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MINIREVIEW

Nitrogenase Metalloclusters: Structures, Organization, and Synthesis

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INTRODUCTION

Biological nitrogen fixation encompasses the portion of the global nitrogen cycle that involves the energy-dependent conversion of inert nitrogen gas to a form that can be utilized by most organisms. A minimal stoichiometry for the primary reaction, reduction of N_2 to NH_3, is usually depicted as follows:

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgATP + 16P_i$$

The catalyst for this reaction is nitrogenase, a metalloenzyme which comprises two component proteins usually referred to as Fe protein and MoFe protein. These designations originate from the metal compositions of the respective component proteins of the conventional, Mo-dependent nitrogenase. Namely, the Fe protein is a γ_2 homodimer ($M_r \approx 60{,}000$; encoded by *nifH*) which contains 4 Fe atoms organized into a single Fe_4S_4 cluster, whereas the MoFe protein is an $\alpha_2\beta_2$ heterotetramer ($M_r \approx 250{,}000$; encoded by *nifDK*) which contains 30 Fe atoms and 2 Mo atoms organized into two pairs of novel metalloclusters, called P clusters and FeMo-cofactors. Alternative nitrogenases which do not contain Mo, but which are otherwise structurally and functionally quite similar to the conventional Mo-containing nitrogenase, have also been described (for a review, see reference 2). During catalysis, electrons are delivered one at a time from the Fe protein to the MoFe protein in a gated process involving the association and dissociation of the component proteins and the hydrolysis of at least two MgATP molecules for each electron transfer. These features are schematically shown in Fig. 1. Because the Fe protein is the obligate electron donor to the MoFe protein, which in turn contains the sites for substrate reduction, the individual component proteins are sometimes designated dinitrogenase reductase and dinitrogenase, respectively (13). It has been known for many years that the nitrogenase metalloclusters play critical roles in electron transfer and substrate reduction. Thus, elucidation of the structures, organization, and biosynthesis of these metalloclusters and determination of their specific functions in electron transfer and substrate reduction have represented major challenges in nitrogen fixation research.

Recent reports on the crystallographic structures of the nitrogenase component proteins and their metalloclusters are significant advances in this area of research (3, 4, 6, 12, 28, 29, 35, 39). These structures now provide a basis for a more sophisticated evaluation of several decades' worth of biophysical, biochemical, and genetic investigations and also permit the design of new biophysical and genetic strategies aimed at determining the details of the molecular mechanism of nitrogenase catalysis. In this brief review, we summarize some of the salient features that have recently emerged concerning the structures, organization, and biosynthesis of the nitrogenase-associated metalloclusters.

ORGANIZATION OF THE NITROGENASE COMPONENT PROTEINS AND THEIR METALLOCLUSTERS

How are the nitrogenase metalloclusters organized to effect electron transfer to a substrate? How is N_2 bound at the active site? What does MgATP hydrolysis have to do with substrate reduction? Although detailed answers are not available, the new structural information, when considered together with the results of biophysical and biochemical-genetic experiments, has provided a fresh basis for consideration of these questions and some insight into possible answers. In this section and in Fig. 1, the structures and organization of the nitrogenase metalloclusters are described. The mechanistic implications of these features are described in detail in the original publications and are briefly summarized below.

A key function of the Fe protein is the integration of nucleotide binding and MgATP hydrolysis with electron transfer between its Fe_4S_4 cluster and the MoFe protein. Both cluster binding and nucleotide binding are located at the interface between the two subunits of the homodimer (12). This interface is generally open and cleft-like except in the vicinity of the cluster-binding site, which is located at one extreme end of the interface and at the surface of the molecule such that one face of the cluster is solvent exposed. Two cysteinyl residues from each subunit, Cys-97 and Cys-132 (residue numbers here and elsewhere in the text refer to the *Azotobacter vinelandii* proteins), provide thiolate ligands to the four Fe atoms of the cluster, which is thus symmetrically bound between the two subunits (12, 15, 20). Because the electrostatic environment of the cluster may contribute to its electrochemical behavior, it is important that each of the thiolate ligands is located at the N-terminal end of an α-helix and that the protein provides six potential NH→S hydrogen bonds to the cluster or its ligands (12). As will be discussed below, specific residues that can be biochemically or genetically associated with formation of the complex between the Fe protein and MoFe protein are also found relatively near the exposed face of the cluster on the same external surface of the protein.

Comparisons of the sequence (43) and polypeptide fold (12) of the Fe protein monomer to other mononucleotide-binding proteins support the conclusion that the Fe protein has one specific MgATP-binding site per subunit located on the surface

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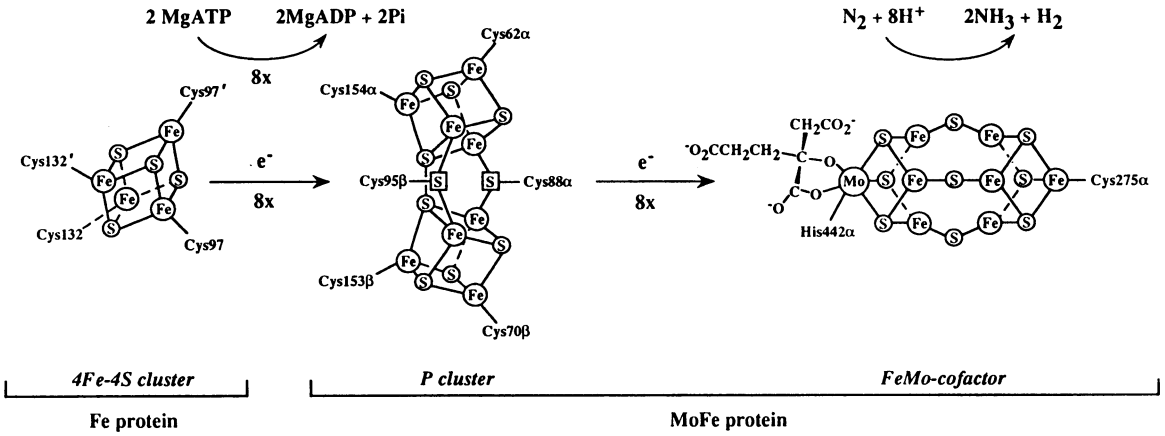

FIG. 1. Organization and structures of the nitrogenase metalloclusters. Arrows indicate the proposed electron path.

of the interfacial cleft. Remarkably, the nucleotide-binding sites are separated from the Fe_4S_4 cluster and its ligands by roughly 20 Å (2.0 nm) (12). Thus, the lowering of the redox potential of the cluster that occurs upon MgATP binding (60), as well as changes associated with nucleotide binding in a number of additional chemical and physical properties (reviewed in reference 8), cannot be a consequence of direct interactions between nucleotide and cluster. Rather, nucleotide binding must transmit a long-distance structural signal, presumably through changes in the conformation of the protein, that ultimately causes an alteration in the environment of the cluster (12, 56) and facilitates electron transfer.

The MoFe protein is roughly four times the size of the Fe protein and binds two types of metalloclusters, commonly referred to as FeMo-cofactor (FeMo-co) and the P cluster. Each cluster contains eight metal atoms, and there are two copies of each type per tetramer. Thus, each αβ dimer holds one FeMo-co paired with a P cluster located at a center-to-center distance of roughly 19 Å (4). The P cluster contains eight Fe atoms and is constructed from two subclusters that are structurally analogous to typical, cuboidal Fe_4S_4 clusters (3, 6, 28, 39). Its binding site is roughly 10 Å from the surface of the protein at a pseudosymmetric interface formed by α and β subunits belonging to the same dimer (28, 29). Six conserved cysteine residues serve as covalent ligands, and the contributions of the individual subunits to cluster binding are equivalent. Four cysteines, two from each subunit, bind as typical cysteinyl thiolate ligands to individual Fe atoms (α-Cys-62 and α-Cys-154; β-Cys-70 and β-Cys-153). In addition, two cysteines link the two subclusters by binding two Fe atoms, one from each subcluster in Fe-S (Cys)-Fe bridges (α-Cys-88 and β-Cys-95). A third link between the two subclusters has also been modeled as a cluster-to-cluster disulfide bond, as indicated in Fig. 1 (3, 6, 28, 39).

FeMo-co consists of a metal-sulfur framework and one molecule of (R)-homocitrate. The framework is constructed from bridged $MoFe_3S_3$ and Fe_4S_3 cluster fragments geometrically analogous to pieces derived from $MoFe_3S_4$ and Fe_4S_4 cuboidal clusters. Three bridging atoms, most likely all inorganic sulfides (3, 6, 28), connect the two fragments by linking pairs of Fe atoms from different fragments; the net stoichiometry is thus $MoFe_7S_9$. A remarkable feature of the cofactor structure is the presence of six trigonal Fe atoms, namely, each of the Fe atoms involved in the Fe-S-Fe bridges between subclusters is apparently bonded to only three S atoms.

The FeMo-co-binding site lies almost wholly within the α-subunit, although some β-subunit residues approach the homocitrate and are indirectly linked to it by water molecules. FeMo-co is anchored to the protein by α-Cys-275 and α-His-442 (28). The former serves as a thiolate ligand to an Fe atom at one end of the cofactor, and the latter binds through a side-chain nitrogen atom to the Mo atom at the opposite end of the cofactor. Homocitrate is coordinated to the Mo atom through its 2-hydroxy and 2-carboxyl groups and interacts with the protein through a number of direct and water-bridged hydrogen bonds (3, 28). For the clostridial enzyme, it has also been noted that several side-chain and main-chain protein groups occupy sites where they are likely to form hydrogen bonds with each of FeMo-co's bridging sulfides (3).

Intermolecular electron transfer

The scheme shown in Fig. 1 depicts a metallocluster-to-metallocluster-to-substrate flow of electrons that is consistent with the available biophysical and biochemical information. The sequence of events probably involves intermolecular delivery of electrons from the Fe protein's Fe_4S_4 cluster to a MoFe protein P cluster in an MgATP-dependent manner. The P cluster then mediates intramolecular transfer of electrons to FeMo-co, which provides the substrate-binding and reduction site. Notice that only one P cluster and one FeMo-co participate in this intramolecular electron transfer pathway. Thus, MoFe protein harbors two independent, but apparently identical, substrate-binding and reduction sites. This feature of the scheme is in accord with both the spatial organization of the metalloclusters and their biophysical properties. For example, the individual FeMo-cos are separated by 70 Å (4), and they are not magnetically coupled (59).

To facilitate transfer of an electron from the Fe protein to the MoFe protein, it is expected that the two components dock in a specific manner such that the Fe protein's Fe_4S_4 cluster is located in reasonable proximity to a MoFe protein P cluster. Modeling studies based on the crystal structures of the separate components show that this can be accomplished by pairing the twofold symmetric surface of the Fe protein that surrounds its Fe_4S_4 cluster with the exposed surface of the MoFe protein's pseudosymmetric αβ interface (21, 29). This docking model is not merely satisfying from the perspective of molecular symmetry, but it is also consistent with biochemical and genetic evidence concerning the involvement of specific residues in complex formation. For example, ADP-ribosylation of the Fe protein's Arg-100 residue, which is located near the

Fe_4S_4 cluster and on the proposed docking surface, prevents electron transfer (31, 32). Moreover, alteration of the Fe protein by substitution of certain amino acids for Arg-100 modifies the activity of the whole enzyme by causing hypersensitivity to salt and/or by uncoupling MgATP hydrolysis from electron transfer (31, 57). Similarly, substitution of an Asn residue for MoFe protein α-subunit Asp-161, which is also located on the proposed docking surface of MoFe protein, uncouples MgATP hydrolysis from productive electron transfer (27). Finally, the current docking model is also consistent with cross-linking studies (55). These results suggest that ionic interactions contribute to the functional nitrogenase complex and may indicate that these residues are involved in the coupling mechanism.

The proposed assignment of the component protein interaction sites could also have relevance to the role of MgATP hydrolysis in electron transfer. Because interaction of the component proteins is required for MgATP hydrolysis, it is likely that the docking event itself elicits hydrolysis. Moreover, it is conceivable that docking and/or hydrolysis could result in reciprocal or independent conformational changes within the component proteins that ultimately influence intermolecular electron transfer. As an example, because some of the residues within the proposed interaction site located on the MoFe protein are linked through short helices to certain of the P cluster's coordinating ligands, any change in structure of the docking site could perturb the structure of the P cluster or its environment. Thus, it is possible that docking could affect the redox chemistry of the P cluster or change its structure by destabilizing one or more of the links between the two Fe_4S_4 subclusters (6). It is also possible that docking could change the proximity of the P cluster to the Fe protein's Fe_4S_4 cluster (6, 27). Such communication at a distance between two proteins, involving nucleotide binding and hydrolysis associated with protein-protein interaction, is a well-known form of signal transduction. Indeed, the primary sequence of the MgATP-binding site (43), the three-dimensional structure of the site (12), and the mechanism of signal transduction by the Fe protein suggested by amino acid substitution studies (47, 56) all show remarkable similarity to the Ras p21 signal transduction system.

FeMo-co is the substrate reduction site

The next step in the electron path, intramolecular delivery of electrons from the P cluster to FeMo-co, is not well understood. There is, however, compelling evidence that FeMo-co is the site of substrate binding and reduction. First, FeMo-co-deficient MoFe proteins produced by certain mutant strains defective in cofactor biosynthesis are inactive but can be reconstituted to an active form by the addition of isolated FeMo-co (48). Second, MoFe protein that contains a structurally altered FeMo-co wherein the organic constituent is citrate rather than homocitrate exhibits altered catalytic properties (16, 30). Third, amino acid substitutions placed in the FeMo-co-binding site also elicit changes in the catalytic and spectroscopic properties of the altered MoFe protein (45, 46).

Although there is no direct evidence about where and how N_2 is bound to FeMo-co, the recently published structural information has provided a new basis for consideration of possible modes of substrate binding and mechanisms of reduction. For example, it has been proposed that the open central cavity of FeMo-co might provide a site for N_2 binding and catalysis of N_2 reduction. In this model, putative weak Fe-Fe interactions between Fe atoms located in the opposing $MoFe_3S_3$ and Fe_4S_3 subclusters are replaced by Fe-N bonds, which in turn promote the initial step in N_2 reduction. It has been noted, however, that the size of the cavity observed in the crystal structure is too small by about 0.5 Å to support N_2 binding in this fashion and that the deficiency in size is more severe when subsequent intermediates in the reaction or other substrates are considered (6).

It should be noted that the proton source and the avenues for substrate entry and product exit are also important aspects of the overall reaction mechanism. At present, there is no direct evidence to identify the actual proton donors involved in substrate reduction or H_2 evolution. However, amino acid residues hydrogen bonded to the homocitrate moiety (29) as well as amino acids that hydrogen bond to FeMo-co sulfur atoms and bound water molecules located in the cofactor-binding cavity (3) are obvious possibilities. If unique paths for substrate entry and product exit exist, they have not yet been identified.

MATURATION OF THE NITROGENASE COMPONENT PROTEINS

The primary translation products of the nitrogenase structural genes (*nifHDK*) are not active. Rather, a consortium of associated *nif*-specific gene products is required for processing immature nitrogenase structural components to active forms. Thus, included among the important questions attached to nitrogen fixation research is understanding how the metalloclusters required for electron transfer and substrate reduction are assembled and inserted into their respective component proteins. In the following sections, we discuss the current understanding concerning the formation and insertion of the nitrogenase metalloclusters.

Biosynthesis of FeMo-co

Experiments which demonstrated that FeMo-co is the active site for nitrogen reduction and, more recently, those which elucidated some details concerning its biosynthesis have provided impressive examples of biochemical detective work. Early studies established that FeMo-co could be extracted from acid-denatured MoFe protein and then used to reconstitute a cofactorless form of the MoFe-protein produced in certain mutant strains (48). Subsequently, a search for the genes whose products are involved in FeMo-co biosynthesis was initiated. These studies, all of which involved biochemical-genetic approaches (i.e., biochemical complementation using extracts prepared from different mutant strains defective in nitrogenase catalysis), have unambiguously identified six players that participate in the process. They include products of the *nifH*, *nifE*, *nifN*, *nifB*, *nifV*, and *nifQ* genes. The *nifH*, *nifE*, *nifN*, and *nifB* gene products are absolutely required for FeMo-co biosynthesis because, in their absence, only the apo form of MoFe protein is produced. Elimination of *nifV* gene product function results in accumulation of MoFe protein having altered substrate reduction properties. In fact, demonstration that apo-MoFe protein (i.e., that produced by a *nifB* mutant) reconstituted by FeMo-co extracted from MoFe protein produced by a *nifV* mutant also has altered substrate reduction properties was the key experiment (16) that confirmed the prediction that FeMo-co occupies the substrate reduction site (48). It is now known that *Klebsiella pneumoniae nifV* mutants produce a FeMo-co that has citrate rather than homocitrate coupled to the Mo site (30). The *nifQ* gene product was identified as having a dispensable role in the mobilization of the Mo required for FeMo-co biosynthesis because its phenotype, production of an apo-MoFe protein,

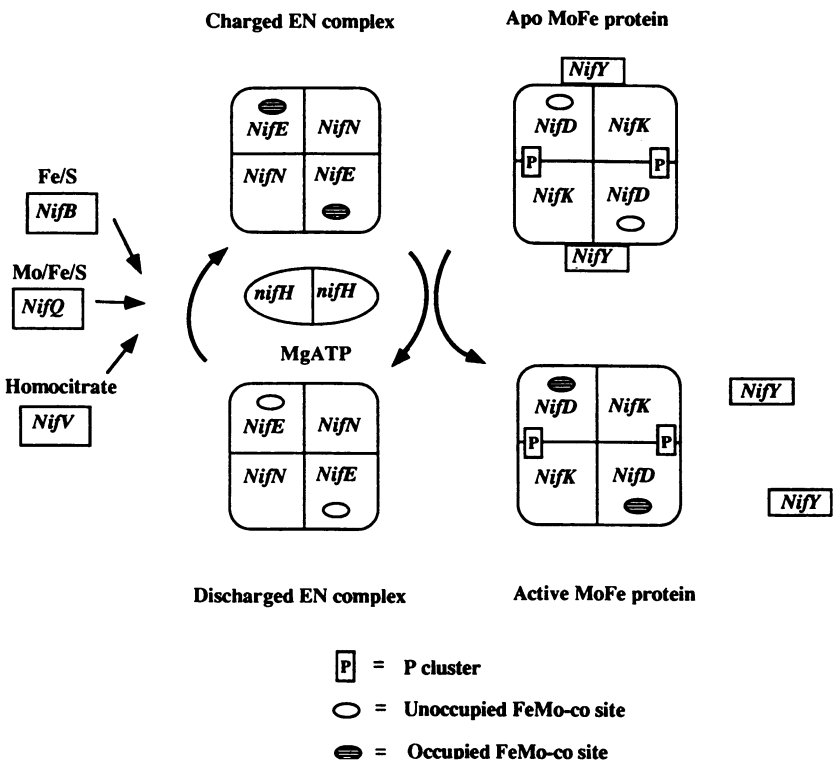

FIG. 2. Gene products involved in FeMo-co biosynthesis. The following features of FeMo-co biosynthesis and insertion are summarized: (i) the *nifB* and *nifQ* gene products are probably involved in an early stage of FeMo-co biosynthesis; (ii) the *nifV* gene product is a homocitrate synthase; (iii) the *nifEN* gene products form a heterotetrameric scaffold required for FeMo-co biosynthesis; (iv) the *nifH* gene product and MgATP are required for both FeMo-co formation and its insertion into the apo-MoFe protein; and (v) the *nifY* gene product is attached to the apo-MoFe protein, might act as a prop to assist FeMo-co insertion, and is released from the apo-MoFe protein upon maturation. Convergent arrows originating from NifB, NifQ, and NifV are for convenience in presentation and are not intended to indicate that these gene products act independently rather than sequentially. This aspect of FeMo-co biosynthesis is not known. Also, it is not known whether FeMo-co is directly released to the apo-MoFe protein from the NifEN complex or whether there is an intermediate carrier.

could be spared by increasing the Mo supplement to the growth medium (52).

Two different pathways for FeMo-co biosynthesis can be imagined. In the first path, units of the FeMo-cofactor could be sequentially assembled into apo-MoFe. In the second path, FeMo-co could be separately synthesized and then inserted into the apo-MoFe protein (Fig. 2). That the latter pathway is the correct one was demonstrated in an elegant series of experiments in which extracts of *nifB*, *nifE*, or *nifN* mutants were mixed with extracts of a *nifDK* mutant to reconstitute MoFe protein activity (51). In other words, the ability of the *nifDK* mutant extract, which contains no MoFe protein polypeptides, to reconstitute apo-MoFe protein produced in *nifB*, *nifE*, or *nifN* mutant extracts demonstrates that FeMo-co can be synthesized in the absence of MoFe protein.

Because FeMo-co is not synthesized directly into the apo-MoFe protein, a reasonable alternative hypothesis was that some other *nif*-specific gene product(s) provides a scaffold for FeMo-co synthesis. It was expected that if such a scaffold exists it would bear some structural similarity, and perhaps some primary sequence identity, to the FeMo-co-binding site located within the MoFe protein. At about the time these questions emerged, serious efforts were underway to determine the nucleotide sequences for all of the *nif*-specific genes from both *A. vinelandii* and *K. pneumoniae* (see references 1 and 24 and references therein). Thus, the above prediction was easily tested by comparing the primary sequences of the *nifD* and *nifK* gene products to the primary sequences of the gene products required for FeMo-co biosynthesis. These comparisons, in fact, revealed striking primary sequence identity when the *nifE* and *nifN* gene products were compared to the *nifD* and *nifK* gene products, respectively (1, 5). Not only did such primary sequence identities give credence to the scaffold hypothesis (i.e., the *nifEN* gene products form a complex upon which FeMo-co is preassembled), but they also permitted the correct prediction of some, but not all, the FeMo-co-binding domains located within the MoFe protein (5). Moreover, the identification of such domains provided, prior to the determination of the crystal structures, a rational strategy for development of amino acid substitution studies aimed at determining the contribution of the FeMo-co polypeptide environment to catalysis (46).

The fact that MoFe protein is not required for FeMo-co formation also permitted the development of an in vitro assay for FeMo-co biosynthesis (49). In this assay, extracts prepared from mutant strains suspected to have complementary defects in FeMo-co biosynthesis are mixed in order to attempt reconstitution of MoFe protein activity. The importance of this system is that it permits a functional assay that can be used in the attempted purification of *nif* gene products which participate in FeMo-co biosynthesis. This approach has led to a number of insights concerning FeMo-co biosynthesis. First, application of the in vitro approach permitted purification of the *nifEN* products complex, which was shown to be an $\alpha_2\beta_2$

heterotetramer (37). Second, it led to the development of a scheme for the purification of the apo-MoFe protein (38). Third, the in vitro system was used to show that ATP (49) and Fe protein (42) were both required for FeMo-co biosynthesis. Finally, the *nifV* gene product was shown to be required for formation of homocitrate, the organic constituent of FeMo-co (19). Concerning this latter aspect, comparison of the primary sequences of various *nifV* gene products to other enzymes that utilize acetyl-CoA indicates that *nifV* is likely to encode a homocitrate synthase which catalyzes the condensation of α-ketoglutarate and acetyl-CoA (33, 53).

The involvement of the Fe protein in FeMo-co biosynthesis, revealed by the in vitro biosynthetic system, was also shown by reconstitution assays using *nifH* mutants (10, 42, 44). Initially, such involvement of Fe protein and ATP in FeMo-co biosynthesis seemed logical; because FeMo-co is probably preassembled on a *nifEN* products complex, which is structurally similar to the MoFe protein, it followed that a step in FeMo-co assembly could involve docking of the Fe protein and the *nifEN* complex in an event coupled to MgATP hydrolysis and electron transfer (42). However, there are a number of experimental observations which argue against this model, and the roles of ATP and Fe protein in FeMo-co biosynthesis remain enigmatic. First, active MoFe protein accumulates in *nifM* mutants (25, 42). The *nifM* gene product is required for processing an immature form of the Fe protein to an active form (22, 25, 36, 40; also, see below). Second, different mutant strains which produce Fe proteins defective in electron transfer or in MgATP binding or hydrolysis all accumulate normal MoFe protein (11, 56). In fact, the only mutant strains defective in Fe protein that are known to also have altered FeMo-co assembly are those in which the entire gene is inactivated (10, 42) or in which one of the Fe_4S_4 cluster ligands has been removed (20).

Formation of the Fe-S-Mo core of FeMo-co is not yet understood. However, it seems likely that both the *nifQ* and *nifB* gene products are involved in an early stage of metal core formation (Fig. 2). The fact that both cysteine, the likely sulfur source for Fe-S cluster biosynthesis (see below), and Mo (52) can suppress the *nifQ* phenotype indicates that the *nifQ* gene product could assist in forming the $MoFe_3S_3$ unit of FeMo-co. The *nifB* gene product could have a complementary role in formation of the Fe_4S_3 unit of FeMo-co. Whether or not individual units of FeMo-co are first preassembled on the *nifB* and *nifQ* gene products and then transferred to the *nifEN* products complex is not yet known. Nevertheless, the potential for formation of FeMo-co precursors on the *nifB* or *nifQ* gene products is supported by the arrangement of potential metallocluster coordinating ligands deduced from their corresponding primary sequences (see discussion in reference 8). It is also probable that the *nifB* gene product contacts the *nifEN* product complex during FeMo-co biosynthesis because, in *Clostridium pasteurianum*, the *nifN* and *nifB* homologs are fused (7). Another presently unknown aspect of FeMo-co biosynthesis concerns the stage at which homocitrate becomes coordinated to the Mo. This question and the specific roles of the *nifQ* and *nifB* gene products should emerge from further application of the in vitro biosynthetic approach.

Insertion of FeMo-co into the apo-MoFe protein

The fact that FeMo-co is separately synthesized and then inserted into the apo-MoFe protein has led to investigations of the nature of this process. A simple and particularly powerful technique that has been applied to this question is native, anaerobic gel electrophoresis. A number of independent studies involving the use of crude extracts, semipurified apo-MoFe protein, and purified apo-MoFe protein have shown that the apo-MoFe protein has a native electrophoretic mobility different from that of the mature MoFe protein (12a, 18, 26, 44, 54). Consequently, it is possible to observe the insertion process by simple gel electrophoresis. These studies have shown that, in addition to their roles in catalysis and FeMo-co formation, ATP and Fe protein are also required for FeMo-co insertion (41, 44). Again, the specific nature of their participation in FeMo-co insertion is not known. Another feature that has emerged from the electrophoretic studies is that the presence of P clusters appears to be a prerequisite for FeMo-co insertion. This conclusion was based on the observation that only apo-MoFe which contained Fe (P clusters) was receptive to FeMo-co, and incorporation of such Fe required the activities of genes postulated to be involved in P cluster assembly (54). This possibility is also substantiated by amino acid substitution studies which have shown that certain altered MoFe proteins having disrupted P cluster environments do not appear to have any associated FeMo-co (9, 26).

The gel electrophoretic technique and the ability to purify the apo-MoFe protein has uncovered a remarkable feature concerning the function of the *nifY* gene product. These studies have shown that apo-MoFe protein produced by certain *K. pneumoniae* mutants has *nifY* gene products, probably two, attached to it (17, 18, 54). Upon reconstitution with FeMo-co, the *nifY* gene products dissociate from the MoFe protein. These results suggest that the *nifY* gene product might act as a "molecular prop" which stabilizes a conformation of the apo-MoFe protein amenable to FeMo-insertion (18, 54). This possibility is supported by the observation that apo-MoFe protein produced in the absence of *nifY* gene product is less stable than apo-MoFe protein produced in the presence of *nifY* gene product (18).

Mobilization of iron and sulfur for Fe-S cluster formation

Our understanding of the formation of MoFe protein P clusters and formation of the Fe protein Fe_4S_4 cluster is considerably less sophisticated than our understanding of FeMo-co assembly. Part of the reason for this deficiency is that much of the current information is apparently contradictory. For example, studies involving *K. pneumoniae* indicate an obligate role for Fe protein in P cluster formation (54), whereas analysis of deletion mutants from *A. vinelandii* clearly show that Fe protein is not required for P cluster formation (42). These differences, which are likely more apparent than real, are probably grounded in the different physiologies of the organisms used and the different approaches used by different investigators. Certain studies of *K. pneumoniae* have involved an additive approach in which the minimum combination of *nif* gene products required to form a FeMo-co reactivatible MoFe protein was assessed by their heterologous, plasmid-directed expression in *Escherichia coli* (14, 54). In contrast, studies of *A. vinelandii* have involved a subtractive approach in which individual *nif*-specific genes were deleted from the chromosome, both singly and in various combinations, and the effects of these deletions on the nitrogenase component protein activities were evaluated (25). Another complication that has recently emerged is that certain *nif*-specific genes are likely to have functional homologs encoded in non-*nif*-specific regions of the genome (58).

In spite of these differences, a common theme that is beginning to emerge is that the *nifU* and *nifS* gene products are likely to function in the acquisition of the inorganic Fe and sulfide required for nitrogenase-specific Fe-S cluster formation

(25, 34, 58). A clear role for the *nifS* gene product in the mobilization of sulfur for Fe-S cluster formation has been established by its recent purification and characterization (58). These studies have shown that the *nifS* gene product is a pyridoxal-phosphate-containing homodimer which catalyzes the specific desulfurization of L-cysteine. Thus, it has been hypothesized that the *nifS* gene product activates sulfur by formation of an enzyme-bound persulfide which serves as the S donor in nitrogenase Fe-S cluster formation. Genes whose products have extraordinary primary sequence identity when compared to the *nifS* gene product have recently been identified in non-nitrogen-fixing organisms (50). Thus, it appears that the function of the *nifS* gene product in the mobilization of sulfur for nitrogenase metallocluster assembly could represent a universal path for Fe-S cluster formation. In this regard, the fact that non-nitrogen fixation-specific gene products might partially fulfill the functions of certain *nif*-specific gene products points to the difficulty in unequivocally assessing functional roles solely on the basis of biochemical-genetic studies. This difficulty is probably more evident in the biosynthesis of the nitrogenase Fe-S clusters than in FeMo-co biosynthesis because all organisms must manufacture Fe-S clusters whereas FeMo-co is unique to nitrogen-fixing organisms.

If the *nifS* gene product is involved in the sulfur side of nitrogenase Fe-S cluster formation, how is Fe mobilized? Although no direct answer is available, there are some observations which bear on this question. We have recently purified the *nifU* gene product (23) and found that it is a homodimer which probably contains one redox-active Fe_2S_2 cluster per subunit. In addition, primary sequence comparisons have shown that there are eight conserved cysteinyl residues in all available *nifU* primary sequences (summarized in reference 8). Thus, in addition to the probable four cysteinyls required to coordinate each Fe_2S_2 cluster, there are four other cysteinyls per subunit which could be involved in sequestering Fe for Fe-S cluster assembly. On the basis of this information, different paths for the participation of the *nifU* gene product in mobilization of Fe can be considered. In one path, the Fe_2S_2 clusters located within the *nifU* gene product could be directly donated to immature forms of the Fe protein or MoFe protein during their respective Fe-S cluster assemblies. An alternative possibility is that the Fe_2S_2 clusters within the *nifU* gene product have redox roles which function to keep Fe bound at other sites, presumably also within the *nifU* gene product, in the proper oxidation state and therefore available for Fe-S cluster assembly. Yet another possibility is that the *nifU* gene product could have a redox role which involves the release of sulfide from the *nifS* gene product persulfide. Determination of whether any of these possibilities is correct will require the development of an in vitro system for nitrogenase Fe-S cluster formation like that described above for FeMo-co biosynthesis.

Maturation of the Fe protein

A variety of biochemical-genetic studies and heterologous gene expression experiments have clearly demonstrated that the *nifM* gene product is required for the activation and stability of the Fe protein (22, 25, 36, 40). Although the true function of the *nifM* gene product is not known, several hypotheses have been extended. These include insertion of the Fe-S cluster (22), conformational isomerization of the Fe-S cluster in a postinsertional event (20), and promotion of the proper assembly of the two subunits of the homodimeric protein such that the Fe_4S_4 cluster ligands are appropriately positioned to receive the Fe_4 S_4 cluster (12). Of course, any combination of these mechanisms also seems plausible. However, the possibility that *nifM* could function in promoting the proper conformation to accept the Fe_4 S_4 cluster now seems particularly attractive because there is precedence in the example of *nifY*, which probably acts as a molecular prop in assisting FeMo-co insertion. As in the cases of the *nifS* and *nifU* gene products, formal proof of the true function of the *nifM* gene product awaits its purification and demonstrated activity in an in vitro reconstitution system.

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