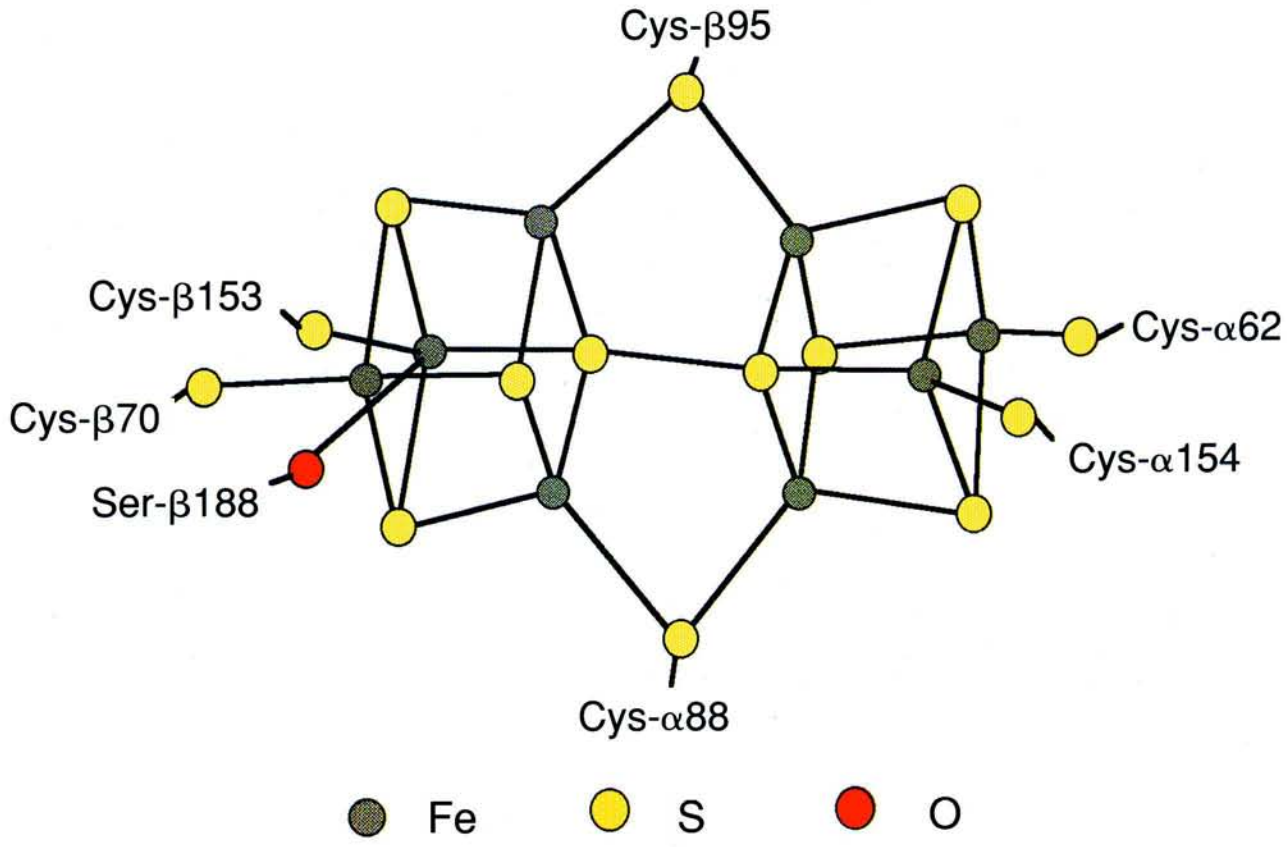


Fig. 1 Schematic representation of a P cluster of Av1[29]

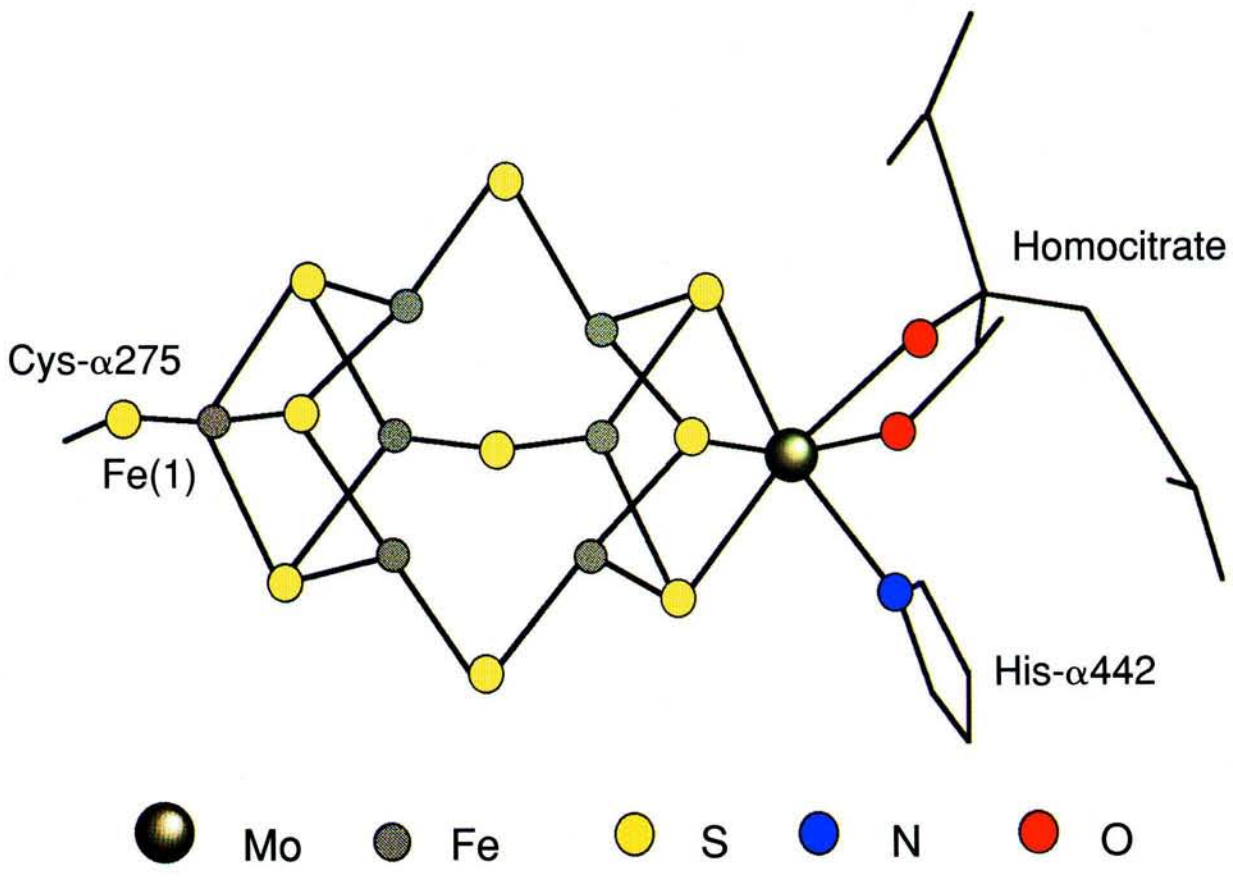


Fig. 2 Schematic representation of the FeMoco cluster of Av1.[29] The cysteine and histidine residues indicated are directly involved in the binding of the cluster to the protein. Figs. 1 and 2 were generated from atomic coordinates kindly supplied by Professor D. C. Rees

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in growth media for nitrogen-fixing bacteria, because of Bortels' demonstration of the molybdenum requirement.

In the absence of molybdenum, and in the presence of vanadium, the V-nitrogenase is expressed but the Fe-nitrogenase is not. The synthesis of Fe-nitrogenase occurs in the absence of both molybdenum and vanadium. How this regulation occurs is not understood in detail, but the expression of Mo-independent nitrogenases in other organisms also appears to be controlled by the availability of vanadium and molybdenum.[36]

Genes Involved in Both Molybdenum and Molybdenum-independent Nitrogenases.—Some *nif* genes are required not only for the activity of the Mo-nitrogenase but also for the function of the Mo-independent nitrogenases. These are, *nif V*, *nif B*, *nif S* and *nif W*. The requirement for *nif B* and *nif V* suggests that similarities in the structures of FeVco (the vanadium analogue of FeMoco) and of the putative FeFe cofactor will be found. Specifically, the requirement of *nif V* implies that homocitrate is a likely component.

Vanadium–Iron Protein Structure and Function.—The structural genes of V-nitrogenase from *A. vinelandii*, *A. chroococcum* and the cyanobacterium *Anabaena variabilis* have been cloned and sequenced.[10,37] The VFe proteins have been purified and characterized from *A. vinelandii*[14] and *A. chroococcum*.[16] The proteins have α and β subunits with amino acid sequences homologous to those of the MoFe protein. In particular, the amino acids known to ligate the P clusters and FeMoco in the MoFe proteins are conserved. However, these nitrogenase proteins also contain an additional small δ subunit which mutational analysis has shown to be essential for activity. No function has been assigned to this subunit, which in *A. variabilis* is fused with the α subunit.[37]

The purified proteins have properties very similar to those of the molybdenum systems and contain vanadium, iron and acid-labile sulfide, but they generally have lower metal contents than MoFe proteins (see Table 1). Mössbauer, EPR and MCD spectroscopies show that clusters analogous to P centres are present,[24] and EXAFS and extrusion studies demonstrate that vanadium is present in a cofactor centre (FeVco) in a chemical environment very similar to that of molybdenum in FeMoco. The EPR spectrum associated with the dithionite-reduced VFe protein has been assigned to an $S = \frac{3}{2}$ spin system arising from FeVco, but is quite distinct from that of FeMoco. Extracted FeVco is capable of activating cofactor-free MoFe protein to reduce C_2H_2 and protons, but N_2 is not reduced. This differential reconstitution of activities was the first indication that the requirements for N_2 reduction by nitrogenase are more stringent than those for the reduction of substrate analogues (see ref. 22).

Substrate Reduction by Vanadium Nitrogenase.—The rate of electron transfer from Fe protein to VFe protein and its ATP dependence are very similar to those of Mo-nitrogenase. However, the substrate specificity of V-nitrogenase differs from that of Mo-nitrogenase, in that C_2H_2 is reduced to C_2H_6 in addition to C_2H_4, and that N_2 does not compete with protons as effectively as is the case with Mo-nitrogenase (see ref. 10). The limiting stoichiometry *in vitro* is as shown in equation (2). Since

$$N_2 + 12\,e + 12\,H^+ = 2\,NH_3 + 3\,H_2 \qquad (2)$$

this is also the effective stoichiometry of substrate reduction by cultures of *A. vinelandii*, it does not arise from the use of sub-optimum assay conditions. In addition N_2H_4 is a minor *product* of dinitrogen reduction by this nitrogenase.[41] Nevertheless, V-nitrogenase is more effective in reducing N_2 at lower temperatures, say 10 °C, than Mo-nitrogenase. This may account for the retention in Nature of what appears at first sight to be a less effective system for supporting growth on N_2. As yet there are no reports on the relative abundances of organisms using V- or Mo-nitrogenase in environments where this property might confer a selective advantage.

Iron–Iron Protein Structure and Function.—The structural genes of Fe-nitrogenase have been cloned from *A. vinelandii*[36] and *Rhodobacter capsulatus*[42] and sequenced. The amino acid sequences are homologous to those of MoFe proteins, with the residues known to ligate P clusters and FeMoco in the MoFe proteins conserved, as with VFe proteins. As with V-nitrogenase, a gene encoding an additional small δ subunit is also present.

However, in biochemical terms the Fe-nitrogenase is the system about which we know the least. The evidence that this system is capable of supporting growth on N_2 is very strong. Strains of *A. vinelandii*, and *R. capsulatus* with deletions of the structural genes of Mo-nitrogenase and, in the case of *A.*

Table 1 Comparison of the physicochemical properties of MoFe, VFe and FeFe proteins

Property	Kp1	$Ac1^V$	$Av1^V$	$Av1^{Fe}$	$Av1^{Fe}$ containing FeMoco	$Rc1^{Fe}$
Native M_r	225 000	240 000	240 000	249 800	249 800	*246 400
Subunit structure	$\alpha_2\beta_2$	$\alpha_2\beta_2\delta_2$	$\alpha_2\beta_2\delta_2$	$\alpha_2\beta_2\delta_2$	$\alpha_2\beta_2\delta_2$	n.d.
Subunit M_r	2 × 54 100	2 × 53 800	2 × 53 800	2 × 58 400	2 × 58 400	59 000
	2 × 58 300	2 × 52 700	2 × 52 700	2 × 51 200	2 × 51 200	50 700
		2 × 13 300	2 × 13 300	2 × 15 300	2 × 15 300	13 400
Metal and S^2 content/atom mol^{-1}						
V	0.06	2 ± 0.3	0.7 ± 0.3	0.01	0.05	0.02
Mo	2	0.06	0.05	0.085	1.07	0.21
Fe	32 ± 3	21 ± 1	9 ± 2	24	24.2	20
S^{2-}	n.d.	19 ± 0.2	21 ± 1	18	17.9	n.d.
EPR g values for dithionite-reduced protein	4.32, 3.73, 2.018	5.6, 4.35, 3.77, 1.93	5.31, 4.34, 2.01, 1.93	n.d.	4.3, 3.66, 2.01	5.44, 1.93
Specific activity/nmol product formed min^{-1} mg^{-1} of protein						
NH_3 formation	990	350	660	38	110	n.d.
H_2 evolution under N_2	648	928	n.d.	213	220	n.d.
C_2H_4 from C_2H_2	1693	608	220	28	58	5.3
H_2 evolution under C_2H_2	304	998	n.d.	202	226	n.d.
H_2 evolution under Ar	2100	1348	1400	253	350	n.d.
Ref.	10	14	16	17	38	39, 40

n.d. = Not determined. * M_r based on gene size.

vinelandii, the genes for V-nitrogenase as well, retain the ability to grow on N_2 in Mo-deficient media (see refs. 36 and 39). Nevertheless, purified preparations of the components of this system have low catalytic activity. Typical activities determined for FeFe protein reducing C_2H_2 are 2% those of the MoFe protein. With N_2 as reducible substrate the only available data are for the *A. vinelandii* protein, and a value of 4% has been reported (Table 1). Since FeFe proteins contain levels of molybdenum ranging from 0.21 to 0.05 atom mol^{-1} of protein for the proteins from *R. capsulatus* and *A. vinelandii*, respectively, the activity may be accounted for if a low level of FeMoco were present. Alternatively, it may be that the conditions under which the activity was measured were not optimal, or that a factor necessary for the reduction of N_2 has been lost during purification. An EPR study of the enzyme from *R. capsulatus* showed no signal associated with FeMoco, but instead the presence of a fully rhombic $S = \frac{3}{2}$ spin system, assigned to a FeFeco species.[40] This latter signal has not been detected in preparations of the FeFe protein isolated from *R. rubrum*.[43]

The low activity of these preparations remains the most urgent problem in this area, since the inference that biological N_2 fixation can occur at a site containing only iron derivatives from the ability of molybdenum and vanadium to repress the synthesis of this system and the fact that purified FeFe protein contains very low amounts of vanadium and molybdenum.

Comparison of the DNA sequences of all three nitrogenases, suggests that the FeFe protein contains a putative cofactor-binding site. Direct evidence for this has been provided by the isolation[43] of a species of the FeFe protein containing FeMoco, purified from a double-deletion strain of *A. vinelandii*, which had been mutated so as to be capable of expressing Fe-nitrogenase in the presence of molybdenum. The purified FeFe protein from this strain contained one FeMoco centre and N_2 was an effective substrate, with a specific activity 15% that of the MoFe proteins (Table 1). In addition, the growth of this strain in metal-free media was stimulated by molybdenum when added at low concentrations (5–20 nmol dm^{-3}). Clearly FeMoco can function in the reduction of N_2 *in vivo* when it is present in the Fe-nitrogenase of *Azotobacter*. A similar hybrid FeFe protein containing FeMoco has been described for *R. capsulatus*, but no activity data were reported.[44] These observations are frustrating since they do nothing to provide an effective biochemical underpinning of the chemical research on the ability of iron sites to reduce N_2 effectively.

In terms of the model structure for FeMoco derived from X-ray studies of the MoFe protein, binding and reduction at an iron site is in no way excluded. In this aspect, the model chemistry of dinitrogen reduction on iron, research stimulated by the discovery of the third nitrogenase, has outpaced the enzymology. The resolution of this problem requires a more rigorous approach to the characterization of the catalytic properties of the FeFe proteins and the redox centres which they contain, in order to establish whether current preparations of these proteins contain a cofactor. The nature of the metals involved at the active site in the third nitrogenase (as in the other two) remains an open question.

Chemical Models for Nitrogenase Function

The three distinct kinds of nitrogenase have given rise to a widely accepted dogma that the active sites should consist principally of molybdenum in the 'classical' Mo-nitrogenase, vanadium in V-nitrogenase, and iron in the third Fe-nitrogenase. There is no conclusive evidence for this since direct proof of interaction of dinitrogen with molybdenum in Mo-nitrogenase has not been obtained, but for almost 30 years the reasonable hypothesis that molybdenum is at the active site of the classical nitrogenase went almost unchallenged.

The idea that dinitrogen complexes should form had been extant for many years[45] before the first announcement of such a complex in 1965.[3] Indeed, it is very likely that the first reported dinitrogen complex was prepared some 20 years before it was ultimately recognized.[46] However, relevant chemical research into nitrogen fixation started a little earlier, in 1964.[47] The ability of a mixture of a transition-metal halide and a strong reducing agent such as an alkyl lithium in anhydrous dioxygen-free solvent to react with dinitrogen (and to yield ammonia upon subsequent hydrolysis) must have been evident to polymer chemists working on Ziegler–Natta catalysis long before 1963.* It is surprising that such observations were not followed up.

After 1965 new chemical nitrogen-fixing systems and new dinitrogen complexes were quickly discovered. The fixing systems fell into two classes. In the first were dinitrogen complexes, most of which contained dinitrogen bound end-on at one end, as might be expected from the structures of comparable carbonyl compounds. Rather unexpectedly, the first complexes showed no reactivity of co-ordinated dinitrogen apart from its replacement. In 1972, the acetylation of dinitrogen co-ordinated to tungsten,[48] and subsequently the protonation[49] and even silylation[50] were demonstrated, and attack by electrophiles remains the commonest mode of reaction of co-ordinated dinitrogen.

These complexes of dinitrogen contain metal atoms in low oxidation states, lower than might be expected for molybdenum or vanadium in a biological environment, and the ligands, often tertiary phosphines, are not very biological. Great efforts have been made to extend the range of complexes to include those with sulfur donors, but without general success. One of the criticisms of the extensive studies of dinitrogen co-ordinated end-on to molybdenum and tungsten has been that these complexes would appear to be so different from any conceivable active site in an enzyme that their reactivities could shed little light on nitrogenase function. The similarities of the structure and function of the three types of nitrogenase strongly suggest that they use similar mechanisms for dinitrogen activation and reduction. Nevertheless, there are also differences, although of questionable significance at present. For example, a small amount of N_2H_4 is formed as a *product* of N_2 reduction by V-nitrogenase but not by Mo-nitrogenase,[41] but it is not known if the formation of N_2H_4 arises from a side-reaction, for example, by dissociation from an intermediate on the route to NH_3.

It is evident from the chemical studies that there is no single pathway for N_2 protonation to ammonia, and, particularly for N–N bond cleavage. There is evidence for different routes, such as disproportionation of hydrazide(1 −), stepwise protonation, and reduction coupled with protonation.[51] The metals which give rise to the dinitrogen complexes most sensitive to this kind of electrophilic attack appear to be those towards the left-hand side of the transition series, from Groups 4 to 6.[11]

Dinitrogen also forms bridging complexes with molybdenum, tungsten and other transition elements. They seem to fall into two classes, one with N–N bond lengths little different from that observed in the end-on singly bonded dinitrogen complexes, and in dinitrogen itself (*ca.* 1.1 Å) and the other with N–N bond lengths which are consistent with a bond order between one and two, rather than three. Until recently, when such compounds showed dinitrogen protonation activity, it was almost invariably found that N_2H_4 was a product. Indeed, in systems with the long N–N bond, it can be proposed arguably that the bonding be written M=N–N=M, rather than M–N≡N–M. Examples are $[\{Nb(S_2CNEt_2)_3\}_2N_2]$[52] (written as containing Nb^V or Nb^{III}) and $[\{Mo(C_5H_5)Me_3\}N_2]$ (written as containing Mo^{IV} or Mo^{VI}).[53] It is now evident[54] that these complexes can give rise to NH_3 upon protonation, and that the accessible oxidation states (and consequently the number of electrons available to reduce N_2) have some influence on the product.

There is a large amount of work associated with the groups of Shilov in Moscow, and Schrauzer in La Jolla, California, which

* The basis for this statement is discussion with polymer chemists active at that time.

does not fall into the categories discussed so far in that dinitrogen complexes have not been isolated, though they are clearly implicated.

Shilov's group[55] has described a range of systems, some of them catalytic, which can reduce N_2 to NH_3 and/or hydrazine in hydroxylic solvents such as methanol. Many of these systems are based on molybdenum, iron or vanadium. The optimum conditions for fixation are often somewhat removed from biological conditions (*e.g.*, high N_2 pressure, extremely high pH) and some have the quirk of operating well only in relatively dilute solution (10^{-4} molar). Semi-mechanistic analyses of these systems have been interpreted consistently as requiring N_2 to bridge end-on between two metal atoms, generally involving moieties like the bridging complexes discussed above. These systems are certainly amongst the most reactive discovered, yet there must still be a doubt about how they actually function.

The group associated with Schrauzer[56] has also carried out extensive research on systems developed by themselves and on some of Shilov's systems. They have also not isolated N_2-carrying intermediates, but they identify the crucial species as binding N_2 sideways to a single metal atom, generally molybdenum.[56]

There has been considerable disagreement between these groups concerning the mechanisms of the reactions involved, and it has not been resolved. However, the argument about the relevance of bridging dinitrogen to a nitrogenase function has to some extent been clarified by the crystal-structure studies of the nitrogenases.

It would be misleading to leave the impression that everyone working in this area has based work on the assumption that molybdenum is at the active site of nitrogenase. For example, Sellmann[57] has developed an extensive chemistry of FeS compounds which has yielded functional models for hydrogenases, nitrate reductases and latterly nitrogenase, based upon iron. His nitrogenase models bind NH_3 and N_2H_4, in the latter case bridging between two Fe atoms. The complexed hydrazine can be oxidized to diazene, bound in the same fashion. The extension, so far unrealized, implies that dinitrogen similarly might bind between two Fe atoms. The demonstration of this kind of binding and the stepwise conversion $N_2 \longrightarrow H\text{–}N{=}NH \longrightarrow H_2N\text{–}NH_2 \longrightarrow H_3N + NH_3$ would be a prize indeed. The latter stages of such a conversion catalysed by a molybdenum–iron cluster with a strong similarity to FeMoco have been described recently.[58]

In any case, regardless of the sceptics, the discovery of V-nitrogenase in 1986 revived interest in Shilov's vanadium systems,[56] which supposedly involved bridging N_2. However, at about the same time Gambarotta and co-workers[19] described the first reasonably stable dinitrogen complex of vanadium, $[\{V(C_6H_4CH_2NMe_2)_2(C_5H_5N)\}_2N_2]$, also with bridging N_2. It reacts with acid to yield ammonia rather than hydrazine. A little later, Woitha and Rehder[18] announced vanadium dinitrogen complexes with singly bound end-on dinitrogen, such as $[V(N_2)_2(Me_2PCH_2CH_2PMe_2)_2]^-$, isoelectronic and isostructural with the well established complexes of molybdenum(0) and tungsten(0). Similarly, they also give ammonia and a little hydrazine upon reaction with acid.

We have suggested[8,59] that the chemistry occurring in a V-nitrogenase might be rather like that in a Mo-nitrogenase. After all, the two elements stand in a diagonal relationship in the Periodic Table and, as discussed above, the structures of FeMoco and FeVco are very similar. However, the discovery of the third nitrogenase containing only iron seemed to render such speculation idle. After all, if iron can mediate the conversion of dinitrogen to ammonia, might it not do so in all three kinds of nitrogenase? There is iron–dinitrogen chemistry, admittedly in phosphine complexes but using iron(0) and iron(II), both reasonably accessible in a biological system, which provides a good functional model (Scheme 3).[59] If this be so, the related question arises concerning the function of the molybdenum and vanadium.

It was expected that determination of the structure of the MoFe protein of nitrogenase would provide the answer to these and related questions. In fact, the proposed structure (Fig. 2) raises as many questions as it settles. The only possibilities for dinitrogen reduction which we can definitely exclude are that N_2 is bound between two Mo atoms or, in the VFe proteins, between two V atoms. Dinitrogen singly bound to Fe, V or Mo are still possible models. Likewise, dinitrogen could bridge between Fe and V, between Fe and Mo, or between Fe and Fe and it has been suggested[60,61] that N_2 binds between two or more of the six central Fe atoms when the cofactor is reduced. In addition, there is no reason to presume that sulfur may not also be involved.

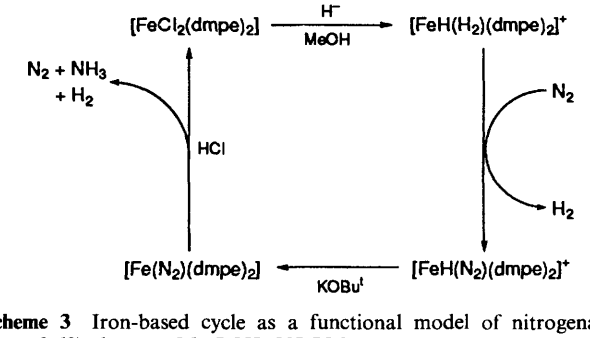
It is to be noted that the crystal structures of the MoFe proteins were determined on the dithionite-reduced species, an oxidation level which does not bind N_2. Although more reduced states of the MoFe protein are currently experimentally accessible in quench and spectroscopic studies, X-ray crystal-structure studies of the MoFe protein with dinitrogen bound to the cofactor are not.

From the point of view of nitrogenase function, many questions are posed by the P cluster and FeMoco structures. Some of these are listed below.

1. The P clusters are undoubtedly basically two $\{Fe_4S_4\}$ cubanes, but they are fused together and the bridges (direct S–S bonds single S atoms, and cysteinyl sulfur atoms) are not reproduced in any of the model structures so far characterized. Are clusters of this type thermodynamically unstable outside the protein environment?

2. Although the hypothesis of hydride involvement is attractive for several reasons, there is no hard physical evidence that hydrido complexes are necessarily involved in binding of dinitrogen. In fact, the Lowe–Thorneley scheme does not require the presence of hydrides in the resting state of the enzyme, the form of the MoFe protein the structure of which has been determined by X-ray crystallography. A recent ENDOR (electron nuclear double resonance) study showed that no new exchangeable protons with hyperfine coupling to FeMoco were introduced on allowing the enzyme to turn over in ^2H_2O and then to relax to the dithionite-reduced level.[62] Thus, the coupled protons seen in the resting enzyme cannot be the source of the H_2 released when N_2 binds to the active site. Can techniques to freeze the enzyme at a specific point during turnover when the hydride concentration is maximal be developed?

3. The molybdenum is six-co-ordinate. It does not achieve an 18-electron full shell, but neither is it co-ordinatively unsaturated by the normal criteria since a Mo^{IV} system can conceivably become seven- or even eight-co-ordinate. However, it does not appear to be a possible site of dinitrogen binding unless the co-ordination changes considerably during turnover. Such changes have not been detected by Mo EXAFS spectroscopy during enzyme turnover in the steady state, but a preliminary report of rapid-freeze Mo EXAFS samples frozen 300 ms after initiation of the reaction indicated a possible



Scheme 3 Iron-based cycle as a functional model of nitrogenase (see ref. 60); dmpe = $Me_2PCH_2CH_2PMe_2$

change in co-ordination sphere, consistent with a N atom at 1.9 Å.[63] These observations clearly indicate the need for further studies of this type. We also now have a model system[35] in which carboxylate is a leaving group which allows dinitrogen to bind, and in which H_2 is evolved after the binding of dinitrogen (although the dinitrogen, once bound, does not seem to be protonated to give NH_3). Does the inability to detect spectroscopically a species containing Mo-bound dinitrogen or a modified co-ordination in nitrogenase arise from its low concentration in the catalytic cycle, or does it simply not exist?

4. The iron atoms may be the site of dinitrogen binding and reduction. There is a precedent in model chemistry for dinitrogen binding to a single metal atom, or it can bridge, or it can bind to several metal atoms at once.[64] In the MoFe protein the co-ordination environment of the Fe atom bound to Cys-275 of the polypeptide is tetrahedral, but there are also six very unusual Fe atoms in the structure, all of which have similar distorted planar-trigonal co-ordination. The 2.2 Å resolution structure of Av1 shows these atoms to be sufficiently close to allow some Fe···Fe bonding interactions, which could provide enhanced co-ordination for these atoms. A role for these atoms in binding N_2 to FeMoco has been suggested. If they do form the binding site, what is the function of molybdenum and vanadium in nitrogen fixation? One possibility is that they modify the mechanism of thiolate substitution at a Fe atom remote from them in the cluster, as has already been demonstrated in some model systems.[65] Could it be that the unique cysteinyl ligated iron is the active site of FeMoco?

5. The conversion of dinitrogen to ammonia requires N_2, electrons and protons. As indicated above, the site at which N_2 binds is uncertain. There is experimental evidence that the pathway of some electrons through MoFe protein during the reduction of N_2 is from the P clusters to FeMoco.[66] The 2.2 Å structure of Cp1 shows that the apparent voids are filled with structured water with potential hydrogen-bonding interactions with amino acid residues close to the homocitrate end of FeMoco,[21,29] but how do protons reach the active site?

6. Secondary interactions of some of amino acid residues close to the FeMoco centres are very important for nitrogenase function. For example, substitution by mutation of Gln-α191 (glutamine) by Lys (lysine) results in a nitrogenase unable to reduce dinitrogen but still able to reduce acetylene and protons.[67] The crystal structure shows Gln-191 to be close to the homocitrate, and hydrogen bonded to it, but it is not bound to the molybdenum. How do these secondary interactions operate?

7. So far FeMoco has eluded chemical synthesis, and outside the protein environment it may not be thermodynamically stable in the absence of *N*-methylformamide which is an awkward solvent for preparative application. Many clusters containing iron, molybdenum and sulfur are formed by a process somewhat grandiosely referred to as 'spontaneous self-assembly'. This really means that their formation is thermodynamically driven, and they presumably represent thermodynamic sinks. This does not appear to be so for FeMoco. However, it is stable under some conditions, because it can be extruded from the protein, and stored in solution for weeks, and then reincorporated to give active MoFe protein. Thus the nature of its stability/instability and the driving force for its formation need to be established.

These recent advances have succeeded in changing the emphasis of work on chemical nitrogen fixation. Chemists now have something much more direct to aim at, both synthetically and mechanistically. However, the questions to be answered are now more numerous and more varied than appeared to be the case only five years ago. Far from refining aspects of molybdenum–dinitrogen chemistry to model nitrogenase function, we have also to consider iron, vanadium and sulfur. We now know that we know far less about the mechanism of biological nitrogen fixation at the atomic level than we thought we did.

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