In Vitro Biosynthesis of Iron-Molybdenum Cofactor and Maturation of the nif-encoded Apodinitrogenase

EFFECT OF SUBSTITUTION FOR NifH WITH SITE-SPECIFICALLY ALTERED FORMS OF NifH*

Priya Rangaraj, Matthew J. Ryle‡, William N. Lanzilotta‡‡, Paul W. Ludden†, and Vinod K. Shah

From the Department of Biochemistry and Center for the Study of Nitrogen Fixation, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706 and the ‡Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322

NiFH has three different roles in the nitrogenase enzyme system. Apart from serving as the physiological electron donor to dinitrogenase, NiFH is involved in iron-molybdenum cofactor (FeMo-co) biosynthesis and in maturation of the FeMo-co-deficient form of apodinitrogenase to a FeMo-co-activable form (apodinitrogenase maturation). The exact roles of NiFH in these processes are not well understood. In the present study, the features of NiFH required for the aforementioned processes have been investigated by the use of site-specifically altered forms of the enzyme. The ability of six altered forms of NiFH inactive in substrate reduction (K15R, D39N, D43N, L127Δ, D129E, and F135Y) to function in vitro FeMo-co synthesis and apodinitrogenase maturation reactions was investigated. We report that the ability of NiFH to bind and not hydrolyze MgATP is required for it to function in these processes. We also present evidence that the ability of NiFH to function in these processes is not dictated by the properties known to be required for its function in electron transfer to dinitrogenase. Evidence toward the existence of separate, overlapping sites on NiFH for each of its functions (substrate reduction, FeMo-co biosynthesis, and apodinitrogenase maturation) is presented.

The MgATP- and reductant-dependent reduction of nitrogen to ammonia is catalyzed by the enzyme nitrogenase, which is composed of two component proteins (1), dinitrogenase (MoFe protein or NiFD) and dinitrogenase reductase (Fe protein or NiFH). Dinitrogenase is an α/β4 tetramer encoded by the nifD and nifK genes, respectively (2), and is associated with two types of metal clusters, the P-clusters and the iron-molybdenum cofactor (FeMo-co)2. NiFH is a homodimer with a single 4Fe-4S cluster ligated to the dimer via Cys97 and Cys132 from each subunit (7).

NiFH has at least three distinct roles in the nitrogenase enzyme system (8). First, it serves as the obligate physiological electron donor to dinitrogenase during nitrogenase turnover (9). The role of NiFH in nitrogenase turnover has been well documented. The sequence of events leading to the transfer of electrons from NiFH to dinitrogenase is known as the “Fe protein cycle” (10). NiFH itself is reduced by either flavodoxin or ferredoxin in vivo or by dithionite in vitro. The reduced NiFH binds two molecules of MgATP and undergoes a conformational change (11) that affects several properties of NiFH, including the reduction of midpoint potential of the 4Fe-4S cluster as well as its ability to interact with dinitrogenase (12). MgATP hydrolysis is then coupled to a one-electron transfer from NiFH to dinitrogenase. The complex dissociates, and the molecules of MgADP bound to NiFH are now exchanged for MgATP. This cycle continues until a sufficient number of electrons accumulate on dinitrogenase for the reduction of N2. The features of NiFH required for this function include the ability of NiFH (i) to interact with low potential electron donors, (ii) to bind MgATP, (iii) to undergo the nucleotide-induced conformational change, (iv) to bind dinitrogenase, (v) to transfer electrons to dinitrogenase, and (vi) to hydrolyze MgATP. Altered forms of NiFH that are unable to perform one or more of the above functions are completely nonfunctional in substrate reduction.

Second, NiFH is required for the biosynthesis of FeMo-co (13–15). Azotobacter vinelandii mutants lacking any one of the nifH, nifN, nifE, or nifB gene products produce a FeMo cofactorless dinitrogenase (2, 5), referred to as apodinitrogenase (this form of apodinitrogenase contains the P-clusters). At least seven nif gene products are known to be involved in FeMo-co biosynthesis: nifQ, nifV, nifX, nifN, nifE, nifB, and nifH (2, 16, 17). The nifQ gene product has been postulated in molybdenum processing during FeMo-co biosynthesis. The nifV gene encodes homocitrate synthetase (18). Recently, NiFX has been demonstrated to be required for in vitro FeMo-co synthesis, although its exact role is not known. The nifN and nifE gene products together form a tetrameric protein that shows a high sequence similarity to the nifK and nifD gene products (20). Thus, NiFNE has been postulated to form a scaffold upon which FeMo-co is synthesized (21, 22). The metabolic product of NiFB, NiFB cofactor (23), has been shown to function as a specific iron and sulfur donor to FeMo-co during FeMo-co biosynthesis (24). NiFH is also required for FeMo-co biosynthesis, but the exact role(s) it plays during cofactor biosynthesis is not clear.

Third, NiFH is involved in the maturation of apodinitrogenase (25). The subunit composition of apodinitrogenase in an A. vinelandii mutant carrying a deletion of nifH is tetrameric (α/β2) and is not FeMo-co-activable (25, 26). It is known that the association of another protein, referred to as γ, with α/β2 apodinitrogenase is required for its in vitro activation by FeMo-co (25). γ has been shown to function as a chaperone-insertase during the biosynthesis of dinitrogenase (27). The

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‡ Present address: Dept. of Molecular Biology and Biochemistry, University of California, Irvine, CA 92697.

¶ To whom correspondence and reprint requests should be addressed. Tel.: 608-262-6859; Fax: 608-262-3453; E-mail: ludden@biochem.wisc.edu.

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processing of αβ, apodinitrogenase to a FeMo-co-activatable, αβγNif species is termed apodinitrogenase maturation. One role of NifH in apodinitrogenase maturation is that it mediates the association of γNif with αβγNif in the presence of MgATP. Interestingly, His-tagged versions of αβγNif apodinitrogenase do not seem to require γ for activation by purified FeMo-co (28).

The roles of NifH in FeMo-co biosynthesis and in apodinitrogenase maturation are not yet well understood, and the features of NifH required for its function in the above processes are not known. Certain A. vinelandii mutants contain altered forms of NifH that are impaired not only in catalysis but also in FeMo-co synthesis and in apodinitrogenase maturation. For example, A. vinelandii strain UW97 (25, 29) produces an altered form of NifH (S44F) that is nonfunctional in all three processes. However, several A. vinelandii mutants containing altered forms of NifH impaired in substrate reduction have been shown to perform both FeMo-co synthesis and apodinitrogenase maturation. The A157S, M156C, R101H, and D125E forms of NifH have been shown to be inactive or substantially impaired in substrate reduction but active in FeMo-co synthesis and apodinitrogenase maturation in vivo as indicated by the presence of active dinitrogenase in these mutant strains (30–34). The A157S NifH has been shown to bind MgATP but is unable to undergo the nucleotide-induced conformational change or to transfer electrons to dinitrogenase and hydrolyze MgATP; yet it is able to synthesize catalytically active dinitrogenase (30). Similarly, D125E NifH is impaired in MgATP hydrolysis but is fully functional in the biosynthesis and insertion of FeMo-co into apodinitrogenase (34). The ability of these forms of NifH, impaired in catalysis and with altered MgATP reactivities, to function in FeMo-co synthesis and apodinitrogenase maturation strongly suggests a role(s) for NifH independent of its role in substrate reduction. We have reported previously that the presence of a redox-active Fe4S4 cluster in NifH is not required for its function in FeMo-co synthesis and in apodinitrogenase maturation (35). This further evidence in support of distinct roles for NifH in nitrogenase turnover, FeMo-co biosynthesis, and apodinitrogenase maturation.

In an attempt to identify the features of NifH required for its function in FeMo-co synthesis and apodinitrogenase maturation, altered forms of the enzyme generated by site-directed mutagenesis were tested for their ability to function in in vitro FeMo-co synthesis and apodinitrogenase maturation reactions. Here, we report that the ability of NifH to perform MgATP hydrolysis (required in electron transfer) is not required for its function in FeMo-co biosynthesis and apodinitrogenase maturation. The features of NifH required for its function in apodinitrogenase maturation seem to be distinct from those required for its function in FeMo-co synthesis. This observation supports the hypothesis that NifH contains distinct domains that enable it to perform each of the above mentioned processes independently. Therefore, a mutation in a region that renders NifH inactive for one function may not affect its ability to function in the other processes.

EXPERIMENTAL PROCEDURES

Strains—Azotobacter vinelandii strains UW97 (S44F NifH; Ref. 29) and CA11.1 (ΔnifHΔDKΔnifDGK::spc; Ref. 36) have been described. Growth, derepression, and cell breakage have been described (37). All strains were grown in the presence of molybdenum and were nif-derpressed.

Materials—Sodium dithionite was from Fluka. Leupeptin, phenylmethylsulfonyl fluoride, phosphocreatine, creatine phosphokinase, and homocitrate lactone were from Sigma. ATP was purchased as a disodium salt from Sigma and was of the highest purity available. Tris base and glycine were from Fisher. Nitrocellulose membrane was from Millipore Corp. Acrylamide/bisacrylamide solution (37.5:1) and the equipment for SDS-polyacrylamide gel electrophoresis were from Bio-Rad.

Ammonium tetrahydroxylodolate (NH₄H₂Mo₅S₄) was a gift from Dr. D. Coucouvanis.

Buffers—Twenty-five mM Tris-HCl (pH 7.4) was used throughout this work. Buffers were sparged with purified N₂ for at least 30 min and were deaerated and flushed repeatedly with purified argon on a gassing manifold. All buffers contained 1.7 mM 2-mercaptoethanol.

Site-directed Mutagenesis, Expression, and Purification of Altered and Wild-type NifH—Oligonucleotide-directed mutagenesis of A. vinelandii nifH was performed as described previously (38, 39). Expression and purification of K15R (40), D99N (41), D43N, L127A (42), D219E (43), and P135Y (44) forms of NifH were carried out as previously reported. All of the altered forms of NifH were purified to homogeneity as judged by analysis on SDS gels stained with Coomassie Blue.

In Vitro Synthesis of FeMo-co (FeMo-co Synthesis Assay)—FeMo-co synthesis reactions were carried out as described by Shah et al. (45). To 9-ml serum vials, flushed with purified argon and rinsed with anaerobic Tris-HCl (pH 7.4) buffer, were added the following: 100 μl of anaerobic Tris-HCl (pH 7.4); 100 nmol of homocitrate, 200 μl of an 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 sodium dithionite), 200 μl of extract (2 mg of protein) of strain UW97 (as a source of NifNE, NifB cofactor, γ, and αβγNif apodinitrogenase), and purified wild-type or site-specifically altered NifH (50 μg of protein). It should be noted that the A. vinelandii strain CA11.1 (ΔnifHΔDKΔnifDGK::spc) that is incapable of either FeMo-co synthesis or apodinitrogenase maturation (25, 29). However, the presence of this altered form of NifH does not interfere with either FeMo-co synthesis or apodinitrogenase maturation reactions carried out by either the wild type or other altered NifHs. After the FeMo-co synthesis/insertion phase of 35 min, 10 ml of (NH₄)₂Mo₅S₄ were added to each of the assays to prevent further FeMo-co insertion into apodinitrogenase, since MoS₄²⁻ has been shown to bind apodinitrogenase irreversibly (46). Acetylene reduction was initiated by the addition of excess MgATP, purified wild-type NifH (0.1 mg of protein), and acetylene. Nitrogenase activity was then quantitated by acetylene reduction as described elsewhere (46).

NifH- and MgATP-dependent Maturation of Tetrameric (αβγ) Apodinitrogenase—NifH-dependent binding of γ to the αβγ forms of apodinitrogenase was monitored by anti-γ immunoblots. The appearance of anti-γ cross-reactive material in the position of αβγNif apodinitrogenase was taken as evidence of association of free γ with αβγ apodinitrogenase. Nine-ml serum vials were evacuated and flushed repeatedly with purified argon and rinsed with anaerobic Tris-HCl. The following were then added to appropriate vials: 100 μl of anaerobic Tris-HCl or 100 μl of an ATP-regenerating mixture (as described above); 200 μl (2 mg of protein) of extract of strain UW97 (as a source of αβγNif apodinitrogenase and γ), and wild-type or site-specifically altered NifH (45 μg of protein) in a total volume of 500 μl. The mixtures were incubated at 30 °C for 30 min, after which they were placed on ice. 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—FeMo-co synthesis assays were carried out as described above, except that 200 μl of desalted extract (2.5 mg of protein) of strain CA11.1 (ΔnifHΔDKΔnifDGK::spc; Ref. 36) was used as a source of the components required for FeMo-co synthesis. The vials were incubated for 30 min at 30 °C, after which they were placed on ice. Aliquots (20 μl) of the reaction mixtures were subjected to anoxic native gel electrophoresis followed by immunoblotting using anti-γ antibody.

Anaerobic Native Gel Electrophoresis—Proteins were separated on anaerobic native gels with a 7–14% acrylamide and 0–20% sucrose gradient as described previously (25).

Antibodies and Immunoblot Analysis—Antibody to γ was a gift from Mary Homer. The protocols for immunoblotting and developing with modifications by Brandner et al. (47) have been described.

Protein Assays—Protein concentrations were determined by the bicinchoninic acid method using bovine serum albumin as a standard (48).

RESULTS

Identification of Features of NifH Required for FeMo-co BioSynthesis and Apodinitrogenase Maturation—Besides serving as the physiological electron donor to dinitrogenase during catalysis, NifH is involved in the biosynthesis of FeMo-co (13–
and in the activation of apodinitrogenase by preformed FeMo-co (25, 26). But the precise role(s) played by NifH in the latter two processes remains largely unknown.

In an attempt to identify the features of NifH required for its function in FeMo-co synthesis and in apodinitrogenase maturation, altered forms of the enzyme generated by site-directed mutagenesis were used in place of the wild-type enzyme in the in vitro FeMo-co synthesis assays and apodinitrogenase maturation reactions. Six altered forms of NifH were used in this study: K15R, D39N, D43N, L127A, D129E, and F135Y. Table I lists the properties of these altered forms of the enzyme, and Fig. 1 shows the positions of Lys15, Asp39, Asp43, Asp129, Leu127, and Phe135 in the NifH structure (49). All of the above mentioned altered forms of NifH accumulate in the “native” form as judged by the presence of a normal 4Fe-4S cluster (40–44). Analysis of the structure of NifH (Fig. 1) (49), shows that (i) Lys15 is a part of the P-loop (residues 9–16; cyan in the right subunit), (ii) Asp39 and Asp43 residues are present in the Switch I region (residues 39–69; orange in the right subunit), and (iii) Leu127, Asp129, and Phe135 form a part of the Switch II region (residues 125–132; purple in the right subunit). The Switch I and II regions play important roles in the communication between the nucleotide binding site of NifH and the 4Fe-4S cluster (42, 50). Both of these regions share common features with Switch I and II regions in other nucleotide switch proteins (51). Given below are the results obtained when each of the site-specifically altered forms of NifH were used independently in place of wild-type NifH in in vitro FeMo-co synthesis and apodinitrogenase maturation reactions.

**K15R NifH—**It has been shown in previous work that Lys15 (15) is a part of the amino acid sequence, termed the “Walker A motif” or the “P-loop,” that is known to function in nucleotide binding (12). Lys15 has been shown to play a major role in both NifH interaction with the γ-phosphate of MgATP and in the MgATP-induced conformational change. K15R NifH showed no detectable binding to MgATP and an extremely low affinity for MgATP-induced conformational change. K15R NifH showed no detectable binding to MgATP and an extremely low affinity for MgATP-induced conformational change.

In order to determine if K15R NifH could function in FeMo-co biosynthesis, it was used in place of wild-type NifH in in vitro FeMo-co synthesis assays. Assays using K15R NifH for FeMo-co synthesis showed minimal (background) acetylene reduction activity when compared with the wild-type NifH (data not shown).

The in vitro FeMo-co synthesis reaction monitors the activity of the newly formed dinitrogenase and thus is a measure of both the amount of FeMo-co synthesized and the amount of FeMo-co inserted into apodinitrogenase. Therefore, these assays monitor the ability of the altered forms of NifH to function in both FeMo-co synthesis and in apodinitrogenase maturation. There exists the possibility that these altered enzymes are impaired in their ability to carry out apodinitrogenase maturation yet are perfectly capable of carrying out FeMo-co synthesis and consequently do not show detectable activity by the in vitro FeMo-co synthesis assay. To address this possibility, the γ gel shift assay was used to monitor the ability of the altered forms of NifH to function in FeMo-co synthesis. In the absence of FeMo-co, the dimeric form of γ (γ2) migrates significantly slower than γ bound to FeMo-co (γ-FeMo-co) on anoxic, native gels. Upon FeMo-co synthesis, γ2 binds FeMo-co (γ-FeMo-co) and migrates at a position distinct from the γ2 position (27). The gel shift of γ can thus be used as a convenient assay for the presence of newly synthesized FeMo-co.

The FeMo-co synthesis assays were performed using extracts of *A. vinelandii* strain CA11.1 (ΔnifHDK ΔnifDGG::xpc) as a source of NifNE, NifB cofactor, and γ2, as described under “Experimental Procedures.” The results of this experiment are shown in the anti-γ immunoblots depicted in Fig. 2. γ in the extract of strain CA11.1 is a dimer (γ2), since the strain is

### Table I

| NifH   | MgATP binding | Conformational change | Binding dinitrogenase | e− transfer | MgATP hydrolysis | FeMo-co synthesis | Apodinitrogenase maturation |
|--------|----------------|-----------------------|----------------------|-------------|------------------|-------------------|--------------------------|
| K15R  | No             | No                    | No                   | No          | No               | No                | No                      |
| D39N  | Yes            | Yes                   | Yes                  | Yes         | Yes              | Yes               | Yes                     |
| D43N  | Yes            | Yes                   | Yes                  | Yes         | Yes              | Yes               | Yes                     |
| L127A | Yes            | MgATP-bound state     | Yes                  | Yes         | Yes              | Yes               | Yes                     |
| D129E | Yes            | Yes                   | Yes                  | No          | No               | Yes               | Yes                     |
| F135Y | Yes            | Yes                   | Yes                  | No          | No               | Yes               | Yes                     |

* Conformational changes brought about in NifH upon binding MgATP.

* Ability of NifH to transfer electrons to dinitrogenase.

* Ability of the site-specifically altered forms of NifH to function in in vitro FeMo-co synthesis.

* Ability of the site-specifically altered forms of NifH to function in the maturation of β3δ2 apodinitrogenase.

* No, inability of the site-specifically altered forms of NifH to function in the properties tested.

* Yes, ability of the site-specifically altered forms of NifH to function in the properties tested.

* “MgATP-bound” conformation brought about in NifH upon the deletion of Leu127.

![FIG. 1. View of NifH highlighting the positions of Asp39, Asp43, Lys15, Leu127, and Phe135 in one of the subunits of NifH.](image).

The P-loop (residues 9–16) is highlighted in cyan, the switch I region (residues 39–69) in orange, and the switch II region (residues 125–132) in purple. The 4Fe-4S cluster is depicted in yellow. The α-carbon backbone is shown as a gray ribbon. Side chains for Asp39, Asp43, Lys15, Leu127, and Phe135 are shown in color. Modeling was performed using RasMol; NifH structure was determined by Georgiadis et al. (49).
impaired in FeMo-co synthesis (Fig. 2, lane 1). Upon the addition of NifH to the reaction mixture (containing all components required for FeMo-co synthesis), FeMo-co is synthesized and accumulates on γ, bound to FeMo-co (γ-FeMo-co) electrophoreses as a faster migrating band on anoxic native gels and can be clearly distinguished from the slower migrating γ species (Fig. 2, compare lanes 1 and 2). The γ gel shift assays with K15R showed no shift of γ upon anoxic native gel electrophoresis of the reaction mixture (Fig. 2, lane 3). These results suggest that the ability to bind MgATP may be a requirement for NifH to function in FeMo-co biosynthesis. However, results below will show that a native form of NifH with the ability to bind MgATP is not necessarily sufficient to allow NifH to function in FeMo-co synthesis. These results also corroborate previously reported results with A157S and D125E altered forms of NifH, respectively. Nevertheless, results with other altered forms of NifH given below suggest that while a form of NifH that can bind MgATP may be necessary, it is still not sufficient for its function in apodinitrogenase maturation.

**D39N NifH**—Asp<sup>39</sup> is part of the Switch I region of NifH that connects the nucleotide binding site to the dinitrogenase docking surface of NifH (50). Asp<sup>39</sup> in NifH has been suggested to play an important role in the dissociation of the NifH-dinitrogenase complex, following electron transfer from NifH to dinitrogenase during catalysis (41). The D39N form of NifH forms a stable complex with dinitrogenase with no detectable dissociation rate. The properties of D39N NifH are summarized in Table I (second line). This altered form of NifH has been shown to bind MgATP and to undergo the nucleotide-induced conformational change, to bind and transfer one electron to dinitrogenase, and also to hydrolyze MgATP; but it shows no substrate reduction activity because of its inability to dissociate from dinitrogenase.

In order to determine if D39N NifH could function in FeMo-co synthesis, the wild-type NifH was replaced by D39N NifH in *in vitro* FeMo-co synthesis assays. These assays showed background FeMo-co synthesis activities, suggesting that this altered form of NifH could not function in *in vitro* FeMo-co synthesis (data not shown). Monitoring FeMo-co synthesis activity of D39N NifH via the *in vitro* FeMo-co synthesis assay may not be accurate, because D39N NifH has been shown to form an irreversible complex with dinitrogenase. Therefore, these results were reconfirmed using the γ gel shift assay, whereby only FeMo-co synthesis can be monitored. The results presented in Fig. 2 show that D39N NifH did not function in the FeMo-co biosynthetic process (Fig. 2, compare lanes 1 and 4). D39N NifH was also tested for function in the apodinitrogenase maturation reaction with the extract of strain UW97 (S44F NifH). The results presented in Fig. 3 (lane 4) show that D39N cannot mediate the association of γ with αβ<sub>2</sub> apodinitrogenase.

**Fig. 2.** Immunoblot of an anoxic native gel developed with antibody to γ, illustrating γ monomerization upon binding FeMo-co. *In vitro* FeMo-co synthesis reactions were performed as described and subjected to anoxic native polyacrylamide gel electrophoresis. Lane 1, reaction excluding NifH; lane 2, reaction including wild-type NifH; lane 3, reaction including K15R NifH; lane 4, reaction including D39N NifH; lane 5, reaction including D43N NifH; lane 6, reaction including L127A NifH; lane 7, reaction including D129E NifH; lane 8, reaction including F135Y NifH. Details of the assay are given under “Experimental Procedures.”

**Fig. 3.** Immunoblot of an anoxic native gel developed with antibody to γ, illustrating NifH- and MgATP-dependent association of γ with αβ<sub>2</sub> apodinitrogenase. Apodinitrogenase activation reactions were performed as described and subjected to anoxic native polyacrylamide gel electrophoresis. Lane 1, reaction including wild-type NifH; lane 2, reaction excluding NifH; lane 3, reaction including K15R NifH; lane 4, reaction including D39N NifH; lane 5, reaction including D43N NifH; lane 6, reaction including L127A NifH; lane 7, reaction including D129E NifH; lane 8, reaction including F135Y NifH. Details of the assay are given under “Experimental Procedures.”
Although D39N NifH can bind MgATP, undergo the MgATP-induced conformational change, bind dinitrogenase, transfer one electron to dinitrogenase, and also hydrolyze MgATP, this form of NifH cannot function in either FeMo-co synthesis or apodinitrogenase maturation. This in turn suggests that the residues in the Switch I region in NifH may play an important role in the above mentioned processes.

D43N NifH—Asp^{43} in NifH is one of the residues that forms a protein chain that connects the nucleotide binding site to the dinitrogenase docking surface of NifH (the proposed Switch I region (41)). This altered form of NifH can bind MgATP and undergo the nucleotide-induced conformational changes but cannot bind dinitrogenase, cannot transfer electrons to dinitrogenase, and cannot hydrolyze MgATP as summarized in Table I (third line).

D39N NifH was tested for FeMo-co synthesis in \textit{in vitro} FeMo-co synthesis assays with extract of strain UW97 (S44F NifH) and in \gamma \text{ gel shift assays with extract of strain CA11.1 (}\textit{p}nifH\textit{D43N/DGK}\textit{::spc}\textit{), as described under \textquotedblleft Experimental Procedures.\textquotedblright} The \textit{in vitro} FeMo-co synthesis assays with D43N NifH showed minimal FeMo-co synthesis activity (data not shown), and the migration of \gamma \text{ on anoxic native gels did not change upon incubation with this altered form of NifH (Fig. 2, lane 5). These results clearly show that D43N NifH cannot function in FeMo-co synthesis. D39N NifH was also tested for apodinitrogenase maturation with extract of strain UW97. The results presented in Fig. 3 (lane 5) show that D43N NifH cannot function in apodinitrogenase maturation. These results, in conjunction with the results obtained with D39N NifH, suggest that the residues in the Switch I region in NifH may play an important role in both FeMo-co synthesis and apodinitrogenase maturation.

L127Δ NifH—Leu^{127} constitutes one of the residues in the Switch II region between Asp^{125} and Cys^{132}, the residues that have been hypothesized to serve as a pathway for signal transduction between the MgATP binding site and the 4Fe-4S cluster in NifH. Deleting Leu^{127} resulted in a form of NifH that approximates the MgATP-bound conformation of NifH (42, 53). This form of NifH has been shown to bind dinitrogenase with extremely high affinity in the absence of MgATP (53). The L127Δ NifH has also been shown to bind MgATP and to transfer one electron to dinitrogenase at a rate of 0.2 s^{-1}, but it is unable to hydrolyze MgATP (Table I, fourth line).

L127Δ NifH was tested for FeMo-co synthesis by \textit{in vitro} FeMo-co synthesis assays and was found to support minimal levels of \textit{in vitro} FeMo-co synthesis activity (data not shown). These results suggest that this altered form of NifH is inactive in FeMo-co synthesis. Even if L127Δ NifH were competent in FeMo-co synthesis, its activity could not be monitored by this assay because of its ability to form a stable and irreversible complex with the newly formed dinitrogenase. Therefore, the \gamma \text{ gel shift assays were employed, using the change in the migration of \gamma \text{ on anoxic native gels as an indicator of FeMo-co synthesis. The results presented in Fig. 2 (lane 6) show that this altered form of NifH could not function in FeMo-co synthesis. L127Δ NifH was used in apodinitrogenase maturation reactions in place of wild-type NifH with extract of strain UW97 (S44F NifH). As seen in Fig. 3 (lane 6), L127Δ NifH was found to be inactive in the apodinitrogenase maturation process.

Although L127Δ NifH can bind MgATP and can bind and transfer one electron to dinitrogenase, it was inactive in both FeMo-co synthesis and apodinitrogenase maturation processes. These data suggest that the conformational change brought about by deleting Leu^{127} in NifH is deleterious to its function in FeMo-co synthesis and apodinitrogenase maturation. There is a possibility that L127Δ NifH could bind NifNE (given the structural similarity between NifNE and dinitrogenase) and form a stable complex as with dinitrogenase, thereby actually inhibiting or slowing down FeMo-co synthesis. Similarly, L127Δ NifH may bind apodinitrogenase irreversibly and therefore not function in apodinitrogenase maturation. Complex formations between L127Δ NifH and either NifNE or apodinitrogenase are currently being tested.

D129E NifH—Asp^{129} is one of the strictly conserved residues in NifH, and analysis of the structure of NifH suggests that Asp^{129} may be involved in nucleotide interactions (49). Asp^{129} has been shown to play an important role in the communication between the 4Fe-4S cluster and the nucleotide binding site in NifH (43). The D129E NifH has been shown to bind MgATP, undergo nucleotide-induced conformational changes, and dock with dinitrogenase but cannot support MgATP hydrolysis, electron transfer to dinitrogenase, or substrate reduction (43).

The \textit{in vitro} FeMo-co synthesis reactions with D129E NifH showed that this altered form of NifH functions in FeMo-co synthesis. The activity generated in reactions with D129E NifH (24.0 nmol of C\textsubscript{2}H\textsubscript{4} formed per min per assay) was comparable with that of the wild-type NifH (24.7 nmol of C\textsubscript{2}H\textsubscript{4} formed per min per assay). These results were reconfirmed by the \gamma \text{ gel shift assay using the extract of strain CA11.1 (}\textit{p}nifH\textit{D129E/DGK}\textit{::spc}\textit{). The results of these assays are presented in Fig. 2 (lane 7). The \textit{in vitro} FeMo-co synthesis reaction that involved D129E NifH shows \gamma \text{ migrating at the \gamma-FeMo-co position, thereby indicating FeMo-co synthesis. These results suggest that the ability of NifH to transfer electrons to dinitrogenase and to hydrolyze nucleotide are properties not required for NifH to function in FeMo-co synthesis. These findings also support previously reported data by Gavini and Burgess (30) and Wolle \textit{et al.} (34).

D129E NifH was tested for function in apodinitrogenase maturation by apodinitrogenase activation reactions. The results of these assays, presented in Fig. 3, clearly show that D129E NifH is competent in mediating the association of \gamma \text{ with apodinitrogenase (Fig. 3, lane 7). These results suggest that nucleotide hydrolytic activity and electron transfer ability are not essential for NifH to function in the apodinitrogenase maturation process.

F135Y NifH—Phe^{135} is one of the conserved residues that is present in the Switch II region and is two residues away from Cys^{132} that ligates the 4Fe-4S cluster in NifH. Analysis of the structure of NifH (49) suggests that Phe^{135} along with Ala^{98} and Val^{130} create a hydrophobic pocket under the 4Fe-4S cluster. Studies on forms of NifH altered at the Phe^{135} site have revealed an important role for Phe^{135} in defining both the spectroscopic properties and the nucleotide-induced changes in the redox potential of the 4Fe-4S cluster (44). The data published by Ryle \textit{et al.} (44) suggest that this residue is critical for the MgADP-induced changes in NifH. The properties of F135Y NifH are listed in Table I (sixth line). The F135Y NifH is able to bind MgATP and to undergo nucleotide-induced conformational changes, but it is unable to bind and to transfer electrons to dinitrogenase and unable to hydrolyze MgATP.

F135Y NifH was tested in FeMo-co synthesis by \textit{in vitro} FeMo-co synthesis assays and was found not to support FeMo-co synthesis (data not shown). These results were reconfirmed by the \gamma \text{ gel shift assay. The results presented in Fig. 2 (lane 8) clearly show no change in the migration of \gamma \text{ in the reaction mixture containing F135Y NifH, corroborating the results obtained in the \textit{in vitro} FeMo-co synthesis assays. These results suggest a role for Phe^{135} in maintaining a form of NifH that is capable of participation in FeMo-co synthesis.

F135Y NifH was added to apodinitrogenase maturation re-
actions in place of wild-type NifH, the results of which are presented in Fig. 3 (lane 8). These results clearly show that F135Y NifH is competent in mediating the association of γ2 with apodinitrogenase (Fig. 3, compare lanes 1 and 8), although less effective than wild-type NifH. These results suggest that the set of properties required for NifH to function in apodinitrogenase maturation is not identical to the set of properties required for NifH to bind dinitrogenase at the site involved in substrate reduction, to transfer electrons to dinitrogenase, and to hydrolyze MgATP. Another important finding in the present work with F135Y NifH is the observation that F135Y NifH can function in apodinitrogenase maturation and not in FeMo-co synthesis. This suggests that the features of NifH necessary for FeMo-co synthesis are not identical to those required in apodinitrogenase maturation. This, in turn, is evidence for the existence of separate sites on NifH involved in substrate reduction, FeMo-co synthesis, and apodinitrogenase maturation.

**DISCUSSION**

In the present study, we have used altered forms of NifH that were generated by site-directed mutagenesis of nifH in *in vitro* FeMo-co synthesis and apodinitrogenase maturation reactions. The six altered forms of NifH (K15R, D39N, D43N, L127Δ, D129E, and F135Y) chosen in this study were completely inactive in substrate reduction and had varied MgATP reactivities. In the present work, we have shown that apart from D129E NifH, the other altered forms did not function in FeMo-co synthesis. From these results, we believe that under the conditions of *in vitro* FeMo-co synthesis, the ability of NifH to transfer electrons to dinitrogenase and to hydrolyze MgATP are properties not required for its function in FeMo-co synthesis. These data are in agreement with the results obtained by Gavini and Burgess (30) and Wolle et al. (34) and extend our understanding of the role of NifH in the FeMo-co biosynthetic process. Nonhydroxylatable analogues of ATP such as β,γ-methylene-ATP, when used in *in vitro* FeMo-co synthesis reactions, have been shown neither to support nor to inhibit FeMo-co synthesis (54). These analogues may not serve as effective ligands to NifH and consequently are unable to support *in vitro* FeMo-co synthesis.

The altered forms of NifH were also tested for their ability to function in apodinitrogenase maturation, which is the MgATP- and NifH-dependent association of γ2 with αβ3 apodinitrogenase. The K15R, D39N, D43N, and L127Δ forms of NifH did not support apodinitrogenase maturation, while the D129E and F135Y forms of NifH were found to be active in this process. These data provide strong evidence that a form of NifH capable of binding MgATP is required for its function in apodinitrogenase maturation. The ability of NifH to perform MgATP hydrolysis is not an essential feature for apodinitrogenase maturation, since the D129E and F135Y altered forms can bind MgATP but cannot carry out MgATP hydrolysis; these forms can support the association of γ with αβ3 apodinitrogenase, and the D129E form can support FeMo-co synthesis.

In this study, we have presented evidence that the ability of NifH to function in FeMo-co synthesis and apodinitrogenase maturation is not dictated by the properties that are known to be required for its function in catalysis. This is illustrated by the analyses of the K15R and D39N forms of NifH, which show that these altered enzymes possess very different properties. While D39N NifH is capable of nucleotide binding, undergoying MgATP-induced conformational change, of binding and transferring electrons to dinitrogenase, and also of MgATP hydrolysis, K15R NifH is not capable of any of the above. However, both of these altered forms of NifH were shown to be inactive in FeMo-co synthesis and apodinitrogenase maturation. Taken together, these observations strongly suggest that the properties required for NifH for FeMo-co synthesis and apodinitrogenase maturation are distinct from those that govern electron transfer for substrate reduction.

Finally, one altered form of NifH, F135Y, was found to function in apodinitrogenase maturation but not in FeMo-co synthesis. This result suggests that the properties of NifH required for apodinitrogenase maturation are different from those required for its function in FeMo-co synthesis. This, in turn supports the hypothesis that NifH contains discrete domains that enable it to independently perform the three known functions of substrate reduction, FeMo-co synthesis, and apodinitrogenase maturation. These domains can be envisioned to partially overlap each other such that, depending on the location of the mutation, the function(s) dictated by that domain will be affected. This is also in agreement with the hypothesis implied by Pierrard et al. (55). Their work with *Rhodobacter capsulatus* nif mutants, in which the Arg102 residue (the site of ADP-ribosylation) (18) was substituted by 13 different amino acids, showed that Arg at the 102-position was optimal but not essential for diazotrophic growth. Their results were consistent with the hypothesis that the Arg at this position belongs to different overlapping functional domains in NifH and that Arg is the ideal residue that optimally satisfies all the requirements dictated by these domains.

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**REFERENCES**

1. Burris, R. H. (1991) *J. Biol. Chem.* **266**, 9339–9342
2. Roberts, G. P., MacNeil, T., MacNeil, D., and Brill, W. J. (1978) *J. Bacteriol.* **136**, 267–279
3. Madden, M. S., Kindon, N. D., Ludden, P. W., and Shah, V. K. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6517–6521
4. Imperial, J., Hoover, T. R., Madden, M. D., Ludden, P. W., and Shah, V. K. (1989) *Biochemistry* **28**, 7796–7799
5. Shah, V. K., and Brill, W. J. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3249–3253
6. Hawkes, T. R., McLean, P. A., and Smith, B. E. (1984) *Biochem. J.* **217**, 317–321
7. Hanifinger, R. P., and Howard, J. B. (1983) *J. Biol. Chem.* **258**, 13486–13492
8. Allen, R. M., Chatterjee, R., Madden, M., Ludden, P. W., and Shah, V. K. (1994) *Crit. Rev. Biotechnol.* **14**, 225–249
9. Ljones, T., and Burris, R. H. (1972) *Biochim. Biophys. Acta* **275**, 93–101
10. Torney, R. N. F., Ashley, G. A., K., and Lowe, D. J. (1993) in *ACS Symp. Ser.* **535**, 290–302
11. Zumb, W. G., Mortensen, E., and Palmer, G. (1974) *Eur. J. Biochem.* **46**, 525–535
12. Seeffeldt, L. C., Morgan, T. V., Dean, D. R., and Mortensen, L. E. (1992) *J. Biol. Chem.* **267**, 6680–6688
13. Shah, V. K., Hoover, T. R., Imperial, J., Paustian, T. D., Roberts, G. P., and Ludden, P. W. (1988) in *Nitrogen Fixation: Hundred Years After* (Bothe, H., de Bruijn, F. J., Newton, W. E., ed) pp. 115–120, Gustav Fischer, Cologne
14. Filler, W. A., Kemp, R. M., Ng, J. C., Hawkes, T. R., Dixon, R. A., and Smith, B. E. (1986) *Eur. J. Biochem.* **160**, 371–377
15. Robinson, A. C., Dean, D. R., and Burgess, B. K. (1987) *J. Biol. Chem.* **262**, 14327–14332
16. Imperial, J., Ugalde, R. A., Shah, V. K., and Brill, W. J. (1984) *J. Bacteriol.* **158**, 187–194
17. Hoover, T. R., Shah, V. K., Roberts, G. P., and Ludden, P. W. (1986) *J. Bacteriol.* **167**, 999–1003
18. Pope, M. R., Murrell, S. A., and Ludden, P. W. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3173–3177
19. Zeng, L., White, R. H., and Dean, D. R. (1997) *J. Bacteriol.* **179**, 5963–5966
20. Deans, D. R., and Brigle, K. E. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5720–5723
21. Paustian, T. D., Shah, V. K., and Roberts, G. P. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6982–6986
22. Roll, J. T., Shah, V. K., Dean, D. R., and Roberts, G. P. (1995) *J. Biol. Chem.* **270**, 4432–4437
23. Shah, V. K., Allen, J. R., Spangler, N. J., and Ludden, P. W. (1994) *J. Biol. Chem.* **269**, 1154–1158
24. Allen, R. M., Chatterjee, R., Spangler, N. J., and Ludden, P. W. (1989) *J. Biol. Chem.* **264**, 10088–10095
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27. Homer, M. J., Dean, D. R., and Roberts, G. P. (1995) J. Biol. Chem. 270, 24745–24752
28. Christiansen, J., Goodwin, P. J., Lanzilotta, W. N., Seefeldt, L. C., and Dean, D. R. (1998) Biochemistry 37, 12611–12623
29. Pulakat, L., Hausman, B. S., Lei, S., and Gavini, N. (1996) J. Biol. Chem. 271, 1884–1889
30. Gavini, N., and Burgess, B. K. (1992) J. Biol. Chem. 267, 4157–4165
31. Bursey, E. H., and Burgess, B. K. (1998) J. Biol. Chem. 273, 29678–29685
32. Lowery, R. G., Chang, C. L., Davis, L. C., McKenna, M. C., Stephens, P. J., and Ludden, P. W. (1989) Biochemistry 28, 1206–1212
33. Wolle, D., Kim, C., Dean, D., and Howard, J. B. (1992) J. Biol. Chem. 267, 3667–3673
34. Wolle, D., Dean, D. R., and Howard, J. B. (1992) Science 258, 992–995
35. Rangaraj, P., Shah, V. K., and Ludden, P. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11250–11255
36. Waugh, S. I., Paulsen, D. M., Mylona, P. V., Maynard, R. H., Premakumar, R., and Bishop, P. E. (1999) J. Bacteriol. 172, 5021–5028
37. Shah, V. K., Davis, L. C., and Brill, W. J. (1972) Biochim. Biophys. Acta 192, 5021–5028
38. Jacobson, M. R., Brigle, K. E., Bennett, L. T., Setterquist, R. A., Wilson, M. S., Cash, V. L., Beynen, J., Newton, W. E., and Dean, D. R. (1989) J. Bacteriol. 171, 1017–1027
39. Seefeldt, L. C., and Mortenson, L. E. (1993) Protein Sci. 2, 93–102
40. Ryle, M. J., Lanzilotta, W. N., Mortenson, L. E., Watt, G. D., and Seefeldt, L. C. (1995) J. Biol. Chem. 270, 13112–13117
41. Lanzilotta, W. N., Fisher, K., and Seefeldt, L. C. (1997) J. Biol. Chem. 272, 4157–4165
42. Ryle, M., and Seefeldt, L. C. (1996) Biochemistry 35, 4766–4775
43. Lanzilotta, W. N., Ryle, M. J., and Seefeldt, L. C. (1995) Biochemistry 34, 10713–10722
44. Ryle, M. J., Lanzilotta, W. N., and Seefeldt, L. C. (1996) Biochemistry 35, 9424–9434
45. Shah, V. K., Imperial, J., Ugalde, R. A., Ludden, P. W., and Brill, W. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1636–1640
46. Shah, V. K., Ugalde, R. A., Imperial, J., and Brill, W. J. (1985) J. Biol. Chem. 260, 3891–3894
47. Brandner, J. P., McEwan, A. G., Kaplan, S., and Donohue, T. (1989) J. Bacteriol. 171, 360–368
48. Smith, P. K., Krohn, R. I., Hermanson, A. K., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 175–179
49. Georgiadis, M. M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J. J., and Rees, D. C. (1992) Science 257, 1653–1659
50. Renner, K. A., and Howard, J. B. (1996) Biochemistry 35, 5353–5358
51. Howard, J. B., and Rees, D. C. (1994) Annu. Rev. Biochem. 63, 255–264
52. Shah, V. K., Davis, L. C., Gordon, J. K., Orme-Johnson, W. H., and Brill, W. J. (1973) Biochim. Biophys. Acta 292, 246–255
53. Lanzilotta, W. N., Fisher, K., and Seefeldt, L. C. (1996) Biochemistry 35, 7188–7196
54. Chatterjee, R., Allen, R. M., Shah, V. K., and Ludden, P. W. (1994) J. Bacteriol. 176, 2747–2750
55. Pierrard, J., Willisen, J. C., Vignais, P. M., Gaspar, J. L., Ludden, P. W., and Roberts, G. P. (1993) Biochem. Biophys. Res. Commun. 192, 1225–1229
In Vitro Biosynthesis of Iron-Molybdenum Cofactor and Maturation of the nif-encoded Apodinitrogenase: EFFECT OF SUBSTITUTION FOR NifH WITH SITE-SPECIFICALLY ALTERED FORMS OF NifH

Priya Rangaraj, Matthew J. Ryle, William N. Lanzilotta, Paul W. Ludden and Vinod K. Shah

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