Characterization of VNFG, the δ Subunit of the vnf-Encoded Apodinitrogenase from Azotobacter vinelandii

IMPLICATIONS FOR ITS ROLE IN THE FORMATION OF FUNCTIONAL DINITROGENASE 2*

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The vnf-encoded apodinitrogenase (apodinitrogenase 2) from Azotobacter vinelandii is an α3β2δ1 hexamer. The δ subunit (the VNFG protein) has been characterized in order to further delineate its function in the nitrogenase 2 enzyme system. Two species of VNFG were observed in cell-free extracts resolved on anoxic native gels; one is composed of VNFG associated with the VNFDK polypeptides, and the other is a homodimer of the VNFG protein. Both species of VNFG are observed in extracts of A. vinelandii strains that accumulate dinitrogenase 2, whereas extracts of strains impaired in the biosynthetic pathway of the iron-vanadium cofactor (FeV-co) that accumulate apodinitrogenase 2 (a catalytically inactive form of dinitrogenase 2 that lacks FeV-co) exhibit only the VNFG dimer on native gels. FeV-co and nucleotide are required for the stable association of VNFG with the VNFDK polypeptides; this stable association can be correlated with the formation of active dinitrogenase 2. The iron-molybdenum cofactor was unable to replace FeV-co in promoting the stable association of VNFG with VNFDK. FeV-co specifically associates with the VNFG dimer in vitro to form a complex of unknown stoichiometry; combination of this VNFG-FeV-co species with apodinitrogenase 2 results in its reconstitution to dinitrogenase 2. The results presented here suggest that VNFG is required for processing apodinitrogenase 2 to functional dinitrogenase 2.

The biological reduction of atmospheric N2 to NH₄⁺ occurs via any one of three genetically distinct nitrogenase enzymes in the aerobe Azotobacter vinelandii (for reviews, see Refs. 1 and 2). Expression of the three nitrogenase enzymes, designated nitrogenase 1, 2, and 3, is regulated by the metal content of the growth medium (3). Nitrogenase 1, a molybdenum (Mo)-containing enzyme, is synthesized in medium containing Mo, while nitrogenases 2 and 3 are repressed by molybdenum. Nitrogenase 2, a vanadium (V)-containing enzyme, is expressed in the absence of molybdenum in medium containing vanadium, expression of nitrogenase 3 (containing only iron) requires medium depleted in both molybdenum and vanadium. Nitrogenases 1, 2, and 3 are encoded by the nif, vnf, and anf operons, respectively; certain nif gene products are necessary for the function of the molybdenum-independent nitrogenases (4–6). All three nitrogenase enzymes are oxygen-labile iron-sulfur proteins comprising two components, dinitrogenase and dinitrogenase reductase. Dinitrogenase contains the active site cofactor of the enzyme, and dinitrogenase reductase serves as the obligate electron donor to dinitrogenase during catalysis in a MgATP- and reductant-dependent process (7, 8). In addition to reducing N₂, all three nitrogenase enzymes reduce C₂H₂ and H⁺ (9).

The component proteins of nitrogenase 2 have been purified from both A. vinelandii and Azotobacter chroococcum (10–14). Dinitrogenase 2 contains an iron-vanadium cofactor (FeV-co) similar to the iron-molybdenum cofactor (FeMo-co) of nitrogenase 1 at the active site (15), and P clusters that are spectroscopically identical to those of dinitrogenase 1 (16). Although the biosynthetic pathway of FeMo-co and the processing of the component proteins of nitrogenase 1 to catalytically active forms are well documented (for review, see Ref. 17), steps in the biosynthesis of the cofactors of nitrogenases 2 and 3 and in the maturation of their component proteins remain uncharacterized.

Certain features of the Mo-independent nitrogenases distinguish them from nitrogenase 1. The formation of C₂H₄ in addition to C₂H₂ as a minor product of C₂H₂ reduction is a characteristic of nitrogenase 2 and 3; in fact, C₂H₄ formation is often taken as indicative of the presence of a Mo-independent nitrogenase (18). Most relevant to this investigation, dinitrogenases 2 and 3 contain an additional subunit, δ, encoded by vnfG and anfG, respectively (19–21), compared to the αβγ structure of the nif-encoded dinitrogenase 1. Kim and Rees (22) suggested that the δ subunits might be involved in stabilization of the quaternary structures of dinitrogenases 2 and 3; subsequent studies, however, illustrated more intriguing functions for the δ subunits of dinitrogenase 2 and 3. Waugh et al. (23) demonstrated that VNFG and ANFG were required for di-zotrophic growth by A. vinelandii but not for C₂H₂ reduction. Recently, VNFG was shown to be required to convert apodinitrogenase 2 (in the presence of FeV-co) to a form capable of substrate reduction (24); the requirement for VNFG could occur in the processing of apodinitrogenase 2 to dinitrogenase 2, or in catalysis by the newly formed holodinitrogenase 2 (24). The role of VNFG and ANFG might be analogous to the function of the γ protein in processing nif-encoded apodinitrogenase 1 (dinitrogenase 1 that does not contain FeMo-co) to dinitrogenase 1. The form of apodinitrogenase 1 that is activable by FeMo-co has an αβγδ composition (25); the γ protein, a non-nif-encoded gene product, has been shown to specifically asso-

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ciliate with FeMo-co and perhaps function as a chaperone-inser-
tase in the maturation of apodinitrogenase 1 to dinitrogenase 1 (26).

In this report, we describe the characterization of VNFG (the β subunit of apodinitrogenase 2) and present results that indi-
cate a role for VNFG in the processing of apodinitrogenase 2 to dinitrogenase 2.

EXPERIMENTAL PROCEDURES

Materials—All reagents used for A. vinelandii growth media were of analytical grade or higher purity. Sodium metavanadate (Na3VO4, 99.995% purity), Tris base, and glycine were purchased from Sigma. Sodium dithionite (DTH) was from Fluka Chemicals. DEAE-cellulose was a Whatman DE-52 product. Octyl-Sepharose CL-4B, Sephadex G-25, and the Superose 12 gel filtration column were from Pharmacia Biotech, Inc. Nicotinamide membrane and acrylamide/bis solution were from Bio-Rad. Citric acid was obtained from Mallinckrodt. All other chemicals were from Sigma. The fast protein liquid chromatography system was a LKB instrument.

A. vinelandii Strains and Growth Conditions—A. vinelandii strains UW45 (nifB−; Ref. 27), CA12 (nifHDK; Ref. 28), CA11.1 (ΔnifHDK:nifDK1::apc; Ref. 23), CA119 (vnfG, Cys-17 → stop; Ref. 23), CA117.30 (ΔnifDKB; Ref. 24), DJ42.48 (ΔnifENXnifE; Ref. 29), CA11.80 (ΔnifHDK::Km; Ref. 19), and CA11.6.82 (W-tolerant, vnfd82::DCA11.80 (ΔnifHDK:nifDK1::Km; Ref. 30) have been described. Strains were grown in Burk's medium prepared in deionized water; all vessels were degassed on a gassing manifold where appropriate) for 10–30 min, and the following components were added to the vials in the order indicated: 200 μl (4 mg of protein) of the appropriate cell-free extract or partially purified apodinitrogenase 2 (0.2 mg of protein), 200 μl of an anoxic solution of 50% glycerol (containing 1.7 mM DTH), 200 μl of a solution of 1.7 mM Na3VO4 and 10 μl of an ATP-regenerating mixture (containing 3.6 mM ATP, 63 mM MgCl2, 51 mM creatine phosphate, 20 units/ml creatine phosphokinase, and 6.3 mM DTH in 0.025 M Tris-HCl, pH 8.0). The reaction-mixtures were incubated at room temperature for 20 min, and samples to be applied onto the native gels were placed on ice. The activity of the newly formed dinitrogenase 2 in the remaining vials was determined by the C2H2 consumption assay for dinitrogenase reductase 1 (31) as follows: 800 μl of the ATP-regenerating mixture and 10 μl (1 mg of protein) of dinitrogenase reductase 1 were added to the vials, which were then brought to atmospheric pressure. C2H2 reduction was ini-
tiated by the addition of 0.5 ml of C2H2 to the vials; following a 30 min incubation at 30 °C in a rotary water-bath shaker, the reactions were terminated by the addition of 0.1 ml of a 4 mM NaOH. When testing the reaction products for a particular component in the FeV-co insertion assay, the given component was excluded and an equal volume of 0.025 M Tris-HCl was added. FeMo-co was added to the reactions (in place of FeV-co) as 40 μl of purified FeMo-co in NMF, or as 50 μl of acid-denatured dinitrogenase 1. Certain reactions contained FeV-co that had been air-oxidized. Reactions testing the requirement for dinitrogenase reductase contained either 40 μl (0.4 mg of protein) of purified dinitrogenase reductase 1, or 40 μl (0.1 mg of protein) of partially purified dinitrogenase reductase 2. Nucleotide (other than ATP) solutions contained the following: 15 mM XTP (or XDP), 30 mM MgCl2, and 63 mM DTH in 0.025 M Tris-HCl, pH 8.0; 200 μl of the appropriate nucleotide solution was added to determine reaction-mixtures in place of the ATP-regenerating solution. The final XTP (or XDP) concentration (other than ATP, which was added in the form of the ATP-regenerating mixture) in the FeV-co insertion reactions was 3.75 mM. When testing various cell-free extracts as a source of FeV-co, 200 μl of the appropriate extract (−3.6 mg of protein) was added to the reactions in place of acid-denature dinitrogenase 2.

Molecular Mass Determination—A Superose 12 column (V0 = 8.5 ml) was used in conjunction with a LKB fast protein liquid chromatography system (model 1250). A p-Cyanomethyl benzylamino acid residue analysis of the VNFG-protein sample was performed by the University of Wisconsin-Madison Medical School. Antibodies to VNFG (the α and β polypeptides of dinitrogenase 2) were produced similarly. Immunoblot analysis was performed as described by Brandner et al. (34).

Partial Purification of Dinitrogenase 2 and Apodinitrogenase 2—Dini
trogenase 2 was partially purified from A. vinelandii strain CA119 (vnfG, Cys-17 → stop) by DEAE-cellulose chromatography, followed by heat treatment as described by Hales et al. (11). Partially purified dinitrogenase 2 was concentrated by ultrafiltration using a XM100-A membrane. Partial purification of apodinitrogenase 2 from A. vinelandii strain CA117.30 (ΔnifDKB) was performed through the octyl-Sepharose chromatography step as described previously (24). Preparation of FeV-co and FeMo-co—A preparation of FeV-co was made by the citric acid treatment of partially purified dinitrogenase 2 with modifications to the method described previously (24). In a typical reaction, 0.4 ml of a solution of 0.4 μM citric acid (made anoxic by diluting a 2.0 M stock solution into N2-sparged water containing 1.7 mM DTH) was added to 2 ml (31.2 mg of protein) of dinitrogenase 2 (partially purified from A. vinelandii strain CA119 (vnfG, Cys-17 → stop) in a screw-capped centrifuge tube (Beckman) fitted with rubber-stoppered inserts to allow evacuation and flushing on a gassing manifold. The solution was mixed thoroughly using a Vortex mixer and incubated on ice for 10 min, following which it was centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and the pellet was suspended in 4 ml of 0.025 M Tris-HCl. This suspension of acid-denatured dinitro-
genase 2 was centrifuged at 10,000 rpm for 20 min, and the supernatant (hereafter referred to as FeV-co) was used as a source of FeV-co in the in vitro activation of apodinitrogenase 2 by the cofactor. Purified FeMo-co was prepared by the method of Shah and Brill (32); when it was necessary to avoid any denaturing effects of N-methylformamide (NMF), FeMo-co was prepared as described in the citric acid treatment of dinitrogenase 1 as described by Allen et al. (35).

Activation of Apodinitrogenase 2 by FeV-co (FeV-co Insertion Assay)—The assay consists of two steps; in the first step, apodinitrogenase 2 is activated by FeV-co to form holodinitrogenase 2, and in the second step, the C2H2 reduction activity of the newly formed holodinitrogenase 2 is monitored. Reactions to be analyzed by native PAGE were only taken through the first phase of the assay (FeV-co insertion). Nine-ml rubber-stoppered serum vials were repeatedly evacuated and 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: 200 μl (4 mg of protein) of the appropriate cell-free extract or partially purified apodinitrogenase 2 (0.2 mg of protein), 200 μl of an anoxic solution of 50% glycerol (containing 1.7 mM DTH), 200 μl of a solution of FeV-co, and 200 μl of an ATP-regenerating mixture (containing 3.6 mM ATP, 63 mM MgCl2, 51 mM creatine phosphate, 20 units/ml creatine phosphokinase, and 6.3 mM DTH in 0.025 M Tris-HCl, pH 8.0). The reaction-mixtures were incubated at room temperature for 20 min, and samples to be applied onto the native gels were placed on ice. The activity of the newly formed dinitrogenase 2 in the remaining vials was determined by the C2H2 consumption assay for dinitrogenase reductase 1 (31) as follows: 800 μl of the ATP-regenerating mixture and 10 μl (1 mg of protein) of dinitrogenase reductase 1 were added to the vials, which were then brought to atmospheric pressure. C2H2 reduction was ini-
tiated by the addition of 0.5 ml of C2H2 to the vials; following a 30 min incubation at 30 °C in a rotary water-bath shaker, the reactions were terminated by the addition of 0.1 ml of a 4 mM NaOH.

When testing the reaction products for a particular component in the FeV-co insertion assay, the given component was excluded and an equal volume of 0.025 M Tris-HCl was added. FeMo-co was added to the reactions (in place of FeV-co) as 40 μl of purified FeMo-co in NMF, or as 50 μl of acid-denatured dinitrogenase 1. Certain reactions contained FeV-co that had been air-oxidized. Reactions testing the requirement for dinitrogenase reductase contained either 40 μl (0.4 mg of protein) of purified dinitrogenase reductase 1, or 40 μl (0.1 mg of protein) of partially purified dinitrogenase reductase 2. Nucleotide (other than ATP) solutions contained the following: 15 mM XTP (or XDP), 30 mM MgCl2, and 63 mM DTH in 0.025 M Tris-HCl, pH 8.0; 200 μl of the appropriate nucleotide solution was added to determine reaction-mixtures in place of the ATP-regenerating solution. The final XTP (or XDP) concentration (other than ATP, which was added in the form of the ATP-regenerating mixture) in the FeV-co insertion reactions was 3.75 mM. When testing various cell-free extracts as a source of FeV-co, 200 μl of the appropriate extract (−3.6 mg of protein) was added to the reactions in place of acid-denature dinitrogenase 2.
anoxic native PAGE.

**Protein Determination**—Protein concentrations of cell-free extracts and partially purified protein fractions were determined by the bicinchoninic acid method (36).

**Association of FeV-co with VNFG (the δ Protein)**—The interaction between FeV-co (in the form of acid-denatured dinitrogenase 2) and VNFG was monitored using the Superose 12 gel-filtration column (used in conjunction with a LKB fast protein liquid chromatography system). Two hundred μl of a solution of FeV-co were applied to the Superose 12 column (equilibrated in buffer containing 0.1 M NaCl in 0.025 M Tris-HCl; the column was eluted with the same buffer, and fractions (0.5 ml, collected anoxically) were tested for FeV-co using the FeV-co insertion assay. Two hundred μl (0.8 mg of protein) of a fraction containing VNFG that contained no VNFDK or FeV-co (and therefore exhibited no activity in the FeV-co insertion assay) were chromatographed on the Superose 12 column (equilibrated and eluted as described above), and 0.5-ml fractions (collected anoxically) were monitored for VNFG by SDS-PAGE. When examining the interaction between FeV-co and VNFG, 200 μl of a solution of FeV-co was incubated with 200 μl of the VNFG-containing fraction for 20 min at room temperature, and 200 μl of the reaction mixture were applied to the Superose 12 column (equilibrated as described above). The column was developed, and fractions were collected as described above; the fractions were monitored for both FeV-co and VNFG by the FeV-co insertion assay and by SDS-PAGE, respectively.

In a control experiment, 200 μl of a solution of FeV-co were incubated with 200 μl (2.8 mg of protein) of cell-free extract of NH₄⁺-grown UW45 (nifB⁻, conditions under which all three nitrogen fixing systems are repressed), and 200 μl of the reaction mixture were chromatographed as described above. Fractions were monitored for FeV-co using the FeV-co insertion assay. In a second control experiment, 200 μl of a solution of FeMo-co in NMF were chromatographed on the Superose 12 column both in the presence and absence of VNFG as described above; fractions were monitored for FeMo-co using the FeMo-co insertion assay (32) and for VNFG by SDS-PAGE.

**RESULTS AND DISCUSSION**

**Accumulation of VNFG (δ) in Various Mutant Backgrounds**—The presence of VNFG was monitored in several A. vinelandii mutant strains by immunoblot analysis of cell-free extracts resolved by SDS-PAGE. Fig. 1 is an immunoblot of a SDS gel developed with antibody to VNFG. A. vinelandii strains CA12 (ΔnifHDK), CA117.30 (ΔnifDKB), and DJ42.48 (ΔnifENXnifE) accumulated VNFG when the strains were grown and derepressed on V-containing medium (Fig. 1, lanes 1, 2, and 4). As reported previously, VNFG is a component of the apodinitrogenase 2 complex (33), and was detected in a purified sample of apodinitrogenase 2 (Fig. 1, lane 8). No VNFG was detected in extract of strain CA117.30 grown and derepressed on molybdenum-containing medium (Fig. 1, lane 5). As expected, strains CA11.1 (ΔnifHDKnifDGK₁::spe) and CA119 (vnfG, Cys-17 → stop) did not accumulate VNFG (Fig. 1, lanes 3 and 6); these data also show that the antibodies to VNFG did not cross-react with VNFDK (present in extract of strain CA119; Ref. 32).

Interestingly, a low molecular weight protein in a partially purified fraction of the anf-encoded dinitrogenase 3 (purified from CA11.1 grown and derepressed on Mo- and V-deficient medium) was also detected with antibody to VNFG (Fig. 1, lane 7). The cross-reactive band seen in lane 7 was not VNFG since dinitrogenase 3 was partially purified from a strain containing a deletion in the unfnf gene (Fig. 1, lane 3); the low molecular weight protein detected was most likely ANFG, the δ subunit of dinitrogenase 3. Given the 39.4% sequence identity at the amino acid level between ANFG and VNFG (1), it is not completely surprising that the antibody to VNFG is cross-reactive toward ANFG.

**Characterization of Electrophoretically Distinct Species of VNFG (δ)**—The forms of VNFG found in cell-free extracts were examined by anoxic native PAGE. Fig. 2A is an immunoblot of an anoxic native gel (1.6 mg of protein/lane). Anoxic native gels were developed with antibody to VNFG (panel A) and with antibody to VNFDK (panel B). All strains were vnf-derepressed unless stated otherwise. Panel A, lane 1, extract of CA12 (ΔnifHDK); lane 2, extract of CA117.30 (ΔnifDKB); lane 3, extract of CA11.1 (ΔnifHDKnifDGK₁::spe); lane 4, extract of DJ42.48 (ΔnifENXnifE); lane 5, extract of CA119 (vnfG, Cys-17 → stop); lane 7, extract of CA119 (vnfG, Cys-17 → stop). Species A consists of VNFG that is tightly associated with VNFDK polypeptides, and species B is a homodimer of VNFG protein. Panel B, lane 1, extract of CA12 (ΔnifHDK); lane 2, extract of CA11.1 (ΔnifHDKnifDGK₁::spe); lane 3, extract of CA117.30 (ΔnifDKB); lane 4, extract of CA119 (vnfG, Cys-17 → stop). Species A consists of VNFG, which will be shown to be tightly associated with the VNFDK polypeptides; species B was determined to be a homodimer of VNFG. Accumulation of dinitrogenase 2 and apodinitrogenase 2 in the various extracts was monitored by the C₃H₄ reduction assay and the FeV-co insertion assay, respectively (Table I).

Strain CA12 (ΔnifHDK) accumulated both VNFG species A and B (Fig. 2A, lane 1) and exhibited dinitrogenase 2 activity (Table I); no apodinitrogenase 2 was detected in the extracts by the FeV-co insertion assay; however, it is possible that a low level of apodinitrogenase 2 is present but cannot be estimated due to the high background activity of dinitrogenase 2 in the strain. The VNFDK polypeptides were shown to migrate to the position of VNFG species A by immunoblot analysis (using antibody to VNFDK) of extracts resolved by native PAGE (Fig. 2B). The antibodies to VNFDK did not cross-react with NIFDK (Fig. 2B, lane 6). Thus, VNFG species A in extract of CA12 constitutes VNFG associated with the VNFDK polypeptides via interactions that are not disrupted under conditions of the native PAGE procedure. The three forms of VNFG observed in species A (Fig. 2A, lane 1) most likely differ from each other in the stoichiometry of the VNFD, -G, and -K polypeptides in each form. Recently, Blanchard et al. reported the purification of two forms of dinitrogenase 2 from A. vinelandii: an α₁β₂ form and an α₂β₂ form; the δ subunit was observed to be associated with...
both forms but was not quantitated (12).

Extractions of strains CA117.30 (ΔnifDKB), CA11.80 (ΔnifHD-KonfH), and DJ42.48 (ΔnifENXvnfE) exhibited VNFG species B only (Fig. 2A, lanes 3, 5, and 6) despite the presence of the VNFDK polypeptides in all three strains (Fig. 2B, lanes 3–5). All three strains exhibited apodinitrogenase 2 activity (as determined by the FeV-co insertion assay) but no dinitrogenase 2 activity (Table I). Therefore, a strain that accumulates dinitrogenase 2 contains both VNFG species A and B, whereas strains impaired in FeV-co biosynthesis that accumulate apodinitrogenase 2 exhibit only VNFG species B. Species B might result from the dissociation of VNFG from the α_2β_2δ_2 apodinitrogenase 2 hexamer under the native PAGE and gel-filtration chromatography conditions; however, the possibility that a population of species B is not part of the apodinitrogenase 2 hexamer cannot be excluded. Species B observed in extract of CA12 might be due to the presence of low levels of apodinitrogenase 2. VNFG did not accumulate in strains CA11.1 (ΔnifHDKonfDGK1::spc) and CA119 (vnfG, Cys-17 → stop), which contain lesions in vnfG (Fig. 2A, lanes 2 and 7).

Previously, we had determined that VNFG did not comigrate with VNFDK as a complex when purified apodinitrogenase 2 (α_2β_2δ_2) was analyzed by anoxic native PAGE (24); it was also demonstrated that VNFG could be dissociated from VNFDK when apodinitrogenase 2 was chromatographed on a gel-filtration column (24). Thus, although the VNFKD and VNFG subunits copurified in a 1:1:1 ratio, suggesting (together with molecular mass analysis) that apodinitrogenase 2 is an α_2β_2δ_2 hexamer (24), the weak interactions between VNFG (the δ subunits) and VNFDK results in the dissociation of VNFG from the apodinitrogenase 2 hexamer under conditions of native PAGE and gel-filtration chromatography. In order to characterize VNFG species B, and to determine whether this species resulted from dissociation of VNFG from VNFDK, partially purified apodinitrogenase 2 (α_2β_2δ_2) was analyzed by gel-filtration chromatography using a Superose 12 column (see “Experimental Procedures”). VNFG reproducibly exhibited an elution profile consistent with a molecular mass of ~26.3 kDa, which is approximately twice the predicted mass (13.8 kDa) for the vnfG gene product (19). Calibration of the Superose 12 column (see “Experimental Procedures”) and the elution profile of VNFG from the Superose 12 column are indicated in Fig. 3A. VNFG in the Superose 12 fractions comigrated with VNFG species B in extract of strain CA12 (ΔnifHD, vnfderepressed) on an anoxic native gel (Fig. 3B, lanes 1–3). Thus, VNFG exists as a homodimer when it is not associated with the VNFDK polypeptides. VNFG in a sample of purified apodinitrogenase 2 (α_2β_2δ_2) coelectrophoresed to the position of VNFG species B in cell-free extract of CA12 (Fig. 3, lanes 1 and 4), indicating that VNFG species B in cell-free extracts of the strains tested is a dimer that is not tightly associated with any other protein(s).

Although apodinitrogenase 2 was purified as an α_2β_2δ_2 hexamer (24), the interactions between VNFG (δ) and VNFDK (αβ) appear to be readily disrupted under conditions of gel-filtration chromatography and native PAGE (24). In this respect, the physical properties of the α_2β_2δ_2 apodinitrogenase 2 complex are quite distinct from those of the α_2β_2γ_2 complex encoded by nifF-encoding apodinitrogenase 1, since the γ protein comigrates with the α and β subunits on native PAGE, and is dissociable only upon treatment of the α_2β_2γ_2 complex with reagents such as urea (37). Dinitrogenase 1 and 2 also differ from each other with respect to the affinities of the γ protein and VNFG for dinitrogenase 1 and 2, respectively. In cell-free extracts, the γ protein dissociates from the α_2β_2γ_2 apodinitrogenase 1 complex upon activation with FeMo-co (26), whereas the δ subunits (VNFG) become tightly associated with the α and β subunits of dinitrogenase 2 in the presence of FeV-co (Fig. 2A, lane 1; results discussed below).

**Requirements for the Stable Association of VNFG (δ) with VNFDK (αβ)—**The in vitro FeV-co insertion assay system together with anoxic native PAGE was employed to investigate conditions under which VNFG species B would associate stably with VNFDK to form species A (VNFG tightly associated with VNFDK). In vitro FeV-co insertion reactions from which components were excluded, one at a time or in combination, were applied onto the native gel, and the VNFG species accumulated in the various reactions were compared to those accumulated in

### Table I
| Strain | Dinitrogenase 2 | Apodinitrogenase 2 |
|--------|-----------------|--------------------|
|        | nmol C_2H_4 formed/min/assay | nmol C_2H_4 formed/min/assay |
| CA12 (ΔnifHD) | 47.4 | 0.0 |
| CA11.1 (ΔnifHDKonfDGK) | 0.0 | 0.0 |
| CA117.30 (ΔnifDKB) | 0.0 | 7.6 |
| CA117.30 (vnfderepressed) | 0.0 | 0.0 |
| CA11.80 (ΔnifHDKonfH) | 0.0 | 3.2 |
| DJ42.48 (ΔnifENXvnfE) | 0.0 | 6.8 |
| CA119 (vnfG, Cys-17 → stop) | 18.0 | 0.0 |

*All strains were vnfderepressed (see “Experimental Procedures”) unless stated otherwise. Reactions contained 200 µl (3.8 mg of protein) of the appropriate cell-free extract.

Dinitrogenase 2 activity was monitored by the C_2H_2 reduction assay, and apodinitrogenase 2 activity was monitored by the FeV-co insertion assay (see “Experimental Procedures”). Activities are expressed as nmol of C_2H_4 formed/min/assay.

Strain CA117.30 was vnfderepressed as described under “Experimental Procedures.”
The VNFG Protein from A. vinelandii

**Fig. 4.** Immunoblot (developed with antibody to VNFG) of an anoxic native gel of various FeV-co insertion reactions. All strains were vnf-derepressed. See “Experimental Procedures” for components included in a complete FeV-co insertion reaction. Desalted extract of CA117.30 (ΔnifDKB) was used as a source of apodinitrogenase 2. Lane 1, extract of CA12 (ΔnifHDK) alone; lane 2, minus FeV-co and MgATP reaction; lane 3, minus MgATP reaction; lane 4, complete FeV-co insertion reaction; lane 5, minus FeV-co reaction; lane 6, minus apodinitrogenase 2 (CA117.30 extract) reaction; lane 7, complete reaction with FeMo-co added in place of FeV-co. Table below figure indicates components added to reaction mixture (+). C2H2 reduction activity (nmol of C2H4 formed/min/assay). Species A consists of VNFG that is tightly associated with VNFDK polypeptides, and species B is a homodimer of VNFG protein.

A complete FeV-co insertion reaction (see “Experimental Procedures”). Fig. 4 is an immunoblot of an anoxic native gel (developed with antibody to VNFG) that illustrates the requirements for the stable association of VNFG with VNFDK. Cell-free extract (desalted by Sephadex G-25 chromatography) of strain CA117.30 (ΔnifHDK) was used as a source of apodinitrogenase 2 in the FeV-co insertion assays. Extract of strain CA12 (ΔnifHDK), which exhibited dinitrogenase 2 activity (Fig. 4, lane 1), shows the presence of both VNFG species A (VNFG tightly associated with VNFDK) and B (dimer of VNFG). A FeV-co insertion reaction that excluded both FeV-co and the ATP-regenerating mixture (defined in “Experimental Procedures”) exhibited VNFG species B, the form exhibited by all strains containing apodinitrogenase 2 (Fig. 4, lane 2). Upon addition of FeV-co to a reaction that excluded only the ATP-regenerating mixture, the formation of VNFG species A was observed (Fig. 4, lane 3). The level of VNFG species A formed was increased in a complete FeV-co insertion reaction (Fig. 4, lane 4) in comparison to the reaction that excluded the ATP-regenerating mixture (Fig. 4, lane 3).

The appearance of species A can be correlated with the formation of holodinitrogenase 2 in the reactions that were tested in the C2H2 reduction assay (see “Experimental Procedures”); the dinitrogenase 2 activity indicated in lane 3 where the ATP-regenerating mixture was excluded from the FeV-co insertion reaction applied onto the native gel is probably due to the addition of excess ATP-regenerating mixture to all the reactions that were monitored for C2H2 reduction activity (see “Experimental Procedures”). When FeV-co was oxidized by exposure to air prior to its addition to the FeV-co insertion reaction, formation of species A was not observed (data not shown). Thus, the presence of FeV-co that is able to reconstitute apodinitrogenase 2 to active dinitrogenase 2 promotes the association of VNFG with VNFDK, and the association is stimulated by the addition of the ATP-regenerating mixture to the FeV-co insertion assay. The stable association of VNFG with VNFDK might arise from a conformational change (induced in the apodinitrogenase 2 complex upon insertion of FeV-co into the active site) that increases the affinity of VNFG for VNFDK; alternatively, VNFG might function in associating with and inserting FeV-co into the active site of apodinitrogenase 1, upon which VNFG forms a stable complex with VNFDK.

**Fig. 5.** Immunoblot (developed with antibody to VNFG) of an anoxic native gel of FeV-co insertion reactions with various nucleotides. Partially purified apodinitrogenase 2 was used in these reactions in place of desalted cell extracts. Lane 1, minus FeV-co and MgATP reaction; lane 2, minus MgXTP reaction; lane 3, minus XTP reaction; lane 4, complete reaction containing ATP; lane 5, complete reaction containing ADP; lane 6, complete reaction containing βγ-CH2-ATP. Table below figure indicates components added to reaction mixture (+).
absence is not a result of any denaturing effects of NMF. A second possibility that could account for the absence of species A formation in the presence of FeMo-co is that VNFG might be involved in specifically associating with FeV-co but not FeMo-co, and inserting the cofactor into the active site pocket of apodinitrogenase 2. This possibility is supported in part by studies described below.

The Requirement of Nucleotide in the Association of VNFG with VNFDK—In order to determine whether nucleotide was absolutely required for the association of VNFG with VNFDK, partially purified apodinitrogenase 2 was used in place of de-salted, cell-free extract of CA117.30 in the FeV-co insertion assay. Fig. 5 is an immunoblot of an anoxic native gel (developed with antibody to VNFG) that illustrates the results of this study. In the absence of both FeV-co and nucleotide, species A was not observed (Fig. 5, lane 1). The addition of FeV-co alone to a reaction that excluded nucleotide did not promote association of VNFG with VNFDK (Fig. 5, lane 2). ATP and its analogs ADP and β,γ-CH₂-ATP functioned in the formation of species A (Fig. 5, lanes 4–6). These data demonstrate that both FeV-co and nucleotide are necessary for the stable association of VNFG with VNFDK. When the components of the ATP-regenerating system excluding ATP were added to a FeV-co insertion reaction, species A formation did not occur (Fig. 5, lane 3), indicating that Mg²⁺, creatine phosphate, and creatine phosphokinasine were not responsible for the stable association of VNFG with VNFDK.

An interesting observation that arose from these studies was that dinitrogenase reductase appeared not to be necessary for the stable association of VNFG with VNFDK. The partially purified fraction of apodinitrogenase 2 used in the FeV-co insertion reactions contained no dinitrogen reductase 1 (or dinitrogen reductase 2) as detected by C₂H₂ reduction activity when complemented with dinitrogenase 1, and also by immunoblot analysis of the fraction using antibody to dinitrogen reductase 1 (dinitrogenase reductase 2 can be detected by antibody to dinitrogen reductase 1; data not shown). Dinitrogenase reductase 1 or dinitrogenase reductase 2 were included in certain FeV-co insertion reactions to determine whether an effect on species A formation would be observed in their presence. Reactions in which dinitrogenase reductase 1 or dinitrogenase reductase 2 were added in the absence of FeV-co did not result in species A formation (data not shown), indicating that the presence of dinitrogen reductase and ATP (added in the form of an ATP-regenerating system) were insufficient for the stable association of VNFG with VNFDK. In a complete FeV-co insertion reaction (containing FeV-co), addition of dinitrogen reductase 1 or dinitrogen reductase 2 did not have an effect on the association of VNFG with VNFDK (data not shown).

The requirements for the stable association of VNFG with the VNFDK polypeptides of apodinitrogenase 2 differ in two notable aspects from the processing of α₁β₂ (NIFDK) apodinitrogenase 1 to the α₁β₂γ₂ form of apodinitrogenase 1: 1) FeV-co is required for the former process whereas the presence of FeMo-co is not necessary for the association of the γ protein with the NIFDK polypeptides of apodinitrogenase 1 (25), and 2) dinitrogenase reductase is not required for the stable associa-
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When a reaction mixture containing both the FeV-co-containing solution and the VNFG-containing fraction was chromatographed on the Superose 12 column, the migration of FeV-co was retarded; FeV-co eluted at \( V/V_a \), 1.59–1.76 (Fig. 6A, peak 2). The Superose 12 fractions that contained FeV-co (as determined by the FeV-co insertion assay) were analyzed by SDS-PAGE, and the presence of VNFG in these fractions was demonstrated (Fig. 6B, lanes 2–5). When the FeV-co-containing Superose 12 fractions were added to apodinitrogenase 2 (in extracts of CA117.30 (\( \Delta \)nif\( DKB \)), the formation of holodinitrogenase 2 was observed (data not shown). Upon fractionation of a mixture containing both the FeV-co solution and extract of strain UW45 (\( nifB^+ \), grown under NH\( _4^- \)-sufficient conditions) on the Superose 12 column, the migration of FeV-co remained unaltered (Fig. 6C, peaks 1 and 2), suggesting that the change in the elution profile of FeV-co upon addition to VNFG is quite likely due to a specific interaction of FeV-co with VNFG.

In a similar set of experiments performed using FeMo-co in place of FeV-co, the elution profile of FeMo-co remained unaffected in the presence of added VNFG (Fig. 6D, peaks 1 and 2), further suggesting that VNFG associates specifically with FeV-co. These data, together with the apparently reversible association of VNFG with the apodinitrogenase 2 complex, suggest that VNFG might function in binding and inserting FeV-co into apodinitrogenase 2 to form the holoenzyme.

Two A. vinelandii strains were examined for the in vivo accumulation of FeV-co, strain CA11.6.82 (W-tolerant, \( \Delta \)nifD82::Tn5B21 ::\( \Delta \)nif\( DKB \)) and strain CA11.1 (\( \Delta \)nif\( HDK\)::spc). FeV-co was not detected in extracts of these strains (unidentified by the FeV-co insertion assay). Given the in vitro data suggesting the specific association of FeV-co with VNFG, it is reasonable to expect that detectable levels FeV-co might accumulate in vivo only in the presence of VNFG; extracts of both strains tested lacked VNFG (as determined by immuno blot analysis using antibody to VNFG). In contrast to the nif system, in which the dinitrogenase 1 polypeptides are not required for the synthesis and accumulation of FeMo-co (38, 39), the presence of the dinitrogenase 2 polypeptides might be necessary for the accumulation of detectable levels of FeV-co. The possibility that FeV-co is assembled on the dinitrogenase 2 polypeptides cannot be excluded. The fact that no FeV-co was observed to accumulate in vivo in strains CA11.1 (\( \Delta \)nif\( HDK\)::spc) and CA11.6.82 (W-tolerant, \( \Delta \)nifD82::Tn5B21 ::\( \Delta \)nif\( HDK\)), despite the presence of significant levels of the \( \gamma \) protein (as detected by immunoblot analysis) in extracts of both strains, suggests that the \( \gamma \) protein is not able to function as a chaperone-insertase in the nitrogenase 2 enzyme system.

Based on our prior (24) and current observations, a model for the formation of holodinitrogenase 2 can be proposed (Fig. 7). Two processes could lead to the association of VNFG with FeV-co: 1) In the presence of FeV-co, the loosely associated VNFG (\( \delta \) subunits of the \( \alpha_2\beta_2\gamma_2 \) apodinitrogenase 2 complex might dissociate (and dimerize) and bind FeV-co; or 2) a VNFG dimer that is not a part of the apodinitrogenase 2 hexamer might associate with FeV-co. The in vitro association of FeV-co with the VNFG dimer does not require the presence of nucleotide; however, nucleotide is required for the stable association of VNFG with VNFDK. Thus, in a subsequent nucleotide-dependent process, the VNFG-FeV-co species could donate FeV-co to apodinitrogenase 2, inducing a conformational change in the overall quaternary structure of the complex that allows the stable association of VNFG with the newly reconstituted dinitrogenase 2. The fact that VNFG remains stably associated with VNFDK polypeptides in holodinitrogenase 2 might have implications for its role in catalysis; studies of Waugh et al. (23)

\footnote{M. J. Homer and G. P. R. Roberts, personal communication.}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7}
\caption{A model for the formation of dinitrogenase 2. The open squares represent empty FeV-co sites in the \( \alpha \) subunit of apodinitrogenase 2, and the boxed "P" represent the P clusters.}
\end{figure}
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indicated that VNFG was required for full catalytic activity of dinitrogenase 2.

The association of FeV-co with VNFG might be further characterized by monitoring the ability of altered forms of VNFG to bind FeV-co. The predicted amino acid sequence of VNFG indicates the presence of a single cysteine residue that is conserved between both VNFG and ANFG (the third subunit of dinitrogenase 3). By analogy to the role of Cys-275 of dinitrogenase 1 (one of the ligands to FeMo-co; Ref. 22), the invariant cysteine residue of VNFG (Cys-17) might serve as a ligand to FeV-co. It will be interesting to site-specifically alter Cys-17 of VNFG (Cys-17) might serve as a ligand to FeV-co. The predicted amino acid sequence of VNFG in vivo and in vitro indicates that VNFG was required for full catalytic activity of dinitrogenase 2.

CONCLUSIONS

In this study, we have characterized the forms of VNFG observed in cell-free extracts of various A. vinelandii mutant strains, and correlated the VNFG forms with the presence of either apodinitrogenase 2 or holodinitrogenase 2. The homodimeric form of VNFG observed under native PAGE and gel-filtration chromatography conditions in extracts of strains that accumulate apodinitrogenase 2 most likely results from VNFG dissociating from the $\alpha_2\beta_2\delta_2$ apodinitrogenase 2 complex due to weak interactions of VNFG with VNFDK. In the presence of FeV-co and nucleotide, VNFG becomes associated stably with the VNFDK polypeptides to yield the VNFG form of dinitrogenase 2. The differences that have emerged between the modes of action of VNFG and the $\gamma$ protein illustrate that the mechanisms utilized in the processing of apodinitrogenase 2 to a catalytically active form are clearly distinct from those involved in the maturation of apodinitrogenase 1.

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