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THE Mo-, V-, AND Fe-BASED NITROGENASE SYSTEMS OF *Azotobacter*

ROBERT R. EADY

AFRC Institute of Plant Science Research, Nitrogen Fixation Laboratory, University of Sussex, Falmer, Brighton BN1 9RQ, England

I. Introduction

Recent research on the genetics, physiology, and biochemistry of nitrogen fixation has revealed that molybdenum does not have an essential role in this process. *Azotobacter* species have been shown to have three genetically distinct nitrogenase systems, one based on molybdenum similar to nitrogenase isolated from a wide range of N_2-fixing organisms, one based on vanadium, and a third that contains iron but only low levels of Mo or V.

In this article the intention is to indicate that despite the differences between these nitrogenase systems there is an underlying functional and structural similarity among them. The discovery and historical development of the work on Mo-independent nitrogenases have been reviewed recently (*1*) and will not be reiterated.

The biological activation of dinitrogen is catalyzed by nitrogenase, the enzyme system that reduces N_2 to NH_3, thereby providing a form of fixed N that is available to higher organisms. Fixation compensates for the steady loss of fixed N to the atmosphere as a consequence of the activities of denitrifying bacteria. Globally, biological nitrogen fixation contributes approximately 60% of the total of 2.5×10^8 tonnes of N per year fixed by both chemical and biological routes.

Molybdenum has long been known to have a role in nitrogen fixation; over 50 years ago Bortels showed that Mo stimulated the N_2-dependent growth of *Azotobacter* (*2*). Subsequently, these observations were rationalized when all purified nitrogenases were shown to be separable into an Fe protein and a MoFe protein that contained Mo as part of an essential iron- and molybdenum-containing cofactor (FeMoco), the probable site at which N_2 is reduced.

The apparently essential role of Mo was further supported by subsequent developments in the genetics of nitrogen fixation. These showed that genes involved in Mo uptake the FeMoco synthesis were closely linked and often coregulated with the genes for the structural polypeptides of Mo nitrogenase, the amino acid sequences of which had been highly conserved in evolution. In addition, kinetic studies on the mechanism of N_2 reduction by Mo nitrogenase and the reactivity of N_2 and likely reduction intermediates at Mo- or W-containing centers provided a plausible scheme for N_2 reduction by nitrogenase.

On chemical grounds, Williams and Wentworth (*3*) proposed that the reduction of N_2 to NH_3 at a transition metal site involving Mo could equally well occur at a site containing V. However, in the context of biological nitrogen fixation, the unequivocal demonstration that V could participate in this process was a surprising development (*4*), as was the subsequent description in 1988 of a nitrogenase that contains Fe and appears to involve neither Mo nor V (*5*).

All three types of nitrogenase have similar requirements for activity—a low-potential electron donor (MgATP) and the absence of oxygen. The Mo-independent enzymes can also be readily separated into two essential protein components, a distinct Fe protein, which together with the VFe protein or a component lacking significant amounts of Mo or V makes up the active enzyme. Which of these enzymes is synthesized depends on the availability of Mo or V in the growth medium. When sufficient Mo is provided, only Mo nitrogenase is synthesized; under Mo-deficient conditions in an otherwise complete medium (i.e., sufficient V and Fe), V nitrogenase is synthesized; when both Mo and V are deficient, the third nitrogenase is formed.

II. The Genetics of Nitrogen Fixation

A brief outline of the genetics of nitrogen fixation, relevant to the structure of the three types of nitrogenases and the types of redox centers they contain, is considered below.

A. Mo Nitrogenase

The genetics of nitrogen fixation is complex (see Ref. *6*). In *Klebsiella pneumoniae*, an organism that only has Mo nitrogenase, 21 *nif*-specific genes (*nif*, *ni*trogen *f*ixation) are found to be clustered on the chromosome in a 23-kb region of sequenced DNA. The function of *nif* genes was first established in *K. pneumoniae*, and many other N_2-fixing organisms have been shown to have homologous genes; in the case of *Azotobacter*, the major *nif* gene cluster has been cloned and sequenced (see Ref. *7*, and references therein).

In addition to those genes whose functions are described below, there are genes involved in generation of low-potential reductant for nitrogenase function *in vivo*, some to which no function has been assigned, and two involved in the specific regulation of the expression of other *nif* genes in response to environmental conditions (e.g., the presence of fixed N or the ambient dissolved oxygen concentration).

The component proteins of Mo nitrogenase are MoFe protein, an $\alpha_2\beta_2$ tetramer encoded by the *nifDK* genes, and an Fe protein, a γ_2 dimer encoded by *nifH*. The formation of an active enzyme requires, in addition to these structural genes, the functions of several other *nif* genes. The *nifM* in some unknown way activates the Fe protein polypeptide and is essential for its function. A number of *nif* genes are involved in FeMoco biosynthesis (*nifHBENVQ*) and are therefore essential for the synthesis of an active MoFe protein.

B. Mo-Independent Nitrogenases

An understanding of the genetics of *nif* gene organization in *Azotobacter* was an essential prerequisite for the unambiguous demonstration that Mo-independent nitrogenases existed. Recombinant DNA technology was used to construct mutant strains of *Azotobacter vinelandii* in which the *nifHDK* genes were specifically deleted (*8*). The demonstration that such mutant strains could grow on N_2 only when Mo was not added to the growth medium established the existence of Mo-independent nitrogenases (*9*), as had earlier been proposed but not generally accepted (*10*). When N_2-dependent growth of a comparable

mutant strain of *Azotobacter chroococcum* was shown to be stimulated by V added at 1–10 n*M*, and a V-containing nitrogenase was isolated, a role for V in biological nitrogen fixation was established (*4*).

Mutant strains of *A. chroococcum* carrying deletions of both Mo and V nitrogenase structural genes are unable to grow on N_2 (*11*). In contrast, comparable strains of *A. vinelandii* can grow provided neither Mo nor V is added to the growth medium (*12*). This ability is due to the presence of the third nitrogenase in *A. vinelandii*.

The similarities among these enzymes, together with the difficulty of removing adventitious Mo from bacterial growth medium to a level at which organisms cannot accumulate it, confounded earlier studies on the involvement of V undertaken before *nifHDK* deletion mutants were available. Nitrogenase isolated from *A. vinelandii* or *A. chroococcum* grown in Mo-deficient medium supplemented with V was of low activity and contained both Mo and V. The slow growth of cultures under these conditions was attributed to vanadium stimulation of the uptake of residual Mo in the medium (see Ref. *1*).

The structural genes of V nitrogenase (*vnf*, *v*anadium-dependent *ni*trogen *f*ixation) have been cloned and sequenced from both *A. chroococcum* (*11*) and *A. vinelandii* (*13*), as have the structural genes of the third nitrogenase of *A. vinelandii* (*anf*, *a*lternative *n*itrogen *f*ixation), a system that is not found in *A. chroococcum* (*14*). These sequences were sufficiently homologous with *nif* genes to allow assignment of *vnfH* and *anfH* to the Fe proteins of these nitrogenases and of *vnfDK* and *anfDK* as subunits of the VFe protein and the third nitrogenase, respectively, and are discussed in more detail in Section III.

The presence of an open reading frame between *vnfD* and *vnfK* led to the identification of an additional small subunit type (δ, encoded by *vnfG*) of the VFe protein; it has the hexameric structure $\alpha_2\beta_2\delta_2$ (*11*). A homologous gene exists between *anfD* and *anfK* (*13a*) and encodes a small subunit of the third nitrogenase (R. Eady and R. Pau, unpublished).

1. Genes Required for the Function of All Three Nitrogenases

Because all nitrogenase systems involve a distinct Fe protein, it is not surprising that *nifM* is essential for activity of all three systems (see Ref. *14*). *NifB* and *nifV* are also essential for all three nitrogenase systems, suggestive of a role in some aspect of cofactor biosynthesis in the Mo-independent systems (*14, 15*). In Mo nitrogenase, *nifV* is involved (probably as a homocitrate synthase) in the biosynthesis of homocitrate (*R*-2-hydroxy-1,2,4-butanetricarboxylic acid; see later, Fig. 2), an organic component of FeMoco (*16*).

NifV mutants are unable to synthesize homocitrate and incorporate citrate into FeMoco. The resulting MoFe protein has altered inhibitor and substrate reduction patterns (see Ref. *16*). As discussed below, the VFe protein contains an iron- and vanadium-containing cofactor (FeVaco) analogous to FeMoco of the MoFe protein. The requirement of *nifV* for V nitrogenase function suggests homocitrate is also a component of FeVaco. At this time of writing, no information is available as to the cofactor(s) present in the third nitrogenase. When purified, this protein contains only low levels of Mo and V (*5*); because *nifV* is also required for the functioning of the third nitrogenase the possibility exists that a cofactor associated with homocitrate, which contains only Fe and lacks a heterometal atom, is a component of this system.

2. *Genes Common to Mo-Independent Nitrogenases*

The reiteration of *nifEN*-like genes, identified in *Azotobacter*, are essential for Mo-independent nitrogenase but not Mo nitrogenase function (*14*). *NifEN* products show sequence homology with *nifDK* and form an $\alpha_2\beta_2$ Fe/S-containing complex analogous to the MoFe Protein. It has been suggested that this complex, although catalytically inactive, provides a scaffold on which FeMoco is synthesized (see Ref. *17*). The reiterations of *nifEN*-like genes may have a role in the assembly of FeVaco and the putitive Fe-containing cofactor of the third nitrogenase.

These genetic data support the suggestion of a parallel route for the synthesis of cofactors of Mo-independent nitrogenase function involving some early steps in common with FeMoco biosynthesis.

III. The Biochemistry of Nitrogen Fixation

Positive DNA hybridization with DNA of some 50 species of N_2-fixing bacteria to a probe of the structural genes of Mo nitrogenase indicates that the structure of this nitrogenase has been highly conserved. Consistent with this, the physicochemical properties of purified Mo nitrogenase components have been shown to be very similar; they have been extensively reviewed and will only be summarized here (see Refs. *17–20* for primary references).

A. The Fe Proteins

The DNA encoding the Fe protein (*nifH*) of some 20 N_2-fixing bacteria, ranging from Archaebacteria and Eubacteria to Cyanobacteria,

have been cloned and sequenced (*21*). The derived amino acid sequences show not less than 45%, and usually greater than 85%, identical structure. There are five invariant cysteine residues that occur in highly conserved regions of these proteins and a consensus nucleotide-binding motif of Gly-X-Gly-X-X-Gly near the NH_2 terminus. The spacing of the invariant Cys residues is different from what is seen in other Fe–S cluster-containing proteins. Site-directed mutagenesis has shown that following Cys → Ser mutation, only two of the conserved Cys residues are essential for activity.

Studies of purified Fe proteins of Mo nitrogenase from eight organisms have shown them to have a γ_2 dimeric structure. Typically their native M_r values are ~62,000 and they contain 4Fe and $4S^{2-}$ atoms per dimer (see Table I). Spectroscopic and redox properties and cluster extrusion data are consistent with the presence of a single [4Fe–4S] cluster that bridges the two subunits and traverses the +1 and +2 oxidation levels with a midpoint potential near ~−320 mV. The binding of MgADP, and to a less marked extent MgATP, results in a conformational change of the protein and a decrease in redox potential of the [4Fe–4S] center by 100 mV.

As isolated, dithionite-reduced Fe proteins exhibit low-temperature EPR spectra consistent with the presence of mixed interconvertible $S = 1/2$ and $S = 3/2$ spin states of the [4Fe–4S] center. The relative intensities of these signals change in the presence of urea or ethane-

TABLE I

COMPARISON OF THE PHYSICOCHEMICAL PROPERTIES OF THE Fe PROTEINS OF DIFFERENT NITROGENASES

	Enzyme		
Fe component property	Mo nitrogenase	V nitrogenase	Third nitrogenase
M_r	62,000	61,000	64,000
Subunit composition	γ_2	γ_2	γ_2
Metal and S^{2-} content (g atom/mol)			
Fe	4	3.7	3.5
S^{2-}	3.8	3.9	4.0
EPR g values for dithionite-reduced protein	2.05, 1.94, 1.86, 4–5	2.035, 1.941, 1.892	ND[a]
Specific activity (nmol NH_4 formed per min/mg protein)	550	337	29

[a] ND, Not determined.

diol, but together they integrate to one spin per dimer. Mixed spin states are unusual for Fe–S proteins but do not arise from noncysteinyl ligation of the FeS center because Fe K-edge EXAFS data indicate S ligation to the Fe atoms. At room temperature, the position and temperature dependence of paramagnetically shifted resonances in the ^1H NMR spectrum indicate the cluster is in the $S = 1/2$ spin state, suggesting the $S = 3/2$ spin state arises on freezing of the samples.

The modification of an exposed arginine residue (Arg 101) by ADP ribosylation modulates the activity of the Fe protein *in vivo*. The conversion of Arg 101 → His by site-directed mutagenesis results in a mutated Fe protein that forms a nitrogenase capable of ATP hydrolysis but not substrate reduction, indicating that this residue is probably required for protein–protein electron transfer.

The Fe Proteins of Mo-Independent Nitrogenases

The structural genes of the Fe proteins of the V nitrogenase of *A. chroococcum* and *A. vinelandii* and of the third nitrogenase of *A. vinelandii* have been cloned and sequenced (*13a, 14, 22*). Comparison of the derived amino acid sequences shows the Fe proteins of Mo and V nitrogenases to be very similar (91% identical) and that of the third nitrogenase to be 61% identical. All sequences show the spacing of the five invariant Cys residues, a conserved Arg residue near position 100, and a consensus nucleotide-binding sequence.

These Fe proteins have been purified to homogeneity (*5, 23, 24*); they are γ_2 dimers containing approximately 4Fe and $4S^{2-}$ atoms and have properties very similar to those of the Fe proteins of Mo nitrogenases (Table I).

The Fe proteins of V nitrogenase exhibit EPR spectra characteristic of $S = 1/2$ and $S = 3/2$ spin states, and bind MgATP, causing a conformational change resulting in enhanced chelation of Fe by α,α'-bipyridyl, as is observed for Fe proteins of Mo nitrogenase. The E_m of the MgADP-bound Fe protein is −463 mV compared with −450 mV of the MgADP–Fe protein complex of Mo nitrogenase of *Azotobacter*. The rate of reduction of MgADP-oxidized Fe protein complexes by dithionite is similar for Fe proteins of Mo and V nitrogenase. The structural homology associated with the [4Fe–4S] center of these Fe proteins allows the formation of functional heterologous nitrogenases between V and Mo nitrogenase components, a property not always shown by components of Mo nitrogenase when isolated from different organisms. In this respect, the Fe protein of the third nitrogenase, which shows lower amino acid sequence homology then do Fe proteins of Mo and V nitro-

genase, shows less than 1% reactivity with MoFe protein compared with 90% for the Fe protein of V nitrogenase.

It is clear from the data discussed above that the Fe proteins associated with Mo-independent nitrogenases of *Azotobacter* are typical members of the highly conserved family of Fe proteins associated with Mo nitrogenases.

B. The MoFe Proteins

The MoFe proteins isolated from all sources are $\alpha_2\beta_2$ tetramers of M_r ~220,000. The DNA sequences encoding the α subunit (*nifD*) and β subunit (*nifK*) have been cloned and sequenced from seven organisms. The derived amino acid sequences indicate considerable homology, in particular five invariant Cys residues in the α subunit and three invariant Cys residues in the β subunit. As in the case with the Fe proteins, the conserved ferredoxin-like Cys-X-X-Cys spacing characteristic of many Fe–S proteins is not observed (Fig. 1). (*25*). Comparison of the sequences of the α and β subunits shows some homology, and X-ray

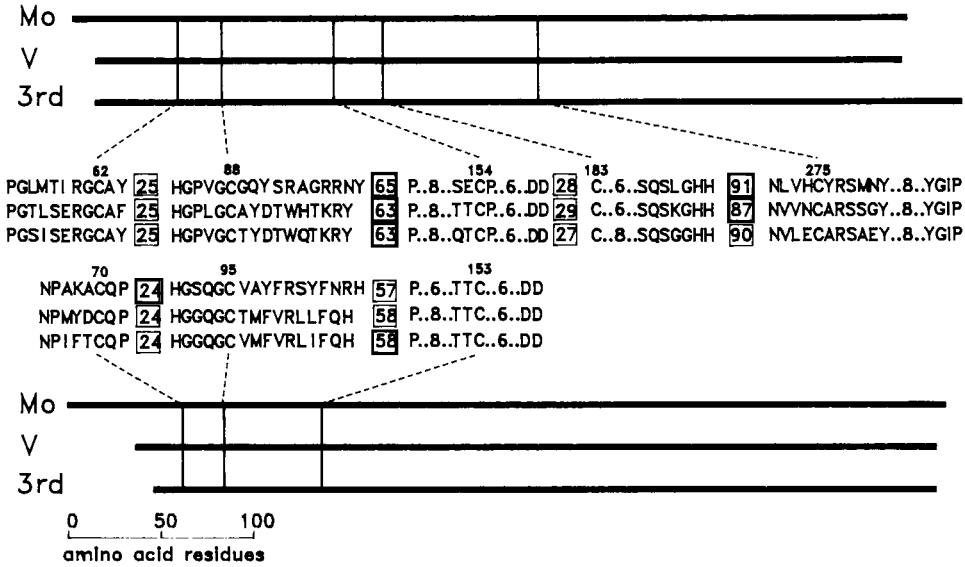


FIG. 1. Schematic representation of the α and β polypeptides of the MoFe protein, the VFe protein, and the third nitrogenase. The vertical lines represent the position of the five invariant Cys residues of the α subunit and the three invariant Cys residues of the β subunit. The flanking amino acid sequences are shown to indicate the degree of sequence homology around these Cys residues. The wide horizontal lines represent the polypeptide chains of the subunits of the three nitrogenases (see Ref. *25*).

diffraction data allow the calculation of a 6-Å rotational function, which suggests there is structural homology between the α and β subunits. A number of MoFe proteins have been purified and characterized. Most active preparations contain 2 Mo atoms and about 30 Fe and S^{2-} atoms per tetramer (Table II) (*26, 27*).

As discussed below, the consensus view of the redox centers present in MoFe proteins is that there are two types (see Ref. *28* for discussion). There are two FeMoco centers (which have the approximate composition one Mo, six to eight Fe, four to nine S, and one homocitrate; this is unique to nitrogenase and different from the Mo cofactors of other Mo-containing enzymes), and four [4Fe–4S] centers (the "P" clusters). If these assignments are correct, then because only 16 Cys residues are invariant among MoFe proteins, conventional Cys ligation to the "P"

TABLE II

COMPARISON OF THE PHYSICOCHEMICAL PROPERTIES OF MoFe AND VFe PROTEINS AND THE THIRD NITROGENASE

Property	MoFe protein in *K. pneumoniae*	VFe proteins		Third nitrogenase of *A. vinelandii*	
		A. chroococcum	*A. vinelandii*	Fast species	Slow species
Native M_r	220,000	210,000	200,000	158,000	216,000
Subunit structure	$\alpha_2\beta_2$	$\alpha_2\beta_2\delta_2$	$\alpha_2\beta_2(\delta_2)$	$\alpha_1\beta_2$	$\alpha_2\beta_2\delta_2$
Subunit M_r	2 × 50,000	2 × 50,000	2 × 52,000	2 × 50,000	2 × 50,000
	2 × 60,000	2 × 55,000	2 × 55,000	1 × 58,000	2 × 58,000
		2 × 13,000			
Metal and S^{2-} content (g atom/mol)					
V	0.05	2 ± 0.3	0.7 ± 0.3	0.015	0.01
Mo	2	0.06	0.05	0.015	0.085
Fe	32 ± 3	21 ± 1	9 ± 2	11	24
S^{2-}	ND	19 ± 0.2	21 ± 1	9	18
EPR *g* values for dithionite-reduced protein	4.3, 3.7, 2.015	5.6, 4.35, 3.77, 1.93	5.31, 4.34, 2.04, 1.93	ND[a]	ND
Specific activity (nmol product formed per min/mg of protein)					
NH_4 formation	990	350	660	30	38
H_2 under N_2	648	928	ND	145	213
H_2 evolution under Ar	2100	1348	1400	203	253
C_2H_4 from C_2H_2	1693	608	220	18	28
H_2 under C_2H_2	304	998	ND	124	202
Reference	*26*	*11*	*27*	*5*	*5*

[a] ND, Not determined.

centers alone cannot occur unless they bridge the α and β subunits. Such a binding mode would utilize all conserved Cys residues, a situation inconsistent with data implicating Cys residues in the binding of the FeMoco centers to the MoFe proteins.

A variety of spectroscopic and physical techniques have been used to investigate the nature of these redox centers. EPR, ^{57}Fe Mössbauer spectroscopy, and Mo and Fe X-ray absorption spectroscopy; ^{95}Mo, ^{33}S, ^{57}Fe, and ^{1}H electron–nuclear double resonance (ENDOR); linear electric field effect; and magnetic circular dichroism (MCD) have provided information about the environment of the Mo and Fe nuclei and their interaction with the unpaired spin of electrons in paramagnetic species of the MoFe proteins.

In addition, site-directed mutagenesis has been used to probe cluster binding, and extrusion studies of FeMoco centers and "P" clusters have provided a considerable insight to their structures.

1. The "P" Clusters

Mössbauer spectroscopy of ^{57}Fe-enriched MoFe protein in dithionite-reduced and dye-oxidized oxidation states were interpreted in terms of approximately 50% of the Fe in the protein being present in cubane clusters similar to [4Fe–4S] clusters of simpler Fe–S proteins, e.g., ferredoxins and *Chromatium* high-potential iron proteins. Spectra of MoFe protein in which "P" clusters were selectively enriched with ^{57}Fe were consistent with two of the clusters having slightly different environments.

The assignment of half of Fe to [4Fe–4S] centers was supported by the extrusion of [4Fe–4S] clusters from denatured protein in the presence of *o*-xylyl-α-α'-dithiol. However, the extrusion technique is unreliable when applied to proteins containing [3Fe–*n*S] centers, when [2Fe–2S] clusters are extruded, as occurs with dye-oxidized MoFe protein.

The spectroscopic properties of "P" clusters are unusual. In the dithionite-reduced MoFe protein all the Fe atoms of the "P" clusters are iron(II), indicating a $[4Fe–4S]^0$ oxidation state, a level difficult to achieve with model complexes. Oxidation gives rise transiently to an EPR-observable ($g_{av} = 1.93$) species, which then relaxes to give a very complex Mössbauer spectrum.

The MCD spectra are also unusual. At low temperature, where magnetic species predominate, the MoFe proteins have distinctive spectra. In the dye-oxidized state the "P" clusters are the only paramagnetic centers and have unusual spin states of 5/2 or 7/2. Recently, dye-oxidized MoFe proteins have been shown to exhibit EPR spectra aris-

ing from "P" clusters, with g values of 10.4, 5.8, and 5.5 consistent with a spin 7/2 system, which integrates to one spin per Mo atom. This is consistent with either that there are only two "P" clusters, which are larger than the proposed [4Fe–4S] centers, or that only two of the [4Fe–4S] centers are associated with the 7/2 spin epr signal. The unusual spectroscopic properties of these clusters may arise from noncysteinyl ligation. Coordination of synthetic Fe–S clusters by Tyr or Ser (*29*) indicates their potential role in biological systems, and in some metalloproteins His provides ligation for an Fe–S center (*30*).

The spatial distribution of metal centers in MoFe proteins has been investigated by X-ray anomolous scattering. Bijvoet difference Patterson maps of Cu–Kα data indicate the presence of four large clusters of approximately equivalent metal contents requiring either that the "P" clusters are organized as two adjacent [4Fe–4S] clusters or that they are larger clusters containing approximately either Fe atoms. Using Co–Kα radiation distinguishes FeMoco from other centers in the protein, and preliminary data are consistent with a 70-Å FeMoco separation and a 19-Å FeMoco–"P" cluster center-to-center separation (*31*).

2. *FeMoco Centers*

Molybdenum is present in MoFe proteins as part of an iron-and molybdenum-containing cluster (FeMoco) that gives rise to the characteristic EPR spectrum of dithionite-reduced MoFE proteins. Strong exchange coupling of this $S = 3/2$ spin system results in a single magnetic entity with g values near 4.3, 3.7, and 2.01. Mössbauer spectroscopy of ^{57}Fe-substituted protein indicates that about seven Fe atoms are associated with each paramagnetic center. ENDOR spectroscopy shows interaction of the unpaired electron giving rise to the EPR signal with ^{57}Fe, ^{95}Mo, ^{1}H, and ^{33}S, nuclei with spins > 0 (see Ref. *17*, and references therein). Five different Fe environments were detectable, and one or two ^{1}H atoms were exchangeable with D_2O, indicating that the cluster is accessible to solvent. Electron spin–echo measurements were consistent with coordination with at least one N atom in the MoFe protein, presumably provided by an amino acid residue, because this interaction was lacking in the extracted cofactor (*32*).

The most detailed information on the environment of Mo in MoFe proteins has been obtained from X-ray absorption spectroscopy. Analyses of the EXAFS spectra are consistent with Fe, S, and O (or N) atoms in the coordination sphere of Mo (Table III) (*33–36*).

Single-crystal polarized X-ray absorption spectroscopy is not consistent with a linear Fe—Mo—Fe structure and Mo=O was only observed in oxygen-inactivated protein. Only very minor changes oc-

TABLE III

COMPARISON OF ENVIRONMENTS OF V IN VFe PROTEINS AND Mo IN MoFe PROTEINS DETERMINED FROM X-RAY ABSORPTION SPECTROSCOPY

	EXAFS			
	Atom	No	R(Å)	Reference
$[Me_4N][VFe_3S_4Cl_3(DMF)_3]^a$	V—O	3	2.12 (2.13)	*33*
	V—S	3	2.35 (2.33)	
	V—Fe	3	2.75 (2.77)	
VFe protein of	V—O (or N)	3 ± 1	2.15	*33*
A. chroococcum$^+$	V—S	3 ± 1	2.31	
	V—Fe	3 ± 1	2.75	
VFe protein of	V—O (or N)	2–3	2.15	*34*
A. vinelandii	V—S	3–4	2.33	
	V—Fe	3 ± 1	2.76	
MoFe protein of	Mo—O (or N)	1.9	2.12	*35*
A. vinelandii	Mo—S	4.5	2.37	
	Mo—Fe	3.5	2.67	

[a] The values in parenthesis were obtained from X-ray crystallographic studies (*36*).

curred on dye oxidation or on superreduction during enzyme turnover. This insensitivity of the Mo K-edge EXAFS to changes in the oxidation level of the cluster is presumably due to spin delocalization onto the iron atoms as indicated by EPR studies of ^{95}-Mo-substituted proteins.

FeMoco can be extracted from acid-treated MoFe protein by treatment with *N*-methylformamide (NMF). The NMF extract contains $MoFe_{5-7}S_{4-9}$ and is capable of activating purified MoFe proteins isolated from strains defective in FeMoco biosynthesis. Mo EXAFS spectra indicate that only very minor changes in the environment of Mo occur on extraction of FeMoco, but electron spin-echo data are consistent with the loss of a ligating N ligand from the cluster. Fe K-edge EXAFS data show two Fe–Fe distances with interactions up to 3.65 Å, indicating long-range order in FeMoco (*37*).

When FeMoco extracted from MoFe protein purified from a nifV mutant is recombined with apo-MoFe protein, the activated protein has the substrate-reducing characteristics of the *nifV*$^-$ enzyme (reduces C_2H_2 effectively but N_2 only poorly). This observation provides the most compelling evidence that FeMoco is, or forms part of, the active site of nitrogenase. Site-directed mutagenesis has implicated one of the conserved Cys residues of the α subunit Cys 275 in binding FeMoco, and also His 196 and Gln 192 (see Refs. *17* and *38* for discussion).

3. *Synthesis of FeMoco in Vitro*

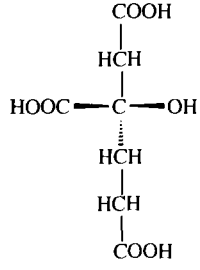
A biosynthetic system for FeMoco has been developed and forms the basis of a good model system for studying the assembly of metal clusters in biological systems (see Ref. *16* for review). Factors necessary for the synthesis of FeMoco *in vitro* are the products of *nifB*, *nifE*, *nifN*, and *nifH* genes, MgATP, molybdate, and homocitrate. Using this system, ^{3}H-labeled homocitrate was shown to be incorporated into FeMoco at a 1 : 1 ratio with Mo. Replacement of homocitrate with citrate in this system resulted in the formation of FeMoco with the substrate reduction patterns characteristic of *nifV* mutants, which are unable to synthesize homocitrate.

A wide range of homologues of homocitrate have been substituted in this system. The reduction of various substrates (N_2, H^+, C_2H_2, or CN) by the reconstituted MoFe proteins was tested, and a range of different specifities was observed. The use of 1-hydroxyacids, e.g., homoisocitrate, isocitrate, or 1-OH citrate, results in the loss of N_2 and C_2H_2 reducing ability, but H^+ and CN are still substrates. Structural features important for N_2 reduction are the 1- and 2-carboxyl groups, the 2-hydroxyl group, and the *R* configuration of the C1 carbon atom (Fig. 2).

Because ^{95}Mo ENDOR studies suggest that in *nifV*$^-$ MoFe protein the Mo site is purturbed by the loss of non-S ligands, it has been proposed that homocitrate provides some or all of the O ligands for Mo in FeMoco (*16*).

C. The VFe Proteins

The DNA encoding the structural genes of the VFe protein (*vnfDGK*) from *A. chroococcum* and *A. vinelandii* have been cloned and



(R)-homocitrate

FIG. 2. Structure of *R*-homocitrate.

sequenced (*11, 13*). The derived amino acid sequences are >95% identical and show ~30% sequence identity with *nifDK*, the structural genes of MoFe protein of *Azotobacter*. Notably, the invariant Cys residues observed in all sequences of MoFe proteins are conserved, five in the α subunit (*vnfD*) and three in the β subunit (*vnfK*) (Fig. 1). A major difference is the presence of an additional gene, *vnfG*, located between *vnfD* and *vnfK*, that encodes an additional small subunit of the VFe protein, which has an $\alpha_2\beta_2\delta_2$ subunit structure. The amino acid sequence of the δ subunit shows no sequence homology with the α or β subunits of the MoFe proteins.

The VFe proteins of *A. chroococcum* and *A. vinelandii* have been purified to homogeneity (*11, 27*). Preparations with the highest activity contain 2 V, ~20 Fe, and ~20 S^{2-} g atoms per hexamer and have specific activities comparable to those of the MoFe protein (see Table II). Since their isolation, attention has naturally focused on the types of redox center they contain, particularly on the involvement of vanadium. Progress in this area has been rapid, and despite the presence of the additional subunit type and the probability that the VFe protein preparations currently available are a mixture of species, the spectroscopic and cofactor extrusion data summarized below are consistent with VFe and MoFe proteins having similar types of redox centers.

1. EPR Spectroscopy

The low-temperature EPR spectra of VFe proteins are more complex than are those of the MoFe proteins because several paramagnetic species are present. In addition, some features of the spectra of VFe proteins from *A. chroococcum* and *A. vinelandii* are different.

Both VFe proteins show an axial signal with g values near 2.04 and 1.93, assigned to an $S = 1/2$ spin system associated with an Fe–S center (*11, 39*). This signal, which is not exhibited by MoFe proteins, integrates to 0.2–0.3 spin mol^{-1}. The redox and pH dependence of the intensity of this signal indicate that the redox potential of the cluster from which it arises is low. However, recent results have shown that the intensity of this signal does not correlate with the activity of the VFe protein and may arise from a protein lacking a full complement of metals (*40*).

The VFe protein of *A. vinelandii* shows a weak, poorly resolved EPR signal with apparent g values of 5.8 and 5.4, which have been assigned to the low-field inflexion of the transition from the ground to the first excited state of the Kramer's doublets of an $S = 3/2$ spin system (*39*). The intensity of this signal integrates to 0.89 spin per V atom and it provided the first spectroscopic evidence for the presence of a V-con-

taining cofactor in the VFe proteins. The VFe protein of *A. chroococcum* shows a more complex spectrum in this region (*11*), with signals at $g = 5.6$, 4.35, and 3.77, assignable to a spin $S = 3/2$ system, but difficulties in simulation of this spectrum are consistent with it arising from a mixture of species (D. J. Lowe, personal communication). These species are apparently resolved on further purification, because VFe protein, which lacks the $S = 1/2$ spin signal discussed above, also has a simpler low-field spectrum and closely resembles that of the *A. vinelandii* VFe protein. A third EPR signal has recently been detected in the VFe protein from *A. vinelandii* (*41*). This signal, which is a broad structureless absorption envelope, is most readily detected at 2 K using the spectrometer in the dispersion mode. It is not found in MoFe proteins and is similar to signals arising from a paramagnetic center coupled to a metal-containing center. The correlation of this signal with VFe protein activity has not yet been established.

2. Magnetic Circular Dichroism (MCD)

Low-temperature MCD spectroscopy has been used to characterize the metal clusters present in the VFe protein of *A. vinelandii* (*42*). The temperature dependence of the MCD transitions of dithionite-reduced protein is similar to those arising from the $S = 3/2$ spin system of FeMoco of the MoFe proteins. The spectra of the VFe proteins have slightly different electronic and magnetic properties but provide strong spectroscopic evidence for the presence of a V–Fe–S cluster. In this oxidation state, only small additional spectral contributions from the two other paramagnetic species ($S = 1/2$ signal and the species detected in the dispersion mode) are observed.

In the dye-oxidized EPR-silent state a cluster that was diamagnetic in the dithionite-reduced state became paramagnetic, with MCD and magnetization characteristics assignable to an $S = 5/2$ spin system with near axial symmetry. These data are very similar, but are not identical with those for the "P" clusters of MoFe proteins and suggest the presence of analogous clusters in the VFe proteins.

3. X-Ray Absorption Spectroscopy

Vanadium K-edge X-ray absorption spectroscopy has provided the most detailed information on the environment of V in the VFe proteins. Data for the VFe proteins of *A. chroococcum* and *A. vinelandii* are very similar (*33, 34*). The edge and near-edge structures of these spectra, which reflect coordination geometry and probable oxidation level of the absorbing atom, are both similar to the complex $\{VFe_3S_4Cl_3[HCON(CH_3)_2]_3\}^-$. The relatively low intensity of the pre-

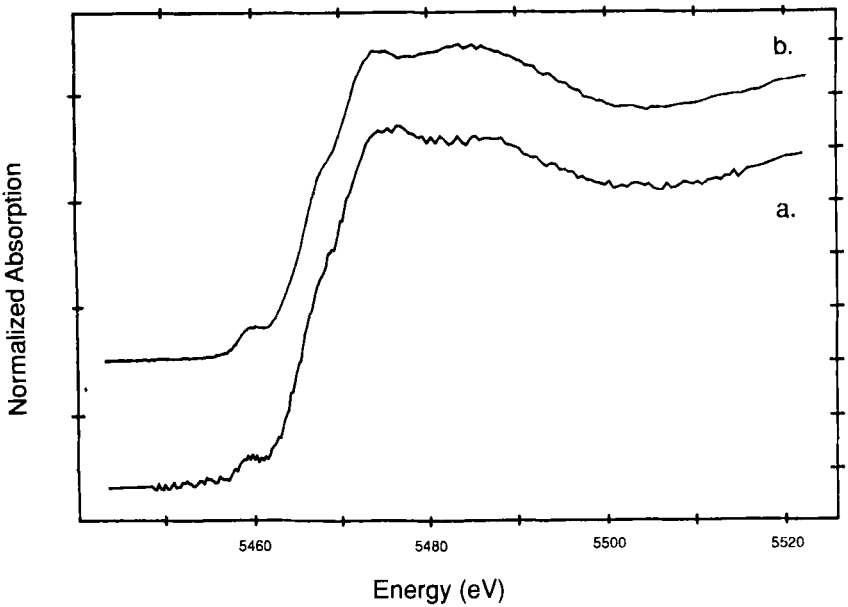


FIG. 3. Vanadium K-edge and XANES of the VFe protein of *A. chroococcum* and a VFe_3S_4 cubane cluster. (a) Dithionite-reduced VFe protein; (b) $[VFe_3S_4Cl_3(DMF)_3]^-$.

edge features (Fig. 3) precludes the presence of V=O bonds or tetrahedral symmetry of the V atom as in $[Et_4N]_3$ $[VS_4(FeCl_2)_2]$, where this pre-edge features is more pronounced. The spectra are consistent with an oxidation level of V(II)–V(IV) in a distorted octahedral coordination of the V atom in the VFe protein, as has been established for the complex $\{VFe_3S_4Cl_3[HCON(CH_3)_2]_3\}^-$ by X-ray crystallographic structure determination. The small changes that occur to the edge spectra of the VFe protein on dye oxidation or on superreduction during enzyme turnover indicate that V, like Mo in the MoFe protein, shows only a limited interaction with the unpaired electrons in the dithionite-reduced state of the protein (*43*).

Analysis of the EXAFS region of the spectra of VFe proteins allowed simulation of the spectra, with backscattering contributions from three compound shells, V–(O or N), V–(S), and V–(Fe). These types of atoms and their distances from the V atom in the VFe protein are very similar to those of Mo in MoFe proteins (see Table III).

4. The Iron and Vanadium Cofactor

The spectroscopic data discussed above are consistent with the VFe protein containing a Fe–V–S cluster analogous to FeMoco of the MoFe

proteins. The presence of such a cluster has been confirmed by the extraction of an iron- and vanadium-containing cluster (FeVaco) from the VFe protein of *A. chroococcum* (*44*).

Treatment of the VFe protein under conditions similar to those used to extract FeMoco gave an NMF solution that contained V, Fe, and S^{2-} in a ratio 1:5.8:4.8, similar to those of Mo:Fe:S^{2-} in FeMoco. The solution exhibited a very weak EPR spectrum with g values near 4.5, 3.6, and 2.01, characteristic of an $S = 3/2$ spin system.

Iron K-edge EXAFS analysis of the NMF solution showed clear evidence for the presence of FeS_2M rhombs (where M = Fe or V) (*45*). There are two Fe–Fe separation distances of ~2.65 and ~3.69 Å; similar data have been reported for FeMoco and indicate that both cofactors have long-range order. The model compound $\{VFe_3S_4Cl_3[HCON(CH_3)_2]_3\}^-$, although a reasonable model for V and Fe EXAFS data, does not show the mixed second shell and long-range structure found in FeMoco and FeVaco.

The close homology of FeMoco and FeVaco extends to the ability of FeVaco to activate apo-MoFe proteins in the FeMoco assay system. The hybrid protein has the characteristic substrate reduction pattern of VFe protein, i.e., C_2H_2 does not effectively inhibit hydrogen evolution and C_2H_6 is formed as a minor product of C_2H_2 reduction. This pattern suggests that FeVaco has been extracted from the VFe protein without major structural changes occurring. However, the hybrid protein does not reduce N_2 to NH_3 or N_2H_4. This is consistent with specific cofactor/polypeptide ligand interactions, necessary for N_2 reduction, but not with the reduction of other substrates, lacking in the FeVaco–MoFe protein complex. This specific requirement has recently been demonstrated in the MoFe protein, where conversion of a single glutmate → glutamine residue by site-directed mutagenesis abolishes the ability to reduce N_2, but C_2H_2 reduction activity is retained (with the atypical formation of C_2H_6) (*46*).

D. The Third Nitrogenase

The structural genes of the third nitrogenase (*anf*) of *A. vinelandii* have been cloned and sequenced (*14*). They are more closely related to those of V nitrogenase than of Mo nitrogenase, although all three systems show the same conserved cysteinyl spacing in the α and β subunits (Fig. 1). The third nitrogenase has a gene (*anfG*) with 40% derived amino acid sequence identity to the product of *vnfG*. The products of *anfD* and *anfK* have 54 and 57% sequence identity with the products of *vnfD* and *vnfK*.

The components of the third nitrogenase, analogous to the MoFe and VFe proteins (dinitrogenase-3) have been purified and partially characterized (*5*). The third nitrogenase can be isolated in two active forms with different subunit configurations, $\alpha_1\beta_2$ and $\alpha_2\beta_2$; there is a δ subunit present at uncertain stoichiometry encoded by *anfG* (R. Eady and R. Pau, unpublished).

The $\alpha_2\beta_2$ purified protein has 24 Fe and 18 S^{2-} atoms per molecule and the $\alpha_1\beta_2$ species has 11 Fe and 8 S^{2-} atoms per molecule. Both species contain only low levels of Mo, V, Cr, Re, and W, but have 0.5 Zn atoms per molecule, the significance of the latter is uncertain. At the present time, no information as to the types of redox centers present in this protein has been published, but possibly only Fe is involved.

The preparations of dinitrogenase-3 currently available have low specific activities for H_2, N_2, and C_2H_2 reduction in comparison with MoFe and VFe proteins (Table II). It is not clear if the reasons for this low activity are the loss of some essential component during purification, or whether the optimum conditions for assay have yet to be established. It should be emphasized that this nitrogenase system is clearly capable of supporting growth of *A. vinelandii* on N_2, because the double mutant lacking the structural genes of both Mo and V nitrogenases grows well (*12*). The effect of metals on the growth of this mutant showed that either Mo or V inhibited growth, a wide variety of metals of possible function had no effect (Co, Cr, Cu, Mn, Ni, Re, W, and Zn), and only Fe stimulated growth. These facts, together with the analytical data for the purified protein, make an involvement of Mo or V in this system very unlikely and by default leave Fe as the functional metal in N_2 reduction. Clearly, this is an interesting area that requires further work.

E. Substrate Reduction

Extensive studies of Mo nitrogenase during enzyme turnover have shown the Fe protein to transfer an electron to the MoFe protein in an ATP-dependent reaction. The probable site of substrate binding and reduction is the FeMoco center of the MoFe protein. In the absence of an added reducible substrate, e.g., under an atmosphere of argon, protons are reduced to dihydrogen. Under an atmosphere of dinitrogen, H_2 evolution still continues even under 50 atm of N_2. For Mo nitrogenase functioning under optimum conditions at 30°C, the limiting reaction is

$$N_2 + 8e + 8H^+ \rightarrow 2NH_3 + H_2 \qquad (1)$$

Each electron transfer results in the hydrolysis of two MgATP to two MgADP, and, following electron transfer, dissociation of the two proteins occurs, a step that is rate limiting in substrate reduction. The apparently energetically wasteful evolution of H_2 for each N_2 reduced is proposed to arise from the requirement for the generation of a reduced site at which N_2 binds to displace H_2.

The proportion of electron flux through the enzyme that results in H_2 evolution rather than N_2 reduction increases as the rate of electron flux decreases. This occurs when reductant or MgATP is limiting so as to decrease the electron flux, and arises as a consequence of partially reduced MoFe protein becoming oxidized by H_2 release before N_2 can bind.

The Mo-independent nitrogenases are less efficient at 30°C in coupling electron flux to the reduction of N_2. For V nitrogenase, 50% (and for the third nitrogenase, 75%) of the electron flux through the enzyme turning over under N_2 results in H_2 evolution. However, pre-steady-state studies of the initial electron transfer from Fe protein to VFe protein indicate that the higher electron allocation to the reduction of H^+ is not due to restricted electron transfer, and the efficiency of coupling of MgATP hydrolysis to electron transfer is as effective as for Mo nitrogenase (*47*).

This inefficiency is not likely to be due to inappropriate suboptimal assay conditions because it occurs *in vivo;* cultures of *Azotobacter,* when growing on N_2 using Mo-independent nitrogenases, evolve considerably more H_2 than when growth depends on Mo nitrogenase (*48*).

In the case of V nitrogenase the efficiency with which N_2 competes with H^+ as reducible substrate increases as the temperature is decreased from 30°C (*47*). Within the temperature range 30–5°C, assays with purified enzymes showed that the activity of Mo nitrogenase toward N_2 as a substrate decreased 10-fold more than for V nitrogenase. This difference may account for the persistence in nature of what, under some conditions, appears to be an inefficient system.

In addition to the reduction of N_2 and H^+, Mo nitrogenase has been shown to reduce a wide range of small molecules that contain triple bonds (e.g., C_2H_2, CN, and N_3). Many of these compounds have yet to be tested as substrates with Mo-independent nitrogenases, but the reduction pattern of one such analog (C_2H_2) has proved interesting.

Mo nitrogenase catalyzes the reduction of C_2H_2 to C_2H_4, and in ^2H_2O forms predominantly [*cis*-$^2H_2$]ethylene, an observation widely interpreted as indicating side-on bonding of C_2H_2 to a metal at the active site. C_2H_2 competes effectively with protons as substrate, and at high

C_2H_2 concentrations (0.1 atm in argon) H_2 evolution is inhibited by 85% compared with the rate under argon alone.

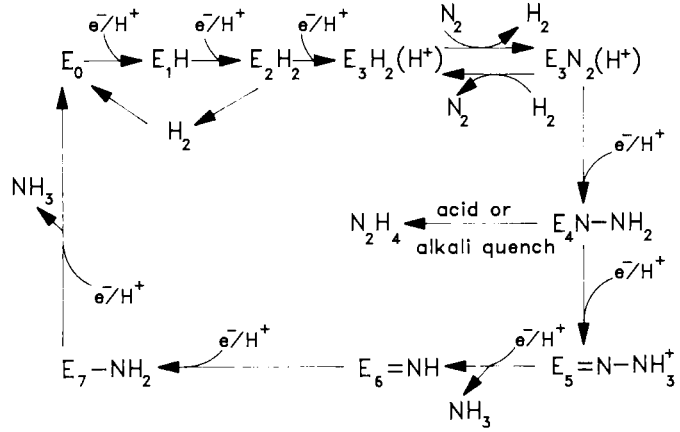
In contrast, the Mo-independent nitrogenases continue to show high rates of H_2 evolution in the presence of C_2H_2 (Table II). In the case of V nitrogenase this is a property of the VFe protein, because the active hybrid enzyme formed between it and the Fe protein of Mo nitrogenase still evolves H_2 in the presence of C_2H_2. In ^2H_2O, V nitrogenase also produces [*cis*-$^2H_2$]ethylene; however, a distinctive property of the VFe protein of V nitrogenase is the formation of C_2H_6 as a minor product of C_2H_2 reduction (*49*). Although both Mo and V nitrogenase slowly reduce C_2H_4 to C_2H_6, the reduction of C_2H_2 to give C_2H_6 has never been reported for Mo nitrogenase, except in the specific case of mutant proteins (*46*). The proportion of C_2H_6 to C_2H_4 as products of C_2H_2 reduction is increased by raising the temperature or at high electron flux, as occurs at high Fe protein to VFe protein ratios.

The kinetics of C_2H_6 formation show that free C_2H_4 is not an intermediate and are consistent with different routes for the formation of C_2H_6 from C_2H_2 and C_2H_4. The difference in reactivity of the cofactor center with substrate to produce different reduction products is presumably a consequence of subtle differences in cofactor polypeptide ligand interactions.

1. The Mechanism of Dinitrogen Reduction

Pre-steady-state stopped-flow and rapid quench techniques applied to Mo nitrogenase have provided powerful approaches to the study of this complex enzyme. These studies of *Klebsiella pneumoniae* Mo nitrogenase showed that a pre-steady-state burst in ATP hydrolysis accompanied electron transfer from the Fe protein to the MoFe protein, and that during the reduction of N_2 an enzyme-bound dinitrogen hydride was formed, which under denaturing conditions could be trapped as hydrazine. A comprehensive model developed from a computer simulation of the kinetics of these reactions and the kinetics of the pre-steady-state rates of product formation (H_2, NH_3) led to the formulation of Scheme 1, the Thorneley and Lowe scheme (*50*) for nitrogenase function.

In this model, the FeMoco centers of the MoFe protein are reduced independently by a series of eight one-electron transfers from the Fe protein with concommitant hydrolysis of MgATP. Following each electron transfer, dissociation of the protein–protein complex occurs in a reaction that is rate limiting when dithionite is used as reductant. Following this dissociation, the oxidized Fe protein, with MgADP



SCHEME 1. The catalytic cycle for the reduction of N_2 by the Mo nitrogenase. E_0 represents the resting state of the MoFe protein of *K. pneumoniae* and species E_1–E_7 represent intermediate forms of this protein following sequential one-electron reduction steps. The arrows between each state represent complex formation between the Fe protein and MoFe protein, electron transfer, and protonation, followed by protein dissociation. N_2 binds to species E_3, accounting for the stoichiometry of Eq. (1); the displacement of N_2 from this species accounts for the competitive inhibition of N_2 reduction by H_2 (see Ref. *50* for a detailed presentation of this scheme).

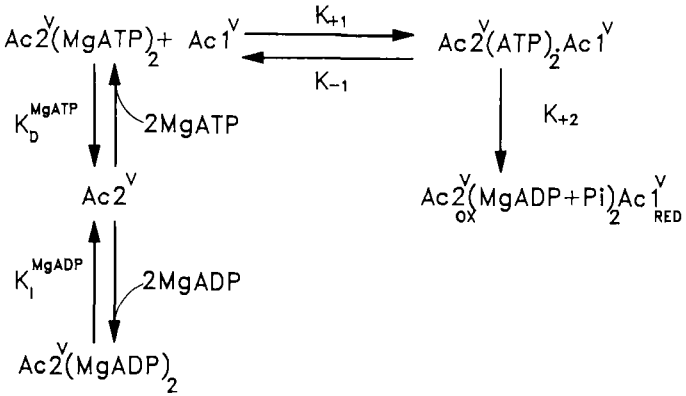
bound, is reduced by SO_2^-, MgADP is released, and MgATP then binds to the reduced Fe protein. Three electrons have to be transferred to a single FeMoco center before N_2 can bind to the MoFe protein to displace H_2, probably through a dihydrogen complex of the type $M—\overset{H}{\underset{H}{|}}$, because dihydrogen is displaced readily from such complexes by a variety of ligands when a third proton is added (*51*). The enzyme-bound dinitrogen is then reduced in a mechanism involving progressive protonation of the β (terminal) N atom as multiple-bond character builds between the α N atom and a metal (putatively Mo) at the substrate-binding site. During the reduction of N_2, *free* N_2H_4 is not an intermediate and reduction through to NH_3 occurs on the enzyme. The species that binds N_2, accounting for the specific and competitive inhibitory effect of H_2 on N_2 reduction.

For Mo-independent nitrogenases, the pre-steady-state rates are restricted to studies of the initial electron transfer reactions of V nitrogenase (*52*). The rate of electron transfer is measured from the increase in absorbance of the Fe protein as it becomes oxidized during turnover.

The dependence of the rate of this reaction on MgATP concentration and its inhibition by MgADP were analyzed in terms of Scheme 2. The values of K_D = 230 ± 10 μM for MgATP and a K_i of 30 ± 5 μM for MgADP are close to those obtained with Mo nitrogenase. The dependence of the first-order rate constant for electron transfer (K_{obs}) on the V nitrogenase or Mo nitrogenase concentrations indicates that the components of V nitrogenase form a weaker electron transfer complex. However, the rate at which the Fe protein and VFe protein associate, and the rate of MgATP-dependent electron transfer, are similar to those of the Fe protein and MoFe protein of Mo nitrogenase.

2. Chemical Studies on the Reduction of N_2

In the majority of dinitrogen transition metal compounds the mode of dinitrogen binding is end-on terminal. The ligated N_2 is generally unreactive to reduction to NH_3. Exceptions include the bis N_2 complexes of Mo and W of the type *trans*-$[Mo(N_2)_2(dppe)_2]$, where dppe = $Ph_2PCH_2CH_2PPh_2$. These complexes are reactive to form NH_3 at ambient temperature and pressure and have been extensively studied (for review and primary references, see Ref. *53*). The most easily observable intermediate is M=N—NH_2 (where M is Mo or W). This compound forms free N_2H_4 on reaction with acid or base, providing a parallel with the enzyme-bound dinitrogen hydride intermediate detectable in nitrogenase turnover.



SCHEME 2. MgATP-dependent electron transfer between components of V nitrogenase. The pre-steady-state electron transfer reactions between the Fe protein and the VFe protein of V nitrogenase of *A. chroococcum* have been analyzed in terms of this scheme (*52*). $Ac2^v$ represents the Fe protein and $Ac1^v$ represents the VFe protein of this system. This scheme is analogous to that used in the detailed study of Mo nitrogenase (see Ref. *50*).

Possible intermediates of the form $M{=}N{-}NH_3^+$ have been observed and characterized; they are stable when M = W but react to form NH_3 when M = Mo. In a cyclic sequence of reactions that can be driven chemically or electrochemically, protonation of bound N_2 occurs in a stepwise manner, with electrons flowing from the metal atom as protons are picked up from solution.

The modeling based on phosphine complexes of Mo and analog chemistry provided the basis against which the scheme for N_2 reduction by Mo nitrogenase was formulated. In the case of vanadium, this chemistry has yet to be developed, because until 1989, stable isolable complexes of dinitrogen vanadium complexes were not known. In that year, a bridging dinitrogen complex $[\{V(2\text{-}Me_2NCH_2C_6H_4)_2Py)\}_2(\mu - N_2)] \cdot 2THF$ was isolated and its structure determined (*54*). Fe–N_2 complexes have been known for some time but their chemistry is relatively unexplored.

In contrast, two of the best known aqueous systems that reduce N_2 are based on vanadium (*55*, *56*) but are difficult to characterize and mechanistic conclusions are often controversial. Of these, the V(II) catechol system, which functions at alkaline pH, provides a good analog for the reactions catalyzed by nitrogenase. In the absence of N_2 this system reduces protons to H_2. As with nitrogenase, this reaction, which is inhibited by N_2 and the limiting stoichiometry that occurs at room temperature and pressure, is

$$8V^{2+} + 8H^+ + N_2 \rightarrow 2NH_3 + H_2 + 8V^{3+}$$

Free N_2H_4 is not an intermediate in this reaction but quenching with acid results in the detection of a small amount of H_2H_4, presumably arising from an intermediate in N_2 reduction.

With regard to C_2H_2 as a substrate, the stereospecificity in producing [*cis*-$^2H_2$]ethylene as a product and small amounts of C_2H_6 provides a parallel with the reactions of V nitrogenase.

IV. Outlook

The discovery of Mo-independent nitrogenases is so recent that their contribution to the global cycling of N is unknown. There is good evidence that they are widely distributed in organisms other than in *Azotobacter*. Recently a *nifHDK* deletion strain of the photosynthetic bacteria *Rhodobacter capsulatus* was shown to grow on N_2 in medium deficient in Mo and V. Nitrogenase with properties similar to the third

nitrogenase described above has been partially characterized (*57*). There is evidence that V nitrogenase is present in the cyanobacterium *Anabaena variabilis* (*58*). It is probably that other established N_2-fixing organisms will be shown to have Mo-independent nitrogenases and it is possible that previously unidentified N_2-fixing species will be identified.

At an early stage in the authors' involvement in research into diazotrophy in *Azotobacter,* experiments with Dr. R. Robson were using continuous culture as a new approach to the old problem of "what metals are required for nitrogen fixation?" In the discussion following a presentation at the conference on recent developments in the chemistry of Cr, Mo and W at Sussex, England, in 1982, Professor R. J. P. Williams suggested that a genetic approach might be fruitful. It was, and a combination of the two techniques, in collaboration with Dr. P. Bishop (who originally postulated the existence of other nitrogenases), led to the general acceptance among biochemists that Mo-independent fixation was a reality.

Although there is compelling evidence that substrate reduction occurs at the FeMoco center of the MoFe protein of nitrogenase, there is no evidence that it occurs directly at Mo. The requirements for N_2 reduction (as might have been deduced from chemical modeling of this process) are more stringent than those necessary for the reduction of other substrates of nitrogenase. Perturbation of interactions between homocitrate in FeMoco and also amino acid ligands of the α polypeptide subunit with FeMoco can result in the selective loss of the ability to reduce N_2. The involvement of V in a cofactor center on which the V atom is in a chemical environment very similar to that of Mo in Mo nitrogenase is suggestive of some common chemistry. If this thesis is extended to the third nitrogenase, the chemistry must invoke Fe, a common feature of all three nitrogenases. Chemical modeling of the reactivity of N_2 at a metal site (Mo or W) has greatly advanced our understanding, and allowed the development of a plausible mechanism for the reduction of N_2 by Mo nitrogenase. The new impetus provided to chemical modeling studies by the discovery of Mo-independent nitrogenases may well produce some surprises.

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