Biosynthesis of the Iron-Molybdenum Cofactor of Nitrogenase

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Key Words

nitrogen fixation, *nif*, FeMo-co, iron-sulfur, NifDK, MoFe protein

Abstract

The iron-molybdenum cofactor (FeMo-co), located at the active site of the molybdenum nitrogenase, is one of the most complex metal cofactors known to date. During the past several years, an intensive effort has been made to purify the proteins involved in FeMo-co synthesis and incorporation into nitrogenase. This effort is starting to provide insights into the structures of the FeMo-co biosynthetic intermediates and into the biochemical details of FeMo-co synthesis.

Contents

INTRODUCTION

Biological nitrogen fixation accounts for roughly two-thirds of the nitrogen fixed globally, whereas the remaining portion is mostly contributed by the industrial Haber-Bosch process. Biological nitrogen fixation is performed, generally at mild temperatures, by diazotrophic microorganisms, which are widely distributed in nature (62). Most biological nitrogen fixation is carried out by the activity of the molybdenum nitrogenase, which is found in all diazotrophs. In addition to the molybdenum nitrogenase, some diazotrophic microorganisms carry alternative vanadium and/or iron-only nitrogenases (7, 53).

NifDK: also designated MoFe protein, dinitrogenase, or component I

FeMo-co: iron-molybdenum cofactor

NifH: also designated Fe protein, dinitrogenase reductase, or component II

The molybdenum nitrogenase enzyme complex has two component proteins (10) encoded by the *nifDK* and the *nifH* genes. The NifDK component is a heterotetrameric ($\alpha_2\beta_2$) protein formed by two $\alpha\beta$ dimers related by a twofold symmetry (**Figure 1**). NifDK carries one iron-molybdenum cofactor (FeMo-co) within the active site in each α-subunit (NifD) (47, 73) and one P-cluster at the interface of the α- and β-subunits in each $\alpha\beta$ pair. FeMo-co is located 10 Å beneath the protein surface. The Mo atom and the distal Fe atom at the other end of the cofactor are coordinated by one histidine and one cysteine residue from the NifD polypeptide, respectively. The P-cluster, also located beneath the protein surface, is coordinated by three cysteine residues from the NifD subunit and three cysteine residues from the NifK subunit.

The NifH component is a homodimer with twofold symmetry. NifH contains sites for Mg · ATP binding and hydrolysis at the dimer interface within each subunit and a single [Fe_4-S_4] cluster at the interface of both subunits (27). The [Fe_4-S_4] cluster of NifH is coordinated by two cysteine residues from each subunit. Binding and hydrolysis of Mg · ATP causes changes in NifH conformation and is coupled to electron transfer from the [Fe_4-S_4] cluster to the P-cluster of the NifDK component (49). The NifH and NifDK components associate and disassociate with each electron transfer cycle (31).

Electrons are subsequently transferred from the P-clusters to the FeMo-co embedded within the NifD subunits where reduction of substrates takes place (**Figure 1**). Dinitrogen (N_2) and protons are the physiological nitrogenase substrates. In the absence of N_2, proton reduction activity is maximal, as more electrons are allocated to this process. However, a minimal 2:1 ratio of proton to N_2 reduction is obligate even at high N_2 pressure (76), indicating that proton reduction is an intrinsic part of the mechanism of N_2 reduction. A number of additional triple-bonded molecules can serve

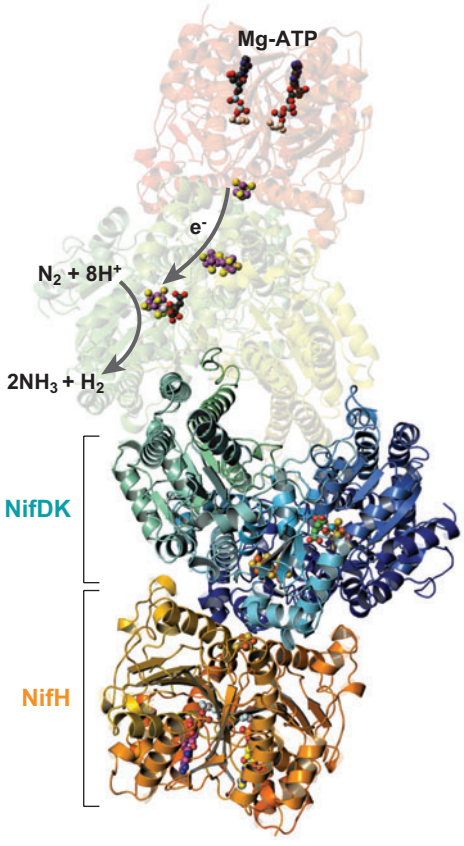

Figure 1

Structure of the molybdenum nitrogenase enzyme complex. The nitrogenase reaction produces ammonia and hydrogen at the expense of ATP. The relative positions of Mg-ATP and the [Fe-S] clusters of the symmetric nitrogenase enzyme complex are shown in the upper half of the figure together with the electron transfer pathway to the active site. The NifH component is orange and the NifDK component is in blue and green.

as substrates for nitrogenase. Among these substrates, acetylene is the most relevant to this review because acetylene and its reduction product, ethylene, are easily detected by gas chromatography and are routinely used to assay nitrogenase activity.

No crystal structure of an alternative nitrogenase has been reported to date. Biochemical and genetic studies have indicated that, although genetically distinct from the molybdenum nitrogenase, the three enzymes have similar subunit and cofactor composition. Attention has been directed to the use of the alternative vanadium and iron-only nitrogenases as catalysts for hydrogen production because they are less efficient in N_2 reduction and allocate more electrons to proton reduction than the molybdenum nitrogenase. However, the ATP dependence and the specific H_2 evolution activities are similar in molybdenum and alternative nitrogenases.

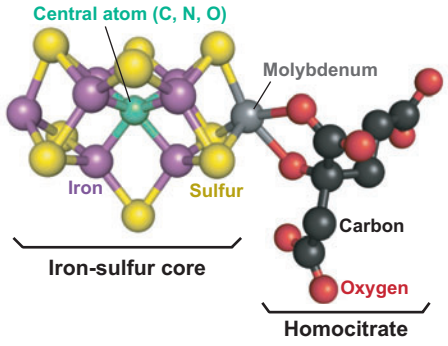

Figure 2

Structure of iron-molybdenum cofactor (FeMo-co) of nitrogenase.

THE FeMo-co OF NITROGENASE

FeMo-co is required by the molybdenum nitrogenase to perform the chemically difficult lysis of the N_2 triple bond (reviewed in Reference 39). FeMo-co is unique and structurally different from all the other molybdenum cofactors known to date. FeMo-co is composed of an inorganic Mo-Fe_7-S_9-X portion and the organic acid *R*-homocitrate, which is coordinated by its C-2 carboxyl and hydroxyl groups to the Mo atom (**Figure 2**) (11, 21, 35, 73). Although there is evidence for participation of the inorganic portion of FeMo-co in substrate binding and catalysis (5, 6), the exact role of homocitrate in catalysis is unknown. Replacement of homocitrate by citrate or other organic acid in FeMo-co drastically changes substrate specificity and results in an impairment in N_2 reduction (40).

The inorganic part of FeMo-co is regarded as one of the most complex iron-sulfur (plus

Molybdenum cofactors: with the exception of FeMo-co, biologically active molybdenum is always found in molybdopterin cofactors (Mo-co). Molybdoenzymes containing Mo-co are widely distributed in nature and participate in essential redox reactions of C, N, and S metabolism

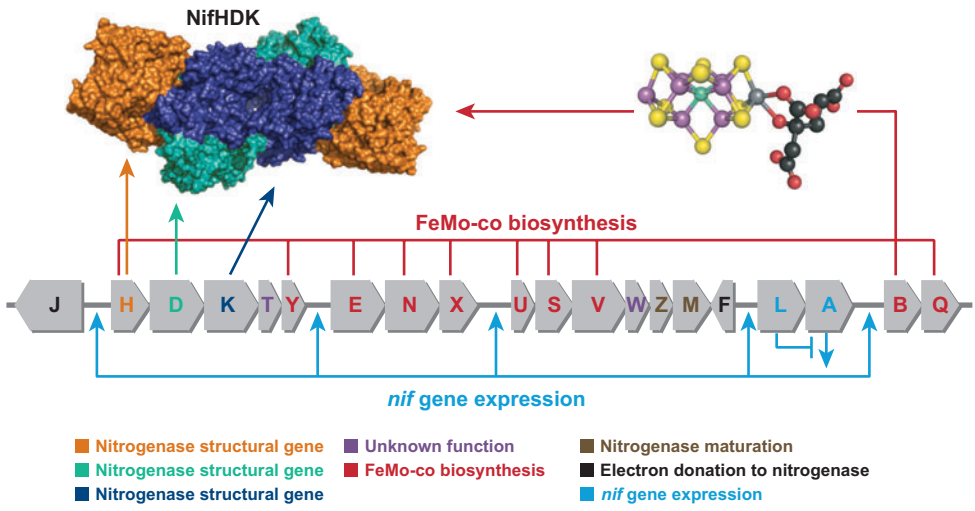

Figure 3

Nitrogen fixation (*nif*) gene cluster of *Klebsiella pneumoniae*. Genes encoding the nitrogenase component proteins, *nifHDK*, are shown on the left. Genes whose products are involved in nitrogen fixation are color coded according to their functions.

a heterometal) clusters found in biology. The structural features of the inorganic part of FeMo-co can be analyzed from two different perspectives relevant to our understanding of FeMo-co biosynthesis and to the design of a synthetic chemistry approach to synthesize this cofactor. It can be described as one $MoFe_3$-S_3X cubane and one Fe_4-S_3X cubane sharing a single atom X at a corner common to both cubanes. These cubanes would be additionally bridged by three sulfur atoms. Alternatively, it can be described as a Fe_6-S_9 core (with the iron atoms symmetrically coordinating a central atom X) that is capped by one Mo and one Fe atom at the end sides of the core.

There are only two protein ligands to FeMo-co (α-Cys^{275} and α-His^{442} in the *Azotobacter vinelandii* nitrogenase enzyme), and the cofactor can be purified by extraction into the organic solvent *N*-methyl formamide after denaturing the NifDK protein (73). The purification of FeMo-co has been an indispensable tool to analyze the processes of FeMo-co biosynthesis and insertion into apo-dinitrogenase (apo-NifDK).

The other complex metallocluster present in the NifDK component of nitrogenase, the [Fe_8-S_7] P-cluster, is structurally similar to FeMo-co in that it is formed by two [Fe_4-S_3] cubanes connected by a central sulfur atom. The structure of the P-cluster and its ligation to the polypeptide changes depending on the oxidation state of the NifDK protein.

nif: genes encoding proteins involved in molybdenum-dependent nitrogen fixation

THE *nif* GENE CLUSTER

Klebsiella pneumoniae was the first diazotroph whose nitrogen fixation (*nif*) genes were analyzed by a combination of genetic and biochemical techniques (4, 51, 64). **Figure 3** shows the *K. pneumoniae nif* gene cluster. Twenty *nif* genes, *nifJHDKTYENXUSVWZMFLABQ*, organized in several transcriptional units, are clustered in a single 23-kb region in the chromosome of *K. pneumoniae*. The *nif* gene cluster is simpler in *K. pneumoniae* than in other model organisms used to study the biochemistry and genetics of nitrogen fixation, such as *A. vinelandii*. This is probably due to the different physiological conditions faced by the oxygen-sensitive nitrogenase in these bacteria. Whereas *K. pneumoniae* fixes nitrogen only under strict anaerobic conditions, *A. vinelandii* combines in a single cell nitrogen fixation activity with strict aerobic metabolism.

The *A. vinelandii nif* genes are clustered into two different chromosomal linkage groups. The major *nif* cluster contains the *nifHDK-TYENX iscA*^{nif} *nifUSV cysE1*^{nif} *nifWZM nifF* genes and several open reading frames interspersed among these genes (42). The minor *nif* cluster contains the *rnfABCDGEH nafY* genes in one transcriptional direction (14, 69) and the *nifLAB fdxN nifOQ* genes in the opposite DNA orientation (44, 65).

The *nifHDK* genes encode the structural components of the molybdenum nitrogenase enzyme complex. The complexity and uniqueness of FeMo-co and the P-cluster demand complex biosynthetic pathways for cofactor biosynthesis and maturation of the NifDK protein. Thus, the products of at least 12 *nif* genes are involved in the biosynthesis of catalytically active molybdenum nitrogenase (68) (see **Figure 3**). Additional gene products are required to provide nitrogenase with electrons. The flavodoxin NifF and the pyruvate:flavodoxin oxidoreductase, NifJ, fulfill this role in *K. pneumoniae* (75). Finally, the products of the *nifA* and *nifL* genes form an activator/antiactivator regulatory system that controls *nif* gene expression to ensure it occurs only under appropriate physiological and environmental conditions (52).

OVERVIEW OF FeMo-co BIOSYNTHESIS

The products of the *nifD* and *nifK* genes do not seem to be required for FeMo-co biosynthesis. FeMo-co is first assembled by specialized biosynthetic machinery and then incorporated into FeMo-co-deficient apo-NifDK, generating the mature NifDK nitrogenase component that is competent for nitrogen fixation (see References 17, 50, and 68 for previous reviews). The proteins involved in FeMo-co biosynthesis can be functionally divided into three classes: molecular scaffolds (NifU, NifB, and NifEN) where FeMo-co is stepwise assembled, metallocluster carrier proteins (NifX and NafY) that carry FeMo-co precursors between assembly sites in the pathway, and enzymes (NifS, NifQ, and NifV) that provide sulfur, molybdenum, and homocitrate as substrates for cofactor synthesis. The exact role of NifH remains controversial.

Figure 4 illustrates a schematic summary of our current model for FeMo-co biosynthesis. This scheme has been centered on NifEN, a scaffold protein that is proposed to function as a central node in the pathway to which [Fe-S]-containing FeMo-co precursors, molybdenum and homocitrate, might converge to complete the assembly of FeMo-co (13, 25, 29, 36, 38, 66, 78). Molybdenum is specifically donated by NifQ (J.A. Hernandez, L. Curatti, C.P. Aznar, Z. Perova, R.D. Britt & L.M. Rubio, unpublished results). The iron-sulfur core is provided by the sequential activities of NifS, NifU, and NifB. The cysteine desulfurase NifS directs the assembly on NifU of simple Fe-S clusters (probably [Fe_2-S_2] or [Fe_4-S_4]) that will serve as metabolic substrates for NifB-cofactor (NifB-co) synthesis (84). The SAM radical protein NifB synthesizes NifB-co in a reaction that requires *S*-adenosylmethionine (SAM) (16). NifB-co comprises the Fe_6-S_9 core of FeMo-co (26) but lacks molybdenum and homocitrate (72). NifX would mobilize NifB-co from NifB to NifEN (32). Homocitrate is generated by the homocitrate synthase NifV. Although we hypothesize that the molybdenum, iron-sulfur, and homocitrate precursors converge on NifEN, it is not clear whether NifEN alone provides a homocitrate binding site. Current evidence indicates that both NifEN and NifH must be present to achieve homocitrate incorporation into the FeMo-co precursor (15, 36, 57). Finally, assembled FeMo-co would be transferred to apo-NifDK via NafY, the product of a non-*nif* gene that also stabilizes the target apo-NifDK protein (67).

Originally, the in vitro FeMo-co synthesis assay was carried out as a biochemical complementation of cell extracts from strains with lesions in different *nif* genes (74). Over the past several years, different laboratories have made extensive efforts to purify all Nif/Naf proteins involved in FeMo-co biosynthesis. **Figure 5** compiles much of this effort by

***nafY*:** gene encoding the nitrogenase accessory factor Y (γ subunit)

NifB-cofactor (NifB-co): the metabolic product of NifB activity. It is an isolatable [Fe-S] cluster of unknown structure that serves as precursor to FeMo-co

SAM: *S*-adenosylmethionine

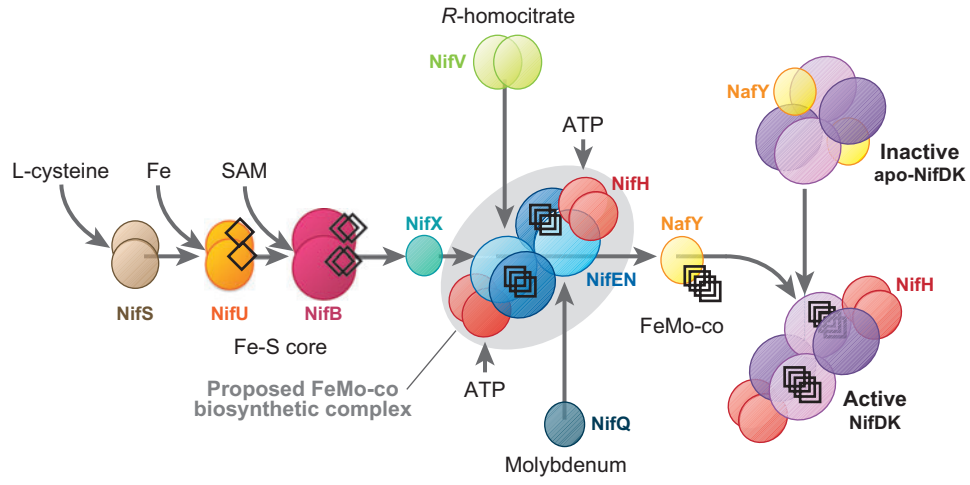

Figure 4

Schematic model of iron-molybdenum cofactor (FeMo-co) biosynthesis illustrating the convergence of FeMo-co precursors into the NifEN/NifH enzyme complex, a central node where FeMo-co synthesis is completed. De novo synthesized FeMo-co is inserted into apo-NifDK to generate catalytically active nitrogenase. The FeMo-co biosynthetic pathway involves enzymes, proteins that act as molecular scaffolds, and carriers of complex metalloclusters. The number of black squares for each FeMo-co precursor represents its level of structural complexity.

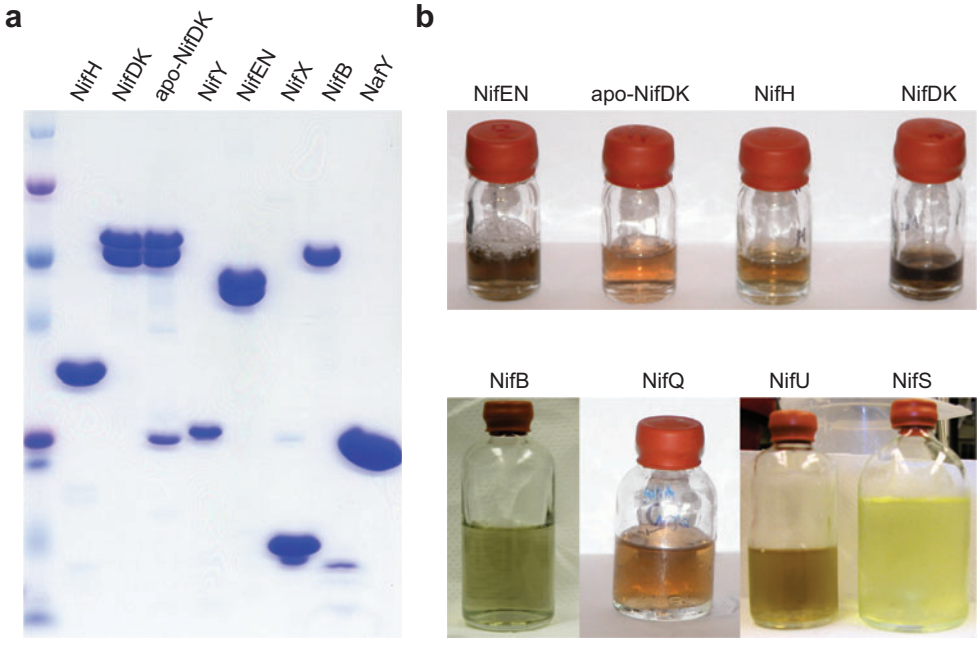

Figure 5

The colorful world of nitrogenase. Many Nif proteins carry [Fe-S] clusters or other cofactors, such as pyridoxal phosphate (PLP), that are essential to their functions. (*a*) SDS-PAGE analysis of purified Nif proteins. (*b*) Anaerobic solutions of purified Nif proteins.

showing purified preparations of most of these proteins, many of which carry [Fe-S] clusters or other colorful cofactors. Purification of all these proteins has allowed in vitro reconstitution of the complete FeMo-co biosynthetic pathway and has demonstrated that the reactions catalyzed by NifB, NifEN, and NifH are necessary and sufficient for FeMo-co synthesis from iron, sulfur, molybdenum, and homocitrate (15). Although not essential, the NifX and NafY proteins increase the synthetic efficiency of the pathway, possibly by providing protection to the oxygen-labile FeMo-co and its intermediates. The in vivo functions of the other Nif proteins involved in FeMo-co synthesis (e.g., NifU, NifS, NifV, and NifQ) can be replaced in vitro by adding the appropriate chemical substrates.

FORMATION OF THE IRON-SULFUR CORE OF FeMo-co

NifU and NifS Proteins

The first suggestion that NifU and NifS were involved in the assembly of [Fe-S] clusters for the nitrogenase component proteins, NifH and NifDK, came from the analysis of *A. vinelandii nifU* and *nifS* mutants. Mutations in either *nifS* or *nifU* affected NifH and NifDK activities, decreasing them by 15- and 4-fold, respectively (43). Because a common feature of NifH and NifDK was the requirement of their [Fe-S] clusters for activity, it was suggested that *nifU* and *nifS* mutants were impaired in the assembly of [Fe-S] clusters for the nitrogenase components. Similar phenotype had previously been observed in *K. pneumoniae nifS* mutants that exhibited negligible NifH activity and a 25-fold reduction in NifDK activity (64), but NifS was proposed to be involved exclusively in NifH processing.

Subsequently, a series of elegant studies in the laboratories of Dean and Johnson demonstrated that NifU and NifS compose cellular machinery for the assembly of [Fe_2-S_2] and [Fe_4-S_4] clusters under nitrogen-fixing conditions (reviewed in Reference 45). NifS is a cysteine desulfurase that provides sulfur for the assembly of transient [Fe-S] clusters onto the molecular scaffold NifU. These transient labile clusters are then transferred to target apoproteins. It was also shown that NifU and NifS were nitrogenase-specific homologs of the IscU and IscS proteins (85), which are involved in general [Fe-S] cluster assembly in a wide range of organisms (45). The special boost in [Fe-S] cluster assembly provided by the activities of NifU and NifS is required to fulfill the high demand imposed by nitrogenase, a catalytically slow enzyme that may represent up to 10% of the total cellular protein under nitrogen-fixing conditions.

NifU is a homodimer of 33-kDa subunits. The most interesting property of NifU is its modular structure. NifU comprises three well-defined highly conserved domains, all of which have the ability to coordinate an [Fe-S] cluster. The second (middle) domain carries four conserved cysteines that coordinate one [Fe_2-S_2] cluster (23). The first (N-terminal) and third (C-terminal) domains contain three and two conserved cysteine residues, respectively, that serve as ligands for the assembly of the transient [Fe-S] clusters that will be further delivered to target apo-proteins. The [Fe_2-S_2] cluster that is permanently bound to NifU has been proposed to have a redox function necessary for NifU to release the labile clusters (1).

The homodimeric NifS protein is a pyridoxal phosphate (PLP)-containing enzyme that catalyzes the desulfurization of L-cysteine, yielding sulfur destined to [Fe-S] cluster formation and L-alanine (87). A reaction intermediate in the form of cysteinyl persulfide is formed in the Cys^{325} residue of NifS (86). Consistently, substitution of Ala by Cys in this position eliminates cysteine desulfurase activity of the enzyme. Formation of a NifUS heterotetramer during cluster assembly has been observed. Within this protein complex, NifS activity directs the assembly of [Fe-S] clusters on the molecular scaffold NifU (77, 83).

In vitro experiments in which transient [Fe_4-S_4] clusters, assembled by NifS on NifU, were

transferred to apo-NifH to reconstitute the dinitrogenase reductase activity of NifH have been reported (19). Additional in vivo experiments showing that NifU and NifS were necessary for the assembly of the [Fe_4-S_4] cluster of NifH (18, 19, 46) provided physiological evidence to further support the roles of NifU and NifS in NifH maturation.

NifU and NifS are also involved in the assembly of the P-cluster and the FeMo-co of the NifDK component of nitrogenase. The involvement of NifU and NifS in P-cluster assembly is supported by the fact that *nifU* and *nifS* mutations cause the accumulation of a form of apo-NifDK whose activity cannot be recovered in vitro by the simple addition of FeMo-co (43). To understand the *nifUS* mutant phenotype, it is important to note that, during NifDK maturation, P-cluster assembly precedes FeMo-co insertion and that apo-NifDK containing P-clusters but lacking FeMo-co is readily activatable by FeMo-co. The synthesis of each P-cluster has been proposed to occur in two steps: (*a*) formation of two [Fe_4-S_4] cluster units at the interface of the NifD and NifK subunits, and (*b*) NifH-dependent condensation of these clusters to generate the mature [Fe_8-S_7] P-cluster (9, 63). It is likely that *nifUS* mutants are impaired in the assembly of the [Fe_4-S_4] cluster units that serve as P-cluster precursors. An alternative interpretation is that the impairment of *nifUS* mutants in the P-cluster assembly is in fact reflecting a deficient assembly of the [Fe_4-S_4] cluster of NifH. We do not favor this alternative interpretation because there is evidence showing that [Fe_4-S_4] cluster-deficient NifH functions in apo-NifDK maturation (14, 61).

Direct participation of NifU and NifS in FeMo-co biosynthesis was demonstrated by analyzing the capability of *nifU* and *nifS* mutants to synthesize active NifB protein and its metabolic product, NifB-co (84). Most of the proteins involved in FeMo-co biosynthesis are [Fe-S] proteins, and therefore mutations in *nifU* and *nifS* were expected to have a pleiotropic effect in this pathway. However, NifB-co synthesis is an early step in the pathway that requires only the activity of the [Fe-S]-containing protein NifB (15, 16). Whereas *K. pneumoniae nifU* and *nifS* mutants synthesized active NifB protein, they were unable to synthesize and accumulate measurable amounts of NifB-co. The simplest explanation for the *nifUS* phenotype is that the NifUS machinery acts as a major provider of [Fe-S] substrates for NifB-co biosynthesis by NifB.

NifB and The Formation of NifB-co

NifB is a homodimeric protein whose activity is essential to the biosynthesis of FeMo-co and those of the cofactors for the alternative vanadium and iron-only nitrogenases. According to its primary sequence, the N-terminal domain of NifB belongs to the SAM radical protein family (79). SAM radical proteins contain an [Fe_4-S_4] cluster coordinated by three cysteine residues and a molecule of SAM. Although SAM radical enzymes catalyze diverse reactions, the mechanism of catalysis starts by reductive cleavage of SAM and generation of a 5′-deoxyadenosyl radical (82). The C-terminal domain of NifB is similar to the NifX/NafY family of proteins (see below) (69) and is hypothesized to be a NifB-co binding site (16).

A functional *nifB* gene is required for the synthesis of NifB-co in vivo, and consistently, the requirement for NifB in the in vitro FeMo-co synthesis assay was satisfied by addition of NifB-co (72). NifB-co, the metabolic product of NifB, is a molybdenum-free and homocitrate-free, oxygen-labile [Fe-S] cluster that serves as a precursor to FeMo-co. Allen et al. (2) have shown the specific incorporation of Fe and S from ^{55}Fe- and ^{35}S-labeled NifB-co into FeMo-co. NifB-co-dependent in vitro FeMo-co synthesis is catalyzed by NifEN and NifH and requires the presence of molybdate, homocitrate, sodium dithionite, and Mg · ATP in the reaction. **Figure 6** illustrates a NifB-co-dependent FeMo-co synthesis assay carried out with purified components. In this assay, FeMo-co is synthesized in vitro and inserted into apo-NifDK to generate active NifDK protein. The amount of synthesized FeMo-co is

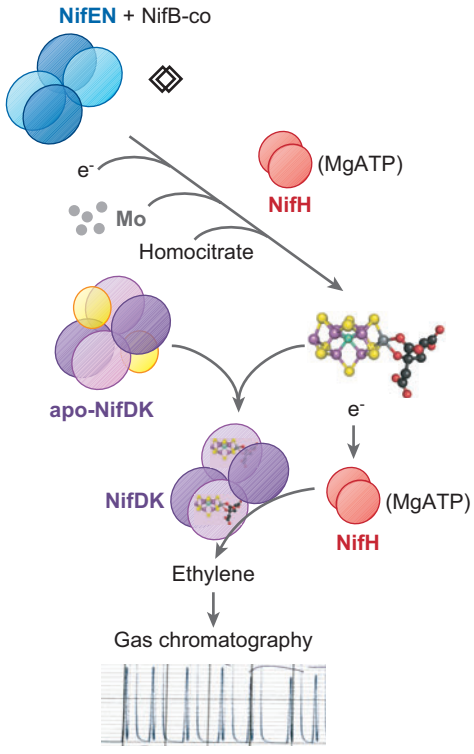

Figure 6

In vitro iron-molybdenum cofactor (FeMo-co) synthesis assay. Purified Nif proteins transform FeMo-co precursors [such as NifB-cofactor (NifB-co)] into FeMo-co upon addition of molybdenum and homocitrate under reducing conditions. Formation of an active NifDK protein is then followed by its acetylene reduction activity in a gas chromatographer.

estimated by the acetylene reducing activity of de novo generated NifDK.

NifB seems to be a molecular scaffold for the assembly of NifB-co. As isolated from *A. vinelandii* cells, NifB did not support in vitro FeMo-co synthesis. However, incubation of purified NifB with ferrous iron, sulfide, and SAM under reducing conditions resulted in the incorporation of additional [Fe-S] clusters into NifB, which acquired the ability to substitute for NifB-co and support in vitro FeMo-co synthesis (16). Radical chemistry is thus required during NifB-co assembly. The reason for this requirement is unclear. Very low-potential radical chemistry could be needed to incorporate the central atom into NifB-co.

The properties of NifB, including its ability to readily support FeMo-co synthesis, were different when purified from *K. pneumoniae* or from *A. vinelandii* cells. The enzyme isolated from *K. pneumoniae* contained more iron and readily substituted for NifB-co in the in vitro FeMo-co synthesis assay without a requirement for iron, sulfide, or SAM (84). Additional evidence that the *K. pneumoniae* NifB carries NifB-co came from the ability of NifB to transfer an iron-containing moiety to NifX, which binds NifB-co but not free iron (32, 58). Comparison of spectroscopic signatures from NifB preparations obtained from *K. pneumoniae* or *A. vinelandii* cells are expected to provide insights into the nature of NifB-co precursors and into the mechanism of the reaction catalyzed by NifB.

MOLYBDENUM PROCESSING FOR FeMo-co BIOSYNTHESIS

Possible Role of NifQ as Physiological Molybdenum Donor

NifQ has been implicated in the processing of molybdenum specifically for the biosynthesis of FeMo-co (41). The *nifQ* mutants accumulate lower levels of molybdenum than the wild-type strain and exhibit a nitrogen-fixation-deficient phenotype, but they are not defective in the synthesis of molybdopterin cofactor (Mo-co).

The primary amino acid sequence of NifQ is unique and unrelated to other proteins involved in molybdenum trafficking, such as molbindins or molybdenum storage proteins (22, 30). NifQ proteins have a conserved $Cx_4Cx_2Cx_5C$ amino acid motif that was proposed to be a binding site for an [Fe-S] cluster, a molybdenum-containing metal cluster, or a Mo-S intermediate for FeMo-co synthesis (17, 67).

Because the phenotype of *nifQ* mutants is leaky, and because molybdate satisfies the molybdenum requirement for FeMo-co synthesis in vitro, the determination of a physiological provider of molybdenum for FeMo-co

EPR: electron paramagnetic resonance

EXAFS: extended X-ray absorption fine structure

biosynthesis has been elusive. High concentrations of molybdate (41) or cysteine (81) (the sulfur source for [Fe-S] cluster biosynthesis) in the growth medium can suppress the *nifQ* phenotype. This was originally interpreted as an indication that a nonenzymatic reaction between molybdenum and sulfur could substitute for the reaction catalyzed by NifQ (81).

Recent results show that, as isolated from *A. vinelandii* cells, NifQ is an iron-sulfur protein with a redox-responsive [Fe-S] cluster. NifQ is also a molybdoprotein that serves as a direct molybdenum donor for FeMo-co synthesis, replacing molybdate in the in vitro FeMo-co synthesis assay (J.A. Hernandez, L. Curatti, C.P. Aznar, Z. Perova, R.D. Britt & L.M. Rubio, unpublished results). NifQ was unable to donate molybdenum for FeMo-co synthesis unless NifH and NifEN were simultaneously present in the reaction. One possible interpretation is that molybdenum delivery proceeds from NifQ to a NifEN/NifH complex. Electron paramagnetic resonance (EPR) spectroscopic studies indicated that NifQ carries a [Mo-Fe_3-S_4] cluster, and that the presence of this metal cluster in NifQ correlates with its ability to support in vitro FeMo-co synthesis. The chemical form of molybdenum donated by NifQ remains unknown.

Possible Role of NifH as Molybdenum Insertase

NifH has been proposed to serve as the entry point for molybdenum incorporation into the FeMo-co biosynthetic pathway based on ^{99}Mo radiolabeling experiments (57) or on extended X-ray absorption fine structure (EXAFS) analysis of FeMo-co precursors associated with NifH in vitro (38). In contrast, substoichiometric amounts of molybdenum were found in NifEN isolated from a Δ*nifH* strain, suggesting the existence of a NifH-independent molybdenum binding site within NifEN (78). The possible role of NifH in the incorporation of molybdenum into FeMo-co is discussed below.

THE HOMOCITRATE SYNTHASE NifV

The NifV protein is as a homocitrate synthase that catalyzes the condensation of acetyl coenzyme A and α-ketoglutarate to form *R*-homocitrate (88). *K. pneumoniae nifV* mutants incorporate citrate in place of homocitrate in FeMo-co (54) and exhibit altered substrate-reducing properties. The *nifV* nitrogenase variant reduces protons and acetylene effectively but is unable to reduce N_2 (55). The phenotype of a *K. pneumoniae nifV* strain could be reverted to wild type simply by the addition of homocitrate to the growth medium during nitrogenase derepression, further correlating the function of NifV in vivo to the synthesis of homocitrate (34).

^{99}Mo-radiolabeling experiments have indicated that addition of molybdenum to the [Fe-S] core of FeMo-co precedes homocitrate incorporation (57). It is not known by which mechanism homocitrate is coordinated to molybdenum and how the FeMo-co synthesis machinery discriminates against other organic acids present in the cell. Although homocitrate analogs can be incorporated into aberrant cofactors by increasing their concentration in the in vitro FeMo-co synthesis assay (40), there is a mechanism for homocitrate incorporation in vivo that appears to be specific enough to discriminate against similar organic acids that are not incorporated into FeMo-co.

The site of homocitrate incorporation into the cofactor is also a matter of debate. On the one hand, Rangaraj & Ludden (57) used ^{99}Mo-labeled molybdate in the purified in vitro FeMo-co synthesis assay to test the effect of various organic acids on ^{99}Mo incorporation into NifH or NifX and suggested that NifX had a role specifying the organic acid incorporated into the cofactor. On the other hand, Hu et al. (38) have recently used EXAFS analysis coupled with in vitro FeMo-co synthesis to propose NifH as responsible for homocitrate insertion into FeMo-co. In any case, the presence of NifEN in the

reactions was required to achieve homocitrate incorporation in vitro, and we propose that the actual incorporation of homocitrate into the FeMo-co precursor would take place in the scaffold protein NifEN while associated with NifH. Given that NifX binds a variety of structurally related cofactors (NifB-co, VK-cluster, and FeMo-co), and exchanges some of them with NifEN, it would not be surprising that NifX accumulated an excess of molybdenum-containing FeMo-co precursor in reactions lacking homocitrate. The proposal of NifH as a homocitrate insertase is more puzzling and is discussed in the context of the FeMo-co biosynthetic factory model.

NifEN IS THE CENTRAL NODE IN FeMo-co BIOSYNTHESIS

Properties of the NifEN Protein

NifEN is a 200-kDa $\alpha_2\beta_2$ heterotetramer that contains [Fe-S] clusters (56). The function of NifEN is essential to FeMo-co biosynthesis. Because of the amino acid sequence similarity between NifEN and NifDK and the observation that NifDK was not required for FeMo-co biosynthesis (80), it was early proposed that NifEN could be a molecular scaffold to assemble FeMo-co (8).

An interesting characteristic of NifEN is the lability of some of its [Fe-S] clusters, which are lost during the purification of the protein, yielding NifEN preparations with different [Fe-S] cluster content. Over years of study, this property has led investigators to different conclusions about the role of NifEN in FeMo-co biosynthesis. From the first reported NifEN purification (4.6 iron atoms per NifEN tetramer) (56) to the last one (24 iron atoms per NifEN tetramer) (78), the iron content of NifEN preparations and the ability of NifEN to support FeMo-co biosynthesis in the absence of externally added iron, sulfur, and molybdenum substrates have increased significantly.

Originally, NifEN was purified from a *nifB* mutant strain, which yielded a NifEN protein able support in vitro FeMo-co synthesis when added to a *K. pneumoniae* cell extract lacking NifEN but containing NifB-co (56). NifEN appeared to carry one single [Fe_4-S_4] per tetramer that was suggested to be involved in electron transfer to a FeMo-co precursor (66). In vitro work demonstrated the incorporation in NifEN of ^{55}Fe or ^{35}S label from radioactive NifB-co (2), and the changes in electrophoretic mobility of NifEN upon NifB-co binding (66). Now we know that Δ*nifB* NifEN lacks the VK-cluster (37) and that its activity is dependent on processing the NifB-co present in the reaction (32).

Overexpression of His-tagged NifEN from a Δ*nifHDK* mutant strain, and application of a faster purification protocol, showed that NifEN contained two identical [Fe_4-S_4] clusters with $S = 1/2$ EPR signal in the reduced state (29). Because the His-tagged NifEN was purified from a strain having a functional *nifB* gene and contained more than eight iron atoms per tetramer, it was inspected for other EPR signals that could account for additional [Fe-S] clusters. Indeed, a different $S = 1/2$ EPR signal was observed in thionine-oxidized NifEN and suggested to arise from a FeMo-co precursor bound to the enzyme (28). This proposal was reinforced in a following study showing that, in contrast to the Δ*nifHDK* NifEN protein, indigo carmine (IDS)-oxidized preparations of the Δ*nifB* NifEN protein (which lacks FeMo-co precursor activity) lacked the $S = 1/2$ EPR signal (37).

The His-tagged NifEN tetramer purified from a Δ*nifHDK* mutant contains 24 iron atoms and, importantly, 0.3 molybdenum atoms (78). Although present in substoichiometric amounts, two lines of evidence suggest that the molybdenum present in NifEN is relevant to the FeMo-co biosynthesis pathway. First, the molybdenum was available for in vitro FeMo-co synthesis and supported apo-NifDK maturation (78). Second, EXAFS analysis showed that this molybdenum was not adventitiously bound molybdate, but that it was embedded in an [Fe-S] cluster ligand environment within the NifEN protein (25).

Vinod K Shah cluster (VK-cluster): represents an intermediate after NifB-co in FeMo-co synthesis and accumulates on NifEN in a Δ*nifH* mutant

IDS: indigo carmine

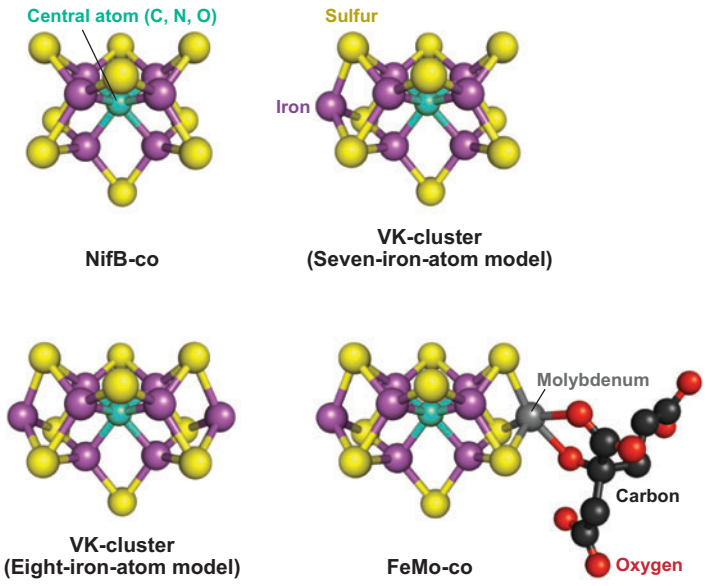

Figure 7

Model of chemical transformations of the Fe-S core during FeMo-co biosynthesis. NifB-co would comprise a six-iron-atom core with a FeMo-co-like central atom signature. The VK-cluster would contain additional iron but no molybdenum or homocitrate.

The EPR signals from the NifB-co-derived FeMo-co precursor within NifEN were further analyzed by transferring it to purified NifX protein in vitro (32). Contrary to NifEN, NifX does not contain [Fe-S] clusters or any other EPR-active species that could interfere with the analysis. The [Fe-S] cluster transferred from NifEN to NifX served as a FeMo-co precursor in vitro and was designated VK-cluster to honor the pioneer Vinod K. Shah. The VK-cluster exhibited isotropic $S = 1/2$ and axial $S = 1/2$ EPR signals in the reduced and the oxidized states, respectively, with two-electron redox transitions that were fully reversible. The EPR signals from an isolated VK-cluster differed significantly from those attributed to the FeMo-co precursor bound to NifEN (37). The reason for this discrepancy is not clear, but it is our hypothesis that the $S = 1/2$ EPR signal detected in IDS-oxidized NifEN arises from an [Fe-S] cluster different from the VK-cluster. Given that this signal has properties resembling those of [Fe_3-S_4] clusters, perhaps the signal could arise from the [$MoFe_3S_{3+X}$] cluster detected by EXAFS, from oxidative damage of NifEN permanent [Fe_4-S_4] clusters, or from additional clusters not yet identified.

Role of NifEN and Its Association with NifH

At least three reactions of the FeMo-co biosynthetic pathway have been proposed to occur within NifEN: incorporation of additional iron, insertion of molybdenum, and incorporation of homocitrate. The first reaction entails the conversion of NifB-co into the VK-cluster. Like NifB-co, the VK-cluster does not contain molybdenum or homocitrate. However, NifB-co and the VK-cluster are electronically different because NifB-co is EPR silent and the VK-cluster shows EPR signals in the reduced and oxidized states (32). Two lines of evidence suggest that additional iron atoms are added to NifB-co to convert it to the VK-cluster. (*a*) When NifDK protein was matured in a NifB-co-dependent FeMo-co synthesis and insertion assay in the presence of additional $^{55}FeCl_3$, it incorporated some ^{55}Fe label, suggesting additional incorporation of iron after the stage of NifB-co (16). (*b*) A comparison of EXAFS data from isolated NifB-co and isolated VK-cluster shows differences that favor a six-iron-atom structure for NifB-co (26) and a seven- (or eight-) iron-atom structure for the VK-cluster (M. Demuez, Y. Guo, B. Soboh, S.J. George, R.Y. Igarashi, et al., unpublished results). Consistently, EXAFS analysis of purified NifEN protein loaded with a FeMo-co biosynthetic intermediate (the VK-cluster) fitted to a seven- or eight-iron-atom model (13). **Figure 7** shows plausible NifB-co and VK-cluster structures based on EXAFS analysis.

The second reaction that takes place within NifEN is the incorporation of molybdenum into the FeMo-co precursor, which appears to occur in two steps. Initially, molybdenum is incorporated into a transient site on NifEN. The presence of molybdenum in the Δ*nifHDK* NifEN protein implies that this step is not absolutely dependent on NifH, although a

role for NifH enhancing molybdenum binding to NifEN cannot be ruled out (78). EXAFS analysis indicates that molybdenum is part of an [Fe-S] cluster at this stage (25). Subsequently, molybdenum is mobilized into the VK-cluster-derived FeMo-co precursor in a reaction that requires NifH and Mg · ATP (36, 38, 57). Reports on whether ATP hydrolysis is required at this step are contradictory. Although there is indirect evidence that NifEN might bind Mg · ATP, no ATP hydrolysis activity was detected in purified NifEN (56) and the attention was turned to the ATP-hydrolyzing protein NifH. In vitro experiments correlate ATP hydrolysis by NifH to molybdenum incorporation into the FeMo-co precursor (38). However, NifH mutant variants that bind but do not hydrolyze Mg · ATP were functional in FeMo-co synthesis in vitro (60) and in vivo (24). Whether the electron transfer ability of NifH is required for this role is also at disagreement. In two different reports, a NifH variant that had its [Fe_4-S_4] cluster removed by chelation was active (61) and inactive (38) in FeMo-co synthesis. If the role of NifH were to promote the transfer of molybdenum from the transient binding site on NifEN to the VK-cluster to generate the next FeMo-co biosynthetic intermediate, then the simplest interpretation fitting most of these data is that this role would be performed by docking with NifEN and exerting some sort of conformational change on it.

The third reaction would be the incorporation of homocitrate into the precursor to generate FeMo-co, which is thought to be NifH dependent. This proposal is speculative because the binding of homocitrate (e.g., ^{14}C-homocitrate) to NifEN has not been demonstrated, and the processing of homocitrate in vitro requires the presence of other substrates (the VK-cluster and molybdenum) and proteins (NifH). It is not clear whether incorporation of homocitrate directly requires NifH. Homocitrate incorporation follows molybdenum incorporation (57), which is dependent on NifH, and the incorporation of homocitrate into a molybdenum-containing precursor in the absence of NifH has not been reported.

The FeMo-co Biosynthetic Factory Model

Complete in vitro synthesis of FeMo-co from its basic constituents has been reported (15). NifB, NifEN, and NifH were sufficient for FeMo-co synthesis from iron, sulfur, molybdenum, and homocitrate under reducing conditions (the in vitro system also required SAM and Mg · ATP). As expected, NifX and NafY addition stimulated the efficiency of FeMo-co biosynthesis, and the addition of the appropriate chemical forms of homocitrate, iron, sulfur, and molybdenum could substitute for the roles played in vivo by NifV, NifS, NifU, and NifQ.

Because NifB, NifEN, and NifH constitute the essential catalytic components of the FeMo-co synthetic machinery, and because FeMo-co and its biosynthetic intermediates are labile (72, 73), the presence in a nitrogen-fixing bacterium of a multi-protein complex involved in FeMo-co biosynthesis seems likely. The structural core of this machinery would be NifEN in close association with NifH and NifB. Other proteins, such as NifX, NafY, NifQ, and NifV, would also interact with NifEN. There is some experimental evidence consistent with the existence of this complex, which we have designated the FeMo-co biosynthetic factory. First, NifEN and a L127Δ variant of NifH form a stable complex in the presence of NifB-co (59). The L127Δ NifH protein also shows strong interaction with NifDK (48), and it is reasonable to postulate that wild-type NifH would interact with NifEN as it does with NifDK. Second, in vitro FeMo-co synthesis experiments show that molybdenum donation by NifQ requires the concomitant presence of NifEN and NifH (J.A. Hernandez, L. Curatti, C.P. Aznar, Z. Perova, R.D. Britt & L.M. Rubio, unpublished results). Third, NifEN and NifH are simultaneously required for incorporation of molybdate and/or homocitrate into a FeMo-co precursor in vitro, and the products resulting from these reactions bind to both NifEN and NifH (57, 58). Fourth, the *nifB* and *nifN* genes are fused in

a single open reading frame in several studied nitrogen-fixing *Clostridium* strains (12). Fifth, the transfer of FeMo-co biosynthetic precursors probably involves protein-protein interactions. Transfer of NifB-co from NifB to NifX (84), or from NifX to NifEN (32), as well as exchange of VK-cluster between NifEN and NifX (32), has been demonstrated in vitro.

The hypothesis of a FeMo-co biosynthetic factory has been formulated before (67) to explain the observed distribution of radioactively labeled FeMo-co precursors between the NifH, NifEN, NifX, and NafY proteins during the biosynthesis of FeMo-co in vitro (2, 3, 57, 58). This hypothesis is also consistent with the recently reported distribution of FeMo-co precursors between NifEN and NifH as analyzed by EXAFS (36, 38).

THE METALLOCLUSTER CARRIER PROTEINS NifX AND NafY

NifX and NafY are members of a family of nitrogenase cofactor binding proteins that would additionally include NifY, the C-terminal domain of NifB, and the VnfX and VnfY proteins [involved in assembly of iron-vanadium cofactor (FeV-co) for the vanadium nitrogenase] (69, 71). These proteins exhibit amino acid sequence conservation and common biochemical properties. NifX is the smallest member of the family and is formed by a single domain. This is also the case for VnfX and VnfY (which are not discussed further in this review). NifB, NifY, and NafY, however, are composed of a C-terminal NifX-like domain and an N-terminal domain that differ in origin and function.

NifX is a 17-kDa monomeric protein that binds structurally related FeMo-co precursors (NifB-co, VK-cluster) and transfers them to the scaffold protein NifEN (32, 58). NifX binds one metal cluster per monomer (32). Binding to NifX increased NifB-co stability in vitro (J.A. Hernandez & L.M. Rubio, unpublished results), and thus it is likely to provide protection to these labile metalloclusters during cofactor synthesis in vivo. Two compatible roles for

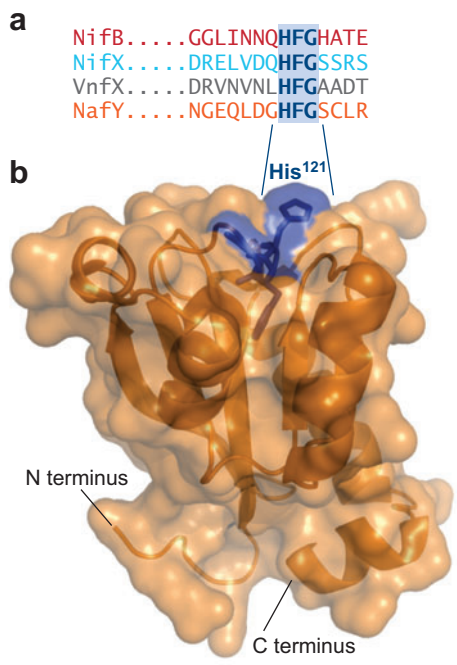

Figure 8

NafY represents a family of metallocluster carrier proteins. (*a*) The conserved sequence HFG proposed to be involved in metallocluster binding. (*b*) The structure of the metallocluster binding domain of NafY, in which residues 121-His-Phe-Gly-123 have been highlighted.

NifX have been proposed. First, NifX would carry FeMo-co precursors from one scaffold (NifB) to the next (NifEN) during cofactor assembly (32, 84). Second, NifX would serve as storage of FeMo-co precursors, buffering the flux of precursors and redirecting them toward NifEN to complete FeMo-co synthesis (32). NifX also binds FeMo-co, but this fact possibly reflects functional overlap between NifX and NafY, which is the major FeMo-co binding protein in the cell other than NifDK (33).

Although not essential, NafY has been proposed to assist during FeMo-co insertion into apo-NifDK. Two roles for NafY have been proposed: (*a*) stabilization of P-cluster-containing apo-NifDK protein in a conformation amenable to FeMo-co insertion, and (*b*) insertion of FeMo-co into apo-NifDK (33, 69). Consistently, purified NafY protein

binds tightly either apo-NifDK or FeMo-co (70). NafY is a 26-kDa monomeric protein composed of two domains that can be separately expressed and purified. The 12-kDa N-terminal domain of NafY is required to bind apo-NifDK (J.A. Hernandez, K.W. Erbil, A. Phillips, D. Zhao, D.E. Wemmer & L.M. Rubio, unpublished results), whereas the 14-kDa C-terminal NifX-like domain is required to bind FeMo-co (20). The three-dimensional structure of the FeMo-co binding domain of NafY has been solved and folds differently than the FeMo-co binding site in NifDK (20) (**Figure 8**). Site-directed mutagenesis studies on NafY suggested the His^{121} residue as a likely ligand to FeMo-co (70). This residue is part of a His-Phe-Gly sequence conserved in NifX, NifB, and VnfX, all of which bind either NifB-co or FeMo-co.

The analysis of the roles of NifX and NafY in vivo has been obscured for two reasons: First, there is functional overlap between members of this family and it is likely that they can partially substitute for each other. Second, neither NifX nor NafY plays an essential role in FeMo-co biosynthesis under standard laboratory growth conditions that provide excess iron and molybdenum (69).

SUMMARY POINTS

1. The biosynthesis of FeMo-co is a multi-step process that involves activities of molecular scaffolds, metallocluster carrier proteins, and enzymes.
2. The availability of all proteins involved in FeMo-co biosynthesis and insertion, in purified form, is providing the first insights into the structures of the FeMo-co biosynthetic intermediates.
3. The SAM radical protein NifB transforms common iron-sulfur clusters, generated by NifUS, into NifB-co, the first isolatable iron-sulfur FeMo-co biosynthetic intermediate with complex structure.
4. The central six-iron-atom cage of FeMo-co already exists at the stage of NifB-co, which also exhibits the spectroscopic signature that has been attributed to a central atom X in FeMo-co.
5. NifB, NifEN, and NifH are sufficient to perform all essential reactions to synthesize FeMo-co in vitro from its basic components: molybdenum, iron, sulfur, and homocitrate.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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