Inhibition of Iron-Molybdenum Cofactor Biosynthesis by L127Δ NifH and Evidence for a Complex Formation between L127Δ NifH and NifNE*

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Besides serving as the obligate electron donor to dinitrogenase during nitrogenase turnover, dinitrogenase reductase (NifH) is required for the biosynthesis of the iron-molybdenum cofactor (FeMo-co) and for the maturation of αβ2 apo-dinitrogenase (apo-dinitrogenase maturation). In an attempt to understand the role of NifH in FeMo-co biosynthesis, a site-specific altered form of NifH in which leucine at position 127 has been deleted, L127Δ, was employed in in vitro FeMo-co synthesis assays. This altered form of NifH has not been shown to inhibit substrate reduction by the wild-type nitrogenase complex, forming a tight protein complex with dinitrogenase. The L127Δ NifH was found to inhibit in vitro FeMo-co synthesis by wild-type NifH as detected by the γ gel shift assay. Increasing the concentration of NifNE and NifB-cofactor (NifB-co) relieved the inhibition of FeMo-co synthesis by L127Δ NifH. The formation of a complex of L127Δ NifH with NifNE was investigated by gel filtration chromatography. We herein report the formation of a complex between L127Δ NifH and NifNE in the presence of NifB-co. This work presents evidence for one of the possible roles for NifH in FeMo-co biosynthesis, i.e. the interaction of NifH with a NifNE/NifB-co complex.

Nitrogenase, which catalyzes the conversion of dinitrogen to ammonium during biological nitrogen fixation, is composed of two component metalloproteins: dinitrogenase and dinitrogenase reductase (1). Dinitrogenase (MoFe protein, NifDK) is an αβ2 tetramer of the nifD and K genes products. Dinitrogenase contains two different metal clusters: the P-clusters and the iron-molybdenum cofactor (FeMo-co), the site of substrate reduction (2–4). Dinitrogenase reductase (Fe protein, NifH), a dimer of the nifH gene product (1), contains two nucleotide binding sites and one Fe4S4 cluster ligated by cysteines 97 and 132, bridging the two identical subunits (5). NifH shares a high degree of structural and functional similarity to a family of proteins called the G-proteins (GTP-binding proteins) (6). The two regions of structural similarity between the G-proteins and NifH include the P-loop (residues 9 to 16 in NifH), which is involved in the binding of ATP, and the Switch II region (residues 125 to 132 in NifH), involved in the Mg2+ coordination (7, 8). The nucleotide binding site in NifH is ~19 Å away from the Fe4S4 cluster (8). However, many changes in the Fe4S4 cluster have been observed upon MgATP binding to NifH, including the accessibility of the cluster to iron chelators like α,α′-bipyridyl, a decrease in the mid-point redox potential of the cluster, and spectral changes in the EPR line shape.

NifH has at least three different functions in the nitrogenase enzyme system. It is required for electron donation to nitrogenase, for apo-dinitrogenase maturation, and for FeMo-co biosynthesis (9). The nitrogenase-catalyzed substrate reduction involves a complex series of reactions including the association of the component proteins, MgATP hydrolysis coupled to electron transfer, followed by the dissociation of the two proteins (10). The role of NifH as the specific electron donor to nitrogenase has been well documented. It serves as the obligate physiological electron donor to dinitrogenase during catalysis, transferring one electron at a time with the concomitant hydrolysis of two MgATPs (11). The electrons transferred to dinitrogenase are channeled to FeMo-co, where the reduction of nitrogen is believed to occur. The features of NifH required for its function in substrate reduction include the ability of NifH to interact with low potential electron donors, to bind MgATP, to undergo the nucleotide-induced conformational change, to bind dinitrogenase, to transfer electrons to dinitrogenase, and to hydrolyze MgATP. Altered forms of NifH that are unable to perform one or more of the above properties result in the enzyme that is completely nonfunctional in electron transfer. Recent studies on these altered forms of NifH generated by site-directed mutagenesis have contributed tremendously toward understanding the role of NifH in nitrogenase turnover (12).

Apart from its indispensable role in catalysis, NifH is involved in two other functions: in the maturation of αβ2 apo-dinitrogenase (NifDK) to the αβ2γ2 form (NifDKγ) (13, 14), and in the biosynthesis of FeMo-co (15). The αβ2 form of apo-dinitrogenase is not FeMo-co-activable, whereas the γ-associated form can be activated upon the addition of preformed FeMo-co. γ is a non-nif protein and has been shown to function as a chaperone insertase during the formation of dinitrogenase (16). NifH mediates the association of γ2 with the αβ2 apo-dinitrogenase in the presence of MgATP. But the exact role of NifH in apo-dinitrogenase maturation is not very well un-
stood. The purification of a His-tagged αβ3 apo-dinitrogenase without the γ subunit that is 80% active by addition of purified FeMo-co has recently been reported (17).

At least seven nif gene products are known to be involved in FeMo-co biosynthesis: nifQ, nifV, nifX, nifB, nifN, nifE, and nifH (18, 19, 22).2 Azotobacter vinelandii mutants carrying a deletion of any of nifH, nifN, nifE, or nifB produce a cofactorless dinitrogenase (this form contains the P-clusters) termed as apo-dinitrogenase. The nifQ gene product has been postulated to play a role in molybdenum processing during FeMo-co biosynthesis (18). The nifV gene encodes homocitrate synthase (23). The nifX gene product has recently been shown to be required for FeMo-co synthesis by the in vitro FeMo-co synthesis assay, although the exact role played by NifX is not known.2 The metabolic product of NifB is NifB-cofactor (NifB-co) (24). NifB-co has been shown to function as a specific iron and sulfur donor to FeMo-co during cofactor biosynthesis (25). The NifN and nifE gene products together form a tetrameric protein that shows a high sequence similarity to the nifK and nifD gene products (26). Thus, NifNE has been postulated to form a scaffold upon which FeMo-co is assembled. An absolute requirement of NifH in FeMo-co biosynthesis has been demonstrated using the in vitro FeMo-co synthesis system (27). However, the precise role(s) of NifH in cofactor biosynthesis is not very clear. Surprisingly, a form of NifH that is active in substrate reduction is not required for its function in FeMo-co biosynthesis. For example, several altered forms of NifH, completely inactive in substrate reduction, have been shown to support cofactor biosynthesis and its insertion into apo-dinitrogenase (28, 29). This supports the hypothesis that NifH contains distinct domains that enable it to perform each of its functions independently.

In an attempt to understand the role of NifH in FeMo-co synthesis, the interaction of NifH with NifNE has been studied. In this study, we have used a site-specific altered form of NifH, L127A. This form of NifH has been shown to bind dinitrogenase with extremely high affinity (30). L127A NifH was found to be inactive in FeMo-co synthesis and inhibited FeMo-co synthesis by wild-type NifH. Here, we report the formation of a complex of L127A NifH with NifNE in the presence of NifB-co. This is the first direct evidence to show the interaction of NifH with NifNE in light of these data, the possible roles for NifH in FeMo-co biosynthesis are discussed.

**EXPERIMENTAL PROCEDURES**

*Strains—* A. vinelandii strains CA11.1 (ΔnifDKΔnifDGKspc) (31), DJ677 (ΔnifDKΔnifB) (32), DJ35 (ΔnifE) (26), and DJ1041 (NifNE overproducer) (33) have been described. Growth, derepression, and cell breakage were performed as previously reported (34). The wild-type and L127A NifHs were purified from the extract of strain CA11.1 (ΔnifDKΔnifDGKspc). As a source of NifNE, NifB-co, and γ3, purified wild-type or L127A NifH (50 μg of protein). The NifHs were used to purify or 100 μg of protein) were added to the assays, and the reaction mixtures were further incubated for 30 min at 30°C. The reactions were then placed on ice, and aliquots (20 μl) of the reaction mixtures were subjected to anoxic native gel electrophoresis followed by immunoblotting using anti-γ antibody, as described below.

**Visualization of γ Gel Shift on Anaerobic Native Gels as an Indication of FeMo-co Synthesis—* In vitro FeMo-co synthesis assays were carried out as described by Shah et al. (37). To 9-ml reaction vials flushed with purified argon and rinsed with anaerobic Tris–HCl (pH 7.5) were added 100 μl of anaerobic Tris–HCl, 10 nmol of sodium molybdate, 100 nmol of homocitrate, 200 μl of an ATP-regenerating mixture (containing 3.6 mM ATP, 6.3 mM MgCl2, 51 mM creatine, 20 units of creatine phosphokinase, and 6.3 mM DTT), 200 μl of extract (2.5 mg of protein) of strain CA11.1 (ΔnifDKΔnifDGKspc); as a source of NifNE, NifB-co, and γ3, and 1.7 mM DTH unless otherwise stated. The metabolic product of NifB is NifB-cofactor (NifB-co) (24). NifB-co was purified from the extract of strain UN1217 (ΔnifHDKΔnifDGKspc) as described by Shah et al. (32). Both NifNE and NifB-co activities were followed by in vitro FeMo-co synthesis assays using extracts of A. vinelandii strains DJ35 (ΔnifE) and UW45 (nifB), respectively (32).

**Inhibition of FeMo-co Synthesis by L127A NifH—* In vitro FeMo-co synthesis assays were performed with the extract of strain CA11.1 (ΔnifDKΔnifDGKspc), as described above. Purified L127A NifH (25 to 250 μg of protein) was added to the assays, and the reaction mixtures were incubated for 30 min at 30°C (preincubation phase). Purified wild-type NifH (50 μg) was then added to the assays, and the reaction mixtures were further incubated for 30 min at 30°C. The reactions were then placed on ice, and aliquots (20 μl) of the reaction mixtures were subjected to anoxic native PAGE followed by immunoblotting using anti-γ antibody, as described below.

**Inactivation of Inhibition of FeMo-co Synthesis by L127A NifH by the Addition of NifNE and NifB-co—* In vitro FeMo-co synthesis assays were performed with the extract of strain CA11.1 (ΔnifDKΔnifDGKspc), as described above. Purified L127A NifH (100 μg of protein), purified NifNE (50 or 100 μg of protein), and an excess of NifB-co (5 nmol of Fe) were added to the assays, and the reaction mixtures were incubated for 30 min at 30°C (preincubation phase). Purified wild-type NifH (50 μg of protein) was then added to the assays, and the reaction mixtures were further incubated for 30 min at 30°C. The reactions were then placed on ice, and aliquots (30 μl) of the reaction mixtures were subjected to anoxic native PAGE followed by immunoblotting using anti-γ antibody, as described below.

**Association of NifH with L127A NifH—* High-resolution, analytical gel filtration chromatography was used to monitor the complex formation between NifNE and L127A NifH. This was performed using a Zebron GF-250 HPLC column equilibrated in degassed 200 mM potassium phosphate buffer (pH 7.3) and 0.5 mM DTT. Before injection onto the HPLC column, the proteins were incubated for 5 min at ambient temperature in degassed 200 mM potassium phosphate buffer (pH 7.3) containing 0.5 mM DTT. The protein concentration of NifH used for each HPLC run was 35 μM and that of NifNE or L127A NifH was 140 μM. The column eluent was monitored from 200 to 800 nm, and data was collected and processed using a Beckman Novapak software package.

The complex formation between NifNE and L127A NifH in the presence of NifB-co was monitored using a Superose 12 gel filtration column. The column was equilibrated in buffer containing 50 mM NaCl in 0.025 M MOPS–NaOH (pH 7.5) and was calibrated using proteins of known molecular masses (dinitrogenase 230 kDa, bovine serum albumin 67 kDa, ovalbumin 45 kDa, myoglobin 18 kDa, and cytochrome c 12 kDa). A hundred μl of a solution containing purified NifNE (0.5 mg of protein) and excess purified NifB-co (5 nmol of Fe) was applied to the column. The column was developed with the same buffer, and fractions (0.5 ml) collected anoxically were tested for NifNE activity by in vitro FeMo-co synthesis assays as described previously (32) and by immunoblotting with anti-NifNE antibody. Two hundred μl of a solution containing L127A NifH (2.0 mg of protein) was then applied to the
column and chromatographed as described above. The fractions (0.5 ml) were analyzed for the presence of NifH by subjecting 10 μl aliquots to SDS-PAGE, followed by NifH immunoanalysis. The protein concentration of L127Δ NifH in the Superose 12 fractions was calculated by a densitometry scan of the NifH immunoblot. When examining the interaction of L127Δ NifH with NifNE, 200 μl of a solution containing purified NifNE (0.5 mg of protein), excess purified NifH-co (5 nmol iron), and L127Δ NifH (2.0 mg of protein; preincubated for 30 min at 30 °C) was applied to the Superose 12 column (equilibrated as described above). The column was developed, and the fractions (0.5 ml) were monitored for NifNE and L127Δ NifH by SDS-PAGE of the fractions, followed by NfNE and NifH immunoblotting. The protein concentrations of NifNE and L127Δ NifH in the Superose 12 fractions were calculated by densitometry scans of the NfNE and NifH immunoblots.

Anaerobic Native Gel Electrophoresis—Proteins were separated on anaerobic native gels with a 7–14% acrylamide and 0–20% sucrose gradient as described previously (14). Antibodies and Immunoblot Analysis—Antibody to γ was a gift from Dr. Mary Homer and Dr. Gary P. Roberts. Anti-NifH antibody was a gift from Dr. Jon Roll and Dr. Gary P. Roberts, Department of Bacteriology, University of Wisconsin-Madison. The anti-NifH antibody was raised in rabbit. The protocols for immunoblotting and developing with antibodies and immunoblot analysis were described previously (40).

Metal Analysis—Iron was quantitated by the bichinonic acid method using bovine serum albumin as standard (39).

RESULTS AND DISCUSSION

In Vitro FeMo-co Synthesis by Wild-type and L127Δ NifH—The site-specific altered form of NifH, L127Δ, was tested in FeMo-co biosynthesis by the γ gel shift assay (16). This assay involves the shift in the migration of γ on anaerobic native gels as an indicator of FeMo-co synthesis. FeMo-co synthesis assays were performed using extracts of A. vinelandii strain CA11.1 (Δnif/ΔDK Δunu/ΔDGK/Δsopc) as a source of NifNE, NifH-co, and γ. The assays were carried out by incubating wild-type and L127Δ NifH (50 μg of protein) with aliquots of extract of strain CA11.1 (2.5 mg of protein), as described under Experimental Procedures. The results of these assays are shown in Fig. 1. γ in the extract of strain CA11.1 is a dimer (γ2) because the strain is impaired in FeMo-co synthesis (Fig. 1, lane 1). Upon addition of NifH to the reaction mixture (containing all components required for FeMo-co synthesis), FeMo-co is synthesized and accumulates on γ, γ bound to FeMo-co (γ−FeMo-co) electrophoreses as a faster migrating band on anaerobic native gels and can be clearly distinguished from the slower migrating γ2 species (Fig. 1, compare lanes 1 and 2). When L127Δ NifH was used in place of wild-type NifH in the assay, no shift of γ was observed upon anaerobic native gel electrophoresis of the reaction mixture (Fig. 1, lane 3). No change was observed upon increasing the concentration of L127Δ NifH or upon increasing the incubation time of the reaction mixtures. These data clearly show that L127Δ NifH is inactive in FeMo-co biosynthesis. A slower migrating species of γ (designated as X) is observed in extracts of strain CA11.1; this species is yet uncharacterized.

Inhibition of in Vitro FeMo-co Synthesis by L127Δ NifH—Leucine 127 in NifH has been shown to play an important role in the MgATP signal transduction pathway from the nucleotide binding site to the Fe4S4 cluster in NifH (36). The deletion of this residue results in a form that greatly resembles the MgATP-bound conformation of NifH. The L127Δ form of NifH is capable of binding two molecules of MgATP, binding dinitrogenase with extremely high affinity, and transferring one electron to dinitrogenase but is incompetent in substrate reduction (30, 36). This altered form of NifH has also been shown to behave as a tight binding inhibitor of dinitrogenase, inhibiting substrate reduction by the wild-type nitrogenase complex (30). L127Δ NifH was found to be impaired in the FeMo-co synthesis reaction, as shown in Fig. 1. Thus, it was of interest to investigate if L127Δ NifH could inhibit FeMo-co synthesis by wild-type NifH.

FeMo-co synthesis was monitored by the γ gel shift assay, as described above. Increasing concentrations of purified L127Δ NifH (25–250 μg of protein) were included in the in vitro FeMo-co synthesis reactions containing the extract of strain CA11.1, as described under Experimental Procedures. After preincubation of 30 min, purified wild-type NifH (50 μg of protein) was added to the reaction mixtures and further incubated for 30 min. The results shown in Fig. 2 reveal that increasing the concentration of L127Δ NifH in the reaction mixture from 25 μg to 250 μg showed inhibition of FeMo-co biosynthesis, as can be seen by the decreasing amounts of the faster migrating γ species (γ−FeMo-co). Increasing the concentration of L127Δ NifH in the preincubation phase to 100 μg completely inhibited FeMo-co synthesis by wild-type NifH (Fig. 2).
2, compare lanes 2 and 5). No inhibition of FeMo-co synthesis was observed when O2-denatured L127Δ NifH (250 µg of protein) was used in the preincubation phase of the in vitro FeMo-co synthesis assay (Fig. 2, lane 8). Thus, the inhibition of FeMo-co synthesis by L127Δ NifH was dependent upon the native conformation of the enzyme. No inhibition of FeMo-co synthesis was observed when a nonspecific protein, bovine serum albumin, was added to the preincubation phase of the reaction in place of L127Δ NifH (data not shown).

Given the specificity of the inhibition, it was of interest to investigate if the inhibition of FeMo-co synthesis by L127Δ NifH was due to its binding to small molecules such as homocitrate, MgATP, or molybdenum, thus making these essential components unavailable to the cofactor biosynthetic machinery. In vitro FeMo-co synthesis assays were performed using extract of strain CA11.1 as a source of all components for FeMo-co synthesis except NifH. Increasing concentrations of homocitrate, MgATP, and molybdenum were added to select assay mixtures to determine if increasing the concentrations of these components would relieve the inhibition of FeMo-co synthesis by L127Δ NifH. The assays were carried out as described under “Experimental Procedures,” with purified L127Δ NifH included in the reaction mixtures containing purified L127Δ NifH (100 µg) during the preincubation phase, as described under “Experimental Procedures.” The results presented in Fig. 3 (lanes 8 and 9) show that the addition of purified NifNE and NifB-co to the reaction mixtures did not relieve the inhibition of FeMo-co synthesis by L127Δ NifH, as indicated by the presence of the faster migrating species of γ. These results suggest that the inhibition of FeMo-co synthesis by L127Δ NifH may be due to a complex formation with NifNE. The addition of purified NifNE alone or purified NifB-co alone to the reaction mixtures did not relieve the inhibition of FeMo-co synthesis by L127Δ NifH (data not shown).

Complex Formation of L127Δ NifH with Purified NifNE in the Presence of NifB-co—The interaction of L127Δ NifH with the NifNE proteins was examined given the structural similarity between NifNE and dinitrogenase. The association of NifNE with L127Δ NifH was monitored by high resolution analytical gel filtration chromatography, as described under “Experimental Procedures.” Fig. 4 shows the elution patterns (measured at 405 nm) resulting from the interactions of NifNE and L127Δ NifH. The elution profile of NifNE resulted in a single peak (Fig. 4, trace 1), which is presumably due to the presence of the Fe4-S4 clusters of NifNE. Similarly, the elution profile of either the wild-type NifH (Fig. 4, trace 2) or L127Δ NifH (data not shown) also resulted in a single peak due to the presence of the Fe4-S4 clusters. When NifNE and the wild-type NifH were incubated together, the elution profile (Fig. 4, trace 3) resulted in two separate peaks, which correspond to the positions of NifNE control (Fig. 4, trace 1) and the wild-type NifH control (Fig. 4, trace 2). However, when NifNE was incubated with the L127Δ NifH, the elution profile showed 3 peaks. The positions of two of the peaks correspond to the positions of the NifNE control and the wild-type NifH control. The third peak (Fig. 4, trace 4, arrow) elutes at a position that is larger in size than...
both NifNE and the wild-type NifH. This third peak elutes at the similar position corresponding to the dinitrogenase-L127ΔNifH complex (data not shown). Based on these elution profile similarities, it seems likely that the third peak represents a complex of NifNE and L127ΔNifH. In contrast to the complex formed between dinitrogenase and L127ΔNifH, the complex formation of NifNE with L127ΔNifH is not complete. As the concentration of L127ΔNifH used in this experiment is four times that of NifNE, one would expect that all the NifNE be complexed with L127ΔNifH and elute at the position of the complex. This is not the case, which can be seen clearly in Fig. 4, trace 4. It has been shown previously that NifNE can exist in two forms, an uncharged form and a charged form (32). In the charged form, it is believed that NifNE is bound to the FeMo-co precursor, NifB-co, or to a processed form of NifB-co. Metal analyses of the overproduced NifNE routinely showed the presence of more iron than is necessary to support the formation of the two Fe₄-S₄ clusters (12–15 iron atoms/NifNE tetramer). Thus it is possible that the preparations of NifNE contain a portion of NifNE bound to NifB-co. It is possible that NifH may interact only with this charged form of NifNE. If this is the case, it would be likely that only a small portion of NifNE would be able to complex with L127ΔNifH, which is consistent with the data presented.

The in vitro formation of a complex between L127ΔNifH and NifNE in the presence of NifB-co was investigated using gel filtration chromatography. A solution containing purified NifNE in the presence of excess NifB-co and a L127ΔNifH-containing solution were fractionated separately on a calibrated Superose 12 gel filtration column. The NifNE-NifB-co complex reproducibly eluted at Ve/Vo 1.31–1.62, with a peak at Ve/Vo 1.5 (Fig. 5, panel A). Elution of purified NifNE in the absence of NifB-co also showed similar Ve/Vo values (data not shown). Purified L127ΔNifH reproducibly eluted at Ve/Vo 1.62–1.8 (values similar to wild-type NifH; data not shown), with a peak at Ve/Vo 1.7 (Fig. 5, panel B). A solution containing both L127ΔNifH and NifNE in the presence of NifB-co was chromatographed on the Superose 12 column, as described under “Experimental Procedures.” The Superose 12 fractions were analyzed for the presence of NifNE and L127ΔNifH proteins by SDS-PAGE and immunoanalyses using anti-NifH and anti-NifNE antibody. The elution of both NifNE and L127ΔNifH under these conditions was found to be altered. NifNE now eluted at Ve/Vo 1.25–1.44, with a peak at Ve/Vo 1.38, whereas L127ΔNifH eluted at Ve/Vo 1.25–1.75 (Fig. 5, panel C). The elution of L127ΔNifH was broader and unlike the sharp peak obtained when chromatographed in the absence of the NifNE-NifB-co complex. Moreover, the elution of L127ΔNifH showed two peaks, one that comigrated with the elution peak of NifNE (Ve/Vo 1.38), and the other peak showing Ve/Vo 1.7, which was identical to the value obtained when L127ΔNifH was chromatographed in the absence of NifNE. Scanning densitometry of the NifNE and NifH immunoblots was used to determine the relative quantities of each protein and to calculate the stoichiometry of the L127ΔNifH to NifNE in the complex. A ratio of 2.5 L127ΔNifH to 1 NifNE was estimated in the L127ΔNifH-NifNE complex.

NifNE activity. Panel B, elution profile of L127ΔNifH from Superose 12 column. Anti-NifH antibody was used for the detection of L127ΔNifH in the Superose 12 column fractions. Panel C, elution profiles of NifNE-NifB-co and L127ΔNifH from Superose 12 column; the circles denote the elution profile of L127ΔNifH, the diamonds denote the elution profile of NifNE, and the squares denote the elution profile for proteins of known molecular weights as described under “Experimental Procedures.” Immunoanalyses with anti-NifNE and anti-NifH was used to detect the presence of NifNE and L127ΔNifH, respectively, in the Superose 12 column fractions.
The fractionation of wild-type NifH and NifNE in the presence of NiFe-co resulted in no change in the elution profiles of either NiFeH or NiFeNE. Upon fractionation of L127Δ NifH in the presence of a nonspecific protein, bovine serum albumin, no change in the elution profile of L127Δ NifH was observed. These data suggest that the change in elution profile of L127Δ NifH is most likely due to a specific interaction of L127Δ NifH with the NiFeNE-NiFeB-co complex.

A similar set of experiments performed without purified NiFeB-co did not show altered elution profiles for L127Δ NifH and NiFeNE (data not shown). These data suggest that the interaction of NiFeH with NiFeNE occurs after the binding of NiFeB-co to NiFeNE and that the interaction is dependent on the presence of NiFeB-co. It has been shown in previous studies that NiFeNE binds NiFeB-co, and a shift in the migration of NiFeNE on anoxic native gels upon binding NiFeB-co has been observed (32). NiFeNE with associated 55Fe and 35S from radiolabeled NiFeB-co has also been observed (25). This reaction has been suggested to be one of the early steps in the FeMo-co biosynthetic pathway, as the binding of NiFeB-co to NiFeNE is not dependent on the presence of NiFeH or MgATP. The conformational change brought about by NiFeB-co binding may expose a region in NiFeNE that serves as the NiFeH recognition site. It seems likely that the binding of NiFeH to NiFeNE is very transient, because no such complex formation was observed with wild-type NiFeH and NiFeNE in the presence or absence of NiFeB-co (data not shown).

The requirement of NiFeH for FeMo-co biosynthesis has been known for the last few years. The exact role of NiFeH in the cofactor biosynthetic pathway is not well understood. That several altered enzymes impaired in catalysis are active in FeMo-co biosynthesis indicates a role for NiFeH that is not analogous to its role in substrate reduction.

Several possible roles for NiFeH in FeMo-co biosynthesis can be envisioned. One role for NiFeH is in the catalysis of a specific redox reaction. However, site-specific-altered forms of NiFeH that show virtually no electron transfer ability have been shown to function in FeMo-co biosynthesis (28). Furthermore, we have shown that the presence of a redox-active Fe4S4 cluster in NiFeH is not required for its function in cofactor biosynthesis (41). These data rule out a role for NiFeH that is analogous to its role in substrate reduction. Another possibility is that NiFeH could be involved in specifying the heteromeric associated with the individual cofactors, namely FeMo-co, FeV-co, and FeFe-co. The evidence in support of this hypothesis lies in the existence of independent proteins for each of the three nitrogenases in A. vinelandii, i.e., NiFeH for the molybdenum-nitrogenase, VnFeH for the vanadium nitrogenase, and AnFeH for the iron-only nitrogenase. However, the substitution of NiFeH with VnFeH in in vitro FeMo-co synthesis reactions has been successfully carried out (21). This result does not support the hypothesis that NiFeH and its analogs specify the heteromeric in the nitrogenase enzymes.

Another role for NiFeH in cofactor biosynthesis could involve its interaction with NiFeNE. The concept of NiFeH interaction with NiFeNE is very appealing, given the structural resemblance between NiFeNE and dinitrogenase. To date, experiments to show the interaction of wild-type NiFeH with NiFeNE were unsuccessful. Cross-linking experiments that involved purified NiFeNE, NiFeH, and the carbodiimide cross-linker EDC (1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride) in the presence or absence of NiFeB-co proved inconclusive.3 In this study, we have shown the interaction of NiFeNE with NiFeH by using a site-specific-altered enzyme, L127Δ, that forms a tight protein complex with dinitrogenase. L127Δ NifH, although im-

3 P. Rangaraj and P. W. Ludden, unpublished data.
4 J. T. Roll and G. P. Roberts, unpublished data.

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