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Structural basis of biological nitrogen fixation

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Biological nitrogen fixation is mediated by the nitrogenase enzyme system that catalyses the ATP dependent reduction of atmospheric dinitrogen to ammonia. Nitrogenase consists of two component metalloproteins, the MoFe-protein with the FeMo-cofactor that provides the active site for substrate reduction, and the Fe-protein that couples ATP hydrolysis to electron transfer. An overview of the nitrogenase system is presented that emphasizes the structural organization of the proteins and associated metallo-clusters that have the remarkable ability to catalyse nitrogen fixation under ambient conditions. Although the mechanism of ammonia formation by nitrogenase remains enigmatic, mechanistic inferences motivated by recent developments in the areas of nitrogenase biochemistry, spectroscopy, model chemistry and computational studies are discussed within this structural framework.

Keywords: nitrogenase; iron–sulphur proteins; nucleotide-switch proteins; biological electron transfer

1. Introduction: nitrogen fixation

In his 1898 address to the British Association, Sir William Crookes presented a remarkable analysis of the sustainability of wheat production by the western world (Crookes 1900). From a comparison of the rates for the production and the consumption of wheat, Crookes concluded that the 'wheat-producing soil is totally unequal to the strain put upon it', so that 'England and all civilized nations stand in deadly peril of not having enough to eat'. Unless Europeans could be 'induced to eat Indian corn or rye bread', the time-scale for famine was estimated to occur in approximately 30 years. Crookes recognized that the yield of wheat per acre was limited by the availability of fixed nitrogen in the soil, and that the crisis would be averted if the soil could be amended with sufficient quantities of nitrogenous fertilizer. At that time, however, the only reasonable

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source of fixed nitrogen for use on a global scale was Chilean saltpeter. Although the size of this sodium nitrate deposit was uncertain, clearly it did not provide an inexhaustible resource, and would only push the day of reckoning further into the future. The uncertainties in these estimates were substantial; by 1921, for example, it was claimed that the Chilean deposits would last for at least 200 years, and that the previous (smaller) estimates were 'alarmist' (Knox 1921). From today's vantage point, the parallels between the 'the wheat problem' of a century ago and the present oil situation (Goodstein 2004) are evident.

The problem with nitrogen, of course, is not one of elemental abundance ('in its free state, nitrogen is one of the most abundant and pervading bodies on the face of the earth'), but rather that most organisms, plants and animals included, are unable to access atmospheric dinitrogen for metabolic purposes. Dinitrogen must be first 'fixed' or converted into a form such as ammonia or nitrate that can be then be consumed. Hence, the challenge was to develop industrial scale processes for the commercial fixation of dinitrogen. In his address, Crookes charged that 'it is the chemist who must come to the rescue of the threatened communities. It is through the laboratory that starvation may be turned into plenty'. Although Crookes favoured the oxidation of dinitrogen in an electric arc, within two decades, his vision was realized by the introduction of the Haber–Bosch process for the reduction of dinitrogen to ammonia under conditions of high temperature and pressure in the presence of an iron-based catalyst. A fascinating account of the history and consequences of the development of the industrial synthesis of ammonia may be found in the work by Smil (2001).

Nitrogen fixation represents one facet of the nitrogen cycle that involves the global interconversion of nitrogen between different oxidation states. A convenient starting point for this process is the reservoir of atmospheric dinitrogen, which contains approximately 4×10^{21} g N (Schlesinger 1991). A fraction of this, estimated as approximately 3×10^{14} g N, is fixed annually to ammonia. After metabolic cycling through the biosphere, fixed N is returned to the atmospheric reservoir through the process of denitrification. This latter process completes the cycle and necessitates the continual replenishment of fixed nitrogen into the biosphere to sustain life. Until the advent of the Haber–Bosch process in the early part of the twentieth century, biological nitrogen almost exclusively provided the entryway for nitrogen into the biosphere (relatively small quantities are produced by terrestrial sources, primarily lightning and volcanic activity). Today, the Haber–Bosch process generates comparable amounts of fixed nitrogen as the biological process (approximately 1×10^{14} g fixed N yr^{-1}), which has fuelled the agricultural impetus behind the increase in world population by approximately 5 billion people over the past century (Smil 2001).

Roughly a decade before Crookes' address, Hellriegl & Wilfarth established that bacteria present in the root nodules of legumes were responsible for biological nitrogen fixation (a contemporary discussion of their discovery is provided by Nutman 1987). Subsequently, free living bacteria catalysing the same reaction were also identified. Crookes recognized the contribution of living processes to nitrogen fixation and its practical implementation through crop rotation, but felt it was too slow to satisfy the agricultural and industrial demands, stating, 'The nitrogen which with a light heart we liberate in a battleship broadside, has taken millions of minute organisms patiently working

for centuries to win from the atmosphere'. Nevertheless, until the Haber–Bosch process was developed, biological nitrogen fixation sustained life by replenishing the supply of fixed nitrogen available to living organisms. Despite decades of study, the mechanism of biological nitrogen fixation remains enigmatic and continues to provide the chemist with scientific challenges. It is the state of our mechanistic understanding of biological nitrogen fixation that will be addressed in this paper.

2. Biological nitrogen fixation: nitrogenase

The catalytic efficiency of biological nitrogen fixation, which proceeds under ambient conditions, contrasts markedly to the Haber–Bosch process and, indeed, any other synthetic system, which either require high temperatures to get reasonable rates or are only able to sustain a limited number of turnovers. Consequently, the molecular mechanism of biological nitrogen fixation has been of great interest in understanding the origins of this catalytic efficiency.

The biochemical machinery required for biological nitrogen fixation is provided by the nitrogenase enzyme system (for representative reviews see Burgess & Lowe 1996; Rees & Howard 2000; Lawson & Smith 2002; Seefeldt *et al.* 2004). Nitrogenase consists of two component metalloproteins designated the iron protein (Fe-protein) and the molybdenum iron protein (MoFe-protein) that catalyse the ATP-dependent reduction of dinitrogen to ammonia. The MoFe-protein contains the active site for substrate reduction, and is organized as an $\alpha_2\beta_2$ tetramer (where the α and β subunits are homologous) of molecular weight approximately 240 kDa. Associated with this protein are 2 Mo, 30 Fe and 32 S organized into two copies each of two extraordinary metalloclusters designated the FeMo-cofactor and the P-cluster. The FeMo-cofactor (or 'cofactor') represents the site of substrate reduction, while the P-cluster is probably the initial acceptor of electrons from the Fe-protein. The Fe-protein mediates the coupling of ATP hydrolysis to electron transfer and is the only known electron donor that can support substrate reduction by the MoFe-protein. The Fe-protein is a dimer of identical subunits (total molecular weight *ca* 60 kDa) that contains one [4Fe : 4S] metallocluster per dimer. In addition to this molybdenum containing nitrogenase, alternative nitrogenases also exist that are homologous to this system, but with the molybdenum almost certainly substituted by vanadium or iron (Eady 1996).

At the protein level, the basic mechanism of nitrogenase (figure 1; Thorneley & Lowe 1985; Burgess & Lowe 1996) involves: (i) complex formation between the reduced Fe-protein with two bound ATP and the MoFe-protein; (ii) electron transfer between the two proteins coupled to the hydrolysis of ATP; (iii) dissociation of the Fe-protein accompanied by re-reduction (via ferredoxins or flavodoxins) and exchange of ATP for ADP and (iv) repetition of this cycle until sufficient numbers of electrons and protons have been accumulated so that available substrates can be reduced. In addition to dinitrogen reduction, nitrogenase catalyses the reduction of protons to dihydrogen, as well as non-physiological substrates such as acetylene that are typically small molecules with unsaturated bonds. The overall reaction stoichiometry of the nitrogenase

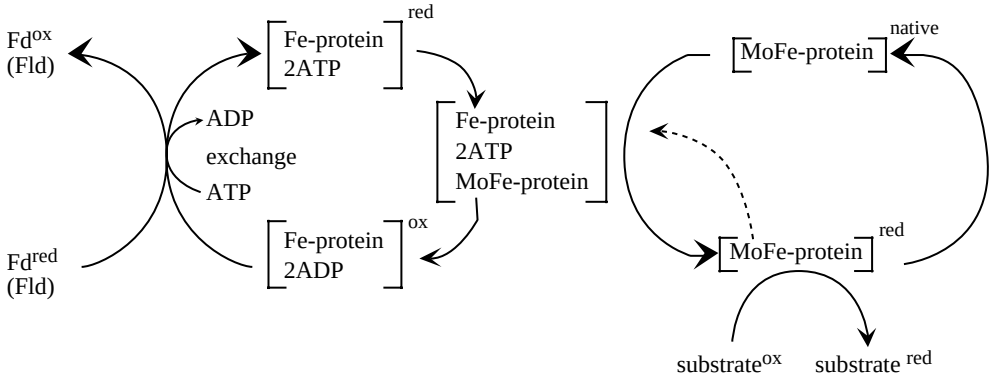

Figure 1. Schematic of the nitrogenase turnover cycle, illustrating the flow of electrons from electron carriers such as ferredoxin (Fd) or flavodoxin (Fld) to the Fe-protein (left), the transfer of electrons from the Fe-protein to the MoFe-protein coupled to the hydrolysis of ATP (centre) and the subsequent reduction of substrates coupled with return of the MoFe-protein to the resting redox state (right).

catalysed reaction has still not been unambiguously determined. These uncertainties may be expressed in the following equation (Rees & Howard 2000):

$$N_2 + (6+2n)H^+ + (6+2n)e^- + p(6+2n)ATP$$
$$\rightarrow 2NH_3 + nH_2 + p(6+2n)ADP + p(6+2n)P_i.$$

In the 'standard' model, the evolution of one molecule of dihydrogen is coupled to the reduction of one molecule of dinitrogen, and an average of two molecules of ATP are hydrolysed per electron transferred so that $n=1$ and $p=2$. Under typical experimental conditions, however, $n>1$ and $p>2$, that is, there is more than stoichiometric evolution of hydrogen relative to dinitrogen reduction, and some ATP hydrolysis appears uncoupled from electron transfer. There are also reports of p values approaching 1 when an all-ferrous form of Fe-protein is utilised as the electron source (Erickson *et al.* 1999).

3. Structural enzymology of nitrogenase

The component proteins of nitrogenase have been extensively characterized by a variety of biochemical and biophysical approaches. A structural perspective is adopted in this paper to provide a molecular-based framework to discuss the mechanism of biological nitrogen fixation. Following the initial structure determinations (Georgiadis *et al.* 1992; Kim & Rees 1992*a*,*b*), a collection of structures from different organisms and in different states are now available for the MoFe-protein (Bolin *et al.* 1993; Kim *et al.* 1993; Peters *et al.* 1997; Mayer *et al.* 1999; Sørlie *et al.* 2001; Einsle *et al.* 2002), Fe-protein (Georgiadis *et al.* 1992; Schlessman *et al.* 1998; Jang *et al.* 2000*a*,*b*; Strop *et al.* 2001; Sen *et al.* 2004), and three complexes between the two proteins (Schindelin *et al.* 1997; Chiu *et al.* 2001; Schmid *et al.* 2002). These structures have established the resting-state

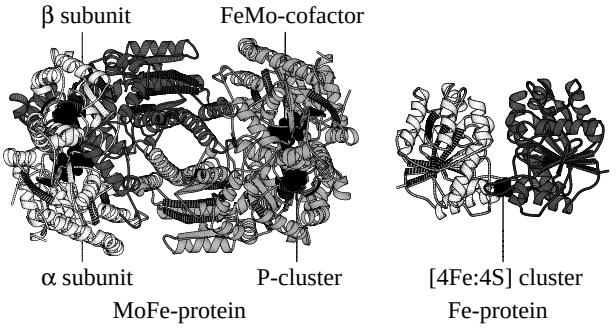

Figure 2. Ribbons diagrams of the polypeptide folds for the nitrogenase MoFe-protein (left) and Fe-protein (right). The α and β subunits to the left of the MoFe-protein are represented by the light and dark shading, respectively, while the metalloclusters are depicted by the dark space-filling models on the right side of the MoFe-protein. The two subunits of the Fe-protein are denoted by light and dark shadings, with the 4Fe : 4S cluster at the dimer interface designated as a space filling model. Ribbons representations were prepared with Molscript (Kraulis 1991).

structures of the nitrogenase component proteins and defined specific modes of interaction.

(*a*) *Structures of the nitrogenase proteins*

The MoFe protein is an $\alpha_2\beta_2$ heterotetramer, where the homologous α and β subunits each contain three domains of the α/β-type (figure 2). The individual domains share common structural elements, in particular, a central four-stranded parallel β-sheet with flanking helices that is also found in the metallocluster binding domains of Fe-hydrogenase and CO-dehydrogenase (Rees 2002). The α and β subunits within a dimer are approximately related by a twofold rotation axis. Each αβ dimer contains one FeMo-cofactor and one P-cluster, with the FeMo-cofactor binding in a cleft at the interface between the three domains of the α-subunit, while the P-cluster is buried at the interface between a pair of α- and β-subunits. The two clusters coordinated to one αβ dimer are separated by approximately 70 Å from the equivalent set of clusters coordinated to the other αβ dimer in the tetramer, and it is commonly considered that the functional unit of the MoFe-protein is an αβ dimer.

The Fe-protein is a dimer of two identical subunits that symmetrically coordinate a single [4Fe : 4S] cluster. The isolated Fe-protein can bind MgADP or MgATP at a stoichiometry of two nucleotides/dimer in a cooperative and oxidation state dependent fashion (Lanzilotta *et al.* 1999). Structurally, Fe-protein adopts the polypeptide fold found in P-loop containing nucleotide binding proteins such as ras p21, G-proteins, myosin and related proteins (Sprang 1997; Vetter & Wittinghofer 2001). The defining characteristic of members of this nucleotide-switch family is that they acquire different conformations depending upon the phosphorylation state of the bound nucleotide. The switch between conformations occurs with the chemical conversion of the triphosphate to the diphosphate form of the nucleotide, so that the rate of nucleotide turnover (the combination of hydrolysis and nucleotide exchange) on these proteins is the ultimate timing event for

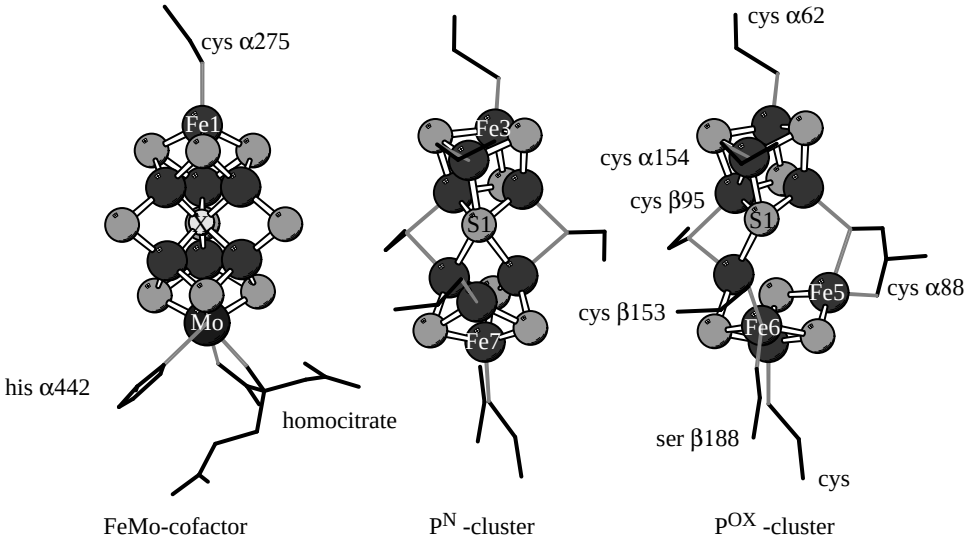

Figure 3. Structures of the nitrogenase FeMo-cofactor and P-clusters in the P^N and P^{OX} states (PDB coordinate sets 1MIN, 2MIN and 3MIN, respectively). Fe and Mo are shaded black, S in dark grey and the interstitial atom ('X') in light grey, while ligands are depicted as black sticks. Liganding residues and selected cluster atoms discussed in the text are labelled.

subsequent cellular processes. Structural studies on nucleotide-switch proteins have identified two regions, termed switch I and switch II, that interact with the terminal phosphate and, hence, are conformationally sensitive to the form of the bound nucleotide. In the Fe-protein, the presence of cluster ligands in the switch II region underscores that the cluster environment will be coupled to the nucleotide state. Furthermore, as the nucleotide binding site and the cluster are positioned at the interface between subunits, the Fe-protein conformation is sensitive to the binding of nucleotides, the state of the cluster and the interaction with the MoFe-protein; changes in the relative positions of Fe-protein subunits corresponding to rotations of 25–100° have been reported (Schindelin *et al.* 1997; Chiu *et al.* 2001; Sen *et al.* 2004).

(*b*) *Metallocluster structures*

The FeMo-cofactor and P-cluster (figure 3) are each composed of eight metals and associated sulphurs organized in distinctive arrangements (Kim & Rees 1992*a*; Bolin *et al.* 1993; Peters *et al.* 1997; Einsle *et al.* 2002). As seen in simpler iron–sulphur clusters of the [2Fe : 2S] and [4Fe : 4S] varieties (Berg & Holm 1982), the basic structural element of these clusters is the Fe_2S_2 rhomb with typical Fe–S and Fe–Fe distances of approximately 2.3 and 2.7 Å, respectively, and average Fe–S–Fe and S–Fe–S angles of 72 and 106°, respectively. The Fe_2S_2 rhomb typically adopts a non-planar, butterfly-like arrangement that is reflected in the deviation of the sum of the Fe–S–Fe and S–Fe–S angles from 180°. Both the biosynthetic mechanism (Dos Santos *et al.* 2004) and the chemical synthesis (Lee & Holm 2004) of the nitrogenase metalloclusters have proven to be challenging, and represent important areas for continued research.

Crystallographic studies have established that the inorganic (heavy-atom) composition of the FeMo-cofactor is 1 Mo, 7 Fe and 9 S organized into an elongated structure. The FeMo-cofactor is linked to the protein through only two residues, Cys α275 and His α442, which coordinate Fe1 and the Mo, respectively, on opposite ends of the cofactor. The octahedral Mo coordination environment is completed through bidendate coordination by homocitrate. The inorganic components exhibit very nearly C_{3v} (threefold) symmetry, which is particularly reflected in the trigonal prismatic arrangement of the six central iron atoms. Viewed perpendicular to the threefold axis, the cofactor exhibits seven alternating layers of metal and sulphur sites. The distances from the Mo to the successive layers of irons average 2.69, 5.06 and 7.00 Å, with an associated standard deviation of approximately 0.02 Å averaged over four crystallographically independent copies of the cofactor (Einsle *et al.* 2002) which are a measure of the close correspondence to threefold symmetry.

Recently, a light atom was identified in the centre of the FeMo-cofactor in a crystallographic analysis at 1.16 Å resolution (Einsle *et al.* 2002). This species had not been detected in previous studies due to electron density ripples emanating from the surrounding atoms, particularly the six irons in the trigonal prism and the nine sulphides of the cofactor, that are separated from the central ligand by 2.0 and 3.3 Å, respectively. Although the atomic identity of this species cannot be unambiguously established crystallographically, the refinement is best fit by a nitrogen; density functional theory (DFT) calculations find that N is the most likely candidate for the interstitial atom (Hinnemann & Nørskov 2003; Lovell *et al.* 2003).

In the dithionite-reduced P^N state, the [8Fe : 7S] P-cluster can be considered to be formed from two [4Fe : 3S] clusters bridged by a hexacoordinate sulphur (defined as atom S1). The overall symmetry of the P-cluster in this state is approximately C_{2v}, that is, a twofold axis passes through the S1 sulphur at the intersection of two perpendicular mirror planes. The coordination spheres of the eight irons are completed by the Sγ of six cysteines, with four cysteines coordinating single irons, while the remaining two cysteines each bridge two irons from the separate cluster sub-fragments. A less symmetrical arrangement is observed in the form of the P-cluster that has been associated with the P^{OX} state (Peters *et al.* 1997), which is oxidized by two electrons relative to P^N (Surerus *et al.* 1992; Pierik *et al.* 1993). In this state, two of the irons, Fe5 and Fe6 in the cluster fragment associated with the β-subunit, move away from the hexacoordinate sulphur. These interactions are each replaced by protein ligands, with the amide nitrogen of the cluster ligand Cys α88 and the side-chain hydroxyl of Ser β188 coordinating Fe5 and Fe6, respectively. Consequently, although there are changes in structure and coordinating ligands, all the irons remain four-coordinate in the P^{OX} structure.

The construction of these clusters from eight redox active metals (in addition to the potential redox activity of the sulphurs) allows the FeMo-cofactor and P-cluster to exhibit a rich set of redox states. Although these states remain to be completely defined, a consensus picture has emerged that the resting forms (as isolated in dithionite) of the FeMo-cofactor and P-cluster correspond to $[1Mo^{4+} : 4Fe^{2+},\ 3Fe^{3+} : 9S : 1N]^0$ for the FeMo-cofactor (Yoo *et al.* 2000; Vrajmasu *et al.* 2003), and to the all-ferrous $[8Fe^{2+} : 7S]^{2+}$ P^N state of

the P-cluster (McLean *et al.* 1987), respectively. Even the apparently prosaic [4Fe : 4S] cluster of the Fe-protein exhibits unusual redox properties, most notably the ability to adopt three different oxidation states, including the all-ferrous $[4Fe : 4S]^0$ in the most reduced form (Watt & Reddy 1994; Angove *et al.* 1997). Although the ability of Fe-protein to potentially serve as a two-electron donor is attractive (since all known substrates of nitrogenase are reduced by multiples of two electrons), the mechanistic relevance of the all-ferrous form of the Fe-protein remains to be definitively established; however, a value for the ATP/electron ratio, p, of approximately 1 with this state has been reported (Erickson *et al.* 1999), which would have important consequences for the energetic efficiency of dinitrogen reduction.

4. The coupling of ATP hydrolysis and interprotein electron transfer

A key intermediate in the nitrogenase mechanism (figure 1) is the formation of the Fe-protein–MoFe-protein complex where ATP hydrolysis is coupled to interprotein electron transfer. Using ADP–aluminum fluoride as a transition state analogue for nucleotide hydrolysis, the nitrogenase proteins can be trapped in a stable complex (Renner & Howard 1996; Duyvis *et al.* 1996). The structure of the ADP–AlF_4^- stabilized Fe-protein–MoFe-protein complex has been determined (Schindelin *et al.* 1997; Schmid *et al.* 2002), and the relative positions of the metalloclusters observed in this complex indicate that electron transfer from the Fe-protein to the FeMo-cofactor proceeds through the P-cluster. The closest distances between the Fe-protein [4Fe : 4S] cluster and the P-cluster and between the P-cluster and FeMo-cofactor are each approximately 12 Å, which are comparable to that typically observed between physiologically relevant redox partners (Page *et al.* 1999), with electron transfer rates at the sub-microsecond scale (Tezcan *et al.* 2001). In contrast, the separation between [4Fe : 4S] cluster and FeMo-cofactor of approximately 28 Å would correspond to an activationless electron transfer time-scale of approximately 0.1 s, which, while still compatible with the turnover time for nitrogenase (*ca* 0.2 s electron^{-1}), involves a significantly greater distance than generally observed in biological systems (Page *et al.* 1999).

The ADP–AlF_4^- stabilized complex also provides mechanistic insights into the coupling between ATP hydrolysis and electron transfer (Rees & Howard 1999). Efficient hydrolysis of the nucleotide requires the presence of several properly positioned groups near the terminal phosphates. One is a residue in the switch II region, corresponding to Gln61 of ras p21 or Asp129 of Fe-protein, that orients the attacking water molecule, while a second is a positively charged residue, Arg in G-proteins and Lys10 in Fe-protein, that may stabilize the leaving products. Short-lived conformational changes in the amino acid side chains lead to the transient activation of the nucleotide for hydrolysis, that is, they stabilize the transition state and control the overall rate. Although most nucleotide-dependent switch proteins have a basal rate of nucleotide hydrolysis, the rate of hydrolysis is greatly accelerated by association with a second protein. Interestingly, 'helper' proteins appear to enhance the rate by one of two mechanisms either by contributing a catalytically important residue to the active

site, for example, GAP (GTPase activating protein) that contributes a catalytic Arg to the ras p21 active site, or by preferential binding to the G-protein transition state, for example, RGS (regulators of G-protein signaling) binding to transducin. In contrast, Fe-protein in the absence of MoFe-protein exhibits little or no ATPase activity. The ADP–AlF_4^- stabilized complex (Schindelin *et al.* 1997) indicates how, in the nitrogenase system, such mechanistic stringency is effected; namely, for nucleotide hydrolysis *both* modes of catalysis are utilised and are apparently required. First, the MoFe-protein serves as an RGS-type protein that stabilizes the catalytically competent conformation of Fe-protein. Second, each Fe-protein subunit contributes the critical Lys10 and Asp129 across the subunit interface, analogous to the GAP-type interactions. The coupling of ATP hydrolysis to inter-protein electron transfer is achieved through the participation of switch II residues in interactions with both ATP and the [4Fe : 4S] cluster, with the result that the protein conformation required for efficient ATP hydrolysis also positions the [4Fe : 4S] cluster appropriately for efficient inter-protein electron transfer.

5. Substrate reduction considerations

Currently missing from our picture of nitrogenase during turnover are the fundamental mechanistic details describing the binding site(s) for substrates on the metalloclusters and the mechanism of substrate reduction. Although it is commonly assumed that substrates and inhibitors of nitrogenase bind to the FeMo-cofactor, direct demonstration of this binding has been exceedingly difficult for any substrate (Igarashi *et al.* 2004), particularly dinitrogen. The straightforward approach to resolving this situation—addition of substrate or inhibitors to MoFe-protein crystals and identifying the binding sites crystallographically—is apparently precluded by the failure of substrate and other ligands to bind to the MoFe-protein in the dithionite reduced states (Thorneley & Lowe 1985). It is possible that these studies could provide information on potential access routes to the FeMo-cofactor and, to that end, we have identified the sites of xenon binding to the MoFe-protein. Xenon sites have previously been utilised to map out substrate access channels in other metalloenzymes (Montet *et al.* 1997; Whittington *et al.* 2001). Although no xenon sites are found adjacent to the cofactor, there are two binding sites near α-helices containing His α195 and Val α71. Mutations in these residues alter the substrate reduction properties of nitrogenase (Scott *et al.* 1990; Christiansen *et al.* 2000; Benton *et al.* 2001), and a binding site for acetylene has been proposed near the latter.

The location of the substrate binding site(s) on the cofactor remains an open issue, even at the most basic level of ‘Fe versus Mo’ (a recent analysis of this issue is provided in Seefeldt *et al.* 2004). Spectroscopic studies, most notably infrared (George *et al.* 1997), EPR and ENDOR experiments (Lee *et al.* 1997, 2004), have demonstrated the binding of inhibitors and poor substrates to Fe sites, and this theme has been explicitly developed in recent DFT calculations of the nitrogenase mechanism (Hinnemann & Nørskov 2004; Huniar *et al.* 2004; Schimpl *et al.* 2004). Model chemistry has not yet provided sufficient guidance to resolve this issue. The most developed systems are based on Chatt-type

chemistry at Mo (Pickett 1996; Leigh 2003), involving a series of electron and proton transfers cycling through Mo^0 to Mo^{VI} in the original scheme, or Mo^{III} through Mo^{VI} in a tris(amido)amine molybdenum complex recently reported by Schrock and coworkers (Yandulov & Schrock 2003). A specific role for Mo (as opposed to Fe) in the fixation of dinitrogen to ammonia could be understood within the framework of a Chatt-type mechanism as reflecting the redox versatility required for this scheme (requiring at least four different oxidation states). However, recent developments in the chemistry of nitrogen species coordinated to mononuclear Fe–tris(phosphino)borate complexes by Peters and colleagues (Betley & Peters 2004) have demonstrated the ability of Fe to adopt formal oxidation states 0 through IV in these compounds, so that the possibility of a Chatt type mechanism based on Fe cannot be excluded.

One potential clue about the interaction of N_2 with the FeMo-cofactor may be provided by the central ligand. Assuming that this interstitial atom is N, it is difficult to imagine how it can be inserted without some relation to dinitrogen reduction. Although a central nitrogen could be a structural, non-exchangeable, component of the cofactor, it is also possible that it could represent an exchangeable species that is germane to the mechanism of dinitrogen reduction. One potentially relevant observation is the evidence from electron spin-echo envelope modulation (ESEEM) studies for one or more ^{14}N nuclei interacting with the FeMo-cofactor (Thomann *et al.* 1987; True *et al.* 1990; Lee *et al.* 1998). To date, it has not been possible to provide an unambiguous assignment of these signals to protein bound nitrogens, suggesting an alternative, non-protein, source for at least some of the signal. Arguing against an exchangeable N, however, are reports that these signals are insensitive to the turnover of $^{15}N_2$ by nitrogenase (Thomann *et al.* 1987; Lee *et al.* 2003), although these negative experiments cannot unambiguously establish that an interstitial ^{14}N would be observed. Perhaps the most accurate way to summarize this topic is that while there is no evidence that the central ligand is an exchangeable nitrogen, this possibility has not been conclusively eliminated either.

The trigonal prismatic Fe sites surrounding the central ligand in the FeMo-cofactor exhibit intriguing parallels to the iron surfaces utilised as catalysts for dinitrogen reduction in the industrial Haber–Bosch process. The iron sites on the catalytically active Fe(111) surface are arranged with threefold symmetry, as are sets of irons surrounding the central ligand in the FeMo-cofactor. Significantly, binding of dinitrogen to the Fe(111) surface is accompanied by dissociation to atomic nitrogen (Jennings 1991), demonstrating that nitrogen–iron binding interactions can be sufficiently strong to weaken and/or break the nitrogen–nitrogen triple bond. However, the distances between these irons in the cofactor are longer (2.63 Å) than in regular metallic iron (2.47 Å), and such strained metal surfaces have been modelled as particularly reactive as catalysts for dinitrogen dissociation (Logadottir & Nørskov 2001). Notwithstanding the enormous disparity of reaction conditions, the parallels between the arrangement of metals in the nitrogenase FeMo-cofactor and the catalyst for the Haber–Bosch process raise the possibility of common mechanistic elements in the reduction of dinitrogen to ammonia, which may be a useful perspective for chemists as they confront the challenge of deciphering the mechanism of biological nitrogen fixation.

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