Purification of a NifEN Protein Complex That Contains Bound Molybdenum and a FeMo-Co Precursor from an Azotobacter vinelandii ΔnifHDK Strain*

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The NifEN protein complex serves as a molecular scaffold where some of the steps for the assembly of the iron-molybdenum cofactor (FeMo-co) of nitrogenase take place. A His-tagged version of the NifEN complex has been previously purified and shown to carry two identical [4Fe-4S] clusters of unknown function and a [Fe-S]-containing FeMo-co precursor. We have improved the purification of the his-NifEN protein from a ΔnifHDK strain of Azotobacter vinelandii and have found that the amounts of iron and molybdenum within NifEN were significantly higher than those reported previously. In an in vitro FeMo-co synthesis system with purified components, the NifEN protein served as a source of both molybdenum and a [Fe-S]-containing FeMo-co precursor, showing significant FeMo-co synthesis activity in the absence of externally added molybdate. Thus, the NifEN scaffold protein, purified from ΔnifHDK background, contained the Nif-Bco-derived Fe-S cluster and molybdenum, although these FeMo-co constituents were present at different levels within the protein complex.

The molybdenum nitrogenase performs the majority of biological nitrogen fixation (N₂ → NH₃) and has two component [Fe-S] proteins: dinitrogenase (also termed MoFe protein) and dinitrogenase reductase (also termed Fe protein) (1, 2). Dinitrogenase is a 230-kDa α₃β₃ tetramer of the nifD and nifK gene products that contains one iron-molybdenum cofactor (FeMo-co) and one P-cluster per dimer (3, 4). Dinitrogenase reductase is a 63-kDa α₃ dimer of the nifH gene product that contains a single [4Fe-4S] cluster coordinated between the two subunits (5). FeMo-co, found within the active site of NifDK, is one of the most complex metalloclusters known in biology, and is composed of seven iron atoms, nine sulfur atoms, one molybdenum, one R-homocitrate, and one light atom (nitrogen, oxygen, or carbon) (6–9).

Several nitrogen fixation (nif) genes have been shown to be involved in FeMo-co biosynthesis and the formation of an active nitrogenase enzyme (see Refs. 10–12 for recent reviews). The current data supports the following model for FeMo-co biosynthesis: (i) The SAM-radical protein NifB generates NifB-co, an [Fe-S] cluster of unidentified structure that serves as FeMo-co precursor (13, 14). Whether NifB-co is the only iron and sulfur source to FeMo-co is not yet known. (ii) NifB-co is then transferred to NifEN, a scaffold protein upon which further steps of FeMo-co synthesis occur. The original proposal of NifEN as scaffold for FeMo-co synthesis (15, 16) arose from the observations that there is significant sequence conservation between NifEN and NifDK, there is a mutual stability requirement by NifE and NifN, and the fact that the structural genes for dinitrogenase (NifDK) were not required for FeMo-co synthesis. NifX has the ability to bind NifB-co and it is possible that NifX is involved in NifB-co transfer. (iii) Molybdenum is then added to a FeMo-co precursor, probably in a NifH-dependent fashion (17). Presumably, molybdenum has been previously processed by the activity of NifQ (18). The physical site of molybdenum incorporation into the FeMo-co precursor has been subject of some debate. Radiolabeling experiments showed [⁹⁹Mo label accumulating on both NifH and NifEN when, NifB-co, MgATP, ²⁹Mo, and DTH were combined with these two proteins in an in vitro reaction mix (19). However, from the inspection of the three-dimensional structure of NifH it is not obvious how this protein could coordinate any type of FeMo-co precursor. On the other hand, no molybdenum has been detected in purified preparations of NifEN, making it difficult to establish it as the site where molybdenum is incorporated into a FeMo-co precursor (20–23). (iv) Homocitrate, the metabolic product of NifV, is incorporated into the precursor to generate FeMo-co (24). (v) FeMo-co then binds to NafY, the product of a non-nif gene that also stabilizes the FeMo-co-deficient apoNifDK, and is finally inserted into apoNifDK to make it catalytically competent for N₂ reduction (25–27).

The first purification of the Azotobacter vinelandii NifEN complex was reported by Roberts and co-workers (20). NifEN was shown to be a 200-kDa α₃β₃ tetramer containing some type of [Fe-S] cluster. Purified NifEN did not function catalytically in an in vitro FeMo-co synthesis system, and the authors suggested that some moiety from NifEN (probably iron of sulfur) was being consumed during the FeMo-co synthesis reaction. A His-tagged NifEN complex was subsequently overexpressed...
Accumulation of Molybdenum in NifEN

in a ΔnifHDK genetic background and purified by Dean and co-workers (21). A faster purification protocol for the His-tagged NifEN was developed allowing a more thorough characterization of the protein. NifEN was shown to contain two identical [4Fe-4S] clusters having an S = 1/2 EPR signal in the dithionite-reduced form that are referred to as the permanent clusters of NifEN. In addition, the authors observed the presence of a different S = 1/2 EPR signal when the enzyme was oxidized by thionine, and suggested it could arise from a FeMo-co precursor accumulated on NifEN (22). Their suggestion was supported by two additional observations: first, NifEN contains more iron than expected to account for the permanent [4Fe-4S] clusters. Second, it had been shown before that NifEN mobility in native gels was dependent on the presence of functional nifB and nifH genes (28). The NifEN species accumulated in a ΔnifHDK genetic background was associated with a FeMo-co precursor activity and had different mobility than the NifEN species accumulated in a ΔnifB ΔnifHDK genetic background. In vitro work with 99Mo labeled NifB-co had showed the specific binding of NifB-co to NifEN (29), and it seemed that further processing of FeMo-co precursors on NifEN required the activity of NiF. Goodwin’s observations on the presence of a FeMo-co precursor on NifEN have been recently confirmed by work at another laboratory (23). In contrast, there is no evidence of molybdenum accumulation on NifEN along with a FeMo-co precursor in a ΔnifHDK genetic background. Rangaraj et al. (19) have recently reported that the incorporation of 99Mo on NifEN in vitro is dependent on the presence of NiF, NifB-co, DTH, and ATP in a FeMo-co synthesis assay mixture.

We show here that the NifEN protein, purified by Co2+ affinity chromatography from a ΔnifHDK A. vinelandii strain, contains a FeMo-co precursor and bound molybdenum. Moreover, in vitro FeMo-co synthesis assays show that the molybdenum accumulated within the purified NifEN protein is suitable for FeMo-co synthesis, suggesting that NifEN serves as the entry point for molybdenum into the FeMo-co synthesis pathway.

EXPERIMENTAL PROCEDURES

Buffers—Buffers for cell breakage, protein purification, and protein assays were made anaerobic by sparging with N2 for 30 min, followed by alternate cycles of vacuum and flushing with argon, and the addition of sodium dithionite (DTH) to a final concentration of 1 mM, unless otherwise specified. Buffers used for obtaining A. vinelandii cell-free extracts contained 0.5 μg/ml leupeptin and 0.2 mM phenylmethylsulfonyl fluoride.

A. vinelandii Strains and Growth Conditions—A. vinelandii strains UW45 (nifB, (30)), DJ35 (ΔnifE, (31)), DJ1143 (ΔnifB his-nifHΔDK) (32), and DJ1041 (ΔnifHDKTYorf1orf2 his-nifE) (21) have been previously described. Strain UW243 (ΔnifHDKTYorf1orf2 ΔnifB::Km’ his-nifE) is a derivative of strain DJ1041 in which the entire nifB gene has been replaced by a Km’ cassette from plasmid pUC4K (Amersham Biosciences). Strains DJ1041, DJ1143, and DJ35 were obtained from D. R. Dean, Virginia Tech. Strains DJ1041 and UW243 were cultivated in a 250-liter fermentor (IF-250; New Brunswick Scientific) in 200-liter batches of modified Burk’s medium with limiting ammonium (80 μg/ml N). Cells were collected by centrifugation 3 h after ammonium exhaustion from the medium.

When tungsten growth conditions were required, A. vinelandii strains DJ35 and UW45 were cultivated in 20-l carboys and derepressed for nitrogenase expression in molybdenum-free medium supplemented with 1 mM Na2WO4 as previously described (33). Molybdenum-free medium was obtained by omitting Na2MoO4, using ultrapure chemicals, and treating all glassware with 4 M HCl followed by extensive washing with nanopure water. Bacterial growth was estimated from the light scattering of culture samples at 600 nm with a Shimadzu UV-1601 spectrophotometer.

Preparation of A. vinelandii Cell Extracts—Cell-free extracts of A. vinelandii were prepared by osmotic shock according to Ref. 34 followed by centrifugation at 30,000 × g for 1 h to remove cell debris. For the removal of low molecular weight components from the A. vinelandii UW45 or DJ35 cell-free extracts, 55-ml aliquots of the extracts were anaerobically desalted by passage through four HiPrep 26/10 columns (Amersham Biosciences) serially connected to an FPLC apparatus (Amersham Biosciences). The desalting chromatography was performed in 25 mM Tris buffer, pH 7.4, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 1 mM DTH.

Purification of NifEN by Co2+ Affinity Chromatography—The poly(His)-tagged NifEN was purified from the DJ1041 cell-free extract by affinity chromatography to a Co2+ resin (Talon resin, Clontech). All purification steps were performed under an anaerobic N2 atmosphere at 4 °C. Cell-free extracts from 250 g of cells of A. vinelandii strain DJ1041 were supplemented with 300 mM NaCl and 10% glycerol, and loaded onto a 1.5 cm × 35 cm column of Co2+ charged resin. The column had been previously equilibrated in buffer A (25 mM Tris buffer, pH 7.9, 300 mM NaCl, 20% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 1 mM DTH). The column was then washed with 150 ml of buffer A followed by 750 ml of buffer A supplemented with 10 mM imidazole. The NifEN protein was eluted with 150 ml of buffer A supplemented with 500 mM imidazole. Imidazole was removed by passage through a series of HiPrep 26/10 desalting columns (Amersham Biosciences) connected to an FPLC apparatus (Amersham Biosciences). The desalting chromatography was performed in buffer A. The fractions containing NifEN protein were pooled and concentrated by ultrafiltration through a 100-kDa cutoff membrane, YM100, in an Amicon cell (Millipore Corp.) inside an anaerobic glove box. NifEN preparations in buffer A were stored under liquid nitrogen until used. The average NifEN purification yield was 700 mg of NifEN protein per 250 g of DJ1041 cell paste. NifEN was estimated to be >98% pure by SDS-PAGE.

To purify NifEN in the presence of W, buffer A was supplemented with 10 mM Na2WO4 during the Co2+ affinity chromatography and a similar purification procedure was used. The eluted NifEN protein was desalted using a HiPrep 26/10 desalting column equilibrated in buffer A, concentrated by ultrafiltration in an Amicon cell, and stored under liquid nitrogen in buffer A.
To purify NifEN loaded with molybdenum in vitro, buffer A was supplemented with 10 mM Na₂MoO₄ during the Co²⁺ affinity chromatography and a similar purification procedure was used. The eluted NifEN protein was desalted using a HiPrep 26/10 desalting column equilibrated in buffer A, concentrated by ultrafiltration as described above and stored under liquid nitrogen in buffer A.

For comparison purposes, the NifEN protein was purified by Zn²⁺ affinity chromatography followed by DEAE-Sepharose chromatography according to Ref. 21 with the modifications introduced by (23). Purified NifEN protein was concentrated by ultrafiltration as described above and stored under liquid nitrogen in buffer A. The average amount of NifEN purified using this procedure was 625 mg of NifEN protein per kg of DJ1041 cell paste.

**Purification of NifEN Not Charged with a FeMo-co Precursor**—Uncharged NifEN was purified from cell-free extracts of *A. vinelandii* strain UW243 by affinity chromatography to a Co²⁺ resin. All purification steps were performed under an anaerobic N₂ atmosphere at 4 °C. The UW243 cell-free extracts were supplemented with 500 mM NaCl and 5% glycerol, and were loaded onto a 20-ml Co²⁺-affinity column equilibrated in buffer A. The column was washed with 200 ml of buffer A supplemented with 10 mM imidazole, and the NifEN protein was eluted from the column by passing with 40 ml of buffer A supplemented with 300 mM imidazole. Eluted NifEN was pooled, concentrated by ultrafiltration through a YM100 membrane in an Amicon cell under a N₂ atmosphere, and then subjected to gel-filtration chromatography on a PD-10 column (Amersham Biosciences) to remove the residual imidazole and to exchange gel-filtration chromatography on a PD-10 column (Amersham Co²⁺ regenerating mixture (containing 3.6 mM ATP, 6.3 mM MgCl₂, A. vinelandii sor) for affinity chromatography as described above and stored under liquid nitrogen in buffer A. The average amount of NifEN purified using this procedure was 625 mg of NifEN protein per kg of DJ1041 cell paste.

**Purification of Other Components**—NifH and NifDK were purified from UW cells as previously described (4). A poly(His)-tagged apoNifDK form was purified from cells of strain DJ1143 as described (32). Methods for the purification of FeMo-co (9) and NifB-co (13) have been previously described.

**In Vitro FeMo-co Insertion and FeMo-co Synthesis and Insertion Assays**—FeMo-co insertion assays were performed as described in (33). NifB-co-dependent in vitro synthesis of FeMo-co assays were performed as described in Ref. 13 with modifications. The reactions were carried out in stopped 9-ml serum vials under argon. The complete reaction mixtures contained: 100 μl of 25 mM Tris-HCl buffer, pH 7.4, 10 μl of 1 mM Na₂MoO₄, 20 μl of 5 mM homocitrate, 200 μl of ATP-regenerating mixture (containing 3.6 mM ATP, 6.3 mM MgCl₂, 51 mM creatine phosphate, 20 units/ml creatine phosphokinase, and 6.3 mM DTH), 10 μl of a solution containing NifB-co (equivalent to 4 nmol of Fe), 200 μl of *A. vinelandii* cell-free extract (~3 mg of protein from UW45 or DJ35 W-grown cells), and 500 μg of purified NifEN in a total volume of 575 μl. The reaction mixtures were preincubated at room temperature for 10 min before the addition of NifB-co and any protein component, and for 30 min at 30°C after addition of protein components to allow for the FeMo-co synthesis and insertion reactions. The resulting activation of apoNifDK present in *A. vinelandii* extract was analyzed by the acetylene reduction assay after adding 0.8 ml of ATP-regenerating mixture and an excess of purified NifH (120 μg of protein) (4). Activities for FeMo-co synthesis and insertion are presented in terms of NifDK activities defined as ethylene formed-min⁻¹-assay⁻¹.

When FeMo-co synthesis assays were performed with only purified components, apoNifDK (100 μg of protein), and NifH (120 μg of protein) replaced the *A. vinelandii* crude extract in the reaction mixture. The total volume of the purified system reaction mixture was 395 μl.

When purified NifEN was used as sole source of molybdenum for FeMo-co synthesis, Na₂MoO₄ was omitted from the complete reaction mixture and 0.5–2.5 nmol (100–500 μg) of heat-denatured NifEN protein (denatured by 1 h of incubation at 70 °C) were added to the mixture. It is important to note that 50 μg of active NifEN was still added to the reaction mixture to perform FeMo-co synthesis. When NifH, uncharged NifEN, or apoNifDK was tested as internal source of molybdenum for FeMo-co synthesis Na₂MoO₄ was omitted from the complete reaction mixture and 0.5–2.5 nmol of heat-denatured protein were added to the mixture.

**EPR Analysis**—EPR spectra were recorded in the laboratory of Professor David Britt, on a Bruker ECS-106 spectrometer equipped with an ER 4116 dual-mode X-band cavity and Oxford Instruments ESR-900 helium flow cryostat. Perpendicular mode EPR spectra were recorded using a microwave frequency of 9.69 GHz, at 11 ± 0.2 K, a modulation frequency of 100 kHz, a modulation amplitude of 1.0 mT (10 Gauss), a sweep rate of 12 mT/s, and a and a microwave power of 10 milliwatt. The program IGOR Pro (WaveMetrics, Lake Oswego, OR) was used for all subsequent handling of spectral data.

**Protein Assays**—Protein concentrations were determined by the bicinchoninic acid method using bovine serum albumin as standard (35). The procedures for SDS-PAGE has been described (36).

**Metal Analysis**—Iron and molybdenum contents in purified NifEN preparations were determined by ICP-OES. Concentrated samples of purified NifEN, NifH or NifDK protein (250 μl, containing ~7.5 mg of protein) were diluted with 250 μl of buffer A (see above) supplemented with 1% NMF and 2% of nitric acid. The mixture was heated at 99 °C for 30 min and subjected to periodical vortexing to ensure full analyte recovery. The precipitated protein was pelleted out by centrifugation at 5,000 rpm for 10 min, and the supernatant was placed into a 1.5-ml microfuge tube and introduced into the ICP-OES system (Optima 5X00™DV ICP-OES) via a peristaltic pump. The sample was aspirated with humidified argon, passed onto the plasma, and analyzed for iron and molybdenum. Commercial calibration standards and multi-element standards were used.

**RESULTS**

**Purification of NifEN**—Poly(His)-tagged NifEN was purified to ~98% homogeneity from *A. vinelandii* strain DJ1041 using Co²⁺ affinity chromatography immediately followed by desalting on a HiPrep gel filtration column (Fig. 1). Approximately 700 mg of NifEN protein can be purified from 250 g of cell paste in a typical purification. As described in more detail below, the
Accumulation of Molybdenum in NifEN

NifEN protein purified by Co$^{2+}$ affinity chromatography has important differences in FeMo-co synthesis activity, the content of bound molybdenum and iron, and its substrate requirements in comparison to previously reported preparations of NifEN purified by Zn$^{2+}$ affinity chromatography (21, 23). These differences are significant in the context of the biochemical pathway for FeMo-co synthesis.

Metal Contents of NifEN—The [Fe-S] clusters of NifEN are labile and the quality of NifEN preparations is very sensitive to the purification procedure employed. For example, the fast purification procedure described in Ref. 23 yielded NifEN with higher iron content than that described in Ref. 20, which was obtained after a longer procedure. The NifEN preparations purified by Co$^{2+}$ affinity were analyzed by ICP-OES and were found to contain $24 \pm 1$ mol of iron per mol of tetrameric NifEN (Table 1). This amount of iron in NifEN is significantly higher than previously reported (20, 21, 23) and may reflect differences in the number or type of [Fe-S] clusters present on this protein.

In addition, the Co$^{2+}$ affinity-purified NifEN contained $0.34 \pm 0.05$ mol of molybdenum per NifEN tetramer, whereas concurrent analyses of buffers were found to have below detectable limits of molybdenum ($<0.001 \text{ nm}$). The presence of molybdenum within the NifEN protein complex purified in this study contrasts with previous reports of metal content analysis of NifEN that did not find molybdenum (21, 23, 37), and thus raises questions about the relevance of the bound molybdenum for FeMo-co synthesis.

To verify that the molybdenum bound to NifEN was not spurious, the NifEN protein was additionally purified by three alternative procedures: (i) in the presence of $10 \text{ mm} \text{ Na}_2\text{WO}_4$ during the Co$^{2+}$ affinity chromatography; (ii) by using Zn$^{2+}$ affinity chromatography as previously reported (21, 23); and (iii) in the presence of $10 \text{ mm} \text{ Na}_2\text{MoO}_4$ during the Co$^{2+}$ affinity chromatography and then desalted to remove unbound molybdate. Tungstate has been shown to inhibit the diazotrophic growth of A. vinelandii by inhibiting molybdate uptake into the cell, and adversely affecting an undetermined step during the course of FeMo-co synthesis or insertion into the immature apoNifDK. The NifEN protein isolated by Co$^{2+}$ affinity chromatography contained very low amounts of bound molybdenum (0.06 mol of molybdenum per tetramer of NifEN). NifEN purified by Zn$^{2+}$ affinity followed by DEAE-Sepharose chromatography, as previously reported (21, 23).

TABLE 1

| Protein            | Metal content | Iron (mol per mol of protein) | Molybdenum (mol per mol of protein) |
|--------------------|---------------|------------------------------|-------------------------------------|
| NifEN (Co)$^{4+}$  | 24 $\pm$ 1    | 0.34 $\pm$ 0.05              |
| + 10 mol Na$_2$WO$_4$ | 27 $\pm$ 2    | 0.12 $\pm$ 0.01              |
| + 10 mol Na$_2$MoO$_4$ | 27 $\pm$ 1    | 4.25 $\pm$ 0.25              |
| NifEN (Zn)$^{2+}$  | 16 $\pm$ 1    | 0.06 $\pm$ 0.01              |
| ApoNifDK$^{5+}$    | 14.5 $\pm$ 0.2 | 0.031 $\pm$ 0.004            |
| NifH$^{+}$         | 4.2 $\pm$ 0.1 | 0.003 $\pm$ 0.001            |
| NifDK$^{+}$        | 32 $\pm$ 1    | 2.20 $\pm$ 0.09              |

$^{4+}$ NifEN was purified by Co$^{2+}$ affinity chromatography.
$^{5+}$ NifEN was purified by Co$^{2+}$ affinity chromatography in the presence of 10 mm Na$_2$WO$_4$ added to the buffers.
$^{5+}$ NifEN was purified by Co$^{2+}$ affinity chromatography in the presence of 10 mm Na$_2$MoO$_4$ added to the buffers.
$^{5+}$ NifEN was purified by Zn$^{2+}$ affinity followed by DEAE-Sepharose chromatography, as previously reported (21, 23).
$^{5+}$ ApoNifDK was purified from strain DJ1143 as reported (32).
$^{5+}$ NifDK and NifH were purified from cells of A. vinelandii UW (wild-type strain).

EPR Spectroscopy of NifEN—Previous EPR spectroscopic analyses of A. vinelandii NifEN have shown that this protein complex is likely to contain more than one type of [Fe-S] clusters. Fig. 2 shows the X-band EPR spectra of NifEN in the DTH-reduced state (Fig. 2, trace 1) and in the indigo Carmine (or thionine) oxidized state (Fig. 2, trace 2), as originally shown by Goodwin et al. (21), and recently reproduced by Hu et al. (23).

The DTH-reduced state exhibits a complex signal having major components in the 100–180 G region (trace 1), as originally shown by Goodwin et al. (21), and recently reproduced by Hu et al. (23). The DTH-reduced state exhibits a complex signal having major components in the 100–180 G region (trace 1), as originally shown by Goodwin et al. (21), and recently reproduced by Hu et al. (23). The DTH-reduced state exhibits a complex signal having major components in the 100–180 G region (trace 1), as originally shown by Goodwin et al. (21), and recently reproduced by Hu et al. (23). The DTH-reduced state exhibits a complex signal having major components in the 100–180 G region (trace 1), as originally shown by Goodwin et al. (21), and recently reproduced by Hu et al. (23). The DTH-reduced state exhibits a complex signal having major components in the 100–180 G region (trace 1), as originally shown by Goodwin et al. (21), and recently reproduced by Hu et al. (23). The DTH-reduced state exhibits a complex signal having major components in the 100–180 G region (trace 1), as originally shown by Goodwin et al. (21), and recently reproduced by Hu et al. (23). The DTH-reduced state exhibits a complex signal having major components in the 100–180 G region (trace 1), as originally shown by Goodwin et al. (21), and recently reproduced by Hu et al. (23).
FeMo-co Synthesis Activity of NifEN Purified by Co\textsuperscript{2+} Affinity Chromatography—We examined the ability of purified NifEN protein to support FeMo-co synthesis and compared it against other reported NifEN preparations. First, NifEN was assayed by its ability to restore FeMo-co synthesis activity in vitro in a reaction containing an A. vinelandii cell-free extract lacking endogenous NifE (strain DJ35). A complete reaction mixture (described in “Experimental Procedures”) contained a cell-free extract from W-grown DJ35 cells, MgATP, R-homocitrate, molybdate, along with purified NifEN, NifH, and NifB-co, and couples the synthesis of FeMo-co to its incorporation into the apoNifDK present in the DJ35 extract. The amount of reconstituted NifDK protein was measured by its activity reducing acetylene to ethylene. The NifEN purified by Co\textsuperscript{2+} affinity was able to complement the DJ35 extract in this reaction and supported FeMo-co synthesis, yielding a NifDK activity of 92.7 ± 2.1 nmol of ethylene formed min\textsuperscript{-1} assay\textsuperscript{-1} (Table 2). A similar result was obtained when NifB-co is omitted from the reaction that used a DJ35 cell-free extract, suggesting that a FeMo-co precursor was present in the reaction that could substitute for NifB-co.

Second, we examined whether the purified NifEN protein contained a bound FeMo-co precursor that serves as a replacement for NifB-co, as previously shown by (22, 23). We performed an analogous analysis with cell-free extracts of A. vine-

TABLE 2
FeMo-co synthesis activity of NifEN purified by Co\textsuperscript{2+} affinity chromatography

The reactions were carried out in stopped 9-ml serum vials under argon as described (13). The resulting activation of apoNifDK was analyzed by the acetylene reduction assay following standard procedures (4). Values are the average of at least two independent determinations ± S.D.

| Assay Condition | Activity | DJ35\textsuperscript{a} | UW45\textsuperscript{a} | Purified system\textsuperscript{b} |
|-----------------|----------|--------------------------|--------------------------|-----------------------------------|
|                 | nmol of C\textsubscript{2}H\textsubscript{4} formed min\textsuperscript{-1} | | | |
| Complete | 92.7 ± 2.1 | 26.5 ± 0.8 | 91.0 ± 1.0 | |
| -NifB-co | 94.8 ± 2.0 | 30.5 ± 2.6 | 94.7 ± 6.3 | |
| -NifEN | 0.0 | 152 ± 0.6 | 0.0 | |
| -Molybdate | 7.4 ± 0.2 | 3.9 ± 0.2 | 7.5 ± 0.2 | |
| -R-homocitrate | 6.5 ± 0.1 | 3.5 ± 0.2 | 4.4 ± 0.3 | |
| -Molybdate, R-homocitrate | 3.5 ± 0.2 | 3.6 ± 0.1 | 4.3 ± 0.4 | |
| -Molybdate, R-homocitrate, NifB-co | 3.3 ± 0.3 | 3.7 ± 0.2 | 4.5 ± 0.3 | |

\textsuperscript{a} The complete reaction mixture contains Na\textsubscript{2}MoO\textsubscript{4}, homocitrate, MgATP, and DH\textsubscript{4}, as described under “Experimental Procedures,” plus 10 µl of a NifB-co solution (containing 4 nmol of Fe). 500 µg of pure NifEN, 120 µg of NifH, and 200 µl of either DJ35 (ΔnifE, 2.4 mg of protein) or UW45 (nifB\textsuperscript{-}, 3 mg of protein) cell-free extract in a total volume of 575 µl.

\textsuperscript{b} Purified components were used in place of cell-free extracts. ApoNifDK (100 µg of protein), NifH (120 µg of protein), and 500 µg of NifEN were added to the reaction mixture in a total volume of 395 µl.

landii strain UW45, which carries a point mutation on nifB and is incapable of synthesizing NifB-co. It is known that the addition of purified NifB-co to an extract of UW45 complements FeMo-co synthesis activity (13). Addition of 0.5 mg of pure NifEN to an UW45 cell-free extract in a reaction mixture lacking NifB-co yielded a NifDK activity of 30.5 ± 2.6 nmol of ethylene formed min\textsuperscript{-1} assay\textsuperscript{-1}, indicating that NifEN purified by Co\textsuperscript{2+} affinity does have a FeMo-co precursor bound and is able to replace the NifB-co activity. Similar values were obtained when this reaction was supplemented with NifB-co, suggesting that purified NifEN was unable to perform multiple turnovers in the in vitro FeMo-co synthesis assay. This observation is in agreement with previous results (20).

Third, FeMo-co synthesis activity was tested in a reaction mixture in which purified NifEN, NifH, and apoNifDK proteins substitute for the A. vinelandii cell-free extract. The purified system reconstitutes NifDK to an activity of 91.0 ± 1.0 nmol of ethylene formed min\textsuperscript{-1} assay\textsuperscript{-1} (equivalent to 910 nmol of ethylene formed min\textsuperscript{-1} mg NifDK\textsuperscript{-1}). Similar NifDK activity (94.7 ± 6.3 nmol of ethylene formed min\textsuperscript{-1} assay\textsuperscript{-1}) was obtained when NifB-co was omitted from the reaction mixture as an additional source of FeMo-co precursor. This confirms that our NifEN preparations can serve as source of FeMo-co precursor.

Along with the absolute protein requirements of apoNifDK, NifH, and a precursor-loaded NifEN, the FeMo-co synthesis reaction requires molybdate, MgATP, and R-homocitrate as co-substrates. Below we show that NifEN purified by Co\textsuperscript{2+} affinity chromatography could serve as a source of both molybdenum and an organic acid (presumably R-homocitrate) in addition to being a source of the iron and sulfur-containing FeMo-co precursor. In FeMo-co synthesis reactions not supplemented with exogenous Na\textsubscript{2}MoO\textsubscript{4} acetyledehyde reduction activities of 7.4 ± 0.2 or 3.9 ± 0.2 nmol of ethylene formed min\textsuperscript{-1} assay\textsuperscript{-1} were obtained for reactions using the DJ35 or UW45 cell-free extracts, respectively (Table 2). To eliminate the possibility that the extracts were serving as a
source of molybdenum, both the DJ35 and UW45 extracts used for these experiments were obtained from A. vinelandii cells repeatedly grown in media devoid of Na₂MoO₄ and instead supplemented with 1 mm Na₂WO₄ to deplete the intracellular molybdenum pools. Both the DJ35 and UW45 cell-free extracts were verified to contain virtually no molybdenum by ICP-OES (less than 0.004 ppm of molybdenum). For the in vitro FeMo-co synthesis reactions using only purified components, the chances of molybdenum contamination from the individual protein components are very small. A NifDK activity of 7.5 ± 0.2 nmol of ethylene formed·min⁻¹·assay⁻¹ was obtained from a FeMo-co synthesis reaction using only purified components and not supplemented with Na₂MoO₄ (Table 2). It appears that the Co²⁺ affinity purified NifEN was serving as source of molybdenum and an Fe-S-containing precursor that became parts of FeMo-co. Furthermore, a reaction performed without adding R-homocitrate yielded 5% of NifDK activity compared with the control reaction (as determined by the acetylene reduction assay). The amount of FeMo-co synthesis was linearly proportional to the amount of NifEN used in reactions without added homocitrate, suggesting that our NifEN preparations also contained a bound organic acid (possibly R-homocitrate), which can be utilized to synthesize FeMo-co in vitro (data not shown). Prior studies have shown that many organic acids can partially substitute for R-homocitrate in the FeMo-co synthesis reactions to produce a cofactor with altered catalytic properties and substrate specificities (41). Similar levels of acetylene reduction were observed when NifB-co, homocitrate, and Na₂MoO₄ were omitted from the reaction. These activities were significantly above the error of measurement or the negative control assay lacking the NifEN protein that shows negligible ethylene production. Additional experiments would be necessary to determine whether the purified NifEN protein contains low amounts of bound R-homocitrate or another organic acid.

As mentioned above, the amount of molybdenum present in purified NifEN preparations could be increased to 4 mol of molybdenum per mol of NifEN tetramer by incubation of the protein with excess Na₂MoO₄ in vitro followed by removal of unbound molybdate. Table 3 shows that the FeMo-co synthesis activity of molybdenum-loaded NifEN in a reaction lacking exogenous Na₂MoO₄ was 80% of a reaction supplemented with an excess of exogenous Na₂MoO₄.

**NifEN as a Source of Molybdenum for FeMo-co Synthesis—**

NifEN was used in increasing amounts in FeMo-co synthesis reactions with purified components, either in the presence of 18 µM Na₂MoO₄ (Fig. 3A) or lacking exogenously added molybdenum (Fig. 3B). There was no difference in acetylene reduction activity when NifB-co was added as a supplement to the reaction (△) in comparison to the reactions with no NifB-co (△), suggesting that all of the active NifEN added to the reaction was loaded with a FeMo-co precursor or that it was unable to process additional NifB-co. When no Na₂MoO₄ was added to the reaction, FeMo-co synthesis still occurred to allow activation of the immature apoNifDK, and the acetylene reduction activity increased with increasing amounts of NifEN used in the reaction (Fig. 3B). While the NifDK activities in the reactions without added Na₂MoO₄ were significantly lower than in the reactions having 18 µM Na₂MoO₄, the proportional increase of acetylene reduction activity with increasing amounts of NifEN indicates that NifEN was a source of molybdenum in these reactions.

We conducted further experiments to conclusively find the molybdenum source that was allowing FeMo-co synthesis in the purified system in the absence of added Na₂MoO₄. Increas-
Accumulation of Molybdenum in NifEN

The final steps of FeMo-co biosynthesis require the incorporation of a molybdenum atom into the cofactor. NifEN and NifH proteins are likely candidates to be involved in the binding of molybdate, its modification, and its eventual ligation to a [Fe-S] cluster destined to become FeMo-co. Although tested repeatedly, a clear demonstration of molybdenum presence within the NifEN protein complex has been elusive. Initial studies using A. vinelandii cell-free extracts incubated with 99Mo failed to detect any radiolabeling on the NifEN position in anoxic-native gel electrophoresis. Likewise, purified preparations of NifEN from a nifB mutant strain or a His-tagged NifEN from a ΔnifHDK strain have been reported not to contain significant amounts of molybdenum. The binding of molybdenum to NifEN has only recently been shown in 99Mo radiolabeling experiments where purified NifEN, NifH, and NifB-co were combined in the presence of 99Mo, DTH, and MgATP. This suggested that the accumulation of molybdenum in NifEN required a reductant and the MgATP-dependent activity of NifH.

The experiments of this report show that, when NifEN is rapidly purified by Co2+ affinity chromatography from cell-free extracts of a ΔnifHDK strain, it contains significant amounts of molybdenum (0.34 mol of molybdenum per mol of NifEN) that can be used for in vitro FeMo-co synthesis. Several experiments were conducted to show that the molybdenum is specifically bound to the NifEN complex and is not a contaminant free in solution or speciously bound to NifEN.

To eliminate the possibility that the molybdenum may be a contaminant present in solution, we purified the NifEN protein from the same ΔnifHDK strain by Zn2+ affinity chromatography and showed that it contained low amounts of molybdenum, reproducing the results published by other laboratories and ruling out the possibility of molybdenum contamination in the buffers. An interesting explanation for the difference in molybdenum content between the Zn2+ and the Co2+ affinity purified NifEN preparation is that the Zn2+ may bind specifically to the same site as molybdenum and displace it. Incubation of the Co2+ affinity-purified NifEN with 1 mM Zn2+, followed by gel filtration, resulted in a NifEN protein showing half of the FeMo-co synthesis activity in the lack of exogenously added molybdenum. The addition of increasing amounts of heat-denatured NifH or apoNifDK did not support FeMo-co synthesis beyond the background level (Fig. 4A). This result indicates that neither NifH nor apoNifDK serve as source of molybdenum in the FeMo-co synthesis assay with purified components and is in agreement with the molybdenum contents of these protein preparations as determined by ICP-OES (Table 1). Altogether, these results demonstrate that the charged NifEN protein complex does contain bound molybdenum, which can be utilized to synthesize FeMo-co in vitro and activate apoNifDK.

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molybdenum (data not shown). The parallel control experiment where NifEN was gel filtered without prior Zn$^{2+}$ treatment showed no loss of activity (data not shown). Importantly, the control experiment where the NifEN apparently lost no molybdenum by gel filtration also indicates that the bound molybdenum was not dissociable during the course of gel filtration. Another significant aspect of the comparison of the NifEN purified by the Co$^{2+}$ or Zn$^{2+}$ affinity protocols is that the NifEN preparations reported here contain higher amounts of iron than previously reported, suggesting a higher cluster content per NifEN, a larger population of NifEN molecules containing [Fe-S] clusters, or both.

Na$_2$WO$_4$ has been shown to inhibit molybdenum-dependent diazotrophic growth of A. vinelandii, and has been suggested to inhibit one or more steps of FeMo-co biosynthesis by competing against molybdate. The purification of NifEN in the presence of 10 mM Na$_2$WO$_4$ rendered a protein with significantly less molybdenum than our standard NifEN preparation, suggesting that Na$_2$WO$_4$ was able to displace the molybdenum bound specifically within the protein complex. Thus, the molybdenum present in the NifEN protein appears to not be a contaminant nor speciously bound, but is still substoichiometric. Importantly, the maximum amount of molybdenum bound to NifEN could be increased to 4 mol of molybdenum per mol NifEN tetramer upon incubation with excess molybdenum in vitro and removal of unbound molybdenum, showing that NifEN can indeed bind stoichiometric amounts of molybdenum.

Additional evidence for the NifEN bound molybdenum being relevant for FeMo-co biosynthesis comes from concurrent XAS studies on the same Co$^{2+}$ affinity-purified NifEN (to be published elsewhere). The quantity of molybdenum present on NifEN has been corroborated by measuring the intensity of K-α fluorescence while exciting with x-ray photons of monochromatic energies above the molybdenum absorption edge. The molybdenum K-edge spectrum indicates that the bound molybdenum is not in the Mo$^{VI}$ oxidation state as in molybdate and the EXAFS analysis show that the molybdenum within NifEN is in a [Fe-S] cluster ligand environment.

Is the molybdenum present in our NifEN preparations part of the NifB-co-derived [Fe-S] cluster that serves as FeMo-co precursor? The data presented in this work do not support this hypothesis. NifEN preparations are loaded with a larger complement of FeMo-co precursor than molybdenum atoms. Metal determinations show that the molybdenum content of NifEN (0.34 mol/mol of NifEN tetramer) is substoichiometric, whereas the iron content (24 mol/mol of NifEN tetramer) is enough to account for the full complement of [Fe-S] clusters, i.e. two permanent [4Fe-4S] clusters and two FeMo-co precursors each one containing 6–8 iron atoms (37). The in vitro FeMo-co synthesis assays confirm this interpretation by showing that the addition of molybdenum, but not NifB-co, to the assay is required to attain maximum synthetic activity. Finally, purification of NifEN by Zn$^{2+}$ affinity chromatography yields a protein preparation that retains the FeMo-co precursor but lacks molybdenum, suggesting their binding are not mutually dependent. Thus, we favor a model in which molybdenum is bound to NifEN at a site that is different from the NifB-co-derived FeMo-co precursor. The molybdenum could be part of an additional [Fe-S] cluster present on NifEN, part of a [3Fe-4S] cluster derived from a permanent [4Fe-4S] cluster, or bound somewhere else in the NifEN protein, and studies are underway to discriminate between these possibilities.

In the context of FeMo-co biosynthesis, the presence of molybdenum in NifEN purified from a ΔnifHDK strain implies that NifH is not essential for the accumulation of molybdenum within the NifEN complex. However, it has been reported that NifH is necessary for the incorporation of molybdenum into the NifB-co-derived FeMo-co precursor (19). These two apparently contradictory observations are compatible if the role of NifH was to catalyze the transfer of molybdenum from one binding site on NifEN into the NifB-co derived FeMo-co precursor. It has also been reported that purified NifH incorporates minimal levels of $^{99}$Mo in the presence of MgATP; NifH accumulates significant levels of $^{99}$Mo only when all the components for FeMo-co synthesis except homocitrate, i.e. NifEN, NifB-co, DTH, and MgATP are present in the reaction mixture (19). Interestingly, we found that pure NifH preparations did not contain molybdenum, as determined by ICP-OES and by in vitro FeMo-co synthesis assays. These observations support a hypothesis in which molybdenum would bind to NifEN independently of NifH while the activity of NifH would possibly be required to incorporate the NifEN-bound molybdenum into the [Fe-S] cluster that serves as FeMo-co precursor.

The FeMo-co synthesis assays with purified components indicate that a fraction of the NifEN protein in the sample contains the [Fe-S] FeMo-co precursor and molybdenum (this fraction is responsible for the 8% of maximum FeMo-co synthesis activity observed in the absence of externally added Na$_2$MoO$_4$) (Table 2). Another fraction of the NifEN protein seems to contain the [Fe-S] FeMo-co precursor and homocitrate (5% of maximum FeMo-co synthesis activity in the absence of externally added homocitrate). If the distribution of homocitrate and molybdenum in the NifEN molecules was random, we would expect that the reaction mixture lacking homocitrate and Na$_2$MoO$_4$ would yield ~0.4% of the maximum FeMo-co synthesis activity. However, the reaction mixture lacking homocitrate and Na$_2$MoO$_4$ exhibited a FeMo-co synthesis activity identical to that of the reaction lacking homocitrate and half of the activity present in the reaction lacking Na$_2$MoO$_4$. Thus, the distribution of homocitrate in the population of NifEN molecules does not seem to be random but it is likely constrained to NifEN that already contain the [Fe-S] FeMo-co precursor and molybdenum. From these data, there is no evidence for a population of NifEN molecules containing homocitrate but no molybdenum. Furthermore, the ΔnifB NifEN protein that is not charged with a NifB-co derived precursor, does not contain bound molybdenum, evidenced by the failure to provide molybdenum in the FeMo-co synthesis assay. While clearly speculative, this suggests that the order of substrate binding to NifEN might be sequential: first the [Fe-S] FeMo-co precursor, second the molybdenum, and finally homocitrate. This pro-

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3 S. George, R. Igarashi, C. Piamonteze, B. Soboh, S. Cramer, and L. M. Rubio, unpublished results.
posed order for substrate binding to NifEN will be probed in future experiments.

In summary, the purification of NifEN was improved by using rapid Co²⁺ affinity chromatography, which yielded a protein having higher amounts of iron than previously reported and significant amounts of bound molybdenum. The finding of molybdenum bound to NifEN supports the previous hypothesis that NiFeN is involved in the incorporation of molybdenum into FeMo-co.

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