Structure–Function Relationships of Alternative Nitrogenases

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Received April 17, 1996 (Revised Manuscript Received August 23, 1996)

Contents

I. Introduction

Biological nitrogen fixation, the conversion of atmospheric N_2 to a form utilizable by higher organisms, is catalyzed by the enzyme nitrogenase. This enzyme is found in relatively few groups of bacteria, but is responsible for the cycling of some 10^8 tons of N per year. All N_2-fixing organisms (diazotrophs) which have been investigated have a nitrogenase system based on Mo and Fe. In addition to Mo-nitrogenase, it is now known that some organisms have alternative nitrogenase systems based on V and Fe, or apparently on Fe alone. It should be emphasized from the outset that the three nitrogenase systems, although clearly related, are genetically distinct and do not simply arise from the substitution of different metals into the same polypeptides.

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A. Mo-Dependent Nitrogen Fixation

The molecular enzymology and genetics of Mo-nitrogenase have been subjected to extensive study for many years. The structure of Mo-nitrogenase component proteins is discussed in detail in the paper by Howard and Rees in this issue and the mechanism of Mo-nitrogenase function in the paper by Burgess and Lowe.

In brief outline, this enzyme consists of two oxygen-sensitive metalloproteins, an Fe protein containing a single [4Fe-4S] center which acts as a specific ATP-dependent electron donor to a MoFe protein. The MoFe protein has two types of redox centers, two P clusters consisting of two bridged [4Fe-4S] cubanes, and two Fe- and Mo-containing cofactor centers (FeMoco), the probable site of binding and reduction of N_2.

Under optimal conditions Mo-nitrogenase catalyses the reaction

$$N_2 + 8e + 8H^+ + 16ATP = 16ADP + 16P_i + 2NH_3 + H_2 \quad (1)$$

Studies of this system have been transformed in the last 4 years by the solution of the three-dimensional X-ray structures of both the Fe protein

S0009-2665(95)00057-4 CCC: $25.00

and the MoFe protein (see the work of Howard and Rees in this issue). The X-ray crystal structures of the MoFe proteins Av1 and Cp1 at 2.2 Å resolution[1,2] have revealed the tertiary structure of the $\alpha_2\beta_2$ tetramer and provided structures for the redox centers that they contain. Of particular relevance to this section, these studies showed that the FeMoco centers are composed of [4Fe-3S] and [1Mo-3Fe-3S] clusters bridged by sulfide and that the Mo atom is coordinated to (*R*)-homocitrate by hydroxyl and carboxyl oxygen atoms. FeMoco is bound to the protein by a cysteine residue ligated to an Fe atom and a histidine residue ligated to the Mo atom. This knowledge enables a more informed interpretation of structural and spectroscopic data obtained for alternative nitrogenases than was possible before these structures were known. In particular, it enables the structure of the V-containing cofactor of the VFe proteins to be predicted with some confidence.

B. Mo-Independent Nitrogen Fixation

The structural genes for Mo-nitrogenases (*nifHDK*) of a wide range of organisms have been sequenced; they are highly conserved and often clustered with genes involved in FeMoco biosynthesis. For many years, the view prevailed that Mo was intimately and essentially involved in the enzymatic reduction of N_2. This situation arose from a combination of physiological and biochemical studies which showed that Mo-nitrogenase was present in all diazatrophs and with the development of defined chemical systems, many based on either Mo or W and capable of reducing dinitrogen. However, some 15 years ago, a study of the genetics and physiology of the N_2-dependent growth of *Azotobacter vinelandii* provided data indicating that Mo was not essential for nitrogen fixation. Strains with mutations in the structural genes for nitrogenase, and therefore unable to use N_2 as a source of nitrogen for growth, surprisingly grew when cultured in media to which no Mo had been added. However, for the reasons indicated above, the proposal by Bishop and co-workers[3] that a Mo-independent route of biological N_2 fixation existed, was not widely accepted.

Initial studies on the involvement of vanadium in nitrogenase function were undertaken before *nif HDK* deletion strains were available, and such studies were inconclusive[4] and eventually led to suggestions of a role for vanadium in "sparing" residual Mo in the growth media.[5] The reality of a Mo-independent route for nitrogen fixation was established beyond question when the structural genes encoding Mo-nitrogenase in *A. vinelandii* were specifically removed and the resulting deletion mutant strains shown to be able to fix N_2 in Mo-deficient medium.[6,7]

Subsequently, vanadium was shown to stimulate the growth of a homologous deletion strain of the closely related organism, *Azotobacter chroococcum*,[8] and V-nitrogenases were purified from strains of *A. chroococcum*[9] and *A. vinelandii*.[10] An unexpected development in 1988 was the demonstration of a third nitrogenase system when *A. vinelandii* was grown under combined Mo and V limitation.[11] This system contained Fe but lacked significant levels of Mo or V.

Molecular genetic studies have shown that Mo-independent nitrogenases are quite widely distributed among diazotrophs,[12] but biochemical studies are restricted to V-nitrogenases of *A. chroococcum* and *A. vinelandii* and the Fe-nitrogenases of *A. vinelandii* and *Rhodobacter capsulatus*.

In this review, my intention is to indicate how the alternative, Mo-independent nitrogenases share a great deal in common both in structure and function with the more extensively characterized Mo-nitrogenases. The different metals in the enzymes appear to modulate the chemical reactivity at the active site. The alternative nitrogenases have similar requirements for activity as Mo-nitrogenase, and for example, the Fe protein and VFe proteins of V-nitrogenase form functional heterologous nitrogenases in combination with Mo-nitrogenase components. The study of these systems presents a fascinating opportunity to gain an insight into the mechanism of biological N_2 fixation, because they provide natural variants containing different metals, which are active in catalyzing the binding and activation of dinitrogen.

Progress in this area of research has been relatively rapid because many of the techniques developed for the study of Mo-nitrogenases are applicable to the study of the alternative systems. It is clear that an understanding of the structure and function of Mo-nitrogenase is applicable in many respects to the Mo-independent nitrogenase systems, and relevant aspects are reviewed here. Recent detailed reviews on the molecular genetics and regulation of nitrogen fixation[13,14] and on the structure, function, and mechanism of action of Mo-nitrogenase have been published[15−17] and should be consulted for primary references. Only in the case of the most recent and directly relevant work are primary citations made.

II. Genetics of Nitrogen Fixation

Molecular genetics, as indicated above, played an essential part in putting alternative nitrogenase research on a firm basis. It has also enabled the types of redox centers involved in these systems to be defined and allowed likely common steps in the biosynthesis of their cofactor centers to be identified.

DNA sequencing established that the structural genes of Mo-independent nitrogenases (*vnf*, *v*anadium-dependent *n*itrogen *f*ixation; *anf*, *a*lternative *n*itrogen *f*ixation—genes for the third nitrogenase) share extensive sequence homology with those of Mo-nitrogenases. The availability of three *vnf* and three *anf* gene sequences from different organisms enabled instructive comparisons with Mo-nitrogenase to be made. First, those amino acid residues for which the X-ray structures of MoFe proteins show to bind P clusters and FeMoco are conserved in the Mo-independent nitrogenases. Secondly, a consensus amino acid sequence around His-α442, a ligand to FeMoco in the MoFe proteins, is different and characterizes the three types of nitrogenase (Table 1). Cp1 is atypical among the MoFe proteins in not conforming to the consensus; however, at present the determination of the sequence in this region would appear to allow the unambiguous assignment of the type of Mo-independent nitrogenase in an organism from DNA sequences.

Table 1. Conserved Amino Acid Sequences Flanking His-442 of the α subunit in MoFe, VFe, and FeFe Proteins

	sequences	ref
	MoFe Proteins[a]	
A. vinelandii	Phe-Arg-Gln-Met-**His**-Ser-Trp-Asp-Tyr	
R. capsulatus	Phe-Arg-Gln-Met-**His**-Ser-Trp-Asp-Tyr	
K. pneumoniae	Phe-Arg-Gln-Met-**His**-Ser-Trp-Asp-Tyr	
C. pasteurianum	Ser-Lys-Gln-Leu-**His**-Ser-Tyr-Asp-Tyr	
	VFe Proteins	
A. vinelandii	Tyr-Val-Asn-Gly-**His**-Gly-Tyr-His	20
A. chroococcum	Tyr-Val-Asn-Gly-**His**-Gly-Tyr-His	23
An. variabilis	Tyr-Val-Asn-Gly-**His**-Gly-Tyr-His	25
	FeFe Proteins	
A. vinelandii	Tyr-Leu-Asn-Ala-**His**-Ala-Tyr-His	22
R. capsulatus	Tyr-Leu-Asn-Ala-**His**-Ala-Tyr-His	24
C. pasteurianum	Tyr-Leu-Asn-Ala-**His**-Ala-Tyr-His	32

[a] Data for the MoFe protein α-subunits are representative of a larger number of sequences (see ref 14).

In addition, as discussed in section II.C.2., mutational analysis showed that some *nif* genes are required for the biosynthesis of both alternative nitrogenases and that the third nitrogenase required some *vnf* genes for activity. The majority of these common genes are those which have a role in cofactor biosynthesis or in activation and insertion of FeS clusters.

An outline of the genetics of nitrogen fixation is considered here from the comparative perspective of what the organization and sequence of the structural genes of the three nitrogenases, and of other genes essential for their function, inform us of the biochemistry of alternative nitrogenases. Since *A. vinelandii* has all three nitrogenase systems, the genes of this organism are discussed in some detail. The *anf* genes of *Rhodobacter capsulatus* have also been cloned and sequenced and *nif* genes involved in the function of the third nitrogenase have been identified, and these data are also discussed. The regulation of these genes by the availability of Mo and V has been reviewed recently[18] and will not be reiterated here.

A. Organization and Comparison of the Structural Genes of Nitrogenases

The genetics of nitrogen fixation is complex. Comparative studies of the nitrogen-fixation genes (*nif* genes) of five well-characterized diazotrophs with quite different physiologies indicate that some 14 genes common to all diazotrophs form the basic set required for Mo-dependent nitrogen fixation. The genes required for Mo-nitrogenase of *A. vinelandii* are located in two unlinked regions of the genome: 29 kb of DNA containing *nifHDKTY, nifENX, nifUSVP, nifWZ, nifM,* and *nifF* and a smaller 6 kb region of DNA containing *nifBQ* and *nifAL*.[19] Genes specific for V-nitrogenase, *vnfA, vnfH, vnfENX,* and *vnfHDGK,* are located in a 14 kb region of the genome,[20] and those for the third nitrogenase, *anfA* and *anfHDGKOR*,[21,22] are located on a different fragment of DNA. The *vnf* genes of *A. chroococcum* show a similar organization to those of *A. vinelandii,* but *anf* genes are not present in this organism.[23]

The situation in *R. capsulatus* is more complex since the *nif* genes are located in three unlinked regions of DNA containing at least 32 genes.[24] The genes for the third nitrogenase have the same organization as the *anfHDGK* operon of *A. vinelandii* and have been cloned and sequenced.

The structural genes of V-nitrogenase from *A. vinelandii*,[20] *A. chroococcum*,[23] and the cyanobacterium *Anabaena variabilis*[25] have been cloned and sequenced. The genes *vnfDK* which code for the α and β subunits of VFe protein and show clear homology with *nifDK* [which encode the α (55 kD) and β (60 kD) subunits of the MoFe proteins] are separated by a gene (*vnfG*) in *A. vinelandii* and *A. chroococcum*. This gene encodes a third small (δ) subunit of the VFe protein in these organisms and is present in a 1:1:1 stoichiometry with the other subunits.[23,26] This coding region in *An. variabilis* is fused with the α subunit encoded by *vnfD*.[25] Although the function of the additional small subunit has not been established, it is essential for Mo-independent N_2 fixation.[27] Small polypeptide subunits are not usually associated with purified MoFe proteins, an exception being the MoFe protein from *Xanthobacter autotrophicus*,[28] as discussed below. Additional subunits have been identified in Kp1 and Av1 isolated from *nifNE* or *nifB* mutants,[29,30] although they copurify with apo MoFe proteins lacking FeMoco, these dissociate after activation with isolated FeMoco and have been proposed to act as chaparonins required for the efficient activation of the apoprotein *in vivo*.[31]

The genes for the Fe-nitrogenase have been cloned and sequenced from *A. vinelandii*,[21,22] *C. pasteurianum*,[32] and *R. capsulatus*.[24] They show a high degree of homology with each other and with the corresponding *vnf* genes (~55% identity). The sequences of VFe and FeFe proteins are more similar to each other than they are to the MoFe proteins with which they show ~30% identity.

B. Amino Acid Sequence Comparisons of MoFe, VFe, and FeFe Proteins

The X-ray crystal structures of Cp1 and Av1 define the residues which are involved in ligation of the redox centers of MoFe proteins (see the work of Howard and Rees in this issue). These structures show that the P clusters are located at the interface of the αβ subunits and are ligated by three Cys residues from each subunit. They also show that each FeMoco center is buried in an α subunit and ligated by single Cys and His residues. DNA sequence data are available for eight MoFe proteins, and the derived amino acid sequences show a high degree of conservation. The Cys and His residues discussed above are invariant in all these MoFe proteins. Early directed mutagenesis studies[33,34] had identified these Cys residues as candidates for potential ligands to the FeS centers, an assignment subsequently confirmed by the X-ray crystal structures.

These advances in our understanding of the structure of MoFe protein enabled significant regions in the sequences of VFe and FeFe proteins to be identified and assigned with some certainty. The invariant cysteine residues, which ligate the P clusters, and the cysteine and histidine residues, which bind FeMoco and are present in all MoFe protein

sequences, are also conserved in *vnfDK* and *anfDK*. As discussed in sections III.B.1. and III.B.2., spectroscopic and extrusion data indicate the presence of homologous metal centers in the VFe proteins.

Additional evidence for the presence of a cofactor-binding site in the FeFe proteins of *A. vinelandii* and *R. capsulatus* is provided by the isolation of hybrid FeFe proteins in which the cofactor binding site is partially occupied by FeMoco.[35,36] These species are synthesized *in vivo* under some conditions and support growth on N_2. An active hybrid species of $Av1^v$ has been made by the incorporation of FeMoco into the apoprotein synthesized by a *nifB* mutant.[37]

C. Genes Involved in both Mo-Nitrogenase and Alternative Nitrogenase Systems

The potential role of *nif* genes in Mo-independent nitrogenase function has been investigated by mutational analysis in *A. vinelandii*, *R. capsulatus*, and *An. variabilis*. This approach established that *nifM*,[38] *nifU*, *nifS*, *nifV*,[24,39] and *nifB*[40−51] are required for activity of V-nitrogenase and also Fe-nitrogenase in *A. vinelandii*. In *R. capsulatus*,[24] *nifB*, *nifV*, *nifR1*, *nifR2*, *nifR4*, ORF9, ORF16, and ORF18 are required for the synthesis of an active Fe-nitrogenase. The function of these ORFs and of the two *anf* ORFs recently assigned as *anfO* and *anfR* in *A. vinelandii* is not known.[21] Potentially, they may cast some light on the subtle interactions which fine-tune the interaction of the cofactor center and the polypeptide residues, because mutation of some of these ORFs results in the selective loss of the ability to reduce N_2 but not other substrates. Although a similar phenotype is seen in Mo-nitrogenase in *nifV* mutants, these differences occur under conditions where this gene would be expected to be functional.

However, an involvement of *nif* genes required for FeMoco biosynthesis in Mo-independent nitrogenase function provides strong presumptive evidence for the presence of similar types of cofactor in these systems. An outline of the steps in FeMoco biosynthesis is presented below and compared with a potential scheme for the biosynthesis of FeVco and FeFeco, the cofactor centers of V-nitrogenase and the third nitrogenase, respectively.

1. The Enzymology of FeMoco Biosynthesis

Studies in *K. pneumoniae* and *A. vinelandii* have shown that six *nif* genes are involved in FeMoco biosynthesis *in vivo*: *nifH*, *nifEN*, *nifQ*, *nifV*, and *nifB*.[14,41] Mutation of any one of these genes results in strains unable to grow on N_2 due to the synthesis of inactive apo-MoFe protein either lacking FeMoco or, in the case of *nifV*, unable to reduce N_2. The development of an *in vitro* system by Shah and coworkers for the MgATP- and MoO_4^{2-}-dependent synthesis of FeMoco was an important development[42,43] and, as described below, has allowed the purification and characterization of some of the proteins involved in this process.

An early step appears to be the formation of NifB-co, a low molecular weight material containing Fe and acid-labile sulfide, which can be solubilized from cell extracts by the detergent *N*-laurylsarcosine.[44] When all other components of the assay are in excess, the amount of FeMoco synthesized is proportional to the amount of NifB-co added to the system, and when NifB-co labeled with ^{55}Fe and ^{35}S is used, both isotopes are incorporated into FeMoco,[45] although quantification of this transfer remains to be established. Since the subunits of the MoFe protein are not required for FeMoco biosynthesis, it was suggested on the basis of sequence homology and the conservation of Cys-275, which ligates FeMoco in the MoFe proteins, that assembly occurs on a complex formed by the *nifNE* products.[14] NifEN has been purified[46] and shown to form an $\alpha_2\beta_2$ tetramer of M_r = 220 kDa which changes electrophoretic mobility in the presence of NifB-co. When radioactively labeled ^{55}FeNifB-co is assayed, label is spontaneously incorporated into NifNE in the absence of MgATP, MoO_4^{2-}, homocitrate, or Fe protein,[45] suggesting that the incorporation of Mo occurs at a late stage in the biosynthetic route, leading to FeMoco. Insertion of FeMoco into apo-MoFe protein involves MgATP, a chaparonin[31] (named γ protein, capable of binding FeMoco and apo-MoFe protein), and Fe protein.

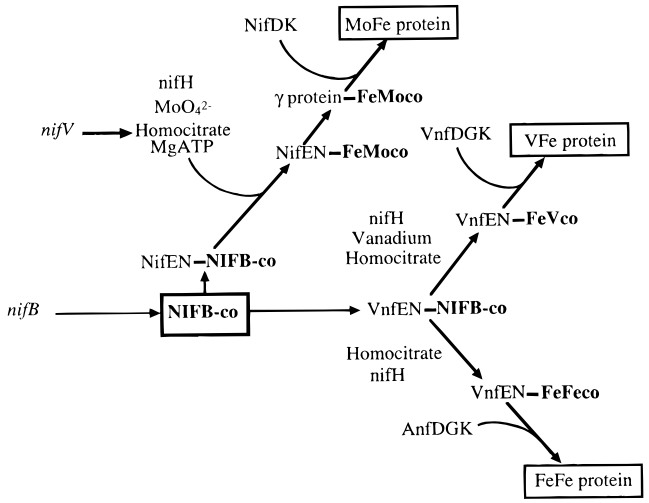
A role for the Fe protein as an essential component[14,41] in the insertion of FeMoco into apo-MoFe protein was unexpected. The effect of mutations on this function have revealed that the Fe protein does not have to be active as an electron donor or in binding MgATP to effectively participate in this biosynthetic role. An intact [4Fe-4S] cluster appears to be required, since mutation of one of the Cys ligands, which normally binds the cluster, results in the synthesis of a form of Fe protein unable to support the insertion of FeMoco.

2. FeMoco Genes Involved in Alternate Nitrogenase Function

The involvement of *nifB*[5b,24,40] in alternative nitrogenase function suggests that a cofactor-specific Fe-S assembly pathway is common to all nitrogenase systems. Other genetic data suggesting the presence of cofactor centers analogous to FeMoco in the Mo-independent nitrogenases is provided by the presence of a reiteration of *nifEN*-like genes in *A. vinelandii*[22] and *A. chroococcum*.[47] The reiterated genes are part of the *vnfAEN* operon and are essential for the function of V- and the third nitrogenase.[48] By homology with the proposed steps in FeMoco biosynthesis, synthesis of FeVco and FeFeco diverge after the formation of a NifB-co−*vnfEN* complex. The requirement of both systems for *nifV*, which amino acid sequence homology indicates most likely encodes a homocitrate synthase, provides very strong genetic evidence for the presence of cofactors in both VFe and FeFe proteins, which are analogous to FeMoco and have homocitrate as a component. A plausible biosynthetic pathway for the cofactors of alternative nitrogenases based on genetic studies and by com-

parison with the proposed steps in FeMoco synthesis is summarized in Scheme 1.

Scheme 1. Potential Routes for the Biosynthesis of Cofactors of the Alternative Nitrogenases



3. Genes Involved in Fe Protein Activity and Mo-Independent Nitrogenase Function

nifM is involved in some way in the maturation of the Fe protein of Mo-nitrogenase, and given the similarity of structure and function of the Fe proteins associated with the Mo-independent systems, their requirement for *nifM* for activity is not surprising.

nifS has been shown to be a pyridoxal phosphate-containing L-cysteine desulfurase, which activates sulfur by the formation of an enzyme-bound persulfide,[49] and shown to catalyze the insertion of the [4Fe-4S] cluster into apo-Fe protein and reconstitute activity.[50] Since mutation of this gene results in a decreased activity of both Fe and MoFe proteins, it is probable that it serves as general sulfide donor in the formation of Fe-S clusters of Mo-nitrogenase components. An analogous role in the formation of active Mo-independent nitrogenases is understandable, given that these systems contain FeS centers which appear homologous to those of Mo-nitrogenase.

III. Structure of Nitrogenase Components

A. Fe Protein—Structure and Function

The X-ray crystal structure of Av2, the Fe protein of Mo-nitrogenase of *A. vinelandii*, is described in detail in the work of Howard and Rees in this issue and is only summarized here. Each of the subunits of the dimeric protein is folded in a single α/β type domain. A single [4Fe-4S] cluster bridges the subunits at one end of the molecule, with the Fe atoms covalently bound by two cysteine residues from each subunit. The potential nucleotide binding sites are located at the subunit interface some 20 Å from the [4Fe-4S] cluster. Since the sequences and physicochemical properties of the Fe proteins of the three nitrogenase systems are very similar, they are considered together here.

The *nifH* gene, which encodes the polypeptide of the Fe protein, appears to be one of the oldest functioning genes, since the outline of its evolutionary tree is largely consistent with 16s rRNA phylogeny.[51] DNA sequence data for some 30 diazotrophs shows proteins to have 45−90% identity and to have some invariant regions. *vnfH* genes of *A. vinelandii*,[20] *A. chroococcum*,[23] and *An. variabilis*[25] has been sequenced. *anfH* has been sequenced in *A. vinelandii*,[21,22] *C. pasteurianum*,[32] and *R. capsulatus*.[24]

Comparison of the DNA sequences of these genes shows them to be typical members of the Fe protein family. The *vnfH* genes show ∼90% identity with *nifH*, and both *vnfH* and *nifH* show ∼60% identity with *anfH*. They all show the characteristic spacing of five Cys residues, two of which are shown by the X-ray structure of Av2 to be involved in ligation the [4Fe-4S] cluster. The *N*-terminal Gly-X-Gly-XX-Gly consensus nucleotide-binding motif characteristic of these proteins is also conserved. The nature of the interaction of Fe protein with MoFe protein leading to ATP hydrolysis and electron transfer from the Fe protein to the MoFe protein is complex and not well-understood. However, chemical cross-linking and directed mutagenesis studies have implicated Arg-100 and Glu-112 residues of Av2 as being likely to be at the contact surface of Fe protein and MoFe protein.[16] These residues are conserved in the Fe proteins of the Mo-independent nitrogenases.

The Fe proteins of V-nitrogenase of *A. vinelandii*[52] and *A. chroococcum*[53] have been purified and shown to have physicochemical properties characteristic of this class of proteins. Fe proteins associated with the alternative nitrogenase systems have not been extensively characterized when compared with the Fe proteins of Mo-nitrogenases, since attention has been focused on the VFe and FeFe components of these systems.

These proteins are dimeric with $M_r \sim 63$ kDa and contain 4 Fe and 4 S^{2-} atoms per dimer. The dithionite-reduced proteins, as isolated, are paramagnetic and, at low temperature, exhibit electron paramagnetic resonance (EPR) spectra with *g* factors characteristic of a $[4Fe\text{-}4S]^{1+}$ center. As has been shown for the Fe proteins of Mo-nitrogenase, this cluster in $Ac2^v$ and $Av2^v$ is a spin mixture of $S = {}^1/_2$ and ${}^3/_2$ states. Binding of MgATP to $Av2^v$ induces a conformational change of the protein, resulting in the Fe atoms of the cluster being more accessible to the chelator α,α′-bipyridyl, and the $g = 1.94$ EPR signal changes from rhombic to axial. Redox potential measurements show that the midpoint potential of the cluster in $Ac2^V$−MgADP of −463 mV compares with that of −450 mV for Ac2−MgADP.[54] The rates of reduction of the phenazine methosulfate-oxidized forms of these nucleotide-bound species by $S_2O_4^{2-}$ are very similar, and correspond to a one-electron reaction.

When Mo-nitrogenase components were first purified in the 1970s, the catalytic properties of combinations of components from Mo-nitrogenases from different organisms was measured to test their relatedness. Despite the high degree of conservation of structure now known to exist, not all combinations were equally effective, and some were nonfunctional. The close relatedness of the Fe protein of V-nitrogenase with 'typical' Fe proteins is shown from the

ability of $Ac2^v$ to form a fully functional nitrogenase with Ac1.

Differences among the Fe proteins of the alternative nitrogenases is indicated by the lack of significant reactivity with any nitrogenase components with the FeFe protein of the Fe-nitrogenase, which specifically requires the Fe protein of this nitrogenase for activity.[55] This Fe protein has not been characterized extensively but is dimeric with $M_r \sim 64$ kDa and contains ca. 4 Fe and 4 S^{2-} atoms per dimer.[11] The protein from *R. capsulatus* has been shown to be subject to modification by ADP ribosylation when cultures are subjected to dark stress,[56] as occurs with Mo-nitrogenase Fe protein from the same organism, and has a characteristic EPR spectrum showing both the $S = {}^1/_2$ and $S = {}^3/_2$ resonances.

B. Mo-Nitrogenase

The properties of Mo-nitrogenase described in this section are not comprehensively reviewed, having been selected for purposes of comparison with the more restricted range of data available for alternative nitrogenases.[15−17]

1. MoFe Protein—Structure and Function

MoFe proteins are $\alpha_2\beta_2$ tetramers of $M_r \sim 230\,000$. They are complex proteins, and preparations with the highest specific activity contain 2 Mo and 30−34 Fe atoms and an approximately equivalent number of acid-labile sulfur atoms (Table 2). Before the X-ray structures were determined, the arrangement of these atoms and the types of redox centers present in MoFe proteins were subjected to extensive spectroscopic studies including Mössbauer, EPR, ENDOR, magnetic CD, and X-ray absorption spectroscopies reviewed in refs 14−17 and 41.

The presence of two types of iron-containing clusters in the MoFe proteins was first demonstrated by Mössbauer spectroscopy. These studies showed that approximately half of the 30−34 Fe atoms present in MoFe proteins, as isolated in the presence of dithionite, were associated with a paramagnetic center characterized by an EPR signal arising from an $S = {}^3/_2$ spin system. This center undergoes a one-electron oxidation to a diamagnetic state in redox titrations with variable E_m values (in the range −30 mV to −350 mV, depending on the source of the protein). Subsequently, procedures were developed for the extraction of an Fe- and Mo-containing cofactor (FeMoco) from the MoFe proteins,[42] and Mössbauer studies showed that the characteristic $S = {}^3/_2$ EPR signal was associated with this Fe-containing cluster.

The remainder of the Fe was shown to be associated with P clusters, which are Fe/S clusters with unusual spectroscopic properties capable of undergoing multielectron redox processes. Characteristic features of these clusters are the unusually steep magnetization curves of the low-temperature MCD transitions in dye-oxidized proteins and a range of EPR signals,[57−61] which arise from oxidized MoFe proteins during oxidative titrations or when poised at redox potentials in the range −500 mV to 100 mV. The Mössbauer parameters of the Fe atoms associated with P clusters of dithionite-reduced MoFe proteins are consistent with an all ferrous oxidation levels reviewed in ref 62. The problems associated with the further reduction of P clusters following electron transfer from the Fe protein during enzyme turnover is discussed in more detail by Burgess and Lowe in this issue.

Of the MoFe proteins, Av1 has been the most extensively characterized by EPR spectroscopy during redox titrations. The reversible dye oxidation of Av1 occurs in two phases. The first four oxidizing equivalents result in the oxidation of the P clusters, the next 2 equiv oxidize the FeMoco centers, and the further addition of two oxidizing equivalents results in the further oxidation of the P clusters. A scheme for the redox states of P centers is shown below:

$$P\ (S = 0) \leftrightarrow P^+\ (S = {}^1/_2, {}^5/_2) \leftrightarrow P^{2+}\ (S = 3 \text{ or } 4) \leftrightarrow P^{3+}\ (S = {}^1/_2, {}^7/_2)$$

P is the diamagnetic species present in dithionite reduced protein, P^+ is a physical spin mixture of $S = {}^1/_2$ and $S = {}^5/_2$, P^{2+} is a non-Kramers paramagnetic state with a $g = 12$ excited state, most easily detected in parallel mode EPR, and P^{3+} is a physical spin mixture of $S = {}^1/_2$ and $S = {}^7/_2$. The signal at $g = 12$ is unusual and provides a spectroscopic signature for the presence of P clusters.

The X-ray structures of Av1 and Cp1 show that the P clusters are located at the α/β subunit interface ligated by three cysteines from both subunits. They are made up of two 4Fe-4S clusters linked by bridging cysteine thiolate groups (see Figure 1A). A bridging sulfur−sulfur bond in the P clusters is observed in a 2.2 Å resolution structure of Av1. In the case of Cp1, a similar structure has been proposed, but so has a structure where a single sulfur atom is shared by the cubanes.[2] In the Av1 structure one of the Fe atoms appears to pentacoordinate, ligating both to Ser-β188 and Cys-β153. Directed mutagenesis of these residues in Av1 changes the redox properties of the P clusters.[63] EPR and MCD studies suggest that ligation by Ser-β188 is largely responsible for the anomalous magnetic properties of P clusters. In addition, mutation of the bridging Cys-α88 residue results in the P clusters of the dithionite-reduced proteins being in the P^+ oxidation state. As discussed below, $Av1^v$ shows similar behavior, and the P clusters are partially oxidized in the protein as isolated in the presence of dithionite. The bridged double cubane structure of the P clusters readily accounts for their unusual spectral properties and their ability to undergo multielectron redox processes. These centers in Kp1 have been shown to become transiently oxidized during enzyme turnover under N_2, providing an experimental basis for their long-suggested role as electron capacitors.[64] Additional evidence for a role for P clusters in mediating intramolecular electron transfer has been obtained in a kinetic study of mutant species of Av1 in which the polypeptide environment between the P clusters and FeMoco centers was perturbed, which left the redox centers unchanged.[65]

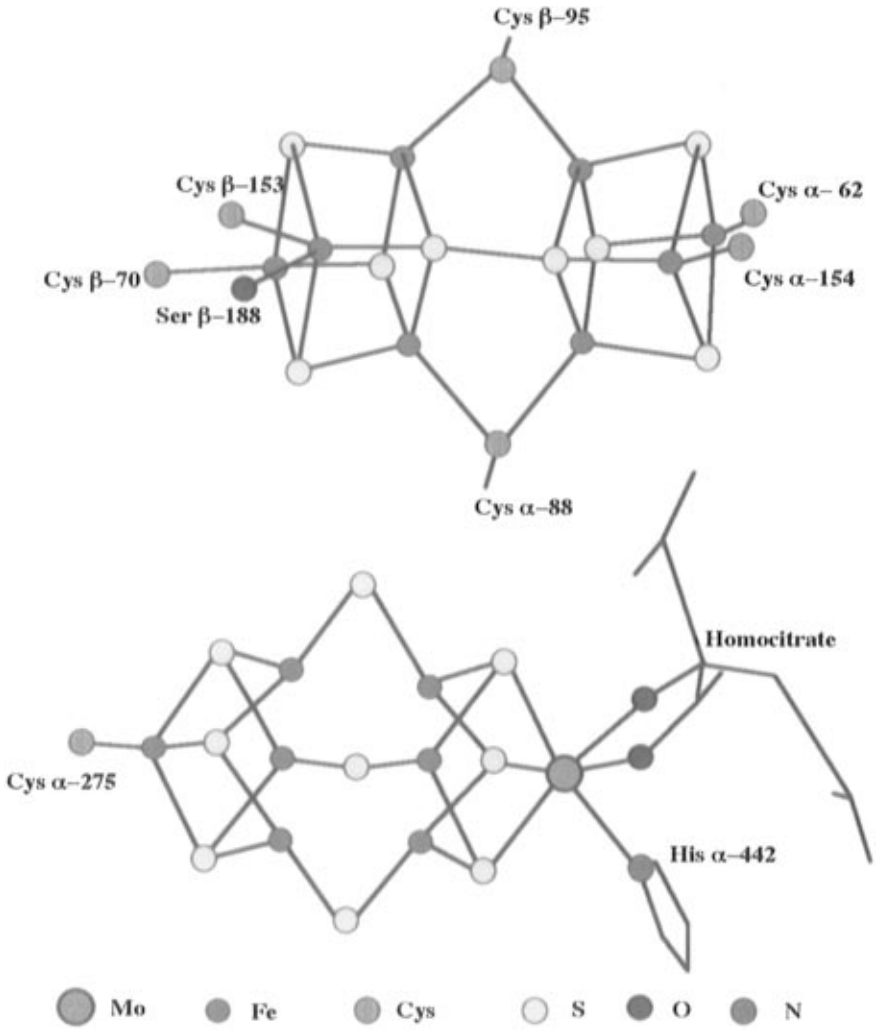


Figure 1. Schematic representation of a P cluster and FeMoco center of Av1. These figures were generated from atomic coordinates kindly supplied by Professor D. C. Rees.

2. FeMoco—Structure and Role in N_2 Reduction

Mo is present in the MoFe proteins as a component of an iron- and molybdenum-containing cofactor (FeMoco) which gives rise to the characteristic $S = {}^3/_2$ EPR spectrum of the dithionite-reduced protein. In 1977, FeMoco was shown to be extractable into *N*-methyl formamide (NMF) in a form capable of activating the inactive apo-MoFe protein isolated from mutant strains unable to synthesize FeMoco.[42] Subsequent studies showed that, in this type of reconstitution experiment, FeMoco transferred the *nifV* phenotype of forming an acetylene-reductase but a poor nitrogenase to the MoFe protein, providing the strongest evidence that FeMoco formed the active site of nitrogenase.[66]

Genetic studies established that MoFe protein was not necessary for the synthesis of FeMoco, a fact which enabled an *in vitro* assay for the gene products and factors necessary for its synthesis to be developed.[67] One important discovery arising from such studies was that (*R*)-homocitrate, (2-hydroxy-1,2,4-butanetricarboxylic acid) was a component of FeMoco and that incorporation of analogues or fluorinated derivatives into FeMoco led to the assembly of a MoFe protein with altered substrate specificity.

Before the X-ray structure of MoFe protein was known, the chemical environment of the Fe and Mo atoms had been investigated by X-ray absorption spectroscopy. These data are presented in Table 3, where they are compared with the data for V in VFe proteins. They provide the most accurate estimate of the nearest neighbor distances of atoms in the first coordination sphere of Mo and V. It is clear from these data that Mo in FeMoco of the MoFe proteins occupies a very similar environment to V in the FeVco center of the VFe proteins.

The X-ray crystal structures of Av1 and Cp1 show that FeMoco is anchored to the protein by α-Cys-275, which is a thiolate ligand to an Fe atom at one end of the cofactor, and by α-His-442, where the imidazole 8-N atom is coordinated to the Mo atom. The initial models for FeMoco at 2.7 Å resolution[67] were constrained to atomic metal−sulfur distances derived from EXAFS studies of the MoFe protein and isolated FeMoco and by model cluster data. The structures of Av1[1] and Cp1[2] have subsequently been refined to a 2.2 Å resolution difference map which confirms that FeMoco is made up of two M-Fe_3-S_3 clusters (where M = Mo or Fe), linked by three bridging sulfides with (*R*)-homocitrate coordinated to the Mo atom by its 2-hydroxy and 2-carboxy groups (Figure 1B). This higher resolution refinement gives average Fe−Fe distances between the bridged Fe sites of 2.5 Å, suggestive of some Fe−Fe bonding interactions that

could provide a fourth coordination interaction for the six, otherwise three-coordinate, bridging iron atoms.[1] The essentially identical structure proposed for the FeMoco center of Cp1 is based on analysis of anomalous dispersion data, where the interpretation of the electron density maps of the clusters was not constrained by other spectral information.[2]

Since the publication of the structure of FeMoco, there have been several sets of calculations as to how N_2 binds to FeMoco.[68,69] It has been suggested that N_2 binds to the center of FeMoco, replacing the weak putative Fe−Fe bonds with multiple Fe−N bonds. To account for the cavity being some 0.5 Å too small to allow this mode of bonding, it is proposed that the more reduced states of the cofactor, generated during enzyme turnover and to which N_2 binds, have an increased FeFe separation distance. However, other alternative substrates and reduction intermediates of N_2 are too large to be bound in this manner, and recent Mo and Fe EXAFS data for nitrogenase during turnover are consistent with a slight contraction rather than expansion of this center.[70]

Directed mutagenesis studies of residues within the FeMoco-binding pocket of Av1 have demonstrated that they have potential functions in fine-tuning the catalytic properties of the enzyme and also the spectroscopic properties of FeMoco.[71] Such studies have shown that substitution of α-His-195 can result in an alteration of the distribution of electrons to C_2H_4 and H_2, the generation of C_2H_6 as an additional product of reduction of C_2H_2, and the uncoupling of ATP hydrolysis from electron transfer in addition to changes in the line shape of the EPR signal of FeMoco. A particularly interesting phenotype is generated by substitution with Gln, which generates a MoFe protein capable of binding, but not reducing, N_2. Although His-195 does not provide direct N-coordination to FeMoco, ESEEM studies have shown that modulation from nitrogen couped to the $S = {}^3/_2$ spin system of FeMoco is sensitive to substitution by Asn. The X-ray crystal structure determination of two MoFe proteins at 2.2 Å resolution is a major achievement, which will change the direction of future research, but it has not yet enabled the nature of subtle interactions of FeMoco and the polypeptide, essential for N_2 reduction, to be identified.

C. V-Nitrogenase

Biochemical studies of V-nitrogenases are restricted to *A. vinelandii* and *A. chroococcum*. As expected from the DNA sequence homology of the structural genes of the V-nitrogenases in these organisms, the purified enzymes are very similar.[12] Much of the work on these proteins has focused on the types of redox centers that the VFe proteins contain and investigated how the presence of V changes the catalytic properties compared with the more extensively studied Mo-nitrogenase.

1. VFe Protein—Structure and Function

VFe proteins have been purified from *A. chroococcum*[9] and *A. vinelandii*[10] and generally have very similar properties. These proteins differ from the MoFe proteins in their subunit structure because they have an additional δ subunit. $Ac1^v$ has been shown to have a $\alpha_2\beta_2\delta_2$ hexameric structure of $M_r \sim 250\,000$. In the case of $Av1^v$, the stoichiometry of the α and β subunits has recently been shown to be variable,[26] and species corresponding to subunit composition $\alpha\beta_2$ and $\alpha_2\beta_2$ have been characterized. The presence of a δ subunit in $Av1^v$ has been demonstrated, but the stoichiometry with the α and β subunits could not be established. This variability in the α and β subunit ratio is similar to the situation with FeFe proteins[11] and suggests that subunit interactions are weaker in the proteins of the Mo-independent nitrogenases. A similar variation in subunit composition has not been detected with $Ac1^v$, even in preparations ranging in specific activity[72] from 550 to 2050.

The V content of VFe proteins is variable, and preparations containing 2 V atoms/mol are the exception rather than the rule. The determination of the absolute metal or acid-labile sulfide content of nitrogenase proteins is difficult and, particularly in the case of acid-labile sulfide analyses, recoveries can seriously underestimate the amount present.

The original values reported for VFe proteins based on a $\alpha_2\beta_2$ structure were 0.7−2 V atoms, 9−23 Fe atoms, and 20 acid-labile sulfur atoms. More recently, the V:Fe:S ratios for different species of $Av1^v$, separable by chromatography on Q-Sepharose, have been reported as 1:19:19 for $\alpha\beta_2$ and 2:30:34 for $\alpha_2\beta_2$ forms of this VFe protein.[26] The analytical data for the V contents were 0.7 ± 0.2 and 1.4 ± 0.2, respectively, and the value ratios given above have been calculated on the basis of an integer V content. The acid-labile sulfur content was determined using MoFe protein as a standard to circumvent the problems of low recovery referred to above. These data indicate that the $\alpha\beta_2$ species has a considerably lower content of acid-labile sulfur atoms, consistent with incomplete Fe-S centers as discussed below. The properties and metal contents of these preparations are summarized in Table 2.

The conservation of the amino acid residues in VFe protein sequences which the X-ray structures show to be involved in binding P clusters and the FeMoco center of the MoFe proteins provides a very strong presumptive case for the presence of homologous redox centers in the MoFe and VFe proteins. The types of redox centers present in VFe proteins have been investigated by EPR, MCD, Mössbauer, and X-ray absorption spectroscopies and by cofactor extrusion studies. These studies, which are summarized below, have shown that $Av1^v$ and $Ac1^v$ contain redox centers which are homologous to the P clusters and FeMoco with V replacing Mo in the cofactor centers.

a. Mössbauer Spectroscopy. As purified, the Mössbauer spectra of $Av1^v$ indicate that some 5−10% of the P clusters are oxidized and do not become reduced in the presence of 10 mM dithionite and methyl viologen.[73] This behavior contrasts with MoFe proteins, where both types of metal clusters are reduced by this treatment. However, reduction of the oxidized P clusters of $Av1^v$ without reduction of the cofactor centers was achieved during enzyme turnover in the presence of limiting Fe protein.

Table 2. Comparison of the Physicochemical Properties of MoFe Proteins, VFe Proteins, and the FeFe Proteins

	Kp1	Ac1^{v}	Av1$^{v}_{A}$	Av1$^{v}_{B}$	Av1^{Fe}	Av1^{Fe} containing FeMoco	Rc1^{Fe}
property							
native M_r	225000	239600		240000[a]	249800[a]		246400[a]
subunit structure	$\alpha_2\beta_2$	$\alpha_2\beta_2\delta_2$	$\alpha\beta_2(\delta)$	$\alpha_2\beta_2(\delta)$	$\alpha_2\beta_2(\delta)$	$\alpha_2\beta\delta_3$	ND
subunit M_r	2 × 54100	2 × 53800	1 × 53800	2 × 53800	2 × 58400	2 × 58400	59000
	2 × 58300	2 × 52700	2 × 52700	2 × 52700	2 × 51200	1 × 51200	50700
		2 × 13300				3 × 15300	13400
metal and S^{2-} content (g atom/mol)							
V	0.06	2 ± 0.3	0.7 ± 0.2	1.4 ± 0.2	0.01	0.05	0.02
Mo	2	0.06	ND	ND	0.085	1.07	0.21
Fe	32 ± 3	21 ± 1	13	21.4	24	24.2	20
S^{2-}	ND	19 ± 0.2	13	24	18	17.9	ND
EPR g values for reduced protein	4.32, 3.73, 2.018	5.6, 4.35, 3.77, 1.93	5.5, 2.03, 1.93, 1.89, 2.05, 1.94	5.5, 2.05, 1.94	ND	4.3, 3.66, 2.01	4.3, 2.05, 1.93
ref	102	8	26	26	11	35	84, 85

[a] M_r based on gene size.

Table 3. Comparison of Mossbauer Parameters of VFe and MoFe proteins

spectral component	Av1^{v}			Av1			Cp1		
	ΔE_Q	δ	area (%)	ΔE_Q	δ	area (%)	ΔE_Q	δ	area (%)
Fe^{2+}	2.99	0.64	12.8	3.02	0.69	13.0	3.00	0.64	12.8
D	0.70	0.65	39	0.82	0.64	42	0.7	0.64	39
S	1.37	0.63	6	1.37	0.64	6	1.37	0.64	6
M	0.94	0.39	48	0.76	0.40	40	0.8	0.41	42
refs		73			103			103	

Low temperature (4.2 K) Mössbauer spectra of the enzymically reduced ^{57}Fe-substituted VFe protein from *A. vinelandii*[73] showed a complex spectrum arising from the overlap of three spectral components. One had magnetic hyperfine structure attributed to a paramagnetic species assigned to the $S = 3/2$ spin species of an M center (FeVco). A second was comprised of three quadrupole doublets (Fe^{2+}, D, and S doublets) assigned to a P component (P clusters) in a diamagnetic state, and a fourth minor quadrupole doublet was assigned to adventitiously bound Fe^{II}. The isomer shifts and quadrupole coupling constants are very similar to those observed in spectra of MoFe proteins (Table 3). In addition, the relative absorption intensities of the various spectral intensities at 4.2 K for the P and M components of 52% and 48% are very close to those reported for Cp1 and Av1 and indicate that iron-containing clusters of VFe and MoFe proteins have similar compositions. These data were interpreted in terms of Av1^{v} containing two 7-Fe-containing FeVco centers and two 8-Fe-containing P clusters. These authors suggest that since the preparation of Av1^{v} used had less than 30 Fe atoms/mol, it was a mixture of apo- and holoproteins.

The P clusters in reduced Av1^{v} were found to be in the diamagnetic P state and therefore the presence of $S = 3/2$ and $S = 1/2$ signals detected in an EPR study (see below) led to the suggestion that the FeVco center could be a mixture of spin states depending on the cofactor environment. The complexity of a superposition of 14 hyperfine split spectra arising from such a mixed spin state precluded detailed analysis and spectral simulation. However, at 80 K, the isomer shift of the FeVco center was the same as that for the FeMoco center of Av1 and Cp1, indicating similar electronic charge distribution over the iron atoms (Table 3).

b. Electron Paramagnetic Resonance Spectroscopy. EPR spectroscopy has been a powerful tool in the characterization of the redox centers of MoFe proteins. Dithionite-reduced MoFe proteins have a characteristic rhombic EPR signal with g factors close to 4.3, 3.7, and 2.01, arising from a transition of the lowest state of an $S = 3/2$ spin system. This signal is a sensitive spectroscopic fingerprint for FeMoco both in MoFe proteins and in the alternative nitrogenases, where hybrid species can be formed *in vivo*[35,36] or synthesized *in vitro*.[37] In addition to this signal, a spin $S = 1/2$ with $g_{av} = 1.92$ is often associated with MoFe proteins and has been assigned to a catalytically inactive, de-molybdo species of the protein. Oxidative redox titrations of MoFe proteins studied by EPR spectroscopy have defined a number of possible oxidation states of the P clusters in these proteins. A signal at $g = 12$, most easily detected in parallel mode EPR, appears to be a unique and characteristic spectroscopic fingerprint for P clusters.[60,61]

The EPR spectra of dithionite-reduced VFe proteins are complex because several paramagnetic species are present. Of the VFe proteins, Av1^{v} has been studied in greater detail. Dithionite-reduced Av1^{v} shows a broad poorly resolved EPR signal with g values at 5.8 and 5.4, which has been assigned to transitions from the ground and first excited state levels of the two Kramers doublets of a spin $S = 3/2$ system, which integrate to 0.9 spins per V atom.[10] The EPR data for Ac1^{v} are more complex and difficult to analyze, since the g values at 5.6, 4.3, and 3.77 appear to arise from a mixture of $S = 3/2$ species, presumably reflecting different environments of the

cofactor center.[9] Oxidative titration showed this center to have a midpoint potential of E_m of −125 mV.[74] By analogy with MoFe proteins, these signals have been assigned to a FeVco center homologous to FeMoco, but which give rise to EPR signals some 10-fold lower in intensity.

Both Ac1^v and Av1^v also exhibit an axial EPR signal with g values of 2.04 and 1.93 assigned to a $S = {}^1/_2$ ground state spin system, which integrates to ~0.2 spin/mol.[9,10] In the case of Ac1^v, the intensity of this signal does not correlate with specific activity, being low in samples of high activity.[72] It has been assigned to an FeS center present in an inactive devanado species. However, this signal in Av1^v remains at a constant ratio to the $S = {}^3/_2$ signal on further purification, which resolved Av1^v_A and Av1^v_B, and it is thought to be an integral part of this VFe protein.[26] Forms of Av1^v which exhibit only the $S = {}^3/_2$ signal, as seen with Ac1^v, have not been observed.

An additional signal,[75] not readily detectable in normal derivative absorption spectra but readily seen in dispersion EPR under conditions of rapid adiabatic passage, is present in the spectra of dithionite-reduced Av1^v. At 2 K and high power to saturate the signals, a broad structureless absorption envelope dominates the spectrum. This signal has been suggested to arise from a paramagnetic center coupled to a metal center. This signal is not seen in samples of MoFe protein under the same conditions.

Subsequent to these EPR studies, a Mossbauer investigation revealed that the P clusters in dithionite-reduced Av1^v are partially oxidized but could be reduced enzymically by enzyme turnover in assays containing limiting Fe protein.[73] EPR signals arising from the paramagnetism of the P clusters in the protein, as isolated, are characterized by $g = 6.67$ and 5.3 of an excited state $S = {}^5/_2$ spin system.

In addition, it has been shown that, as isolated, Av1^v is a mixture of two species with different subunit composition and metal compositions[26] (see above). These species, designated Av1^v_A and Av1^v_B, corresponding to the $\alpha\beta_2$ and $\alpha_2\beta_2$ subunit species, respectively, and have been shown to have different redox behaviors. The V:Fe:S^{2-} ratio and EPR properties of Av1^v_A are consistent with this species containing only one FeVco center, one P cluster, and a [4Fe-4S]-like center. Av1^v_B has a V:Fe:S^{2-} ratio consistent with a species having a full complement of FeVco centers and P clusters based on 2 g atoms of vanadium per molecule.[26]

As isolated, Av1^v_A and Av1^v_B have very similar EPR features[26,76] with both forms having the $S = {}^3/_2$ and $S = {}^1/_2$ states centered at $g = 5.5$ and 1.94, respectively, as was observed in earlier preparations of Av1^v and described above. They differ in that Av1^v_A has an additional $S = {}^1/_2$ slightly rhombic signal with g values of 2.03, 1.93, and 1.89, characteristic of reduced ferredoxins. As in the case of Av1^v, as isolated in the presence of dithionite, both Av1^v_A and Av1^v_B show features in their EPR spectra assignable to an $S = {}^5/_2$ spin system corresponding to 1-equiv-oxidized P clusters.

Enzymic reduction of Av1^v_B results in loss of the EPR signals associated with oxidized P clusters. The EPR difference spectra of Av1^v_B, as isolated and the protein reduced by enzyme turnover in the presence of limiting Fe protein, shows features with g values of 6.67, 5.3, and 4.3. Temperature dependence studies showed the 6.67 and 5.3 signals to arise from an excited state, which has been tentatively assigned to an $S = {}^5/_2$ spin system with $D = -3.2$ cm^{-1} and $E/D = 0.029$. Since partially oxidized Av1 shows analogous signals associated with oxidized P clusters, these signals of Av1^v_B indicate the presence of P clusters. A minor sharp first-derivative-shaped signal at $g = 4.31$ is also observed which is not present in spectra of reduced Av1^v_B. As isolated, Av1^v_B shows the $S = {}^3/_2$ signal of FeVco with g values of 5.68 and 5.45 ($E/D = 0.29$), these shift to 5.71 and 5.42 ($E/D = 0.28$) on enzymic reduction of the P centers. The change in E/D value indicates that the rhombicity of the cofactor signal decreases as the P centers are oxidized, implying a structural change about the cofactor as the P centers undergo redox processes.

Oxidative titration of enzymically reduced Av1^v_B has been reported and, as indicated below, the data are consistent with concurrent oxidation of both the FeVco centers and P clusters. This behavior contrasts with that of Av1, where a two-electron oxidation of each P cluster occurs before the FeMoco centers are oxidized.

At the start of the titration of Av1^v_B, EPR signals associated with FeVco ($g = 5.71$ and 5.42) and a small sharp inflexion at $g = 4.34$ are observed. After the addition of 1.5 equiv of thionine, the $g = 6.67$ and broad $g = 4.3$ signals seen in the protein as isolated are restored, and the FeVco signal is slightly bleached. The sharp inflexion signal at $g = 4.34$, which underlies the broad $g = 4.3$ feature, increases in intensity on further oxidation. After the addition of three oxidizing equivalents of thionine, the FeVco signal is 50% bleached and the $g = 6.67$ and 4.3 signals are at their maximum intensity. Further oxidation results in the decrease of all these signals except for the sharp inflexion signal at $g = 4.34$. During this phase of further oxidation, a signal at $g = 11.9$ in the parallel mode and 11.6 in the perpendicular mode assignable to a two-electron-reduced P cluster is observed. This signal has been interpreted as being associated with the excited state of an integer $S = 3$ spin system. A similar signal[74] has been observed during oxidative titrations of Ac1^v.

In redox titrations, Av1^v_A exhibits EPR signals which are consistent with redox-induced intramolecular electron transfer between reduced P cluster and the oxidized FeVco center. Initially, changes occur which are consistent with oxidation of only the FeVco center (in contrast to MoFe proteins, where P clusters are oxidized first). The signal at $g = 4.31$ increases in amplitude to a maximum at one reducing equivalent. Further oxidation by 0.2 equiv results in a dramatic increase of signal intensity associated with dithionite-reduced FeVco ($g = 5.68$, 4.45), oxidized P clusters ($g = 6.67$), and the broad $g = 4.3$ signal, with concomitant loss of the $g = 4.31$ signal. This abrupt change is consistent with a coupled transfer of an electron from a P cluster to the oxidized FeVco center. To account for these observations, the authors[76] have proposed a model in which Av1^v_A is capable of existing in two redox forms, one which is

generated in the initial stages of oxidation, where the midpoint potential of the FeVco center is more negative than that of the P clusters. The second is formed as a consequence of the addition of further oxidant. The resulting rise in redox potential is sensed by a redox center (proposed to be a P cluster), resulting in a conformational change in the protein coupled to changes in redox potential. The addition of further thionine resulted in the monotonic decrease of the signals associated with FeVco, P clusters, and the broad $g = 4.3$ signal. During this process, a first-derivative-shaped inflexion at $g = 4.3$ appears, which is different from a similar signal seen in the early stages of the oxidation of $Av1^{v}{}_{A}$.

In summary, EPR studies of the VFe proteins in the dithionite-reduced state indicate the presence of a cofactor center containing vanadium (FeVco), whereas studies on the proteins in different oxidation states indicate that they contain P clusters. The significance of other signals, at least one of which has no counterpart in the MoFe proteins, remains to be established. A study of a form of VFe protein which lacks an α subunit and contains one FeVco and P cluster center and a [4Fe-4S] center has provided evidence for a redox-mediated conformational change that triggers electron flow from reduced P clusters to the cofactor center.

c. Magnetic Circular Dichroism (MCD) Spectroscopy. Low-temperature MCD spectroscopy monitors magnetically induced dichroism of the Fe-S charge-transfer band of paramagnetic Fe-S centers. Studies of Kp1 and Av1 were facilitated by the fact that the paramagnetism of FeMoco results in it dominating the spectrum of the $S_2O_4^{2-}$-reduced protein. On dye oxidation of the protein, the FeMoco centers become diamagnetic and no longer contribute significantly to the spectra. However, oxidation of the protein results in P clusters assuming various paramagnetic states, depending on the redox potential and, therefore, dominating the spectra.

Initial MCD studies on the P clusters of oxidized Kp1 and Av1 used the dye thionine as an oxidant. Subsequently, it has been shown from EPR and Mössbauer studies that, although oxidation can proceed in one-electron steps, under the conditions of the early experiments, the proteins would have been oxidized by the removal of two electrons to the P^{2+} state.

The MCD spectra of $S_2O_4^{2-}$-reduced $Av1^{v}$ between 300 and 1000 nm at 4.2 K showed differences[77] in the intensity and frequency of the temperature-dependent MCD transitions when compared to data for Av1. Because EPR spectroscopy indicates the presence of several paramagnetic centers, MCD magnetization curves at 800 and 520 nm at different temperatures were constructed to establish the nature of the paramagnetic species contributing to the spectrum. The plots at these wavelengths for Av1 and $Av1^{v}$ were similar in form, consisting of sets of nested curves. The pronounced nesting indicated that these transitions originate mostly from the $S = {}^{3}/_{2}$ species with only minor contributions from the paramagnetic component with an $S = {}^{1}/_{2}$ ground state. Differences between the curves for Av1 and $Av1^{v}$ were attributed to changes in the zero-field splitting parameters.

Table 4. Comparison of the Environment of V and Fe in VFe Proteins and Mo and Fe in MoFe Proteins and Extracted Cofactors Determined from EXAFS Measurements

	type of interaction	*N*	*R* (Å)
	Mo−O(N)	2	2.12
Av1	Mo−S	4.5	2.37
	Mo−Fe	3.5	2.67
	Fe−Fe	1.4	3.78
	V−O(N)	2−3	2.15
$Av1^{v}$	V−S	3−4	2.33
	V−Fe	3 ± 1	2.76
	Fe−Fe	1	3.76
	V−O(N)	3 ± 1	2.15
$Ac1^{v}$	V−S	3 ± 1	2.31
	V−Fe	3 ± 1	2.75
	Fe−Fe	nd[b]	nd
FeMoco	Mo−O(N)	2	2.09
	Mo−S	3	2.36
	Mo−Fe	3.5	2.68
	Fe−Fe	1.3	3.68
FeVco	Fe−S	3	2.24
	Fe−V	1	3.69
	Fe−Fe	2	2.65
	Fe−Fe	1	3.69

[a] Data for $Ac1^{v}$ and extracted FeVco are from refs 78 and 82; those for $Av1^{v}$, from refs 79 and 80; and those for Av1 and FeMoco, from ref 80. [b] nd = not determined.

A unique feature of the MCD characteristics of oxidized P clusters of MoFe proteins is their unusually steep magnetization curves when compared with data for other FeS-containing proteins. Thionine oxidation of $Av1^{v}$ resulted in steep magnetization curves being obtained, which were very similar to those of P clusters of Av1. These spectra indicate that the paramagnetic chromophores present in these oxidized proteins have very similar magnetic properties. These data were interpreted as arising from a paramagnetic site of $S = {}^{5}/_{2}$ or $S = {}^{7}/_{2}$ spin state. The intensity of the MCD spectrum of oxidized $Av1^{v}$ is approximately half that of Av1 and it was suggested that this was due to a lower content of P centers in $Av1^{v}$. This situation may have been due to the presence of the $\alpha\beta_2$ form of $Av1^{v}$, which has recently been identified and has a lower content of P clusters.

d. X-ray Absorption Spectroscopy. Before the X-ray structures of Cp1 and Av1 were available, X-ray absorption spectroscopy had provided the most detail of the chemical environment of Mo in the MoFe proteins. The assignments, based on this technique, were subsequently confirmed by the X-ray crystal structures of Cp1 and Av1. This technique was applied to investigate the environment of V in the VFe proteins very soon after they were first isolated. In addition, Fe EXAFS of $Av1^{v}$ and of extracted FeVco have been reported. This technique is particularly powerful when applied to proteins where metal atoms are in a single environment, as is the case with Mo and V in MoFe and VFe proteins. It is more difficult when the metal atom is in a number of different environments, as is the case with Fe in MoFe, VFe, and FeFe proteins, where an average environment is seen.

Both $Av1^{v}$ and $Ac1^{v}$ have been studied at the V K-edge, and the data and assignments are in excellent agreement[78,79] (Table 4). The features of the absorption edge are consistent with V^{II} or V^{IV} in a

distorted octahedral coordination. The EXAFS region can be simulated by a three-component fit with Fe, S, and O or N as nearest neighbor atoms to the V atom. The types of atom and their distances from the V atom are very similar to those of Mo in the FeMoco center of MoFe proteins, except for a longer V−Fe distance compared with the Mo−Fe distance in MoFe proteins (see Table 4). In the case of the MoFe proteins, the X-ray crystal structure has removed the ambiguity as to the nature of the light atoms. The Mo is octahedrally coordinated to carboxyl and hydroxyl groups of homocitrate and a N atom of the imidazole ring of His-442. Since homocitrate is a likely component of FeVco (see section III.C.2.) and His-442 is conserved in the sequences of the VFe proteins (Table 1), the light atoms assigned in V EXAFS analysis are most likely oxygen atoms of homocitrate and a histidine residue nitrogen.

Fe K-edge EXAFS measurements[80] have been made on Av1 and $Av1^{v}$ and on the extracted cofactor[81] of the protein of $Ac1^{v}$. The EXAFS is dominated by Fe−S and Fe−Fe interactions, at 2.32 and 2.64 Å, respectively. A longer Fe−Fe distance of 3.7 Å is also required in the simulation in all three cases. The two Fe−Fe separations observed are consistent with the structure proposed for FeMoco from the X-ray crystallographic study and again emphasize the structural similarity between the cofactor centers of MoFe and VFe proteins.

Little change in Mo or V EXAFS data is seen following oxidation or reduction of the cofactor centers. The Mo edge and XANES of MoFe proteins are unchanged on dye oxidation or super-reduction of the FeMoco center during enzyme turnover, and only small angular rather than radial changes in the environment of the V atom are observed in $Ac1^{v}$.[82] Fe EXAFS data showed that on oxidation a slight contraction of the average first coordination sphere Fe−S distances occurred.[70]

2. FeVco—Extraction and Properties

Using methods developed for the extraction of FeMoco from the MoFe proteins a cofactor center has been extracted[83] from $Ac1^{v}$. Preparations of extracted FeVco in NMF contained V, Fe, and acid-labile sulfide in the ratio $\sim$1:(5.8 $\pm$ 0.4):(4.8 $\pm$ 0.8), within the range determined for isolated FeMoco, with V replacing Mo. The EPR spectra of the isolated material exhibited signals near 4.3, 3.6, and 2.0, characteristic of a spin $S = {}^{3}/_{2}$ system but some 8-fold less intense than FeMoco at an equivalent concentration. As discussed in section 2.4 the Fe EXAFS data for extracted FeVco are very similar to those of FeMoco.

FeVco was shown to be capable of binding to the FeMoco-binding site in MoFe protein, because incubation with the inactive MoFe protein isolated from a *nifB* mutant of *K. pneumoniae* resulted in the formation of an active hybrid protein. In typical extractions, some 20% of the initial activity of $Ac1^{v}$ was recovered. The substrate reduction pattern of the hybrid protein had the characteristic property of V-nitrogenase,[12] in producing some C_2H_6 (3%) in addition to C_2H_4 as a product of C_2H_2 reduction. Although C_2H_2 and protons were effective substrates, N_2 was not reduced either to NH_3 or N_2H_4. This observation provided the first indication that specific interactions of the cofactor center with amino acid residues in the vicinity of the cofactor-binding site are required for the reduction of N_2. This appears to be a general property for nitrogenase systems since directed mutagenesis of residues in the cofactor-binding pocket of Av1 results in the selective loss of the ability to reduce N_2.

C. Fe-Nitrogenase

1. FeFe Protein—Structure and Function

The FeFe protein of the third nitrogenase was first purified from a *nifHDK*-deletion strain of *A. vinelandii*,[11] and subsequently this protein was characterized from *R capsulatus*[84,85] and isolated from *R. rubrum*.[56,86] Spectroscopic studies on these proteins are limited to EPR spectroscopy, which been used extensively to probe the types of redox center that these proteins contain.

Fractionation of crude preparations of the FeFe protein of *A. vinelandii* by preparative gel electrophoresis resulted in the isolation of two species, termed 3^{F} and 3^{S}, denoting fast- and slow-migrating, respectively. Staining of SDS−PAGE gels with Coommassie Blue showed both to have α and β subunits of M_r = 58 and 54 kDa but with different relative amounts. The species 3^{F} corresponded to $\alpha_1\beta_2$ and species 3^{S} to $\alpha_2\beta_2$ subunit compositions assuming a native M_r = 158 and 216 kDa, respectively.[11] No small polypeptide correspond to the δ subunit encoded by *anfG* was detected in these experiments, although subsequent work demonstrated its presence in FeFe protein.[35]

Metal contents measured by neutron activation analysis for Fe, Mo, V, W, Cr, Re, and Zn were reported. Apart from Fe, only Zn was considered to be present at significant levels, but the values obtained for Mo and V are also included in Table 2. The ratio of Fe to S^{2-} is similar for the two species.

A strain of *A. vinelandii* lacking the genes for Mo- and V-nitrogenases has been constructed, and growth is inhibited by both Mo and V when these metals are added to the growth medium.[87] The addition of Co, Cr, Cu, Mn, Ni, Ti, W, and Zn did not stimulate growth of cultures which had a doubling time of $\sim$15 h. Together with the biochemical data for purified FeFe protein, these data provide support for the suggestion that dinitrogen reduction can occur at a center lacking Mo or V. However, as discussed below, a form of this enzyme which contains 1 FeMoco center/mol and has a subunit stoichiometry corresponding to $\alpha_2\beta\gamma_3$ has been isolated from a double mutant lacking Mo- and V-nitrogenases.[35] This species of FeFe protein contained Mo:Fe:S^{2-} in the ratio 1:24:18, showed a characteristic EPR signal of FeMoco, and effectively reduced N_2. During enzyme turnover, this EPR signal decreases in intensity, as is observed for MoFe protein. FeMoco could be extracted from this species and was active in the *in vitro* assay system. The potential of FeMoco to be incorporated into the cofactor binding site in this protein highlights the problem with preparations of

Table 5. Comparison of the Specific Activities of VFe, FeFe, and MoFe Proteins

	specific activity (nmol of product/mg of protein/min)						
	NH_3 formation	H_2 evolution under N_2	H_2 evolution under Ar	H_2 evolution under C_2H_2	C_2H_4 formation	C_2H_6 formation	ref
			MoFe Proteins				
K. pneumoniae	990	648	2100	304	1693	0	102
A. vinelandii	1040	nd[a]	2220	303	2000	nd	10
R. capsulatus	470	210	1300	140	1200	>0.05	35
			VFe Proteins				
A. chroococcum (Δ*nifHDK*)	350	928	1348	998	608	15	9
A. vinelandii (Δ*nifHDK*)	660	nd	1400	nd	220	nd	10
			FeFe Proteins				
A. vinelandii 3^S	38	213	253	202	28	nd	11
(Δ*nifHDK* W^r) 3^F	30	145	203	124	18	nd	11
A. vinelandii (Δ*nifHDK*; Δ*vnfHDK*)	110	220	350	226	58	26	35
R. capsulatus (Δ*nifHDK, modB*)	350	1300	2400	1700	260	5	85

[a] nd = not determined.

low activity which contain Fe and appear to lack a heterometal. A similar hybrid FeFe protein containing FeMoco has been isolated from a *nifHDK*-deletion strain of *R. capsulatus*,[36] but no metal contents were reported.

The substrate reduction pattern of Fe-nitrogenase is more like V-nitrogenase than Mo-nitrogenase in that C_2H_2 is a poor substrate compared with protons and N_2 and high levels of H_2 evolution occur in the presence of these substrates (Table 5). The overall activities of these preparations were lower than for V-nitrogenase, but the component ratio with respect to Fe protein was not optimized.

The FeFe protein has also been isolated and characterized from a *nifDH* deletion mutant of *R. capsulatus*. The doubling time for cultures of this strain growing on N_2 in Mo-deficient medium was 9 h. The addition of V had no effect on the growth or activity of the cultures. Isolated FeFe protein was unstable to freeze−thaw cycles and very O_2 sensitive. Initial work used Fe protein of *Xa. autotrophicus* for assay because of the lability of the Fe protein of the Fe-nitrogenase in *R. capsulatus*, and this may have contributed to the low activity observed. Assuming $M_r = 228$ kDa for this protein, the metal content is shown in Table 2.

An improved purification for this protein has recently been reported,[85] and the activity now approaches that of the VFe proteins (see Table 5). These preparations were isolated from a *nifHDK*, *modB* double mutant, defective in Mo uptake and unable to synthesize MoFe protein. The FeFe protein has a $\alpha_2\beta_2\gamma_2$ subunit structure of 268 kDa and M_r values of 61, 58, and 15 kDa for the α, β, and γ subunits, respectively. No analytical data for the metal content of these more active preparations were reported, but the earlier preparations contained low levels of Mo (Table 2), which were not considered to be significant because they did not correlate with activity during purification.[84]

EPR spectra of the dithionite-reduced protein shows features at $g = 4.3$, 2.05, and 1.93. The weak 4.3 signal was assigned to adventitious ferric iron contamination, and the other signals to an $S = {}^1/_2$ spin system similar to that observed in preparations of VFe proteins. Integration of this signal corresponded to 0.05 spins per FeFe protein and was suggested to arise from inactivated protein. These spectra differ from those reported earlier[88] for less active preparations in that the $g = 5.44$ resonance peak, which integrated to 1 spin/mol, was absent.

The characteristic $S = {}^3/_2$ signal associated with FeMoco, which is readily detectable with high sensitivity, was also absent, indicating that the increased activity is not due to the presence of FeMoco in these preparations. The absence of an EPR signal assignable to FeFeco centers in the protein was suggested to be a consequence of the replacement of Mo by Fe in these centers, resulting in an integer spin ground state (probably $S = 0$) arising from antiferromagnetic coupling of the Fe atoms.

A preliminary report on the FeFe protein of *R. rubrum*[86] indicated that this protein has a $\alpha_2\beta_2\gamma_2$ subunit structure and contains 16−20 Fe atoms per hexamer and no detectable Mo, V, or W. These preparations had low activity toward C_2H_2. EPR analysis showed the presence of a strong signal around $g = 2$ and the absence of the $g = 3.7$ signal characteristic of FeMoco. A factor capable of restoring activity of *nifB*-apo MoFe protein and also of replacing homocitrate in the *in vitro* FeMoco synthetic system could be extracted from the FeFe protein.

IV. Electron Transfer and Substrate Reduction

A. Electron Transfer

The alternative nitrogenases have similar requirements for the reduction of substrates as Mo-nitrogenase: MgATP, usually supplied from an ATP-regenerating system to avoid inhibition by MgADP, the absence of dioxygen, and a low potential reductant, usually dithionite ($S_2O_4^{2-}$). In addition to N_2 and protons (eq 1), Mo-nitrogenase will reduce C_2H_2, HCN, and a number of other small molecules containing C, N, or O multiple bonds. These reactions, and their inhibition by H_2 and CO, have been studied extensively in order to gain insight to the mechanism of the coupled proton and electron transfer reactions, which are catalyzed by nitrogenase. CO inhibits the reduction of all substrates except protons, and H_2 only inhibits the reduction of N_2.

The most comprehensive scheme for the catalytic cycle for N_2 reduction by Mo-nitrogenase is that of

Thorneley and Lowe. This scheme is based on steady-state kinetics and computer modeling of pre-steady-state stopped-flow and rapid-quench data for the rates of electron transfer, the time courses of the generation of enzyme-bound intermediates, and product formation. The current status of the mechanism of N_2 reduction by Mo-nitrogenase is reviewed in detail in the paper by Burgess and Lowe in this issue.

In the case of V-nitrogenase, only the reactions forming the first two steps of the catalytic cycle of nitrogenase turnover, i.e., the binding of MgATP to the Fe protein and subsequent electron transfer from $Ac2^v$ to $Ac1^v$, have been studied.[54,89] The rate of electron transfer for the $Ac2^v-Ac1^v$ complex ($k_{+2} = 46 \pm 2\ s^{-1}$) is significantly slower than for Ac2−Ac1 (k_{+2} 220 s^{-1}). The dependence of this reaction on MgATP concentration ($K_D = 230 \pm 10\ \mu M$) and its competitive inhibition by MgADP ($K_i^{MgADP} = 30 \pm 5\ \mu M$) were very similar to those of Mo-nitrogenase. The rate of formation of the $Ac2^v-Ac1^v$ complex ($k_{+1} \geq 3.0 \times 10^7\ M^{-1}\ s^{-1}$) is very similar to that for Mo-nitrogenase components. The rate of the dissociation of the two proteins following electron transfer, which is the rate-limiting step in Mo-nitrogenase turnover, has not been determined.

These similarities, together with the ability of the components of V-nitrogenase to form catalytically competent enzymes with the complementary protein of Mo-nitrogenase, even for N_2 as a substrate, strongly suggest a broad similarity with regard to the mechanism of the two nitrogenase systems (as described for Mo-nitrogenase by the Thorneley−Lowe scheme).

B. Substrate Reduction

The flux of electrons through Mo-nitrogenase is essentially independent of the reducible substrate, but electrons are partitioned depending on the rate of enzyme turnover and the reducible substrate. In assays under an inert atmosphere, usually argon, protons are reduced to H_2. Under the same conditions except under an atmosphere of N_2, H_2 evolution is inhibited and the balance of electron flux is utilized for the reduction of N_2. Optimum conditions of high electron flux result in one H_2 being evolved for each N_2 molecule reduced. As discussed by Burgess and Lowe in this issue, this stoichiometry is thought to be mechanistically significant, arising as a consequence of the binding of N_2 via a hydride displacement mechanism.

Although substrate reduction by the alternative nitrogenases has not been investigated as extensively, it is clear that differences in the cofactor centers and their interaction with neighboring amino acid residues modulate the reactivity of these systems. Both V- and Fe-nitrogenase share similar substrate reduction patterns. C_2H_2 is a poor substrate and N_2 does not compete as effectively with protons for the electrons available during turnover. Consequently, high rates of H_2 evolution are observed in the presence of these substrates. Both alternative systems catalyze the reduction of C_2H_2 to form C_2H_4 and, as a minor product, some C_2H_6. The limited data available on C_2H_2 and N_2 reduction by Fe-nitrogenase is shown in Table 5.

1. Coupling of ATP Hydrolysis to Electron Transfer

The efficiency of the coupling of ATP hydrolysis to electron flux through nitrogenase is one measure of the efficiency of the enzyme. In the case of Mo-nitrogenase, it has been shown that, under optimum conditions, the ratio of ATP hydrolyzed for two electrons transferred to proton reduction is ~4.5. Very similar values were found for Mo- and V-nitrogenases of *A. chroococcum* of 4.32 ± 0.16 and 4.87 ± 0.03, respectively.[90] However, under N_2, significant differences were seen when total electron flux into NH_3 and H_2 was used to calculate the ratio. Under these conditions, the ratios were 5.71 ± 0.41 for Mo-nitrogenase and 7.14 ± 0.14 for V-nitrogenase.[90] The significance of these differences, and whether it is a general property of nitrogenase, remains to be established, since no data for purified Mo-nitrogenase components during turnover under N_2, rather than argon, have been published.

2. Reduction of N_2

Under conditions of optimum electron flux (saturating concentrations of reductant, MgATP, and a high Fe protein to MoFe protein ratio) under N_2, the distribution of electrons to N_2 and protons are significantly different for Mo- and V-nitrogenases (eqs 2 and 3, respectively):

$$N_2 + 8e + 8H^+ = 2NH_3 + H_2 \qquad (2)$$

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

This difference is not due to different affinities for N_2, since the apparent K_m for N_2 as a reducible substrate for Mo- and V-nitrogenases of *A. chroococcum* at 30 °C is similar, 19 and 29 kPa, respectively.[91] Under conditions of saturating N_2 (339 KPa), limiting values for the H_2/N_2 ratio of 1.13 ± 0.13 for Mo-nitrogenase and 3.5 ± 0.03 for V-nitrogenase were obtained.[91] The higher proportion of electrons utilized for the reduction of protons is not due to restricted electron transfer, since the pre-steady-state rate of electron transfer from $Ac2^v$ to $Ac1^v$ ($k_{+2} = 46 \pm 2\ s^{-1}$) is 10-fold faster than the rate-limiting step in the catalytic cycle.[54] Consistent with this, the ratio of H_2/NH_3 formed does not decrease as the electron flux through V-nitrogenase is increased at high $Ac2^v$ to $Ac1^v$ ratios.[9] It has been suggested that, as with Mo-nitrogenase, the rate-limiting step of turnover is the dissociation of oxidized Fe protein−MgADP from the electron transfer complex.

The lower efficiency of V-nitrogenase when compared with Mo-nitrogenase is not due to the use of suboptimum assay conditions, because it is also observed in bacterial cultures.[7] Chemostat cultures, which readily allow the rate of N_2 fixation *in vivo* to be quantified, have been used to compare the efficiency of the Mo- and V-nitrogenases of *A. chroococcum*. The ratio of H_2 evolved to N_2 reduced at 30 °C (the H_2/N_2 ratio) was 1 for cultures using Mo-nitrogenase and 3 for those using V-nitrogenase (stoichiometries corresponding to eqs 2 and 3). A further indication of the lower efficiency of V-dependent nitrogen fixation was a higher requirement for

the carbon energy source to achieve the same yield of biomass during V-dependent growth.

During the reduction of N_2 by Mo-nitrogenase, N_2H_4 is only detected in solution when the reaction mixture is quenched with acid or alkali. In the Thorneley−Lowe scheme, dinitrogen is suggested to bind to a metal at the active site through one end only. Protonation of the terminal N atom of the bound N_2 then proceeds, with electron flow from the metal to the dinitrogen. This process results in the formation of a dinitrogen hydride intermediate, which generates N_2H_4 when the enzyme is denatured. There are no reported instances with wild-type or mutant proteins, of the formation of N_2H_4 as a *product* of N_2 reduction by Mo-nitrogenase.

In contrast, the reduction of N_2 by V-nitrogenase results in the formation of a small amount of N_2H_4 as a product.[92] Assays of combinations of Mo- and V-nitrogenase components of *A. chroococcum* showed that N_2H_4 formation was a property of the VFe protein. The amount of N_2H_4 formed at 30 °C is small (0.5% of the electron flux to N_2 as a substrate) but increases 15-fold as the assay temperature is raised[91] from 30 to 40 °C. Further increasing the temperature to 50 °C resulted in the reversible loss of the ability of V-nitrogenase to form NH_3 from N_2, but N_2H_4 as a product increased 3-fold. This observation was interpreted as arising from a temperature-induced conformational change occurring in $Ac1^v$ such that, at the higher temperature, the enzyme was largely unable to reduce N_2 to NH_3. This change in conformation of $Ac1^v$ has been proposed to prevent interaction of FeVco with residues of the polypeptide required for the reduction of N_2 to NH_3, but not to N_2H_4. This would suggest that such residues are involved in the final protonation step, resulting in cleavage of the N−N bond to yield NH_3. The release of N_2H_4 is the first direct evidence for an enzyme intermediate at the four-electron reduced level during the reduction of N_2 by nitrogenase.

The activity of Mo- and V-nitrogenases of *A. chroococcum* toward N_2 as a substrate as the assay temperature is decreased below 30 °C shows different behavior.[93] As the temperature is lowered, V-nitrogenase continues to reduce N_2 in contrast to Mo-nitrogenase, which diverts an increasing proportion of electron flux into the reduction of protons rather than N_2. For example, over the temperature range 30 to 5 °C, the specific activity of Mo-nitrogenase for N_2 decreases 10-fold more than V-nitrogenase. Experiments with combinations of Mo- and V-nitrogenase components showed the behavior to be conferred by $Ac2^v$.

Similar behavior was observed with cultures of *A. chroococcum*[94] in which V-nitrogenase, but not Mo-nitrogenase, functioned at temperatures below 10 °C. In addition, V-nitrogenase supported growth at 30 °C at pH values as low as 6.2, but Mo-nitrogenase did not. These studies suggest that the possession of a V-nitrogenase would confer a selective advantage to nitrogen-fixing organisms under conditions of low temperature or in an acidic environment. In this context, it has been shown that repression of V-nitrogenase by Mo observed at 30 °C in Azotobacter does not occur at lower temperatures.[95]

3. Reduction of C_2H_2

C_2H_2 is a relatively poor substrate[9−11] for Fe- and V-nitrogenases due to the continued reduction of protons (60% of the total flux) in the presence of C_2H_2. This contrasts with Mo-nitrogenases, for which proton reduction is limited to 15−20% of the electron flux under similar conditions. It has been shown in the case of V-nitrogenase that this is a property of the VFe protein[9] and is not due to a higher apparent K_m for C_2H_2, which at 5.9 kPa compares with the range for Mo-nitrogenase of 0.2−0.6 kPa. As with Mo-nitrogenase, the reduction is highly stereospecific,[96] giving [*cis*-$^2H_2$]C_2H_4 when the reaction is carried out in ^2H_2O.

The reduction of C_2H_2 by Fe- and V-nitrogenases produces a small amount of C_2H_6 in addition to C_2H_4. In the case of V-nitrogenase, $Ac1^v$ has been shown to confer this property to a functional enzyme. The amount of C_2H_6 is small, typically 0.02−7.4% of the electrons utilized for C_2H_2 reduction, but it is readily detected and is applicable to whole organisms.[97] The ratio of these products is independent of the partial pressure of C_2H_2 up to 30 kPa but increases with electron flux through the enzyme as either the temperature or the $Ac2^v$:$Ac1^v$ ratio is increased. The apparent K_m for C_2H_2 for the formation of both products is the same, suggestive of a single binding site in V-nitrogenase. However, the inhibitory effect of CO on the Fe-nitrogenase (see section IV.B.4.) has been interpreted as arising from two binding sites.

The mechanism by which C_2H_6 is formed is not clear. There is a lag of several minutes before its rate of formation becomes linear, whereas the rates of concomitant C_2H_4 and H_2 formation are linear.[96] Although V-nitrogenase catalyzes the slow reduction of C_2H_4 to C_2H_6, free C_2H_4 has been shown not to be involved as an intermediate in C_2H_6 formation from C_2H_2. The reduction of C_2H_2 to form C_2H_6 in addition to C_2H_4 by cultures of microorganisms grown under conditions of Mo-deficiency is presumptive evidence for the functioning of a Mo-independent nitrogenase.[97] This test was the first indication that *An. variabilis* had such a system[98] and, subsequently, the genes for V-nitrogenase were cloned and sequenced form this organism.[25]

However, it has subsequently been demonstrated that mutagenesis[71] of MoFe protein in the vicinity of the FeMoco binding site can result in significant levels of C_2H_6 being formed from C_2H_2. In addition, very small amounts are formed by the wild-type enzyme[87] when assayed at 50 °C.

C. Inhibitors of Substrate Reduction

Extensive steady-state studies of the mutual inhibition by alternative reducible substrates have been made with Mo-nitrogenase. No data are available for alternative nitrogenases except for the inhibition of proton reduction by C_2H_2 discussed above. For V-nitrogenase of *A. chroococcum*,[96] it has been reported that H_2 (at 85 kPa) completely inhibits the reduction of N_2 but not the reduction of C_2H_2. No analysis of kinetic data have been presented and the competitive nature of the inhibition of N_2 reduction by H_2 remains to be established.

In the case of inhibition by nonsubstrates, in the Mo-nitrogenase system, H_2 is a specific competitive inhibitor of N_2 reduction but does not affect the reduction of any other substrates. The specific competitive inhibition of N_2 reduction by H_2 has been interpreted as arising from them binding to a common site on the enzyme (see the work of Burgess and Lowe in this issue). CO inhibits the reduction of all substrates except protons and, under enzyme turnover conditions, elicits the formation of one or two $S = {}^1/_2$ EPR signals, depending on the CO concentration. Recent ^{13}C ENDOR studies of these signals are consistent with them arising from the sequential binding of CO to the same metal cluster[99] of MoFe protein. These signals are not observed with V-nitrogenase under turnover conditions.[37]

Inhibition data for V-nitrogenases of *A. chroococcum*[92] and *A. vinelandii*[100] are broadly similar. Under conditions of high electron flux, CO inhibits C_2H_2 reduction by V-nitrogenase less strongly than it does for Mo-nitrogenase. Assuming that binding occurs at V, this difference correlates with the bond energies of metal carbonyls, which increase with the atomic number of the metal. However, a recent study[100] of V-nitrogenase of *A. vinelandii* has shown surprising differences in the effect CO has on C_2H_4 and C_2H_6 formation from C_2H_2. First, at low partial pressures of CO and low electron flux ($Av2^v$:$Av1^v$ of 1:5), instead of inhibition, a doubling in the rate of C_2H_4 formation is observed. Only under conditions of high flux ($Av2^v$:$Av1^v$ of 8:1) is the expected inhibition observed. Secondly, a differential effect on C_2H_6 formation from C_2H_2 is seen, since the enhanced rate of C_2H_6 formation at low CO levels occurs under both high and low electron flux. These data differ from those reported earlier for V-nitrogenase of *A. chroococcum*, for which under conditions of high flux, the inhibition of both C_2H_4 and C_2H_6 behaved similarly and no stimulation of activity was seen.[92]

In the case of Fe-nitrogenase[101] and CO, a different pattern of inhibition was observed for C_2H_4 and C_2H_6 formation. The inhibition of C_2H_4 formation was monophasic as the partial pressure of CO was increased, but that for C_2H_6 formation was biphasic. At high CO partial pressures, up to 80% more C_2H_6 was formed than C_2H_4. These authors proposed two sites for C_2H_6 formation, one of which was not subject to inhibition by CO.

V. Outlook

It is clear from the extensive body of data reviewed above that Mo- and V-nitrogenases have very similar structures and types of redox centers. In particular, the cofactor centers have very similar structures and ligand geometry with V replacing Mo in FeVco. It is not clear what role the essential δ subunit plays in the V-system, and it appears that the subunit interactions in VFe protein are weaker and can lead to the formation of active species with a single cofactor center.

Early work unequivocally established that Fe-nitrogenase is capable of supporting growth of organisms on N_2, but the low activity of purified Fe-nitrogenase in reducing N_2 did not preclude the involvement of a heterometal present in low concentrations. The recent isolation of preparations of FeFe protein with N_2-reducing activity comparable to that of V-nitrogenases strongly suggests that FeFeco is a reality, and the molecular enzymology of its biosynthesis leads to the expectation that its structure will be found to be similar to the other cofactors. As is observed with VFe protein, the subunit interactions in this system are also weak and result in preparations with variable stoichiometry. The limited spectroscopic data available for these systems is consistent with the presence of P clusters, and the further spectroscopic characterization of this system should prove very interesting.

Although the chemistry developed to model N_2 reduction at a Mo center can plausibly be extended to V, the isolation of a functional Fe-nitrogenase raises the possibility that reduction at an Fe site may be common to all three nitrogenases. The structure of FeMoco derived from X-ray studies of the MoFe proteins does not exclude the binding of N_2 to Fe, and several theoretical models for this type of binding, including formation of multiple bonds to the central Fe atoms, have been proposed. The discovery of alternative nitrogenases has stimulated chemical research on modeling biological nitrogen fixation. Such studies, so long dominated by Mo because of a perceived essential role, are now being extended successfully both to V and Fe centers.

VI. Abbreviations

Fe protein, the iron-containing protein of nitrogenase; MoFe protein, the molybdenum- and iron-containing protein of Mo-nitrogenase; VFe protein, the vanadium- and iron-containing protein of V-nitrogenase; FeFe protein, the iron only containing protein of Fe-nitrogenase; Cp1, Av1, Kp1, and Xa1 denote the MoFe proteins of the Mo-nitrogenase of *Clostridium pasteurianum*, *Azotobacter vinelandii*, *Klebsiella pneumoniae*, and *Xanthobacter autotrophicus*, respectively; Av2, the Fe protein of *A. vinelandii* Mo-nitrogenase; Ac2, the Fe protein of *A. chroococcum* Mo-nitrogenase; $Ac1^v$ and $Ac2^v$, the VFe protein and Fe protein of the V-nitrogenase of *Azotobacter chroococcum*; $Av1^v$ and $Av2^v$, the corresponding proteins of the V-nitrogenase of *Azotobacter vinelandii*. The structural genes of the nitrogenase systems are denoted by *nif* for Mo-nitrogenase, *vnf* for V-nitrogenase, and *anf* for the third Fe-nitrogenase. ORF, open reading frame (a DNA sequence encoding an unknown protein); NMF, *N*-methylformamide; XANES, X-ray absorption edge and near edge structure; EXAFS, extended X-ray absorption fine structure, ESEEM, electron spin-echo envelope modulation.

VII. Acknowledgments

I thank Brian Hales for communicating his research results prior to publication, Antonio Pierik for useful discussions on sequence comparisons, and the BBSRC for funding of my research on nitrogen fixation discussed here.

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CR950057H