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Molybdenum Trafficking for Nitrogen Fixation†

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Abstract

The molybdenum nitrogenase is responsible for most biological nitrogen fixation, a prokaryotic metabolic process that determines the global biogeochemical cycles of nitrogen and carbon. Here we describe the trafficking of molybdenum for nitrogen fixation in the model diazotrophic bacterium *Azotobacter vinelandii*. The genes and proteins involved in molybdenum uptake, homeostasis, storage, regulation, and nitrogenase cofactor biosynthesis are reviewed. Molybdenum biochemistry in *A. vinelandii* reveals unexpected mechanisms and a new role for iron-sulfur clusters in the sequestration and delivery of molybdenum.

The scarcity of molybdenum in the Earth's crust belies its importance for the metabolism of living organisms and for the global biogeochemical cycles of major elements such as nitrogen, sulfur and carbon (1,2). In contrast, many elements that are present in considerably larger amounts have not apparent biological function (e. g. Al, Ti or Zr) (3). Despite its low abundance - Mo ranks 53rd in the Earth's crust - molybdenum is more available to biological processes than many other metals, which are found in chemical forms difficult to assimilate. Molybdate is the predominant source of Mo^{VI} at neutral and basic pH. Other common sources of molybdenum are the highly insoluble molybdenite (MoS_2) and wulfenite ($PbMo_4$) ores (4). In marine environments, molybdenum is present at around 110 nM, being the most abundant transition metal in the sea (1). In terrestrial environments, molybdenum distribution is irregular, usually lower than in the marine systems, and estimated to be 50 nM on average. The importance of molybdenum in soil ecosystems has recently been highlighted by a study showing that molybdenum scarcity severely limits biological nitrogen fixation in tropical forests (5). Limitation of nitrogen fixation by molybdenum might be common in highly weathered acidic soils, which would hinder the ability of some forests to balance carbon and nitrogen (6).

Comparative genomic studies reveal that molybdenum metabolism is widespread in nature (7). Molybdenum is utilized - to different extents - by most organisms belonging to the three domains of life: archea, bacteria, and eukaryotes. Phylogenetic analyses reveal that most prokaryotes (archea and bacteria) and higher eukaryotes utilize molybdenum, whereas many unicellular eukaryotes, including parasites and some yeast, have lost their ability to use this metal.

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In addition to being the only second row transition metal essential for life, molybdenum provides an extremely versatile building tool for the coordination chemist. The element has a [Kr] $4d^5$ $6s^1$ electron configuration, and its chemical properties thus center on its half-filled 4d shell. Molybdenum is able to adopt oxidation states from (-II) to (+VI) together with ligand coordination numbers ranging from 4 to 8 and a variety of coordination geometries. It forms compounds with most inorganic and organic ligands, and a wide range of metal and mixed-metal clusters have been synthesized. The chemistry of the higher molybdenum oxidation states (Mo^{VI}, Mo^{V}, Mo^{IV}, Mo^{III}) is dominated by oxo species, such as molybdates and polymolybdates, and terminal oxygen containing species like $Mo^{VI}O_2$, $Mo^{V}O$, $Mo^{IV}O$ and $Mo^{IV}O_2$ comprise the central cations in a range of complexes (8). Molybdenum also has a rich and diverse sulfur chemistry, which comprises ligand-based redox behavior, internal electron-transfer processes and 'intermediate' redox states (9), all largely a consequence of the small energy gap between the sulfur 3p and the Mo 4d orbitals (10). The importance of molybdosulfur complexes in biological and industrial catalysis has led to the study of an array of monomeric (11), dimeric (12) and cluster complexes (13,14).

Biologically active molybdenum is found in the cofactors of molybdoenzymes. Much of our understanding of molybdenum sites in enzymes is derived from combining the results of EPR[1] (15-17) and ENDOR (18,19) spectroscopies on paramagnetic enzyme intermediates, with structural information from extended X-ray absorption fine structure (EXAFS) spectroscopy (16,20-24) and X-ray crystallography (25-28). In addition, the chemical behavior of related inorganic compounds has been used to calibrate the spectroscopies and to suggest or confirm structural and mechanistic possibilities for the enzymes (11,14). These studies have shown that all molybdenum cofactors, with the exception of the FeMo-co of nitrogenase, are based on a unique tricyclic pterin (molybdopterin) and are generically termed as Mo-co (16,24,29). Mo-co containing enzymes catalyze a range of oxidation/reduction reactions in carbon, sulfur and nitrogen metabolism, such as the oxidation of hypoxanthine to xanthine, the reduction of nitrate to nitrite and the oxidation of formate to carbon dioxide (16,24).

Biological Nitrogen Fixation

Fixed nitrogen is an essential component of amino acids, proteins, and nucleic acids in all organisms. It is also present in other essential molecules that are abundant in the biosphere, such as chlorophyll and heme groups. Although nitrogen gas (N_2) constitutes 78% by volume of Earth's atmosphere, it is unusable by most organisms, which can only assimilate fixed nitrogen molecules. The low reactivity of N_2 limits its conversion into fixed nitrogen molecules. Nevertheless, a special group of prokaryotic organisms has developed the ability to fix N_2 at moderate temperature and pressure conditions in a process known as biological nitrogen fixation. Nitrogen-fixing organisms (diazotrophs) reduce N_2 into NH_4^+ that is subsequently assimilated by themselves and by other organisms, such as plants, fungi, and animals. Thus, the global balance of nitrogen on Earth's biosphere relies on the capacity of diazotrophic organisms to serve as primary input of fixed nitrogen into the ecosystems (30).

The enzyme responsible for all biological nitrogen fixation activity is termed nitrogenase. The catalytic site of nitrogenase contains a complex metallocluster where N_2 binding and reduction into NH_4^+ takes place. There are four classes of nitrogenase enzymes characterized so far. Three of them are homologous enzymes with similar - not identical - protein subunit composition and metal cofactor structure (31,32); these are the Mo-

[1]FeMo-co, iron-molybdenum cofactor; FeV-co, iron-vanadium cofactor; FeFe-co, iron-only cofactor; Mo-co, molybdenum cofactor; NifB-co, NifB-cofactor; VK-cluster, Vinod K. Shah cluster; *nif*, genes encoding proteins involved in nitrogen fixation; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine spectroscopy; ENDOR, electron nuclear double resonance.

nitrogenase, V-nitrogenase, and Fe-only nitrogenase. The Mo-nitrogenase, which contains the iron-molybdenum cofactor or FeMo-co, is the most commonly distributed nitrogenase; it is also the most efficient in the conversion of N_2 into NH_4^+. Although most diazotrophs only have the Mo-nitrogenase, some of them also synthesize alternative V-nitrogenase and/or Fe-only nitrogenase enzymes, which contain the FeMo-co-like FeV-co or FeFe-co metalloclusters at their active sites, respectively. There are no reported diazotrophs lacking a Mo-nitrogenase and carrying uniquely an alternative nitrogenase. Regulation of nitrogenase expression in bacteria carrying alternative nitrogenases is dependent on the availability of molybdenum, vanadium or iron in the medium (33). These three nitrogenases consist of two component proteins that, in the case of the Mo-nitrogenase, are denoted as NifDK[2] and NifH. The NifDK component accommodates the active site cofactor whereas the NifH component serves as specific electron donor to NifDK.

The FeMo-co active site of the Mo-nitrogenase is a complex Mo-Fe-S metallocluster comprising an inorganic Fe_6-S_9 core that coordinates a central light atom X (C, N or O) and is capped by external Fe and Mo atoms. A molecule of *R*-homocitrate coordinates the molybdenum atom through its C-2 carboxyl and hydroxyl groups to complete the cofactor (Figure 1) (27,28,34,35). The role of the molybdenum sub-site in FeMo-co is unclear (31,36). For some time, molybdenum was considered a likely locus for substrate binding and catalysis as the Mo-nitrogenase is substantially more active in N_2 reduction than its V or Fe analogs, and the *R*-homocitrate ligand is essential for catalysis (31,32). In addition, molybdenum compounds can readily coordinate N_2 and its reduced forms, and this chemistry has been systemized into a putative mechanism termed the “Chatt cycle” (37). However, recent combined spectroscopic and substrate/inhibitor binding studies strongly indicate that the [Fe_6-S_9-X] core is the initial substrate binding site (31,32,36) whereas current mechanistic models do not include a direct function for the molybdenum sub-site (31).

The fourth type of nitrogenase is a Mo-nitrogenase phylogenetically unrelated to the other three classes that has only been found in the bacterium *Streptomyces thermoautotrophicus*. This Mo-nitrogenase exhibits completely different biochemical features that consist of different protein composition, insensitivity to O_2, low Mg·ATP requirement, and a Mo-co type of cofactor at the active site (Mo-molybdopterin cytosine dinucleotide or Mo-MCD) (38).

The bacterium *Azotobacter vinelandii* has traditionally been one of the preferred models to study molybdenum metabolism in prokaryots because it carries several Mo-co-containing enzymes and a Mo-nitrogenase. The genetic components involved in molybdenum capture, trafficking, storage, metabolism, and regulation that are encoded in this organism have been studied for decades (Figure 2). Although studies in other diazotrophs such as *Klebsiella pneumoniae* and *Rhodobacter capsulatus* have provided significant insights into the metabolism of molybdenum, *A. vinelandii* will be used as the reference organism for discussion in this review. The recent release of the *A. vinelandii* genome sequence (39) has revealed the presence of new genes related to molybdenum, for example those encoding an anaerobic Mo-co-dependent formate dehydrogenase. This review will focus on those proteins and processes related - albeit not necessarily in an exclusive way - to molybdenum-dependent nitrogen fixation.

[2]The NifDK component of nitrogenase is also referred to as dinitrogenase, component I, or MoFe protein. The NifH component is also referred to as dinitrogenase reductase, component II, or Fe protein.

Molybdenum Trafficking for Nitrogen Fixation: The Path to the Nitrogenase Active Site

(i) Binding of extracellular molybdenum

The average molybdenum concentration in soils (ca. 15 μmol/kg or 1-2 ppm) is the lowest among all transition metals having a biological role (40). Molybdate (MoO_4^{2-}, Mo^{VI}) is the main source of molybdenum because it is soluble at neutral and basic pH. Other forms of molybdenum described to efficiently serve as assimilable molybdenum sources are MoO_3, Mo-cysteine dimer, Mo-glutathione dimer, $MoCl_6$, $MoCl_4$ dipyridine, and MoS_2 (41-43). In contrast to iron, which is poorly available in oxic soils due to precipitation of iron oxides and iron hydroxides, the oxoanionic molybdate is highly soluble. Its negative electric charge prevents adsorption onto particle surfaces at neutral and basic pH resulting in the formation of weak complexes with most organic ligands with the exception of catechols (44). The catechol groups of organic matter are able to bind molybdate over a wide pH range retaining molybdenum in the top layer of the soil (45).

Many bacteria are known to produce and secrete siderophores. Siderophores are small, high-affinity Fe^{III}-chelating compounds secreted to the extracellular medium by organisms subjected to low iron availability (46). Besides chelating Fe^{III}, siderophores are able to chelate molybdate, vanadate, and tungstate. The genome of *A. vinelandii* encodes the genes for the production of five different siderophores: the mono-chatecols aminochelin and 2, 3-dihydroxybenzoic acid (DHBA), the bis-chatecol azotochelin, the tris-chatecol protochelin, and the pyoverdin-like azotobactin (47). Genes involved in the production and excretion of catechol siderophores, including *csbC* (*Avin21220*) (48), and *csbX* (*Avin21230*) (49) had been previously reported. The siderophore DHBA is the one most abundantly excreted by *A. vinelandii*, but has low affinity for metals and its participation in sequestering molybdenum is limited (50). Protochelin, also abundantly excreted, dominates the speciation of iron, molybdate, vanadate and tungstate present in the medium, whereas aminochelin and azotobactin become dominantly excreted at extremely low iron concentrations (48,51). Catechol siderophores play a crucial role in trace metal nutrition by binding oxoanions of molybdenum, vanadium or tungsten present either in solution, or attached to naturally occurring ligands, or bound to organic matter (47,52). In addition, catechol siderophores have been proposed to be involved in detoxification of those metals that are deleterious to cell metabolism (53).

(ii) Molybdate transport inside the cell

Bacteria scavenge molybdate from the environment by using high affinity ABC-type transport systems (54-58). These specialized Mo-uptake systems are required by bacteria to efficiently discriminate between molybdate and tungstate (53). Average soil tungstate concentrations are around 1 ppm, in the range of molybdate concentrations. Tungstate has similar structural properties to molybdate, but its uptake and incorporation into active-site cofactors yield inactive W-nitrogenase (59) and W-nitrate reductase (60) enzymes.

In addition to the high-affinity transporter specific for molybdate, the sulfate transporter has been suggested to serve as low affinity molybdate transport system, although this role has not been extensively characterized (33).

The high affinity ABC transporter is encoded by the *modA*, *modB* and *modC* genes present in the *mod* operon. The *modA* gene encodes a high-affinity molybdate-binding protein located in the periplasm; molybdate reaches the periplasm, presumably through porins, and then binds to ModA. The *modB* gene encodes an integral cytoplasmic membrane protein that provides the transport channel. The *modC* gene encodes the so-called conserved component,

a membrane associated protein that binds and hydrolyzes Mg·ATP to provide energy for the active transport of molybdate. Unlike most microorganisms, *A. vinelandii* contains three copies of the *modABC* operon, revealing the importance and complexity of molybdenum metabolism in this microorganism. Even the closely related nitrogen-fixing strain *Pseudomonas stutzeri* contains a single copy of the *modABC* operon (61). The products of the *A. vinelandii* $modA_1B_1C_1$ operon (*Avin50670 to Avin50650*) have been shown to be required for growth under conditions of molybdate limitation (58). The *A. vinelandii* genome sequence showed that the $modA_1B_1C_1$ operon is located near the so-called minor *nif* cluster, which contains the *nifB* and *nifQ* genes among others. The second known *mod* operon, $modA_2B_2C_2$ (*Avin01300* to *Avin01280*), is located near the major *nif* operon. The genome sequence also revealed the existence of a third *mod* copy, $modA_3B_{3a}B_{3b}C_3$ (*Avin50730 to Avin50700*) located next to the $modA_1B_1C_1$ operon (39).

In addition to the use of soluble molybdate ions, *A. vinelandii* cells are able to use up molybdate-siderophore complexes as molybdenum source (44). The mechanism of molybdate-siderophore utilization is not clear. Yet, on the basis of the known mechanism of iron transport systems, it has been suggested that siderophores transfer molybdate to the ModA protein located in the periplasm (47). Similarly, *A. vinelandii* cells are able to take up vanadate-siderophore complexes. On the other hand, the tungstate-siderophore complexes, which are deleterious to bacterial growth, are either poorly taken up (53) or not taken up at all (47).

(iii) Molybdenum cellular homeostasis

The *A. vinelandii* ModG protein has been proposed to be responsible for the homeostasis of molybdenum in the cytoplasm (58). The *modG* mutants exhibit pleiotropic effects in nitrate reductase and nitrogenase activities that suggest a role for ModG in balancing molybdenum availability for the biosyntheses of Mo-co and FeMo-co (58). The *A. vinelandii* ModG protein is encoded by the *Avin50690* gene, which is clustered with and divergently transcribed from $modEA_1B_1C_1$. Each ModG monomer consists of a tandem repeat of two 65-amino-acid-long Mop domains, a structural fold that specifically binds molybdate and discriminates against other oxoanions. In its native conformation, ModG is a trimer that binds up to eight molecules of molybdate (62).

Mop domains can be found as stand-alone structures or in combination with other types of domains as part of larger proteins, such as in ModA and ModE (see below). The Mop domains are widespread in bacteria and have been thoroughly studied in *A. vinelandii*, *Clostridium pasteurianum*, *Escherichia coli* and *R. capsulatus* (63,64).

(iv) Molybdenum storage

The capacity of *A. vinelandii* to scavenge molybdate from the medium is remarkable. This bacterium can accumulate 25 times more molybdenum than it requires for maximum nitrogenase activity (42). The molybdenum storage protein (MoSto) is responsible for these high levels of molybdenum accumulation (65) and, to our knowledge, it has only been described in *A. vinelandii*.

The MoSto protein is an $\alpha_3\beta_3$ hexamer of the *mosA* (*Avin43200*) and *mosB* (*Avin43210*) gene products that can store up to 100 molybdenum atoms per hexamer (66). MoSto can also incorporate tungsten. Metal storage within MoSto occurs in the form of a variety of compact polynuclear oxoanions (67). The incorporation of molybdate into MoSto is a nucleotide-dependent process (68). The release of molybdate from MoSto is, however, ATP-independent and appears to be pH-dependent and to occur stepwise, suggesting the involvement of several amino acid groups in the release mechanism (66,69). No additional

proteins seem to be required to load or unload molybdate from MoSto. Although MoSto expression is not controlled by *nif* regulatory factors (65), the level of molybdenum incorporation into MoSto increases in nitrogen fixing cells, consistent with a proposed role as a molybdenum reservoir destined to FeMo-co synthesis. Nitrogen fixation is a process requiring considerable amounts of molybdenum given that NifDK may represent up to 10% of the total protein content during diazotrophic growth.

(v) Molybdate-dependent gene regulation

The function and structure of the molybdate-responsive transcriptional regulator ModE has been well characterized in *E. coli*. ModE binds molybdate and represses the expression of *modABC* genes under molybdenum-replete growth conditions (70-72). In addition, ModE has been shown to coordinate expression of a number of molybdenum-dependent genes by repressing or activating their expression. The molybdate transporter genes, Mo-co biosynthesis genes (73), and several molybdoenzyme structural genes, such us the periplasmic and respiratory nitrate reductases (74,75) or the dimethylsulfoxide reductase (76), are known to be regulated by ModE.

Each subunit of a ModE dimer consists of two Mop domains and a helix-turn-helix (HTH) DNA-binding domain (77,78). Efficient DNA binding requires protein dimerization and molybdate binding; both functions are mediated by the Mop domains of ModE (78). Binding of molybdate to ModE drives extensive conformational changes in both the Mop domain and the DNA-binding domains that largely increase its affinity for specific operator sequences (72,77,79,80).

ModE has been identified in a number of bacteria, including *A. vinelandii* (54). Moreover, ModE binding sequences have been found widespread in bacteria and archea (81). The *A. vinelandii* ModE is encoded by the *Avin50680* gene located within the $modEA_1B_1C_1$ operon. The *modE* mutant exhibits normal diazotrophic growth rates. However, *A. vinelandii* strains carrying mutations in both *modE* and *modG* genes are severely impaired in diazotrophic growth (58), indicating their involvement in nitrogenase biogenesis or regulation. The *A. vinelandii* genome carries an additional *modE* copy (*Avin33430*) adjacent to a V-nitrogenase transcriptional regulator *vnfA2* (*Avin33440*). The role of this ModE homolog has not yet been characterized. The presence of putative ModE-binding sites upstream the $vnfA_2$ and *anfA* genes, encoding regulatory proteins for the V-nitrogenase and the Fe-only-nitrogenase respectively, suggests that ModE coordinates the expression of all three nitrogenases in *A. vinelandii* in response to molybdenum availability (33).

(vi) FeMo-co biosynthesis

The biosynthesis of the complex FeMo-cofactor involves the activities of a large battery of *nif* and non-*nif* gene products, including proteins that exhibit catalytic activity, proteins that act as molecular scaffolds, and proteins which role is to carry and protect FeMo-co intermediates between the sequential assembly sites (82). The genes encoding proteins involved in FeMo-co biosynthesis in *A. vinelandii* are located within two chromosomal regions equidistant from the origin of replication denoted as the major *nif* region (*Avin01360* to *Avin01710*) and the minor *nif* region (*Avin50990* to *Avin51060*) (39,83-85).

The model for the FeMo-co biosynthetic pathway depicted in Figure 3 illustrates the convergence of the FeMo-co building blocks towards a central catalytic node consisting of NifEN and NifH. The NifU/NifS/NifB/NifX branch of the pathway provides NifB-co, a FeMo-co biosynthetic intermediate proposed to comprise the [Fe_6-S_9-X] core of the cofactor (86). At this stage, molybdenum has not yet been incorporated into the precursor (87). The specific role of the cysteine desulfurase NifS and the scaffold protein NifU is to provide

[Fe_2-S_2] and/or [Fe_4-S_4] clusters that would serve as metabolic substrates for NifB to synthesize NifB-co (88). The synthesis of NifB-co by NifB is a redox and *S*-adenosyl methionine-dependent process that involves radical chemistry (89). It has been proposed that very low-potential radical chemistry carried out by NifB is responsible for the incorporation of the X atom into NifB-co (89). Although not essential, the carrier protein NifX has been shown to optimize the transfer of NifB-co from NifB to NifEN (90). NifEN transforms NifB-co into the VK-cluster, which is proposed to be the next intermediate in the biosynthetic pathway (90). Similar to NifB-co, the VK-cluster does not contain molybdenum or homocitrate. However, these two intermediates are electronically and structurally different: while NifB-co is EPR silent (91), the VK-cluster exhibits EPR signals in the dithionite-reduced and the thionine-oxidized states (90). EXAFS and NRVS analyses indicate that NifB-co is an [Fe_6-S_9-X] cluster (86) whereas VK-cluster structures containing this core plus one or two additional terminal Fe atoms are favored (92).

In the second branch, NifQ specifically donates molybdenum to the NifEN/NifH proteins (93). The molybdenum of NifQ is present in an [Fe-S] cluster environment (see below). It is currently unknown whether NifQ transfers its entire [$MoFe_3S_4$] cluster, a portion of the cluster or simply the Mo atom. The third branch of the pathway provides homocitrate synthesized by the condensation of acetyl coenzyme A and α-ketoglutarate, a reaction catalyzed by NifV (94).

The putative NifEN/NifH complex would integrate the building components provided by the three biosynthetic branches to complete the synthesis of FeMo-co in a redox-dependent reaction or series of reactions that also require Mg·ATP. The newly synthesized FeMo-cofactor is transferred to the cofactor deficient apo-NifDK protein to generate catalytically active NifDK. The transfer process is mediated by the nitrogenase accessory factor NafY, a non-*nif* metallochaperone that is thought to protect FeMo-co from degradation and to stabilize the FeMo-co deficient apo-NifDK (95,96).

(vii) Molybdenum in NifQ

The *nifQ* gene product is an iron-sulfur protein known by genetic evidence to be involved in the incorporation of molybdenum into nitrogenase. NifQ is present in *A. vinelandii* cells grown in the presence of molybdenum and the absence of ammonium. At concentrations of molybdate in the medium around the nM range, *nifQ* mutants of *A. vinelandii* are impaired in molybdenum-dependent nitrogen fixation (97). It is significant that *nifQ* mutants are not defective in the activities of other molybdoenzymes (98) or the alternative V- or Fe-only-nitrogenases (85). Although *nifQ* mutant strains are not defective in molybdate uptake, it has been observed that they accumulate lower levels of molybdenum than the wild-type strain. The *nifQ* phenotype is suppressed by increasing the molybdate concentrations to μM levels or by adding excess cysteine to the medium, suggesting that the reaction catalyzed by NifQ might also occur non-enzymatically when the levels of the reactants are high. Similar *nifQ* phenotype and suppression profiles have been observed in *K. pneumoniae* (99,100).

All nitrogen-fixing Proteobacteria –except some species of Rhizobia- contain NifQ homologues. Amino acid sequence alignments of NifQ proteins show a conserved $Cx_4Cx_2Cx_5C$ motif at the C-terminus of the protein that could be capable of coordinating the [Fe-S] cluster of NifQ. The native molecular weight of *A. vinelandii* NifQ (25.7 kDa) is similar to the weight deduced from the *nifQ* sequence (19.7 kDa), indicating a monomeric structure and precluding the possibility of having one [Fe-S] cluster coordinated by two NifQ subunits (93).

Recent biochemical and EPR spectroscopic evidence has shown that NifQ carries a metal cluster comprising a [$MoFe_3S_4$] core, and that the presence of this metal cluster in NifQ is

correlated with its ability to support *in vitro* FeMo-co synthesis (93). As-isolated NifQ exhibits EPR properties similar to those of $[Fe_3\text{-}S_4]^+$-containing proteins, whereas the EPR signals of reduced NifQ resemble those of $[MoFe_3S_4]$ metal clusters prepared synthetically. Metal analysis, and the observation that $[Fe_3\text{-}S_4]$ to $[MoFe_3S_4]$ cluster conversion could be achieved *in vitro* by incubating NifQ with molybdate and sulfide under reducing conditions, confirmed that each NifQ molecule contains a single [Fe-S] cluster with the capability to carry a Mo atom.

Molybdate must undergo at least three chemical transformations before being incorporated into FeMo-co: replacement of O-ligands by S ligands, reduction of molybdenum from the Mo^{VI} oxidation state to the Mo^{IV} state found in the cofactor (101,102), and insertion of molybdenum into an [Fe-S] cluster environment. The $[Fe_3S_4]$ cluster observed in as-isolated NifQ could be the site for molybdenum binding and reduction. The reduced $[Fe_3\text{-}S_4]^0$ clusters are known to be able to coordinate heterometals to complete $[MFe_3\text{-}S_4]$ clusters in reversible equilibria (103). An attractive hypothesis is that NifQ would have its role in molybdenum trafficking through a cyclic process of molybdenum binding to the $[Fe_3S_4]$ cluster by reductive coupling to molybdate and molybdenum releasing from the $[MoFe_3S_4]$ cluster in response to an oxidation event (Figure 4). Consistently, EXAFS analysis strongly suggests that the molybdenum within NifQ is in the (IV) oxidation state (George, Hernandez and Rubio, unpublished results).

The $[MoFe_3S_4]$-loaded form of NifQ serves as specific molybdenum donor for the NifEN/NifH proteins during FeMo-co biosynthesis. A puzzling fact is that molybdate too can serve as a Mo source for FeMo-co biosynthesis *in vitro*. However, genetic evidence indicates that NifQ has an essential role when the levels of available molybdenum are in a physiological range. Mobilization of molybdenum from NifQ requires the simultaneous participation of NifH and NifEN, suggesting that NifQ would be the physiological molybdenum donor to a putative NifEN/NifH complex.

(viii) Molybdenum in NifEN and NifH

The NifEN protein, as isolated from a strain lacking *nifH*, contains substoichiometric amounts of molybdenum (104). The lack of NifH impedes the final steps of FeMo-co biosynthesis causing accumulation of biosynthetic intermediates within the NifEN scaffold. The presence of molybdenum in NifEN has been a rather contentious finding because it appears to depend on the purification method used to isolate this protein (104,105). However, two independent observations support that the molybdenum within NifEN is indeed relevant to FeMo-co synthesis: (*i*) the purified preparations of NifEN served as a sole molybdenum source for the *in vitro* FeMo-co biosynthesis assay in a defined reaction mixture containing only purified components (104). (*ii*) The EXAFS analysis showed that molybdenum in NifEN is part of a $[MoFe_3S_{3+n}]$ cluster but not adventitiously bound molybdate (106).

Although the quality of EXAFS data was not high enough as to discard the possibility of the $[MoFe_3S_{3+n}]$ being part of a larger [Mo-Fe-S] cluster, the lack of apparent long-range (5Å) metal interactions supports the hypothesis that this cluster is in fact a $[MoFe_3S_4]$ cubane. In such a case, this cluster would be very similar to the one carried by NifQ, and it is possible to envision at least three biosynthetic origins. First, NifEN contains a “receiving” $[Fe_3S_4]$ cluster that accepts a reduced Mo atom or a Mo-S molecule extracted from the $[MoFe_3S_4]$ cluster of NifQ, which would act as a donor. This would be a redox dependent metal transfer similar to the ones reported to occur in reduced $[Fe_3\text{-}S_4]^0$ clusters (103). The observation of EPR signals that show similarity to signals from $[Fe_3S_4]^+$-containing proteins in preparations of NifEN lacking molybdenum is consistent with this scenario (105,107). Second, the complete $[MoFe_3S_4]$ cluster is transferred from NifQ to NifEN. Transfer of

complete [Fe_4S_4] clusters from scaffold proteins to target apo-proteins is widespread in nature (108). Third, the NifEN [$MoFe_3S_4$] cluster could have its origin in the reductive coupling of molybdate into a "receiving" [Fe_3S_4] cluster in a NifQ-independent pathway. It is important to note that the NifEN was isolated from a strain that contains NifQ but lacks NifH, and that molybdenum transfer from NifQ to NifEN is not efficient in the absence of NifH (93). This pathway could be responsible for the suppression of the *nifQ* mutant phenotype observed by increasing the concentration of molybdate or cysteine in the medium (99,100). In any case, the [$MoFe_3S_{3+n}$] cluster of NifEN has been shown to be a molybdenum donor for the VK-cluster, which is the other FeMo-co biosynthetic intermediate carried by NifEN (104).

NifH too has been proposed to serve as the entry point for molybdenum incorporation into the FeMo-co biosynthetic pathway. ^{99}Mo radiolabeling experiments showed the incorporation of ^{99}Mo into NifH in FeMo-co biosynthesis reaction mixtures containing purified NifEN, NifH, NifB-co and Mg·ATP (109). However, it should be noted that incorporation of ^{99}Mo occurred also in NifEN, and that the presence of all components (NifEN, NifH, NifB-co and Mg·ATP) were required for ^{99}Mo incorporation into both NifEN and NifH. Similar results were observed by EXAFS analysis of FeMo-co precursors associated with NifH after *in vitro* FeMo-co biosynthesis reactions (110). Since the presence of the [$MoFe_3S_{3+n}$] cluster of NifEN is not absolutely dependent on NifH (104), a possible explanation for the role of NifH would be that its activity is required to mobilize molybdenum from the [$MoFe_3S_{3+n}$] cluster into the NifB-co-derived VK-cluster within the NifEN protein thus generating a molybdenum-containing FeMo-co biosynthetic intermediate.

Perspectives

The finding that the [Fe-S] cluster of NifQ was involved in the sequestration and delivery of molybdenum during FeMo-co synthesis has triggered many questions (111). Perhaps, the most intriguing one is how would NifQ exert all the biochemical transformations upon molybdenum that are required for such trafficking. A molybdate-reducing system that replaces Mo-O bonds by Mo-S bonds, reduces molybdenum, and inserts the heterometal into the [Fe3-S4] cluster of NifQ must exist in order to generate a [$MoFe_3S_4$] cluster. We hypothesize that NifQ is the central protein of an enzymatic system with molybdate reductase activity. Adjacent to *nifQ* in the minor *nif* region are genes encoding a ferredoxin (*Avin51020*), NifO (*Avin51030*), a putative rhodanase (*Avin51050*), and a monothiol glutaredoxin (*Avin51060*).

Some glutaredoxins have been described to be involved in [Fe-S] cluster assembly (112). Thus, it is possible that the *nif* glutaredoxin has a role in the formation of the [Fe_3-S_4] cluster of NifQ, which serves as scaffold for molybdenum binding. Rhodanases have sulfur transferase activity and have been shown to direct the reconstitution of [Fe-S] clusters in ferredoxins and NifH (113,114). Thus, it is possible that the *nif* rhodanase could be involved in the synthesis of the [Fe_3-S_4] cluster of NifQ. The generation of the [$MoFe_3S_4$] cluster could be achieved by reductive coupling of molybdate to the [Fe_3-S_4] cluster. The *nif*-specific ferredoxin could be involved in electron donation to reduce the [Fe_3-S_4] cluster of NifQ. Finally, the NifO protein is similar to the arsenate reductase ArsC, which uses reduced glutathione to convert arsenate to arsenite. Whether or not NifO could be involved in molybdate reduction remains open.

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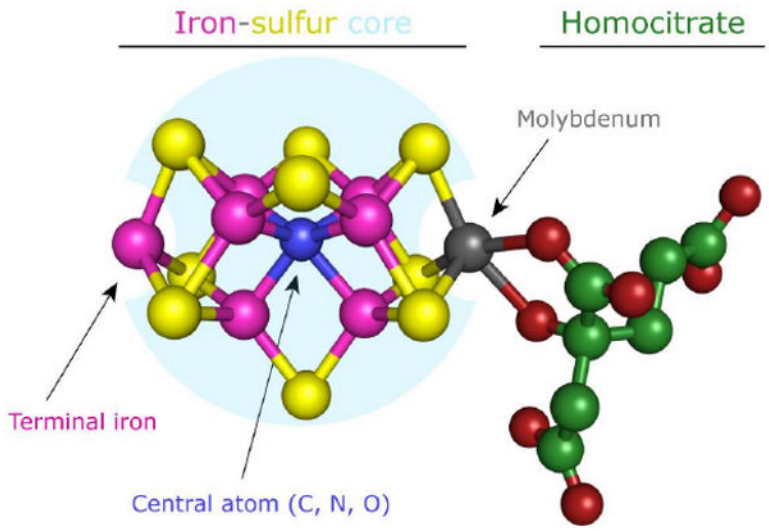

FIGURE 1.

The iron-molybdenum cofactor (FeMo-co) of nitrogenase structured from a biosynthetic perspective. The location of the Fe_6-S_9-X cofactor core corresponding to the NifB-co precursor is highlighted by a cyan sphere. Terminal Fe and Mo atoms capping the open sites at the cofactor core are labeled. Atom colors: iron, magenta; molybdenum, grey; sulfur, yellow; oxygen, red; carbon, green; and central atom X, blue.

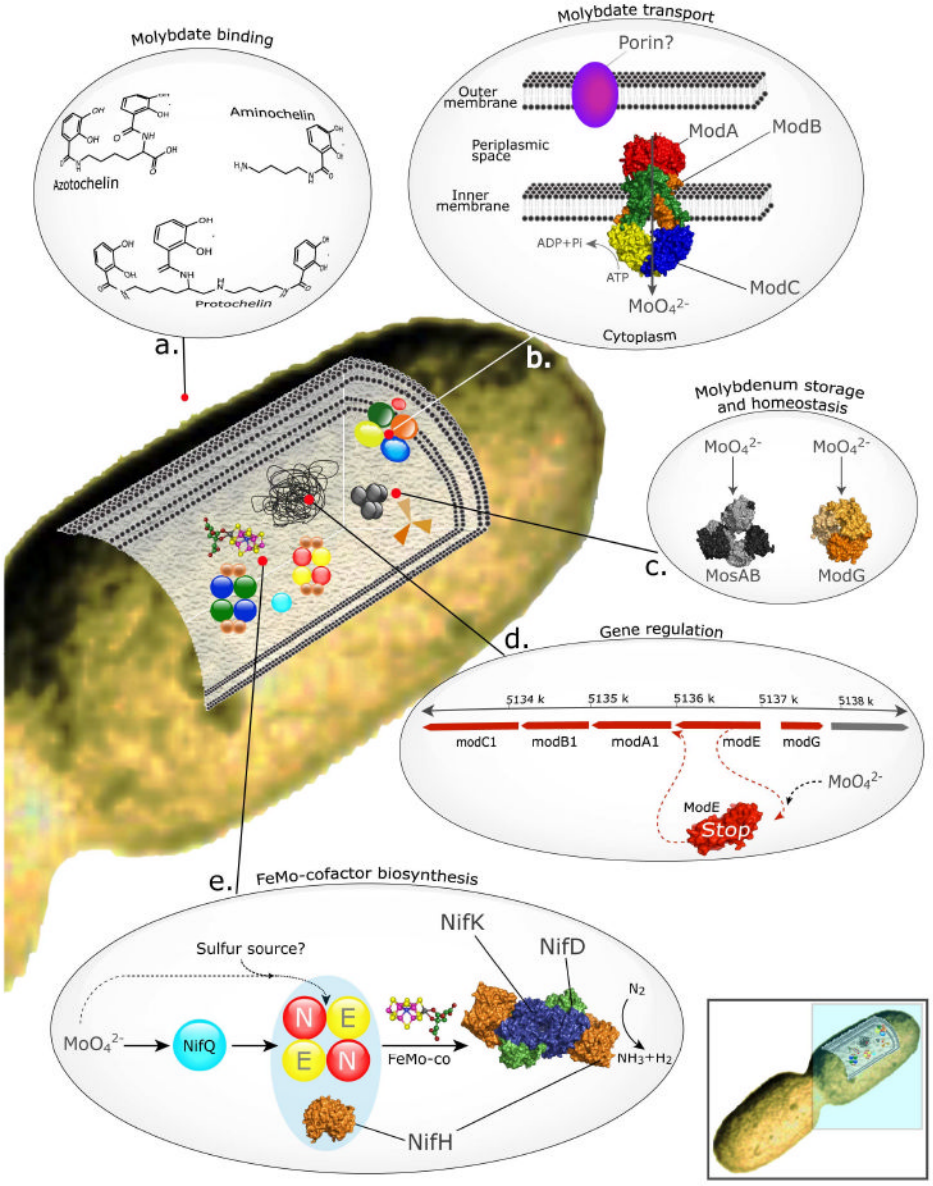

FIGURE 2.

Molybdenum trafficking for nitrogen fixation in the bacterium *Azotobacter vinelandii*. The figure shows a pathway of molybdenum towards the molybdenum-nitrogenase enzyme and the protein components involved in this pathway. Some of these proteins are exclusively dedicated to the nitrogenase biogenesis (e.g. NifQ and NifEN); some other have general roles in the metabolism of molybdenum (e.g. the ModABC molybdate transport system and the molybdenum-dependent transcriptional regulator ModE). Excreted siderophores with capacity to bind molybdate anions are also depicted. The *modABC* structure corresponds to the molybdate transport system of *Archaeoglobus fulgidus*. The ModE, NifH, and NifHDK structures shown are from *A. vinelandii*. Panel *e* shows a simplified FeMo-co biosynthetic pathway illustrating the two putative pathways for molybdenum incorporation into the Mo-nitrogenase cofactor. A complete FeMo-co biosynthetic pathway is shown in Figure 3.

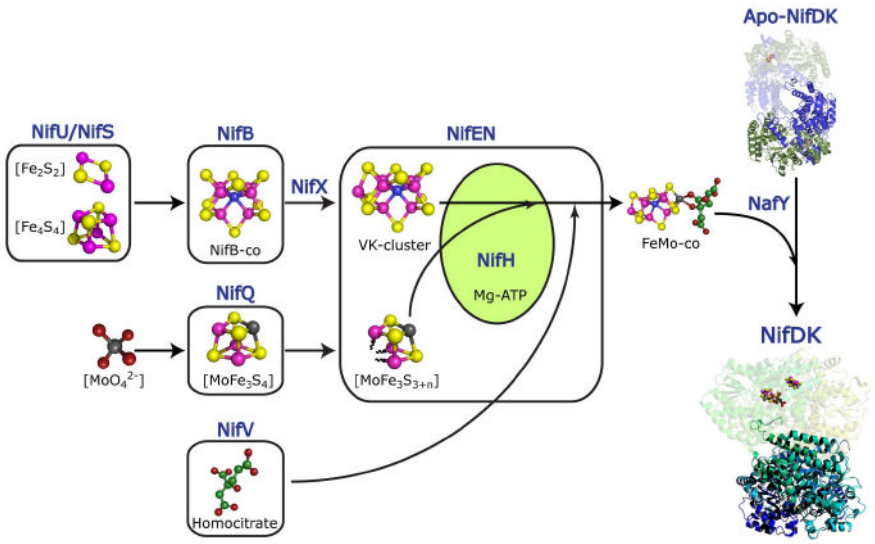

FIGURE 3.

Schematic model of the FeMo-co biosynthetic pathway showing the convergence of FeMo-co biosynthetic precursors ([Fe_6-S_9-X], [$MoFe_3S_4$] and homocitrate) into a central node composed of the NifEN and NifH proteins. The [Fe-S] cluster biosynthetic branch involves the activities of NifS, NifU, NifB and NifX; the molybdenum branch involves the activity of NifQ; and the homocitrate is synthesized by NifV. The NifEN protein catalyzes the conversion of NifB-co into the VK-cluster, whereas the incorporation of molybdenum into FeMo-co – and probably that of homocitrate– requires the concerted activities of NifEN and NifH. Completed FeMo-co is then transferred by NafY to the cofactor-deficient apo-NifDK protein to generate active Mo-nitrogenase.

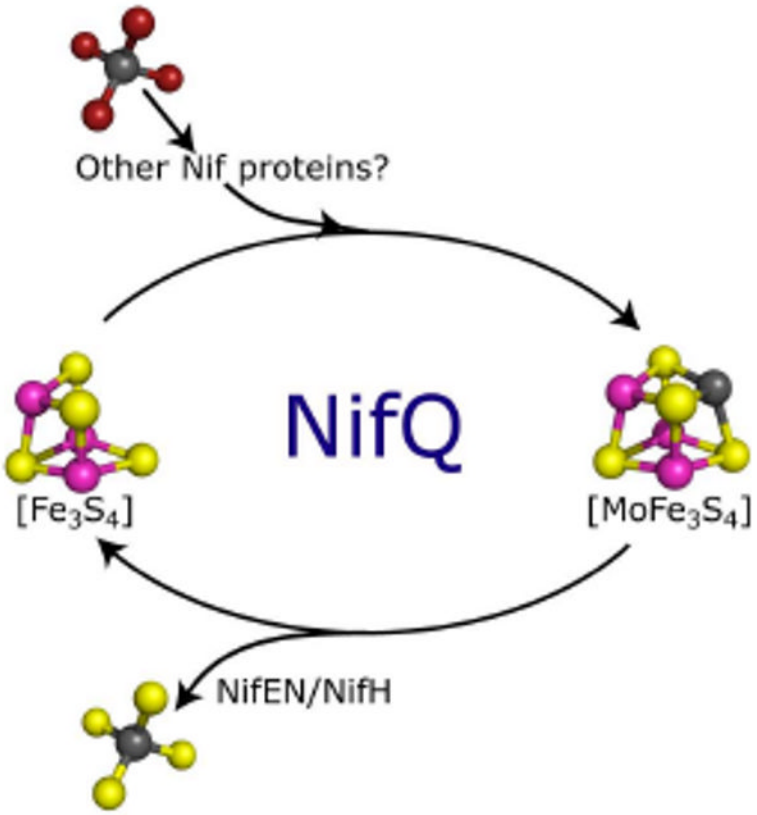

FIGURE 4.

Hypothetical model for a redox-dependent cycle of cluster interconversion in NifQ. A [$MoFe_3S_4$] structure based on recent spectroscopy data was used for this model (93). The [Fe_3-S_4] cluster of NifQ serves as molybdenum binding site. Substitution of Mo-S for Mo-O bonds and reduction of molybdate would be required; other Nif proteins might assist NifQ during this process *in vivo*. The presence of NifEN/NifH promotes the release of molybdenum from NifQ. A sulfur donor might be required to release Mo from the [$MoFe_3S_4$] cluster. Atom colors: iron, magenta; sulfur, yellow; molybdenum, grey; oxygen, red.

Table 1

A. vinelandii genes, described in this review, related to molybdenum metabolism and nitrogen fixation.

Gene number	Annotation	Function
Binding of extracellular molybdenum		
Avin21220	*csbC*	Isochorismate synthase
Avin21230	*csbX*	Efflux pump
Molybdate transport inside the cell		
Avin50670	$modA_1$	Periplasmic molybdate-binding protein
Avin50660	$modB_1$	Membrane channel protein
Avin50650	$modC_1$	Cytoplasmic ATPase
Avin01300	$modA_2$	Periplasmic molybdate-binding protein
Avin01290	$modB_2$	Membrane channel protein
Avin01280	$modC_2$	Cytoplasmic ATPase
Avin50730	$modA_3$	Periplasmic molybdate-binding protein
Avin50720	$modB_{3a}$	Membrane channel protein
Avin50710	$modB_{3b}$	Membrane channel protein
Avin50700	$modC_3$	Cytoplasmic ATPase
Molybdenum cellular homeostasis		
Avin50690	*modG*	Molybdenum homeostasis
Molybdenum storage		
Avin43200	*mosA*	Subunit of the Mo-storage protein
Avin43210	*mosB*	Subunit of the Mo-storage protein
Molybdate-dependent gene regulation		
Avin50680	*modE*	Mo-responsive transcriptional regulator
Avin33430	*modE* copy	Mo-responsive transcriptional regulator
Avin33440	$vnfA_2$	Transcriptional activator of the *vnf* genes
FeMo-co biosynthesis and nitrogen fixation		
Avin01360 to Avin01710	Major *nif*	
Avin01380, Avin01390, Avin01400	*nifHDK*	Mo-nitrogenase structural genes
Avin01450, Avin01470, Avin01480, Avin01640	*nifENXV*	FeMo-co biosynthesis
Avin01620, Avin01630,	*nifUS*	Fe-S cluster biosynthesis
Avin50990 to Avin51060	Minor *nif*	
Avin50910	*nafY*	FeMo-co insertion and apo-NifDK stability
Avin51000, Avin50990	*nifAL*	Transcriptional regulation of *nif* genes
Avin51010	*nifB*	FeMo-co biosynthesis
Avin51020	*fdxN*	Ferredoxin
Avin51030	*nifO*	Unknown function
Avin51040	*nifQ*	Incorporation of Mo into FeMo-co
Avin51050	*rhdN*	Putative rhodanase
Avin51060	$grx5^{nif}$	Monothiol glutaredoxin