Incorporation of Iron and Sulfur from NifB Cofactor into the Iron-Molybdenum Cofactor of Dinitrogenase*

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NifB-co is an iron- and sulfur-containing precursor to the iron-molybdenum cofactor (FeMo-co) of dinitrogenase. The synthesis of NifB-co requires at least the product of the nifB gene. Incorporation of $^{55}$Fe and $^{35}$S from NifB-co into FeMo-co was observed only when all components of the in vitro FeMo-co synthesis system were present. Incorporation of iron and sulfur from NifB-co into dinitrogenase was not observed in control experiments in which the apodinitrogenase (lacking FeMo-co) was initially activated with purified, unlabeled FeMo-co or in assays where NifB-co was oxygen-inactivated prior to addition to the synthesis system. These data clearly demonstrate that iron and sulfur from active NifB-co are specifically incorporated into FeMo-co of dinitrogenase and provide direct biochemical identification of an iron-sulfur precursor of FeMo-co.

Under different in vitro FeMo-co synthesis conditions, iron and sulfur from NifB-co were associated with at least two other proteins (NIFNE and gamma) that are involved in the formation of active dinitrogenase. The results presented here suggest that multiple FeMo-co processing steps might occur on NIFNE and that FeMo-co synthesis is most likely completed prior to the association of FeMo-co with gamma.

The conversion of dinitrogen to ammonium by biological systems is catalyzed by nitrogenase. Nitrogenase is composed of two oxygen-labile metalloproteins: dinitrogenase (also called MoFe protein or component I) and dinitrogenase reductase (also called NIFH, Fe protein, or component II; Refs. 1 and 2). Dinitrogenase is an $\alpha_2\beta_2$ tetramer of the nifD and nifK gene products, and it contains two pairs of unique metal clusters, known as the iron-molybdenum cofactor (FeMo-co)1-4 Refs. 3 and 4 and the P-cluster (4, 5). Dinitrogenase is specifically reduced by dinitrogenase reductase. Dinitrogenase reductase, which contains a single Fe$_2$S$_2$ cluster, is an $\alpha_2$ dimer of the nifH gene product (6). The electrons transferred to dinitrogenase are ultimately channeled to FeMo-co, the site of substrate reduction (see Ref. 7 for a concise review).

FeMo-co is composed of molybdenum, iron, sulfur, and homocitrate ((R)-2-hydroxyl-1,2,4-butanetricarboxylic acid) in a ratio of 1:7:9:1 (4, 8). The products of at least six nitrogen fixation (nif) genes, including nifQ, nifV, nifB, nifH, nifN, and nifE, are required for the biosynthesis of FeMo-co (8–11). Interestingly, the genes that encode dinitrogenase (nifD and nifK) are not required for FeMo-co biosynthesis, suggesting that FeMo-co is assembled elsewhere in the cell and is then inserted into FeMo-co-deficient dinitrogenase (apodinitrogenase; Refs. 12 and 13). The high degree of sequence similarity between the nifN and nifK sequences and the nifE and nifD sequences suggests that NIFNE might serve as a scaffold for FeMo-co biosynthesis (14). This hypothesis is supported by the recent observation that the mobility of NIFNE on native (non-denaturing) gels changes specifically upon the addition of NifB-co, a likely FeMo-co precursor (described below; Ref. 15). An in vitro FeMo-co synthesis system that requires an ATP-regenerating system, molybdate, homocitrate, and at least NIFB, NIFNE, and NIFH has been described (11). Although use of the in vitro system has yielded significant information concerning FeMo-co biosynthesis, the nature of the iron and sulfur donor(s) for the biosynthesis of FeMo-co remains unknown.

NifB-co is one potential source of iron and sulfur for FeMo-co biosynthesis. In the course of attempting to purify the NIFB protein from Klebsiella pneumoniae, Shah et al. (16) isolated and purified the apparent product of NIFB as a detergent-solubilized, small molecule termed NIFB-cofactor (NifB-co). Solutions of NifB-co exhibit certain characteristics that are similar to solutions of purified FeMo-co, including color, stability in N-methylformamide, and oxygen lability. The requirement for NIFB in the in vitro FeMo-co synthesis assay is satisfied by the addition of NifB-co, and the amount of FeMo-co synthesized in vitro is proportional to the amount of NifB-co added to the system in which all other components are present in excess. The stoichiometric requirement of NifB-co is consistent with the hypothesis that NifB-co is an iron-sulfur precursor of FeMo-co. Because a functional nifB gene is also required for the molybdenum-independent nitrogen fixation systems (17), it has been proposed that NifB-co is the basic iron-sulfur cluster for the synthesis of FeMo-co, the vanadium-containing cofactor (FeV-co) of the nif-encoded nitrogenase, and the iron-only cofactor of the anf-encoded nitrogenase (16).

In vitro activation of apodinitrogenase by FeMo-co apparently requires the presence of a protein designated as gamma (18). Recent studies show that addition of purified FeMo-co to crude extracts and partially purified fractions containing gamma results in a shift in the electrophoretic mobility of gamma on native gels. The mobility change correlates with the incorporation of iron into gamma (19). In addition, crude extracts that contain this faster migrating form of gamma (with associated FeMo-co) are capable of activating apodinitrogenase in vitro (19).

To date there has been no direct evidence for the incorporation of iron and sulfur from NifB-co into FeMo-co or for the flow of iron and sulfur from NifB-co through the NIFNE and gamma
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proteins. This report directly demonstrates that iron and sulfur from NifB-co can become associated with NIF NE and gamma, and ultimately become incorporated into apodinitrogenase as FeMo-co.

**EXPERIMENTAL PROCEDURES**

Materials—Sephadryl S-200 and Sephadex G-25 were from Pharmacia Biotech Inc. Sodium dithionite (DTH), Zwittergent 3-12 (SB-12), ultrapure calcium chloride dihydrate, and magnesium sulfate heptahydrate were from Fluka products. \( ^{56}\)FeCl\(_3\) and Na\(_3\)S\(_2\)O\(_4\) were obtained from Du Pont NEN. Analytical grade sucrose was from Serva. Sodium phosphates, potassium phosphate, and sodium carbonate were purchased from Fisher Scientific. Ultrapure ammonium acetate was from Aldrich.

Chelex-100 was obtained from Bio-Rad. Glass beads were obtained from Thomas Scientific. All other chemicals were from Sigma.

**Azobacter vinelandii Strains and Growth Conditions—**A. vinelandii strains UW45 (nifIB; Ref. 17), D767 (nifIB-kan nifDK; Ref. 15), CA11.1 (nifHDK nifDGK1; spec; Ref. 20), and D768 (nifIDK \( \Delta \)ENYK; kan) were grown, derepressed, and extracts were prepared as described (3). Strain D768 (\( \Delta \)ifDK \( \Delta \)ENYK; kan) was constructed by transforming strain D3 (21) with pDB583. Plasmid pDB583 was derived from pDB42 by replacing an internal BglII fragment with a 1.6-kilobase BamHI fragment that carries a kanamycin resistance cartridge from pUC4-KIXX (from Pharmacia). D768 resulted from a double mutant (nifDK, \( \Delta \)ENYK). All necessary, small molecules (i.e. homocitrate) were removed from the cell extracts by Sephadex G-25 column chromatography.

K. pneumoniae Strain and Growth Conditions—K. pneumoniae strain UN1217 (nifN4536) has been described (22). The minimal medium (23) was modified as described previously (16). When derepressing K. pneumoniae, the presence of Na\(_3\)S\(_2\)O\(_4\) 2.5 g of Mg\(_2\)SO\(_4\) \( \cdot \)7H\(_2\)O was substituted for Mg\(_2\)SO\(_4\) \( \cdot \)7H\(_2\)O (the only source of sulfur in the medium). FeCl\(_3\) was excluded from medium used for derepression of strain UN1217 in the presence of \( ^{56}\)FeCl\(_3\). Contaminating metals were removed from the phosphate salts by passage over a Chelex-100 column and all glassware was rinsed overnight with 4 \( \times \) HCl.

For sulfur donation studies, a starter culture of strain UN1217 was grown aerobically on a rotary shaker at 30°C for 20–24 h in 275 ml of medium (minimal medium as described previously; Ref. 16) containing 15.6 mM ammonium acetate (filter-sterilized). One ml of this culture was added to 10 ml of the equilibration buffer and the NifB-co was eluted with 0.1M NaCl in 25 mM MOPS (pH 7.5) containing 1 mM DTT and 0.4% N-lauroyl sarcosine. NifB-co binds to Sephacyr S-200 under these conditions. The column was washed with two column volumes of the equilibration buffer and the NifB-co was eluted with 0.1M NaCl in 25 mM MOPS (pH 7.5) containing 1 mM DTT and 2% SB-12. The column flow rate during the load and elution was approximately 19 ml/h. Fractions were collected anoxically and assayed for NifB-co activity using the in vitro FeMo-co synthesis assay (described below). The supernatants were stored at –20°C.

**NifB-co Purification**—All buffers used throughout the purification of NifB-co contained 1.7 mM DTH. The NifB-co was purified according to the published protocol with some key modifications to accommodate small scale purification of radiolabeled NifB-co (16). The supernatants from the high-speed centrifugation were combined and diluted 3-fold into 0.1M Tris-HCl (pH 7.4). DTT was added to a final concentration of 5 mM, and the diluted sample was incubated for 1 h at room temperature. The preparation was applied to a 1 \( \times \) 8.5-cm Sephacyr S-200 column equilibrated with 0.1 mM NaCl in 25 mM MOPS (pH 7.5) containing 1 mM DTT and 0.4% N-lauroyl sarcosine. NifB-co binds to Sephacyr S-200 under these conditions. The column was washed with two column volumes of the equilibration buffer and the NifB-co was eluted with 0.1M NaCl in 25 mM MOPS (pH 7.5) containing 1 mM DTT and 2% SB-12. The column flow rate during the load and elution was approximately 19 ml/h. Fractions were collected anoxically and assayed for NifB-co activity using the in vitro FeMo-co synthesis system (described below). 35S-Labeled and 35Fe-labeled NifB-co were successfully purified from 4 and 3 kg wet wt lots of cells, respectively.

**In Vitro FeMo-co Synthesis Assay**—Nine-ml serum vials were flushed with purified argon and rinsed with anoxic 25 mM Tris-HCl (pH 7.4). A complete FeMo-co synthesis reaction mixture was prepared by combining the following: 100 \( \mu \)l of 25 mM Tris-HCl (pH 7.4), 10 \( \mu \)l of 1 mM Na\(_3\)MoO\(_4\), 20 \( \mu \)l of 5 mM homocitrate (that had been treated with base to cleave the lactone, pH 8.0), and 200 \( \mu \)l of an ATP-regenerating mixture (containing 3.6 mM ATP, 63 mM Mg\(_2\)SO\(_4\), 51 mM phosphocreatine, 20 units/ml creatine phosphokinase, and 63 mM DTH). The reaction mixtures were incubated at room temperature for 10–15 min. Two hundred \( \mu \)l of the appropriate A. vinelandii cell-free extract (except in FeMo-co activated samples), 25 \( \mu \)l of the Sephacyr S-200 fraction described above (NifB-co), and 10 \( \mu \)l of purified dinitrogenase reductase (0.1 mg of protein) were added to the reaction mixtures. The vials were incubated in a rotary water-bath shaker at 30°C for 35 min. After this incubation, samples to be applied onto native polyacrylamide gels were placed on ice. The activity of the newly formed dinitrogenase was measured in the remainder of the vials using the C\(_2\)H\(_4\) reduction assay as described previously (24). Activation with any iron limitation was performed with an iron limitation.

In control experiments, various components of the complete reaction mixture were excluded as indicated under “Results and Discussion.” Where indicated, apodinitrogenase in 200 \( \mu \)l of the appropriate extract was activated by incubation with an excess of purified, unlabeled FeMo-co for 10 min before performing the in vitro FeMo-co synthesis reaction with the labeled NifB-co. Activation of Apodinitrogenase by FeMo-co (FeMo-co Insertion Assay)—The preparation of FeMo-co and the FeMo-co insertion assays were performed as described previously (3). Where indicated, NifB-co
was added to the reaction mixture and incubated for 20 min prior to the addition of FeMo-co.

Anoxic Native Gel Electrophoresis—One hundred μl of anoxic 50% glycerol were added to reaction mixtures that would be applied onto the gels. Unless otherwise indicated, 100 μl of the reaction mixtures (with added glycerol) were applied onto the gels. Proteins were separated on an anoxic native gel with a 5–10% acrylamide (37.5 acrylamide:1 bisacrylamide) and 0–20% sucrose gradient and 400 μM Tris-HCl buffer (pH 9.0). Gel dimensions were approximately 14 × 10 × 0.15-cm. The reservoir buffer was N2-sparged 65 mM Tris-glycine (pH 8.6) containing 1.7 mM EDTA. Gels were prerun for at least 45 min at 122 V for initial reduction and proteins were electrophoresed for 2,300 V × h (approximately 122 V) or 900 V × h (approximately 60 V). Gels were run at 4 °C. ImmunobLOTS—The protocols for immunoblotting and developing (24) with modifications by Brandner et al. (25) have been described. Native gels were equilibrated in the transfer buffer for at least 15 min before blotting. Polyconal antibodies to dinitrogenase were raised in rabbits. Visualization of Radioactivity—Gels were exposed to a phosphor screen for 1–3 days and scanned using a Molecular Dynamics model 425e PhosphorImager.

RESULTS AND DISCUSSION

Identification of an Iron and Sulfur Donor for FeMo-co Synthesis—In the initial study, purified NifB-co was reported to contain iron as the only metal (16). The similarities between solutions of NifB-co and FeMo-co suggested that NifB-co was an iron-sulfur cluster, although the presence of acid-labile sulfide was not demonstrated. Prior to investigating the role of NifB-co as a sulfur donor for FeMo-co synthesis, the presence of acid-labile sulfide in NifB-co was confirmed (data not shown). Various components of the buffer used to purify NifB-co, including N-lauroylsarcosine, DTT, and DTH, interfered with the colorimetric assay for acid-labile sulfide, and therefore precise quantitation of the iron:sulfur ratio of NifB-co was not possible. The data revealed, however, that purified NifB-co preparations contained significant amounts of acid-labile sulfide. Modifications, such as the buffer pH, purification scheme (37), and dithionite oxi- dation to acid-labile sulfide are currently being investigated to accurately quantitate the iron and sulfur content of NifB-co.

To investigate the role of NifB-co as an iron-sulfur donor for FeMo-co biosynthesis, NifB-co was independently labeled in vivo with 55Fe or 35S. The labeled cofactor was purified as described under “Experimental Procedures.” The 55Fe- and 35S-labeled NifB-co fractions contained an average of 2,200,000 and 300,000 cpm/ml, respectively. The average activities of the 55Fe and 35S-labeled NifB-co fractions were 770 and 400 nmol of C2H2 reduced/min by dinitrogenase formed/ml of the NifB-co-containing fraction, respectively.

The in vitro FeMo-co synthesis system together with anoxic, native gel electrophoresis was employed to monitor the incorporation of iron and/or sulfur from NifB-co into the FeMo-co of dinitrogenase. A complete reaction mixture that included all of the components known to be required for FeMo-co biosynthesis was used to monitor donation of iron and sulfur from NifB-co to FeMo-co. The complete reaction mixtures contained molybdenum, homocitrate, an ATP-regenerating system, and cell-free extract from strain UW45 (rifB), which served as a source of NIFNE, dinitrogenase reductase, apodinitrogenase, and any other unidentified factors required for in vitro FeMo-co biosynthesis. Purified 55Fe- or 35S-labeled NifB-co was added to complete the reaction mixture. A number of control reactions (in which iron and sulfur from NifB-co were not expected to be incorporated into dinitrogenase) were performed to demonstrate the specificity of incorporation of radiolabel from active NifB-co into dinitrogenase. Homocitrate, molybdenum, and MgATP were omitted from some in vitro FeMo-co synthesis reaction mixtures to prevent FeMo-co synthesis. In other control reaction mixtures, the oxygen-labile, labeled NifB-co was inactivated by exposure to air prior to addition to the reactions. In other control assays, all of the apodinitrogenase present in the UW45 extract was activated in vitro with unlabeled, purified FeMo-co prior to the addition of labeled NifB-co to the complete reaction mixture. In this system, all of the available FeMo-co binding sites on the apodinitrogenase should be occupied by unlabeled FeMo-co, and therefore iron and sulfur from labeled NifB-co were not expected to be associated with dinitrogenase.

To investigate the incorporation of 55Fe and 35S from NifB-co into FeMo-co, the proteins in the various in vitro FeMo-co synthesis reaction mixtures were separated on anoxic native gels. The position to which dinitrogenase migrated was determined by immunoblot analysis (data not shown). The data in Fig. 1A revealed that incorporation of 55Fe from labeled NifB-co into dinitrogenase required the presence of all of the components known to be required for in vitro FeMo-co synthesis. A prominently labeled band that comigrated with dinitrogenase was only detected in the lanes to which the complete FeMo-co synthesis reaction mixture (plus apodinitrogenase) was applied (Fig. 1A, lanes 7 and 8). The species that migrated slightly faster than dinitrogenase has been identified as NIFNE and is discussed in detail below. At least five lines of evidence suggested that in the complete in vitro FeMo-co synthesis reaction mixture, iron from NifB-co was specifically incorporated into the FeMo-co of dinitrogenase. (i) Only very low levels of iron were associated with dinitrogenase when FeMo-co synthesis was prevented in the reaction mixtures by omission of MgATP, molybdenum, or homocitrate (each of which is a known requirement for in vitro FeMo-co synthesis; see Fig. 1A, lanes 2, 6, and 9, respectively). (ii) The absence of a band that co-migrated with dinitrogenase in samples where the air-inactivated 55Fe-NifB-co was utilized demonstrated that active NifB-co was required for incorporation of the 55Fe into FeMo-co of dinitrogenase (Fig. 1A, lane 5). (iii) Activation of apodinitrogenase with unlabeled FeMo-co prior to synthesizing FeMo-co using the 35S-labeled NifB-co resulted in almost no association of 55Fe with dinitrogenase (Fig. 1A, lane 3). (iv) No radiolabel was detected at the dinitrogenase position in the lane containing only free 55Fe-NifB-co (Fig. 1A, lane 4). (v) Labeled dinitrogenase was not observed when the complete reaction mixture was oxidized following the FeMo-co synthesis reaction, but prior to being applied to the gel (data not shown). Note that the C2H2 reduction activities of the various assays (Fig. 1A) are consistent with the conclusion that holodinitrogenase was only formed in the complete system (by FeMo-co synthesis using 55Fe-NifB-co; Fig. 1A, lanes 7 and 8) and in the FeMo-co activated sample (by activation of apodinitrogenase with unlabeled FeMo-co; Fig. 1A, lane 3). Similar results were obtained when 35S-labeled NifB-co was used in the various in vitro FeMo-co synthesis reaction mixtures (Fig. 1B, compare lanes 1, 2, and 4 (control experiments) with lane 3 (complete system)). The differences in the band intensities in reactions that used 35S-labeled NifB-co compared to those that used 55Fe-labeled NifB-co (Fig. 1, compare A and B) are most likely because the cpm/ml of the 35S-labeled NifB-co was only 14% that of the 55Fe-labeled NifB-co. Together these data show that iron and sulfur from NifB-co were only incorporated into dinitrogenase under conditions where FeMo-co was synthesized and demonstrate that iron and sulfur from NifB-co were specifically incorporated into the FeMo-co of dinitrogenase. These data provide direct biochemical identification of an iron and sulfur precursor of FeMo-co.

The lack of 55Fe associated with dinitrogenase in the sample
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Table I

| FeMo-co insertion system | Activity* |
|-------------------------|-----------|
| UW45 + FeMo-co         | 14.5      |
| UW45 + NifB-co + FeMo-co| 14.4      |
| UW45 + inactivated NifB-co + FeMo-co | 14.2 |

*FeMo-co insertion assays contained 0.1 ml of 25 nm Tris-HCl (pH 7.4) and 0.1 ml of desalted UW45 extract as a source of apodinitrogenase (1.5 mg of protein). Where indicated, 20 µM of purified NifB-co (in buffer containing 2% N-lauroylsarcosine) was added and the mixtures were incubated for 20 min at room temperature. Purified FeMo-co (in NMF) was then added. After further incubation, 0.8 ml of ATP-regenerating mixture and an excess of purified dinitrogenase reductase (0.1 mg) were added and the C2H4 reduction activities were monitored.

Expressed as nanomoles of C2H4 formed/min/assay.

In the in vitro FeMo-co synthesis assay (with all other components in excess), 20 µM of NifB-co supported a dinitrogenase activity of 20 nmol of ethylene formed/min/assay.

NifB-co was oxidized in air for >15 min prior to addition to the assay.

Iron and Sulfur from NifB-co Associate with NIFNE—NIFNE has been proposed to serve as a scaffold for FeMo-co biosynthesis (16).

In the complete in vitro FeMo-co synthesis reaction mixture (Fig. 1A, lanes 7 and 8), the iron from NifB-co was obviously associated with more than one protein. The slowest migrating species is currently unidentified (Fig. 1A, lane 8). However, the species was also present in extracts of wild type cells grown on NH4Cl (data not shown) and was likely binding iron from denatured NifB-co because it was also observed in samples containing oxidized NifB-co (Fig. 1A, lanes 5 and 12) and, in fact, became more prominent as the labeled NifB-co samples lost activity, most likely due to oxygen inactivation. The slowest migrating species was not detected when 35S-labeled NifB-co was used (Fig. 1B).

Iron and Sulfur from NifB-co—NIFNE—NIFNE has been proposed to serve as a scaffold for FeMo-co biosynthesis (14, 15). This hypothesis is based on several lines of evidence. There is a high degree of sequence similarity between the nifN and nifK sequences and the nifE and nifD sequences. Most notably, Cys-275 of NIFD, which serves as a ligand to FeMo-co in the dinitrogenase protein, is conserved in NIFNE (14). In addition, the mobility of NIFNE on native gels has been shown to respond specifically to the addition of NifB-co (15). NIFBE activity has been shown to initially copurify with NIFNE in extracts from certain mutant backgrounds. It has been suggested that this reflects a direct interaction between NifB-co and NIFNE (15). It was therefore of interest to specifically address whether iron and sulfur from NifB-co could associate with NIFNE under various in vitro FeMo-co synthesis reaction conditions.

The labeled species that migrated slightly faster than dinitrogenase (Fig. 1A, lanes 7 and 8) was identified as NIFNE by immunoblot analysis (data not shown) and by use of purified NIFNE protein (Fig. 1A, lane 1). Purified NIFNE with associated 55Fe from NifB-co (Fig. 1A, lane 1) comigrated with the labeled species observed in a number of the in vitro FeMo-co synthesis reaction mixtures including: (i) reaction mixtures from which MgATP (Fig. 1A, lane 2), molybdenum (Fig. 1A, lane 6), homocitrate (Fig. 1A, lane 9), or dinitrogenase reductase (data not shown) were excluded, (ii) complete in vitro FeMo-co synthesis reaction mixtures in which all of the apodinitrogenase was activated with unlabeled FeMo-co prior to the addition of the 55Fe-NifB-co (Fig. 1A, lane 3), and (iii) the complete reaction mixture (Fig. 1A, lanes 7 and 8). Active NifB-co was required for this association, as determined by the absence of this species when air-inactivated 55Fe-NifB-co was used. However, based on previous activity and iron analysis studies, NifB-co has been predicted to donate all of the iron for FeMo-co biosynthesis (16).

in which apodinitrogenase was initially activated with purified, unlabeled FeMo-co suggested that, once bound to the protein, there was not a significant amount of turnover of FeMo-co in this system. The absence of a labeled dinitrogenase band indicated that the synthesized 55Fe-FeMo-co did not displace the FeMo-co that originally activated the apodinitrogenase.

The failure of NifB-co to form a complex with apodinitrogenase was also tested by investigating its possible ability to inhibit the insertion of FeMo-co into apodinitrogenase. As shown in Table I, preincubation of apodinitrogenase with an excess of NifB-co did not result in any detectable inhibition of FeMo-co insertion, consistent with the labeling results in Fig. 1.

accurate quantitation of the number of iron and sulfur atoms donated by NifB-co for FeMo-co synthesis remains to be accom-
NIFNE. The Fe-NifB-co data presented here definitively demonstrated that FeMo-co might be completed on NIFNE and suggest that FeMo-co synthesis requires MgATP, or dinitrogenase reductase (each of which is a requirement for FeMo-co synthesis). NifB-co is associated with NIFNE. These results are consistent with a model where these components are needed for FeMo-co synthesis reactions (Fig. 2, compare lanes 2 and 3). Extracts of strain CA11.1 depleted for the Nif proteins exhibited high levels of NIFB and NIFNE activities as monitored by the FeMo-co synthesis assay. Immunoblot analysis of a native gel containing crude extract from this strain showed that the NIFNE present migrated at the position for NIFNE with bound NiFe-co (data not shown; Ref. 15). We hypothesized that the majority of NIFNE in lane 3 (Fig. 2) had bound NiFe-co (unlabeled, of in vivo origin), and therefore little association of the added 55Fe-NifB-co with NIFNE was observed in the absence of a complete in vitro FeMo-co synthesis reaction system. When dinitrogenase reductase was added to complete the reaction mixture containing CA11.1 extract, molybdenum, homocitrate, and MgATP, all of the components known to be required for FeMo-co biosynthesis were present and therefore FeMo-co was synthesized. Thus, the unlabeled NiFe-co (of in vivo origin) was apparently incorporated into FeMo-co and proceeded along the biosynthetic pathway (discussed below). The NIFNE protein was then available to interact with the 55Fe-NifB-co that had been added to the system, and therefore an increase in 55Fe associated with NIFNE was observed upon addition of dinitrogenase reductase to the reaction mixture (Fig. 2, lane 2). These results demonstrate the relevance of the interaction of NiFe-co with NIFNE. The apparent ability to chase NiFe-co from NIFNE in CA11.1 extracts by addition of dinitrogenase reductase to the reaction mixture suggests that NIFNE is not only capable of binding NiFe-co, but these components can be chased by dinitrogenase reductase under in vivo conditions.

It is currently unclear if FeMo-co synthesis is completed on NIFNE, however there is evidence that NIFNE can bind to FeMo-co in crude extracts. When purified FeMo-co was added to extracts of strain DJ677 (ΔnifB::kan ΔnifKD), which lacks apodinitrogenase and is unable to synthesize NiFe-co, and the mixture was applied to a Sephacryl S-200 sizing column, the NIFNE-containing fraction contained FeMo-co as determined by the ability to activate apodinitrogenase in extracts of strain UW45 (NiFe-co; data not shown). A similar experiment was done by adding purified FeMo-co to extracts of strain DJ678 (ΔnifDK ΔYENX::kan), which lacks both apodinitrogenase and NIFNE, no NiFe-co was associated with the Sephacryl S-200 column fraction that corresponded to the NIFNE-containing fraction in the DJ 677 experiment (data not shown). These data suggest that NIFNE is capable of associating with the completed FeMo-co molecule and are consistent with the hypothesis that FeMo-co is completed on NIFNE. The observation that 55Fe from NiFe-co was associated with NIFNE in extracts of strain UW45 that had been activated with purified FeMo-co (Fig. 1A, lane 3) might suggest that NIFNE has a greater affinity for NiFe-co than for FeMo-co.

Iron and Sulfur from NiFe-co to NIFNE. Gamma was first identified as an additional subunit associated with purified apodinitrogenase from an A. vinelandii nifB strain (28). Subsequent studies have revealed that dinitrogenase reductase and MgATP are required to associate gamma with the apodinitrogenase which then allows in vitro activation by FeMo-co (18). Gamma might also be the 65-kDa protein-FeMo-co complex isolated by Ugalde et al. (12). Gamma has recently been shown to specifically incorporate iron upon addition of purified FeMo-co (19). It was therefore of interest to examine the association of iron and sulfur from NiFe-co with gamma under a variety of FeMo-co synthesis conditions.

Iron and sulfur from NiFe-co to NIFNE. Gamma—Gamma was first identified as an additional subunit associated with purified apodinitrogenase from an A. vinelandii nifB strain. Gamma—Gamma was first identified as an additional subunit associated with purified apodinitrogenase from an A. vinelandii nifB strain. Gamma—Gamma was first identified as an additional subunit associated with purified apodinitrogenase from an A. vinelandii nifB strain.
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**Fig. 3.** Phosphorimage of a native, anodic gel of various in vitro FeMo-co synthesis reactions that included ^55^Fe-labeled-NifB-co. Arrows indicate the positions of dinitrogenase and gamma (with bound FeMo-co) as determined by immunoblot analysis. The unknown species is also indicated by an arrow. Synthesis reactions used extracts of strain DJ 677 (ΔnifB::kan ΔnifDK), except in lane 6. Lane 1, minus apodinitrogenase with ^55^Fe-NifB-co inactivated by air prior to addition to the assay; lane 2, minus apodinitrogenase and homocitrate; lane 3, minus apodinitrogenase and MgATP; lane 4, minus apodinitrogenase with purified, unlabeled FeMo-co added; lane 5, minus apodinitrogenase (all other FeMo-co synthesis requirements present); lane 6, complete in vitro FeMo-co synthesis reaction (UW45 [nifB]). Where appropriate, the C2H2 reduction activities of the assays (nmol of C2H4 formed/min/assay) are reported. Table below the figure indicates components present (+) in the reaction mixture.

The accumulation of FeMo-co on other proteins to be detected. To visualize dinitrogenase and gamma on the same system (Fig. 3), samples were electrophoresed on a gel for significantly fewer V × h than the gels shown in Figs. 1 and 2. The fastest migrating species (marked "unknown" in Fig. 3) is unidentified (discussed below). The smear on the gel (that obscures ^55^Fe associated with NIFNE) is attributed to ^55^Fe from oxygen-inactivated NifB-co (see Fig. 3, lane 1). The position of gamma (with bound FeMo-co) on the gel was determined by immunoblot analysis (data not shown).

The data in Fig. 3 revealed that significant amounts of ^55^Fe from ^55^Fe-NifB-co accumulated on a protein that co-migrated with gamma in extracts of strain DJ 677 only when all of the components required for FeMo-co biosynthesis were present (Fig. 3, lanes 2). ^55^Fe was not associated with gamma when FeMo-co synthesis could not occur due to omission of the MgATP (Fig. 3, lanes 3) or homocitrate (Fig. 3, lane 2) from the reaction mixture or when purified, unlabeled FeMo-co was added prior to the synthesis reaction (Fig. 3, lane 4). In addition, no label was associated with this protein when the ^55^Fe-NifB-co was inactivated by exposure to air prior to addition to the FeMo-co synthesis reaction (Fig. 3, lane 1). Because all components of the in vitro FeMo-co synthesis system were required to observe ^55^Fe-NifB-co-dependent labeling of gamma, these data support the conclusion that the iron associated with gamma was in the form of FeMo-co and definitively demonstrate that the iron previously observed to be associated with gamma (in the form of FeMo-co) was from NifB-co.

A ^55^Fe-labeled species that migrated slightly faster than gamma was observed in some reaction mixtures (Fig. 3, lanes 2, 3, and 5). The band did not correspond to free ^55^Fe-NifB-co and was dependent on active NifB-co because the species was absent in samples where air-inactivated ^55^Fe-NifB-co was utilized (Fig. 3, lane 1). The species was most prominent in samples that contained all of the components required for FeMo-co synthesis (Fig. 3, lane 5) and in samples that contained all of the components required for FeMo-co synthesis except homocitrate (Fig. 3, lane 2). Immunoblot analysis indicated that this species did not correspond to gamma or NIFNE. The possibility that this is another relevant protein involved in FeMo-co biosynthesis is currently under investigation. A similar species appears to have been observed when ^99^MoO4^2− was used to investigate incorporation of molybdenum into dinitrogenase in the presence of various homocitrate analogs (see Fig. 3 of Ref. 27).

Model of FeMo-co Biosynthesis—The results of this study suggest that the iron and sulfur from NifB-co associate with the NIFNE prior to being incorporated into FeMo-co and being transferred to gamma and ultimately to dinitrogenase. The results presented here are consistent with a biosynthetic model in which molybdenum, MgATP, and homocitrate, in addition to dinitrogenase reductase, enter the biosynthetic pathway at the level of NIFNE. Observation of the unidentified species that migrated slightly faster than gamma (and was prominently labeled with ^55^Fe in a reaction mixture that lacked homocitrate), however, somewhat complicates these conclusions, and therefore definitive proof of where molybdenum and homocitrate enter the biosynthetic pathway will require use of ^99^MoO4^2− and labeled homocitrate. The gamma protein apparently associates with the completed FeMo-co molecule.

A major question that remains concerns the source of iron and sulfur for NifB-co biosynthesis. Exciting results from the laboratory of Dean and colleagues indicate that the nifU and nifs gene products are likely to be involved in the mobilization of inorganic iron and sulfur for the synthesis of the nitrogenase iron-sulfur clusters (see Ref. 7 for a recent review). A system that utilized NIFS for the reconstitution of the FeS4 cluster of dinitrogenase reductase was recently described (29). It will be interesting to attempt to generate NifB-co using a similar system.

Conclusions—The results of this study demonstrate that iron and sulfur from NifB-co are incorporated into the FeMo-co of dinitrogenase and provide direct biochemical identification of an iron-sulfur donor for FeMo-co biosynthesis. Under different in vitro FeMo-co synthesis conditions, iron and sulfur from NifB-co were associated with at least two other proteins (NIFNE and gamma) that are involved in the formation of active dinitrogenase. The results presented here suggest that multiple FeMo-co processing steps might occur on NIFNE. The ability to "chase" NifB-co from NIFNE by altering the in vitro reaction conditions suggests that the NIFNE-NifB-co complex is a physiologically relevant precursor along the FeMo-co biosynthetic pathway. Gamma appears to associate with a completed FeMo-co molecule. These results are consistent with a model in which the iron and sulfur from NifB-co associate with NIFNE (where additional steps of FeMo-co biosynthesis likely occur) at some point prior to FeMo-co being associated with gamma and ultimately being incorporated into dinitrogenase. Studies are currently under way to establish where in the biosynthetic pathway molybdenum and homocitrate are incorporated into the FeMo-co.

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