Cloning and Mutational Analysis of the γ Gene from Azotobacter vinelandii Defines a New Family of Proteins Capable of Metallocluster Binding and Protein Stabilization*

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Dinitrogenase is a heterotetrameric (αβγδ) enzyme that catalyzes the reduction of dinitrogen to ammonium and contains the iron-molybdenum cofactor (FeMo-co) at its active site. Certain Azotobacter vinelandii mutant strains unable to synthesize FeMo-co accumulate an apo form of dinitrogenase (lacking FeMo-co), with a subunit composition αβγδγ′ which can be activated in vitro by the addition of FeMo-co. The γ protein is able to bind FeMo-co or apodinitrogenase independently, leading to the suggestion that it facilitates FeMo-co insertion into the apoenzyme. In this work, the non-nif gene encoding the γ subunit (nafY) has been cloned, sequenced, and found to encode a NifY-like protein. This finding, together with a wealth of knowledge on the biochemistry of proteins involved in FeMo-co and FeV-co biosyntheses, allows us to define a new family of iron and molybdenum (or vanadium) cluster-binding proteins that includes NifY, NifX, VnfX, and now γ. In vitro FeMo-co insertion experiments presented in this work demonstrate that γ stabilizes apodinitrogenase in the conformation required to be fully activable by the cofactor. Supporting this conclusion, we show that strains containing mutations in both nafY and nifX are severely affected in diazotrophic growth and extractable dinitrogenase activity when cultured under conditions that are likely to occur in natural environments. This finding reveals the physiological importance of the apodinitrogenase-stabilizing role of which both proteins are capable. The relationship between the metal cluster binding capabilities of this new family of proteins and the ability of some of them to stabilize an apoenzyme is still an open matter.

Nitrogenase catalyzes the reduction of nitrogen gas to ammonium, in an ATP- and reductant-dependent reaction. It is one of the best characterized metalloenzymes and is an excellent model for elucidating metalloprotein assembly. Nitrogenase is composed of two oxygen-labile metalloproteins: dinitrogenase and dinitrogenase reductase (1, 2). Dinitrogenase (also termed component I or molybdenum-iron protein) is a 240-kDa αβγδ tetramer of the nifD and nifK gene products (3). Each αβ nitrogenase dimer contains an iron-molybdenum cofactor (FeMo-co) and a P cluster (3, 4). Dinitrogenase reductase (also termed component II or iron protein) is a 60-kDa αδ dimer of the nifH gene product which contains a single 4Fe-4S center coordinated between the two subunits (5). NifH has at least three roles in the nitrogenase enzyme system (6): first, it serves as electron donor to nitrogenase; second, it participates in the biosynthesis of FeMo-co; and third, it is required for maturatory activation of apodinitrogenase to a FeMo-co-activable form.

The genes that encode dinitrogenase (nifD and nifK) are not required for FeMo-co biosynthesis, suggesting that FeMo-co is assembled elsewhere in the cell and is then inserted into apodinitrogenase (7). It is known that the products of at least seven nitrogen fixation (nif) genes, nifB, nifE, nifH, nifN, nifQ, nifV, and nifX, are involved in the biosynthesis of FeMo-co (8). Azotobacter vinelandii or Klebsiella pneumoniae strains with mutations in nifB, nifN, or nifE produce a FeMo-co-deficient hexameric (αβγδγ′) apodinitrogenase that can be activated in vitro by the simple addition of purified FeMo-co (9, 10). On the other hand, apodinitrogenase from ΔnifH mutants has a tetrameric composition (αβγδ) and requires some type of NifH- and MgATP-dependent maturation that, in turn, promotes the association of the γ subunit and leads to the form that is competent for FeMo-co activation (11, 12).

In vitro studies on crude extracts of an A. vinelandii nifB mutant demonstrated that the γ protein specifically binds to free FeMo-co and to apodinitrogenase, consistent with a role in FeMo-co insertion (13). However Christiansen et al. (14) have reported that pure preparations of His-tagged apodinitrogenase from a nifB mutant strain lacked the γ subunit but still could be activated to 80% of the theoretical value by the simple addition of FeMo-co, which suggests a role for γ other than that of a FeMo-co insertase. As they pointed out, the solution to this controversy would require the inactivation of the γ gene.

In K. pneumoniae the third subunit in the hexameric apodinitrogenase is the product of the nifY gene (10, 15). However, this is not the case in A. vinelandii, where the γ-encoding gene is not in the nifγ gene cluster. Although A. vinelandii contains a nifY gene there is no mutant phenotype associated with its inactivation (16).

In this work, we have cloned and inactivated the A. vinelandii gene encoding the γ protein. In vivo and in vitro results presented here indicate that γ stabilizes apodinitrogenase in an open (FeMo-co activable) conformation; by contrast, our

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† The abbreviations used are: FeMo-co, iron-molybdenum cofactor; FeFe-co, iron-only cofactor; FeV-co, iron-vanadium cofactor; kbp, kilo-base pair(s); ORF, open reading frame.

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results do not support a role for γ as an essential FeMo-co insertase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sodium dithionite was from Fluka. Leupeptin, phenylmethylsulfonyl fluoride, phosphocreatine, creatine phosphokinase, and ATP were from Sigma. Tris and glycine were from Fisher Scientific. Nitrocumene and polyvinylidene difluoride membranes were from Millipore. Acrylamide/bisacrylamide and the equipment for SDS-PAGE were from Bio-Rad. Ammonium tetrathiomolybdate ([NH₄]₂MoS₄) was a gift from D. Coucouvanis (University of Michigan, Ann Arbor).

**Buffers**—25 mM Tris-HCl, pH 7.5, was used throughout this work. All buffers for protein analysis were sparged with purified N₂ for 20–30 min, and sodium dithionite was added to a final concentration of 1 mM. Buffers used for obtaining A. vinelandii cell-free extracts contained 0.5 µg/ml leupeptin and 0.2 mM phenylmethylsulfonyl fluoride.

Strains and Growth Conditions—A. vinelandii strains DJ (wild type), DJ166 (∆nafY), and DJ208 (∆nafY) were obtained from D. R. Dean, Department of Biochemistry, Virginia Technical Institute, Blacksburg, Virginia. Strain UW45 (nafY) has been described previously (17). Growth in the presence of molybdate, nif derepression, and cell breakage have been described (18). For growth on plates, the medium was solidified with separately autoclaved 1.5% agar solution. Growth was estimated from the absorbance of the culture at 37 °C. Growth in the presence of molybdate, nif derepression, and cell breakage have been described (18). For growth on plates, the medium was solidified with separately autoclaved 1.5% agar solution. Growth was estimated from the absorbance of the culture at 37 °C.

**For growth rate determinations, strains were grown at 30 °C on Burk’s modified medium (designated as standard growth conditions) or at 37 °C on Burk’s modified medium not supplemented with molydate (designated as stressing growth conditions). When a fixed nitrogen source was required, ammonium acetate was added to a final concentration of 29 mM. Growth was estimated from the absorbance of the culture at 37 °C.

**Cloning of the γ-Encoding Gene (nafY).**—The N-terminal amino acid sequence for γ (VTPVNMRTAILRIRAILARALPTGGVQLL) was obtained from a preparation of partially purified αβγ complex apo-nitrogenase (see below). Degenerate oligonucleotides 5 ’-GTACCGGCGGTATTACAGT-3 ’ and 5 ’-CTGCTGGACGTGGTTCG-3 ’ were designed, based on the N-terminal amino acid sequence, and were used as primers in a PCR amplification of 81-bp DNA fragments from strain A. vinelandii. After sequencing the 81-bp fragment, oligonucleotides 5 ’-GCACCCGGTCCCGGGTTC-3 ’ (complementary to nucleotides 42–63 with respect to the translation start of the γ gene, termed nafY for nitrogenase accessory factor Y) and 5 ’-ATGCGCAAGACGCGGTTCTTCGGAGC-3 ’ (complementary to nucleotides 41–20 with respect to the translation start of the γ gene) were used as primers in a reverse PCR. SalI-digested and religated chromosomal DNA from A. vinelandii was used as template for this reaction. A 1.5-kbp PCR product was obtained, ligated into pGEM-T, sequence, and digested with BglII and SalI to generate a 1,236-bp DNA fragment that was finally ligated into pUC21 to render plasmid pHBB20. Plasmid pHBB20 contains sequences 5 ’ of nafY from the A. vinelandii chromosome. A. vinelandii strain UW139 was generated by transforming DJ strain with plasmid pHBB20 and selecting for single recombinants on plates containing ammonium acetate- and kanamycin-supplemented Burk’s medium. Genomic DNA from strain UW139 was digested with either BamH1 or XhoI, ligated, and used to transform E. coli DH5α, rendering plasmids pHBB21 and pHBB24, respectively. Plasmid pHBB21 contains 14 kbp of A. vinelandii DNA sequences 5 ’ of nafY, whereas plasmid pHBB24 contains 1.2 kbp of sequences 5 ’ of nafY along with nafY and ~16 kbp of 3 ’-sequences (see Fig. 1).

**Plasmid Constructions and DNA Manipulations.—**Plasmid constructions, PCR, and transformation of E. coli were carried out by standard methods (19). A computer search for homologies was made using the BLAST algorithm (20). Isolation of DNA from A. vinelandii strains was carried out using the RNeasy™ Tissue Kit (Qiagen). For Northern analysis, 10-µg portions of RNA samples were electrophoresed in a denaturing formaldehyde gel and transferred to a positively charged nylon membrane (Millipore). Prehybridization and hybridization were performed in the presence of 50% formamide at 42 °C. A PCR-amplified DNA fragment, covering the entire nafY, was used as probe after labeling with Prime-It II Random Primer Labeling Kit (Strategene) and [γ-32P]CTP.

**Mutagenesis of A. vinelandii Genes.—**Procedure for A. vinelandii transformation (22) and gene replacement (23) have been described. Plasmid pHBB25 contains a 3.4-kb II fragment DNA from plasmid pHBB24 which includes a portion of rnfH, nafY, and additional 3 ’-sequences. Plasmids pHBB29a and pHBB29b were generated by sub-stititution of a kanamycin resistance cassette for a 231-bp SalI fragment from pHBB25. The kanamycin resistance gene in the cassette, obtained from plasmid pUC4K, was in the same orientation as nafY in plasmid pHBB29a and in the opposite orientation from nafY in plasmid pHBB29b. UW146 was generated by transformation of strain UW45 with plasmid pHBB29b. Strains UW141, UW147, and UW154 were generated by transformation of strains DJ, DJ166, and DJ208 with plasmid pHBB29a, respectively, followed by selection of a Kan phenotype. Plasmid pHBB28 was generated after removing the 231-bp SalI fragment from pHBB25 described above, to produce an in-frame deletion within nafY. Strains UW149, UW156, and UW158 were generated by transformation of strains UW141, UW145, and UW147 with plasmid pHBB28, respectively, and scored for the Kan phenotype. UW166 was generated by transformation of UW156 with plasmid pHBB28, which retains only the nafY DNA fragment, and scored for a Kan phenotype. UW167 was generated by transformation of strain DJ with plasmid pHBB43. All generated mutations were checked by PCR to confirm that double recombination and segregation events occurred.

**In Vitro Dinitrogenase and Dinitrogenase Reductase Activities.—**Dinitrogenase and dinitrogenase reductase activities in cell-free extracts were obtained after titration with an excess of the complementary component as described (24). The specific activity of each protein is defined as nmol of ethylene formed/min/mg of protein.

**Activation of Apodinitrogenase with Purified FeMo-co** (in Vitro FeMo-co Insertion Assay)—FeMo-co was prepared in N-methylformamide as described (25). 9-ml serum vials were evacuated and flushed repeatedly with purified argon and rinsed with anaerobic 25 mM Tris-HCl, pH 7.5. Then 0.7 ml of Tris-HCl buffer, 200 µl of UW45 or UW146 cell-free extracts (~3 mg of protein), and 2 or 10 µl of a solution containing FeMo-co (equivalent to 0.1 and 0.5 nmol of Mo, respectively). The mixtures were incubated for 30 min at 30 °C after which 0.8 ml of ATP-regenerating mixture (containing 3.6 mM ATP, 6.3 mM MgCl₂, 51 mM phosphocreatine, 20 units/ml creatine phosphokinase, and 6.3 mM sodium dithionite) was added. An excess of purified NfH (0.2 mg of protein) were added. Nitrogenase activity was then quantitated by a acetylene reduction as described (26).

For the in vitro FeMo-co insertion time course experiments, 21-ml serum vials were evacuated and flushed repeatedly with purified argon and rinsed with anaerobic 25 mM Tris-HCl buffer, 0.7 ml of UW45 or UW146 cell-free extracts (~21 mg of protein), and 70 µl of a solution containing FeMo-co (equivalent to 3.5 nmol of molybdenum). The mixtures were incubated at 30 °C, and 0.2-ml aliquots were removed at different times and injected into anaerobic 9-ml serum vials containing 40 nmol of (NH₄)₂MoS₄ to prevent further insertion of FeMo-co into apodinitroge-nase. The vials were incubated for at least 10 min at room temperature, and 0.8 ml of ATP-regenerating mixture and an excess of purified NfH (0.2 mg of protein) were added. Nitrogenase activity was then quantitated by acetylene reduction as described (26).

**SDS-PAGE and Immunoblot Analysis—**The procedure for SDSPAGE has been described (27). Immunoblot analysis was performed as described by Brandner et al. (28).

**Preparation of γ Protein for Terminal Sequencing.—**A preparation of partially purified hexameric apodinitrogenase (αβγγγγγ) from strain UW45, containing about 12 µg of protein, was subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The γ protein band was excised from the membrane and used for N-terminal sequence at the Protein/Peptide Micro Analytical Laboratory of the California Institute of Technology.

**Protein Assays—**Protein concentrations were determined by the
bicinchoninic acid method using bovine serum albumin as standard (29).

RESULTS AND DISCUSSION

Cloning of the Gene Encoding the γ Protein from *A. vinelandii*—A combination of PCR and reverse PCR techniques was used to isolate the gene encoding γ from *A. vinelandii* genomic DNA (for details see “Experimental Procedures”). The cloning procedure resulted in the recovery of plasmids pRHB21 and pRHB24. pRHB21 contains ~14 kbp of *A. vinelandii* DNA sequence 5’ of the γ-encoding gene (*nafY*), whereas pRHB24 contains 1.2 kbp of sequence 5’ of *nafY* along with *nafY* and ~16 kbp of 3’-sequences (Fig. 1). Sequencing of the 1.2-kbp *Sal*I-*Bgl*II fragment located 5’ of *nafY* revealed the presence of three ORFs, designated ORF1, ORF2, and ORF3, which would encode polypeptides showing homology to RnfG, RnfE, and RnfH polypeptides from *Rhodobacter capsulatus*, respectively (30). In *R. capsulatus*, the Rnf polypeptides are required in vivo for nitrogen fixation and are proposed to constitute a membrane complex involved in electron transport to nitrogenase (30, 31). Thus, *rnf* genes and *nafY* are clustered in the *A. vinelandii* chromosome (Fig. 1). No other ORFs were found in the 500 bp 3’ of *nafY* in plasmid pRHB24, strongly suggesting that *nafY* is the last gene of the cluster.

*nafY* Encodes a NifY-like Protein—Fig. 2 compares the amino acid sequences of γ with some γ homologs found in protein data bases. It is clear that *nafY* encodes a protein that is similar to NifY, NixX, VnxX, and the C-terminal half of NifB from *A. vinelandii* and other bacterial sources. The actual role of NifY in the *A. vinelandii* nif system is not known. However, *K. pneumoniae* NifY is found instead of γ as the third subunit in the hexameric apoprotein (10, 15). NifX and VnxX are proteins involved in the biosynthesis of FeMo-co and FeV-co, respectively (8, 32). FeV-co is an iron-vanadium cofactor contained in the *nrf*-encoded dinitrogenase and functions analogously to FeMo-co of the molybdenum system (33). NifB is required for the three nitrogenase systems (molybdenum-, vanadium-, and iron-only-containing nitrogenases) (34), and its role may be the reconstitution of NifB-co, an iron-sulfur cluster that serves as precursor for FeMo-co, FeV-co, and FeFe-co biosyntheses (35). Sequence identity between *nifY* and *nixX* gene products from *A. vinelandii* and *K. pneumoniae* have been noted before (16), but no functional relationships could be inferred at that time. When sequence identity between NifY/NixX and NifB proteins was noted, a role for NifY/NixX proteins in FeMo-co maturation or stabilization was suggested based on the known role of NifB in FeMo-co biosynthesis (36). Interestingly, the most similar protein in data bases is the recently reported product of a *nifY*-like gene in the bacterium *Pseudomonas stutzeri* (GenBank accession no. AJ297529). The physical organization of the *rnf* region in *P. stutzeri* (*rnfCDG*EH-*nifY*-like-ORF13-ORF12-*nifH*) somewhat resembles the *A. vinelandii* organization, where two separate *rnf*GEH-*nafY* (this work) and ORF13-ORF12-*nifH* regions have been found (GenBank accession no. AF014048). Although it is reasonable to believe that there might be a physical linkage between the two regions in the *A. vinelandii* chromosome, this is unlikely because we have not been able to complement the mutation in the *A. vinelandii* strain DJ54 (∆*nifH*) by transformation with plasmids pRHB21 or pRHB24.

Consistent with the sequence similarity among γ, NifY, NixX, and VnxX polypeptides, in vitro studies provide supporting evidence that they are functionally related because they are able to interact with some intermediates of the FeMo-co or FeV-co biosynthetic pathways. First, the γ protein binds FeMo-co (13). Second, NixX and VnxX from *A. vinelandii* are also able to bind FeMo-co (37) as well as NifB-co (32, 37). Third, VnxX is also able to bind a FeV-co precursor (vanadium-containing iron-sulfur cluster) lacking homocitrate (32). Taken together, these lines of evidence indicate that γ, NifY, NixX, and VnxX constitute a family of iron and molybdenum (or vanadium) cluster-binding proteins.

*nafY* and *rnfH* Are Transcribed Independently—It has been reported that the *rnf* gene cluster from *R. capsulatus* is subject to nif regulation and that their products are required in vivo for nitrogen fixation (30). In contrast, nothing is known about regulation of the expression of the *rnf* gene cluster in *A. vinelandii*, but it is known that γ (NafY) is not the product of a *nif* gene, nor is it *nif* coregulated (13). Because *rnfH* and *nafY* are clustered in the *A. vinelandii* chromosome, having an intergenic region of only 24 bp, we wondered whether they are cotranscribed. As a preliminary approach to address this question, the Ω interposon was inserted into the *Bgl*II site located within *rnfH* to generate a polar mutation (Fig. 1). The Ω interposon carries transcriptional terminators and has been shown to impair gene expression from promoters 5’ of the site of Ω.

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2 P. J. Goodwin, D. R. Dean, and C. Rüttimann-Johnson, personal communications.
insertion in a wide range of Gram-negative bacteria (38). Crude extracts were prepared from nif-derepressed cells of wild-type and mutant strain UW165 (rnfH::H9024) and analyzed by immuno-blot of an SDS-gel developed with antibody to H9253 (Fig. 3). The presence of H9253 in the extracts of strain UW165 suggests that a promoter 3' of the H9024 interposon insertion site is driving nafY expression. However, the 80-bp region between the H9024 insertion site and the nafY start codon lacks any obvious promoter sequences and is unable to promote H9253 accumulation when present in a plasmid in E. coli (data not shown). Given the substantial amount of H9253 protein that A. vinelandii cells are able to accumulate (9, 13), the lack of an obvious promoter sequence 5' of nafY is unexpected; nevertheless the result still suggests independent regulation of rnfH and nafY.

In addition, a 1.1-kb RNA species was identified by Northern analysis using a nafY probe (Fig. 4). The observed transcript is of sufficient length to encode the whole NafY and is present in both ammonium-grown and nif-derepressed cells (compare lanes 1 and 2), although it appears to be more abundant in nif-derepressed cells. Consistent with the immunoblot results, cells from strain UW165 (rnfH::H9024, lanes 3 and 4) also contain the 1.1-kb nafY transcript, indicating independent transcription from rnfH. Our Northern analysis does not, however, rule out the presence of additional longer transcripts that could cover part or the whole rnf-nafY cluster.

**Mutational Analysis of the nafY Gene**—The nafY gene was mutated by creation of a nonpolar in-frame deletion, termed ΔnafY, and the mutation was transferred to the chromosome of A. vinelandii wild-type strain to generate strain UW149 (see "Experimental Procedures"). Immunoblot analysis showed the absence of γ in extracts of UW149 (Fig. 3). As presented in Table I, molybdate-dependent diazotrophic growth rate was minimally affected in strain UW149. Also, crude extracts of strain UW149 exhibit normal levels of in vitro dinitrogenase and dinitrogenase reductase activities (Table II). These results suggest that the role of H9253 is not essential for diazotrophic growth under standard laboratory conditions, perhaps because some other protein is performing the same function when H9253 is not present.

To test the possibility of functional redundancy because of the presence of NifY or NifX (which have some sequence and functional similarity as noted above), ΔnafY mutation was transferred to the chromosome of strains DJ208 (ΔnifY) and DJ166 (ΔnifX), generating UW156 (ΔnifYΔnafY) and UW158.
Acetylene reduction activities in extracts of A. vinelandii nafY, nifX, and nifY strains

Values are the averages of at least two assays performed separately. Activities are expressed as nmol of ethylene formed/min/mg of protein. ND means not determined.

| Source of extract | Genotype | Activity in extract | Dinitrogenase activity | Standard N2 fixation conditions | Stressing N2 fixation conditions |
|-------------------|----------|---------------------|------------------------|---------------------------------|---------------------------------|
| DJ                | Wild-type| 26.2 ± 7.4          | 29.7 ± 6.0             | 17.9 ± 5.7                      |
| DJ166             | ΔnafY    | 27.7 ± 5.4          | 31.1 ± 4.0             | ND                              |
| DJ208             | ΔnifY    | 24.4 ± 9.3          | 32.4 ± 11              | ND                              |
| UW149             | ΔnafY    | 27.3 ± 3.6          | 28.3 ± 2.2             | 1.5 ± 0.1                       |
| UW156             | ΔnafYΔnifY| 24.5 ± 4.8          | 29.1 ± 4.8             | ND                              |
| UW158             | ΔnafYΔnifX| 25.6 ± 2.1          | 27.5 ± 3.8             | 0.5 ± 0                         |
| UW166             | ΔnaYΔnifYnifX::kan| 27.2 ± 3.6 | 38.0 ± 7.2 | 2.0 ± 1.2 |

*a Extracts from A. vinelandii cells grown under standard nitrogen fixation conditions are shown. Portions (0.2 ml) of each extract used for the reactions contained ~3 mg of protein. Acetylene reduction assays were carried out for 15 min at 30 °C.

*b Acetylene reduction assays were carried out for 15 min at 30 °C after tiration with an excess of dinitrogenase reductase as described (22).

*Stressing conditions refer to cells grown at 37 °C and molybdenum stress.

Following this logic, we attempted to force A. vinelandii cells to maintain a stable apodinitrogenase at a higher temperature and in an “open” conformation by reducing FeMo-co availability inside the cell. Thus, A. vinelandii strains carrying mutations in nafY, nifY, and nifX genes were tested for their abilities to grow under conditions of elevated temperature (37 °C) and molybdenum stress. Molybdenum stress refers to cells growing in Burks’s medium not supplemented with the standard 10 μM NaMoO₄. A. vinelandii is known to be extremely efficient in scavenging traces of molybdenum from the culture medium (39), and the short-term lack of a molybdenum supplement should not be interpreted as a Mo-free medium. Under these conditions, the combination of mutations in nafY and nifX (UW158) and nafY, nifY, and nifX (UW166) had a profound effect on diazotrophic growth rates (5-fold lower than wild type), whereas UW149 (ΔnaYΔnifY), DJ166 (ΔnifX), and UW156 (ΔnaYΔnifYΔnifX) strains exhibited decreases of ~35% in their growth rates, and no effect was observed for DJ208 (ΔnifY) (Table I). In contrast, all mutant strains exhibited wild-type growth rates when using ammonium as nitrogen source under the same culturing conditions at 37 °C (data not shown).

The mutation in nafY also had an effect on the levels of extractable dinitrogenase activity when mutant strains were grown under stressing nitrogen fixation conditions (Table II). Dinitrogenase activity in the wild-type strain (DJ) was 60% of the value observed in standard conditions. However, it was severely diminished (2–5% of the values observed in standard conditions) in strains carrying mutations in nafY (UW149), nafY and nifX (UW158), and nafY, nifX, and nifY (UW166). This decrease in the activity of dinitrogenase in nafY mutant strains correlated with the accumulation of lower levels of NiDK polypeptides, as checked by immunoblot (data not shown). It is clear that the function of nafY is very important for dinitrogenase activity when cells are under stressing nitrogen fixation conditions. It is important to note that no ethane was detected during the acetylene reduction assays performed to determine nitrogenase activities under molybdenum stressing conditions.

The production of ethylene and ethane from acetylene by nitrogenase is characteristic of alternative molybdenum-independent nitrogenases and, therefore, the result indicates that only the molybdenum-dependent nitrogenase system is present.

Table III shows that the addition of purified FeMo-co to cell
extracts of *A. vinelandii* strains grown under stressing nitrogen fixation conditions did not increase the levels of nitrogenase activity already present in the extracts (compare with values in Table II). The lack of activation by FeMo-co was not fixed by the activity already present in the extracts (compare with values in Table II). The reaction mixtures were incubated at 30 °C for 30 min, after which 0.8 ml of a MgATP-regenerating system and an excess of purified dinitrogenase reductase (0.2 mg of protein) were added, and the acetylene reduction activity was assayed.

* Dinitrogenase reductase (0.2 mg of protein) was added to the FeMo-co insertion phase and to the activity phase. Values are the averages of at least two assays performed separately. Activities are expressed as nmol of ethylene formed/min/mg of protein.

Taken together, these results are consistent with a model in which NaFY and Nfx, but not NifX, are capable of playing a role in either insertion of FeMo-co into apodinitrogenase or stabilization of apodinitrogenase in an open activatable form. These two possible roles are likely to be very important under natural environmental conditions, where limiting amounts of molybdenum are available and apodinitrogenase must be maintained and protected in an open (FeMo-co-deficient) conformation. Although we cannot conclusively rule out a role for NaFY in the stabilization of FeMo-co inside the cell, no substantial dinitrogenase reactivation is observed when purified FeMo-co is added to cell extracts of *nafY* mutants grown under stressing conditions.

**In Vitro Activation of Apodinitrogenase in Extracts of Strains**

* *UW45* and *UW146* by Purified FeMo-co—Because of its ability to bind independently to FeMo-co and to apodinitrogenase, both FeMo-co insertase and chaperone roles have been proposed for the γ protein (13). A tetrameric (α4β2γ2) His-tagged apodinitrogenase is, however, competent for FeMo-co activation despite lacking γ (14). We have compared the FeMo-co-dependent activation properties of γ-deficient (α2β2γγ) and hexameric (α2β2γγγγ) apodinitrogenases present in extracts of strains *UW45* (*nifBnaFY*) and *UW45* (*nifB*), respectively. In this work, we will use a question mark in the description of the γ-deficient apodinitrogenase because we do not yet know its exact submitochondrial composition. To demonstrate that the mutation in *nafY* is not affecting the accumulation of apodinitrogenase in the extracts of *UW146*, the same amount of total protein from extracts of *UW45* and *UW146* was resolved by SDS-PAGE and analyzed by immunoblot, developed with antibodies to dinitrogenase (Fig. 5). The amount of anti-NifDK-reacting material in the immunoblot was then quantified by densitometric analysis and shown as relative numbers in Fig. 5. These data indicate that under standard nitrogen fixation conditions, the *nafY* mutation does not result in decreased levels of apodinitrogenase in extracts of *UW146*.

30 min after the addition of FeMo-co, apodinitrogenase lacking γ (*UW146*) could only be activated to a value of 9.2 nmol of ethylene formed/min/mg of protein, which is 57% of the value seen for the γ-containing apodinitrogenase from *UW45* (see Table IV). *UW46* and *UW45* extracts contained similar levels of apodinitrogenase (see above) and dinitrogenase reductase activities (23.5 ± 2.8 nmol of ethylene produced/min/mg of protein). These data considered, the evident difference in activation could only be caused either by a lower insertase activity or a lower level of activable apodinitrogenase in extracts of strain *UW146*. These results are in contrast to the case in *K. pneumoniae*, where a *nifB*γ mutant is severely affected in the levels of FeMo-co-activable apodinitrogenase (10) and in apodinitrogenase antigen as well. Fig. 6 illustrates a time course of FeMo-co insertion into apodinitrogenase over a period of 30 min. Apodinitrogenase in extracts of *UW45* and *UW146* presented a similar activation profile, except that the maximum level of activation is halved in extracts of *UW146*. It is clear that the lack of γ in extracts of *UW146* does not limit the rate of FeMo-co insertion during the reaction. Moreover, no enhancement of FeMo-co insertion was obtained by combining extracts of *nif*-derepressed *UW146* cells and ammonium-grown wild-type cells containing γ protein, as expected if γ were involved in the insertion reaction (data not shown). Thus, the addition of γ to the *in vitro* insertion reaction mixture is not enough to restore the levels of activable apodinitrogenase in *UW146* extracts. Results presented in Table IV and Fig. 6 indicate that a lower level of activable apodinitrogenase, and not a defect in FeMo-co insertion, is the main cause of reduced holodinitrogenase reconstitution in extracts of strain *UW146*. It is very interesting that the value of holodinitrogenase formation from γ-free apodinitrogenase is about 50% of the value obtained from the γ-containing enzyme. Experiments to distinguish whether there is only one nonactivable FeMo-co pocket per α2β2 apodinitrogenase tetramer or if half of its population is nonactivable by FeMo-co are currently being developed.

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**TABLE III**

**Effect of the addition of purified FeMo-co to extracts of *A. vinelandii* nafY, nafYnifX, and nafYnifYnifX strains grown under stressing nitrogen fixation conditions**

| Source of extract | Genotype | Activity |
|------------------|----------|----------|
|                  |          | Extract only | Plus dinitrogenase reductase |
|                  |          | nmol/min/mg of protein | nmol/min/mg of protein |
| DJ               | Wild-type | 16.5 ± 2.2 | 25.4 ± 1.3 |
| UW149            | ΔnaFY    | 3.5 ± 0.9 | 3.1 ± 1.4 |
| UW158            | ΔnaFYΔnifX | 0.73 ± 0.02 | 0.4 ± 0.03 |
| UW166            | ΔnaFYΔnifX:kan | 1.9 ± 1.1 | 3.8 ± 0.2 |

* The FeMo-co activation assay system contained 0.2 ml of extract (0.6–1.4 mg of protein) and purified FeMo-co in N-methylformamide.

* Dinitrogenase reductase (0.2 mg of protein) was added to the FeMo-co insertion phase and to the activity phase. Values are the averages of at least two assays performed separately. Activities are expressed as nmol of ethylene formed/min/mg of protein.

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In vitro activation of apodinitrogenase by purified FeMo-co in extracts of UW45 and UW146 strains

| Source of extracta | Activityb |
|-------------------|-----------|
| nmol Mo          | nmol/min/mg of protein |
| UW45 (nifB) (αβγδ) | 3.0±0.5  |
| 0.1              | 3.0±0.5  |
| 0.5              | 15.8±4.2 |
| UW146 (nifBnafY) (αβγδ) | 1.7±0.1  |
| 0.1              | 1.7±0.1  |
| 0.5              | 9.2±0.2  |

a The FeMo-co activation assay system contained 0.2 ml of extract from UW45 or UW146 strain (~3.0 mg of protein) and purified FeMo-co in N-methylformamide. The reaction mixtures were incubated at 30 °C for 30 min, after which 0.8 ml of a MgATP-regenerating system and an excess of purified dinitrogenase reductase (0.2 mg of protein) were added, and the acetylene reduction activity was assayed (see “Experimental Procedures”).

b Activity is expressed as nmol of ethylene formed/min/mg of protein. Values are the averages of at least five assays.

CONCLUSION

In vitro studies with A. vinelandii crude extracts showing that the γ protein is able to bind either to free FeMo-co or to apodinitrogenase, and to dissociate from the αβγδ subunits after holodinitrogenase reconstitution, led to the proposal of a chaperone insertase role for γ (13). In this study, we have cloned the gene encoding γ, termed nafY, and showed that it encodes a NifY-like protein. Based on sequence and functional similarities, the existence of a NafY/NifY/NifX/VnfX family of iron and molybdenum (or vanadium) cluster-binding proteins is proposed. By mutational inactivation of genes of this family, we have established that both γ and NifX are playing a role important for diazotrophic growth when culturing conditions are set to simulate a real environmental situation, in which molybdenum limitation is likely to occur and be of physiological relevance. In principle, that role could be the stabilization of apodinitrogenase or a role during FeMo-co insertion. In vitro FeMo-co insertion experiments indicate that γ stabilizes apodinitrogenase in the conformation required for being fully activable by the cofactor. However, the data presented in this study do not support an essential role for γ as a FeMo-co insertase and concur with the results of Christiansen et al. (14), which showed that a His-tagged γ-deﬁcient apodinitrogenase could be activated by the simple addition of FeMo-co. The relationship between the metal cluster binding capabilities of this new family of proteins and their ability to stabilize an apoenzyme has yet to be explained.

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Cloning and Mutational Analysis of the γ Gene from Azotobacter vinelandii Defines a New Family of Proteins Capable of Metallocluster Binding and Protein Stabilization

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