A Vanadium and Iron Cluster Accumulates on VnfX during Iron-Vanadium-cofactor Synthesis for the Vanadium Nitrogenase in Azotobacter vinelandii*

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The *vnf*-encoded nitrogenase from *Azotobacter vinelandii* contains an iron-vanadium cofactor (FeV-co) in its active site. Little is known about the synthesis pathway of FeV-co, other than that some of the gene products required are also involved in the synthesis of the iron-molybdenum cofactor (FeMo-co) of the widely studied molybdenum-dinitrogenase. We have found that VnfX, the gene product of one of the genes contained in the *vnf*-regulon, accumulates iron and vanadium in a novel V-Fe cluster during synthesis of FeV-co. The electron paramagnetic resonance (EPR) and metal analyses of the V-Fe cluster accumulated on VnfX are consistent with a VFe8S8 precursor of FeV-co. The EPR spectrum of VnfX with the V-Fe cluster bound strongly resembles that of isolated FeV-co and a model VFe8S8 compound. The V-Fe cluster accumulating on VnfX does not contain homocitrate. No accumulation of V-Fe cluster on VnfX was observed in strains with deletions in genes known to be involved in the early steps of FeV-co synthesis, suggesting that it corresponds to a precursor of FeV-co. VnfX purified from a *nifB* strain incapable of FeV-co synthesis has a different electrophoretic mobility in native anoxic gels than does VnfX, which has the V-Fe cluster bound. NifB-co, the Fe and S precursor of FeMo-co (and presumably FeV-co), binds to VnfX purified from the *nifB* strain, producing a shift in its electrophoretic mobility on anoxic native gels. The data suggest that a precursor of FeV-co that contains vanadium and iron accumulates on VnfX, and thus, VnfX is involved in the synthesis of FeV-co.

Interest in vanadium as a biologically relevant element has increased in the last two decades. Although it had been known for some time that vanadium occurs in a highly enriched form in marine organisms and mushrooms (for a review, see Ref. 1), the discovery of its presence and functional role in haloperoxidases (reviewed by Butler (2)) and nitrogenases (reviewed by Eady (3)) is recent. Nitrogenase, the bacterial enzyme that catalyzes the conversion of N2 into NH4+, contains a unique iron-sulfur-heterometal cofactor, which is the site of substrate reduction. The most widely studied nitrogenases contain molybdenum as the heterometal in their active site cofactor (the iron-molybdenum cofactor (FeMo-co)1). The discovery of molybdenum-independent nitrogenases by Bishop et al. (4, 5) has led to the isolation and characterization of nitrogenases containing an iron-vanadium cofactor (FeV-co) (see Ref. 3 for a review), as well as nitrogenases in which no metal other than iron has been detected in the active site cofactor (6). These three nitrogenase systems are genetically distinct, the genes encoding them being contained in different regulons (designated *nif*, *vnf*, and *anf* genes for the molybdenum, vanadium, and iron-only nitrogenases, respectively). *Azotobacter vinelandii* harbors all three nitrogenase systems, and their expression is regulated by the metal content of the culture medium (7). Each nitrogenase consists of two easily separable protein components: dinitrogenase and dinitrogenase reductase. The reduction of substrate is catalyzed by dinitrogenase, whereas dinitrogenase reductase serves as the obligate electron donor to dinitrogenase.

The *nif*, *vnf*, and *anf* regulons encode not only the nitrogenases but also products involved in cofactor biosynthesis and insertion in the cofactorless dinitrogenases. Among the genes known to be required for FeMo-co synthesis are *nifB*, *nifE*, *nifN*, *nifV*, and *nifH*. The protein encoded by *nifB* synthesizes NifB-co, a precursor that is the iron and sulfur donor to FeMo-co (8, 9) and most probably to FeV-co, because *nifB* is required for vanadium-dependent diazotrophic growth (10). The gene products of *nifE* and *nifN* form a tetrameric protein (NifEN), that is able to bind NifB-co (11). Analogs of the *nifE* and *nifN* genes exist in the *vnf* regulon (vnfEN (12)), probably serving a similar role during FeV-co synthesis. The gene product of *nifV* is homocitrate synthase (13). Homocitrate is a structural component of FeMo-co (14) and most probably FeV-co, because *nifV* is also required for full functionality of the *vnf*-dinitrogenase (15). The product of *nifH*, dinitrogenase reductase, plays multiple roles in the nitrogenase system. In addition to reducing dinitrogenase during nitrogenase turnover, NifH (VnFH) is required for FeMo-co (and presumably FeV-co) biosynthesis (16, 17), but its exact role in this process remains to be established. It is important to note that the structural gene products for dinitrogenase (nifDK) are not required for accumulation of FeMo-co and not involved in its synthesis (18).

Any other gene products involved in FeMo-co/FeV-co biosynthesis remain unknown, and the complete biosynthetic pathway is yet to be understood. An important unanswered question regards the gene product(s) providing the specificity for the incorporation of the heterometal (vanadium, molybdenum, or iron) into the cofactor. We report here that a V-Fe-containing cluster accumulates on the gene product of *vnfX* during syn-

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1 The abbreviations used are: co, cofactor; Mops, 4-morpholino-d-propanesulfonic acid; EPR, electron paramagnetic resonance.
thesis of FeV-co. Our data suggest that this cluster is a precursor of FeV-co.

**EXPERIMENTAL PROCEDURES**

**Materials**—All materials used for growth medium preparation were analytical grade. Tris base and glycine were purchased from Fisher. DEAE-cellulose (DE-52) was from Whatman. Sephadex G-75 and phenyl-Sepharose were obtained from Amersham Pharmacia Biotech. 49V (VVI) in 6 N HCl, 0.1–1.0 M NaCl/ml was purchased from Los Alamos National Laboratories. Sodium metavanadate (99.995% purity) and all other chemicals were from Sigma. Nickelsulfonate membranes and acrylamide/bisacrylamide solution were obtained from Mallinkrodt.

**Bacterial Strains and Growth Conditions**—A. vinelandii strains CA12 (ΔmifHDK) (19), CA11.1 (ΔmifHDK::DGK-spc) (20), CA11.7 (ΔmifDBK) (21), CA85 (ΔmifDNfVinEnK-570::kn) (12), D248.2 (ΔmifDVnInEnK-570::kn) (12), and CA11.5 (ΔmifHDK::ΔHif) (22) have been described. All glassware used to prepare the culture medium and for cell growth was washed with 4 N HCl and rinsed thoroughly with deionized water. The strains were grown in Burk’s modified medium lacking molybdenum and containing Na4VO3 (0.1 μM) spiked with radioactive 49VCl3 (0.5 μC/ml) and 400 μg/ml nitrogen in the form of ammonium acetate at 30 °C with shaking. In order to ensure maximum incorporation of 49V into the cells, starter cultures that had been depleted of vanadium by subculturing three times in molybdenum-free, vanadium-free medium were used to inoculate the 49V-containing medium. Cells were grown overnight, collected by centrifugation, and resuspended in nitrogen-free medium containing Na4VO3 and 49VCl3 (same concentrations as above). The cultures were incubated then for 5 h at 30 °C for derepression of the vanadium nitrogenase system. Cells were harvested by centrifugation and frozen at −80 °C. For large scale purification of VnX, A. vinelandii strain CA11.1 was grown in 2-liter carboys containing 15 liters of medium containing 0.1 μM NaVO3 and 40 μg/ml nitrogen in the form of ammonium acetate. The cultures were incubated at 30 °C and aerated vigorously. Cultures were monitored for the depletion of ammonium, following which, derepression of nitrogenase was allowed for 5 h. Cells were collected using a temperature controller.

**Preparation of VnX for N-terminal Sequencing**—One ml of partially purified VnX was subjected to preparative anoxic native gel electrophoresis, the region of the gel containing the radioactivity was sliced, and the protein was eluted from the acrylamide. SDS gel electrophoresis of this sample showed a 20-kDa protein. The protein was transferred to a polyvinylidene difluoride membrane (Problot, Applied Biosystems, Foster, CA). N-terminal sequencing and N-terminal sequence analysis were done at the Department of Biochemistry, Medical College of Wisconsin (Milwaukee, WI).

**Antibodies and Immunoblot Analysis**—Pure VnX was cut out of preparative SDS-polyacrylamide gel electrophoresis gels. The protein was eluted from the acrylamide and injected into a rabbit to produce polyclonal antibodies at the Animal Care Unit of the University of Wisconsin-Madison. Immunoblot analysis was performed as described by Brandner et al. (24).

**Purification of VnX from a nifB Strain**—VnX was partially purified from A. vinelandii CA117.3 (ΔmifDBK) grown and derepressed in the absence of molybdenum and presence of vanadium. Extract of 40 g of cells was chromatographed on a DE-52 column (2.5 × 17 cm) equilibrated with 25 mM Tris-HCl, pH 7.4, containing 20% glycerol. The column was washed with 80 ml of the same buffer, and protein was eluted stepwise with 0.075, 0.15, and 0.30 M NaCl in 25 mM Tris, pH 7.4, containing 20% glycerol. The presence of VnX in the fractions was assessed using immunoblot analysis of anoxic native gels. The fractions containing VnX were pooled and concentrated to 15 ml by ultrafiltration using a PM10 membrane (Amicon). The concentrated fraction was loaded on a Sephadex G-75 column (2.5 × 95 cm) equilibrated with 25 mM Tris-HCl, pH 7.4, 10% glycerol, 50 mM NaCl. The fractions from Sephadex G-75 containing VnX were pooled and frozen in liquid N2.

**Incubations with NifB-co**—NifB-co was obtained from Klebsiella pneumoniae UN1217 (ΔnifFGK) (25) as has been described (9). NifB-co and 2Fe-2Fe-NifB-co were in 25 mM Tris-HCl, pH 7.4, containing 2% SB12 (3-(dodecyl-dimethylammonio)propane-1-sulfonate, Fluka). Incubations of VnX with NifB-co were done for 30 min at 30 °C.

**Analysis of VnX for Homocitrate**—Approximately 80 ml of 49V-VnX partially purified from A. vinelandii CA11.1 (48 nmol of vanadium, as estimated by specific activity of 49V; 15 mg of total protein) was added dropwise to 900 ml of acetone containing 6.75 ml of 5 N HCl at 4 °C with continuous stirring over a 10-min period. The mixture was stirred for an additional 15 min, and the precipitated protein was removed by filtration. The filtrate was evaporated to approximately 8 ml using a rotary evaporator, and the pH was adjusted to 8.0–9.0 before chromatography on an AG-1X8 (formate form, Bio-Rad) column (1 × 41 cm) equilibrated with water. The column was eluted with a gradient of 0–6 N formic acid (400 ml total). In order to determine in which fractions of the AG-1X8 column the homocitrate elutes, a standard of [3H]homocitrate (0.1 mmol) was added. The concentration of homocitrate expected in the sample of VnX used for homocitrate extraction (considering 1 mol of homocitrate/mol of vanadium). The recovery of standard homocitrate was assessed using immunoblot analysis of anoxic native gels. The fractions containing VnX were pooled and concentrated to 15 ml by ultrafiltration using a PM10 membrane (Amicon). The concentrated fraction was loaded on a Sephadex G-75 column (2.5 × 95 cm) equilibrated with 25 mM Tris-HCl, pH 7.4, 10% glycerol, 50 mM NaCl. The fractions from Sephadex G-75 containing VnX were pooled and frozen in liquid N2.

**RESULTS AND DISCUSSION**

In order to study which proteins play a role in FeV-co biosynthesis, mutant strains of A. vinelandii were grown in medium depleted of molybdenum and containing 49V spiked with 15N (radioactive). The labeled proteins present in cell extracts were analyzed through phosphorimaging of anoxic native gels (Fig. 1, lane 1). In a separate FeV-co study that is a wild-type for the vnf-nitrogenase system (CA12 (ΔmifHDK)), this mutant major protein band containing 49V was the vnf-dinitrogenase, as expected (Fig. 1, lane 1). In extracts of a mutant strain unable to synthesize the structural proteins of either the molybdenum nitrogenase or the vanadium nitrogenase (CA11.1 (ΔmifHDK::DGK-spc)), one prominent radiolabeled protein band was observed (Fig. 1, lane 2,
Acrylamide. This step is not shown in the purification table, in an anoxic native gel, the region of the gel containing the after phenyl-Sepharose chromatography was electrophoresed in order to obtain pure protein, 1 ml of the fraction obtained following the radioactivity associated with it. Purification steps included DEAE-cellulose, Sephadex G-75, and phenyl-Sepharose chromatography, as shown in Table I. Purified VnfX homolog is proposed to play a similar role in FeV-co synthesis. A minority species that is of maximum observed intensity at 4.8 K (Fig. 1, lane 3) was also seen in all other extracts used. This band did not stain for protein by Coomassie Blue or ammonium. 

The purified protein was identified as VnfX, the major band present in extracts of A. vinelandii mutant strains grown and derepressed in the presence of ammonium. Isolated NifB-co contains only Fe and S, and is paramagnetic, it would not be readily interpreted as arising from a majority species. Analogous signals have been seen in the aged version of the majority species. Magnetic hyperfine structure due to $^{51}$V ($I = 7/2$; the majority isotopic species of vanadium present) is apparent in the g = 2 region under these conditions (Fig. 3B, inset). Because the observation of this hyperfine structure is maximized at the same conditions as is the intensity of the S = 3/2 species, it is probable that the hyperfine structure is due to $^{51}$V incorporated into the species that produces the S = 3/2 resonances, rather than due to a protein-bound $^{51}$V (S = 1/2) species. The ground state spin and spectral line shapes of the majority species resemble those of isolated FeV-co (28), the reduced nif-dinitrogenase of A. chroococcum (29), and a synthetic [Fe$_5$S$_9$] cubane cluster (30). The minority S = 1/2 component (g = 2.05, 1.96, 1.89) was most clearly observable at higher temperatures (Fig. 2A), and its relative abundance varied from sample to sample. The minority species may represent a damaged version of the majority species. Analogous signals have been seen in the nif-dinitrogenases of A. chroococcum (31) and A. vinelandii (32) and have been suggested to represent damaged FeV-co in those proteins. Therefore, the spectroscopic properties of the S = 3/2 species are consistent with an [Fe-V-S] cluster with similar properties to both FeV-co (proposed to be [Fe$_5$S$_9$]), and a [Fe$_5$S$_9$] cluster. Metal analysis revealed that there are $8 \pm 1$ mol of Fe and 0.5 mol of vanadium bound per mol of VnX. The levels of vanadium and Fe are more consistent with a model in which a [Fe$_5$S$_9$] cluster is bound to VnX, as opposed to a [Fe$_6$S$_9$] cluster. However, it does not appear that FeV-co itself accumulates on VnX, as vanadium- and iron-containing VnX (as purified) is not able to donate $^{49}$V label to FeV-co-deficient dinitrogenase (VnfDGK) (see below). The low level of vanadium (0.5 mol/mol of VnX) may indicate that some vanadium was lost during purification or that a fraction of the cluster on the isolated VnX had not yet acquired an atom of vanadium. It is possible that the minority species arises from a damaged [Fe-S] cluster in which vanadium has been lost. As discussed below, NifB-co is able to bind to VnX in the absence of vanadium. Isolated NifB-co contains only Fe and S, and is diamagnetic in the presence of dithionite. Whereas it is possible that VnX-bound NifB-co is paramagnetic, it would not

Hamiltonian, $H_p$, with $D$ and $E$ as the axial and rhombic splitting parameters, and an isotropic g-tensor, $g_0$, as given by Equation 1.

$$H_p = g_0 b H \cdot S + (S^2 + 1/3) (E/D) (S^2 - S^3)$$  \hspace{1cm} (Eq. 1)

On the basis of their temperature dependence behavior, the features at $g = 4.82$, $g = 3.36$, and $g = 1.90$ are attributed to resonances from the lower doublet of the $S = 3/2$ system with a rhombicity parameter $(E/D)$ of 0.15 and $D > 0$, using $g_0 = 2.00$. An accurate assessment of the high field g value is hindered by the diffuse nature of the resonance and the presence of the minority species. As Fig. 2B demonstrates, power-saturation of the majority species (determined by following the intensity of the $g = 4.82$ resonance) only becomes evident at temperatures below 10.0 K when using a 10.0 mW microwave power. A plot of the normalized intensity of the $g = 4.82$ resonance divided by the square root of the microwave power versus the log of microwave power at a temperature of 4.8 K, according to Equation 2, is shown in Fig. 3A (27).

$$S = P_{1/4}/(1 + P_{1/2})^{1/2}$$  \hspace{1cm} (Eq. 2)

$P_{1/2}$ is the power for half-saturation, and $b$ is the inhomogeneity parameter. Analysis of the data reveals a $P_{1/2}$ of 4.3 mW using $b = 1$, which provided the best fit. Fig. 3A shows that at 4.8 K and 1.0 mW, the S = 3/2 species is largely not power-saturated. Because the minority S = 1/2 species was not observed at 4.8 K and 1.0 mW (Fig. 3B), it can be assumed that the g = 2 region of the spectrum is arising from only the majority species. Magnetic hyperfine structure due to $^{51}$V ($I = 7/2$; the majority isotopic species of vanadium present) is apparent in the g = 2 region under these conditions (Fig. 3B, inset). Because the observation of this hyperfine structure is maximized at the same conditions as is the intensity of the S = 3/2 species, it is probable that the hyperfine structure is due to $^{51}$V incorporated into the species that produces the S = 3/2 resonances, rather than due to a protein-bound $^{51}$V (S = 1/2) species. The ground state spin and spectral line shapes of the majority species resemble those of isolated FeV-co (28), the reduced nif-dinitrogenase of A. chroococcum (29), and a synthetic [Fe$_5$S$_9$] cubane cluster (30). The minority S = 1/2 component (g = 2.05, 1.96, 1.89) was most clearly observable at higher temperatures (Fig. 2A), and its relative abundance varied from sample to sample. The minority species may represent a damaged version of the majority species. Analogous signals have been seen in the nif-dinitrogenases of A. chroococcum (31) and A. vinelandii (32) and have been suggested to represent damaged FeV-co in those proteins. Therefore, the spectroscopic properties of the S = 3/2 species are consistent with an [Fe-V-S] cluster with similar properties to both FeV-co (proposed to be [Fe$_5$S$_9$]), and a [Fe$_5$S$_9$] cluster. Metal analysis revealed that there are $8 \pm 1$ mol of Fe and 0.5 mol of vanadium bound per mol of VnX. The levels of vanadium and Fe are more consistent with a model in which a [Fe$_5$S$_9$] cluster is bound to VnX, as opposed to a [Fe$_6$S$_9$] cluster. However, it does not appear that FeV-co itself accumulates on VnX, as vanadium- and iron-containing VnX (as purified) is not able to donate $^{49}$V label to FeV-co-deficient dinitrogenase (VnfDGK) (see below). The low level of vanadium (0.5 mol/mol of VnX) may indicate that some vanadium was lost during purification or that a fraction of the cluster on the isolated VnX had not yet acquired an atom of vanadium. It is possible that the minority species arises from a damaged [Fe-S] cluster in which vanadium has been lost. As discussed below, NifB-co is able to bind to VnX in the absence of vanadium. Isolated NifB-co contains only Fe and S, and is diamagnetic in the presence of dithionite. Whereas it is possible that VnX-bound NifB-co is paramagnetic, it would not

![Phosphorimage of a native anoxic gel of extracts of A. vinelandii mutant strains grown and derepressed in the presence of 49VCl-](image-url)
A V-Fe Cluster Is Associated with VnfX

**TABLE I**

<table>
<thead>
<tr>
<th>Purification of VnfX from A. vinelandii CA11.1</th>
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<tbody>
<tr>
<td><strong>Fraction</strong></td>
</tr>
<tr>
<td>CA11.1 extract</td>
</tr>
<tr>
<td>DE-52</td>
</tr>
<tr>
<td>Sephadex G-75</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
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*The total cpm in fractions was determined by scintillation counting, and the fraction of cpm associated with VnfX was estimated from phosphorimages of anoxic native gels using ImageQuant (Molecular Dynamics).*

As indicated above, vanadium- and iron-containing VnfX is not able to donate the V-Fe cluster to FeV-co-deficient dinitrogenase (VnfDGK) under the conditions tested to date. Assay mixtures used for this purpose contained 25 mM Tris-HCl, pH 7.4, extract of *A. vinelandii* CA11.3 (*ΔnifKDB*) grown in the presence of vanadium (as a source of FeV-co-deficient vnf-dinitrogenase, VnfH, and other vnf proteins), an ATP generating system, MgCl2 (1.8 mM), and partially purified VnfX with the 49V-Fe cluster bound (122,000 cpm/assay). Assays were performed in the absence or presence of homocitrate (104 μM). Purified NiIF and partially purified NiIFEN were added to some assays, in case the vnf counterparts of these proteins were limiting in the extract of *A. vinelandii* CA11.3. Under none of the conditions used was the 49V-Fe cluster donated to apo-dinitrogenase, as estimated by phosphorimaging of anoxic native gels, nor could nitrogenase activity be detected.

The V-Fe Cluster Accumulating on VnfX Does Not Contain Homocitrate—Experiments were conducted to determine whether the V-Fe cluster associated to VnfX contains homocitrate. Partially purified VnfX was extracted with acidified acetone, and the extract was purified by AG-1X8 chromatography. The data was fit using Equation 2, affording a $P_{1/2} = 4.3$ mW. Best fits were obtained with $b = 1$. Microwave power levels used were 0.10, 0.20, 0.50, 1.0, 2.0, 3.5, 5.0, 10, 15, and 20 mW. B) EPR spectrum of VnfX obtained at 4.8 K and 1.0 mW. The inset expands the region of the spectrum containing the nuclear hyperfine resonances (2600–3800 Gauss). The spectrum shown is the sum of six scans, with a six-scan cavity spectrum performed at the same experimental conditions subtracted. VnfX purified from *A. vinelandii* CA11.1 (*ΔnifKDB*::spc) was at a final concentration of 23 μM.

**TABLE II**

<table>
<thead>
<tr>
<th>Detection of homocitrate by in vitro FeMo-co synthesis assay</th>
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<tr>
<td>R-HC standard</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>nmol/assay</td>
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<tr>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
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<tr>
<td>1.0</td>
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<tr>
<td>2.0</td>
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<tr>
<td>4.0</td>
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<tr>
<td>12.5</td>
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<tr>
<td>50</td>
</tr>
<tr>
<td>0.5</td>
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<tr>
<td>1.0</td>
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<tr>
<td>2.0</td>
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<tr>
<td>4.0</td>
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<tr>
<td>12.5</td>
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</table>

As shown in Table II, no homocitrate could be detected in the extract of VnfX, as assessed by in vitro FeMo-co synthesis assays. Table II also shows the activities obtained in vitro FeMo-co synthesis assays when adding amounts of homocitrate equivalent to the amounts expected to be in the VnfX extract if...
this organic acid was a part of the cluster associated to VnfX. Homocitrate is probably added to FeV-co at a later step than the one occurring on Vnfx.

VnfX Is Not Absolutely Required for FeV-co Synthesis—Surprisingly, A. vinelandii CA48 (vnfET05::kan), a strain that does not express VnfX (as shown by immunoblot analysis, apparently due to polarity of the mutation in vnfE on vnfX) is able to grow diazotrophically in the presence of vanadium, although after a prolonged lag period (12). This strain does produce vnf-encoded dinitrogenase, as shown in Fig. 1, lane 8. As expected, no label was seen in this extract at the position of VnfX. It is possible that NiIX or some other protein replaces VnfX in the synthesis of FeV-co. Wollfinger and Bishop (12) have suggested that NifEN replaces VnfEN in this strain during vanadium-dependent diazotrophic growth. Immunoblot analysis shows that both NiIX and NiIFN are expressed in vanadium-grown CA48 (data not shown). Nevertheless, no $^{55}$V was associated to NiIX in extract of this strain (Fig. 1, lane 8). Association of $^{55}$V to NiIFN may be weaker than to VnfX and not withstand the electrophoresis conditions. No $^{55}$V radioactivity was seen in extracts of strain DJ424.8 ($^{55}$NiIFN/ET05::kan) (Fig. 1, lane 7). The mutations in this strain are polar on nifX and vnfX.

Two Electrophoretically Distinct Species of VnfX—Polyclonal antibodies to VnfX were generated by purifying VnfX, which was extracted from an SDS gel. Immunoblot analysis of anoxic extracts of the partially purified VnfX (from A. vinelandii CA11.1) showed a major band cross-reacting with the antibody, which comigrated with the $^{49}$V-labeled VnfX (Fig. 4, lanes 2 and 5). A minor band of slower electrophoretic mobility also cross-reacted with the VnfX antibody (labeled V-deficient VnfX in Fig. 4, lane 5). After treatment of VnfX with air, the band corresponding to $^{49}$V-VnfX could no longer be seen, but the band migrating in the higher position remained (Fig. 4, lane 6).

Treatment of VnfX with air resulted in loss of vanadium and iron (data not shown). The slower migrating band in lane 5 probably arose from dissociation of the V-Fe cluster from VnfX during purification (see below). In extracts of a mutant strain incapable of NiIX-co production (A. vinelandii CA117.3 ($^{55}$NifDKnifIF)), there was no vanadium or iron associated with VnfX (as estimated by phosphorimaging (Fig. 1, lane 3) and iron staining of anoxic native gels, not shown). The vanadium-deficient species cross-reacting with the anti-VnfX antibody in extracts of this strain had a slower mobility in anoxic native gel electrophoresis (Fig. 4, lane 7) than the species that has the V-Fe cluster bound. Vanadium-deficient VnfX was partially purified from an extract of A. vinelandii CA117.3. When subjected to gel filtration electrophoresis using Sephadex G-75, this form of VnfX eluted at a volume that corresponds to an apparent molecular mass of 33 kDa, compared with an elution volume corresponding to an apparent molecular mass of 20 kDa for the species that has the V-Fe cluster bound (the molecular mass predicted from the DNA sequence of vnfX is 19 kDa (12)). The subunit molecular mass of both vanadium-deficient VnfX and V-Fe-containing VnfX was determined to be 20 kDa by SDS-polyacrylamide gel electrophoresis. We hypothesize that a dimeric form of VnfX monomerizes upon binding a V-Fe precursor of FeV-co. This would be analogous to the monomerization of the FeMo-co-binding protein, $\gamma$, which is involved in insertion of FeMo-co into the cofactorless nif-apodinitrogenase (33). Other interpretations are also consistent with our observations, such as a dramatic conformational change in a monomeric VnfX upon metal cluster binding.

Vanadium-deficient VnfX (as Purified from the nifB’ Strain) Is Able to Bind NiIX-co—Treatment of vanadium-deficient VnfX with purified NiIX-co caused a shift in the migration of VnfX in anoxic native gels (Fig. 4, lane 8). The VnfX-NiIX-co complex migrated at the same position as does VnfX that has the V-Fe cluster bound to it (e.g. the VnfX purified from A. vinelandii strain CA11.1 (Fig. 4, lane 2). Oxygen-inactivated NiIX-co did not produce the shift of VnfX (Fig. 4, lane 9), nor did a solution of SB12 (Fig. 4, lane 10), a detergent that was present at that concentration in the NiIX-co solution. The binding of NiIX-co to VnfX was confirmed by incubating the partially purified protein with $^{55}$Fe-NiIX-co. When this incubation mixture was subjected to anoxic native gel electrophoresis and the gel was analyzed by phosphorimaging, the $^{55}$Fe label was found associated with VnfX (Fig. 4, lane 3).

Immunoblot analysis was also used to confirm that VnfX was not expressed in A. vinelandii grown in the presence of molybdenum (Fig. 4, lane 11) or in the presence of ammonium acetate (Fig. 4, lane 12), which represses synthesis of all three nitrogenase systems. Furthermore, anti-VnfX antibody did not cross-react with NiIX (data not shown). No association that is stable to anoxic native gel electrophoresis was observed between VnfX and dinitrogenase (VnFDGK) or VnFX (data not shown).

A homolog of vnfX exists in the nif regulon (nifX). The product of nifX is required for in vitro FeMo-co synthesis with purified components (34). Nevertheless, nifX is not absolutely required in vivo for FeMo-co synthesis, because strains of A. vinelandii and K. pneumoniae with mutations in nifX are Nif$^-$(35, 36). The same seems to be true for VnfX. A. vinelandii CA48 (vnfET05::kan) is able to grow diazotrophically in the presence of vanadium, although after a prolonged lag period. It is possible that some other gene product replaces VnfX (or NiIX) in vivo, although with lower efficiency.

As stated before, in extracts of strains with mutations in nifB or a double mutation in vnfH and nifH, no V-Fe cluster accumulates on VnfX. Thus, the involvement of NiIX and VnFX in...
FeV-co biosynthesis must occur earlier in the biosynthetic pathway than the step occurring on VnfX. We speculate that the gene products believed to play a role in FeV-co synthesis would do so in the following order (Scheme I).

\[
\text{NifB} \rightarrow \text{NifB-co} \rightarrow \text{VnfX(NifB-co)} \rightarrow \vdots
\]

\[
+ V \rightarrow \text{VnfX-V-Fe} \rightarrow \text{FeV-co}
\]

Scheme 1

**Conclusions**—We have identified and characterized what appears to be a precursor of FeV-co that contains the heterometal (vanadium). This intermediate is associated with VnfX, which suggests that this protein is involved in FeV-co synthesis. The step occurring on VnfX could be the addition of vanadium to the partially formed cluster, because both a vanadium-containing cluster and NifB-co, its Fe-S precursor, can accumulate on VnfX. Alternatively, a cluster already containing vanadium could be transferred to VnfX, and a further processing step could occur on this protein.

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A Vanadium and Iron Cluster Accumulates on VnfX during Iron-Vanadium-cofactor Synthesis for the Vanadium Nitrogenase in *Azotobacter vinelandii*

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