

Formation and Insertion of the Nitrogenase Iron–Molybdenum Cofactor

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1. Introduction to Nitrogenase

The nitrogenases represent a class of complex metalloenzymes that catalyze the key reductive step in the global biological nitrogen cycle—nucleotide-dependent reduction of dinitrogen to ammonia. The best-studied member of this group is the Mo-dependent nitrogenase, which is composed of two component proteins usually designated the Fe protein and the MoFe protein (Figure 1), names that were derived from the compositions of their respective metallocluster complements.¹ The Fe protein is an agent of electron transfer that sequentially delivers single electrons to the MoFe protein in a process coupled to MgATP hydrolysis. During the catalytic cycle, nucleotide binding to the Fe protein elicits a conformational change that primes the Fe protein for complex formation with the MoFe protein. Such nucleotide-induced interaction of the component proteins subsequently triggers nucleotide hydrolysis, electron transfer, and complex dissociation.² A schematic representation of this process is shown in Figure 1. No artificial source of reducing equivalents has been shown capable of substituting for the function of the Fe protein in electron transfer necessary for substrate reduction.³ This feature is gener-

ally believed to reflect obligate reciprocal conformational signaling between the Fe protein and the MoFe protein as a way to accomplish the accumulation of the multiple electrons required for substrate reduction.^{4,5} In this respect it is emphasized that, during the catalytic cycle, electrons are delivered to the MoFe protein one at a time, but multiple electrons are required for substrate reduction. Nitrogenase catalysis is complicated, and the exact mechanism has remained elusive for two important reasons. First, the MoFe protein does not bind substrate in the resting state, but must first accumulate two or more electrons to effect substrate binding. Second, in the absence of other substrates, all electrons accumulated within the MoFe protein become diverted to proton reduction, which returns the protein to the resting state. Thus, although attempts have been made to biophysically characterize the intractable semi-reduced forms of the MoFe proteins, with or without substrate or inhibitors bound,^{6,7} intermediate states of the protein have not been clearly defined so far. The reader is referred to comprehensive reviews on the structure and catalytic mechanism of nitrogenase.^{2,3,8–11}

2. Nitrogenase-Associated Metalloclusters

The metalloclusters contained within the Mo-dependent nitrogenase include a typical [4Fe–4S] cluster bridged between the identical subunits of the Fe protein, and two novel clusters contained within the MoFe protein, designated the P cluster and FeMo-cofactor. Electron transfer is believed to proceed from the Fe protein [4Fe–4S] cluster to the P cluster, and then to FeMo-cofactor, which provides the substrate reduction site (Figure 1). Isolated MoFe protein is an $\alpha_2\beta_2$ tetramer, but individually paired $\alpha\beta$ units are usually considered as separate catalytic entities, and each of these contains one P cluster and one FeMo-cofactor. The P cluster is located at the pseudosymmetric $\alpha\beta$ interface and is positioned near the surface that interacts with the Fe protein during complex formation. In the as-isolated, “reduced” form of the MoFe protein, the [8Fe–7S] P cluster (referred to as P^N in this state) comprises two fused [4Fe–4S] subclusters that share a μ_6 -sulfide. These subclusters are further linked, and are connected to the MoFe protein subunits, by two μ_2 -cysteinate bridges, one each provided by an individual α - and β -subunit. There are four other typical cysteinate ligands, two provided by each subunit, that also attach the P

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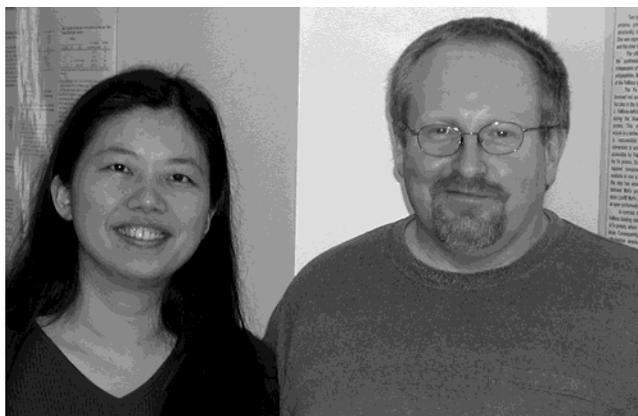
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Dennis R. Dean (right) was born in Indianapolis and is a graduate of Wabash College (B.A.). He received a Ph.D. working under the direction of Arthur I. Aronson at Purdue University and performed postdoctoral studies at the University of Wisconsin in the laboratory of Masayasu Nomura. He is currently a Professor of Biochemistry at Virginia Tech. His research interests include the biogenesis of simple and complex Fe–S clusters and the mechanism of biological nitrogen fixation.



Yilin Hu (left) was born in Shanghai, China. She earned her B.S. at FuDan University (Shanghai, P.R. China), where she studied genetics and genetic engineering. In 1999, she received her Ph.D. from the Department of Biochemistry at Loma Linda University (Loma Linda, California). She then joined the research group of Professor Barbara K. Burgess at the University of California, Irvine, as a postdoctoral fellow, where she worked on questions related to oxidative stress response of *Azotobacter vinelandii*. She is currently a postdoctoral researcher in the Department of Molecular Biology and Biochemistry, University of California, Irvine, working together with her husband, Markus Ribbe, on questions related to the assembly process of the nitrogenase MoFe protein of *A. vinelandii*.

Markus Ribbe (right) was born in Marktredwitz, Germany. He studied biology at the University of Bayreuth, where he earned his B.S., M.Sc., and doctorate (Dr. rer. nat.) degrees at the Department of Microbiology under thesis advisor Professor Ortwin Meyer. In 1998, he joined the research group of Professor Barbara K. Burgess at the University of California, Irvine, as a postdoctoral fellow. During his postdoctoral training, he focused on nitrogenase-related questions, with emphasis on the assembly process of the nitrogenase MoFe protein of *Azotobacter vinelandii*. He is currently continuing this line of research as an assistant researcher in the Department of Molecular Biology and Biochemistry, University of California, Irvine.

cluster to the MoFe protein. Upon treatment of the as-isolated MoFe protein with chemical oxidants, the P cluster rearranges to give an open, asymmetrical structure—referred to as P^{OX}—that has alterations in

amino acid coordination including an oxygen ligand and a nitrogen ligand, respectively provided by a serine side-chain alkoxide and a backbone cysteine amide.^{12,13} The P^{OX} form of the P cluster is oxidized by two electrons with respect to the P^N state. Although there is good evidence that the P cluster undergoes changes in redox state during turnover,¹⁴ it is not yet known whether P^{OX} represents a catalytically relevant state. Nevertheless, such significant redox-dependent rearrangements highlight the plasticity of [Fe–S] clusters, even when they are anchored within a polypeptide matrix, a feature that is relevant to structural rearrangements that are likely to occur during complex metallocluster assembly.

Like the P cluster, FeMo-cofactor has an unusual structure not recognized so far in other biological systems. The metal–sulfur core of FeMo-cofactor is constructed from [4Fe–3S] and [3Fe–Mo–3S] substructures linked by three μ_2 -sulfide bridges (Figures 1 and 2). A recent high-resolution crystal structure of the MoFe protein revealed that the central cavity of FeMo-cofactor, previously thought to be unoccupied, contains an interstitial atom, presumably μ_6 , whose identity is not yet known.¹⁵ In addition to its metal–sulfur core, FeMo-cofactor contains an organic constituent, homocitrate, which is attached to the Mo atom through its 2-hydroxy and 2-carboxyl groups. FeMo-cofactor is covalently attached to the MoFe protein through a cysteinate ligand (provided by α -Cys²⁷⁵) to an Fe atom at one end and by a side-chain nitrogen atom (provided by α -His⁴⁴²) to the Mo atom, located at the opposite end (Figure 2).¹⁶ In addition to covalent ligands, FeMo-cofactor is tightly held within the MoFe protein through a variety of direct and water-bridged hydrogen bonds.

There is compelling genetic and biochemical evidence that FeMo-cofactor provides the substrate reduction site. First, certain mutant strains that are unable to synthesize FeMo-cofactor produce an “apo” MoFe protein¹⁷ that contains a normal complement of P clusters but does not contain FeMo-cofactor.^{18,19} Such apo-MoFe proteins can be activated by the addition of FeMo-cofactor that is extracted from the intact MoFe protein by using a chaotropic solvent such as *N*-methylformamide. Second, FeMo-cofactor produced in a mutant strain that is defective in the gene required for homocitrate biosynthesis contains citrate rather than homocitrate.^{20,21} This form of the MoFe protein has altered catalytic activities; for example, it remains capable of relatively efficient proton reduction, but is not capable of efficient dinitrogen reduction.²² If citrate-substituted FeMo-cofactor is used to activate apo-MoFe protein, then the reconstituted protein is also capable of efficient proton reduction but reduces dinitrogen very poorly.²³ Third, certain mutant strains having substitutions for those amino acids that provide the first shell of noncovalent interactions with the FeMo-cofactor exhibit dramatic alterations in substrate reduction.^{24,25} One recent example is substitution of the α -Gly⁶⁹ residue by Ser, which results in an altered MoFe protein that retains an ability to effectively reduce dinitrogen but is severely altered in its ability

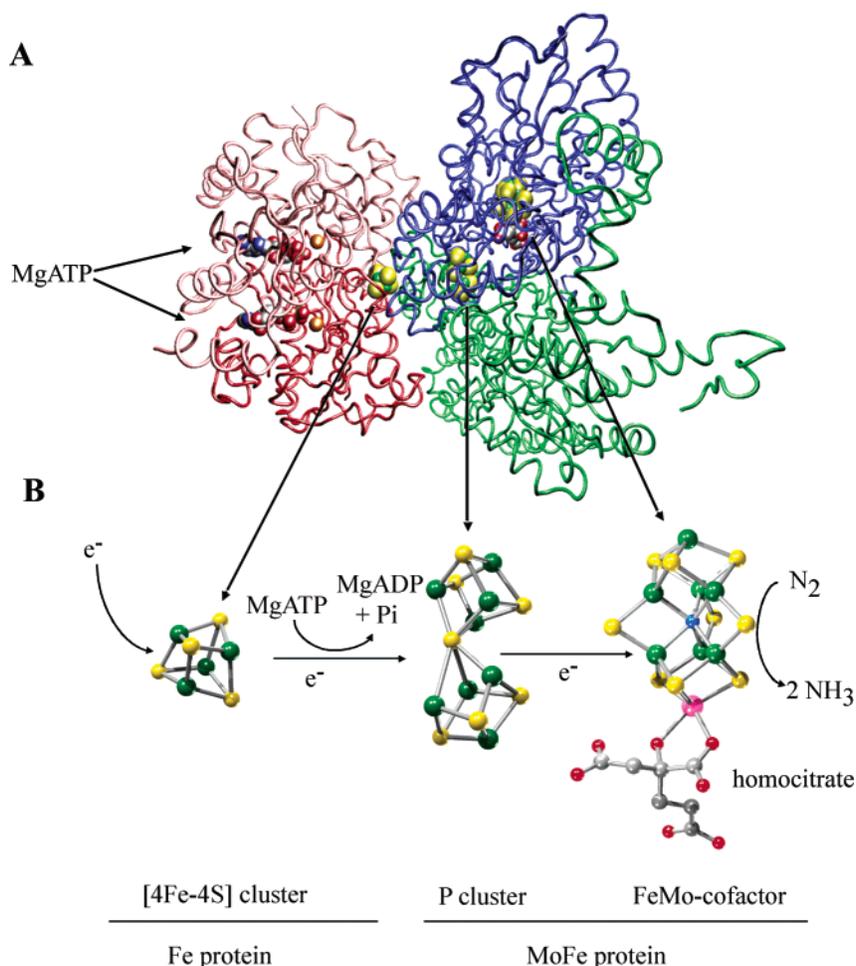


Figure 1. Nitrogenase component proteins and their associated metal clusters. (A) Fe protein is shown on the left (identical subunits in pink and red), and one catalytic $\alpha\beta$ dimer of the MoFe protein is shown on the right (α -subunit in blue and β -subunit in green). The associated metal clusters and MgATP located within the nitrogenase complex are shown as space-filling models. Note that the nitrogenase complex structure was solved in the presence of MgADP-AlF_4^- , which is analogous to MgATP binding. (B) The structures of nitrogenase metal clusters are shown in ball-and-stick models. The direction of electron flow and the associated reactions are indicated by arrows. Electrons flow in an ATP-dependent reaction from the [4Fe-4S] cluster of Fe protein to the P cluster and FeMo-cofactor of the MoFe protein, where the reduction of N_2 to ammonia occurs. Figures were generated in VMD¹¹⁴ (A) and SWISS PDB VIEWER¹¹⁵/POVRAY (B) using 1N2C and 1M1N PDB coordinates. Atom colors: carbon in gray, nitrogen in blue, oxygen in red, phosphorus in dark green, sulfur in yellow, magnesium in orange, iron in green, and molybdenum in pink.

to reduce the alternative substrate acetylene.^{26,27} Moreover, substitution of the MoFe protein α -Val⁷⁰ residue by Ala or Gly expands the ability of nitrogenase to reduce small-chain alkynes, propyne and butyne, which are not effectively reduced by the wild-type enzyme.²⁵ Thus, not only is it proven that FeMo-cofactor provides the substrate reduction site, but the available evidence now points to initial substrate binding, at least, occurring at a specific location within FeMo-cofactor.

The recent identification of an atom within the central Fe–S cage of FeMo-cofactor has led to speculation that this atom is a mechanistically relevant monoatomic nitrogen atom (nitride) that might become inserted into the metal–sulfur cage as an initial step in the activation of dinitrogen. Although the central atom could well be a nitride, and there are now theoretical calculations that support this possibility,^{28,29} it is very unlikely that it becomes inserted within the inner core as a consequence of MoFe protein-dependent dinitrogen reduction. One reason for this is that FeMo-cofactor is separately synthe-

sized and then inserted into the apo-MoFe protein.³⁰ Namely, FeMo-cofactor can be synthesized in mutant strains that produce no MoFe protein.³¹ Also, there are a number of mutant strains that are completely defective in their ability to reduce dinitrogen due to a defective Fe protein, for example a *nifM* deletion strain,³² yet these mutant strains produce a fully active MoFe protein that contains a complete complement of FeMo-cofactor. Thus, if insertion of the interstitial atom requires nitrogenase catalysis, it would not seem possible that intact FeMo-cofactor could be assembled in mutants that are incapable of catalysis. Finally, arguments that the occurrence of six coordinately unsaturated Fe atoms present in the original FeMo-cofactor structure does not make chemical sense³³ would also apply to the structure of any precursor molecule. Even if the central atom is a nitride, recent spectroscopic experiments have demonstrated that it is not exchangeable by substrate nitrogen as the enzyme turns over.³⁴ Thus, there are three important questions with respect to the central atom within FeMo-cofactor that remain to be ans-

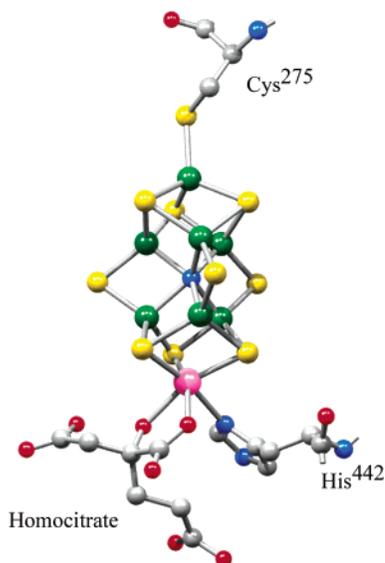


Figure 2. FeMo-cofactor and α -subunit ligands. FeMo-cofactor is attached to MoFe protein by α -Cys²⁷⁵ and α -His⁴⁴². This figure was generated in SWISS PDB VIEWER¹¹⁵/POVRAY using 1M1N PDB coordinates. Atom colors: carbon in gray, nitrogen in blue, oxygen in red, sulfur in yellow, iron in green, and molybdenum in pink.

wered—what is it, how does it get there, and what does it do?

3. Structure of the Apo-MoFe Protein

Over the past 10 years, a number of MoFe protein crystal structures have been reported, including those from *Azotobacter vinelandii*,^{9,12,15,35–41} *Clostridium pasteurianum*,^{42–44} and *Klebsiella pneumoniae*,¹³ and all of these are highly conserved on the basis of both primary sequence and three-dimensional structure. An important achievement toward understanding how the MoFe protein is activated by FeMo-cofactor was recently realized by crystallographic determination of the three-dimensional structure of an apo-MoFe-protein produced by *A. vinelandii*.⁴⁵ A comparison of the MoFe protein and apo-MoFe protein is described here to provide a platform for a discussion of what is known, or suspected, concerning the biosynthesis and insertion of FeMo-cofactor.

One of the gene products required at an early stage in FeMo-cofactor biosynthesis is NifB, and inactivation of NifB results in synthesis of apo-MoFe protein that can be activated *in vitro* by the addition of FeMo-cofactor, without the requirement of any other factors.^{19,46–48} A NifB-deficient strain was the source of apo-MoFe protein for crystallographic analysis. Like MoFe protein,^{9,12,15,35–41} apo-MoFe protein⁴⁵ is an $\alpha_2\beta_2$ heterotetramer consisting of a pair of $\alpha\beta$ dimers related by a molecular two-fold rotation axis (Figure 3A,B). The homologous α - and β -subunits of both structures include three domains, designated α I, α II, α III, and β I, β II, β III, respectively, all of which exhibit parallel β -sheet/ α -helical polypeptide folds. In both MoFe protein and apo-MoFe protein, domains of the α - and β -subunits contribute ligands to the P clusters. These ligands are located in a common core of a four-stranded, parallel β -sheet flanked by α -helices and additional β -strands.¹⁰ The P clusters of

both MoFe protein and apo-MoFe protein are located between domains α I and β I. FeMo-cofactor occupies a cavity within the MoFe protein formed among domains α I, α II, and α III (Figure 3A), but this same cavity is not present in the apo-MoFe protein (Figure 3B). Instead, domain α III of the apo-MoFe protein undergoes substantial structural rearrangement when compared to the same region of the MoFe protein, whereas domains α I and α II, and the β -subunit of apo-MoFe protein, remain mostly unchanged, relative to those of the MoFe protein. In general, the β -strands and α -helices of domain α III in apo-MoFe protein are shorter toward their C- and N-termini, respectively, in comparison to those of the MoFe protein.

A comparison of the molecular surfaces of the MoFe protein (Figure 4A) and apo-MoFe protein (Figure 4B) reveals that the structural rearrangement of domain α III in the apo-MoFe protein creates an “FeMo-cofactor insertion funnel” that does not exist in the MoFe protein. Despite the prevailing negative surface charge of apo-MoFe protein, there are a number of positive surface charges that line the entire length of the proposed insertion funnel. This positively charged path includes residues α -Lys³¹⁵, α -Lys⁴²⁶, α -Arg⁹⁶, α -Arg⁹⁷, α -Arg²⁷⁷, α -Arg³⁵⁹, α -Arg³⁶¹, α -His²⁷⁴, α -His³⁶², α -His⁴⁴², and α -His⁴⁵¹ (Figure 5B). Given the dominant contribution of homocitrate to the overall negative charge of FeMo-cofactor,³ this feature could help steer the negatively charged FeMo-cofactor down the funnel toward its correct position in the mature MoFe protein. A comparison of the positions of residues in the apo-MoFe protein with the corresponding positions in the MoFe protein reveals key residues that undergo substantial structural rearrangement upon cofactor insertion and hence are implicated as participating in the FeMo-cofactor insertion process (Figure 5). All of these residues are located in the α III domain involved in the formation of the proposed FeMo-cofactor insertion funnel in the apo-MoFe protein (see above). Some of these residues form a loop at the entrance of the funnel in the apo-MoFe protein. This loop contains positively charged residues, α -Arg³⁵⁹, α -Arg³⁶¹, and α -His³⁶², which could provide the first contact point for entry of FeMo-cofactor. It also contains a number of highly conserved residues, such as α -Gly³⁵⁶, α -Gly³⁵⁷, and α -Arg³⁵⁹, which normally surround the FeMo-cofactor and form hydrogen bonds to the cofactor sulfurs in the MoFe protein (Figure 5A). A comparison between the positions of this stretch in the MoFe protein and the apo-MoFe protein (Figure 5B) reveals a repositioning of residues by distances up to 20 Å, indicating that this loop might serve as a gate that is open for FeMo-cofactor entry, and closes upon FeMo-cofactor insertion. FeMo-cofactor is covalently attached to the MoFe protein by α -His⁴⁴² to the Mo atom at one end and α -Cys²⁷⁵ to an Fe atom at the opposite end of the cofactor (Figures 2 and 5A). In the apo-MoFe protein, α -Cys²⁷⁵ occupies the same position. However, the C $_{\alpha}$ of α -His⁴⁴² in the apo-MoFe protein shifts \sim 5 Å during the rearrangement of the α III domain and joins two other residues, α -His²⁷⁴ and α -His⁴⁵¹, to form a striking “His triad” (Figure 5B) which could also help guide the negatively

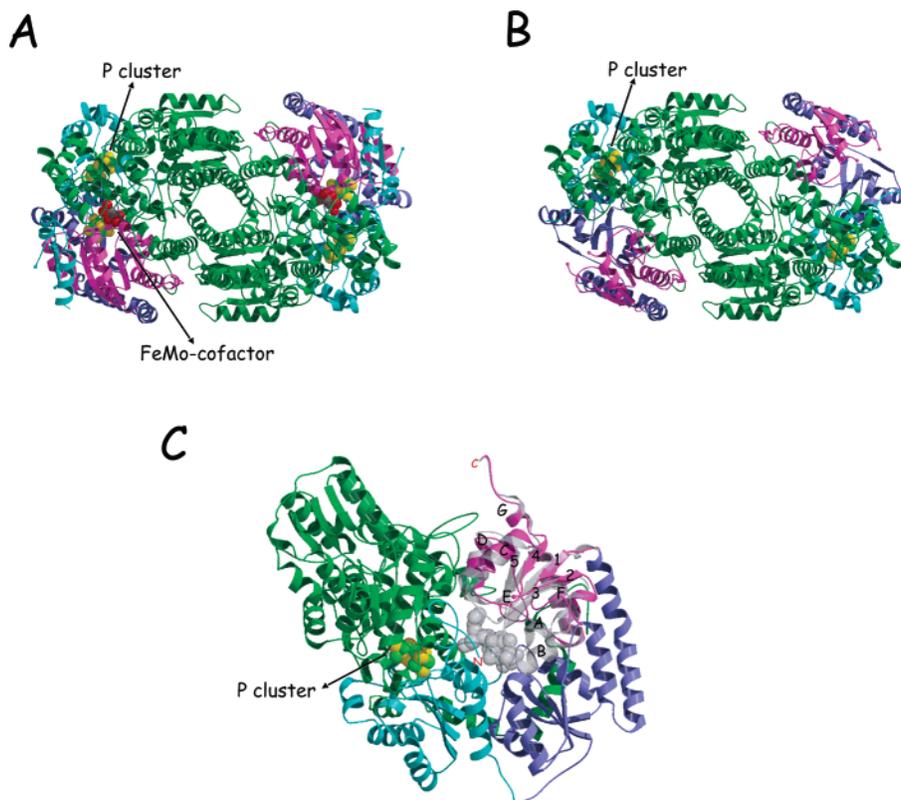


Figure 3. The $\alpha_2\beta_2$ tetrameric structure of MoFe protein (A) and apo-MoFe protein (B). Domains of the α -subunit are light blue (α I), dark blue (α II), and purple (α III), whereas those of the β -subunit are green. Residues α -380 and α -408 of apo-MoFe protein are shown as small purple spheres, indicating the disordered region ranging from α -381 to α -407 in domain α III. FeMo-cofactor (MoFe protein) and P cluster (MoFe protein and apo-MoFe protein) are shown as space-filling models, with molybdenum, iron, sulfur, oxygen, and carbon atoms colored in purple, green, yellow, red, and gray, respectively. (C) Front-side view of one apo-MoFe protein $\alpha\beta$ subunit pair with the superposition of the α III domain of MoFe protein that includes the FeMo-cofactor in light gray. Subunits, domains, and atoms of the P cluster are colored the same way as those in A and B. The α -helices A–F and β -strands 1–5 are labeled in black. The visible termini of the α -subunit of apo-MoFe protein are labeled N (α 49) and C (α 480) in red. In general, the β -strands and α -helices of domain α III in apo-MoFe protein (purple) are shorter toward their C- and N-termini than those in MoFe protein (gray). Programs MOLSCRIPT¹¹⁶ and RASTER3D¹¹⁷ were used to prepare this figure.

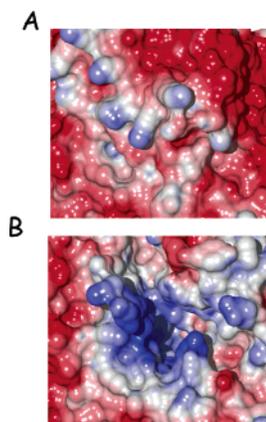


Figure 4. Electrostatic surface potential of the “FeMo-cofactor insertion funnel”. The figure shows a comparison between the same regions of MoFe protein (A) and apo-MoFe protein (B). During insertion of FeMo-cofactor, the α III domain in apo-MoFe protein undergoes a structural rearrangement that closes the funnel shown in B. Negative and positive potentials, which are calculated by the Poisson–Boltzmann equation, are shown in red ($-10.0kT$) and blue ($10.0kT$), respectively, with k = Boltzmann constant (1.38×10^{-23} J/K) and T = temperature (K). Programs MSMS,¹¹⁸ SWISS PDB VIEWER,¹¹⁵ and POVRAY were used to prepare this figure using 3MIN and 1L5H PDB coordinates.

charged FeMo-cofactor to the appropriate binding site during the insertion process. Residues α -His⁴⁴² and α -Trp⁴⁴⁴ also switch their relative positions in the respective structures of apo-MoFe protein and MoFe protein. This structural rearrangement could serve to “lock” the FeMo-cofactor at its final location in the mature MoFe protein. In addition to the structural rearrangements located in close proximity to the Mo site, there is also structural rearrangement when the homocitrate environment is examined. For example, α -Lys⁴²⁶, which is hydrogen bonded—through water—to homocitrate in the MoFe protein (Figure 3A), is shifted by ~ 5 Å in the apo-MoFe protein, indicating that α -Lys⁴²⁶ could also anchor and orient FeMo-cofactor during the insertion event. All of the residues noted here are highly conserved among all known MoFe protein primary sequences.⁴⁵

On the basis of the structural comparisons described above, a general *in vitro* mechanism for FeMo-cofactor insertion can be proposed. The negatively charged FeMo-cofactor accesses the α -subunit through a positively charged funnel, led by the homocitrate/Mo end. The FeMo-cofactor continues to move into the funnel until it encounters α -His⁴⁴² at the bottom of the funnel, which serves as the initial Mo atom docking point, and α -Lys⁴²⁶, which serves

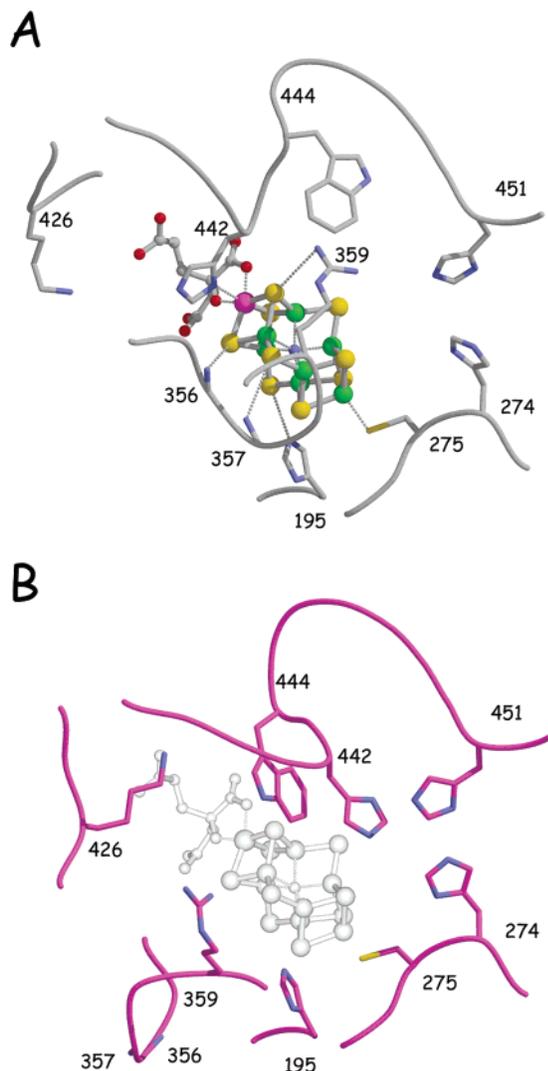


Figure 5. Protein environment of MoFe protein (A) and apo-MoFe protein (B) in the vicinity of the FeMo-cofactor binding area. Parts of the C_{α} backbone and important side-chain residues in the α -subunit are shown. FeMo-cofactor is represented by a ball-and-stick model. Gray dotted lines indicate hydrogen-bonding interactions between the residues and the FeMo-cofactor. The theoretical position of FeMo-cofactor in apo-MoFe protein is indicated in light gray after superposition of the C_{α} positions of the α -subunits in apo-MoFe protein and MoFe protein. The following key residues for FeMo cofactor insertion are shown: (1) α -His²⁷⁴, α -His⁴⁴², α -His⁴⁵¹ (“His triad” in apo-MoFe protein); (2) α -Trp⁴⁴⁴ (part of the FeMo-cofactor “lock”); (3) α -355 through α -359 (part of the “lid” loop from α -353 through α -364); and (4) α -Lys⁴²⁶ (“anchor” for the homocitrate of the FeMo-cofactor). Programs MOLSCRIPT¹⁶ and RASTER3D¹⁷ were used to prepare the figure. Atoms are colored the same as those described earlier.

to anchor and orient FeMo-cofactor. Entry of the negatively charged FeMo-cofactor into the positively charged funnel is also likely to break/form hydrogen bonds, which could trigger a rearrangement in the positions of α -His⁴⁴² and α -Trp⁴⁴⁴, leading to the capture of FeMo-cofactor, which is accomplished by the structural rearrangement of a loop containing residues α -353 to α -364, ultimately leading to covalent attachment of FeMo-cofactor through residues α -His⁴⁴² and α -Cys²⁷⁵.

In addition to the availability of a structure of apo-MoFe protein⁴⁵ produced by a NifB-deficient strain,

this same protein has been extensively characterized by spectroscopic and biochemical methods.^{19,46–49} Early studies on the apo-MoFe protein indicated that a significant conformational change must occur during FeMo-cofactor insertion, because the α -Cys²⁷⁵ residue, which provides a covalent ligand to FeMo-cofactor, is hypersensitive to alkylating reagents in apo-MoFe protein but is not susceptible to alkylation in the mature MoFe protein.^{19,50} This prediction, also suggested by the original observation that apo-MoFe can be activated by simple addition of FeMo-cofactor,^{18,19,46} was confirmed by the crystallographic analysis.⁴⁵ Biochemical studies also indicated that apo-MoFe protein contains intact P clusters—having nearly the same electronic features as the MoFe protein—which again was confirmed by the structure.^{19,45,47} Of particular interest with respect to the potential role of the Fe protein in the process of FeMo-cofactor biosynthesis and insertion—discussed later—was the finding that apo-MoFe protein can support Fe protein-dependent nucleotide hydrolysis at a rate nearly the same as that which occurs under normal turnover of the intact MoFe protein, yet apo-MoFe protein is unable to support reduction of any substrate.¹⁹

4. General Aspects of FeMo-Cofactor Biosynthesis

Although it is clear that FeMo-cofactor is separately synthesized and then inserted into an apo-MoFe protein,^{30,31} and there is general agreement about the identity of proteins involved in that process, the exact sequence of events, as well as the specific roles of the individual players, is not well understood (Table 1). In fact, there is not even a consensus among the authors of this review with respect to some aspects of FeMo-cofactor biosynthesis. These different views have emerged from the complexity of the system and the likely redundant functions of the involved players that cause difficulty in making unambiguous assignments. Here we describe some general aspects of the process, we suggest a comprehensive, albeit speculative, pathway for FeMo-cofactor biosynthesis (Figure 6), and we point out some of the more contentious issues. Our goal is to provide a framework for the chemist to appreciate the complexity of the biological process and perhaps provide some insight for the rational design of the chemical synthesis of FeMo-cofactor or its precursors on the basis of what is known, or suspected, about the biosynthetic process. It is noted that, in addition to the Mo-dependent nitrogenase, *A. vinelandii* also produces a closely related V-dependent nitrogenase^{51–53} that is structurally and functionally quite similar to the Mo-dependent nitrogenase, although it is genetically distinct. A major difference between these two nitrogenases is that the Mo-dependent system has Mo as the heterometal contained within FeMo-cofactor, whereas the V-dependent system contains V in its complementary cofactor—designated VFe-cofactor. It is believed, although not yet proven crystallographically, that FeMo-cofactor and VFe-cofactor are identical, except for their respective heterometal. The biosynthesis of both cofactors shares some common steps involving the same biosynthetic

Table 1. *nif* Gene Products and Other Components Involved in the Overall FeMo-Cofactor Biosynthesis

gene	product/function(s)
<i>nif</i> gene products	
<i>nifH</i>	Fe protein subunit
<i>nifD</i>	MoFe protein α -subunit
<i>nifK</i>	MoFe protein β -subunit
<i>nifB</i>	involved in the production of an Fe/S-containing FeMo cofactor precursor, designated NifB-cofactor
<i>nifQ</i>	involved in FeMo-cofactor biosynthesis, probably at an early step
<i>nifV</i>	homocitrate synthase
<i>nifX</i>	probably an intermediate carrier in FeMo-cofactor biosynthesis
<i>nifY</i>	probably an intermediate carrier in FeMo-cofactor biosynthesis
<i>nifN</i>	subunit of NifN ₂ E ₂ ; appears to provide a transient site upon which one or more events related to FeMo-cofactor assembly occur
<i>nifE</i>	subunit of NifN ₂ E ₂
<i>nifU</i>	complements NifS in the mobilization of Fe and S for metalocluster assembly; required for the synthesis of active Fe protein and MoFe protein
<i>nifS</i>	pyridoxal-dependent cysteine desulfurase; required for the synthesis of active Fe protein and MoFe protein
<i>nifW</i>	required for the synthesis of a fully active MoFe protein
<i>nifZ</i>	required for the synthesis of a fully active MoFe protein
<i>nifT</i>	function unknown
<i>nifF</i>	flavodoxin
<i>nifJ</i>	pyruvate:flavodoxin oxidoreductase
<i>nifA</i>	positive regulatory element
<i>nifL</i>	negative regulatory element
others	
<i>nafY</i>	probably an intermediate carrier in FeMo-cofactor biosynthesis

apparatus; other steps, which are apparently functionally equivalent but are probably related to heterometal specificity, are encoded by separate genes. Another significant difference between the MoFe protein and the VFe protein is that the latter has another small subunit designated the δ -subunit. Because the respective genetic organizations^{54,55} of the two systems (Figure 6C) and the biochemical characterization of some of the VFe-cofactor biosynthetic components^{56–59} are relevant to the biosynthesis of FeMo-cofactor, certain aspects of VFe-cofactor will also be considered.

Four key features can be considered with respect to FeMo-cofactor biosynthesis: (1) formation of an Fe–S core, (2) rearrangement of the Fe–S core to form an entity that is topologically similar to the metal–sulfur core of FeMo-cofactor, (3) insertion of Mo and attachment of homocitrate, and (4) trafficking of FeMo-cofactor or its precursors among the various sites at which these events occur. In our model, several aspects of which have also been suggested by others,⁵⁵ we propose that the flow of Fe and S through the biosynthetic pathway is as follows: NifUS \rightarrow NifB \rightarrow NifX \rightarrow NifEN \rightarrow NifY/Gamma \rightarrow MoFe protein. As schematically shown in Figure 6A, NifUS complexes are involved in the initial mobilization of Fe and S and serve to assemble Fe–S fragments⁶⁰ that are subsequently delivered to NifB. These fragments are proposed to become linked within NifB to form an Fe–S core⁶¹ (Figure 6B) that also contains the unknown atom (N, O, or C) that ultimately occupies

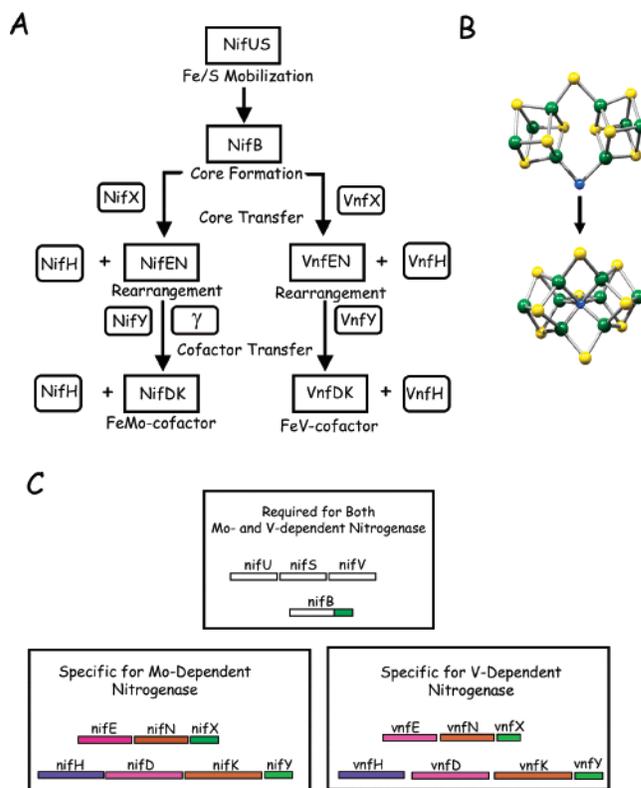


Figure 6. Proposed model for FeMo-cofactor biosynthesis. (A) Flowchart diagram of the proposed pathway for the FeMo-cofactor and VFe-cofactor biosynthesis. The boxes represent the proteins involved in this process, and the arrows represent the transfer of precursor forms through the process. The proteins shown in the squared-edge boxes presumably participate in major cluster modifications, while the proteins shown in the rounded-edge boxes carry out accessory functions. (B) Hypothetical Fe–S core rearrangement that could occur during biosynthesis. (C) Genetic organization of the genes involved in FeMo-cofactor and VFe-cofactor biosynthesis. Genes or regions that show sequence homology or similar functions between the two systems are presented in the same color code. Note that *nafY*, which encodes the gamma protein, is not shown in the figure. This gene is located upstream of *nifB* and is located in a gene cluster encoding a series of electron-transfer proteins. The specific roles for these proteins in nitrogen fixation in *A. vinelandii* are not known, but a homologous cluster of genes present in *Rhodobacter capsulatus* is known to be required for nitrogen fixation.¹⁰⁰

the presumably hexacoordinated central site within the completed FeMo-cofactor. This core is then transferred from NifB to the NifEN complex by an “escort” protein designated NifX.⁶² It is possible that both Mo and homocitrate could be incorporated while the core is attached to NifX. A subsequent rearrangement of the FeMo-cofactor precursor is then proposed to occur within the NifEN complex to give either a completed FeMo-cofactor or perhaps a precursor that is identical to FeMo-cofactor—but without the attachment of either Mo or homocitrate.⁶³ The final step of MoFe protein maturation involves the delivery of FeMo-cofactor, or its precursor, by one of two possible escort proteins designated NifY⁶⁴ and Gamma.⁶⁵ If Mo and homocitrate attachment does not occur during a previous step, then it is likely that such attachment occurs at this stage. A complication of FeMo-cofactor biosynthesis is that the Fe protein, which is the obligate electron donor for nitrogenase catalysis, is

also required for both the *in vivo* synthesis of FeMo-cofactor and the *in vivo* insertion of FeMo-cofactor into the apo-MoFe protein.^{31,66–74} As discussed in a later section, it is proposed that involvement of the Fe protein is related to its ability to interact with both the apo-MoFe protein¹⁹ and the NifEN complex^{75,76} to effect conformational changes necessary for both assembly and insertion of FeMo-cofactor. The model as shown in Figure 6A also suggests that FeMo-cofactor and VFe-cofactor synthesis involves initial formation of an identical core with a subsequent branch point involving parallel pathways for completion and insertion of the corresponding cofactors. This suggestion is based on the genetic organization of the genes encoding the respective cofactor assembly and trafficking components (Figure 6C) from these two systems, as well as primary sequence comparisons among these proteins.

5. Formation of the FeMo-Cofactor Core—Proposed Roles for NifU, NifS, and NifB Proteins

Genetic experiments have shown that NifU, NifS, and NifB are all required for production of active MoFe-protein and VFe-protein, indicating these proteins are involved in the early stages of the Fe–S core required for formation of both FeMo-cofactor and VFe-cofactor.⁷⁷ Furthermore, NifU and NifS are required for the accumulation of an active Fe protein and an active MoFe protein.⁷⁸ Because the only feature common to both nitrogenase component proteins is that they both contain Fe and S, it was initially proposed that NifS and NifU have complementary functions related to the general mobilization of S and Fe for nitrogenase metallocluster formation. This suggestion was experimentally verified by the demonstration that NifS is a pyridoxal-dependent cysteine desulfurase and NifU provides an *in vitro* scaffold for the formation of [2Fe–2S] clusters destined for nitrogenase metallocluster formation.^{79–81} A likely pathway involves the formation of a protein-bound cysteine persulfide on NifS that is subsequently donated to NifU, upon which, in the presence of Fe, a labile [2Fe–2S] cluster is formed.⁶⁰ These labile [2Fe–2S] clusters probably represent building blocks used for assembly of the Fe–S core on the NifB protein. The details concerning the assembly of [2Fe–2S] units on the NifU scaffold are not yet known. For example, neither the source of Fe that is supplied to NifU nor the sequence of events related to Fe and S sulfur delivery has been established, although it has been shown that NifU and NifS are able to form a macromolecular complex.⁶⁰ Also, it is not yet known whether it is [2Fe–2S] or [4Fe–4S] units that are ultimately formed on the NifU scaffold prior to their release for FeMo-cofactor formation or Fe protein maturation. We favor the latter possibility because (1) there is preliminary data that [4Fe–4S] clusters can be formed on NifU—perhaps by reductive coupling of two [2Fe–2S] units (Smith and Johnson, personal communication), (2) Fe protein maturation requires a [4Fe–4S] cluster, and (3) an attractive model for FeMo-cofactor core formation involves linking two [4Fe–4S] clusters.

The NifEN complex, discussed in detail in the next section, and the NifB protein are differentiated from all of the other proteins involved in MoFe protein maturation because their inactivation results in accumulation of an apo-MoFe protein that can be activated by the simple addition of purified FeMo-cofactor.^{19,46} This feature led to the development of a biochemical complementation strategy where crude extracts of NifB-deficient cells and crude extracts of NifEN-deficient cells, neither of which has nitrogenase activity, could be mixed to produce an extract that has nitrogenase activity.⁸² This result indicated that NifB and NifEN have complementary biochemical functions involving FeMo-cofactor biosynthesis and provided the basis for an assay that could be used for the attempted purification of NifEN and NifB. While this strategy proved successful for the purification of NifEN,⁶³ it has not yet been possible to isolate an active form of NifB by this or any other method. However, during the attempted purification of NifB, it was found that an “Fe–S core” could be isolated from detergent-treated membranes, and this entity could be added to NifB-deficient extracts to achieve activation of the apo-MoFe protein, providing that homocitrate, Mo, and MgATP were also added.⁶¹ Importantly, this Fe–S core cannot be purified from NifB-deficient strains, indicating that its formation requires NifB. Because of the involvement of NifB in its formation, the Fe–S core has been designated NifB-cofactor. The only metal detected in NifB-cofactor preparations is Fe, and it was also shown that addition of ⁵⁵Fe- or ³⁵S-labeled NifB-cofactor to apo-MoFe protein activation assays resulted in the incorporation of ⁵⁵Fe or ³⁵S into FeMo-cofactor contained in the activated protein.⁸³

Although there is compelling evidence that NifB-cofactor is an Fe–S-containing entity necessary for FeMo-cofactor (or VFe-cofactor) assembly and that NifB is necessary for its formation, neither the structure of NifB-cofactor nor the reaction catalyzed by NifB is understood. There are two possibilities that can be considered with respect to a possible NifB-cofactor structure. One possibility is that NifB-cofactor represents a fragment of the FeMo-cofactor Fe–S core, which becomes fused to another fragment at a later step in the assembly process. The other possibility, and the one we favor, is that the entire Fe–S complement required for FeMo-cofactor assembly is formed by the action of NifB and that rearrangement of this core, followed or preceded by Mo and homocitrate insertion, occurs at later stages in FeMo-cofactor assembly. In addition to comprising the FeMo-cofactor Fe–S core, we also suggest that NifB-cofactor formation could be the step at which the as-yet unidentified atom located in the center of the finished FeMo-cofactor enters the pathway. Figure 6B shows a plausible NifB-cofactor structure that summarizes our thoughts concerning these issues. This structure contains two separate [4Fe–4S] clusters that are linked by a bridging S and N, the latter being the atom proposed to occupy the center of the FeMo-cofactor. As shown in Figure 6B, a NifB-cofactor having this structure could rearrange at a later step in FeMo-cofactor biosynthesis, either before

or after Mo and homocitrate attachment, to give an intact FeMo-cofactor. Our basis for proposing the structure shown in Figure 6B was based on three criteria: (1) it has the correct metal and sulfur stoichiometry—assuming later replacement of a corner Fe atom by Mo; (2) it is topologically similar to FeMo-cofactor—which is relevant to a later discussion on the role of escort proteins; and (3) radical chemistry could be involved in the insertion of the interstitial atom, and, as discussed below, NifB is a member of the radical *S*-adenosylmethionine-dependent enzyme superfamily.

Because NifB has not been purified, speculations about its possible functions are confined to knowledge of its primary sequence.⁸⁴ Alignment of the many available NifB primary sequences shows that there are nine conserved Cys residues as well as eight conserved His residues among most of them. Thus, there is an excess of potential ligands available to coordinate the entire complement of Fe atoms necessary for FeMo-cofactor assembly in a single NifB molecule, and this does not even include other conserved amino acids, such as Asp residues, that also have the potential of serving as metallocluster ligands. Inspection of the N-terminal region shows that three of the conserved Cys residues are contained within a primary sequence signature that is typical for a family of [Fe–S] cluster-containing *S*-adenosylmethionine-dependent enzymes.⁸⁵ This family of enzymes catalyzes a diverse number of reactions, including methylation, isomerization, sulfur insertion, ring formation, and anaerobic oxidation, all of which are unified only by their dependence on radical chemistry.^{86,87} Clearly, given that we do not even know the identity of the central atom located within FeMo-cofactor, it cannot be certain that radical chemistry is involved in its insertion into an FeMo-cofactor precursor. However, it is difficult to imagine that any other aspect of the formation of the FeMo-cofactor core would require radical chemistry, so we have provisionally assigned that function to the SAM-dependent signature in the NifB primary sequence.

6. Rearrangement of NifB-Cofactor—Proposed Role of the NifEN Complex

The concept of the possible involvement of molecular scaffolds in complex Fe–S cluster assembly was originally formulated on the basis of the discovery that FeMo-cofactor is synthesized separately from the MoFe protein subunits and then inserted into the apo-MoFe protein.^{30,31} This model suggested that certain assembly proteins would have a primary sequence that is similar to the FeMo-cofactor binding site within the MoFe protein, which could provide a template, or scaffold, for FeMo-cofactor formation. This possibility was supported when the primary sequence of NifE was found to be similar to NifD (MoFe protein α -subunit) and the primary sequence of NifN was found to be similar to NifK (MoFe protein β -subunit),⁸⁸ leading to the prediction that NifEN forms an $\alpha_2\beta_2$ complex, structurally analogous to the MoFe protein,^{63,89} upon which FeMo-cofactor is assembled stepwise. Because the NifD and NifE se-

quences exhibited the most striking conservation, particularly within a region previously targeted as an FeMo-cofactor binding site,⁸⁸ it was also predicted that FeMo-cofactor would be contained substantially or entirely within the MoFe protein α -subunit. It was also recognized that certain of the Cys residues within both the α - and β -subunits of MoFe protein are also conserved in the corresponding positions in the respective NifE and NifN primary sequences. This led to the prediction that the proposed NifEN complex would also contain an Fe–S cluster at a location similar to where the P cluster is located within the MoFe protein. Although all of these predictions would be substantially confirmed and extended by the crystal structure of the MoFe protein^{9,12,15,35–41} and purification of the NifEN complex,^{63,89} the concept of the NifEN complex as a molecular scaffold, in its original incarnation, needed to be modified. Namely, with the advent of the identification of NifB-cofactor,⁶¹ it does not appear that FeMo-cofactor is sequentially assembled on a template provided by NifEN. Rather, NifEN appears only to provide a transient site upon which one or more events related to FeMo-cofactor assembly occur.

Now that the structures of both MoFe protein^{9,12,15,35–40} and apo-MoFe protein⁴¹ are known, a more informed basis for speculation on the role of the NifEN complex in FeMo-cofactor assembly can be offered. What is particularly important is to compare the primary sequence of the region within the MoFe protein that provides the FeMo-cofactor-binding site with the corresponding NifE sequence, as well as to compare the sequence within the apo-MoFe protein that comprises the access funnel to the corresponding region in the NifE sequence. With respect to the former, it can be appreciated that certain of those residues that either provide a covalent ligand or tightly pack FeMo-cofactor within the polypeptide matrix are not duplicated in the corresponding NifE primary sequence. For example, α -His⁴⁴², which coordinates the Mo atom of FeMo-cofactor, is substituted by Asn at the corresponding NifE sequence position. However, α -Cys²⁷⁵, which also covalently attaches FeMo-cofactor to the mature MoFe protein, is also a Cys in the corresponding NifE sequence (NifE-Cys²⁵⁰). It is also striking that some of the residues providing the access funnel to the FeMo-cofactor binding site are also positively charged in the corresponding NifE residues, indicating the possibility for an analogously charged funnel in the NifEN complex. MoFe protein residues that participate in forming this funnel (with the corresponding residue in the NifE primary sequence given in parentheses) include the following: α -Lys³¹⁵ (Leu), α -Lys⁴²⁶ (Arg), α -Arg⁹⁶ (Trp), α -Arg⁹⁷ (Arg), α -Arg²⁷⁷ (His), α -Arg³⁵⁹ (Lys), α -Arg³⁶¹ (Trp), α -His²⁷⁴ (Val), α -His³⁶² (Ser), α -His⁴⁴² (Asn), and α -His⁴⁵¹ (Gly). These similarities lead us to speculate that an FeMo-cofactor precursor accesses the NifEN complex in a way analogous to FeMo-cofactor insertion into the apo-MoFe protein. Because there are no obvious places within this region for coordination of a metallocluster other than NifE-Cys²⁵⁰, it does not appear likely that an FeMo-cofactor precursor could be

formed on the NifEN complex by joining separate fragments. Instead, we favor a model where the role of the NifEN complex is not related to an assembly scaffold function as originally proposed, but rather NifEN provides a site for the rearrangement of NifB-cofactor—or a processed form of NifB-cofactor—to produce an entity that is either topologically identical to the metal–sulfur core of FeMo-cofactor or actually is the completed FeMo-cofactor having homocitrate attached.

The conservation of some but not all positively charged residues when the apo-MoFe protein FeMo-cofactor access funnel residues are compared to the corresponding NifE residues can lead to two opposing but reasonable views with respect to FeMo-cofactor assembly. One interpretation is that conservation of certain positively charged residues favors the notion that homocitrate and Mo are already attached to the FeMo-cofactor precursor as it engages the NifEN complex. Namely, like the apo-MoFe protein, such positively charged residues could interact with the negatively charged homocitrate to channel the precursor to the rearrangement site. It could also be considered that ionic interactions between these residues and homocitrate actually participate in triggering one or more cluster rearrangement events. Another related possibility is that homocitrate and Mo are already attached to the NifEN complex, presumably within the proposed “assembly funnel”, before engagement by NifB-cofactor and that heterometal/homocitrate insertion occurs at this stage. In either case, it appears that the α -His⁴⁴² residue, which anchors FeMo-cofactor to the MoFe protein, is not duplicated in the NifE primary sequence because FeMo-cofactor, or its precursor, must ultimately escape from the NifEN complex. This same “escape” requirement could account for the circumstance that some positively charged residues found in the access funnel for MoFe protein maturation are not duplicated in the corresponding NifE primary sequence. The alternative and opposing interpretation of the conservation of some positively charged residues within the NifE sequence, which correspond to the FeMo-cofactor entry funnel residues, is that the FeMo-cofactor precursor that accesses the NifEN complex will not have Mo and homocitrate already attached to it. The rationale for this model is that the presence of any positively charged residues in this region might prevent exit of the FeMo-cofactor precursor from the assembly site if homocitrate is already attached. We favor the possibility that either homocitrate and Mo are attached to an FeMo-cofactor precursor before engagement with the NifEN complex or homocitrate and Mo are incorporated into an FeMo-cofactor while on the NifEN complex. The reason for this suggestion is that NifEN and VnfEN represent branch points with respect to channeling NifB-cofactor toward FeMo-cofactor or VFe-cofactor biosynthesis (Figure 6A). Namely, if homocitrate and heterometal insertion occurs at a step after NifEN/VnfEN involvement, then there would be no apparent reason for the cell to produce two separate gene sets whose products catalyze the exact same function.

7. Homocitrate Formation and Mo Mobilization

As already mentioned, the entry point for homocitrate and Mo incorporation into FeMo-cofactor is not yet known. However, the protein responsible for homocitrate formation, NifV, has been identified, isolated, and characterized.⁹⁰ NifV catalyzes the condensation of acetyl-CoA and α -ketoglutarate to form homocitrate. Early biochemical and genetic complementation experiments indicating that NifV-deficient cells produce an altered FeMo-cofactor that contains citrate, rather than homocitrate,²⁰ have now been confirmed by crystallographic analysis of MoFe protein produced by a NifV-deficient strain of *K. pneumoniae*.²¹

Very little is known about the mobilization of Mo for FeMo-cofactor synthesis except that a Mo transport pathway, common for both FeMo-cofactor and molybdopterin biosynthesis, is required.⁹¹ A branch point in targeting Mo specifically for FeMo-cofactor formation appears to involve the NifQ gene product.^{84,92,93} Although the number and spacing of Cys residues contained in NifQ indicate that it is likely to contain an Fe–S cluster of some type, it is unlikely that such a cluster could be an obligate precursor for FeMo-cofactor biosynthesis because strains deficient in NifQ can still form FeMo-cofactor, providing that these cells are supplemented with either cysteine or Mo.^{94,95} It therefore seems more plausible that NifQ, and perhaps other proteins associated with nitrogen fixation, are involved in sequestering Mo specifically for delivery to an FeMo-cofactor assembly site, and could also be involved in a process that places Mo in the correct oxidation state for FeMo-cofactor formation. Along these lines, it is interesting that in *A. vinelandii*, NifQ is encoded in a gene cluster that includes a ferredoxin and a protein having high sequence similarity to ArsC.⁸⁴ The ArsC protein is an arsenate reductase involved in reducing arsenate to an oxidation state that favors its efflux from cells.⁹⁶ However, ArsC does not have an endogenous reductase function, but receives reducing equivalents from a separate donor. Perhaps the nitrogen fixation-related ArsC homologue could be involved in binding Mo to facilitate a reductive event, catalyzed by some other redox-active protein (for example, NifQ or its associated ferredoxin).⁸⁴

Another unknown aspect of FeMo-cofactor biosynthesis concerns when and how homocitrate and Mo become attached to each other. For example, it is not known whether Mo and homocitrate are separately inserted into an FeMo-cofactor precursor, or if a Mo-homocitrate complex is inserted. Nevertheless, some insight about the process can be gained from the consideration of the coordination environment of the [4Fe–4S] cluster contained in aconitase. In this case, in the resting state of the enzyme, three Fe atoms are coordinated by three typical cysteinate ligands, whereas the fourth Fe is coordinated by a hydroxyl group. In the presence of substrate, the 2-carboxyl and 2-hydroxyl groups of citrate become coordinated to this Fe in the same way that the 2-carboxyl and 2-hydroxyl groups of homocitrate are coordinated to Mo in FeMo-cofactor. It is particularly relevant that, in the absence of citrate, the fourth Fe atom of the

aconitase [4Fe–4S] cluster is highly labile, leading to the formation of a [3Fe–4S] cluster.^{97,98} Thus, not only is there biological precedent for coordination of an Fe–S cluster by an organic constituent, but the reversible interconversion of [4Fe–4S] and [3Fe–4S] species within aconitase suggests an attractive mechanism for removal of an Fe with subsequent incorporation of a heterometal at one corner of the proposed NifB-cofactor structure during FeMo-cofactor assembly. As far as the attachment of Mo to homocitrate is concerned, there does not appear to be any Nif-encoded protein that is required for this function. Nevertheless, modestly lower MoFe protein activities are found in extracts of NifW- or NifZ-deficient strains.^{32,99} This, along with the observation that higher levels of homocitrate are required to correct the deficiency of a NifVW double mutant, when compared to a NifV-deficient strain, indicates that NifV and NifW could have some function related to the coupling of Mo and homocitrate.⁹⁹

8. Role of Escort Proteins in FeMo-Cofactor Assembly

Although isolated FeMo-cofactor can be used to activate apo-MoFe protein *in vitro*, it is not likely that “free” FeMo-cofactor in solution activates apo-MoFe protein *in vivo*. Rather, it appears that an intermediate carrier serves that function. During purification of apo-MoFe protein produced by an *A. vinelandii* NifB-deficient strain, a small protein, designated gamma, was found to be associated with the apo-MoFe protein.⁴⁶ When isolated FeMo-cofactor is added to apo-MoFe protein having gamma attached, a fully active MoFe protein is produced from which gamma becomes dissociated.⁶⁵ It was also shown that crude extracts of mutant strains that do not contain MoFe protein accumulate a form of gamma that has FeMo-cofactor attached to it.^{65,100} These observations have led to a model where gamma could serve dual roles—one where it serves to stabilize a form of the apo-MoFe protein in a conformation amenable to FeMo-cofactor insertion, and another where gamma acts as an intermediate carrier of FeMo-cofactor. Similar results were found with *K. pneumoniae*, where apo-MoFe protein was also found to contain a small protein that dissociates upon the addition of FeMo-cofactor.^{49,64} However, in this case the small protein was identified as the product of *nifY*, a gene contained in the same transcription unit encoding the MoFe protein subunits.⁷⁸ These findings did not initially appear compatible because gamma is not the product of the *A. vinelandii* *nifY* gene, which is also contained in the same transcription unit encoding the MoFe protein subunits. This issue was resolved when the gene encoding gamma, designated *nafY*, was found to have a high degree of sequence similarity when compared to NifY¹⁰⁰ (Figure 7B). Why *A. vinelandii* produces two proteins that apparently serve the same function is not obvious.

Another unresolved issue with respect to the role of gamma is that the apo-MoFe protein produced by a NifB-deficient strain, and whose structure was solved crystallographically, has neither gamma nor NifY attached to it.⁴⁵ The apo-MoFe protein used for

the structural analysis contains a polyhistidine tag at the N-terminal-region, a feature that was exploited to aid isolation of highly purified protein,¹⁹ whereas purified apo-MoFe that has gamma attached to it does not have a polyhistidine tag. An apo-MoFe protein that carries a polyhistidine tag at the carboxyl end, rather than the N-terminus, also does not contain gamma when purified.¹⁹ The carboxyl and N-terminal regions within the apo-MoFe protein structure are far removed from each other, so it is improbable that the absence of gamma in polyhistidine-tagged apo-MoFe proteins arises from insertion of the tag sequence. A more reasonable explanation is that gamma is dissociated from the apo-MoFe protein under conditions of high salt and high imidazole used for the purification of polyhistidine-tagged proteins.

Comparison of the primary sequence of NifY and gamma with the primary sequences of other Nif-specific proteins shows that NifX and the carboxyl end of NifB also bear significant sequence similarity to NifY, to gamma, and to each other. Some pairwise comparisons of the primary sequences of these proteins are shown in Figure 7. These primary sequence similarities, the ability of gamma to bind FeMo-cofactor⁶⁵ or NifB-cofactor *in vitro*, and the ability of NifX to bind FeMo-cofactor or NifB-cofactor *in vitro*⁶² indicate that NifY, gamma, and NifX are all structurally related proteins (Figure 7) that likely bind FeMo-cofactor or one of its intermediates during the assembly and insertion process. Important questions related to this family of proteins concern their specific functions and entry points with respect to FeMo-cofactor biosynthesis. A model that we and others⁵⁵ prefer is that they are escort proteins that sequentially deliver FeMo-cofactor or its precursors from one assembly site to another. The genetic organization of these genes, as well as their analogous counterparts involved in VFe-cofactor biosynthesis (Figure 6C), supports a pathway where assembled NifB-cofactor eventually becomes located in the carboxyl end of NifB that exhibits sequence similarity to the escort proteins. There is no evidence to indicate whether this region actually participates in NifB-cofactor assembly or is simply an exit site. In this model, NifB-cofactor is subsequently released to NifX, which carries it to the NifEN complex. As already mentioned, this step represents a logical place for the incorporation of Mo and homocitrate because this is the stage at which FeMo-cofactor and VFe-cofactor biosyntheses diverge.

Following the assembly step that takes place on the NifEN complex, the intermediate—or perhaps the completed cofactor—is then released to NifY, or gamma, which carries it to the apo-MoFe protein. Considering the sequence similarity between the carboxyl end of NifB and the various escort proteins, it is likely that NifB-cofactor, other possible assembly intermediates, and FeMo-cofactor are all topologically related, as they apparently bind to structurally related proteins. It is this consideration that leads us to speculate on the possible structure of NifB-cofactor (Figure 6B), which is proposed to be topologically similar to FeMo-cofactor, and to suggest that

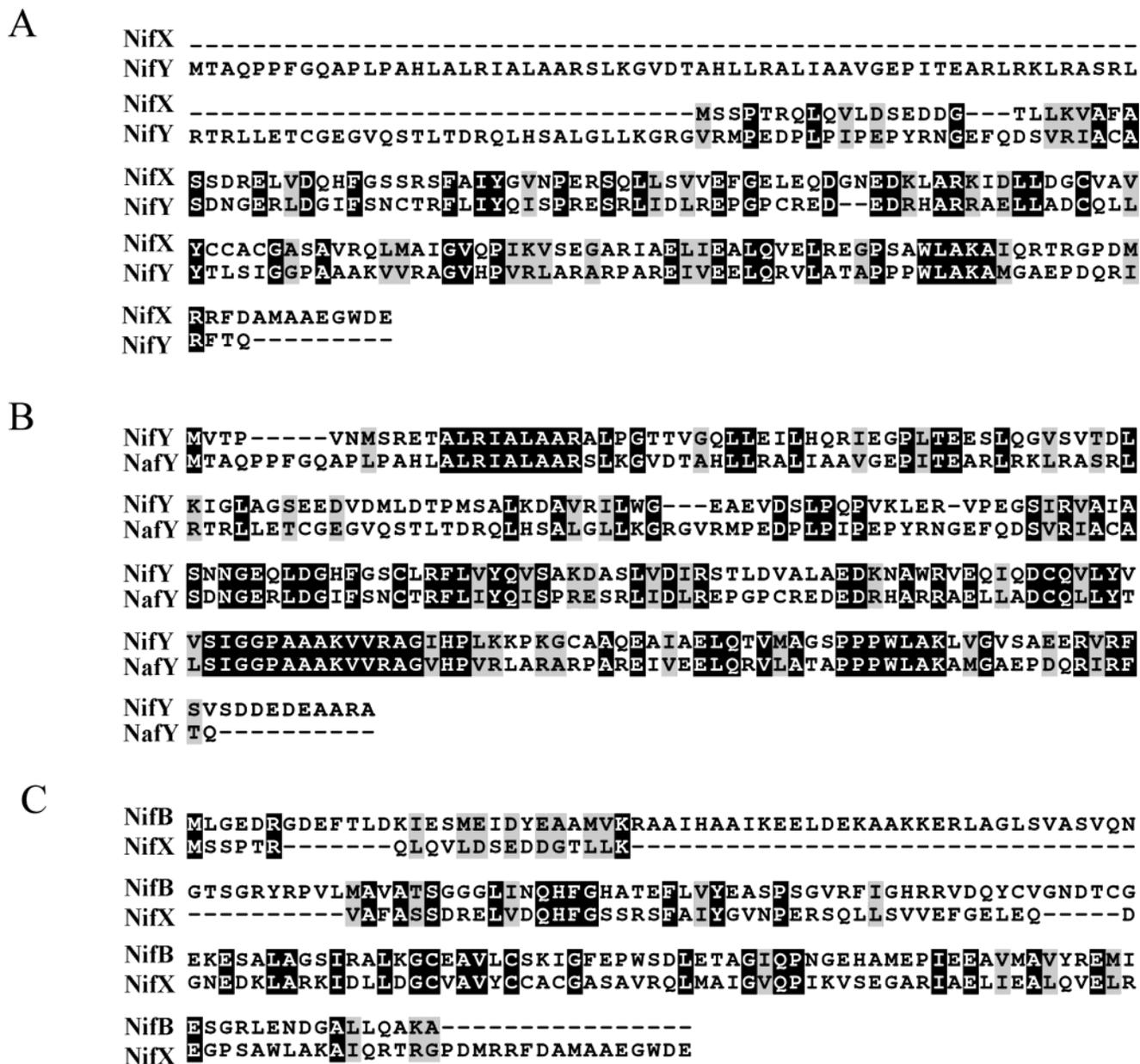


Figure 7. Pairwise comparisons of the primary sequences of *A. vinelandii* proteins involved in FeMo-cofactor biosynthesis. Sequence alignments are shown between (A) NifX and NifY, (B) NifY and NafY, and (C) C-terminal portions of NifB and NifX. The amino acid sequences are indicated by one-letter code abbreviation. Identical residues are shaded in black and similar residues in gray. The alignments were generated using CLUSTAL W alignment with Gonnet encoding a series matrix.¹¹⁹

the entire complement of Fe and S necessary for FeMo-cofactor is delivered to the NifEN complex as one entity, rather than as fragments. The reason that we associate NifX with delivery to the NifEN complex, and NifY with delivery to the apo-MoFe protein, is that *nifENX* are contained in a single transcription unit and that *nifHDKY* are contained in a separate transcription unit (Figure 6C). This genetic organization could permit the physiological adjustment of the amount of NifX or NifY with the amount required for delivery to their respectively proposed partners. It is noted that the gamma-encoding gene, designated NafY, is not associated with either transcription unit, which again points to the peculiarity of an apparent redundancy in the capacity for delivery of FeMo-cofactor to the apo-MoFe protein in *A. vinelandii*.¹⁰⁰ Also, the conservation in primary sequences among

the proposed escort proteins and the carboxyl region of NifB probably accounts for the reason that it has not yet been possible to make clear functional assignments by complementary biochemical and genetic experiments. Namely, in the absence of one or more escort proteins,¹⁰⁰ either the NifB carboxyl domain or one of the other escort proteins could serve the necessary function. Sorting these issues out will require making loss-of-function mutations in various combinations of all of these proteins, and some progress along these lines has already been reported.¹⁰¹

9. Role of Fe Protein in FeMo-Cofactor Biosynthesis and Insertion

It was first recognized some years ago that FeMo-cofactor biosynthesis requires participation of the Fe

protein.^{31,66–74} It was initially proposed that Fe protein could have a redox function related to FeMo-cofactor assembly on the NifEN complex because *in vitro* FeMo-cofactor biosynthesis requires MgATP,¹⁰² and because the NifEN complex is structurally analogous to the MoFe protein.⁸⁸ In this model, MgATP-induced interaction of the Fe protein with the NifEN complex, analogous to Fe protein–MoFe protein docking during catalysis, could occur with electron-transfer participating in FeMo-cofactor biosynthesis rather than substrate reduction.⁸⁸ Although it has now been shown that Fe protein is capable of interaction with the NifEN complex,⁷⁵ this model cannot be correct because neither the binding of nucleotides nor the capacity for electron transfer is required for Fe protein to participate in FeMo-cofactor biosynthesis.¹⁰³ Nevertheless, as shown in Figure 6A, a logical step for participation of the Fe protein in FeMo-cofactor biosynthesis involves interaction of Fe protein with the NifEN complex.⁷⁵ If the rearrangement of NifB-cofactor does occur on the NifEN complex as we have proposed, a docking event between Fe protein and the NifEN complex could trigger conformational changes necessary either for this event or, perhaps, in effecting release of FeMo-cofactor or its intermediate from the NifEN complex. Another possibility is that docking between the NifEN complex and Fe protein is necessary for the NifEN complex to adopt a conformation amenable to accept NifB-cofactor, or a related intermediate, when delivered by the proposed NifX escort protein.

Like the apo-MoFe protein produced by NifB-deficient cells, MoFe protein produced by Fe protein-deficient cells does not contain FeMo-cofactor.^{31,66–74} Also, purified “apo-MoFe protein” produced by Fe protein-deficient cells (hereafter referred to as Δ NifH-apo-MoFe protein) remains able to support Fe protein-dependent MgATP hydrolysis at high rates.⁷² However, there are other features that distinguish NifB-deficient apo-MoFe protein and Δ NifH-apo-MoFe protein. One striking difference is that Δ NifH-apo-MoFe protein, present in crude extracts, has a different electrophoretic mobility during native gel electrophoresis when compared to either apo-MoFe protein or MoFe protein.^{71,104} Thus, it appears that the Δ NifH-apo-MoFe protein has some other protein or factor attached to it that is not also attached to the apo-MoFe protein produced by NifB-deficient cells.^{71,104} This difference is highlighted by the observation that Δ NifH-apo-MoFe protein cannot be activated *in vitro* by the simple addition of isolated FeMo-cofactor.^{66,71,72,74,102,104–106} Rather, activation of the Δ NifH-apo-MoFe protein, which occurs only at low levels when compared to apo-MoFe protein activation, also requires Fe protein, MgATP, and the molecular chaperone GroEL.^{46,105} This low level of activation could be attributed to the absence of some other necessary protein,¹⁰⁴ but a recent detailed characterization of Δ NifH-apo-MoFe protein now points to an alternative explanation involving the P clusters.⁴⁵ Although the purified Δ NifH-apo-MoFe protein has a sufficient number of Fe atoms to account for a full complement of P clusters, spectroscopic characterization reveals these clusters to be

unusual when compared to P clusters contained in the MoFe protein or the apo-MoFe protein. For example, the as-isolated Δ NifH-apo-MoFe protein exhibits an unusually strong $S = 1/2$ EPR signal in the $g = 2$ region, which on integration accounts for up to 0.7 spin/mol of protein. This signal has not been assigned to any known P cluster oxidation state and is recognized as only a very minor component of apo-MoFe protein produced by NifB-deficient cells. Also, the parallel mode $g = 11.8$ EPR signal, observed in indigo disulfonate-oxidized P clusters from MoFe protein and apo-MoFe protein, is absent in purified Δ NifH-apo-MoFe protein. Finally, although Δ NifH-apo-MoFe protein is able to support Fe protein-dependent MgATP hydrolysis, electron transfer from the Fe protein to the Δ NifH-apo-MoFe protein has not been observed.⁷² This result also contrasts with the apo-MoFe protein, which is able to accept an electron from the Fe protein.^{19,72} The differences between the P cluster contained in the apo-MoFe and the Δ NifH-apo-MoFe protein can be attributed to two different possible sources. One possibility is that lack of *in vivo* Fe protein interaction with an otherwise normal apo-MoFe protein results in irreversible damage of the P clusters. This possibility emerges from the observation that low *in vivo* flux through nitrogenase causes increased sensitivity of nitrogenase to oxidative damage^{107,108} and could explain why it has not yet been possible to achieve full reconstitution of the Δ NifH-apo-MoFe protein. A second possibility is that Fe protein is required for the maturation of the P clusters. For example, if the [8Fe–7S] P clusters are formed by the fusion of two separate [4Fe–4S] clusters, then an Fe protein-induced conformational change could be required as part of this process.

A final complicated aspect of FeMo-cofactor insertion during maturation of the MoFe protein is the apparent involvement of the molecular chaperone GroEL. Evidence for participation of GroEL in nitrogenase maturation was first suggested in the early 1990s.^{109–112} More recently, it has been demonstrated that GroEL is necessary for full activation of an FeMo-cofactor-deficient MoFe protein produced by a mutant having an altered Fe protein (α -Glu¹⁴⁶ residue substituted by Asp).¹⁰⁵ The interesting aspect of this finding is that it might explain the requirement of MgATP for FeMo-cofactor assembly or insertion. It has long been known that *in vitro* FeMo-cofactor assembly/insertion assays require the addition of MgATP, and this requirement was initially assigned to the participation of the Fe protein. It is now known, however, that the ability of Fe protein to hydrolyze MgATP can be dissociated from its obligate participation in FeMo-cofactor assembly/insertion.

10. Concluding Comments

The biosynthesis of FeMo-cofactor is an enormously complicated process that involves its sequential assembly on scaffold proteins (NifU, NifB, and the NifEN complex). Formation of FeMo-cofactor does not involve participation of the apo-MoFe protein; rather, FeMo-cofactor is separately formed and then inserted into the apo-MoFe protein. This process is further

complicated by the involvement of escort proteins (NifX, NifY, gamma), which appear to transport FeMo-cofactor or its precursors from one site to another and eventually to the apo-MoFe protein. Even though complementary genetic and biochemical strategies have been successful in identifying the principal players in these processes, as well as the associated proteins involved in targeting Mo for FeMo-cofactor formation (NifQ) and formation of homocitrate (NifV), most details concerning how these assembly proteins work together remain unknown. Although there is general consensus about the overall involvement of individual players in FeMo-cofactor biosynthesis, detailed insights have been denied for two principal reasons. First, some of the participants, for example, the escort proteins, could have overlapping functions. Second, it has not yet been possible to isolate assembly proteins trapped in forms that contain FeMo-cofactor or its precursors in quantities that are amenable to detailed biophysical and structural characterization. In our view, this latter aspect represents the major challenge in this research area. Although there is a plethora of molecular genetic techniques that have been used to unravel the assembly of certain other complex metalloclusters, such as molybdenum cofactor, these are not easily applied to FeMo-cofactor biosynthesis. The reasons for this are that assembly of FeMo-cofactor is oxygen sensitive, and because it is so complicated, the system cannot be simply transferred to *Escherichia coli*. Also, sophisticated methods for controlled high-level expression of recombinant proteins in *A. vinelandii* are only now just beginning to emerge. These opportunities should advance rapidly now that a draft version of the *A. vinelandii* genome sequence is available. We feel that the most important challenges in this area of research will involve the isolation of NifB (with and without NifB-co bound), isolation of a NifEN complex (with an FeMo-cofactor precursor bound), and isolation of the escort proteins NifX, NifY, and gamma (with FeMo-cofactor and its precursors bound). Although significant progress has already been made with respect to several of these issues, in most cases, pure samples in concentrations amenable for detailed biophysical and structural characterization are not yet available. Nevertheless, given the success with respect to crystallographic analysis of the MoFe protein¹⁵ and the apo-MoFe protein,⁴⁵ as well as recent success in solving the structure of a fragment of gamma¹¹³—together with new opportunities for genetic manipulation of *A. vinelandii*—we are optimistic that these challenges will be met.

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12. References

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- The numbering of amino acids in this article corresponds to positions within the relevant proteins from *A. vinelandii*.
- The term "apo-MoFe protein" has historically been used to designate MoFe proteins produced by *nifE*, *nifN*, *nifB*, or *nifH* mutants that do not contain FeMo-cofactor. However, the term "apo" is a misnomer because these proteins still retain some form of P cluster. It has previously been shown that the properties of apo-MoFe proteins produced by *nifE*, *nifN*, or *nifB* mutants are not the same as the properties of apo-MoFe protein produced by a *nifH* mutant. In this review, apo-MoFe protein refers to the form produced by a *nifB*-deficient strain, and the form produced by a *nifH*-deficient strain is designated Δ nifH-apo-MoFe protein.
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