Mechanism of Molybdenum Nitrogenase

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Contents

I. Introduction

Reduced nitrogen is an essential component of nucleic acids and proteins, and thus, all organisms require this nutrient for growth. Unfortunately, even though elemental dinitrogen (N_2) comprises 79% of the earth's atmosphere, this abundant source is effectively inert and can only be used for biosynthesis following conversion to a useable form like ammonia. In nature, this ability to fix N_2 is restricted to a small but diverse group of diazotrophic microorganisms that contain the enzyme nitrogenase. This enzyme catalyzes the MgATP-dependent reduction of N_2 to ammonia and, as shown in Figure 1, is composed of two separately purified proteins. We believe it is one of the most interesting and complex metalloenzymes so far isolated.

Recently, a major breakthrough has occurred in this field with the publication of the crystallographic structures of both component proteins of nitrogenase and their metal centers (see review in this issue by Howard and Rees).[1−8] Now that the structural problems are being solved so rapidly, the focus of investigation can shift toward detailed mechanistic studies and toward understanding the assembly of this multicentered metalloenzyme. These studies in turn are expected to have an impact on our understanding of a variety of other fundamental issues in biology. For example, the Fe protein of nitrogenase is one example of a large family of proteins (e.g. H-Ras p21, RecA, myosin) that have energy transduction mechanisms involving switching between conformational states upon nucleotide binding or hydrolysis.[9−12] The system is also expected to yield information about the processes of gated electron transfer, long-range electron transfer, multielectron transfer, and proton transfer within proteins. Finally, although metalloproteins play a variety of essential roles in catabolism, metabolic regulation, and metal storage, very little is known about the biosynthesis of metal centers and their incorporation into proteins. Because the numerous genes involved in nitrogenase assembly have already been cloned and sequenced, this is expected to be among the first completely defined metal cluster assembly systems.

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Barbara K. Burgess was born in Cleveland and is a graduate of Purdue University (B.S., Ph.D.). She served on the research staff at the Charles F. Kettering Research Laboratory in Yellow Springs, OH. Since 1984 she has been at the University of California, Irvine, where she is currently a Professor of Molecular Biology and Biochemistry. Her research interests are in the area of bioinorganic chemistry, with emphasis on the structural organization, function, and reactivity of the protein-bound [FeS] and [MoFeS] clusters in nitrogenase and ferredoxin. She has three sons, Tristan, Erin, and Devin.

David J. Lowe was born in Stoke-on-Trent, UK, and gained a Ph.D. from London University after graduating from Cambridge University (M.A.). He subsequently worked in the School of Molecular Sciences at Sussex University and in the AFRC Nitrogen Fixation Laboratory, both in Brighton, UK, and is now a Senior Research Scientist at the Nitrogen Fixation Laboratory in the John Innes Centre, Norwich, UK. His research interests include the use of spectroscopy and kinetics to study the bioinorganic chemistry of metalloenzymes, especially nitrogenase. He is married to Anne, and they have four children, Lucie, Jon, Ruth, and Peter.

In this article we will focus narrowly on the mechanism of molybdenum nitrogenase. This subject was last reviewed in detail by these authors in 1985,[13,14] so we will concentrate on what has been learned since then about the numerous partial reactions that occur during nitrogenase turnover beginning with the reduction of the Fe protein and ending with N_2 reduction.

II. The Fe Protein

The Fe protein of nitrogenase is the only known redox-active agent that is capable of transferring electrons to the MoFe protein in such a way that the latter can reduce substrates. In addition to that function, the Fe protein has at least two and possibly three other functions. First, it is required for the initial biosynthesis of FeMo cofactor. Second, it is required for the insertion of preformed FeMo cofactor into a FeMo cofactor-deficient MoFe protein, a process that may involve modification of the latter. Third, it has been implicated as being possibly important in the regulation of the alternative systems. It is important to note that to be active in FeMo cofactor biosynthesis and insertion the Fe protein does not have to transfer electrons to the MoFe protein. In this section we will discuss only the electron transfer function of the Fe protein. We note however that because it is a multifunctional protein we believe it is inaccurate to refer to the Fe protein as dinitrogenase reductase and that nomenclature will therefore not be used here.

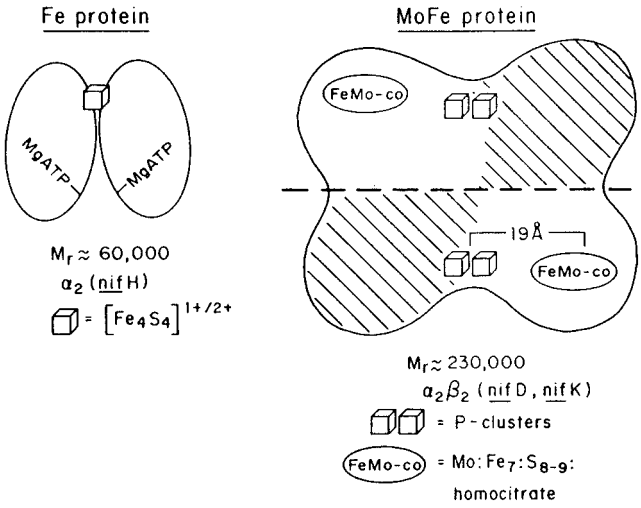


Figure 1. The two-component proteins of molybdenum nitrogenase.

A. Redox Properties

1. Properties of the [4Fe-4S]$^+$ Cluster

As originally proposed by Howard and co-workers[15,16] and confirmed by X-ray crystallography[1] the Fe protein of nitrogenase contains a single [4Fe-4S]-Cys_4 cluster bridged symmetrically between two identical subunits. When the protein is isolated in the presence of excess dithionite the [4Fe-4S] cluster is in the 1+ oxidation state. It has now been very firmly established using EPR, Mössbauer, and MCD spectroscopies that in frozen buffer solution the [4Fe-4S]$^+$ exists in a mixture of $S = {}^1/_2$ and $S = {}^3/_2$ spin states (Figure 2).[17−23] The Fe protein therefore appears to be poised close to a spin crossover point where two chemically different [4Fe-4S]$^+$ structures are of equal energy. This situation is common for [Fe-S] clusters in general.[24,25] The chemical changes required to switch between the $S = {}^1/_2$ and $S = {}^3/_2$ states are expected to be subtle, for example the gain or loss of a hydrogen bond, the lengthening or shortening of a bond, or the binding of OH^- or H_2O to a vacant coordination site.

It may not be a coincidence that nature has positioned the cluster at a point where the two spin states can be easily interconverted. This situation is common in hemeproteins where the change in spin state may facilitate electron transfer by allowing

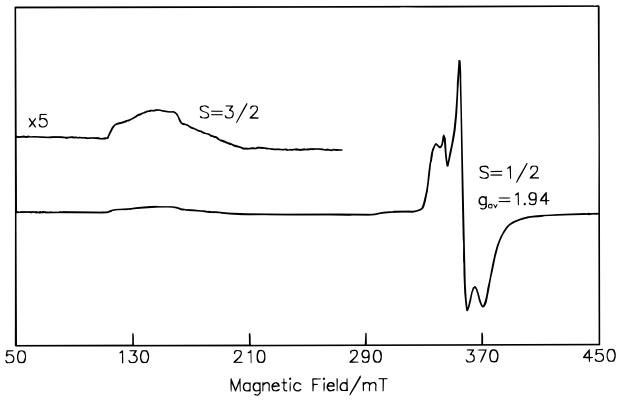


Figure 2. EPR spectra of the $S = {}^1/_2$ and $S = {}^3/_2$ spin states for the $[4Fe\text{-}4S]^+$ cluster from *A. vinelandii* nitrogenase Fe protein. Fe protein (~40 mg mL^{-1}) was in 25 mM HEPES buffer, pH 7.4, containing 1 mM sodium dithionite. The spectrum was a sum of 20 scans at a temperature of 10 K with a microwave frequency of 9.645 GHz and power of 20 mW using 0.94 mT modulation depth at 100 kHz.

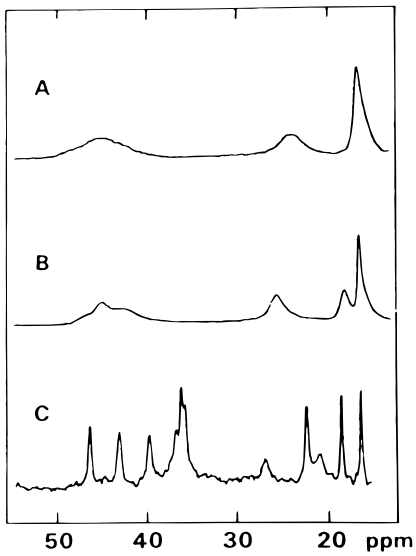


Figure 3. Low-field parts of the 250 MHz ^1H NMR spectra of reduced *C. pasteurianum* Fe protein and of reduced *B. stearothermophilus* ferredoxin:[28] (A) native Fe protein at 295 K; (B) native Fe protein at 325 K; (C) ferredoxin at 325 K.

bond lengths to contract and expand without significant changes in energy thus minimizing the movement necessary for the electron transfer.[26,27] In the case of the Fe protein, which is clearly designed to exist in a variety of conformations (see below), a spin-state change involving, for example, expansion of a bond length could be a trigger for a conformational change at a remote location.

In contrast to this suggestion, however, Meyer et al. have argued that the spin-state mixture observed for the Fe protein is unlikely to be mechanistically significant.[28] Their ^1H NMR data are illustrated in Figure 3 which shows the paramagnetically shifted resonances in the ^1H NMR spectra of *Clostridium pasteurianum* Fe protein compared to *Bacillus stearothermophilus* ferredoxin both at 325 K. The latter is known to display only the $S = {}^1/_2$ spin state at low temperature. Meyer et al. argue that because the number of proton resonances exhibited by *C. pasteurianum* Fe protein, their positions and temperature dependencies are similar to those of *B. stearothermophilus* ferredoxin the $[4Fe\text{-}4S]^+$ cluster of the Fe protein must also be only $S = {}^1/_2$ under NMR conditions. Further, the room temperature ^1H NMR spectra were not perturbed significantly by the addition of solvents that are known[17,20] to dramatically change the relative concentrations of the $S = {}^1/_2$ and $S = {}^3/_2$ spin states displayed by the Fe protein $[4Fe\text{-}4S]^+$ at low temperature. Analysis of these data led the authors to conclude that at the mechanistically significant temperature the clusters were all $S = {}^1/_2$ while the $S = {}^3/_2$ state was mainly a consequence of the freezing process.[28]

In spite of the study just described it may be too early to dismiss the significance of the Fe protein's spin-state mixture. One weakness of the ^1H NMR study, for example, is that no data were presented to establish what the ^1H NMR of a pure $S = {}^3/_2$ system should look like. One possibility for a model of this state would be the Fe protein purified from *Azotobacter vinelandii* grown on vanadium, because this protein has much less $S = {}^1/_2$ than Fe protein from cells grown with molybdenum even in the presence of solvents known to favor the $S = {}^1/_2$ state.[20] In addition, it should be noted that even if the clusters are all $S = {}^1/_2$ at room temperature the data still show that the cluster is poised at a point where the two spin states can be easily interconverted. This leaves open the possibility that some other mechanistically significant event, like binding to the MoFe protein, could also perturb that equilibrium, triggering another event.

Two other features of the $[4Fe\text{-}4S]^+$ cluster are notable. First the $g_{av} = 1.94$ EPR signal exhibited by the $S = {}^1/_2$ state is very broad relative to the signals exhibited by simple ferredoxins. The X-ray structure now makes it clear that this broadness cannot be explained by the interaction of the $[4Fe\text{-}4S]^+$ with another paramagnetic center and it has been suggested that it may represent a mixture of molecules with different $S = {}^1/_2$ ground states.[17] Second, ESEEM studies of D_2O exchange near the $[4Fe\text{-}4S]^+$ cluster indicate that its protons exchange much faster than the water protons near the $[4Fe\text{-}4S]^+$ clusters of ferredoxin.[29] Thus it appears that the Fe protein's $[4Fe\text{-}4S]^+$ cluster is normally exposed to solvent.

2. Oxidation of the $[4Fe\text{-}4S]^+$ Cluster

The Fe protein can be reversibly oxidized from the 1+ to the 2+ state in four ways: (1) by O_2; (2) by self-oxidation; (3) using redox active dyes; and (4) enzymatically by forward electron transfer to the MoFe protein.

Although the purified Fe protein is rapidly inactivated by O_2, Thorneley and co-workers have recently demonstrated that O_2 can initially react reversibly with the reduced Fe protein to form $[4Fe\text{-}4S]^{2+}$ with a k_{obs} of 2.6 s^{-1}.[30] Their data indicate that the protein is inactivated initially not because the $[4Fe\text{-}4S]^{2+}$ cluster is attacked by O_2 but rather because the protein is attacked by superoxide or some other reactive species.

Self-oxidation of the Fe protein occurs when the purified protein is incubated anaerobically at room temperature in the presence of excess dithionite.[31−33] Over a period of a few hours all of the dithionite in the solution is consumed.[31] Once the dithionite is gone the Fe protein slowly self-oxidizes to the $[4Fe\text{-}4S]^{2+}$ state. This reaction is accelerated greatly by the addition of methyl viologen to the Fe protein solutions[32] or by the addition of MgATP.[33,34] This self-oxidation process is not mechanistically significant but has been shown to cause numerous problems in interpreting spectroscopic (e.g. CD, ^{1}H NMR) and other room temperature experiments on the reduced Fe protein in the presence of dithionite.[34−41] For example, because of this self-oxidation phenomenon the oxidation state of the published Fe protein structure is not known.[1] Given the amount of time required for crystallization, however, it is likely that the protein is in the $[4Fe\text{-}4S]^{2+}$ state. Because self-oxidation is a serious technical problem a number of groups have attempted to understand it in order to prevent it from occurring.[31−33] Most recently it has been discovered that self-oxidation can be prevented by pretreatment of buffer solutions with Chelex.[33] Thus it appears that self-oxidation is caused by some small molecule, probably a metal, that contaminates the buffers and the problem can be avoided in future experiments by removal of that species.

The most common method for producing oxidized Fe protein for kinetic and spectroscopic experiments is to treat the protein with redox active dyes (e.g. thionine, methylene blue, indigodisulfonate) followed by chromatography to remove the dye. The resulting protein has been characterized by absorbance, CD, RR, and Mössbauer spectroscopies.[17,31,34,40,42−44] It should be noted that at least one paper has suggested that the $[4Fe\text{-}4S]^{2+}$ protein produced by dye oxidation is different from the protein produced by self-oxidation.[45] Some studies have also used enzymatically oxidized Fe protein produced by addition of the MoFe protein and MgATP[17] a system that will be discussed in detail below. In many of the most recent studies[17,42,44,46] it has been observed that regardless of oxidation conditions, the $[4Fe\text{-}4S]^{2+}$ is unstable and can lose two Fe atoms to form $[2Fe\text{-}2S]^{2+}$. This is a significant reaction with 10−30% of the oxidized protein ending up in the $[2Fe\text{-}2S]^{2+}$ state. It has further been shown that once the $[2Fe\text{-}2S]^{2+}$ cluster is present it can be reduced by either one or two electrons to the $[2Fe\text{-}2S]^{+}$ or $[2Fe\text{-}2S]^{0}$ states.[42,46] It is surprising that the $[4Fe\text{-}4S]^{2+}$ cluster of the Fe protein appears to be so unstable even when it is produced by enzymatic oxidation. Clearly something must prevent this loss of Fe reaction from occurring under natural turnover conditions.

3. Reduction of the $[4Fe\text{-}4S]^{2+}$ Cluster

The $[4Fe\text{-}4S]^{2+}$ cluster of the free oxidized Fe protein can be readily reduced to the $[4Fe\text{-}4S]^{+}$ state using a variety of reductants (e.g. dithionite, reduced ferredoxin, reduced flavodoxin). Since most of the kinetic data that are available for the enzyme have used dithionite this review will focus on that reductant. Although dithionite is commercially available it contains a number of contaminants and a recent report considers a simple method for dithionite purification that is applicable to the nitrogenase system.[47] As shown in eqs 1 and 2 the actual reductant for nitrogenase is not the two-electron dithionite ion itself but rather the one-electron dissociation product $SO_2^{\bullet-}$.[48,49]

Table 1. Reduction Potentials of Fe Protein $[4Fe\text{-}4S]^{+/2+}$

organism	$E°'$, mV/SHE	ref
K. pneumoniae	−200	30
A. chroococcum	−230	30
A. vinelandii	−290	63
C. pasteurianum	−295	74

$$S_2O_4^{2-} \rightleftharpoons 2SO_2^{\bullet-} \quad (1)$$

$$SO_2^{\bullet-} + H_2O \rightleftharpoons HSO_3^{-} + H^+ + e^- \quad (2)$$

$$e^- + FeP_{ox} \rightleftharpoons FeP_{red} \quad (3)$$

In experiments involving the Fe protein, the kinetics observed for $[4Fe\text{-}4S]^{2+}$ reduction by $SO_2^{\bullet-}$ vary depending upon whether the reduction of the Fe protein (eq 3) does or does not perturb the equilibrium shown in eq 1.[13] If it does not, then there is a half-order dependence on dithionite concentration.[48] This situation has been observed for the reduction of the Fe protein under a variety of conditions including turnover conditions.[13] If the reaction shown in eq 3 does perturb the equilibrium for the reaction shown in eq 1, then the reaction is first order in dithionite concentration. This situation has been reported for the free Fe protein from *A. chroococcum*.[13]

It should be noted that in contrast to the situation with the MoFe protein that will be discussed below, redox titrations of the Fe protein in either the oxidizing or reducing directions for the $[4Fe\text{-}4S]^{2+/+}$ couple are very well behaved and show no signs of hysteresis. Titrations of these types have resulted in the pH-independent reduction potential data shown in Table 1.

4. Reduction of $[4Fe\text{-}4S]^{+}$ to $[4Fe\text{-}4S]^{0}$

A very recent report has indicated that the $[4Fe\text{-}4S]^{+}$ cluster of the Fe protein can be reversibly reduced by one electron to the $[4Fe\text{-}4S]^{0}$ state which contains four ferrous Fe atoms.[32] This reaction is reported to be pH independent and, as shown in Figure 4, has an $E°'$ of −460 mV vs NHE which is within the physiological range. The $[4Fe\text{-}4S]^{0}$ cluster appears to be readily produced using methyl viologen, Ti(III), or the hydroquinone form of flavodoxin as reductants but is not produced when dithionite is used as a reductant (Gary Watt, personal communication).[32] As will be discussed below, this report opens up the possibility that under some conditions the Fe protein might serve as a two-electron donor to the MoFe protein. Given the significance of this, a Mössbauer study of the fully reduced Fe protein would be extremely valuable in directly determining the oxidation states and electronic structure of the Fe atoms.

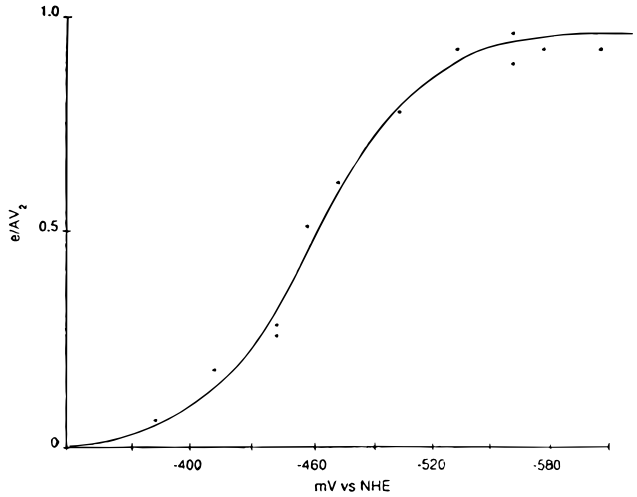


Figure 4. Microcoulometric reduction of $[4Fe\text{-}4S]^{+}$ to the $[4Fe\text{-}4S]^{0}$ state at pH 8.0 with 0.1 mM methylviologen as mediator.[32] An $E^{\circ\prime}$ value of −460 mV vs NHE and an $N = 1$ value were obtained from fitting the data to the Nernst equation.

B. Reaction of the Fe Protein with Nucleotides

1. Initial Binding of MgATP to the Fe Protein

The bulk of the evidence that is currently available strongly supports the long-held view that the Fe protein has two binding sites for MgATP, one on each identical subunit, and that it binds two MgATPs in both its oxidized and reduced states.[50,51] There are conflicting data concerning whether or not the wild-type Fe protein can bind ATP in the absence of Mg^{2+} or other divalent cations.[52,53] Repeated attempts to grow crystals of the Fe protein in the presence of MgATP have failed.[1] Consequently, there is no structure available to show how MgATP binds initially to the Fe protein. There is however a great deal of information available to support the model shown in Figure 5 for the approximate location of the γ-phosphate of MgATP.

Robson and co-workers were the first to suggest that the Fe protein might share a common MgATP binding motif with other proteins.[54] These proteins share what are commonly referred to as the Walker A (GXXXXGKTS; X can be varied) and Walker B (ZZZZD; Z is a hydrophobic residue) motifs that are associated with nucleotide binding.[55] Table 2 compares the sequence of the Fe protein to the sequences of a number of other ATP binding proteins in these two regions and Figure 5 shows a model for the topology of the ATP-binding domain based on X-ray analysis of some of the proteins.[56] The location shown for the terminal γ-phosphate is in the general area of the position of a molybdate ion in the X-ray structure of crystals of the Fe protein that were grown in the presence of molybdate.[1] However, the exact location of the phosphate in Figure 5 and the molybdate ion are significantly different. The Fe protein preparation that was used to grow the Fe protein crystals also appears to have contained a small amount (~0.4 occupancy) of a tightly associated nucleotide that has been modeled as ADP.[1] The position of the α and β phosphates in that model are also roughly similar to those shown in Figure 5 but again their exact location is significantly different.

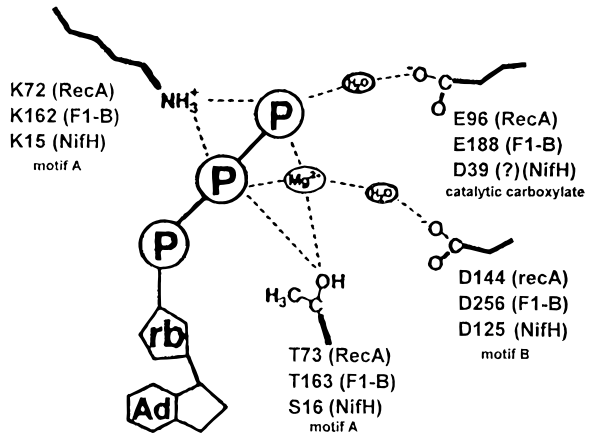


Figure 5. Schematic illustration of residues interacting with a bound ATP molecule in the ATP-binding domain of proteins which catalyze ATP-triggered reactions.[56] Numbering of residues are for *E. coli* RecA, bovine mitochondrial F_1-ATPase β subunit and *A. vinelandii* Fe protein.

The Fe protein X-ray structure shows that in the absence of MgATP there is a salt bridge between D125 of motif B and K15 of motif A.[1] Analysis of a K15Q site-directed mutant variant of the Fe protein shows that its affinity for MgATP is decreased to ~35% of wild-type.[57] These data suggest that when MgATP binds the salt bridge is broken to allow the lysine to bind to the terminal phosphate of MgATP. This view is also supported by analysis of a K15R variant which shows no detectable binding of MgATP. In that case R15 is suggested to form too strong an interaction with D125, preventing the salt bridge from breaking.[58] Support for D125 coordination to Mg^{2+} either through a water molecule as shown in Figure 5 or by direct coordination also comes from analysis of a D125E Fe protein variant that has altered reactivity toward both nucleotides and divalent cations.[59] A study of mutations at S16 clearly demonstrates that a hydroxyl is required at that site for MgATP binding.[59] A S16T Fe protein variant bound MgATP more tightly than the wild-type but unlike the wild-type could not use Mn^{2+} in place of Mg^{2+}.[60] This study supports the requirement for S16 coordination to Mg^{2+} (as shown in Figure 5) and leads to a model whereby S16 remains associated with Mg^{2+} following MgATP hydrolysis,[60] when the Mg^{2+} must move from its β- and γ-phosphate position in ATP (Figure 6) to an α- and β-phosphate position in ADP.[61] It should be noted that the movement of Mg^{2+} is an essential aspect of MgATP hydrolysis that could trigger a long-range conformational change.

As just discussed, there is sufficient evidence to conclude that the location of the Mg^{2+} and β- and γ-phosphates of ATP are roughly similar to those shown in Figure 5. Unfortunately, there is not sufficient evidence to suggest how the ribose and adenine portions of MgATP bind. As shown in Figure 7 two binding modes have been suggested. The first, which will be called the cross-subunit mode, is based on the position of the modeled ADP in the Fe protein structure.[1] In that binding mode, from the phosphate site the nucleotide extends across the subunit interface so that the adenine ring contacts residues Y159, A160, and N163 in the opposite subunit while the ribose group interacts with K41 in the same subunit

Table 2. Nucleotide Binding Motifs

NifH family	MOTIF A		MOTIF B	
	15	39		125
NifH	R Q I A F Y G K G G I G K S T T S Q N T L A A M A E M G Q R I M I V G C D P K A D S T R L M L H S K	NifH	E N G A Y Q D L D F V S Y D V L G D V V	
MinD	I I V V T S G K G G V G K T T S S A A I A T G L A Q K G K K T V V I D F D I G L R N L D L I M G C E	MinD	D D L K A M D F E F I V C D S P A G I E	
ArsA (N)	P Y L F F T G K G G V G K T S I S C A T A I R L A E Q G K R V L L V S T D P A S N V G Q V F S Q T I	ArsA(N)	Q L S G A C T T E T A A F D E F T G L L	
ArsA (C)	G L I M L M G K G G V G K T T M A A A I A V R L A D M G F D V H L T T S D P A A H L S M T L N G S L	ArsA(C)	R V I R E A G K R F V V M D T A P T G H	
FrxC	L T L A V Y G K G G I G K S T T S C N I S T A L A K R G K K V L Q I G C D P K H D S T F T L T G F L	FrxC	E L N A F D E Y D V I L F D V L G D V V	
IncC	T L V T A N Q K G G V G K T S T L V F L A F D F F E R G L R V A V I D L D P Q G N A S Y T L K D F A	IncC	K A L A N Q G F D V C L I D T A P T L G	
RecA		RecA		
E.coli	R I V E I Y G P E S S G K T T L T L Q V I A A A Q R E G K T C A F I D A E H A L D P I Y A R K L G V	E.coli	A L A R S G A V D V I V V D S V A A L T	
H^+-ATPase		H^+-ATPase		
F_1-β		F_1-β		
bovine	G K I G L F G G A G V G K T V L I M E L I N N V A K A H G G Y S V F A G V G E R T R E G N D L Y H E	bovine	F R D Q E G Q D V L L F I D N I F R F T	
spinach	G K I G L F G G A G V G K T V L I M E L I N N I A K A H G G V S V F G G V G E R T R E G N D L Y M E	spinach	F R D V N E Q D V L L F I D N I F R F V	
E.coli	G K V G L F G G A G V G K T V N M M E L I R N I A I E H S G Y S V F A G V G E R T R E G N D F Y H E	E.coli	F R D E - G R D V L L F V D N I Y R Y T	
F_1-α		F_1-α		
Bacillus PS3	Q R F L I I G D R Q T G K T S V A I D T I I N Q K - D Q N M I C I Y V A I G Q K E S T V A T V V E T	Bacillus PS3	Y F M I M G K H V L V V I D D L S K Q A	
FliI family		FliI family		
FliI	Q R I G I F A G S G V G K S T L M G M I A K Q T E A D L N V I A L V G E R G R E V R E F I E K D L G	FliI	Y F R D K G Q N V M F M M D S V T R V A	
SecA protein		SecA protein		
E.coli	E R C I A E M R T G E G K T L T A T L P A Y L N A L T G K G V H V V T V N D Y L A Q R D A E N N R P	E.coli	E E R V Q R K L H Y A L V D E V D S I L	
Rho Protein		Rho protein		
E.coli	Q R G L I V A P P K A G K T M L L Q N I A Q S I A Y N H P D C V L M V L K I D E R P E E V T E M Q R	E.coli	R L V E H K K D V I I L L D S I T R L A	
DnaA protein		DnaA protein		
E.coli	N P L F L Y G G T G L G K T H L L H A V G N G I M A R K P N A K V V Y M H S E E R F V Q D M V K A L Q	E.coli	F K R Y Y R S V D A L L I D D I Q F F A	
DnaB protein		DnaB protein		
E.coli	D L I I V A A R P S M G K T T F A M N L V E N A A M L Q D K P V L I F S L E M P S E Q I M M R S L A	E.coli	I A R E H G G I G L I M I D Y L Q L M R	
Lon Protease		Lon Protease		
E.coli	P I L C L V G P P G V G K T S L G Q S I A K A T G R K Y V R M A L G G V R D E A E I R G H R R T Y I	E.coli	M A K V G V K N P L F L L D E I D K M S	

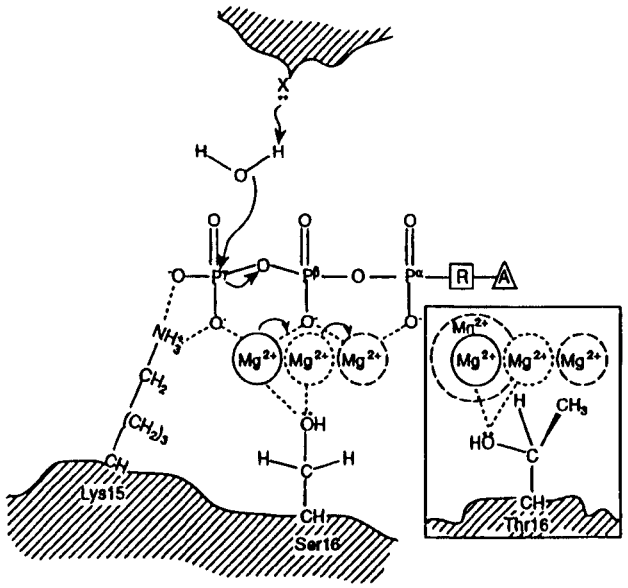


Figure 6. Model for the role of lysine 15 and serine 16 in nucleotide hydrolysis by the Fe protein.[57] Adenine is represented by an R inside a box. The transition state is represented by the dotted line around Mg^{2+}. Note that the movement of Mg^{2+} itself is an essential part of MgATP hydrolysis that may also trigger long distance conformational changes.

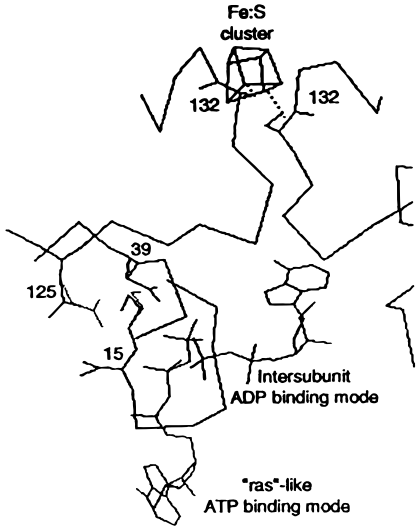


Figure 7. Two possible binding modes for nucleotides.[59] As described in the text the intersubunit mode is based on the X-ray structure of the *A. vinelandii* Fe protein while the "ras"-like mode is based on analogy to H-Ras p21.

and D129 in the opposite subunit. This binding mode is consistent with the observation that an A157S Fe protein variant has altered nucleotide reactivity.[62] The second binding mode shown in Figure 7 has been suggested on the basis of analogy to the known binding mode for GTP in the monomeric G-protein H-Ras p21. That binding mode would position the adenine along the subunit interface at the opposite end of the Fe protein from the cluster near residues 186 and 211 of the same subunit.[1,59] Whether one or the other or both of the binding modes shown in Figure 7, or some different binding mode for the adenine and ribose portions of ATP function in nitrogenase turnover is not known at present.

Another confusing aspect of the literature on MgATP binding has to do with the reported dissocia-

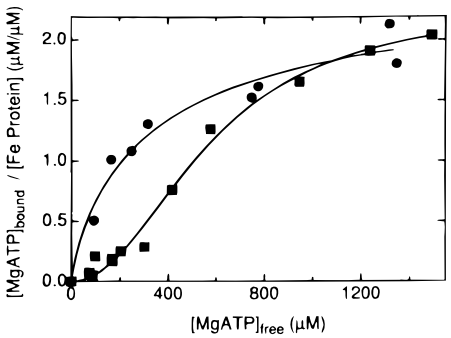


Figure 8. The binding of MgATP to the wild-type reduced Fe protein (■) shows positive cooperativity which can be eliminated in some mutants (●), in this case a D129E mutant.[63]

tion constants which vary from ~17 to 1700 μM for MgATP.[50] The variability is due to a variety of factors. For example, the source of the Fe protein, the technique used, whether the technique is directly measuring binding vs some other change that occurs in the Fe protein subsequent to binding, whether it is measuring binding to free Fe protein vs Fe protein complexed with the MoFe protein, etc. The most recent measurements of direct binding have been made on uncomplexed, reduced *A. vinelandii* Fe protein using a modified equilibrium column technique.[58] Two conclusions can be drawn from those data. First, as shown in Figure 8, the binding of MgATP appears to show strong positive cooperativity such that the binding of the first molecule of MgATP increases the protein's affinity for the second molecule.[58,60,63] That this cooperativity is real is supported by the observation that the cooperativity is lost for some mutations in the MgATP binding pocket.[60,63] A second observation is that the apparent K_d for MgATP binding to wild-type reduced *A. vinelandii* Fe protein is of the order of 580 μM ATP. This is only an apparent K_d because it represents an average of the two sites but it is useful in comparing wild-type and mutant proteins.

A final aspect of the initial binding of MgATP that deserves continued investigation is the observation that MgATP has a greater affinity for oxidized Fe protein than it does for reduced Fe protein.[41,64] This result is surprising because it suggests that the structure of the oxidized protein is substantially different from the structure of the reduced protein not just at the cluster but 19 Å away where the terminal phosphate of MgATP binds.[1] Indeed reported changes in the affinity upon oxidation/reduction[41,64] are greater than some of the reported changes in affinity obtained by directly modifying residues in the MgATP binding pocket.[57,58,60,63] This is also consistent with different proton ENDOR spectra being observed from VOATP bound to reduced compared with oxidized Fe protein. In the absence of these data there is no reason to assume that there would be a substantial structural difference between the oxidized and reduced forms of the Fe protein because such long distance structural changes are not observed for other [Fe-S] proteins whose X-ray structures are available in both the oxidized and reduced states.[65] Again this result points out the unique nature of the dimeric Fe protein that is clearly

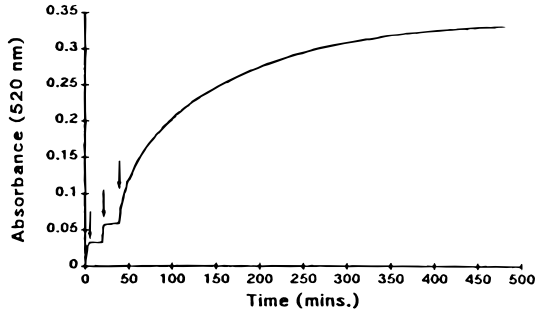


Figure 9. Time course of MgATP-induced chelation of Fe from the reduced Fe protein.[69] At the first arrow 10 mM Fe protein was added, at the second arrow α,α′-bipyridyl was added to a final concentration of 2.4 mM and allowed to stand for 20 min. At the third arrow MgATP was added to a final concentration of 0.6 mM. A_{520} corresponds to the absorbance maximum of the α,α′-bipyridyl ferrous complex.

designed to exist in a variety of conformations. Unfortunately, as indicated above, we do not know the oxidation level of the Fe protein whose structure has been determined.[1] Now that the self-oxidation problem has been solved, however, it should be possible to repeat the crystallization experiments under more controlled conditions.[33] Conformational changes on reduction to the newly described [4Fe-4S]^0 level may well be just as profound.

2. The MgATP-Induced Conformational Change

A critically important step in the overall nitrogenase mechanism is the change in the conformation of the Fe protein that occurs upon MgATP binding. As will be discussed below, there are site-directed mutant variants of the Fe protein available that bind MgATP but do not undergo the conformational change so that the two steps can now be separated. In this section we briefly review the many ways that the conformational change can be observed and consider what is known about the signal transduction pathway that connects the MgATP binding site over a 19 Å distance to the [4Fe-4S]$^{+/2+}$ cluster.

a. The Chelation Reaction. As shown in Figure 9 one of the most striking effects of MgATP binding by the Fe protein is the change in the reactivity of the [4Fe-4S] cluster with chelators. In the absence of MgATP the [4Fe-4S]$^+$ cluster is not accessible to attack by α,α′-bipyridyl[66,67] and is only very slowly attacked by bathophenanthrolinedisulfonate.[68] When MgATP binds,[66−69] both chelators are able to rapidly remove all of the Fe from the Fe protein.[66−69] The kinetics of Fe removal from the Fe protein are complex,[66,67] and the kinetics of Fe removal from the oxidized Fe protein[42] are quite different from those of Fe removal from the reduced Fe protein.[69] For oxidized protein the reaction is biphasic and involves the formation of a [2Fe-2S] cluster. Also for the oxidized protein the reaction with either chelator occurs slowly in the absence of MgATP.[42] For the reduced protein the reaction is also biphasic but the kinetics are very different and do not involve formation of a [2Fe-2S] intermediate.[68] Also for the reduced protein the reaction with α,α′-bipyridyl is completely dependent upon the addition of MgATP. These data provide evidence that the structure of the oxidized and reduced Fe proteins may be different

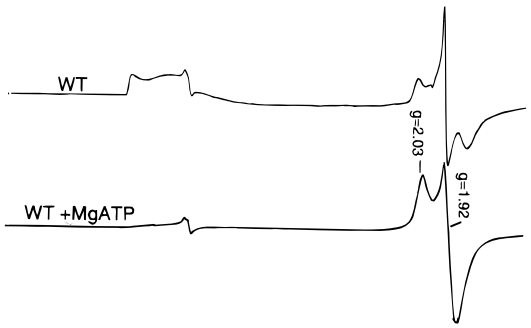


Figure 10. EPR spectra of purified dithionite reduced wild-type *A. vinelandii* Fe protein before and after the addition of MgATP.[58]

not only before but also after MgATP binds although interpretation of the chelation data are complicated by the fact that the chelators have different affinities for Fe^{2+} and Fe^{3+}.

The chelation results just described are generally interpreted to mean that the [4Fe-4S] cluster of the Fe protein becomes more exposed upon MgATP binding. This view is also supported by observations that the Fe protein becomes more O_2 sensitive when MgATP is bound. As discussed above, EPR and LEFE studies of D_2O exchange have shown that in the absence of MgATP, D_2O exchange is rapid and that the cluster is therefore already exposed to water.[29] Further, the D_2O exchange rate is not affected by the addition of MgATP, suggesting that the site is fully saturated with water prior to MgATP binding.

b. Changes in EPR Properties. As shown in Figure 10 the EPR spectrum of the reduced $[4Fe\text{-}4S]^+$ cluster in the Fe protein has a rhombic line shape with signals arising from both spin $^1/_2$ ($g = 2$ region) and spin $^3/_2$ ($g = 5$ region) states. Addition of MgATP results both in a change in the shape of the $S = {}^1/_2$ signal from rhombic to axial[70−72] and in a change in the proportion of the $S = {}^3/_2$ signal (Figure 10).[19,58,73] One study that quantitated the changes in the proportions of the $S = {}^3/_2$ and $S = {}^1/_2$ states upon MgATP binding found that, surprisingly, the proportion of the $S = {}^3/_2$ state decreased with no concomitant increase in the amount of the $S = {}^1/_2$ signal,[19] while another group did find such an increase.[73] The latter group additionally found a new $g = 4.3$ signal arising from an $S = {}^5/_2$ state of the reduced Fe protein/MgATP complex but the quantitation of that signal showed that it was a very minor component.[73] This issue appears to need further investigation.

It should be noted that studies of spin-state mixtures in the absence of nucleotides showed that the proportion of each spin state could be altered by the addition of solvents. For example about 85% of the clusters are $S = {}^3/_2$ in samples containing 0.5 M urea while 90% are $S = {}^1/_2$ in solvents containing 50% ethylene glycol.[17] The binding of MgATP appears to override this solvent effect such that the spin-state proportions observed for the Fe protein in the presence of MgATP are much less solvent dependent.[73] In addition to the EPR changes just described the addition of MgATP also causes a pronounced decline in the quadruple splitting of the Mössbauer spectrum of oxidized $[4Fe\text{-}4S]^{2+}$ containing protein.[17,73]

Table 3. Changes of Reduction Potential of the Fe Protein $[4Fe\text{-}4S]^{+/2+}$ Observed Upon Nucleotide Binding

	$E°'$, mV/SHE			
organism		+MgATP	+MgADP	ref
K. pneumoniae	−200	−320	−350	30
A. chroococcum	−230	−350	−463	30
A. vinelandii	−290	−430	−430	63
C. pasteurianum	−295	−400	−380	74

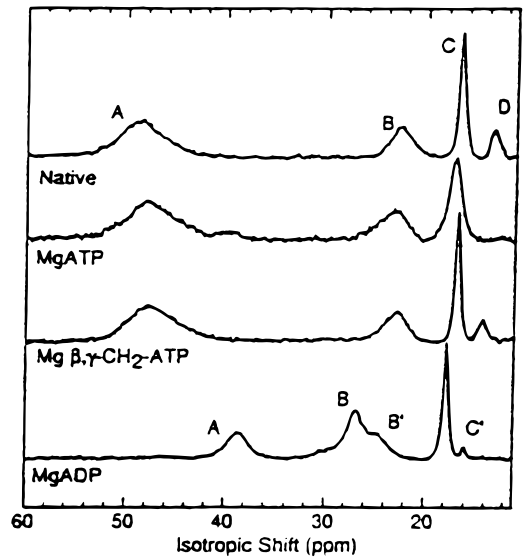


Figure 11. Effects of nucleotide binding on the isotropically shifted ^1H NMR resonances of the dithionite reduced *A. vinelandii* Fe protein.[33]

c. Changes in Reduction Potential. As shown in Table 3 the addition of MgATP causes the reduction potential to become more negative by ∼120 mV.[41,74] This 120 mV shift in potential could be a direct result of a protein conformational change and a reflection of the observation discussed above that MgATP binds more tightly to the oxidized protein than it does to the reduced protein.[35,41,45,64] Although it is tempting to suggest that this lowering of the reduction potential is critically necessary for subsequent electron transfer, there are data in the literature that would argue against that conclusion. Thus, as shown in Table 3 the reduction potential of *A. vinelandii* Fe protein is ∼100 mV lower than that of *Klebsiella pneumoniae* Fe protein both before and after the addition of MgATP. Nonetheless *in vitro* mixtures of *K. pneumoniae* MoFe protein and *A. vinelandii* Fe protein or *K. pneumoniae* Fe protein and *A. vinelandii* MoFe protein are close to 100% active.[75]

d. Changes in ^1H NMR of Reduced Fe Protein on MgATP Binding. Fe-K edge X-ray absorption spectroscopy studies have revealed that nucleotide binding to the Fe protein does not change the Fe−Fe or Fe−S bond distances of the [4Fe-4S] cluster significantly.[46,76] This suggests that the changes in the properties of the [4Fe-4S] cluster must be the result of changes in the protein environment near the cluster. ^1H NMR is one method that has been used to look at changes that occur to the $[4Fe\text{-}4S]^+$ cysteinyl ligands upon MgATP binding.[28,33]

Figure 11 shows the paramagnetically shifted ^1H NMR resonances exhibited by *A. vinelandii* Fe protein in the reduced $[4Fe\text{-}4S]^+$ state before and after

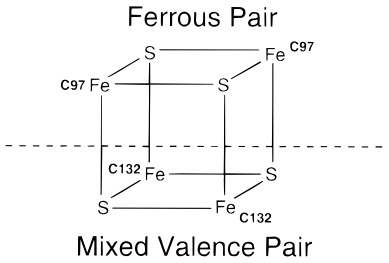


Figure 12. Suggested arrangement of cysteine ligands in the Fe protein.[33]

the addition of MgATP. In the absence of MgATP four signals are observed in the 60−100 ppm chemical shift region. Signals A (49 ppm) and B (23 ppm) each arise from four β-CH_2 protons of cysteinyl ligands while signals C (17 ppm) and D (14 ppm) each arise from two cysteinyl α-CH protons.[33] Further characterization of these ^{1}H NMR data[33] combined with prior Mössbauer studies[17] have led to the assignment of signals A and C to two cysteinyl ligands bound to the mixed-valence iron pair (Fe^{3+}−Fe^{2+}) while signals B, C, and D are assigned to cysteinyl ligands bound to the ferrous iron pair (Fe^{2+}−Fe^{2+}), which, as shown in Figure 12 are suggested to be C132 and C97, respectively. It is important to note that the spectra obtained for *C. pasteurianum* Fe protein (Figure 3) are similar to but not identical with the *A. vinelandii* Fe protein spectra both before and after MgATP binding.

The addition of MgATP to the *A. vinelandii* Fe protein causes only small shifts for each of the four resonances with signal D shifting such that it is no longer observable (Figure 11). Quantitation of these data suggests that signal D has shifted downfield and is concealed beneath signal C.[33] Thus the large conformational change observed upon MgATP binding does not appear to give rise to major changes in the orientations of the cluster ligands. It is also interesting to note that the changes observed for MgATP were not identical to those observed for the nonhydrolyzable ATP analogue Mg$\beta\gamma$-CH_2 ATP (Figure 11).[33] This result is somewhat surprising because previous studies indicated that this analogue resulted in identical EPR spectral changes in the $g = 2$ region for *A. vinelandii* Fe protein to those seen for MgATP binding[72] and identical changes in the ^{1}H NMR spectrum for *C. pasteurianum* Fe protein.[28]

e. Changes That Occur in CD and RR upon MgATP Binding to the Oxidized [4Fe-4S]$^{2+}$ Protein. CD spectroscopy in the visible wavelength region is a useful way to monitor the type of environment of [Fe-S] clusters in proteins. Studies of Fe proteins purified from three organisms have shown that the CD is measurable but weak in both the oxidized [4Fe-4S]$^{2+}$ and reduced [4Fe-4S]$^{+}$ states and that the CD is independent of the bacterial source of the Fe protein.[34,36,37,43,77] Because of the self-oxidation problem discussed in section II.A.2 above no detailed reports have appeared on CD studies of nucleotide binding to the reduced protein. The early studies did demonstrate that the oxidized Fe protein CD spectrum changes upon binding nucleotides. However, the conclusion from these early studies that the effects of MgATP and MgADP are the same is in error probably because MgATP had been hydrolyzed to

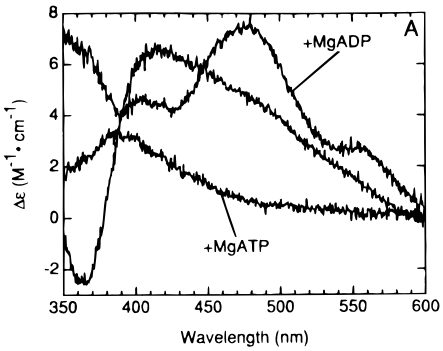


Figure 13. Circular dichroism spectra of wild-type oxidized *A. vinelandii* Fe protein with and without nucleotides.[63]

MgADP during the course of the experiments.[46] (The K_ds for nucleotide binding reported in the early studies were also too low by ∼1 to 2 orders of magnitude.) Figure 13 which comes from a recent study[63] of *A. vinelandii* Fe protein clearly illustrates that the changes that occur upon MgATP binding and MgADP binding are different.

The resonance Raman spectra obtained for oxidized Fe protein samples are similar to solution spectra of synthetic cubane clusters such as $[Fe_4S_4(SCH_2Ph)_4]^{2-}$.[44] Like the bands observed in the synthetic cluster spectra, the Raman spectrum of oxidized Fe protein can be completely assigned under T_d symmetry. However, the bands for the Fe protein are much broader than those found for either synthetic clusters or for simpler ferredoxins. This broadening has again been suggested to arise from numerous slightly different conformational states in frozen solution.[44] The presence of MgATP did not affect the frequencies or intensity patterns of the Raman bands associated with the [4Fe-4S]$^{2+}$ clusters, again suggesting that little, if any, structural perturbation of the cluster itself accompanies MgATP binding. Two additional bands at 289 and 391 cm^{-1} were observed upon MgATP addition; however, these were shown to be artifacts of the [4Fe-4S]$^{2+}$ to [2Fe-2S]$^{2+}$ cluster conversion reaction discussed in section II.A.2 above. It should be noted that this artifactual cluster conversion reaction did not occur during the CD experiments shown in Figure 13 and that those spectra therefore represent real changes that occur in the environment of the [4Fe-4S]$^{2+}$ cluster upon MgATP binding.[46]

f. What Is the Nature of the MgATP-Induced Conformational Change? At present there is no structure available for the Fe protein with MgATP bound. The general direction of the MgATP-induced conformational change, however, has been addressed by small-angle X-ray scattering studies of the wild-type Fe protein and an A157S *A. vinelandii* Fe protein variant that binds MgATP but does not undergo the conformational change.[78] As shown in Table 4 these experiments reveal that the binding of MgATP induces a significant conformational change that is observed as a decrease of about 2.0 Å in the Fe protein radius of gyration. Thus the Fe protein contracts when MgATP binds.

Site-directed mutagenesis experiments have identified three different regions of the Fe protein sequence that are somehow involved in this conforma-

Table 4. Radii of Gyration for the Wild-Type and an A157S Variant of Fe Protein in the Absence and Presence of Nucleotides[80]

	R_g, Å		
Fe protein	in the absence of nucleotides	in the presence of MgATP	in the presence of MgADP
wild-type Fe protein	27.3 ± 0.2	25.2 ± 0.2	27.2 ± 0.2
A157S Fe protein	27.6 ± 0.3	27.1 ± 0.2	NA

tional change. The first region is the Walker A motif shown in Table 2 and discussed in section II.B.1 above. Analysis of a K15Q variant of the Fe protein shows that although it binds MgATP it does not undergo the MgATP induced conformational change.[57] This suggests that the breaking of the salt bridge between K15 and D125 may be a trigger that initiates the conformational change. The second region involved in that conformational change is the polypeptide extending from D125 up to [4Fe-4S] cluster ligand C132. Mutations in this region appear to have the opposite effect in that the resulting Fe proteins tend to be in a MgATP-like or partial MgATP-like conformation under conditions where the wild-type protein does not undergo the conformational change. For example, the Fe atoms of the [4Fe-4S] cluster of a D125E variant can be chelated to some extent without added nucleotide, with ADP and without Mg^{2+}, conditions where little chelation occurs for the wild-type protein.[59] A D129E variant binds MgATP more tightly than the wild-type and without cooperativity. That reduced protein also exhibits an EPR signal at $g = 2.03$ which is observed for the wild-type Fe protein only in the presence of MgATP, it releases Fe^{2+} much faster than the wild-type protein, and it has certain features of both the CD and ^{1}H NMR that are indicative of a MgADP bound state (see section II.B.2.f.4 below).[63] By far the most interesting mutation in this region is a form of the *A. vinelandii* Fe protein that has a L127 deletion.[53] In the absence of MgATP, that protein appears to be stuck in a conformation very close to the one induced in the wild-type upon MgATP binding.[53]

The picture that emerges from these studies is that the binding of the γ-phosphate of MgATP initiates a reaction that allows communication with the [4Fe-4S] cluster over a 19 Å distance via a D125 to C132 signal transduction pathway. The movement of these residues alone however is apparently not sufficient to cause a global 2.0 Å decrease in the radius of gyration because there is a third region of the protein that is also required for the conformational change. This highly conserved region which is shown in Table 5 includes A157. An A157S variant of the Fe protein binds MgATP but does not undergo the MgATP-induced conformational change.[62] This residue is close to the region of the protein that is proposed to bind adenine in the cross-subunit mode[1] and its involvement in the conformational change implies that the region between the two subunits is somehow critically involved in the reaction.

3. Initial Binding of MgADP

MgADP is not only a product of MgATP hydrolysis by nitrogenase but it is also a potent physiological

Table 5. Comparison of NifH, VnfH, and AnfH Sequences in the Region of A157[61,a]

	REGION NEAR A157
	A157 ↑
	* * ** ***** *
A. vinelandii #1	AQEIYIVJSG EMMAMYAANN IARGILKYAH
Frankia A	AQEIYIVTSG EMMAMYAANN IARGILKYAH
Frankia B	AQEIYIVTSG EMMAMYAANN IARGILKYAH
Anabaena 7120	AQEIYIVTSG EMMAMYAANN IARGILKYAH
A. chroococcum #2	AQEIYIVCSG EMMAMYAANN IAKGIVKYAH
A. vinelandii #3	AQEVYIVASG EMMAIYAANN ICKGLVKYAK
K. pneumoniae	AQEIYIVCSG EMMAMYAANN ISKGIVKYAK
Rh. capsulatus	AQEIYIVCSG EMMAVYAANN IPKGILKYAN
T. ferrooxidans	AQEIYIVMSG EMMAMYAANN ISKGVLKYAN
R. meliloti	AQEIYIVMSG EMMALYAANN IAKGILKYAH
R. phaseoli	AQEIYIVMSG EMMALYAANN IAKGILKYAH
R. trifoli	AQEIYIVMSG EMMALYAANN IARGILKYAS
B. japonicum	AQEIYIVMSG EMMAMYAANN ISKGILKYAN
R. parasponia	AQEIYIVMSG EMMAMYAANN ISKGILKYAN
A. caulinodans	AQEIYIVMSG EMMAMYAANN ISKGILKYAN
C. pasteurianum #1	AQEIYIVASG EMMALYAANN ISKGIOKYAK
C. pasteurianum #2	AQEIYIVASG EMMALYAANN ISKGIQKYAK
C. pasteurianum #3	AQEIYIVASG EMMAVYAANN ICKGLVKYAN
M. voltae	AEDVYIVTTC DPMAIYAANN ICKGIKRYGN
Mt #1	AQEIYIVTSG EMMALYAANN IAKGILKYAE
Mt #2	AEQIYVVTSS DYMAIYAANN ICRGISEFVK
M. ivanovi	ADEVYIVTSG EYMALYAANN ICRGI----K

[a] The asterisks indicate completely conserved residues.

inhibitor of nitrogenase turnover. The majority of the information that is currently available supports the view that the Fe protein has two binding sites for MgADP, that it binds MgADP in both its oxidized and reduced states, and that because MgADP is a competitive inhibitor of MgATP binding, it appears to bind to the same sites as MgATP.[50,51,79] Although the binding of MgATP and MgADP are mutually exclusive their binding modes are not necessarily the same.[59] As discussed above, the Fe protein structure does show partial (~0.4) occupancy of a nucleotide in one site that has been modeled as ADP in the cross-subunit binding mode.[1] Studies of mutations at K15 of the *A. vinelandii* Fe protein additionally show that K15 is not needed for interaction with MgADP which is consistent with its binding to the γ-phosphate of MgATP as shown in Figure 5.[58,59]

As was the case for MgATP binding there is great variability in the reported dissociation constants for MgADP;[50,51] however, two conclusions appear to be clear from the available data. First, MgADP binds more tightly to the Fe protein than does MgATP and second, MgADP binds more tightly to oxidized [4Fe-4S]$^{2+}$ containing Fe protein than it does to reduced [4Fe-4S]$^{+}$ containing protein.[50] For example, using the equilibrium column binding method the K_ds for binding to reduced *A. vinelandii* Fe protein are 590 μM for MgATP and 128 μM for MgADP while the K_ds for binding to the oxidized Fe protein are 440 μM for MgATP and 60 μM for MgADP. Also as was the case for MgATP binding, the binding of MgADP shows positive cooperativity.

4. The MgADP-Induced Conformational Change

All currently available information is consistent with the conclusion that MgADP binding to the Fe protein causes a conformational change and that this change is more mild than and quite different from the change caused by MgATP binding. Evidence that some conformational change occurs upon MgADP binding that affects the [4Fe-4S] cluster includes the

observations that (a) the reduction potential is lowered (Table 3);[41,74] (b) the ^{1}H NMR spectra of reduced Fe protein are altered (Figure 11);[28,33] (c) both the $S = {}^3/_2$ and $S = {}^1/_2$ EPR signals are altered;[17,71,73] (d) the high-temperature Mössbauer spectra are altered;[17,73] and (e) the CD spectrum of oxidized Fe protein is altered (Figure 13).[34,36,37,43,46,77] Evidence that this conformational change is different from, and more mild than, that caused by MgATP include the observations that (a) there is no change in the radius of gyration of the Fe protein upon MgADP binding;[78] (b) the cluster remains stable in the presence of chelators when MgADP is bound;[15,42] (c) Fe protein crystals are quite stable in MgADP solutions;[80] (d) the observed changes in the EPR spectrum with MgADP are more minor than those observed for MgATP binding;[73] (e) the $S = {}^1/_2$ and $S = {}^3/_2$ mixture is still solvent sensitive when MgADP is bound;[73] and (f) the CD (Figure 13)[46] and ^{1}H NMR (Figure 11)[33] spectral changes caused by MgADP are different from those caused by MgATP.

At present there is no structure available of the Fe protein with two MgADP molecules bound in either oxidation state. It is also important to remember that the structure of the Fe protein with MgADP bound may not be the same as the structure of the Fe protein following MgATP hydrolysis because the latter initially contains phosphate and is complexed with the MoFe protein.

III. Complex Formation

As shown in Figure 1 the MoFe protein can be viewed as being composed of two identical halves that do not communicate with each other.[50] Each half has one α and one β subunit, one FeMo cofactor center, one P cluster, and one binding site for the Fe protein. For the sake of this discussion the term complex will refer to one Fe protein binding to one-half of the MoFe protein but obviously for the holo MoFe protein both 1:1 and 2:1 Fe protein/MoFe protein complexes are possible. The bulk of available data support the view that during the course of a normal cycle of nitrogenase turnover complex formation is rapid, occurring at close to the diffusion controlled limit, and reversible.[22] The normal reaction also appears to involve reduced Fe protein with two MgATP molecules bound, and it is believed to be independent of the level of reduction of the MoFe protein.[81,82] This section will discuss different approaches that have been used to try to determine the factors that are important for complex formation and to locate the docking sites on the Fe and MoFe proteins.

A. Chemical Cross-Linking

One approach to studying complex formation has involved use of the chemical cross-linking reagent, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide.[83] In the reaction of this cross-linking reagent with nitrogenase one of the identical subunits of the Fe protein dimer is linked by an isopeptide bond to the β subunit of the MoFe protein.[83,84] For *A. vinelandii* nitrogenase this reaction is highly specific involving attachment of Glu112 of the Fe protein to Lys399 of the β subunit of the MoFe protein.[84] It should be noted that although Glu112 is part of a highly conserved patch of acidic residues, Glu112 itself is not conserved. Lys399 is neither conserved nor located in a highly conserved region of the β subunit of the MoFe protein. As discussed in section II.B.2 above it is clear that the structure of the Fe protein changes substantially when MgATP binds. It is therefore surprising that the amount of cross-linked product formed between *A. vinelandii* MoFe and Fe proteins and the rate of complex formation are essentially independent of whether or not nucleotides are present.[83]

B. Mutant Studies That Reveal the Importance of the MgATP-Induced Conformational Change

Two types of experiments are generally done to examine whether or not an altered form of the Fe protein can form a complex with the MoFe protein. One type is based on the observation that the MoFe protein can protect the $[4Fe\text{-}4S]^+$ cluster of the wild-type [Fe protein $(MgATP)_2$] complex from chelators.[85] This observation suggests that normally the MoFe protein binds to the Fe protein in such a way that the Fe protein's $[4Fe\text{-}4S]^+$ is covered up and is no longer accessible to the chelators. Another possible interpretation, however, is that binding to the MoFe protein might cause an additional conformational change in the Fe protein that results in its $[4Fe\text{-}4S]^+$ cluster being made inaccessible to chelators. The second type of experiment is designed to answer the question: can an inactive mutant form of the Fe protein compete with wild-type Fe protein in an activity assay? If the inactive mutant form can still bind normally to wild-type MoFe protein then it should be able to compete such that 50% activity should be observed at a 1:1 ratio of wild-type Fe protein to inactive mutant Fe protein.

Among the first site-directed mutant variants of the Fe protein to be tested for their ability to bind normally to the MoFe protein were K15Q[57] and A157S.[62] Although these mutation sites are far apart the proteins have in common the fact that they bind MgATP but do not undergo the MgATP-induced conformational change. Surprisingly, although both altered Fe proteins appeared to form normal chemical cross-linking complexes with the MoFe protein neither was able to compete with wild-type Fe protein in an activity assay. As discussed in section II.B.2.f above there are now other variants in this general category and a pattern has emerged. Thus, if the protein does not undergo the MgATP-induced conformational change it cannot compete with the wild-type Fe protein in the chelator protection or activity assay. Conversely, Fe protein variants that are stuck in a MgATP-like or partial MgATP-like conformation in the absence of MgATP (e.g. D129E, ΔL127) appear to bind more tightly to the MoFe protein than does the nucleotide free wild-type Fe protein.[53,63]

The inescapable conclusion from these experiments is that the MgATP-induced conformational change is required for the formation of an active Fe protein/MoFe protein complex. It is therefore possible that the specific nature of that conformational change has evolved not because it results in a 100 mV change in reduction potential but rather because it is a neces-

sary prerequisite to form a complex that optimally positions the enzyme for MgATP hydrolysis and electron transfer.

C. Mutant Studies That Examine Regions of the Fe Protein That May Be Directly Involved in Complex Formation

There are numerous reports in the literature illustrating the fact that salts inhibit the overall rate of nitrogenase catalysis and affect the early steps in the nitrogenase catalytic cycle.[39,86] Although there are many points during the nitrogenase cycle where salt could potentially inhibit the reaction (e.g. MgATP binding), there is little doubt that a major reason for the negative effects of salt is that salt inhibits complex formation.[86] This in turn leads to the obvious conclusion that complex formation involves, in part, the formation of critical salt bridges between negatively charged residues on one protein and positively charged residues on the other.

One positively charged residue in the Fe protein that has been extensively studied is an arginine at position 100. This invariant surface residue has been identified in the structure as being close to the [4Fe-4S] cluster on the "top" surface of the protein on the same face as Q112, the residue identified as being involved in the chemical cross-linking reaction.[1] In addition, in some organisms, R100 is reversibly ADP-ribosylated in a process that regulates nitrogenase activity.[87−89] Fe protein variants of R100 are either completely inactive or have greatly reduced activity.[90,91] Studies of two *A. vinelandii* Fe protein variants R100Y and R100H reveal that both are extremely salt sensitive and that they have a decreased affinity for the MoFe protein. This in turn implicates R100 as being one contact point between the two component proteins during normal complex formation. Two other charged Fe protein residues, R140 and K143, have also been shown to be important for complex formation.[85] Thus R140Q and K143Q variants of *A. vinelandii* Fe protein have greatly reduced affinities for binding to the MoFe protein as deduced from salt inhibition and protection from Fe^{2+} chelation experiments.[85]

Another observation that may give clues to the docking site is that a heterologous mixture of the Fe protein from *C. pasteurianum* and the MoFe protein from *A. vinelandii* is known to form a very tight catalytically inactive complex.[92] Two studies have examined portions of the Fe protein that might be responsible for this tight binding by making hybrid *A.vinelandii*/*C. pasteurianum* Fe proteins. In one case the hybrid *A. vinelandii* Fe protein had its carboxyl terminal 18 residues replaced with the five analogous residues from the clostridial protein leading to the conclusion that the tight complex was unlikely to be primarily due to residues in the carboxy terminus.[93] The second study directly tested the proposal, based on the structure,[94] that surface residues in a loop region 59−67 along the top face of the Fe protein were critical for docking.[95] That *A. vinelandii* Fe protein hybrid which had residues 59−67 substituted from the clostridial protein, formed a relatively tighter complex with the MoFe protein providing support for the proposal[94] that a region defined by residues 59−67 within the Fe protein is involved in component protein interaction.[95]

D. Summary: Complex Formation

At present the available data support a model whereby the reduced Fe protein must be in its contracted MgATP conformation prior to formation of an active complex with the MoFe protein. Because the structure of the Fe protein in this conformation is not known, computer-generated models for what the complex might look like must be viewed with caution because they are based on the "relaxed" Fe protein structure.[94] A recently reported variant of the Fe protein that is stuck in a MgATP-like conformation and which forms a tight complex with the MoFe protein may eventually be used to overcome these structural limitations.[53] It has also very recently been demonstrated that a stable complex can be formed between the Fe protein, the MoFe protein, MgADP, and AlF_4^-. This complex can be regarded as a transition-state analogue, and its crystal structure would also provide great insight into the nature of the active complex itself.[96,97] In the meantime, the data support models that involve charged Fe protein residues R100, R140, and K143 as being somehow involved in the complex formation and are consistent with the proposal that a residue 59−67 loop is also somehow involved. These data in turn are consistent with the logical proposal[94] that the $[4Fe\text{-}4S]^+$ cluster of the Fe protein should be positioned in the complex as close as possible to an electron acceptor in the MoFe protein.

IV. MgATP Hydrolysis and Electron Transfer or Electron Transfer and MgATP Hydrolysis

At the beginning of this step in the first cycle of nitrogenase turnover the reduced Fe protein with MgATP bound has formed a complex with one-half of the dithionite-reduced MoFe protein. As indicated above, the structure of that complex is not known and the structure of the conformation of the [Fe protein $(MgATP)_2$] that participates in the formation of that complex is not known. In the next "step" in nitrogenase turnover an electron is transferred from the Fe protein to the MoFe protein and 2MgATPs are hydrolyzed to 2MgADPs and $2P_i$s. Although these reactions are often thought of as occurring concomitantly[50,51,98,99] it is of critical importance to our understanding of how this enzyme works to determine the sequence of events. In other words, is the point of MgATP hydrolysis to overcome a thermodynamic or physical barrier that allows electron transfer from the Fe protein to the MoFe protein or, does the electron transfer depend solely on the formation of the correct complex with MgATP hydrolysis driving some subsequent reaction? In this section we discuss what is known about the mechanism of MgATP hydrolysis, the mechanism of electron transfer and the sequence of events.

A. MgATP Hydrolysis

In general, when MgATP is hydrolyzed three products are formed, MgADP, H^+, and HPO_4^{2-} each of which may be released at different times. This is

in itself a multistep process that occurs via different mechanisms in different enzymes. For example, in some cases the reaction proceeds via phosphorylation of an amino acid in the enzyme and in other cases the reaction involves direct cleavage by H_2O. To try to distinguish between these possibilities Mortenson et al.[100] used ^{31}P NMR to characterize the stereochemistry of the thiophosphate released from nitrogenase when it hydrolyzes chiral MgATP-γ-S labeled with ^{18}O, ^{17}O, and ^{16}O in the γ-phosphate position. They concluded from these experiments that MgATP-γ-S hydrolysis occurs by direct displacement of MgADP by water oxygen without the formation of an enzyme bound phosphorylated intermediate. It was subsequently demonstrated using ^{18}O-labeled ATP that it is the $P_{(\gamma)}-{}^{18}OP_{(\beta)}$ bond that is cleaved in the nitrogenase reaction.[101] A similar one-step mechanism for nucleotide triphosphate hydrolysis occurs for a number of other enzymes including H-Ras p21.[102]

An extremely important observation about MgATP hydrolysis by nitrogenase is that even though the Fe protein binds MgATP no hydrolysis occurs until the Fe protein forms a complex with the MoFe protein. Thus, either the MoFe protein participates directly in MgATP hydrolysis by providing a catalytic group, or binding to the MoFe protein causes a conformational change in the Fe protein that brings an essential group on the Fe protein into the correct position to catalyze MgATP hydrolysis. The only data suggesting that the MoFe protein might be directly involved in MgATP hydrolysis have recently been reviewed elsewhere and show that the oxidized MoFe protein does appear to have binding sites for MgADP.[50,103] It should be noted, however, that the assembly of an active MoFe holoprotein is also a MgATP requiring reaction and that the observed MgADP sites may participate in the assembly reaction rather than in normal nitrogenase turnover.[62] Suggestions that MgATP hydrolysis occurs using catalytic groups only within the Fe protein are based primarily on analogy to H-Ras p21.[94] That protein does hydrolyze GTP alone so presumably all required catalytic groups are on H-Ras p21 itself. It should be noted, however, that the rate of GTP hydrolysis is greatly stimulated by the binding of H-Ras p21 to another protein and the role that the other protein plays in GTP hydrolysis is not known.[104] Theoretical studies suggest that the activation of the nucleophilic water which hydrolyzes the MgGTP or MgATP needs a general base. For H-Ras p21 extensive mutagenesis has still not identified what that base might be and it has even been suggested that GTP itself might function as the base.[105] For nitrogenase, as indicated in Figure 5, Fe protein D39 has been suggested as a candidate for the catalytic residue on the basis of analogy to other proteins, and D129 has been suggested on the basis of its location in the structure but experimental evidence to support these proposals is not yet available.[1,106]

Regardless of the details of the mechanism of MgATP hydrolysis it is clear that once the appropriate complex has formed MgATP is hydrolyzed whether or not electron transfer occurs. This reductant-independent MgATP hydrolysis is most easily illustrated by experiments that utilize dye-oxidized Fe protein (Figure 14);[107] however, there are a number of other situations where it also occurs. For example, MgATP hydrolysis continues when electron flow out of the MoFe protein is blocked by addition of inhibitors like cyanide[108] or by modification of the FeMo cofactor site.[109] MgATP hydrolysis also continues when electron transfer is blocked in certain Fe protein variants, discussed in section III.C above (e.g. R100), that have defects in portions of the Fe protein that are believed to be important for complex formation. MgATP hydrolysis also continues at certain temperatures, component protein concentration ratios, and pH values where electron transfer is not optimized.[110−112] There is no reason to believe that the basic mechanism of this MgATP hydrolysis is any different from the mechanism of reductant-dependent MgATP hydrolysis. Indeed the K_i for MgADP competitive inhibition of reductant-dependent and reduction-independent MgATP hydrolysis are identical, indicating that both reactions occur at the same site.

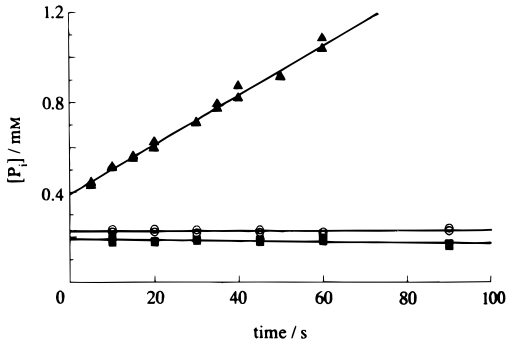


Figure 14. Reductant-independent MgATP hydrolysis is a property of the Fe protein/MoFe protein complex (▲) and not of the isolated MoFe protein (■) or Fe protein (○).[107]

The picture that emerges from the available data is that the only thing that is really critical for turning nitrogenase into an ATPase is the formation of the correct complex, a reaction that in turn requires the MgATP-induced conformational change discussed in section II.B.2 above. Finally, there are two other features of MgATP hydrolysis that have been addressed by studying the reductant-independent reaction. First, Thorneley et al.[107] have shown using a $[^{18}O_4]P_i$−water exchange technique that the ATP cleavage reaction is reversible and secondly, there is good evidence that MgATP can exchange without complex dissociation.[113] The latter point strongly suggests that the MoFe protein does not "cover up" the MgATP-binding site during complex formation.

B. The Relationship between MgATP Hydrolysis and Electron Transfer

During the course of a normal *in vitro* nitrogenase reaction using dithionite as an electron donor, following complex formation, both MgATPs that are bound to the reduced Fe protein are hydrolyzed, and one electron is transferred to the MoFe protein. Where that electron goes in the MoFe protein will be discussed below. At present there are no data that compel us to accept the early proposal that once an electron is in the MoFe protein it can fall back into the Fe protein in a futile cycle.[114] Rather, it seems

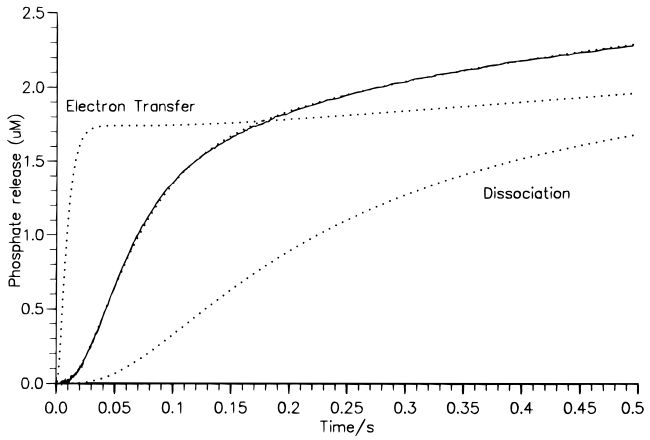


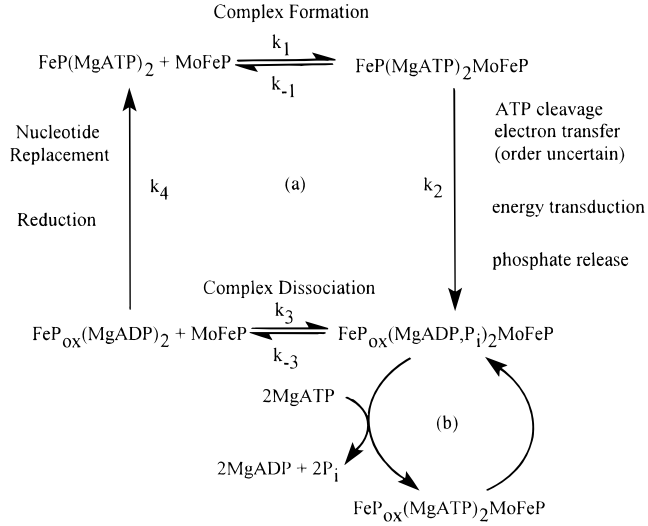
Figure 15. Pre-steady-state phosphate release from nitrogenase.[107] The solid line shows the data, and the dotted lines show simulations with various assumptions.[117]

more likely that this is an example of a unidirectional gated electron-transfer reaction.

In recent years a number of studies have attempted to determine in what order the MgATP hydrolysis and electron-transfer reactions occur and controversy has arisen over the interpretation of results. Techniques used include (a) monitoring phosphate release during pre-steady-state rapid quench of the turning over enzyme;[98,99] (b) monitoring proton production using pH indicators;[115] (c) monitoring heat release by stopped-flow calorimetry;[116] and (d) using a fluorescent probe to monitor phosphate release under pre-steady-state conditions.[117] There are problems with the interpretation of these data. For example, rapid quench cannot distinguish between on enzyme cleavage of MgATP and subsequent phosphate release, whereas heat and pH changes are difficult to assign to a specific reaction. Pre-steady-state real time experiments have shown that P_i is released subsequent to electron transfer and before complex dissociation but do not determine when MgATP hydrolysis occurs (Figure 15).

In reviewing the literature in this area it seems increasingly probable that the order of events may well be different under different sets of conditions. In other words, just as MgATP hydrolysis can be independent of electron transfer it is possible that electron transfer may be independent of MgATP hydrolysis. Both reactions have in common the fact that they require the formation of a specific active complex. However, even though the requirements for electron transfer appear to be more stringent than those for MgATP hydrolysis it is possible that neither reaction is directly coupled to the other. Very recently two lines of evidence have begun to emerge which may support this view. First as discussed in section II.A.4 above it has been reported that a two-electron-reduced form of the Fe protein can serve as an electron donor to the MoFe protein with both electrons being transferred to substrate.[32] More recently, the stoichiometry of this reaction has been investigated and it appears that 2MgATPs are hydrolyzed for every two electrons transferred, which would reduce what was always thought to be the limiting stoichiometry.[118] Second, a mutant of the Fe protein ΔL127 that is "stuck" in a MgATP-like conformation and forms a tight complex with the

Scheme 1. The Fe Protein Cycle of Nitrogenase: Cycles a and b Show Reductant-Dependent and -Independent ATP Hydrolysis



MoFe protein (see section III.B above) appears to be able to transfer electrons to the MoFe protein (albeit at a greatly reduced rate) in the absence of MgATP.[119] It should be noted, however, that these experiments involved monitoring oxidation of the Fe protein rather than directly observing reduction of the MoFe protein. In those experiments only one electron is transferred and no substrates are reduced.

The picture that emerges from all of the studies discussed above is that (a) the role of MgATP hydrolysis is unlikely to be to facilitate electron transfer from the Fe protein to the MoFe protein; (b) the energy transduction step is likely to be phosphate release from the $FeP_{ox}(MgADP,P_i)_2MoFeP_{red}$ complex and/or the accompanying movement of Mg^{2+} (from the γ-β to the α-β position) rather than the initial hydrolysis of MgATP; and (c) the energy transduction step is likely to drive some reaction that occurs within the MoFe protein. Since phosphate bound to the $FeP_{ox}(MgADP)_2$ complex is not required to obtain the complex dissociation rates observed during turnover it is unlikely that energy transduction drives the dissociation process.[117] It should be noted that if the MgATP binding site is far removed from the MoFe protein, as has been suggested,[1] the interface between the Fe and MoFe proteins must be an important part of the energy transduction pathway which must involve conformational changes within both component proteins. Again the structural consequences of those conformational changes are not yet known.

V. Summary Fe Protein Cycle

Following MgATP hydrolysis, electron transfer, and the release of inorganic phosphate, we are left with a complex that contains the oxidized Fe protein, two MgADPs, and the MoFe protein that has been reduced by one electron relative to the dithonite reduced state. As shown in Scheme 1, the next step is the slow dissociation of the $FeP_{ox}(MgADP)_2$ from the reduced MoFe protein. This reaction is the rate-limiting step for nitrogenase activity when all components are at saturating concentrations.[13] As shown

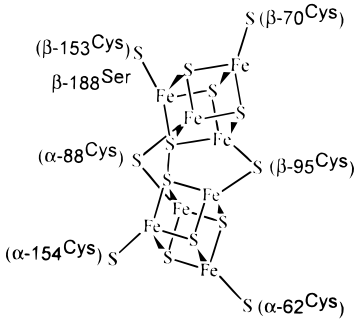


Figure 16. Current model for the structure of the P clusters of the nitrogenase MoFe protein. The clusters are represented with their ligating cysteinyl residues in parentheses and the nearby β-188Ser.[3]

in Scheme 1 it has been proposed that reductant-independent MgATP hydrolysis does not require dissociation but rather depends only upon the steady-state concentration of the $FeP_{ox}(MgADP)_2MoFeP_{red}$ complex. This in turn depends upon many things including the pH, temperature, and the ratio of the two-component proteins. The final steps in the cycle are in order: the reduction of the $FeP_{ox}(MgADP)_2$ complex by $SO_2^{\bullet-}$, the release of 2MgADP, and the subsequent binding of 2MgATP. This sequence appears to be due to the tight binding of 2MgADPs to the oxidized Fe protein as discussed in section II.B.3 above. Once the Fe protein is reduced, MgADP release and MgATP binding appear to be rapid.[81]

Following the first round of electron transfer we are left with the free reduced MoFe protein. As discussed above this protein can go on to form a complex with $FeP_{red}(MgATP)_2$ and the cycle can be repeated over and over again. This leads to the concept discussed below that several reduced states of the MoFe protein exist during turnover which will be referred to as E_0, E_1, E_2, E_3, etc. with the numbers indicating the level of reduction relative to the dithionite reduced state. Under conditions of limited availability of reduced Fe protein, often referred to as low flux, the lesser reduced states will be more prevalent while when Fe protein is in excess the more reduced states will be more populated.

VI. The MoFe Protein

A. General

Some of the most exciting developments over the last few years have concerned our knowledge of the detailed structure of the MoFe protein of nitrogenase. The conclusions have confirmed or explained many previous results but also have given us a number of surprises. They come primarily from the X-ray crystallographic work of the groups of Rees[2−4,6,120] and Bolin[5,7,8,121] and have largely superseded the results from other structural methods like ultracentrifugation and electron microscopy.[122−125] Much attention has naturally focused on the nature of the two kinds of metal- and sulfur-containing clusters and their immediate environments. The models of these two clusters are shown in Figures 16 and 17 and are discussed in fine detail in the accompanying paper on nitrogenase studies by Rees. We will concentrate on those features which we find most interesting but

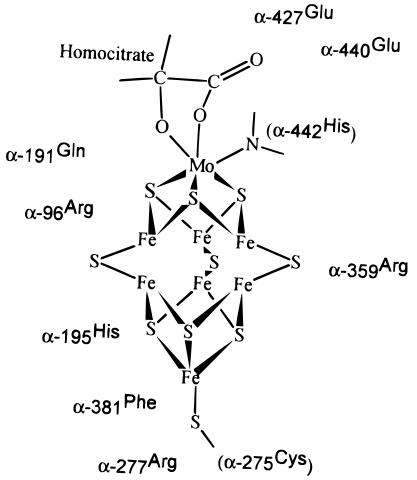


Figure 17. Current model for the structure of the FeMo cofactor clusters of the nitrogenase MoFe protein. FeMo cofactor is represented with the ligating α-275Cys and α-442His in parentheses together with nearby residues, that could interact with the cluster.[3]

note that although there are independent determinations from two groups, they are models based on much spectroscopic information as well as fitting to the X-ray data. This means that they do not have the status of X-ray crystallographic structure determinations of small molecules and although the coarse details must be taken as correct, a number of points may need fine tuning. Other methods, such as EXAFS,[126] can still define interatomic distances with greater accuracy (see below).

We had all hoped that once we knew these structures, we would understand much of how the enzyme works. However, although many ideas, such as the possibility that dinitrogen can bind in a bridging mode between two molybdenum atoms, can be ruled out, we seem to be little closer to knowing what actually happens. We do however finally have a roadmap on which to base ideas and experiments.

The X-ray studies have revealed that the MoFe proteins are symmetrical $\alpha_2\beta_2$ tetramers with the very similar folds of the α and β subunits being related by pseudo 2-fold axes of symmetry. Contacts between the $\alpha\beta$ pairs are almost exclusively between the two β subunits. The P clusters bridge the interface between dissimilar subunits whereas the FeMo cofactor clusters are totally enclosed within α subunits. In addition two cations, probably Ca^{2+} or Mg^{2+}, bind at related sites with ligands from both β subunits. The edge-to-edge distance between FeMo cofactor and the closest P cluster is about 14 Å with this space largely occupied by the homocitrate ligand to the FeMo cofactor and a network of hydrogen-bonded water molecules.

We now consider details of the properties of the clusters before looking at the functioning of the complete enzyme.

B. The P Clusters

1. What Is Their Structure?

Until the last 10 years, P clusters had been thought of as comprising two pairs of "unusual" [4Fe-4S] cubanes. Hagen and co-workers,[127] on the basis of

Table 6. Redox Levels of P Clusters[135]

name	redox potential	spin state	comments
P^N		$S = 0$	oxidation level as isolated in the presence of dithionite
$P^{semi-ox}$	−307 mV	$S = {}^1/_2, {}^5/_2$	observed transiently and in titrations;[134,238] redox potentials close with the value given reported for $P^N \leftrightarrow P^{Ox1}$ [135]
P^{Ox1}	+90 mV	$S = 3, 4$	excited state, parallel mode EPR usually at $g = 12$; spin state controversial[132,135]
P^{Ox2}	+345 mV	$S = {}^1/_2, {}^7/_2$	spin-state admixture of unknown origin
$P^{superox}$		$S > 2$	irreversible damage
P^U		$S = {}^1/_2$	observed in a protein without FeMoco; redox state relative to P^N unknown[137]

EPR studies, contested this concept by proposing that 8Fe clusters could be present. This was confirmed by the X-ray crystallographic work, and the detailed geometry of the 8Fe clusters was revealed, according to Chan et al.,[6] as shown in Figure 16. This model, in which two [4Fe-4S] cubanes are joined by a disulfide bond at one corner plus two cysteinyl residues bridging pairs of iron atoms, is however not fully established. In particular, Bolin's group[7] indicates that the "disulfide" may in fact be a single shared sulfur atom. Although the Rees group has observed loss of a sulfide from the P clusters under some conditions, they did not concomitantly see sufficient movement of the remaining atoms to be consistent with the Bolin structure.[6] A biochemist might question whether the extensive manipulation required by X-ray studies has resulted in damage being done to this delicate protein, whether the proteins remained active in the X-ray beams, and therefore which, if either, alternative is more correct. In addition, the oxidation level of the clusters in these experiments is not precisely defined.

Clusters like that in Figure 16 will have some interesting properties. For example, relative to classical [4Fe-4S] clusters, the bridging cysteinyl sulfides will make more positive the average net charge on each cluster, as would the formation of an S−S bond, thus making the iron atoms more stable in lower oxidation states. Indeed it has been suggested[120] that the reversible reduction and cleavage of the proposed disulfide bond could provide a mechanism for a P cluster to act as a two-electron reducing agent.

Selective mutagenesis has been used to probe the P cluster environment, and the putative cysteinyl ligands were correctly identified before the publication of the X-ray crystallographic results.[128−130] In addition to the cysteinyl thiol ligands, there is a potential interaction with the hydroxyl of a serine residue at position β-S188 and a single Fe atom that is ligated by β-C153.[2−4,6] A β-S188G variant of the MoFe protein gives a protein with a roughly halved specific activity, the same result that is obtained for a β-C153S mutation.[106] A surprising result is that for *K. pneumoniae* MoFe protein, if either bridging cysteine is replaced by alanine, nitrogenase activity is abolished, whereas if both are replaced, some dinitrogen-reducing activity is seen to be retained.[129] In this case other interactions, perhaps from neighboring residues, must be able to maintain the integrity of the cluster. Mutations at all other cysteinyl P cluster ligands give proteins that either do not assemble or are inactive.[106]

2. What Do We Know about Their Oxidation Levels?

P clusters can achieve a number of oxidation levels, the electronic structures of some of which have generated a considerable amount of enthusiastic discussion over recent years; we prefer not to contribute to this. Mössbauer spectroscopy has long provided evidence that, when the MoFe proteins are isolated in the presence of dithionite, P clusters are at an oxidation level called P^N in which all the Fe atoms are essentially ferrous, although a small amount of delocalized ferric character cannot be excluded. Note that we do not in fact know the net charge on the cluster at any oxidation level.

There is as yet no direct experimental evidence for reduction of P clusters below the P^N level. However, if the hypothesis is correct that part of their function is to transfer electrons between the Fe protein and FeMo cofactor, they must either become further reduced during this process or they must reduce FeMo cofactor before accepting an electron from the Fe protein. There is evidence that in a variant of the MoFe protein that has a homocitrate-less species called MoFe cluster substituted for FeMo cofactor, electron transfer involving P clusters can still take place.[109] When the Fe protein and MgATP are added to this protein no substrate reduction occurs, but MgATP is hydrolyzed and a $g_{av} = 1.94$ EPR signal, which integrates to 1 spin per P cluster, develops. Whether the appearance of this signal represents an oxidation or a reduction of the P clusters remains to be decided.

A number of groups have looked at potentiometric and chemical oxidation of P clusters[127,131−134] using EPR, MCD, or Mössbauer spectroscopy. Although the use of solid thionine as a reductant by Hagen et al.[127] has generated controversy,[131] the paper was noteworthy because it contained the suggestion that P clusters might be 8Fe centers. The overall results are summarized in Table 6 which is based largely on[134] where extensive redox titrations are reported. It should be noted that exact redox potentials vary between organisms.[135] If each reaction involves a one-electron change, this would be consistent with the report that a FeMo cofactor-deficient protein undergoes only a four-electron oxidation above the dithionite reduced level,[136] compared with six-electron oxidations shown by the holoproteins.[135] There are inconsistencies, regarding the occurrence of hysteresis in these processes, between the results of different groups of workers. Certainly the conversion of P^{Ox2} to $P^{Superox}$, which results in irreversible damage to the cluster, is not thermodynamically revers-

ible but the other reactions seem to be reversible when a variety of redox dyes are used to establish equilibrium between the protein and the electrodes. Perhaps the inconsistencies are a result of slow equilibration and poor accessibility when using single dyes as electron-transfer mediators. This possibility would also explain different, unexpected numbers of electrons being seen to be taken up in consecutive oxidation waves using different proteins, in particular the *A. vinelandii* MoFe protein being oxidized by 3 + 3 electrons and *C. pasteurianum* MoFe protein by 1 + 5 electrons.[135] The inconsistencies could also arise from varying proportions of eight vs seven sulfide forms of the P clusters being present in different samples (see section VI.B.1 above).

An alternative nomenclature using P^{n+} has been suggested[133] for the various oxidation levels, using $n = 0$ for P^n. A description based on the latter will clearly be better than the phenomenological scheme we use in Table 6, but confusion could arise at the moment because it might be taken as implying a known charge on the clusters. Tittsworth and Hales[133] also achieve a simulation of many features of the dependence of the intensities of the $S = {}^1/_2$, $S = {}^5/_2$, and $S = 3$ (or 4) signals on adding oxidant, using a random distribution of electrons between P clusters that give the $S = {}^1/_2$ or $S = {}^5/_2$ signal after accepting one electron and the $S = 3$ (or 4) state after two. Their data is not extensive, however, and the quantitative agreement between experiment and simulation is therefore insufficiently complete to be totally convincing.

Interestingly, the various oxidation levels generally have multiple spin states with facile interconversions[137] and closely spaced energy levels dominating their electronic structures. Perhaps this is because such relationships are useful in satisfying the Frank–Condon principle for a center involved in multiple electron transfers. Despite the undoubted intellectual interest in the detailed electronic structures of each oxidation level, whether these are relevant to the functioning of nitrogenase remains to be seen. This will depend upon whether P clusters behave as capacitors and are involved in multiple electron transfer reactions, or simply operate between two oxidation levels. If the latter is the case their complex and, as far as we know, unique structure would seem to be an eccentric design feature.

There is an intriguing indication,[138] from the observation of an increase in A_{430}, that an oxidation of Fe-S clusters occurs within the MoFe protein when and only when the enzyme is at the point in its catalytic cycle when it is primed for the initial reduction of dinitrogen—see below and Figure 18. A simultaneous observation of EPR signals similar to those given by oxidized P clusters led the authors to suggest that at this point an oxidation of these centers may result in the generation of the reducing equivalents needed for the difficult initial reduction of dinitrogen. Because the increase in A_{430} is observed specifically with dinitrogen these are the first spectroscopic observations of different effects during the turnover of nitrogenase with different substrates. We await with interest confirmation (or not) of the suggestion that the P clusters become more oxidized than the P^N state during turnover. Unfortunately, such experiments are made particularly difficult since only at most ~15% of the MoFe protein is ever in this state.

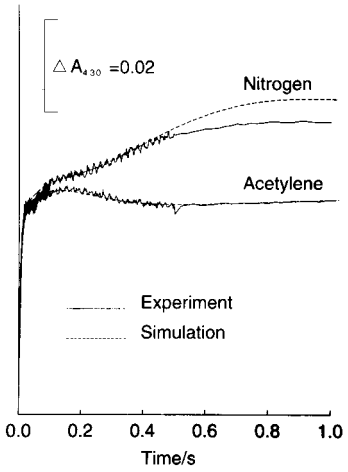


Figure 18. Time course of absorbance changes for nitrogenase turning over in the presence of nitrogen and acetylene. The changes in A_{430} are shown, together with simulations based on the Thorneley–Lowe scheme, demonstrating a spectroscopic effect of the substrate being reduced.[138]

Table 7. Comparison of Bond Lengths Obtained from EXAFS and Current Resolution X-ray Structure Information

sample	method	Mo–S	short Mo–Fe	long Mo–Fe
MoFe protein	X-ray	2.42–2.47	2.92–2.95	5.13–5.39
MoFe protein	EXAFS	2.37	2.70	5.06

C. The Iron–Molybdenum Cofactor

1. What Is Its Structure?

Until 1992, there were many publications describing work on trying to understand the make up of the FeMo cofactor.[139–143] The cluster structure, shown as finally revealed in Figure 17 and discussed in detail in Howard and Rees' review in this issue, was a combination of fitting various spectroscopic results[144–146] to the electron density map and has a number of features which we, among others, find interesting. These include (1) the six-coordination of the Mo atom, which some authors have considered saturating despite the existence of seven[147] and even eight[148]-coordinate molybdenum compounds; (2) the unusually distorted coordination of the six trigonal Fe atoms; (3) the extensive hydrogen-bonding networks in the region of the homocitrate and the cluster sulfides; (4) the neighboring residues, which profoundly affect spectroscopic features of the FeMo cofactor[149] and the protein's ability to reduce substrates (especially dinitrogen[150]), despite not being directly bonded to the cluster.[149,151] All these structural details must be taken as correct, at this level of resolution, although the comparison of bond length information from the X-ray crystallographic studies with those obtained with the more accurate EXAFS[126] technique (Table 7) shows significant discrepancies. It is generally assumed that FeMo cofactor contains the binding site for dinitrogen; but where? The

possibilities seem only to be limited by the imagination of the observers; perhaps one of the extant suggestions is correct.

2. What Redox Levels Can It Adopt?

As prepared, with the MoFe protein in dithionite, FeMo cofactor is in the widely studied $S = {}^3/_2$ state with a characteristic EPR spectrum (g values close to 4.3, 3.7, and 2.0) that can even be recognized in whole cells. It is relatively easy to oxidize it reversibly, chemically, or potentiometrically, by one electron to a diamagnetic state.[134,135] It can be removed intact from the protein by various solvent extractions in weakly acid solution[135,142,152−154] and in this state is still capable of single electron redox chemistry to a perhaps more reduced as well as a more oxidized level.[155−158] Since the cofactor becomes EPR silent during enzyme turnover, following reduction by the Fe protein, it is normally assumed that FeMo cofactor becomes reduced in enzyme that is actively reducing substrates. An engaging recent report of EXAFS on the MoFe protein, maintained partially in a turning over state by addition of limiting Fe protein,[159] shows that each time the MoFe protein becomes reduced by one-electron equivalent a significant number of metal−metal distances contract by about 0.04 Å. Presumably this result indicates that electrons do not go into antibonding orbitals as the FeMo cofactor is reduced. This behavior is unusual among iron−sulfur clusters which generally expand on reduction. In terms of the discussion below, about where substrates might bind, it could be that a net contraction of the two four-metal halves of FeMo cofactor results in an expansion of the central cavity.

VII. Substrate Binding and Reduction

A. Binding

1. Spectroscopy

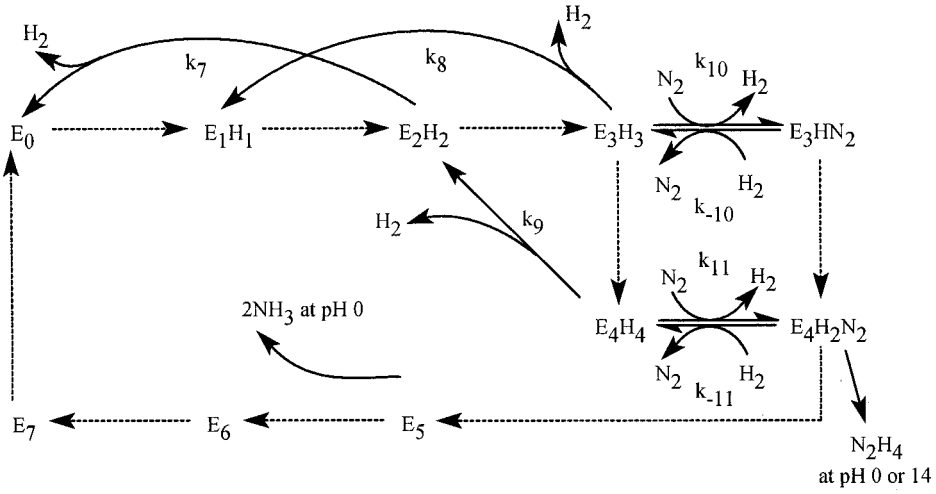
Although it is generally accepted that substrates (with the possible exception of protons) always bind and are reduced at the FeMo cofactor center of the MoFe protein, this implies nothing about the redox level(s) of this protein with which they interact. ENDOR studies (Nelson, M. K.; True, A. E.; Orme-Johnson, W. H.; Hoffman, B. M., personal communication)[160] of the $S = {}^3/_2$ level of FeMo cofactor within the MoFe protein have revealed no associations, affecting hyperfine interactions with protons or ^{57}Fe, of FeMo cofactor upon the addition of a wide variety of substrates. The only exception appeared to be a small effect of methyl isocyanide on the ^{57}Fe ENDOR of a single class of iron nuclei. In addition, no interactions have been seen between Mo and CO, cyanide, azide, or CH_3NC, using EXAFS of isolated MoFe protein at this redox level, although a recent ENDOR paper has shown that the inhibitor CO can bind to the FeMo cofactor site of the turning over enzyme.[161] We have the old account[162] that a pK_a, observed using EPR of the $S = {}^3/_2$ signal, is shifted in the presence of the substrate acetylene which makes it surprising that nothing is seen by ENDOR. Perhaps the EPR effect is due to a change in general hydrophobic interactions rather than a direct observation of substrate binding. Note that there is of course nothing magic about the $S = {}^3/_2$ redox level, except its experimental accessibility; it is merely the level at which the MoFe protein normally presents itself to us. Thus if only particular redox levels interact with substrates there is no a priori reason why the $S = {}^3/_2$ level should be one of these. George et al.[163] have shown that it is possible to simulate the EPR spectrum of free FeMo cofactor using a Gaussian distribution of both axial distortions and rhombicity, indicating that in solution the isolated FeMo cofactor is extremely flexible. This may mean that when it is bound to protein, by only two covalent bonds, it is able to adopt a variety of conformations depending upon its redox level and ligation by substrates.

2. Kinetics

The kinetics of nitrogenase action are best understood in terms of the MoFe protein cycle introduced by Thorneley and Lowe[13] and shown in Scheme 2. This comprises the eight Fe protein cycles (Scheme 1) that are required to transfer eight electrons and eight protons to one-half of the MoFe protein in order to reduce N_2 to $2NH_3$ and to evolve one H_2 (for a discussion of concommitant H_2 evolution see section VII.C.5, below). There is abundant kinetic evidence that various substrates bind to the MoFe protein at a level more reduced than that with $S = {}^3/_2$ EPR[13] and that the exact level depends upon the nature of the substrate. Consequently the substrates binding to relatively more oxidized levels will be noncompetitive inhibitors of those binding to more reduced levels and will also be preferentially reduced when there is a low electron flux though the MoFe protein. In particular N_2 binds after a three- or four-electron reduction below the dithionite-reduced level (E_3 or E_4 of Scheme 2), so that it is only efficiently reduced at high electron flux. It is an essential feature of the Thorneley−Lowe scheme that substrates bind only to, and products are released only from, free MoFe protein, and that the site(s) at which this occurs is protected from access by solvent in the relatively long-lived complex between the Fe and MoFe proteins. These assumptions were necessary to explain quantitatively the observations that (1) dihydrogen is only released from nitrogenase, on quenching in acid, after two protein−protein dissociations had occurred, even though two electrons had been transferred to the MoFe protein before the second dissociation; and (2) the percentage of electron flux diverted from ammonia formation into dihydrogen evolution depends on the electron flux through the MoFe protein.[13] This mathematical feature of the scheme may indicate that protons are only able to move within the highly ordered waters, observed by X-ray crystallography in the FeMo cofactor environment, as the component proteins dissociate. It is of note that the crystal structures were done on free protein so that the bound water structure may well be different in the protein−protein complex.

The kinetic evidence for how substrates bind has been discussed before.[14,164] All we note here is that they vary in size from the proton up to CH_2=CHNC and 1-butyne (~5.5 Å), so that the binding site must

Scheme 2. The MoFe Protein Cycle of Nitrogenase[a]



[a] In this scheme[13] the E_n represent a functional half of the MoFe protein (MoFeP), comprising one $\alpha\beta$ polypeptide pair plus one FeMo cofactor and one P cluster, which has been reduced by n electrons. Each dotted arrow represents a complete Fe protein cycle (Scheme 1) that transfers one electron from FeP to MoFeP. Rate constants are found in Table 8.

Table 8. Rate Constants of the Reactions in Schemes 1 and 2[a]

rate constant	value	comment
k_1	5×10^7 M^{-1} s^{-1}	responsible for lower activity at low protein concentrations
k_{-1}	15 s^{-1}	
k_2	200 s^{-1}	electron transfer from FeP to MoFeP
k_3	4.4×10^6 M^{-1} s^{-1}	responsible for lower activity at high protein concentrations
k_{-3}	6.4 s^{-1}	rate-limiting step when substrates and FeP are saturating
k_4	3×10^6 M^{-1} s^{-1}	rate of reduction of $FeP(MgADP)_2$ complex
k_6	1.2×10^9 M^{-1} s^{-1}	rate of dissociation of $S_2O_4^{2-}$ into $2SO_2^{\bullet-}$
k_{-6}	1.75 s^{-1}	rate of association of $2SO_2^{\bullet-}$ to $S_2O_4^{2-}$
k_7	250 s^{-1}	gives increased H_2 evolution at low electron flux
k_8	8 s^{-1}	slow to maximize E_3 concentration and hence N_2 binding
k_9	400 s^{-1}	rapid H_2 evolution from most reduced hydridic species
k_{10}	4×10^5 M^{-1} s^{-1}	determine K_m^{N2} and K_I^{H2} at low electron flux
k_{-10}	8×10^4 M^{-1} s^{-1}	
k_{11}	2.2×10^6 M^{-1} s^{-1}	determine K_m^{N2} and K_I^{H2} at high electron flux
k_{-11}	3×10^6 M^{-1} s^{-1}	

[a] The values are for *K. pneumoniae* nitrogenase at 23 °C, pH 7.4.[13]

have significant flexibility. There is also evidence that in some cases more than one molecule can bind simultaneously since additive effects of substrates and inhibitors can be observed. It is important in this context, and when considering the way in which various substrates and inhibitors compete for sites on nitrogenase, to consider exactly what is meant by a site. It should be remembered that fully competitive kinetics will not always be observed when two molecules bind at the same coordination position of the same metal. This would happen if they bound to different oxidation levels (e.g. E_2 and E_3 of Scheme 2) or when other ligands have changed. In this case the kinetic behavior would suggest that they bind to different sites.

3. Other Nitrogenases

While these are discussed in detail in Eady's article in this issue, the mere fact of the existence of vanadium and (perhaps) iron only nitrogenases must influence our perceptions of substrate binding. There is clear genetic and biochemical evidence that cofactors similar to FeMo cofactor occur in the other two classes. Presumably dinitrogen, and other substrates, bind in a similar manner to all of them. This has led to suggestions that only the iron (and/or sulfur!) atoms are involved in substrate binding and that the role of the heterometal is to subtly modulate the properties of the atoms at the binding site in the way seen in model systems.[165] It is also plausible that the rest of the cofactor modulates the properties of the atom at the "molybdenum" site to effectively bind dinitrogen; there may be no reason in principle why molybdenum, vanadium, or iron cannot do this job.[166]

What we need is good structural evidence about the binding site from some really imaginative experiment. Perhaps this could be a new spectroscopic probe, or maybe a suicide inhibitor that is reduced and then binds irreversibly at the same site which can then be characterized structurally. If we were sure that the inhibitor carbon monoxide binds at the substrate reduction site, then the observation of its binding to FeMo cofactor[161] would help us in this search.

B. Reduction—General Considerations

1. Electron Allocation

In most circumstances, when nitrogenase is reducing substrates, the electron flux through the MoFe protein depends only on the concentrations of reduced

and oxidized Fe protein and of MgATP and MgADP. In particular it is independent of the substrate being reduced (apart from some substrates that also inhibit total electron flux). Thus the effect of varying the relative concentrations of competitive substrates is only to change the allocation of reducing equivalents between them.

2. Protons and Electrons

It does not escape the attention of students of this enzyme that the reductions it catalyzes all (with the possible exceptions of nitrite and azide) involve the addition of an equal number of pairs of protons and electrons to the substrate. Considerable effort has been put into following the electronic part of the process, but relatively little into trying to understand how the protons are delivered. It might be thought that protons are plentiful in aqueous solution so that their arrival at the substrate reduction site is facile. But perhaps this is the problem. Certainly the Thorneley−Lowe scheme[13] suggests that many of the properties of the enzyme constitute a considerable effort to exclude unwanted protons from the substrate reduction site, in order to prevent the build up of reducing power being used solely to produce dihydrogen before dinitrogen can bind at the highly reduced E_3 or E_4 levels (see Scheme 2). If this is so, the protons must be delivered one (or two) at a time, perhaps via one of the linked hydrogen-bonding networks around the FeMo cofactor, or perhaps a job of ATP hydrolysis is to allow such a controlled transfer of protons?

An extensive study of the variation of substrate reduction with pH has attempted to address this problem.[167] Dihydrogen evolution under argon needs a group with a pK ~6.3 to be deprotonated, and one with a pK ~9.0 to be protonated. CO and C_2H_2 shift the pK of the 9.0 group to about 8.5 whereas N_2 does not affect it; the acidic group is not affected by CO and N_2, but C_2H_2 shifts it by 0.4 in the acid direction. Thus CO appears to inhibit dihydrogen evolution at pHs around 9.0 in the same way as for the nifV$^-$ phenotype, in which the homocitrate moiety of FeMo cofactor is replaced, typically by citrate. Presumably the ionizable groups seen here are close to the FeMo cofactor and are perhaps involved in proton delivery. It may be that this is the mechanism by which mutating residues in the region of FeMo cofactor binding pocket causes subtle alterations in the sensitivity of H_2 evolution (and C_2H_2 reduction) to CO inhibition.[151] MoFe protein α subunit residue H195 is a good candidate for the more acidic group because of its known importance in cyanide inhibition (see below).[168] Similar differential effects on H_2 and C_2H_2 reduction can be produced with various antibodies.[169]

In addition to the issue of how protons are transferred through the protein matrix to the FeMo cofactor site, it is important to ask where those protons reside at FeMo cofactor prior to substrate reduction. A recent study of the sequential three-electron reduction of the [3Fe-4S]$^+$ cluster of *A. vinelandii* ferredoxin I to the [3Fe-4S]$^{2-}$ state[170] showed that following the addition of the first electron, a proton apparently had to be added to neutralize the charge before the second electron could be added. In that system there is compelling evidence that the first proton is added directly to the [3Fe-4S]^0 cluster, most likely on a sulfide atom, and that the proton transfer is slow relative to the electron transfer.[170] It is possible that this type of sequential electron/proton transfer (i.e. a net hydrogen atom transfer) occurs for the FeMo cofactor site of nitrogenase as well, with the three bridging sulfide atoms being likely candidates for the sites of protonation. This process could provide an explanation for why the system is set up to add electrons from the Fe protein at such a slow rate. Thus, time may be needed for the reactions, giving a slow transfer of protons through the protein matrix, and for proton transfer to FeMo cofactor, before the next electron can be added. Obviously this is not the only possibility. For example, metal hydrides have long been proposed to be involved in substrate reduction by nitrogenase.[13] Unfortunately at present, there is no physical evidence to support any hypothesis for proton transfer.

Following electron transfer from the Fe protein, the $S = {}^3/_2$ FeMo cofactor center of the MoFe protein is reduced to an EPR-silent, but paramagnetic state.[70,71,171,172] The disappearance of this EPR signal occurs following the addition of one electron per FeMo cofactor center, indicating that FeMo cofactor is the final location for the first electron that enters the MoFe protein from the Fe protein.[172] Although it seems likely that the electrons are transferred from the Fe protein to the P clusters and then to FeMo cofactor, there is currently no compelling experimental evidence to suggest that this is the case. Because all products that leave nitrogenase have been reduced by multiples of two electrons, the MoFe protein must be able to accumulate several electrons before products are released. At present it is not clear if the electrons are stored in P clusters, in FeMo cofactor, or if when they enter the MoFe protein one at a time, they are immediately used to produce enzyme-bound substrate reduction intermediates. An insight into this problem of how electrons are transferred within the MoFe protein is given by the MoFe protein variant, discussed above in the section on oxidation levels of P clusters, which can be constructed to contain a version of the cofactor, called the MoFe-cluster, without homocitrate.[109,173] This protein cannot reduce protons or acetylene, but is capable of supporting MgATP hydrolysis in the presence of the Fe protein and dithionite as well as undergoing redox at the P clusters.[109] In the future this variant should provide a useful probe of electron-transfer mechanisms within the MoFe protein.

Substitution of the cysteine ligands to P clusters by residues also capable of forming bonds to Fe, especially serine, have supported the proposal that P clusters are involved in electron transfer through the MoFe protein.[168] Most substitutions at the terminal cysteines abolish activity, although β-C153 can be replaced by serine or glycine to give a 50% lowering of electron flux as well as affecting the spectroscopic characteristics of the P clusters. Similar effects occur after changing one of the bridging cysteines (α-C88) to glutamine, glycine, or threonine. The electron flux can be also lowered by replacing

β-Y98, in the region of the MoFe protein partway between the P clusters and FeMo cofactor, by histidine although spectroscopic properties of the clusters remained unchanged.[174] This was interpreted as indicating that this MoFe protein variant had an impaired ability to transfer electrons between its clusters and hence that the electron-transfer pathway involved this region. It should be noted, however, that no X-ray structures have yet appeared of any site-directed mutant variants of the MoFe protein so that interpretation of kinetic data on these proteins must be viewed with caution.

C. Reduction—Individual Substrates

It is not our intention to give a complete account of the effects of all substrates, rather the reader should refer to previous reviews for the wider scene.[14,164] Instead we focus on recent information, older work we find newly relevant, new substrates, and especially on the effects of subtle variations around the FeMo cofactor site, such as changes in the homocitrate and adjacent amino acids. For the sake of completeness, the known substrates not discussed in some detail below are N_2H_4, N_3^-, $CH_3C{\equiv}CH$, $C_2H_5C{\equiv}CH$, $H_2C{=}C{=}CH_2$, $CH_2(HC{=}CH)$, $CH_2(N{=}N)$, C_2H_5CN, C_3H_7CN, C_2H_5NC, $H_2C{=}CHNC$.

1. Methyl Isocyanide, Cyanide, and Cyanamide

These interact with nitrogenase as both substrates and inhibitors.[108,175−177] In the second of these roles, CH_3NC and CN^- inhibit the total electron flux through the enzyme far more than they inhibit ATP hydrolysis (this latter activity has not been studied in the presence of cyanamide). Competition experiments are consistent with binding as substrates and inhibitors, being at the same site for each compound.[175,176] Note that the earlier conclusion that cyanide and HCN bind independently, with one being the substrate and the other the inhibitor,[108] is inconsistent with later results.[176] Surprisingly, mutating MoFe protein α subunit residue H195 to glutamine abolishes cyanide inhibition without affecting substrate reduction.[168] If the proposal[176] that cyanide acts as an inhibitor only when it is not protonated is correct, this result strongly implicates α-H195 in proton transfer to FeMo cofactor and/or to substrate.

CH_3NC is reduced to methane, methylamine, and dimethylamine (plus a small amount of ethylene and ethane). CN^- and $N{\equiv}CNH_2$ give methylamine, ammonia, and methane. The ratios of these various products depend on electron flux and substrate concentration with the more reduced ones favored at higher flux. There is an interesting fully characterized chemical parallel for reduction of bound cyanide to aminocarbyne ($-CNH_2$) at molybdenum on a four-electron reduction path to methylamine.[178]

These substrates can completely suppress dihydrogen evolution and are optimally reduced at a relatively low Fe protein to MoFe protein ratio of about 2.5:1. These observations have been interpreted as indicating that they are reduced by MoFe protein at a redox level more oxidized even than the one from which dihydrogen is evolved, E_2. Since an essentially equimolar mixture of E_0 and E_1 can be prepared at high MoFe protein to Fe protein ratios, biophysical measurements on such a mixture in the presence of, say, methyl isocyanide may give us the first real information on how a substrate binds to nitrogenase. Our optimism about the success of such experiments is tempered by the realization that, strictly speaking, the results do not necessarily mean that these substrates must bind (not necessarily be reduced) at E_0 or E_1. Another possibility is that they could also bind to E_2, but much faster than H_2 is evolved at this level.

The only one of these substrates to have been studied using pre-steady-state kinetics is cyanide.[176] This has revealed the intriguing property that both product formation and inhibition of dihydrogen evolution on quenching in acid, are delayed by about 3 s after the start of enzyme turnover. A similar delay in the inhibition, by cyanide, of dihydrogen evolution without quenching has also been seen using a membrane-leak reaction chamber linked to a mass spectrometer.[179] The lengths of these delays are inconsistent with their being due to the need to reduce the MoFe protein by a reasonable number of electrons. It would not be surprising if such a delay also occurs with the other substrates in the group. The proposal is[176,179] that some covalent modification of the protein, perhaps a displacement of a metal ligand, is necessary before the substrate binding site is fully formed.

Interestingly, ^{19}F NMR of a fluorinated thiol ligand has been used to show that methyl isocyanide and cyanide bind to isolated FeMo cofactor in the $S = {}^3/_2$ state, with parallel EXAFS work on the MoFe protein, demonstrating no interaction with the Mo.[180] Does this mean that they do not bind at Mo during turnover? Or perhaps the binding site observed in these experiments is not the functional one in the protein. Indeed EPR and MCD studies,[181] taken together with the NMR work,[180] show that cyanide can bind to isolated FeMo cofactor at two sites and that it is displaced from one of these by thiols. Since EXAFS experiments have shown that selenol, as a model for thiols, binds to an iron atom,[182,183] it seems reasonable to propose that the other site could be at Mo. This is consistent with the application of a new high-resolution EXAFS technique, demonstrating a change of Mo coordination consistent with cyanide binding.[126]

2. Acetylene and Ethylene

Acetylene is one of the longest established nitrogenase substrates and had been thought to be reduced exclusively to ethylene by molybdenum-containing nitrogenases. It is routinely used for estimating the activity of preparations of the enzyme since this reduction is characteristic of nitrogenase and ethylene is relatively easy to quantitate, especially when compared with ammonia.

The reduction of protioacetylene to *cis*-deuterioethylene in D_2O by nitrogenase has been used to support models that imply sideways binding and reduction of substrates. However, in a sterically restricted site, it is quite possible for side-on binding to be followed by a rotation to the end-on position and protonation to *cis*-deuterioethylene if the initial protonation occurs at the metal.[184]

Recent chemical kinetic work on relatively simple metal sites[184] has shown that whether the product is exclusively ethylene, or whether this can be reduced further to ethane, depends on where the initial protonation or hydrogenation occurs. It is therefore not surprising, in retrospect, that under different conditions, some ethane can be produced and/or ethylene can be a substrate in the biological systems. For example: (1) ethylene inhibits total electron flux and ATP hydrolysis by *K. pneumoniae* nitrogenase; (2) ethylene is reduced to ethane in a reaction consuming <1% of the total electron flux at Fe protein to MoFe protein ratios of five although no ethane could be detected when acetylene was being reduced to ethylene;[185] (3) ethane is a product of acetylene reduction by vanadium nitrogenase, by a MoFe protein varient with residues close to the FeMo cofactor mutated,[150,151] by an unusual wild-type molybdenum-containing nitrogenase[186] and by conventional MoFe protein at elevated temperatures.[187]

Steady-state and pre-steady-state kinetic work on the enzyme has indicated that ethylene formed from acetylene reduction is not released on quenching the enzyme with acid until the E_3 level of reduction of the MoFe protein has been reached.[188] C_2H_2 binds, however, at more oxidized levels,[188] explaining the diversion of electron flux from C_2H_2 reduction into N_2 reduction at higher Fe protein to active MoFe protein ratios.[189] At high protein concentrations, C_2H_2 inhibits nitrogenase by increasing the association rate between MoFe protein and oxidized Fe protein. Although CO inhibits the reduction of C_2H_2 it does not affect this concentration effect, showing that both must bind simultaneously.[188] It is not clear whether CO displaces C_2H_2 from the site at which it is reduced, with another C_2H_2 site affecting total activity, or whether CO binds to a different site but prevents the transfer of electrons to C_2H_2. Nonquenching experiments with *A. vinelandii* nitrogenase showed a significant burst, close to 1 mol of H_2 per mole of Mo, in the presence of C_2H_2 at the very low Fe protein to MoFe protein ratio of 0.76.[179] In contrast, quenching experiments showed no such burst with *K. pneumoniae* nitrogenase at the higher Fe protein to MoFe protein ratio of four.[188] The kinetic schemes presented in refs 107 and 179 cannot explain all these data unless reasonable assumptions are made about the nitrogenases from the two organisms having different rate constants for a number of partial reactions, especially for hydrogen release and interprotein complex formation; there is evidence that such differences exist.[190]

3. Nitrogen Oxides and Nitrite

Although NO was long ago established as an inhibitor of nitrogenase, it is difficult to work with because of its reactivity with dioxygen and because its inhibitory effects are seen at very low partial pressures. Nevertheless it has been shown to inactivate the Fe protein,[191] and possibly the MoFe protein,[192] irreversibly as well as being a noncompetitive inhibitor of proton, C_2H_2, and N_2 reduction. The inactivation of nitrogenase *in vivo* by hydroxylamine is probably caused by the production of small amounts of nitric oxide.[193] NO reacts with many metals and there is good evidence that it reacts with the iron−sulfur cluster of the Fe protein, needing less than a 2-fold molar excess of the gas over the protein to complete the inactivation.[192] At these low concentrations it oxidizes the cluster and abolishes the ability of the protein to bind nucleotides. At high concentrations a characteristic iron−nitrosyl EPR signal develops. Contrary to previous views,[14,164] there is now kinetic evidence indicating that NO is reduced when it acts as a competitive inhibitor[192] but no products have yet been identified.

Nitrous oxide was the first compound, other than dinitrogen itself, that was shown to be reduced by nitrogenase. It acts as both a substrate and a competitive inhibitor.[194] The reaction catalyzed is the two-electron reduction of N_2O to N_2 and water although a significant amount of ammonia is also formed. There is an inconsistency in trying to understand the ammonia formation data. Jensen and Burris' proposal is that NH_3 comes from the further reduction of the product N_2,[194] yet when they saw the expected inhibition of NH_3 formation by H_2 there was no corresponding increase in N_2 production. This suggests that at least some of the ammonia arises from a different pathway. When data are extrapolated to infinite N_2O partial pressure, it appears to be capable of completely suppressing H_2 evolution. It is competitive with N_2 reduction but does not support HD formation from D_2 in H_2O; indeed it inhibits this reaction in the presence of N_2 with the same kinetics as it inhibits N_2-dependent NH_3 formation.[194] HD formation is discussed in more detail in section VII.C.5, below. It would be interesting to know whether the protons giving rise to the water formed from N_2O reduction are the same as those putatively bound at the N_2 reduction site. If this were so, T_2 inhibiting N_2 reduction could be incorporated into the water product in the presence of all three gases.

Nitrite is a relative newcomer on the scene.[195] It inactivates the Fe protein irreversibly, probably by binding to the iron−sulfur cluster, in a reaction that is enhanced by MgATP. It has been suggested that the true inactivator is NO since this would be produced in small amounts in these experiments as a result of the reduction of nitrite by dithionite[192] The work of Vaughn and Burgess,[195] however, indicates that this is not the case. Also, in a similar fashion to nitric oxide, it is a competitive inhibitor of proton and acetylene reduction, being itself reduced by six electrons to ammonia, without inhibiting total electron flux.[195] Such six-electron reductions are extremely rare in biology and are only catalyzed by three enzymes: nitrogenase, assimilatory nitrite reductase (also converting nitrite to ammonia), and sulfite oxidase (reducing sulfite to sulfide). Since nitrogenase has been shown to catalyze the first two of these processes, it would be interesting to see whether it could catalyze the third, although the assays would have to be done in the absence of the usual reductant, dithionite, whose oxidation products would interfere with any measurements.

We do not know how any of these N−O bond-containing molecules react with nitrogenase. Our prejudices, from the known chemistry of such mol-

ecules, are that the linear N_2O binds via its O and that NO and nitrite via their N. This could explain the similar effects of the latter two. It is possible that N_2O could also bind via its N and that in this case it is alternatively reduced to hydroxylamine plus ammonia in a reaction which would not be inhibited by dihydrogen, thus providing an alternative route for ammonia formation (see above). Note that the harder, less polarizable, and more thermodynamically stable nitrate is neither an inhibitor nor a substrate.[195]

4. Carbonyl Sulfide, Carbon Dioxide, and Carbon Monoxide

The report that carbonyl sulfide could inhibit acetylene reduction by nitrogenase[196] led Seefeldt and co-workers[197] to look very carefully for carbon monoxide as a product of COS and CO_2 reduction. They used the sensitivity of the optical changes occurring as CO binds to hemoglobin and clearly demonstrated the reality of these reductions at a very slow rate. They also showed that carbon disulfide is an inhibitor of the reduction of acetylene and protons (the latter in contrast to CO at pH 7.3) but did not examine whether it can be a substrate. CO_2, COS, N_3^-, and N_2O were considered as perhaps having similar reactivities on a four-iron face of FeMo cofactor, by analogy with some models for N_2 reduction.[198]

Since carbon monoxide is isoelectronic with dinitrogen it is surprising that it is not reduced by nitrogenase. It is, as discussed above, a well-known inhibitor of the reduction of all substrates except protons. However, disruption of the hydrogen bonding network around homocitrate, either by replacing it by citrate (as in the $NifV^-$ phenotype) or by making substitutions at the arginines presumed to be involved in such a network[168] makes proton reduction sensitive to inhibition by CO. Similar effects are seen at high pH,[167] where a residue close to FeMo cofactor is presumably deprotonated.

EPR studies of turning over enzyme have established that CO interactions can produce two different EPR signals one observed at high [CO] and one at low [CO] suggesting two binding sites.[199] ^{13}C ENDOR has recently been seen from two ^{13}CO's interacting with paramagnetic centers in the MoFe protein.[200] Since the associated EPR signals show ENDOR interactions with ^{57}Fe only when the FeMo cofactor is labeled, but not when the ^{57}Fe is in the P clusters, the CO binding site must be on FeMo cofactor.[161] This is important since it proves that at least one inhibitor binds at the FeMo cofactor site of the enzyme. Note in this context that CO and C_2H_2 can bind to nitrogenase simultaneously,[188] emphasizing that it is not clear whether CO binds at the same site as (all) substrates. An unexpected feature of the CO interactions that produce the high-CO and low-CO EPR signals is revealed on quenching the turnover of CO-inhibited nitrogenase by adding ethylene glycol.[199] Although the signals only appear initially after a delay, they can still be interconverted after quenching by varying the partial pressure of CO. The enzyme must therefore be held in some altered state, able to bind CO and form the EPR signals rapidly. This must be a different way of binding from that giving inhibition of acetylene reduction, since the latter occurs with no delay after initiating turnover.[188] The K_i of CO also varies with electron flux, indicating that different states of the enzyme, perhaps different oxidation levels, interact differentially with CO.[199]

5. Dinitrogen, Protons, Dihydrogen, and HD Formation

We now come to perhaps the most crucial set of problems about nitrogenase: where and how is dinitrogen bound, activated, and reduced? Since this reduction is intimately linked to that of protons we consider them together. Protons are, of course, always with us in aqueous solution and are reduced when no other substrate is present. The possibility that dihydrogen could be evolved from P clusters has been discussed but we are aware of no direct evidence for this. In particular we note that MoFe proteins containing no FeMo cofactor do not reduce protons.

Dihydrogen reduction can be detected by observing the formation of HD from D_2 gas and protons derived from water. A critical feature of this reaction, which is usually referred to as HD formation, is that it takes place essentially only in the presence of dinitrogen. Another well-studied dihydrogen reaction of nitrogenase is the inhibition of N_2 reduction by H_2. Since the steady-state kinetics of HD formation and the inhibition of N_2 reduction by H_2 are the same, and since both reactions are specific for N_2, it is reasonable to assume that these are different manifestations of the same process. It is significant that no D_2 is produced when HD is being reduced and that no atoms from T_2 gas enter the aqueous phase; thus the hydrogen atoms coming from the gas phase must always remain distinguishable from those from the water. There remains the intriguing report that isolated MoFe protein can catalyze H_2 uptake slowly,[201] although at rates far slower than could have been observed in the T_2 uptake experiments above. The significance of this reactivity is obscure at present.

We have known since 1969 that H_2 evolution could not be completely eliminated during N_2 reduction.[202] This observation was later confirmed by a number of studies that attempted to eliminate H_2 evolution by extrapolation of N_2 concentration-dependence data[203] or by direct measurement of the H_2 evolved to N_2 reduced at extremely high (6−50 atm) N_2 partial pressures.[204,205] Others have presented data showing that the ratio of H_2 evolved to N_2 reduced varies with the Fe protein/MoFe protein ratio,[206,207] pH,[208] and the ADP/ATP ratio.[209] None of these reports, however, demonstrate that H_2 evolution can ever be completely eliminated during N_2 reduction. Thus it is generally accepted that, during N_2 reduction catalyzed by nitrogenase, some electrons will always be lost to H_2 evolution.

Hadfield and Bulen interpreted their observation as evidence that H_2 evolution was, in some way, an intimate part of the chemical mechanism of N_2 reduction.[202] Expanding on this concept, Newton et al. suggested that the minimum stoichiometry of the reaction might be one H_2 evolved per N_2 reduced.[210] Rivera-Ortiz and Burris give values of 0.56−0.9 H_2 evolved per N_2 reduced by extrapolation to infinite N_2, which could be used to argue either for or against

a 1:1 stoichiometry.[203] Others have reported ratios of >1:1 at high N_2 concentrations.[204,205,211] Mortenson and Upchurch showed that the ratio approached 1:1 with increasing ADP/ATP ratios and suggested that it might be <1 under some conditions.[209] Thus the data that support the obligatory evolution of one H_2 for every N_2 reduced are much less compelling than the data that require us to believe that some H_2 will always be evolved during N_2 reduction.

Before discussing mechanistic explanations for the observation that H_2 evolution cannot be eliminated there are at least two other observations that are worth noting. First vanadium nitrogenases (which are discussed in the review in this issue by Eady) evolve at least three dihydrogens per dinitrogen reduced. Is this a case of a nonobligatory waste of reducing equivalents by a less efficient nitrogenase or does it represent a fundamental mechanistic difference?[187] We prefer the former option. Second, dinitrogen is not the only substrate that fails to eliminate hydrogen evolution. Thus azide also cannot eliminate H_2 evolution, and the reaction appears to be stoichiometric with N_3^- reduction.[212] In that case, however, N_3^- reduction does not catalyze HD formation under D_2, and H_2 does not inhibit N_3^- reduction, indicating that in at least this case the residual H_2 evolution reaction is not related to the HD formation reaction.

Over the years a number of explanations have been offered to rationalize the observation that H_2 evolution cannot be eliminated during N_2 fixation. The simplest of these is that H_2 evolution represents either a leakage of electrons at a more oxidized state of the enzyme or a simple competition for electrons and protons at a highly reduced state of the enzyme.[13,206,207] These explanations do not require a minimum 1:1 stoichiometry. It has also been suggested that H_2 evolution might arise by decomposition of a partially reduced bound N_2 intermediate to $N_2 + H_2$ which again does not require a minimum 1:1 stoichiometry.[51,210] Another possibility is that H_2 evolution is intimately coupled to N_2 reduction and is needed to achieve a thermodynamically unfavorable, partially reduced, bound N_2 intermediate.[213] This explanation does require a minimum 1:1 stoichiometry. A final related suggestion is that N_2 can only bind to nitrogenase by displacement of dihydrogen (possibly bound as a dihydride) to give H_2 evolution;[13,207] this also requires a minimum 1:1 stoichiometry. This explanation is a pivotal part of the Thorneley−Lowe scheme that rationalizes not only a limiting stoichiometry, but also the action of dihydrogen as an inhibitor and the formation of HD as shown in Figure 19 (see above).[13] From chemical parallels they consider that this reaction is likely to be associative, i.e. N_2 binds before H_2 leaves. The observation of a pre-steady-state burst of dihydrogen evolution of about one molecule per Mo[179] with a number of substrates can be qualitatively explained if reasonable assumptions, consistent with steady-state data, are made about the differences in rate constants of partial reactions between different organisms. Their simulations indicate that the size of such bursts should decrease significantly at Fe protein to MoFe protein ratios of about four, which is higher than the ratios that have been used to date.[179] There are still no other suggested schemes[207,214] that are consistent with all the data.[13]

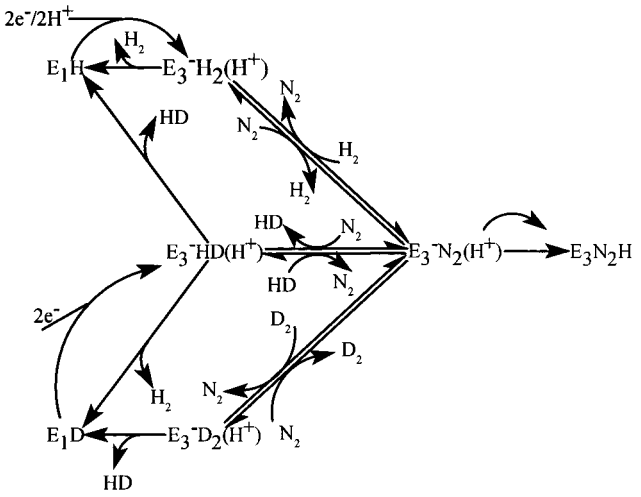


Figure 19. Scheme for the formation of HD by nitrogenase. This scheme provides a mechanism by which D_2 in the gas phase can inhibit N_2 reduction and be itself reduced to HD with the same binding constant and at the same time no D^+ can enter solution. Also no D_2 can be produced if HD is used as the inhibitor. This is a subset of the Thorneley−Lowe scheme with the single additional assumption that the proton in parentheses cannot exchange with the hydrogens derived from the gas phase.[13]

Over the years the reduction of dinitrogen by this enzyme has been proposed to occur by one of two general mechanisms. In one case the reduction is suggested to be symmetrical, yielding diazine and hydrazine as enzyme-bound intermediates. In the other case the reduction is suggested to occur via the initial release of one ammonia, leaving a nitride attached to the enzyme. In fact, there are only two clear examples of experimental evidence for the formation of any intermediates during the reduction of N_2 to NH_3. First, pre-steady-state quenching of molybdenum nitrogenase actively reducing dinitrogen, gives hydrazine in acid or base.[13] This was interpreted, in the light of chemical parallels, as indicating that the two-electron reduced hydrazido-(2−), i.e. bound $=N-NH_2$, was present, although this cannot be regarded as a rigorous proof of the existence of such an intermediate. Second, although hydrazine is not a free product during molybdenum nitrogenase action, it has subsequently been demonstrated that this is not a necessary attribute of nitrogenases since small amounts are formed by a vanadium nitrogenase, especially at elevated temperature.[187,215] Since in the range 45 to 50 °C, NH_3 formation decreased dramatically, whereas N_2H_4 production continued to increase (although still at about only 1% of total electron flux), Dilworth et al.[187] suggested that two competing pathways gave NH_3 or N_2H_4, and that the partial reactions leading to these pathways had different activation energies. It is also possible that bound hydrazine lies on the direct route from dinitrogen to ammonia. Note that hydrazine is a known substrate of molybdenum nitrogenase. We await direct spectroscopic or structural measurements on bound intermediates with great interest.

Although diazine is a conceivable intermediate in a symmetrical reduction of dinitrogen, it is not possible to study whether it can be a substrate since it is unstable in water. However, McKenna and Simeonov[216] have shown that both *cis*- and *trans*-dimethyldiazine are substrates of nitrogenase, giving ammonia, methane, and methylamine in roughly equal proportions. This clearly indicates that symmetrical reduction of just C—N or N═N bonds does not occur. The possibilities are that both symmetrical reductions take place at equal rates, or that methane is released initially followed by a breaking of the N═N double bond; the latter, indicating end-on binding of the substrates, seems more probable.

The activity of nitrogenase that is most susceptible to disruption is its ability to catalyze the reduction of dinitrogen. Changes in the homocitrate moiety of FeMo cofactor have profound effects. NifV$^-$ MoFe protein is ineffective in reducing N_2 and N_2O, and CO is partly able to inhibit its proton reduction activity.[217,218] This mutation was shown to result in an altered FeMo cofactor which carried its phenotype with it when it was transferred to protein deficient in FeMo cofactor.[219] This is still perhaps the best evidence that substrate reduction occurs at FeMo cofactor. Spectroscopic changes can be detected by ENDOR in the protons around the cluster and at the Mo of NifV$^-$ FeMo cofactor.[220] In a series of papers, the Madison group established that the *nifV* gene almost certainly encodes a homocitrate synthase and that the homocitrate is part of FeMo cofactor.[221−224] The X-ray crystallographic results revealed that homocitrate provides two ligands for Mo (Figure 17). In the absence of homocitrate other anions of organic acids, especially citrate, can take its place and produce the NifV$^-$ phenotype; similar effects can be produced at high pH[167] and with various mutants, see above. A method for *in vitro* reconstitution of FeMo cofactor using a wide variety of carboxylic acids has proved invaluable for probing the role of the homocitrate.[225] The minimum structural requirement was shown to be the hydroxyl and carboxyl groups that coordinate to Mo plus an additional carboxyl and an R configuration of the chiral center. Only homocitrate itself proved capable of supporting significant reduction of dinitrogen, with acetylene and proton reduction being successively less stringent. All of these changes must disrupt the hydrogen-bonding network around the homocitrate, but exactly how they produce their effects is not yet clear. Again no X-ray structures are yet available for the MoFe protein substituted with any organic acid other than homocitrate.

Now that we know the environment of the FeMo cofactor, a fruitful area of work lies in studying the effects of changing residues in this region of the α subunit of the MoFe protein. Dean, Newton, and their co-workers have led progress in these analyses,[149−151,168,226] looking particularly at potential hydrogen-bonding interactions from α-Q191 and α-E440 with the homocitrate, α-H195 with a central bridging sulfide, α-R277 with α-H195, α-R359 with a sulfide ligand to Mo and α-R96 with two sulfides, plus α-F381 which lies over a face of FeMo cofactor.[168] Substitution of α-Q191 by lysine abolishes the ability to reduce dinitrogen as well as affecting acetylene reduction (see above).[151] Note the persistence in some of this literature of an early error in which α-Q191 was misassigned as a glutamate. When α-H195 is changed to asparagine, but not to glutamine, the coupling of the paramagnetism of the $S = {}^3/_2$ state to a ^{14}N from a different (as yet unknown) residue is dramatically changed as seen by ESEEM.[149,227] It has been proposed that this is because glutamine is capable of forming the same hydrogen bond as histidine, whereas the shorter asparagine is not. Nitrogenase activities are altered by substitutions at this site.[150] Its critical role is shown by asparagine, tyrosine, glutamine, leucine, threonine, and glycine, all producing Nif$^-$ phenotypes although all the corresponding proteins can reduce protons and acetylene to different extents.

Of the MoFe protein varieties that have been reported so far the α-H195Q derivative, which disturbs the spectroscopic parameters least, is perhaps the most interesting. Despite N_2 not being a substrate it must still bind to this altered MoFe protein since it can be shown to inhibit proton and acetylene reduction.[150] Importantly this protein still supports the characteristic N_2-dependent HD formation (C.-H. Kim, D. R. Dean, and W. E. Newton, personal communication), implying that N_2 binds in the same way as to effective enzyme and providing support for the proposal that the mechanism of HD formation involves D_2 displacement of N_2 and not D_2 interception of a N_2 reduction intermediate. Acetylene and dinitrogen binding to this mutant are very much more sensitive to CO than with the wild-type protein.[150] This species must be capable of revealing a great deal about how dinitrogen is activated and reduced and is a prime candidate for spectroscopic and structural investigation.

There are various suggestions, mainly based on speculations about how FeMo cofactor might function, as to how dinitrogen could be reduced. Some interesting theoretical work has given us insights into various ways of binding N_2 as a result of analyses of which modes are likely to be most activated.[228−230] These studies challenge the experimental biochemists to find ways of distinguishing among such possibilities. One of the more intriguing proposals is that N_2 could bind in the center of the FeMo cofactor, roughly along the Mo to end-Fe axis, with bonds to all six central Fe atoms.[230] This possibility was first suggested by Chan, Kim, and Rees,[6] even though they pointed out that the space is ~0.5 Å too small for dinitrogen with FeMo cofactor at the $S = {}^3/_2$ oxidation level. If this proposal indeed represents how N_2 binds, then most of the other substrates mentioned above are far too large to fit into this space and must therefore bind in different ways. More reduced dinitrogen intermediates are also considerably larger than N_2 itself and would have to expand the cluster considerably, or move to an external binding position. A major problem with the theoretical work in this area[232] is that the calculations are done on the $S = {}^3/_2$ redox level of the cluster, apparently ignoring the kinetic and spectroscopic evidence given above that dinitrogen does not bind at this oxidation level, but only after at least a three electron reduction. The

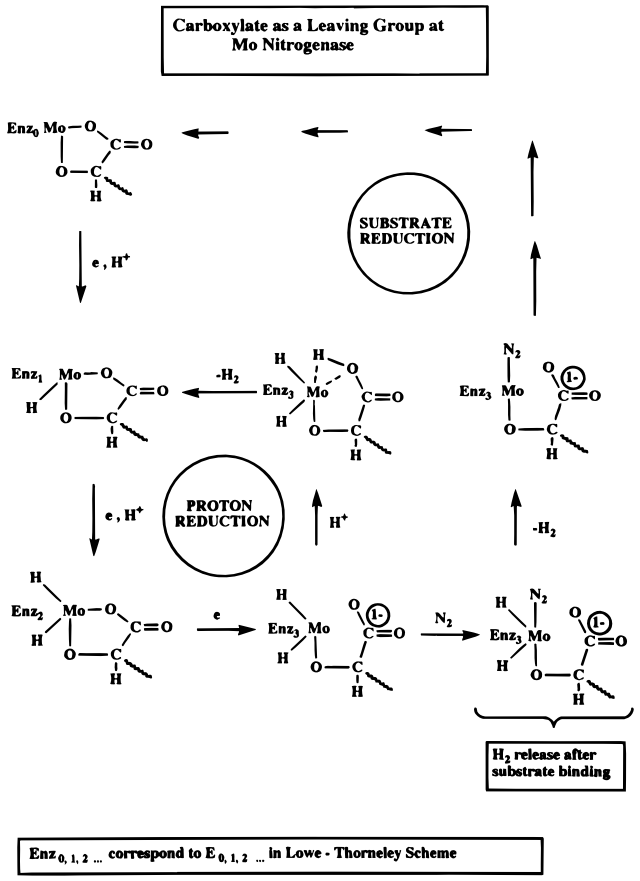


Figure 20. Proposed scheme for H_2 evolution, H_2 binding, and reduction at a Mo site. This scheme is consistent with the Thorneley−Lowe mechanism, is based on known chemical reactions, and suggests a role for homocitrate as the provider of a carboxylate leaving group.[233]

work also does not consider that the energy transduction step in nitrogenase turnover could result in a major conformational change to the MoFe protein. A useful test of the calculations would be to see whether they are consistent with the observed unusual contraction of the cluster as it is reduced.[159] Nevertheless, the work of Stavrev and Zerner[230] is a powerful aid to intuition about the routes by which protons and electrons could be supplied to FeMo cofactor, suggesting that protons are supplied through the homocitrate, perhaps via α-H195, and electrons through α-H442. Kim and co-workers[226] felt that α-H195 was more likely to be involved in electron transfer or positioning the substrate since acetylene and proton reduction were hardly affected in the α-H195Q MoFe protein variant. On the other hand the possibility that α-H195 is involved in proton transfer is perhaps supported by the effect of a mutation at this point on inhibition by cyanide.

Synthetic chemists have shown us how dinitrogen can bind and be reduced at single metal centers[231−233] and how metallosulfur clusters interact with various nitrogenase substrates.[234,235] The suggestion by Hughes et al.[233] describing binding of dinitrogen, which is then reduced after dihydrogen release (Figure 20), elegantly contributes a role for homocitrate, which provides a carboxylate leaving group at Mo, and fits very well with the Thorneley−Lowe scheme. It would be interesting to know what theoretical models, such as that of Zerner and Stavrev,[230] predict about changes in the strength of the bond between Mo and this carboxylate oxygen as electrons are added to the cluster.

VIII. Conclusions

We have now generated a sound base from which to use the tools of molecular genetics to subtly modify the chemistry occurring during nitrogenase action, together with structural and physicochemical methods to answer some of the most important questions about how nitrogenase works. These include the nature of the exact processes going on during ATP hydrolysis, how the energy released is used to control the transfer of electrons and protons to the substrate reduction site, how the two-component proteins interact, and precisely how dinitrogen binds, is activated and subsequently reduced. We confidently look forward to answers to many of these questions in the next few years. Their impact will spread beyond the immediate field of this most discriminating and intricate of enzymes to give us a deeper understanding of the many vital processes which it exemplifies.

IX. Abbreviations

ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; CD, circular dichroism; ENDOR, electron−nuclear double resonance; EPR, electron paramagnetic resonance; ESEEM, electron spin−echo envelope modulation; EXAFS, extended X-ray absorption fine structure; Fe protein or FeP, the iron protein component of nitrogenase; FeMo cofactor, the iron−molybdenum cofactor; GTP, guanosine 5′-triphosphate; LEFE, linear electric field effect; MCD, magnetic circular dichroism; MoFe protein or MoFeP, the molybdenum−iron protein component of nitrogenase; NMR, nuclear magnetic resonance; RR, resonance Raman; SHE, standard hydrogen electrode. Note: All amino acids are referred to by their one letter codes and all residue numbers are with reference to the *A. vinelandii* Fe and MoFe protein sequences.

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References

(1) Georgiadis, M. M.; Komiya, H.; Chakrabarti, P.; Woo, D.; Kornuc, J. J.; Rees, D. C. *Science* **1992**, *257*, 1653.
(2) Kim, J.; Rees, D. C. *Nature* **1992**, *360*, 553.
(3) Kim, J.; Rees, D. C. *Science* **1992**, *257*, 1677.
(4) Kim, J.; Woo, D.; Rees, D. C. *Biochemistry* **1993**, *32*, 7104.
(5) Bolin, J. T.; Ronco, A. E.; Morgan, T. V.; Mortenson, L. E.; Xuong, N.-H. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1078.
(6) Chan, M. K.; Kim, J.; Rees, D. C. *Science* **1993**, *260*, 792.
(7) Bolin, J. T.; Campobasso, N.; Muchmore, S. W.; Minor, W.; Morgan, T. V.; Mortenson, L. E. In *New Horizons in Nitrogen Fixation*; Palacios, R., *et al.*, Eds.; Kluwer Academic Publishers: Boston, MA, 1993; p 89.

(8) Bolin, J. T.; Campobasso, N.; Muchmore, S. W.; Morgan, T. V.; Mortenson, L. E. In *Molybdenum Enzymes, Cofactors and Model Systems*; Stiefel, E. I., Coucouvanis, D., Newton, W. E., Eds.; American Chemical Society: Washington, DC, 1993; p 144.
(9) Pai, E. F.; Krengel, U.; Petsko, G. A.; Goody, R. S.; Kabsch, W.; Wittinghofer, A. *EMBO J.* **1990**, *9*, 2351.
(10) Jurnak, F. *Science* **1985**, *230*, 32.
(11) Story, R. M.; Steitz, T. A. *Nature* **1992**, *355*, 374.
(12) Karkaria, C. E.; Chen, C. M.; Rosen, B. P. *J. Biol. Chem.* **1990**, *265*, 7832.
(13) Thorneley, R. N. F.; Lowe, D. J. In *Molybdenum Enzymes*; Spiro, T. G., Ed.; Wiley-Interscience: New York, 1985; p 221.
(14) Burgess, B. K. In *Molybdenum Enzymes*; Spiro, T. G., Ed.; Wiley-Interscience: New York, 1985; p 161.
(15) Hausinger, R. P.; Howard, J. B. *J. Biol. Chem.* **1983**, *258*, 13486.
(16) Howard, J. B.; Davis, R.; Moldenhauer, B.; Cash, V. L.; Dean, D. *J. Biol. Chem.* **1989**, *264*, 11270.
(17) Lindahl, P. A.; Day, E. P.; Kent, T. A.; Orme-Johnson, W. H.; Münck, E. *J. Biol. Chem.* **1985**, *260*, 11160.
(18) Hagen, W. R.; Dunham, W. R.; Braaksma, A.; Haaker, H. *FEBS* **1985**, *187*, 146.
(19) Hagen, W. R.; Eady, R. R.; Dunham, W. R.; Haaker, H. *FEBS* **1985**, *189*, 250.
(20) Onate, Y. A.; Finnegan, M. G.; Hales, B. J.; Johnson, M. K. *Biochim. Biophys. Acta* **1993**, *1164*, 113.
(21) Lowe, D. J.; Smith, B. E. *Biochem. Soc. Trans.* **1985**, *13*, 579.
(22) Lowe, D. J.; Thorneley, R. N. F. *Biochem. J.* **1984**, *224*, 895.
(23) Watt, G. D.; McDonald, J. W. *Biochemistry* **1985**, *24*, 7226.
(24) Carney, M. J.; Papaefthymiou, G. C.; Spartalian, K.; Frankel, R. B.; Holm, R. H. *J. Am. Chem. Soc.* **1988**, *110*, 6084.
(25) Carney, M. J.; Papaefthymiou, G. C.; Witener, M. A.; Spartalian, K.; Frankel, R. B.; Holm, R. H. *Inorg. Chem.* **1988**, *27*, 346.
(26) Maltempo, M. M.; Moss, T. H. *Q. Rev. Biophys.* **1976**, *9*, 181.
(27) Maltempo, M. M.; Ohlsson, P.-I.; Paul, K.-G.; Petersson, L.; Ehrenberg, A. *Biochemistry* **1979**, *18*, 2935.
(28) Meyer, J.; Gaillard, J.; Moulis, J. M. *Biochemistry* **1988**, *27*, 6150.
(29) Morgan, T. V.; McCracken, J.; Orme-Johnson, W. H.; Mims, W. B.; Mortenson, L. E.; Peisach, J. *Biochemistry* **1990**, *29*, 3077.
(30) Thorneley, R. N. F.; Ashby, G. A. *Biochem. J.* **1989**, *261*, 181.
(31) Dao, C. J.-F. Self-oxidation of dithionite-Av2 solutions. A Ph.D. Dissertation from the graduate school of University of Southern California, 1990.
(32) Watt, G. D.; Reddy, K. R. N. *J. Inorg. Biochem.* **1994**, *53*, 281.
(33) Lanzilotta, W. N.; Holz, R. C.; Seefeldt, L. C. *Biochemistry* **1995**, *34*, 15646.
(34) Stephens, P. J.; McKenna, C. E.; McKenna, M. C.; Nguyen, H. T.; Morgan, T. V.; Devlin, F. In *Current Perspectives in Nitrogen Fixation*; Gibson, A. H., Newton, W. E., Eds.; Elsevier/North-Holland Biomedical Press: New York, 1981; p 357.
(35) Cordewener, J.; Haaker, H.; Veeger, C. *Eur. J. Biochem.* **1983**, *132*, 47.
(36) Stephens, P. J.; McKenna, C. E.; Smith, B. E.; Nguyen, H. T.; McKenna, M. C.; Thomson, A. J.; Devlin, F.; Jones, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2585.
(37) Stephens, P. J.; McKenna, C. E.; Nguyen, H. T.; Lowe, D. J. In *Electron Transport and Oxygen Utilization*; Ho, C., Ed.; Elsevier North Holland: 1981; p 405.
(38) Braaksma, A.; Haaker, H.; Grande, H. J.; Veeger, C. *Eur. J. Biochem.* **1982**, *121*, 483.
(39) Burns, A.; Watt, G. D.; Wang, Z. C. *Biochemistry* **1985**, *24*, 3932.
(40) Nguyen, H. T. Ph.D. Thesis, University of Southern California, 1982.
(41) Watt, G. D.; Wang, Z.-C.; Knotts, R. R. *Biochemistry* **1986**, *25*, 8156.
(42) Anderson, G. L.; Howard, J. B. *Biochemistry* **1984**, *23*, 2118.
(43) Gutheil, W. G. Ph.D. Thesis, University of Southern California, 1989.
(44) Fu, W.; Morgan, T. V.; Mortenson, L. E.; Johnson, M. K. *FEBS* **1991**, *284*, 165.
(45) Larsen, C.; Christensen, S.; Watt, G. D. *Arch. Biochem. Biophys.* **1995**, *323*, 215.
(46) Ryle, M. J.; Lanzilotta, W. N.; Seefeldt, L. C.; Scarrow, R. C.; Jensen, G. M. *J. Biol. Chem.* **1996**, *271*, 1551.
(47) McKenna, C. E.; Gutheil, W. G.; Song, W. *Biochem. Biophys. Acta* **1991**, *1075*, 109.
(48) Cruetz, C.; Sulin, N. *Inorg. Chem.* **1974**, *13*, 2041.
(49) Lambeth, D. O.; Palmer, G. *J. Biol. Chem.* **1973**, *248*, 6095.
(50) Yates, M. G. In *Biological Nitrogen Fixation*; Stacey, G., Burris, R. H., Evans, H. J., Eds.; Chapman and Hall: New York, 1992; p 685.
(51) Burgess, B. K. In *Advances in Nitrogen Fixation Research*; Veeger, C., Newton, W. E., Eds.; Nijhoff/Junk Publishers: Dordrecht, 1984; p 103.
(52) Thorneley, R. N. F. *Biochim. Biophys. Acta* **1974**, *358*, 247.
(53) Ryle, M. J.; Seefeldt, L. C. *Biochemistry* **1996**, 35, 4766.
(54) Robson, R. L. *FEBS* **1984**, *173*, 394.
(55) Walker, J. E.; Saraste, M.; Runswick, M. J.; Gay, N. J. *EMBO J.* **1982**, *1*, 945.
(56) Yoshida, M.; Amano, T. *FEBS Lett.* **1995**, *359*, 1.
(57) Seefeldt, L. C.; Morgan, T. V.; Dean, D. R.; Mortenson, L. E. *J. Biol. Chem.* **1992**, *267*, 6680.
(58) Ryle, M. J.; Lanzilotta, W. N.; Mortenson, L. E.; Watt, G. D.; Seefeldt, L. C. *J. Biol. Chem.* **1995**, *270*, 13112.
(59) Wolle, D.; Dean, D. R.; Howard, J. B. *Science* **1992**, *258*, 992.
(60) Seefeldt, L. C.; Mortenson, L. E. *Protein Sci.* **1993**, *2*, 93.
(61) Cohn, M.; Hughes, T. R. *J. Biol. Chem.* **1962**, *237*, 176.
(62) Gavini, N.; Burgess, B. K. *J. Biol. Chem.* **1992**, *267*, 21179.
(63) Lanzilotta, W. N.; Ryle, M. J.; Seefeldt, L. C. *Biochemistry* **1995**, *34*, 10713.
(64) Cordewener, J.; Haaker, H.; van Ewijk, P.; Veeger, C. *Eur. J. Biochem.* **1985**, *148*, 5499.
(65) Stout, C. D. *J. Biol. Chem.* **1993**, *268*, 25920.
(66) Walker, G. A.; Mortenson, L. E. *Biochemistry* **1974**, *13*, 2382.
(67) Mortenson, L. E.; Walker, M. N.; Walker, G. A. In *Proceedings of the First International Symposium on Nitrogen Fixation*; Newton, W. E., Nyman, C. J., Eds.; Washington State University Press: Pullman, WA, 1976; p 117.
(68) Ljones, T.; Burris, R. H. *Biochemistry* **1978**, *17*, 1866.
(69) Deits, T. L.; Howard, J. B. *J. Biol. Chem.* **1989**, *264*, 6619.
(70) Orme-Johnson, W. H.; Hamilton, W. D.; Jones, T. L.; Tso, M. Y.; Burris, R. H.; Shah, V. K.; Brill, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 3142.
(71) Smith, B. E.; Lowe, D. J.; Bray, R. C. *Biochem. J.* **1973**, *135*, 331.
(72) Zumft, W. G.; Palmer, G.; Mortenson, L. E. *Biochim. Biophys. Acta* **1973**, *292*, 413.
(73) Lindahl, P. A.; Gorelick, N. J.; Münck, E.; Orme-Johnson, W. H. *J. Biol. Chem.* **1987**,*4262*, 14945.
(74) Zumft, W. G.; Mortenson, L. E.; Palmer, G. *Eur. J. Biochem.* **1974**, *46*, 525.
(75) Emerich, D. W.; Burris, R. H. *J. Bacteriol.* **1978**, *134*, 936.
(76) Lindahl, P. A.; Boon-Keng, T.; Orme-Johnson, W. H. *Inorg. Chem.* **1987**, *26*, 3912.
(77) McKenna, C. E.; Stephens, P. J.; Eran, H.; Luo, G.; Zhang, F. X.; Nguyen, H. T. In *Advances in Nitrogen Fixation Research*; Veeger, C., Newton, W. E., Eds.; Martinus Nijhoff/Dr. W. Junk Publishers: Dordrecht, 1984; p 115.
(78) Chen, L.; Gavini, N.; Tsuruta, H.; Eliezer, D.; Burgess, B. K.; Doniach, S.; Hodgson, K. O. *J. Biol. Chem.* **1994**, *269*, 3290.
(79) Mustafa, E.; Mortenson, L. E. *Nature* **1967**, *216*, 1241.
(80) Rees, D. C.; Howard, J. B. *J. Biol. Chem.* **1983**, *258*, 12733.
(81) Thorneley, R. N. F. *Phil. Trans. R. Soc. Lond. B* **1992**, *336*, 73.
(82) Fisher, K.; Lowe, D. J.; Thorneley, R. N. F. *Biochem. J.* **1991**, *279*, 81.
(83) Willing, A. H.; Georgiadis, M. M.; Rees, D. C.; Howard, J. B. *J. Biol. Chem.* **1989**, *264*, 8499.
(84) Willing, A.; Howard, J. B. *J. Biol. Chem.* **1990**, *265*, 6596.
(85) Seefeldt, L. C. *Protein Sci.* **1994**, *3*, 2073.
(86) Deits, T. L.; Howard, J. B. *J. Biol. Chem.* **1990**, *265*, 3859 and references therein.
(87) Pope, M. R.; Murrell, S. A.; Ludden, P. W. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3173.
(88) Gotto, J. W.; Yoch, D. C. *Arch. Microbiol.* **1985**, *141*, 40.
(89) Lowery, R. G.; Saari, L. L.; Ludden, P. W. *J. Bacteriol.* **1986**, *166*, 513.
(90) Wolle, D.; Kim, C.-H.; Dean, D.; Howard, J. B. *J. Biol. Chem.* **1992**, *267*, 3667.
(91) Lowery, R. G.; Chang, C. L.; Davis, L. C.; McKenna, M. C.; Stephens, P. J.; Ludden, P. W. *Biochemistry* **1989**, *28*, 1206.
(92) Emerich, D. W.; Ljones, T.; Burris, R. H. *Biochem. Biophys. Acta* **1978**, *527*, 2359.
(93) Jacobson, M. R.; Cantwell, J. S.; Dean, D. R. *J. Biol. Chem.* **1990**, *265*, 19429.
(94) Howard, J. B.; Rees, D. C. *Annu. Rev. Biochem.* **1994**, *63*, 235.
(95) Peters, J. W.; Fisher, K.; Dean, D. R. *J. Biol. Chem.* **1994**, *269*, 28076.
(96) Duyvis, M. G.; Wassink, H.; Haaker, H. *FEBS Lett.* **1996**, *380*, 233.
(97) Renner, K. A., Howard, J. B. *Biochemistry* **1996**, *35*, 5353.
(98) Eady, R. R.; Lowe, D. J.; Thorneley, R. N. F. *FEBS Lett.* **1978**, *95*, 211.
(99) Hageman, R. V.; Orme-Johnson, W. H.; Burris, R. H. *Biochemistry* **1980**, *19*, 2333.
(100) Mortenson, L. E.; Webb, M.; Bare, R.; Cramer, S. P.; Morgan, T. V. In *Nitrogen Fixation Research Progress*; Evans, H. J., Bottomley, P. J., Newton, W. E., Eds.; Martinus Nijhoff: Boston, 1985; p 577.
(101) McKenna, C. E.; Gutheil, W. G.; Kenyon, G. L.; Matsunaga, T. O. *Bioorg. Chem.* **1989**, *17*, 377.
(102) Feuerstein, J.; Goody, R. S.; Webb, M. R. *J. Biol. Chem.* **1989**, *264*, 6188.
(103) Eady, R. R.; Miller, R. W.; Gormal, C. In *Nitrogen Fixation: Fundamentals and Applications*; Tikhonovich, I. A., Provorov, N. A., Romanov, V. I., Newton, W. E., Eds.; Kluwer Academic Publishers: The Netherlands, 1995; p 147.
(104) Bourne, H. R.; Sanders, D. A.; McCormick, F. *Nature* **1990**, *348*, 125.
(105) Schweins, T.; Geyer, M.; Scheffzek, K.; Warshel, A.; Kalbitzer, H. R.; Wittinghofer, A. *Struct. Biol.* **1995**, *2*, 36.

(106) Peters, J. W.; Fisher, K.; Dean, D. R. *Annu. Rev. Microbiol.* **1995**, *49*, 335.
(107) Thorneley, R. N. F.; Ashby, G. A.; Julius, C.; Hunter, J. L.; Webb, M. R. *Biochem. J.* **1991**, *277*, 735.
(108) Li, J.-G.; Burgess, B. K.; Corbin, J. L. *Biochemistry* **1982**, *21*, 4393−4402.
(109) Ma, L.; Brosius, M. A.; Burgess, B. K. *J. Biol. Chem.* **1996**, 271, 10528.
(110) Eady, R. R.; Postgate, J. R. *Eur. J. Biochem.* **1974**, *162*, 265.
(111) Imam, S.; Eady, R. R. *FEBS Lett.* **1980**, *110*, 35.
(112) Watt, D. G.; Bulen, W. A.; Hadfield, K. L. *Biochemistry* **1975**, *14*, 4266.
(113) Thorneley, R. N. F.; Cornish-Bowden, A. *Biochem. J.* **1977**, *165*, 255.
(114) Orme-Johnson, W. H.; Davis, L. C. In *Iron-Sulfur Proteins*; Lovenberg, W., Ed.; Academic: New York, 1977; p 15.
(115) Mensink, R. E.; Wassink, H.; Haaker, H. *Eur. J. Biochem.* **1992**, *208*, 289.
(116) Thorneley, R. N. F.; Ashby, G.; Howarth, J. V.; Millar, N. C.; Gutfreund, H. *Biochem. J.* **1989**, *264*, 657.
(117) Lowe, D. J.; Ashby, G. A.; Brune, M.; Knights, H.; Webb, M. R.; Thorneley, R. N. F. In *Nitrogen Fixation: Fundamentals and Applications*; Tikhonovich, I. A., Provorov, N. A., Romanov, V. I., Newton, W. E., Eds.; Kluwer Academic Publishers: 1995; p 103.
(118) Thiriot, D. M.S. Thesis, Brigham Young University, Provo, UT, 1995.
(119) Lanzilotta, W. N., Fisher, K., Seefeldt, L. C. *Biochemistry* **1996**, *35*, 7188.
(120) Rees, D. C.; Kim, J.; Georgiadis, M.; Chan, M. K.; Komiya, H.; Woo, D.; Chirino, A. J.; Schlessman, J.; Hsu, B. T. In *New Horizons in Nitrogen Fixation;* Palacios, R., Mora, J., Newton, W. E., Eds.; Kluwer Academic Publishers: Boston, 1992; p 83.
(121) Dean, D. R.; Bolin, J. T.; Zheng, L. *J. Bacteriol.* **1993**, *175*, 6737.
(122) Voordouw, G.; Haaker, H.; van Breemen, J. F. L.; van Bruggen, E. F. J.; Eady, R. R. *Eur. J. Biochem.* **1983**, *136*, 397.
(123) Tsuprun, V. L.; Mitsova, I. Z.; Blazhchuk, I. S.; Gvozdev, R. I.; Orlova, E. V.; Kiselev, N. A. *Eur. J. Biochem.* **1985**, *149*, 389.
(124) Diemann, E.; Branding, A.; Müller, A. *Naturwissenschaften* **1992**, *79*, 267.
(125) Müller, A.; Diemann, E.; Knüttel, K.; Branding, A.; Schneider, K. *Naturwissenschaften* **1991**, *78*, 507.
(126) Liu, H. I.; Filipponi, A.; Gavini, N.; Burgess, B. K.; Hedman, B.; DiCicco, A.; Natoli, C. R.; Hodgson, K. O. *J. Am. Chem. Soc.* **1994**, *116*, 2418.
(127) Hagen, W. R.; Wassink, H.; Eady, R. R.; Smith, B. E.; Haaker, H. *Eur. J. Biochem.* **1987**, *109*, 457.
(128) Kent, H. M.; Ioannidis, I.; Gormal, C.; Smith, B. E.; Buck, M. *Biochem. J.* **1989**, *264*, 257.
(129) Kent, H. M.; Baines, M.; Gormal, C.; Smith, B. E.; Buck, M. *Mol. Microbiol.* **1990**, *4*, 1497.
(130) May, H. D.; Dean, D. R.; Newton, W. E. *Biochem. J.* **1991**, *277*, 457.
(131) Lindahl, P. A.; Papaefthymiou, V.; Orme-Johnson, W. H.; Münck, E. *J. Biol. Chem.* **1988**, *263*, 19412.
(132) Surerus, K. K.; Hendrich, M. P.; Christie, P. D.; Rottgardt, D.; Orme-Johnson, W. H.; Münck, E. *J. Am. Chem. Soc.* **1992**, *114*, 8579.
(133) Tittsworth, R. C.; Hales, B. J. *J. Am. Chem. Soc.* **1993**, *115*, 9763.
(134) Pierik, A. J.; Wassink, H.; Haaker, H.; Hagen, W. R. *Eur. J. Biochem.* **1993**, *212*, 51.
(135) Morgan, T. V.; Mortenson, L. E.; McDonald, J. W.; Watt, G. D. *J. Inorg. Biochem.* **1988**, *33*, 111.
(136) Gavini, N.; Ma, L.; Watt, G.; Burgess, B. K. *Biochemistry* **1994**, *33*, 11842.
(137) Mouesca, J.-M.; Noodleman, L.; Case, D. A. *Inorg. Chem.* **1994**, *33*, 4819.
(138) Lowe, D. J.; Fisher, K.; Thorneley, R. N. F. *Biochem. J.* **1993**, *292*, 93.
(139) Nelson, M. J.; Levy, M. A.; Orme-Johnson, W. H. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 147.
(140) Newton, W. E.; Gheller, S. F.; Hedman, B.; Hodgson, K. O.; Lough, S. M.; McDonald, J. W. *Eur. J. Biochem.* **1986**, *159*, 111.
(141) McLean, P. A.; Wink, D. A.; Chapman, S. K.; Hickman, A. B.; McKillop, D. M.; Orme-Johnson, W. H. *Biochemistry* **1989**, *28*, 9402.
(142) Wink, D. A.; McLean, P. A.; Hickman, A. B.; Orme-Johnson, W. H. *Biochemistry* **1989**, *28*, 9407.
(143) Madden, M. S.; Krezel, A. M.; Allen, R. M.; Ludden, P. W.; Shah, V. K. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6487.
(144) Arber, J. M.; Flood, A. C.; Garner, C. D.; Gormal, C. A.; Hasnain, S. S.; Smith, B. E. *Biochem. J.* **1988**, *252*, 421.
(145) Hoffman, B. M.; Venters, R. A.; Nelson, M.; Roberts, J. E.; Orme-Johnson, W. H. *J. Am. Chem. Soc.* **1982**, *104*, 4711.
(146) Eidsness, M. K.; Flank, A. M.; Smith, B. E.; Flood, A. C.; Garner, C. D.; Cramer, S. P. *J. Am. Chem. Soc.* **1986**, *108*, 2746.
(147) Durrant, M. C.; Hughes, D. L.; Richards, R. L.; Baker, P. K.; Harris, S. D. *J. Chem. Soc., Dalton Trans.* **1992**, *23*, 3399.
(148) Pombeiro, A. J. L.; Hills, A.; Hughes, D. L.; Richards, R. L. *Acta Crystallogr.* **1995**, *C51*, 23.
(149) DeRose, V. J.; Kim, C.-H.; Newton, W. E.; Dean, D. R.; Hoffman, B. M. *Biochemistry* **1995**, *34*, 2809.
(150) Kim, C.-H.; Newton, W. E.; Dean, D. R. *Biochemistry* **1995**, *34*, 2798.
(151) Scott, D. J.; Dean, D. R.; Newton, W. E. *J. Biol. Chem.* **1992**, *267*, 20002.
(152) Huang, H. Q.; Kofford, M.; Simpson, F. B.; Watt, G. D. *J. Inorg. Biochem.* **1993**, *52*, 59.
(153) Lough, S. M.; Jacobs, D. L.; Lyons, D. M.; Watt, G. D.; McDonald, J. W. *Biochem. Biophys. Res. Commun.* **1986**, *139*, 740.
(154) Shah, V. K.; Brill, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 3249.
(155) Schultz, F. A.; Gheller, S. F.; Newton, W. E. *Biochem. Biophys. Res. Commun.* **1988**, *152*, 629.
(156) Newton, W. E.; Gheller, S. F.; Sands, R. H.; Dunham, W. R. *Biochem. Biophys. Res. Commun.* **1989**, *162*, 882.
(157) Newton, W. E.; Gheller, S. F.; Feldman, B. J.; Dunham, W. R.; Schultz, F. A. *J. Biol. Chem.* **1989**, *264*, 1924.
(158) Schultz, F. A.; Feldman, B. J.; Gheller, S. F.; Newton, W. E. *Inorg. Chem. Acta* **1990**, *170*, 115.
(159) Christiansen, J.; Tittsworth, R. C.; Hales, B. J.; Cramer, S. P. *J. Am. Chem. Soc.* **1995**, *117*, 10017.
(160) Howes, B. D.; Fisher, K.; Lowe, D. J. *Biochem. J.* **1994**, *297*, 261.
(161) Christie, P. D.; Lee, H.-I.; Cameron, L. M.; Hales, B. J.; Orme-Johnson, W. H.; Hoffman, B. M. *J. Am. Chem. Soc.* **1996**, in press.
(162) Smith, B. E.; Lowe, D. J.; Bray, R. C. *Biochem. J.* **1973**, *135*, 331.
(163) George, G. N.; Prince, R. C.; Bare, R. E. *Inorg. Chem.* **1996**, *35*, 434.
(164) Burgess, B. K.; In *Molybdenum Enzymes, Cofactors, and Model Systems*; Stiefel, E. I., Coucouvanis, D., Newton, W. E., Eds.; American Chemical Society: Washington, DC, 1993; p 144.
(165) Henderson, R. A. In *Nitrogen Fixation: Fundamentals and Applications*; Tikhonovich, I. A., Provorov, N. A., Romanov, V. I., Newton, W. E., Eds.; Kluwer Academic Publishers: Boston, 1995; p 117.
(166) Leigh, G. J. *Eur. J. Biochem.* **1995**, *229*, 14.
(167) Pham, D. N.; Burgess, B. K. *Biochemistry* **1993**, *32*, 13725.
(168) Newton, W. E.; Fisher, K.; Kim, C.-H.; Shen, J.; Cantwell, J. S.; Thrasher, K. S.; Dean, D. R. In *Nitrogen Fixation: Fundamentals and Applications*; Tikhonovich, I. A., Provorov, N. A., Romanov, V. I., Newton, W. E., Eds.; Kluwer Academic Publishers: Boston, 1995; p 91.
(169) Abe, N.; Onodera, K.; Maruyama, Y. *Agric. Biol. Chem.* **1990**, *54*, 1961.
(170) Shen, B.; Martin, L. L.; Butt, J. N.; Armstrong, F. A.; Stout, C. D.; Jensen, G. M.; Stephens, P. L.; LaMar, G. N.; Gorst, C. M.; Burgess, B. K. *J. Biol. Chem.* **1993**, *268*, 25928.
(171) Mortenson, L. E.; Zumft, W. G.; Palmer, G. *Biochim. Biophys. Acta* **1973**, *292*, 422.
(172) Orme-Johnson, W. H.; Münck, E. In *Molybdenum and Molybdenum Containing Enzymes*; Coughlan, M. P., Ed.; Pergamon Press: Oxford, 1980; p 427.
(173) Ma, L.; Gavini, N.; Liu, H. I.; Hedman, B.; Hodgson, K. O.; Burgess, B. K. *J. Biol. Chem.* **1994**, *269*, 18007.
(174) Peters, J. W.; Fisher, K.; Newton, W. E.; Dean, D. R. *J. Biol. Chem.* **1995**, *270*, 27007.
(175) Rubinson, J. F.; Corbin, J. L.; Burgess, B. K. *Biochemistry* **1983**, *22*, 6260.
(176) Lowe, D. J.; Fisher, K.; Thorneley, R. N. F.; Vaughn, S.; Burgess, B. K. *Biochemistry* **1989**, *28*, 8460.
(177) Miller, R. W.; Eady, R. R. *Biochim. Biophys. Acta* **1988**, *952*, 290.
(178) Hughes, D. L.; Mohammed, M. Y.; Pickett, C. J. *J. Chem. Soc., Chem. Commun.* **1989**, *18*, 1399.
(179) Liang, J.; Burris, R. H. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 9446.
(180) Conradson, S. D.; Burgess, B. K.; Vaughn, S. A.; Roe, A. L.; Hedman, B.; Hodgson, K. O.; Holm, R. H. *J. Biol. Chem.* **1989**, *264*, 15967.
(181) Richards, A. J. M.; Lowe, D. J.; Richards, R. L.; Thomson, A. J.; Smith, B. E. *Biochem. J.* **1994**, *297*, 373.
(182) Conradson, S. D.; Burgess, B. K.; Newton, W. E.; DiCicco, A.; Filipponi, A.; Wu, Z. Y.; Natoli, C. R.; Hedman, B.; Hodgson, K. O. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 1290.
(183) Gormal, C. A.; Harvey, I. A.; Hasnain, S. S.; Richards, R. L.; Schneider, R.; Smith, B. E., Strange, R. W. In *Nitrogen Fixation: Fundamentals and Applications*; Tikhonovich, I. A., Provorov, N. A., Romanov, V. I., Newton, W. E., Eds.; Kluwer Academic Publishers: The Netherlands, 1995; p 149.
(184) Henderson, R. A. *Angew. Chem.* **1996**, 35, 946.
(185) Ashby, G. A.; Dilworth, M. J.; Thorneley, R. N. F. *Biochem. J.* **1987**, *247*, 547.
(186) Schneider, K.; Müller, A.; Krahn, E.; Hagen, W. R.; Wassink, H.; Knüttel, K.-H. *Eur. J. Biochem.* **1995**, *230*, 666.
(187) Dilworth, M. J.; Eldridge, M. E.; Eady, R. R. *Biochem. J.* **1993**, *289*, 395.

(188) Lowe, D. J.; Fisher, K.; Thorneley, R. N. F. *Biochem. J.* **1990**, *272*, 621.
(189) Sadkov, A. P.; Likhtenshtein, G. I. *Biochemistry-USSR* **1990**, *55*, 446.
(190) Jacobs, D.; Mitchell, D.; Watt, G. D. *Arch. Biochem. Biophys.* **1996**, *324*, 317.
(191) Liang, J.; Burris, R. H. *Indian J. Biochem. Biophys.* **1988**, *25*, 636.
(192) Hyman, M. R.; Seefeldt, L. C.; Morgan, T. V.; Arp, D. J.; Mortenson, L. E. *Biochemistry* **1992**, *31*, 2947.
(193) Caballero, F. J.; Igeno, M. I.; Quiles, R.; Castillo, F. *Arch. Microbiol.* **1992**, *158*, 14.
(194) Jensen, B. B.; Burris, R. H. *Biochemistry* **1986**, *25*, 1083.
(195) Vaughn, S. A.; Burgess, B. K. *Biochemistry* **1989**, *28*, 419.
(196) Madden, M. S.; Kindon, N. D.; Ludden, P. W.; Shah, V. K. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 6517.
(197) Seefeldt, L. C.; Rasche, M. E.; Ensign, S. A. *Biochemistry* **1995**, *34*, 5382.
(198) Dance, I. G. *Aust. J. Chem.* **1994**, *47*, 979.
(199) Cameron, L. M.; Hales, B. J. In *Nitrogen Fixation: Fundamentals and Applications*; Tikhonovich, I. A., Provorov, N. A., Romanov, V. I., Newton, W. E., Eds.; Kluwer Academic Publishers: The Netherlands, 1995; p 109.
(200) Pollock, R. C.; Lee, H.-I.; Cameron, L. M.; DeRose, V. J.; Hales, B. J.; Orme-Johnson, W. H.; Hoffman, B. M. *J. Am. Chem. Soc.* **1995**, *117*, 8686.
(201) Wang, Z. C.; Watt, G. D. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 376.
(202) Hadfield, K. L.; Bulen, W. A. *Biochemistry* **1969**, *8*, 5103.
(203) Rivera-Ortiz, J. M.; Burris, R. H. *J. Bacteriol.* **1975**, *123*, 537.
(204) Bulen, W. A. In *Proceedings of the First International Symposium on Nitrogen Fixation*; Newton, W. E., Nyman, C. J., Eds.; Washington State University Press: Pullman, WA, 1976; p 177,
(205) Simpson, F. F.; Burris, R. H. *Science* **1984**, *224*, 1095.
(206) Wherland, S.; Burgess, B. K.; Stiefel, E. I.; Newton, W. E. *Biochemistry* **1981**, *20*, 5132.
(207) Guth, J. H.; Burris, R. H. *Biochemistry* **1983**, *22*, 5111.
(208) Hageman, R. V.; Burris, R. H. *Biochim. Biophys. Acta* **1980**, *591*, 63.
(209) Mortenson, L. E.; Upchurch, R. G. In *Current Perspectives in Nitrogen Fixation*; Gibson, A. H., Newton, W. E., Eds.; Australian Academy of Sciences: Canberra, 1981; p 75.
(210) Newton, W. E.; Corbin, J. L.; McDonald, J. W. In *Proceedings of the First International Symposium on Nitrogen Fixation*; Newton, W. E., Nyman, C. J., Eds.; Washington State University Press: Pullman, WA, 1976; p 53.
(211) Dilworth, M. J.; Thorneley, R. N. F. *Biochem. J.* **1981**, *193*, 971.
(212) Rubinson, J. F.; Burgess, B. K.; Corbin, J. L.; Dilworth, M. J. *Biochemistry* **1985**, *24*, 273.
(213) Burgess, B. K.; Wherland, S.; Newton, W. E.; Stiefel, E. I. *Biochemistry* **1981**, *20*, 5140.
(214) Li, J.-L.; Burris, R. H. *Biochemistry* **1983**, *22*, 4472.
(215) Dilworth, M. J.; Eady, R. R. *Biochem. J.* **1991**, *277*, 465.
(216) McKenna, C. E.; Simeonov, A. M. In *Nitrogen Fixation: Fundamentals and Applications*; Tikhonovich, I. A., Provorov, N. A., Romanov, V. I., Newton, W. E., Eds.; Kluwer Academic Publishers: The Netherlands, 1995; p 158.
(217) McLean, P. A.; Smith, B. E.; Dixon, R. A. *Biochem. J.* **1983**, *211*, 589.
(218) Liang, J.; Burris, R. H. *J. Bacteriol.* **1989**, *171*, 3176.
(219) Hawkes, T. R.; McLean, P. A.; Smith, B. E. *Biochem. J.* **1984**, *217*, 317.
(220) True, A. E.; McLean, P.; Nelson, M. J.; Orme-Johnson, W. H.; Hoffman, B. M. *J. Am. Chem. Soc.* **1990**, *112*, 651.
(221) Hoover, T. R.; Robertson, A. D.; Cerny, R. L.; Hayes, R. N.; Imperial, J.; Shah, V. K.; Ludden, P. W. *Nature* **1987**, *329*, 855.
(222) Hoover, T. R.; Imperial, J.; Liang, J.; Ludden, P. W.; Shah, V. K. *Biochemistry* **1988**, *27*, 3647.
(223) Hoover, T. R.; Imperial, J.; Ludden, P. W.; Shah, V. K. *J. Bacteriol.* **1988**, *170*, 1978.
(224) Hoover, T. R.; Imperial, J.; Ludden, P. W.; Shah, V. K. *Biochemistry* **1989**, *28*, 2768.
(225) Imperial, J.; Hoover, T. R.; Madden, M. S.; Ludden, P. W.; Shah, V. K. *Biochemistry* **1989**, *28*, 7796.
(226) Kim, C.-H.; Zhen, L.; Newton, W. E.; Dean, D. R. In *New Horizons in Nitrogen Fixation*; Palacios, R., et al., Eds.; 1993; p 105.
(227) Thomann, H.; Bernardo, M.; Newton, W. E.; Dean, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6620.
(228) Deng, H.; Hoffmann, R. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1062.
(229) Shestakov, A. F. In *Nitrogen Fixation: Fundamentals and Applications*; Tikhonovich, I. A., Provorov, N. A., Romanov, V. I., Newton, W. E., Eds.; Kluwer Academic Publishers: The Netherlands, 1995; p 162.
(230) Stavrev, K. K.; Zerner, M. C. *Chem. Eur. J.* **1996**, *2*, 83.
(231) Laplaza, C. E.; Cummins, C. C. *Science* **1995**, *268*, 861.
(232) Wagenknecht, P. S.; Norton, J. R. *J. Am. Chem. Soc.* **1995**, *117*, 1841.
(233) Hughes, D. L.; Ibrahim, S. K.; Pickett, C. J.; Querne, G.; Lauoenan, A.; Talarmin, J.; Queiros, A.; Fonesca, A. *Polyhedron* **1994**, *13*, 3341.
(234) Coucouvanis, D.; Mosier, P. E.; Demadis, K. D.; Patton, S.; Malinak S. M.; Kim, C. G.; Tyson, M. A. *J. Am. Chem. Soc.* **1993**, *113*, 12193.
(235) Demadis, K. D.; Coucouvanis, D. *Inorg. Chem.* **1995**, *34*, 3658.
(236) Orme-Johnson, W. H. In *Molybdenum Enzymes, Cofactors, and Model Systems*; Stiefel, E. I., Coucouvanis, D., Newton, W. E., Eds.; ACS Symposium Series 535; American Chemical Society: Washington, DC, 1993; p 257.

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