Metal trafficking for nitrogen fixation: NifQ donates molybdenum to NifEN/NifH for the biosynthesis of the nitrogenase FeMo-cofactor

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The molybdenum nitrogenase, present in a diverse group of bacteria and archea, is the major contributor to biological nitrogen fixation. The nitrogenase active site contains an iron–molybdenum cofactor (FeMo-co) composed of 7Fe, 9S, 1Mo, one unidentified light atom, and homocitrate. The *nifQ* gene was known to be involved in the incorporation of molybdenum into nitrogenase. Here we show direct biochemical evidence for the role of NifQ in FeMo-co biosynthesis. As-isolated NifQ was found to carry a molybdenum–iron–sulfur cluster that serves as a specific molybdenum donor for FeMo-co biosynthesis. Purified NifQ supported *in vitro* FeMo-co synthesis in the absence of an additional molybdenum source. The mobilization of molybdenum from NifQ required the simultaneous participation of NifH and NifEN in the *in vitro* FeMo-co synthesis assay, suggesting that NifQ would be the physiological molybdenum donor to a hypothetical NifEN/NifH complex.

nif | iron-sulfur cluster | *Azotobacter vinelandii*

Biological nitrogen fixation performed by microorganisms that have nitrogenase(s) accounts for roughly two-thirds of the nitrogen fixed globally. Most nitrogen fixation is carried out by the activity of molybdenum nitrogenase (1), which is widely distributed in nature (2). The molybdenum-nitrogenase enzyme is composed of two proteins (3): a heterotetrameric NifDK-protein component ($\alpha_2\beta_2$ dinitrogenase) and a homodimeric NifH-protein component (dinitrogenase reductase). NifDK contains one iron–molybdenum cofactor (FeMo-co) within the active site of each α-subunit (NifD), and one [8Fe–7S] P-cluster at the interface of the α- and β-subunits in each $\alpha\beta$ pair (4). NifH contains a [4Fe–4S] cluster bridging the two subunits and one site for Mg-ATP binding and hydrolysis in each subunit (5).

FeMo-co is a unique cofactor‖ composed of a [Mo–3Fe–3S] partial cubane bridged to a [4Fe–3S] partial cubane by three sulfur ligands and one unidentified light atom in the center of the cofactor (6). The molybdenum atom is also coordinated to the organic acid homocitrate. The biosynthesis of FeMo-co is a complex process that involves the activities of several nitrogen fixation (*nif*) gene products that function as molecular scaffolds, enzymes, or escort proteins that carry FeMo-co precursors between assembly sites in the pathway (7, 8). Following assembly, FeMo-co is inserted into apo-NifDK, a P-cluster-containing but FeMo-co-deficient form of NifDK that is matured into functional NifDK simply by FeMo-co insertion, to generate the mature dinitrogenase enzyme that is competent for nitrogen fixation.

The NifEN scaffold protein is believed to function in the pathway as a central node to which an [Fe–S]-containing FeMo-co precursor, molybdenum, and homocitrate might converge to complete the assembly of FeMo-co (9–12). In this model, the [Fe–S]-containing FeMo-co precursor, NifB-co**, would be initially assembled by NifB and then transferred to NifEN for its conversion into the [Fe–S] VK-cluster (13, 14). Homocitrate would be provided by the homocitrate-synthase

activity of NifV (15, 16). Although molybdate satisfies the molybdenum requirement for FeMo-co synthesis *in vitro*, the physiological source of molybdenum remains unknown.

Genetic evidence from *Azotobacter vinelandii*, *Klebsiella pneumoniae*, and other nitrogen-fixing bacteria has suggested the involvement of NifQ in molybdenum processing for nitrogenase. In all cases tested, *nifQ* has been shown to be essential for diazotrophic growth, although excess molybdate in the growth medium alleviated the *nifQ* phenotype (17–19). The role of NifQ is specifically related to the Mo-nitrogenase because *nifQ* mutants were not affected in either the capacity to grow by using the alternative vanadium or iron-only nitrogenase systems (20) or in the activity of molybdopterin-containing enzymes (21).

NifQ homologues are present in all diazotrophic species of the Proteobacteria phylum, with the exception of some *Rhizobium* species. NifQ proteins have a conserved $Cx_4Cx_2Cx_5C$ amino acid motif that has been suggested to bind a molybdenum-containing metal cluster or a Mo-S intermediate for FeMo-co synthesis (22). However, NifQ proteins are unique and their primary sequences are unrelated to other proteins shown to be involved in molybdenum trafficking. NifQ proteins do not carry molbindin-like domains that are involved in molybdate binding (23). Molbindin domains are found in the ModA component of molybdate-transport systems, in the molybdate-responsive ModE transcriptional regulator, and in the *A. vinelandii* ModG protein involved in molybdenum homeostasis. Additionally, NifQ does not exhibit significant sequence similarity to the molybdenum-storage-protein (Mosto) MosA and MosB polypeptide components, which are able to bind large amounts of molybdenum, presumably as heptamolybdate (24).

In this work, we show that NifQ carries a molybdenum–iron–sulfur cluster that serves as a specific molybdenum donor for the synthesis of FeMo-co *in vitro*. Mobilization of molybdenum from NifQ required the simultaneous presence of the NifH and NifEN
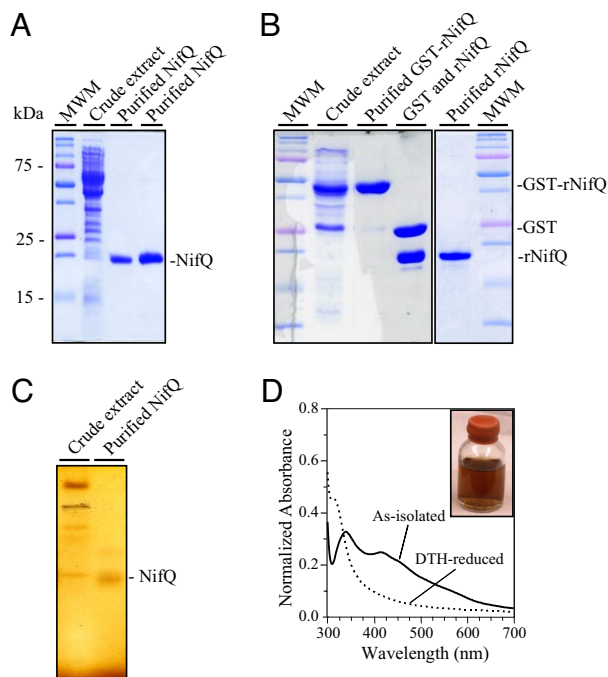

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Fig. 1. Purification and UV-visible spectra of native *A. vinelandii* NifQ and recombinant rNifQ. (*A*) SDS/PAGE analysis of *A. vinelandii* NifQ purification steps (cell-free extract lane contains 40 μg of protein; purified NifQ lanes contain 5 μg and 15 μg of protein, respectively). Molecular weight markers are indicated to the left. The position of NifQ is indicated to the right. (*B*) SDS/PAGE analysis of recombinant rNifQ purification steps (cell-free extract lane contains 25 μg of protein; purified protein lanes contain 8 μg of protein). Protein positions in the gel are indicated to the right. (*C*) Fe staining of NifQ purified from *A. vinelandii* cells after anoxic native PAGE (cell-free extract lane contains 100 μg of protein; purified NifQ lane contains 25 μg of protein). (*D*) UV-visible spectra of as-isolated NifQ (solid line) and DTH-reduced NifQ (dotted line). DTH-treated NifQ samples (33 μM NifQ) were gel-filtered on a Sephadex G-25 resin to remove excess DTH before recording the spectrum. Spectra were normalized by absorbance at 280 nm.
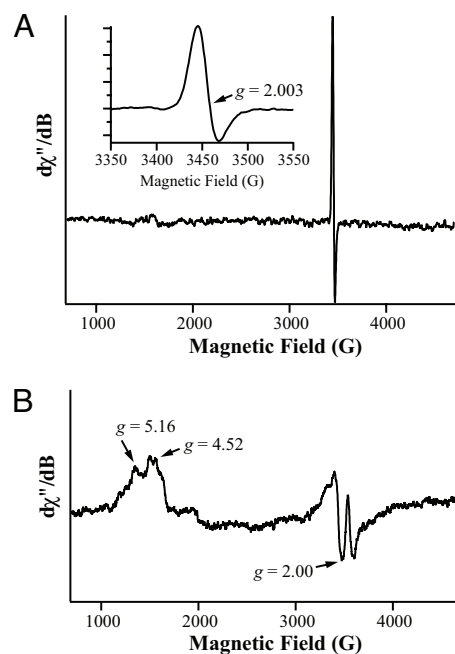

Fig. 2. CW-EPR analysis of NifQ from *A. vinelandii*. Samples contained ≈1 mM NifQ. (*A*) As-isolated NifQ. The magnification is an enlarged image in the g = 2 region. (*B*) NifQ protein (1 mM) incubated with 5 mM DTH, 1 mM Na_2MoO_4, and 1 mM Na_2S. EPR spectra were recorded under the following experimental conditions: temperature, 12 K; microwave frequency, 9.70 GHz; microwave power, 0.03 mW for as-isolated NifQ and 5 mW for reduced NifQ; modulation amplitude, 8 G; modulation frequency, 100 kHz; time constant, 80.97 ms; conversion time, 10.24 ms; and resolution, 4096 points.

proteins. These findings strongly support a role for NifQ as the physiological molybdenum donor to a putative NifEN/NifH complex during FeMo-co biosynthesis.

Results

NifQ Is an Iron-Sulfur Molybdoprotein. Expression of a His-tagged version of NifQ in UW300 cells was 50-fold higher than NifQ expression in wild-type cells. His-NifQ overexpression did not alter *in vitro* nitrogenase activity [supporting information (SI) Table S1] or the regulation by ammonium of the expression of NifQ or any other essential *nif* gene analyzed in UW300 (data not shown). Hereafter, we refer to the *A. vinelandii* His-tagged NifQ variant as NifQ. The NifQ protein was purified under strict anaerobic conditions from UW300 cells (Fig. 1*A* and *Experimental Procedures*). The average purification yield was 280 mg NifQ/kg UW300 cell paste. The *A. vinelandii* NifQ was also purified from recombinant *Escherichia coli* cells overexpressing GST-NifQ fusion protein (Fig. 1*B*). NifQ protein purified from *E. coli* cells was denominated as rNifQ. The average purification yield was 4 g rNifQ/kg *E. coli* cell paste.

NifQ and rNifQ preparations were reddish brown and exhibited a positive signal in gels stained for Fe after anoxic native PAGE (Fig. 1*C*). Exposure of the purified proteins to air resulted in color bleaching indicative of sensitivity to oxygen (data not shown). The native molecular weight of as-isolated NifQ (M_r = 25.7 ± 0.4 kDa) is similar to the molecular weight deduced from the *A. vinelandii nifQ* primary sequence (M_r = 19.7 kDa) and

thus consistent with a monomeric structure. As-isolated NifQ contained 3.1 ± 0.1 Fe atoms and 0.30 ± 0.04 Mo atoms per NifQ molecule as determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Interestingly, ICP-OES analysis showed that rNifQ contained 4.6 ± 0.2 Fe atoms but no molybdenum. NifQ displayed a UV-visible spectrum typical of [Fe–S] proteins with a sharp peak at 340 nm, a broad peak at 410 nm, and an A_{410}/A_{280} ratio of 0.2 (Fig. 1*D*). This ratio decreased after incubating NifQ with 5 mM sodium dithionite (DTH), which is characteristic of [Fe–S] cluster reduction by DTH.

The Molybdenum Within NifQ Is in a [Mo–3Fe–4S] Cluster Environment. Fig. 2*A* shows the X-band continuous wave electron paramagnetic resonance (CW-EPR) spectrum of the as-isolated NifQ protein. The spectrum exhibits a fairly isotropic signal centered at g = 2.00 with a peak-to-peak line width of ≈27 G (Fig. 2*A* magnification). This EPR signal has an asymmetric shape that is characteristic of a S = 1/2 $[3Fe–4S]^+$ cluster (25, 26); it exhibits line width similar to that of aconitase (25) and line shape similar to that of ferredoxin II from *Desulfovibrio gigas* (27). It has been proposed that the EPR signal of oxidized $[3Fe–4S]^+$ clusters arises from the antiferromagnetic exchange coupling of three high-spin (S = 5/2) ferric ions to form an S = 1/2 ground state (26). Power- and temperature-dependence profiles of the EPR signal from NifQ are also consistent with the presence of $[3Fe–4S]^+$ clusters in the as-isolated NifQ (Fig. S1).

Treatment of NifQ with DTH gave rise to a different EPR-active species resembling spectra of chemically synthesized compounds comprising S = 3/2 $[Mo–3Fe–4S]^{3+}$ clusters (28–30) (data not shown). This new EPR signal did not arise from reduction of the $[3Fe–4S]^+$ cluster to the $[3Fe–4S]^0$ state (S = 2), because such a species would be EPR silent in the perpendicular mode of CW-EPR. When NifQ treatment with DTH was
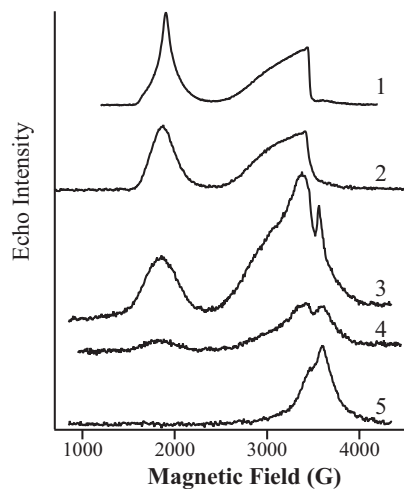
Fig. 3. Two-pulse ESE-EPR field-sweep spectra recorded at 4.2 K of DTH-reduced NifDK protein (Line 1); DTH-reduced NafY:FeMo-co complex generated *in vitro* (44) (Line 2); NifQ protein (1 mM) incubated with 5 mM DTH, 1 mM Na_2MoO_4, and 1 mM Na_2S (Line 3); DTH-reduced NifQ (1 mM NifQ and 5 mM DTH) (Line 4); and DTH-reduced rNifQ (1 mM rNifQ and 5 mM DTH) (Line 5). EPR spectra were recorded under the following experimental conditions. Trace 1: microwave frequency, 9.74 GHz; microwave power, 25 W; t = 140 ns; and repetition time, 8 ms. Trace 2: microwave frequency, 9.74 GHz; microwave power, 39 W; t = 140 ns; and repetition time, 8 ms. Trace 3: microwave frequency, 9.73 GHz; microwave power, 63 W; t = 136 ns; and repetition time, 10 ms. Trace 4: microwave frequency, 9.73 GHz; microwave power, 63 W; t = 136 ns; and repetition time, 10 ms. Trace 5: microwave frequency, 9.72 GHz; microwave power, 79 W; t = 144 ns; and repetition time, 20 ms.

carried out in the presence of molybdate and sulfide, the intensity of the $[Mo–3Fe–4S]^{3+}$-like signal increased and the features at the g = 4 region of the spectra were sharpened (Fig. 2*B*).

Electron spin echo (ESE) field-sweep EPR spectrum of DTH-reduced NifQ showed a line shape (Fig. 3 Line 4) with features similar to that of FeMo-co associated with the NifDK component of nitrogenase (Fig. 3 Line 1) or with the carrier protein nitrogenase accessory factor Y (NafY) (Fig. 3 Line 2). This line shape is consistent with the presence of an S = 3/2 [Mo–Fe–S] cluster in NifQ. Treatment of NifQ with DTH, molybdate, and sulfur markedly increased the [Mo–Fe–S] cluster signal (Fig. 3 Line 3). On the other hand, the molybdenum-free and DTH-reduced rNifQ lacked the [Mo–Fe–S] cluster signal in the ESE field-sweep EPR spectrum (Fig. 3 Line 5), as expected.

In conclusion, CW-EPR and ESE field sweep EPR analysis, combined with Fe and Mo determinations, indicate that NifQ is able to coordinate a redox responsive [Mo–3Fe–4S] cluster.

NifQ Serves as a Molybdenum Donor for FeMo-co Synthesis *in Vitro*. The assay for *in vitro* FeMo-co synthesis with purified components used in this work requires a FeMo-co precursor (NifB-co or VK-cluster[††]), NifH, NifEN, homocitrate, Mg-ATP, DTH, and a source of molybdenum (either Na_2MoO_4 or NifQ). Synthesized FeMo-co binds and activates FeMo-co-deficient apo-NifDK protein, generating active NifDK that is routinely assayed by its acetylene-reducing activity.

Fig. 4*A* shows the titration with NifQ of VK-cluster-dependent *in vitro* FeMo-co biosynthesis reactions. Because no molybdate was added to the reactions, this result shows that as-isolated NifQ substituted for Na_2MoO_4 in the assay. Similar results were

[††]The VK-cluster is the [Fe–S] cluster precursor to FeMo-co that accumulates on NifEN in a Δ*nifH* mutant strain and is thought to represent an intermediate after NifB-co in FeMo-co synthesis (13).
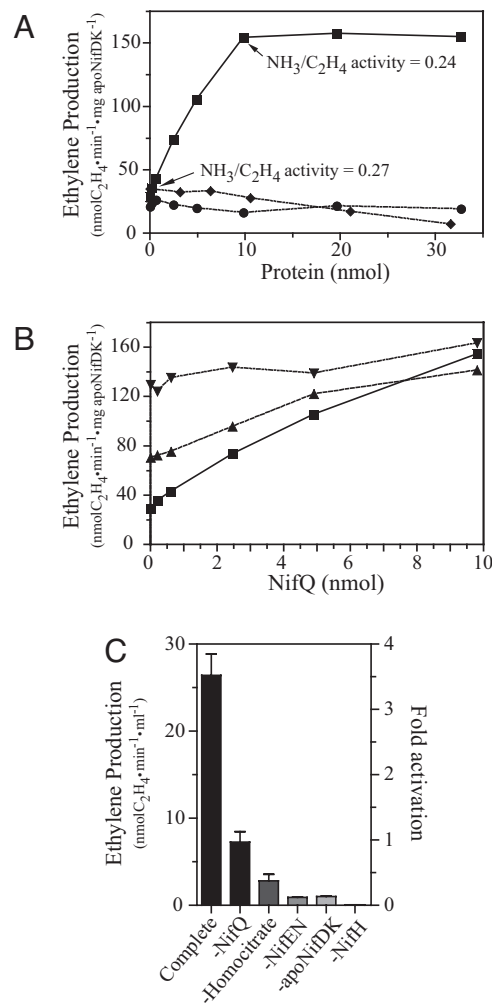

Fig. 4. NifQ serves as a molybdenum donor for *in vitro* synthesis of FeMo-co with purified components. FeMo-co synthesis was determined by the acetylene reduction activity of matured NifDK. In addition, the dinitrogen reduction activity of matured NifDK was determined in selected samples. All vials were acid-washed and thoroughly rinsed with Milli-Q water to remove traces of molybdenum. FeMo-co synthesis reactions contained 0.43 nmol of apo-NifDK, 0.48 nmol of VK-cluster loaded NifEN, 2.4 nmol NifH, and the indicated amount of NifQ (0–30 nmol). (*A*) Titration of FeMo-co synthesis reactions with increasing amounts of purified NifQ (squares), purified rNifQ (circles), or purified cyanobacterial nitrate reductase (NarB) (diamonds). No molybdate was added to the reactions. (*B*) Titration of FeMo-co synthesis reactions with increasing amounts of purified NifQ in the presence of 18 μM molybdate (inverted triangles), 0.18 μM molybdate (triangles), or no molybdate (squares). (*C*) Requirement for different components in the FeMo-co synthesis reaction with purified proteins. No molybdate was added to the reactions. All reactions contained 4.9 nmol of NifQ except the reaction lacking NifQ. Fold activation with respect to the reaction lacking NifQ is indicated in the right *y* axis. In the experiments lacking NifH, FeMo-co synthesis was stopped by the addition of 0.3 mM $(NH_4)_2MoS_4$ before assaying the activity of reconstituted NifDK. Data are the average of two to four independent determinations.

obtained when NifB-co and precursor-free NifEN [purified from UW243 cells (13)] were substituted for VK-cluster-loaded NifEN in the assay (data not shown). We note that the background level of apo-NifDK activation observed in the reaction lacking NifQ was because of mobilization of the molybdenum present in purified NifEN preparations (11).

The level of NifDK-specific activity reconstituted *in vitro* was limited by several factors. First, at saturating levels of NifQ (10 nmol), the amount of NifEN present in the assay (0.48 nmol)

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became limiting. When 2.4 nmol of NifEN were used in the assay, the level of reconstituted NifDK activity and the amount of NifQ required to saturate the assay increased accordingly (data not shown). Second, molybdenum donation by NifQ was incomplete under the *in vitro* FeMo-co biosynthesis assay conditions. *In vitro* FeMo-co synthesis reactions in which denatured NifQ protein served as the molybdenum source yielded 2-fold higher NifDK activity levels than reactions in which the same amount of native NifQ served as the molybdenum donor (data not shown). Third, saturating amounts of apo-NifDK were added to the assay to obtain higher absolute levels of apo-NifDK activation at the expense of obtaining lower specific activity per mg of NifDK, as described in ref. 14.

NifQ supported the formation of fully functional NifDK protein. *In vitro* reconstituted NifDK reduced dinitrogen to ammonia with a ratio of specific activity of ammonia-to-ethylene production similar to native NifDK (Fig. 4*A*). This ratio was different from those of the alternative nitrogenases (especially the iron-only nitrogenase), demonstrating that NifQ mostly supported the synthesis of FeMo-co and not that of the analogous cofactor FeFe-co.

Neither purified rNifQ, determined to be molybdenum-free by ICP-OES, nor the molybdoprotein nitrate reductase (NarB) were able to activate the apo-NifDK in parallel assays (Fig. 4*A*). Purified NarB contained 1 mol of Mo per mol of protein and was fully active in a methyl-viologen-dependent nitrate reductase activity assay (31) (data not shown). The fact that the molybdenum-free NifQ and the molybdoprotein NarB could not activate apo-NifDK suggested that (*i*) the molybdenum-loaded form of NifQ was specifically required for FeMo-co synthesis and (*ii*) NarB could not act as a source of molybdenum for FeMo-co synthesis.

The effect of NifQ in apo-NifDK activation was less pronounced when a subsaturating amount of molybdate was additionally included in the reaction mixture and was very small in a reaction additionally containing a saturating amount of molybdate (Fig. 4*B*). Thus, NifQ could serve as a molybdenum donor as well as molybdate in the *in vitro* FeMo-co synthesis assay.

Fig. 4*C* shows the effect of omitting specific protein components from the apo-NifDK activation mixture in reactions lacking molybdate. The reaction containing NifQ showed maximal NifDK activity, whereas the reaction lacking NifQ exhibited 20% of such a maximum. The NifDK activity observed in the reconstituting reaction lacking NifQ has been reported (11) and attributed to the presence of substoichiometric amounts of molybdenum in purified NifEN that are incorporated into FeMo-co. Reactions carried out in the absence of either NifEN, NifH, apo-NifDK, or homocitrate showed very low NifDK activities, indicating their requirement for apo-NifDK activation using NifQ as the molybdenum donor.

Mobilization of Molybdenum from NifQ Requires the Simultaneous Presence of NifH and NifEN Proteins. The features of the stimulatory effect of NifQ on apo-NifDK activation shown in Fig. 4*A* demonstrate that the molybdenum carried by NifQ was used for FeMo-co synthesis *in vitro*. However, the identity of the molybdenum acceptor protein(s) in the reaction remained unclear. To investigate this aspect, as-isolated NifQ samples were incubated under FeMo-co synthesis conditions in the presence of either (*i*) NifH, (*ii*) NifEN, (*iii*) NifH and NifEN, or (*iv*) NifH, NifEN, and apo-NifDK. After incubation, the protein components of each reaction mixture were reisolated by chromatography (Fig. S2) and their molybdenum contents were determined by ICP-OES. The result of this analysis is shown in Table 1.

The molybdenum content of NifQ was not significantly affected after incubation with NifH or NifEN. Likewise, the molybdenum content of either NifEN or NifH was unaltered by incubation with NifQ. These results suggest a lack of molybde-

Table 1. Mobilization of molybdenum from NifQ under conditions of FeMo-co synthesis

Protein	Molybdenum, mol
NifQ	
Before incubation (as isolated)	0.36 ± 0.03
After incubation with NifEN	0.28 ± 0.02
After incubation with NifH	0.29 ± 0.01
After incubation with NifEN and NifH	0.11 ± 0.01
After incubation with NifEN, NifH and apo-NifDK	0.15 ± 0.02
NifEN	
Before incubation (as isolated)	0.24 ± 0.03
After incubation with NifQ	0.19 ± 0.02
After incubation with NifQ and NifH	0.45 ± 0.06
NifH	
Before incubation (as isolated)	0.009 ± 0.002
After incubation with NifQ	0.013 ± 0.001
After incubation with NifQ and NifEN	0.060 ± 0.010

Purified as-isolated NifQ was incubated with purified (*i*) NifH, (*ii*) NifEN, (*iii*) NifH and NifEN, or (*iv*) NifH, NifEN and apo-NifDK in reaction mixtures that also contained Mg-ATP, DTH, and homocitrate. No external source of molybdenum was added to the mixtures. NifQ, NifH, and NifEN proteins were reisolated by chromatography after incubation. Molybdenum content was quantified by ICP-OES and is shown in mol of molybdenum per mol of protein (i.e. NifQ monomer, NifH dimer, or NifEN tetramer). Values are the average of at least two independent determinations $\pm$ SD.

num exchange between NifQ and NifH or between NifQ and NifEN.

On the other hand, a major alteration in the molybdenum distribution among NifQ, NifH, and NifEN occurred when all three proteins were incubated together under FeMo-co synthesis conditions and then reisolated (Table 1). Upon reisolation, the molybdenum content of NifQ was >3-fold lower, with a concomitant significant increase in the molybdenum content of both NifEN and NifH. The addition of apo-NifDK as a "sink" of *in vitro* synthesized FeMo-co did not seem to further stimulate mobilization of molybdenum from NifQ toward NifEN/NifH.

In conclusion, significant mobilization of molybdenum from NifQ required simultaneous incubation with NifH and NifEN under FeMo-co synthesis conditions. Donation of molybdenum from NifQ contributed to FeMo-co synthesis, as demonstrated by its ability to activate apo-NifDK when included in the reaction mixtures (Fig. 4 and Table 1).

Discussion

Molybdenum is normally present in the environment as trace amounts of molybdate (<50 nM) or as highly insoluble minerals such as molybdenite (MoS_2) or wulfenite ($PbMoO_4$) (32). Consequently, microorganisms have developed different strategies to cope with molybdenum limitation. A model microorganism with which to study molybdenum uptake and metabolism is *A. vinelandii*, which carries Mo-co-containing enzymes and nitrogenase. *A. vinelandii* expresses several high-affinity molybdate-transport systems, a Mosto, and additional proteins involved in molybdenum trafficking for the biosynthesis of FeMo-co and Mo-co. One such protein is NifQ, for which genetic evidence has suggested a role in molybdenum processing for nitrogenase.

Alignment of NifQ amino acid sequences from a variety of bacteria shows a conserved C-terminal region including a $Cx_4Cx_2Cx_5C$ motif suggested to be a metal cluster binding site (Fig. S3). Herein, we provide definitive evidence for the presence of metal cluster(s) on NifQ.

The CW-EPR spectrum of as-isolated NifQ resembles a $[3Fe-4S]^+$-containing protein (25), whereas the EPR spectrum of DTH-reduced NifQ resembles model compounds having a

[Mo–3Fe–4S]$^{3+}$ cluster unit (28–30). Two interpretations can be considered to rationalize these observations. First, each NifQ molecule carries two types of [Fe–S] clusters: a [3Fe–4S] and a [Mo–3Fe–4S] cluster. Second, a percentage of the NifQ molecules in a purified preparation contains a [3Fe–4S] cluster (observable in the as-isolated state of NifQ), whereas the rest of the NifQ molecules contain a [Mo–3Fe–4S] cluster (observable in the DTH-reduced state of NifQ). We favor the second interpretation because of the average contents of iron (3.1 mol Fe/mol NifQ) and molybdenum (0.3 mol Mo/mol NifQ) in NifQ preparations and because the [Mo–3Fe–4S] signal in NifQ could be enhanced by incubation with sulfur and molybdenum under reducing conditions, indicating that *in vitro* cluster reconstitution was taking place. Coordination of a heterometal by a reduced [3Fe–4S]^0 cluster to complete a [M–3Fe–4S] cubane has been reported before (33).

In any case, the results presented herein indicate that NifQ is able to coordinate a redox responsive [Mo-3Fe-4S] cluster. Consistently, extended X-ray absorption fine structure (EXAFS) analysis of NifQ, to be reported elsewhere, indicates the presence of a [Mo-3Fe-4S] cluster in this protein (J.A.H. *et al.*, unpublished results). Although a significant percentage (≈70%) of NifQ molecules in a purified preparation carry [3Fe–4S] clusters, incubation of NifQ with molybdate and sulfide under reducing conditions appears to increase [Mo-3Fe-4S] cluster occupancy.

Why is the as-isolated NifQ protein loaded with substoichiometric amounts of molybdenum? There are three nonexclusive possible explanations. First, as-isolated NifQ might represent a snapshot of molybdenum trafficking through NifQ during active FeMo-co synthesis. Second, molybdenum binding to NifQ might be labile under purification conditions. Third, because NifQ is present at very low levels in nitrogenase-derepressed *A. vinelandii* wild-type cells (data not shown), strain UW300, which overexpresses a his-tagged variant of NifQ, was used in this study. NifQ overexpression in UW300 cells might have overwhelmed the capacity of the cells to load NifQ with molybdenum.

Interestingly, NifQ expressed and purified from *E. coli* cells (rNifQ) was found to be a molybdenum-free [4Fe–4S]-cluster-containing protein. The CW-EPR spectrum of DTH-reduced rNifQ, recorded at 12 K, showed an axial EPR signal ($g_1 = g_2 = 2.05$ and $g_3 = 1.92$) with a line shape typical of [4Fe–4S]$^+$ clusters (Fig. S4). In addition, temperature- and power-dependence profiles of this EPR signal were indicative of the presence of a [4Fe–4S]$^+$ cluster in rNifQ (power dependence shown in Fig. S5). These observations suggest that the loading of molybdenum into NifQ is carried out by a specific mechanism in *A. vinelandii* (a mechanism that is absent in *E. coli*) rather than by a process in which NifQ captures free molybdate from the cytoplasm.

The present study shows a clear linkage between NifQ and molybdenum trafficking for nitrogen fixation. NifQ served as direct source of molybdenum for FeMo-co biosynthesis *in vitro*. The role of NifQ appears to be in substituting sulfur by oxygen ligands to the molybdenum atom by incorporating it into an [Fe–S] cluster frame. This role is consistent with previous genetic evidence showing suppression of a *nifQ* mutant phenotype by cystine in the growth medium (34). It is not clear why molybdate can substitute for NifQ in the *in vitro* FeMo-co biosynthesis assay, but it is possible that molybdate reacts *in vitro* with excess DTH (35), thus generating Mo–S compounds suitable for FeMo-co synthesis.

The requirement for the simultaneous presence of NifH and NifEN in the reaction suggests that a NifEN/NifH complex could be required to unload the molybdenum from NifQ. Neither NifH nor NifEN alone were capable of mobilizing molybdenum from NifQ. These results also suggest specificity in the process of molybdenum transfer rather than leakage of molybdenum from NifQ into the solvent followed by capture of molybdenum by another protein. The ability of NifEN to associate with wild-type

NifH can be inferred from the observation that a NifH mutant variant (L127Δ) forms a stable complex with a form of NifEN that is loaded with a FeMo-co precursor (36). Such a FeMo-co precursor, denominated as the VK-cluster, appears to be a molybdenum-free complex [Fe–S] cluster (13, 37).

Determination of the entry point of molybdenum into the pathway for FeMo-co biosynthesis has been elusive. *In vitro* FeMo-co synthesis reactions using Na$_2$MoO$_4$ as source of molybdenum showed mutual NifH and NifEN dependence in the incorporation of molybdenum into FeMo-co precursors (38, 39). Based on these results, NifH was suggested to be an entry point for molybdenum (38) or even asserted to be a molybdenum/homocitrate insertase (39). On the other hand, the existence of a NifH-independent molybdenum binding site within NifEN was proposed based on the presence of substoichiometric amounts of molybdenum in NifEN purified from a Δ*nifH* strain (11). Molybdenum within NifEN was shown to be part of a [Mo–Fe–S] cluster different from the previously characterized FeMo-co precursor VK-cluster (12) and thought to represent a transient site for intramolecular molybdenum donation toward the VK-cluster. However, these observations could not rule out a NifH's possible role in enhancing molybdenum binding into this transient site in NifEN.

This study provides strong evidence indicating that molybdenum trafficking for FeMo-co synthesis involves the specific donation of molybdenum from NifQ to NifEN/NifH. Although genetic and biochemical evidence shows that excess molybdate can substitute for NifQ, this protein has an essential role when the levels of molybdenum available for FeMo-co synthesis are low. This result accredits NifQ as a physiological molybdenum donor and suggests that a putative NifEN/NifH complex might be the molybdenum acceptor during FeMo-co biosynthesis. The chemistry underlying this process and specifically whether or not NifQ transfers the entire [Mo–3Fe–4S] cluster remains to be elucidated.

Experimental Procedures

Expression and Purification of NifQ from *A. vinelandii*. NifQ was purified from cell-free extracts of *A. vinelandii* strain UW300 (P$_{nifH}$::*his$_9$-nifQ*) by affinity chromatography to a Co^{2+} resin (Talon resin, Clontech) under anaerobic conditions inside a glove box. Buffers for protein purification and analysis were sparged with purified N$_2$ for 20–30 min. The *A. vinelandii* cell-free extracts were prepared by osmotic shock according to ref. 40, followed by centrifugation at 30,000 × *g* for 1 h to remove cell debris. Cell-free extracts were loaded onto a 20-ml Co^{2+}-affinity column equilibrated in buffer A [10 mM sodium phosphate, 1.8 mM potassium phosphate buffer (pH 7.3), 140 mM NaCl, 2.7 mM KCl, 10% glycerol]. The column was washed with 200 ml of buffer B [50 mM Tris-HCl buffer (pH 7.9), 500 mM NaCl, 25 mM imidazole], and the NifQ protein was eluted from the column by applying 40 ml of buffer C [50 mM Tris-HCl buffer (pH 7.9), 150 mM NaCl, 300 mM imidazole]. Eluted NifQ was concentrated by ultrafiltration through a YM10 membrane in an Amicon cell under a N$_2$ atmosphere and then subjected to a cycle of dilution-concentration in buffer D [50 mM Tris-HCl buffer (pH 7.9), 150 mM NaCl] to remove the residual imidazole. A typical purification procedure yielded 100 mg of NifQ from 340 g of UW300 cell paste. NifQ preparations were estimated to be >95% pure based on SDS/PAGE analysis. The preparations of purified NifQ were frozen as droplets into liquid nitrogen until use.

***In Vitro* FeMo-co Synthesis Assays with Purified Components.** The *in vitro* FeMo-co synthesis reactions were performed in 9-ml serum vials sealed with rubber stoppers. All of the vials were acid-washed overnight in 4 M HCl and thoroughly rinsed with Milli-Q water (Millipore) before use. The vials were repeatedly evacuated and flushed with argon gas and finally rinsed with 0.3 ml of anaerobic 25 mM Tris-HCl (pH 7.5), 1 mM DTH. The complete reactions contained 25 mM Tris-HCl (pH 7.5), 0.182 mM homocitrate, 2.92 mM DTH, 3.3% glycerol, an ATP-regenerating mixture (1.32 mM ATP, 18.7 mM phosphocreatine, 2.27 mM MgCl$_2$, 45.4 μg/ml creatine phosphokinase), 150 μg of NifH (2.4 nmol), 100 μg of apo-NifDK (0.43 nmol), and 100 μg of NifEN (0.48 nmol) as a source of FeMo-co precursor (purified NifEN protein carries the VK-cluster). When indicated, Na$_2$MoO$_4$, purified NifQ, rNifQ, or NarB were used as a source of molybdenum in the reaction. The reactions (total volume

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of 350 μl) were incubated at 30°C for 35 min to allow for the FeMo-co synthesis and insertion reactions. The resulting activation of apo-NifDK was analyzed by the acetylene reduction assay after addition of 0.2 mg of NifH and 0.8 ml of ATP-regenerating mixture (41). Ammonia production by activated apo-NifDK was determined as described (14).

Analysis of Molybdenum Transfer Among NifQ, NifH, and NifEN. *In vitro* FeMo-co synthesis mixtures were scaled up ≈20-fold to a volume of 7 ml, keeping the concentration of all chemicals constant. Na$_2$MoO$_4$ was omitted from the reaction mixtures, and all vials were acid-washed and thoroughly rinsed with Milli-Q water to remove molybdenum traces. Two hundred nanomoles of NifQ were mixed with either (*i*) 112 nmol of NifH, (*ii*) 9.6 nmol of NifEN, (*iii*) 112 nmol of NifH plus 9.6 nmol of NifEN, or (*iv*) 112 nmol of NifH, 9.6 nmol of NifEN, and 8.6 nmol of apo-NifDK. Reactions mixtures were incubated for 35 min at 30°C under conditions of *in vitro* FeMo-co synthesis, and then the proteins in each mixture were separated by chromatography as follows. NifQ and NifH mixtures were separated by chromatography in Co^{2+} affinity resin, which binds NifQ but not NifH. NifH was then desalted by gel filtration on a PD-10 column (GE Healthcare). NifQ and NifEN were separated by preparative gel filtration on a HiLoad 16/60 Sephadex 200 PG column (GE Healthcare). NifQ, NifH, and NifEN were separated by sequential chromatography in Co^{2+} affinity resin and preparative gel filtration on a HiLoad 16/60 Sephadex 200 PG column. Finally, when the mixture contained NifQ, NifH, NifEN, and apo-NifDK, NifQ was separated by affinity chromatography on a Co^{2+} resin followed by preparative

gel filtration on a HiLoad 16/60 Sephadex 200 PG column. In all cases, reisolated proteins were concentrated in Centricon devices (Amicon) and stored as droplets in liquid nitrogen.

To determine the molybdenum content associated with each isolated component, protein samples were heat-denatured and centrifuged, and the supernatant was analyzed by ICP-OES as described in *SI Experimental Procedures*.

EPR Spectroscopy. X-band CW-EPR spectra were obtained by using a Bruker ECS106 spectrometer equipped with an ER4116DM dual-mode X-band cavity. Cryogenic temperature was obtained by using an Oxford Instrument ESR900 liquid helium flow cryostat, and temperature was controlled by using an ITC503 temperature controller (Oxford Instruments). ESE-detected EPR spectra were recorded on the Bruker Elexsys using the EN4118x-MD4 probe. ESE-EPR was collected by using the two-pulse sequence $\pi/2-\tau-\pi-\tau-$ echo. The $\pi/2$ pulse was set to 16 ns, and τ ranged from 136–144 ns.

Additional methods, including the strains used in this work (Table S2), are detailed in *SI Experimental Procedures*.

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