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

EFFECT OF SUBSTITUTION OF VNFH FOR NIFH*

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NIFH (the nifH gene product) has several functions in the nitrogenase enzyme system. In addition to reducing dinitrogenase during nitrogenase turnover, NIFH functions in the biosynthesis of the iron-molybdenum cofactor (FeMo-co), and in the processing of \( \alpha_2 \beta_2 \) apodinitrogenase 1 (a catalytically inactive form of dinitrogenase 1 that lacks the FeMo-co) to the FeMo-co-activatable \( \alpha_2 \beta_2 \gamma_2 \) form. The molybdenum-independent nitrogenase 2 (vnf-encoded) has a distinct dinitrogenase reductase protein, VNFH. We investigated the ability of VNFH to function in the in vitro biosynthesis of FeMo-co and in the maturation of apodinitrogenase 1. VNFH can replace NIFH in both the biosynthesis of FeMo-co and in the maturation of apodinitrogenase 1. These results suggest that the dinitrogenase reductase proteins do not specify the heterometal incorporated into the cofactors of the respective nitrogenase enzymes. The specificity for the incorporation of molybdenum into FeMo-co was also examined using the in vitro FeMo-co synthesis assay system.

The reduction of atmospheric \( \text{N}_2 \) to ammonium by biological systems is catalyzed by the nitrogenase enzymes. The aerobe Azotobacter vinelandii harbors three genetically distinct nitrogenase enzymes that are regulated by the metal content of the growth medium, among other factors (1–3). Nitrogenases 1, 2, and 3 are encoded by the nifH genes, respectively. Nitrogenases 1, 2, and 3 are encoded by the nifH genes, respectively.

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that protein a likely candidate for specifying the heterometal incorporated into the respective cofactors of the nitrogenase enzymes.

NIFH has multiple roles in the nitrogenase 1 enzyme system. In addition to MgATP-dependent electron transfer to dinitrogenase during substrate reduction, NIFH is required for the biosynthesis of FeMo-co (10, 11) and for the maturation of apodinitrogenase 1 (a catalytically inactive form of dinitrogenase 1 that lacks FeMo-co) to its FeMo-co-activatable form (27, 28). In the latter process, NIFH is required for the association of the γ protein (a non-nif-encoded protein) (28) with αβ2 apodinitrogenase 1 to form the FeMo-co-activatable αβ2γ hexamer (27). Some altered forms of NIFH that are unable to function as a reductant for nitrogenase-dependent substrate reduction are fully functional in FeMo-co biosynthesis and in the maturation of apodinitrogenase 1 (29–31), indicating that the characteristics of NIFH that enable it to function in nitrogenase turnover are not necessary for its role in the formation of active dinitrogenase.

In vivo studies by Joerger et al. (23) and Gollan et al. (32) suggest that NIFH supports FeV-co synthesis and that ANFH (the anfh gene product) supports FeMo-co synthesis. We utilized the in vitro FeMo-co synthesis assay system to definitively determine whether VNFH would function in FeMo-co biosynthesis; the ability of VNFH to replace NIFH in the formation of the FeMo-co-activatable αβ2γ form of apodinitrogenase 1 was also examined. Studies on the specificity of the incorporation of molybdenum into FeMo-co are discussed.

EXPERIMENTAL PROCEDURES

Materials—DEAE-cellulose was a Whatman DE52 product. Sephacryl S-100 and the Mono Q anion exchange column were from Pharmacia Biotech Inc. The fast protein liquid chromatography instrument was from LKB. Sodium dithionite (DTH) was purchased from Fluka Chemicals. Sodium metavanadate (NaVO3, 99.995% purity), Tris base, and glycine were Fisher products. Acrylamide/bisacrylamide solution was obtained from Bio-Rad. All reagents used for A. vinelandii growth medium were of analytical grade or higher purity. Tetrathiomolybdate ((NH4)2MoS4) was a gift from D. Coucouvanis, and (NH4)2Mo3S8 was obtained from Bio-Rad. All reagents used for the purification of VNFH—except for the extraction buffer—were purchased from Sigma.

Purification of VNFH—All column chromatography steps except for the extraction buffer contained 0.1 M NaCl in 0.025 M Tris-HCl, pH 7.4. Following application of the extraction buffer, VNFH-containing fractions that exhibited the highest activity were concentrated by ultrafiltration (described above) and purified further on a Mono Q anion exchange column used in conjunction with a fast protein liquid chromatography system. Two ml (6.8 mg of protein) of the VNFH-containing retentate from the ultrafiltration cell was applied onto the Mono Q column that had been equilibrated with 0.15 M NaCl in 0.025 M Tris-HCl. The column was washed with 1 bed volume of the equilibration buffer, following which VNFH was eluted using a 20-ml increasing linear gradient from 0.15 to 0.4 M NaCl in 0.025 M Tris-HCl, pH 7.4. VNFH eluted with 0.32 M NaCl in 0.025 M Tris-HCl. Active fractions were stored in 9-ml serum-stoppered vials at −80 °C. The ability of VNFH to transfer electrons to dinitrogenase 1 was tested using the acetylene reduction assay for nitrogenase activity, and the results were consistent with the published results. VNFH was equally effective as NIFH in transferring electrons to dinitrogenase 1, consistent with the results of Chisnell et al. (34).

Activation of Apodinitrogenase 1 by FeMo-co (FeMo-co Insertion Assay)—FeMo-co was prepared in N-formylmethionine as described previously (6). The reactions were performed in 9-ml serum-stoppered vials that were repeatedly evacuated, flushed with argon, and rinsed with 0.3 ml of 0.025 M Tris-HCl containing 1.7 mM DTH. The following components were added to the vials in the order indicated: 100 μl of 0.925 M Tris-HCl; 200 μl of an ATP-regenerating mixture (containing 3.6 mM ATP, 6.3 mM MgCl2, 20 units/ml creatine phosphokinase, and 6.3 mM DTH); 200 μl (3.8 mg of protein) of extract of strain DJ1030 (ΔnifHΔnifB, nif-depressed) as a source of αβ2γ apodinitrogenase 1 and the γ protein; and 10–50 μl (0.1 mg of protein) of the appropriate dinitrogenase reductase. The vials were incubated for 10 min at room temperature to allow the formation of αβ2γγ protein apodinitrogenase 1. One hundred μl of anoxic 50% glycerol were added to the reactions to be analyzed by native PAGE, and these vials were placed on ice. Ten μl of a solution containing an excess of FeMoco were added to each vial, which were incubated for 1 h at 30 °C at room temperature during which αβ2γγ protein apodinitrogenase 1 was activated by FeMo-co to form dinitrogenase 1. Fifty nmol of (NH4)2Mo5S8 (prepared in N-formylmethionine containing 1.7 mM DTH) were added to the vials to prevent further FeMo-co insertion into apodinitrogenase 1. Activity of the newly reconstituted dinitrogenase 1 was monitored by the C2H2 reduction assay for nitrogenase (12). (NH4)2Mo5S8 was excluded in certain incubations of the apodinitrogenase reductase (that used in the insertion phase of the assay) was added in place of 0.1 μl of NIFH normally added during the C2H2 reduction phase of the assay (12).

In Vitro FeMo-co Synthesis Assay—Nine-ml serum vials were repeatedly evacuated, flushed with argon, and rinsed with buffer containing 1.7 mM DTH. Components were added to the vials in the following order: 100 μl of 0.025 M Tris-HCl, 10 μl of 1 mM Na2MoO4, 20 μl of 5 mM homocitrinate (that had been treated with base to cleave the lactone, pH 8.0), and 200 μl of the ATP-regenerating mixture (defined above). The vials were incubated at room temperature for 10–20 min. Two hundred μl of extract (−3.8 mg of protein) of either DJ1030 (ΔnifHΔnifB, nif-depressed) or CA12 (ΔnifHΔDK, nif-depressed), 25 μl of a solution containing NifB-co, and 10–50 μl (0.1 mg of protein) of the appropriate dinitrogenase reductase were added to the vials. The vials were incubated at 30 °C for 30–90 min. Following this incubation, 100 μl of anoxic 50% glycerol were added to the reactions to be analyzed by anoxic native PAGE, and these vials were placed on ice. Five nmol of (NH4)2Mo5S8 (prepared as described above) were added to the remaining vials to prevent further FeMo-co synthesis during the subsequent C2H2 reduction assay. (NH4)2Mo5S8 was excluded from certain reactions to which 0.1 mg of the appropriate dinitrogenase reductase (that used in the synthesis phase of the assay) was added in place of 0.1 μl of NIFH normally added during the C2H2 reduction phase of the assay.

Native Gel Electrophoresis—Proteins were resolved on anoxic native
Dinitrogenase Reductases in FeMo-co Synthesis

**TABLE I**

| Dinitrogen reductase | FeMo-co insertion<sup>a</sup> | FeMo-co synthesis<sup>b</sup> |
|----------------------|-----------------------------|-----------------------------|
|                      | −MgATP                     | +MgATP                      |
| None                 | 0.3                         | 0.4                         | 0.03                         |
| NIFH                 | 0.5                         | 14.6                        | 29.8                         |
| VNFH                 | 0.4                         | 14.5                        | 8.2                          |
| VNFH (R. rubrum)<sup>c</sup> | 0.4                        | 13.7                        | **ND**                      |

<sup>a</sup> Assays contained 0.1 mg of the appropriate dinitrogenase reductase protein and 3.8 mg of extract of strain DJ1030 (ΔnifHΔnifBΔnifD, nif derepressed) as a source of αβ₂₂ apodinitrogenase and γ protein.

<sup>b</sup> FeMo-co insertion assays were performed as described under "Experimental Procedures"; activities are expressed as nanomoles of C₂H₄ formed/min/assay.

<sup>c</sup> FeMo-co synthesis assays were performed as described under "Experimental Procedures"; activities are expressed as nanomoles of C₂H₄ formed/min/assay.

<sup>d</sup> NIFH was purified from R. rubrum as described in Ludden and Burris (42).

<sup>e</sup> Not determined.

**RESULTS AND DISCUSSION**

**Ability of VNFH to Support the Maturation of Apodinitrogenase 1 to the FeMo-co-activatable αβ₂₂γ₂ Form**—The association of the γ protein with αβ₂₂ apodinitrogenase 1 to form the αβ₂₂γ₂ FeMo-co-activatable species requires the presence of NIFH and nucleotide (27, 28), as diagrammed in Reaction 1.

\[
\text{αβ₂₂} \rightarrow \gamma \rightarrow \text{αβ₂₂γ₂} \rightarrow \text{FeMo-co} \rightarrow \alphaβ₂⁺γ \rightarrow \text{Dinitrogenase 1 (catalytically active)}
\]

**Reaction 1**

The FeMo-co insertion assay and anoxic native PAGE were employed to test whether VNFH might replace NIFH in the maturation of apodinitrogenase 1. The results in Table I show that treatment of extract containing αβ₂₂ apodinitrogenase 1 and the γ protein with equivalent levels of purified NIFH or VNFH resulted in similar levels of activity in the FeMo-co insertion assay, indicating that VNFH is as effective as NIFH in the conversion of αβ₂₂ apodinitrogenase 1 to the αβ₂₂γ₂ form. Nucleotide is necessary for the VNFH-dependent maturation process as is maturation supported by NIFH (27). Control reactions in which (NH₄)₂Mo₃S₃ was not added to quench further FeMo-co insertion and which contained VNFH in both insertion and C₂H₄ reduction phases of the assay exhibited similar levels of activity as reactions to which NIFH was added (following (NH₄)₂Mo₃S₃ addition) during the C₂H₄ reduction phase. Thus, the activities reported in Table I for reactions that contained VNFH in the insertion phase alone were not a result of NIFH functioning to attach the γ protein to αβ₂₂ apodinitrogenase 1 during the C₂H₄ reduction phase of the assay. NIFH from another organism (Rhodosporillum rubrum) also supported activity in the FeMo-co insertion assay (Table I).

To confirm the results of the FeMo-co insertion assays, we employed anoxic, native PAGE to monitor the association of the γ protein with αβ₂₂ apodinitrogenase 1 in extracts of strain DJ1030 (ΔnifHΔnifBΔnifD, nif derepressed) in the presence of nucleotide and the different dinitrogenase reductase proteins. Fig. 1, an immunoblot of an anoxic, native gel (developed with antibody to the γ protein), illustrates that VNFH functions in the association of the γ protein with αβ₂₂ apodinitrogenase 1 (Fig. 1, lane 3). These results are consistent with the activities observed in the FeMo-co insertion assays testing the different dinitrogenase reductase proteins (Table I).

The high degree of amino acid sequence identity between NIFH and VNFH (91%) (1) is consistent with the effectiveness of VNFH in both substrate reduction (when complemented with dinitrogenase 1) and in the maturation of apodinitrogenase 1. The domain(s) of NIFH required for both the above functions are quite likely highly conserved in VNFH. At present, the role(s) of the dinitrogenase reductase protein in the maturation of apodinitrogenase 1 remains under investigation.

**Ability of VNFH to Function in in Vitro FeMo-co Synthesis**—VNFH was tested in the in vitro FeMo-co synthesis assay in place of NIFH (Table I). VNFH typically exhibited 25–30% of the FeMo-co synthesis activity (in our fixed time assay) observed with an equivalent level of NIFH, despite exhibiting similar levels of activity in the C₂H₄ reduction assay. Addition of increasing levels of VNFH and increasing the time allowed for in vitro FeMo-co synthesis did not result in a linear increase in activity (data not shown). The limiting step(s) in the assay is not the maturation of apodinitrogenase 1, because VNFH functions as effectively as NIFH in the maturation process (discussed above). The reasons for the lower level of FeMo-co synthesis observed with VNFH are not known. It is possible that VNFH is unable or slow to dissociate from a nif protein(s) with which it interacts during the course of FeMo-co synthesis, thus limiting further turnover of the protein(s) involved.

Homer et al. (28) demonstrated that the γ protein dimer (present in extracts of A. vinelandii) strains unable to synthesize FeMo-co monomerized upon associating with FeMo-co, and thus it was possible to employ the monomerization of the γ protein (detected by anoxic native PAGE) as an alternate assay for the completion of FeMo-co synthesis. Thus, FeMo-co synthesized in vitro in reaction mixtures containing an extract of strain CA12 (ΔnifHΔDKD, nif derepressed) would accumulate on the γ protein (resulting in the monomerization of the γ protein dimer) due to the absence of apodinitrogenase 1 in extracts of this strain. Fig. 2 is an immunoblot (developed with antibody to the γ protein) of an anoxic native gel that demonstrates the results of this study. When dinitrogenase reductase is excluded
from the in vitro FeMo-co synthesis reaction, the γ dimer and a slow migrating species of γ that is uncharacterized (indicated by X on Fig. 2) are observed (Fig. 2, lane 1); the dimeric form of the γ protein is observed in extracts of strains that are impaired in FeMo-co biosynthesis (33). That both NIFH and VNFH support FeMo-co biosynthesis is illustrated by the monomerization of the γ protein observed as the faster migrating γ protein-FeMo-co form in reactions that included NIFH or VNFH (Fig. 2, lanes 2 and 3).

Does dinitrogenase reductase specify the heterometal contained in the nitrogenase cofactors? Two lines of evidence suggest that the dinitrogenase reductases do not specify or select against the heterometal that is incorporated into the cofactors of the nitrogenase enzymes: 1) the ability of VNFH to function in in vitro FeMo-co synthesis (albeit less effectively than NIFH), and 2) the observation by Joerger et al. (23) that NIFH supported vanadium-dependent diazotrophic growth of A. vinelandii strain containing a deletion in the vnfH gene, indicating that, in vivo, NIFH functions in FeV-co biosynthesis. Gollan et al. (32) demonstrated the in vivo synthesis and incorporation of FeMo-co into the dinitrogenase 3 polyepitides of a Rhodobacter capsulatus strain containing deletions in the nifHDK genes; the synthesis of FeMo-co in the absence of a nifH gene suggests that ANFH most likely replaced NIFH in the synthesis of FeMo-co. Our results demonstrating the ability of VNFH to function in the in vitro biosynthesis of FeMo-co suggest that the dinitrogenase reductase protein quite likely does not select against the incorporation of molybdenum into FeV-co and FeFe-co.

The Specificity for Molybdenum of the in Vitro FeMo-co Synthesis System—Cofactor structures of the three nitrogenases are proposed to be essentially similar with vanadium and iron atoms replacing the molybdenum atom in FeV-co and FeFe-co, respectively (2, 21, 40). The requirement of the nifB and nifV gene products for the biosynthesis of all three cofactors suggests that certain steps in the biosynthesis of FeMo-co are shared in the biosynthetic pathways of all three cofactors. Although FeV-co is largely characterized, extended x-ray absorption fine structure studies on dinitrogenase 2 indicate that FeV-co is similar in structure to FeMo-co with the octahedral vanadium atom surrounded by 3 oxygen atoms and 3 sulfur atoms as is the molybdenum atom in FeMo-co (41). Other similarities between FeMo-co and FeV-co include the ability to extract FeV-co into N-methylformamide (20) and its probable ligation to the dinitrogenase 2 polypeptide via the conserved cysteine and histidine residues (analogous to Cys-275 and His-442 of NIFD) that ligate FeMo-co to dinitrogenase 1 (8, 23).

To determine whether the FeMo-co synthesis system would utilize vanadium and iron in the synthesis of FeV-co and FeFe-co, respectively, we tested various vanadium- and iron-containing compounds in place of molybdenum in the in vitro FeMo-co synthesis assay. Extract of A. vinelandii strain UW45 (nifB–, tungsten-grown) was used as a source of all the nif-encoded proteins necessary for the synthesis of FeMo-co. Active dinitrogenase 1 was formed only when molybdenum (in the form of NaMoO4) was included in the in vitro reactions (Table II). Molybdenum added to in vitro FeMo-co synthesis reactions in the form of (NH4)2MoO2S2, K2MoO3S, and MoS2 also supported FeV-co synthesis assay. Extract of strain CA117.30 (ΔnifDKB) in vitro FeMo-co synthesis reactions containing vanadium (in the form of NaVO3, V2O5, VCl3, VOPO4, or [K2(H2O)5][(VO2)2(R,S-homocitrate)]2H2O). Iron was included in the assays as FeNO3 or FeCl3.

Activities are expressed as nanomoles of C2H4 formed/min/assay.
that steps and precursors unique to the synthesis of FeV-co quite likely exist. The identification of additional unfv genes and the characterization of phenotypes of strains carrying lesions in unfv genes might enable the elucidation of steps involved in the biosynthesis of FeV-co.

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