

Purification and Characterization of NafY (Apodinitrogenase γ Subunit) from *Azotobacter vinelandii**

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The formation of an active dinitrogenase requires the synthesis and the insertion of the iron-molybdenum cofactor (FeMo-co) into a presynthesized apodinitrogenase. In *Azotobacter vinelandii*, NafY (also known as γ protein) has been proposed to be a FeMo-co insertase because of its ability to bind FeMo-co and apodinitrogenase. Here we report the purification and biochemical characterization of NafY and reach the following conclusions. First, NafY is a 26-kDa monomeric protein that binds one molecule of FeMo-co with very high affinity ($K_d \approx 60$ nM); second, the NafY-FeMo-co complex exhibits a $S = 3/2$ EPR signal with features similar to the signals for extracted FeMo-co and the M center of dinitrogenase; third, site-directed mutagenesis of *nafY* indicates that the His¹²¹ residue of NafY is involved in cofactor binding; and fourth, NafY binding to apodinitrogenase or to FeMo-co does not require the presence of any additional protein. In addition, we have obtained evidence that suggests the ability of NafY to bind NifB-co, an FeS cluster of unknown structure that is a biosynthetic precursor to FeMo-co.

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 iron-molybdenum protein) is a 240-kDa $\alpha_2\beta_2$ tetramer of the *nifD* and *nifK* gene products (3, 4). Each $\alpha\beta$ nitrogenase dimer contains an iron-molybdenum cofactor (FeMo-co)¹ and a P cluster (4, 5). Dinitrogenase reductase (also termed component II or iron protein) is a 60-kDa α_2 dimer of the *nifH* gene product that contains a single 4Fe-4S center coordinated between the two subunits (6). In contrast to the $\alpha_2\beta_2$ subunit composition of the mature dinitrogenase, apodinitrogenase (FeMo-co-deficient) from *Klebsiella pneumoniae* or *Azotobacter vinelandii* strains with mutations in *nifB*, *nifN*, or *nifE* have a hexameric composition ($\alpha_2\beta_2\gamma_2$). The hexameric apodinitrogenase can be activated *in vitro* by the simple addi-

tion of purified FeMo-co (7, 8). In *K. pneumoniae*, the γ subunit has been identified as the product of the *nifY* gene (7, 9), whereas in *A. vinelandii* it is the product of the non-*nif* gene termed *nafY* (nitrigenase accessory factor γ) (10). On the other hand, apodinitrogenase from a $\Delta nifH$ mutant strain has a tetrameric composition ($\alpha_2\beta_2$) and is not readily activated by purified FeMo-co (11, 12).

There are three forms of NafY present in extracts of *A. vinelandii*: an apodinitrogenase-associated form, an unassociated form proposed to exist as a homodimer, and a FeMo-co-associated form (13). NafY serves as a “molecular prop” whose function is to maintain the conformation of the FeMo-co-deficient apodinitrogenase competent for FeMo-co insertion (7, 9, 13, 14). FeMo-co is found buried, and thus inaccessible, within the protein in the mature $\alpha_2\beta_2$ dinitrogenase. It is believed that the maturation of apodinitrogenase occurs in several steps. The $\alpha_2\beta_2$ form of apodinitrogenase is synthesized having an inaccessible FeMo-co site. The FeMo-co site in the $\alpha_2\beta_2$ form is then made accessible through a reaction that requires dinitrogenase reductase and MgATP (11). The dinitrogenase reductase-dependent maturation of apodinitrogenase promotes the binding of γ (NafY) and its conversion to the stabilized $\alpha_2\beta_2\gamma_2$ form. Finally, FeMo-co is inserted, and NafY disassociates from the mature dinitrogenase. Consistent with this notion, it has been recently shown that a deletion in *nafY* halves the competency of apodinitrogenase for FeMo-co insertion in *Azotobacter* extracts (10). The ability of NafY to bind separately to free FeMo-co or to apodinitrogenase led Roberts and co-workers (13) to propose that NafY was also a FeMo-co insertase. This proposal remains controversial because it has been shown that NafY is dispensable for the insertion of FeMo-co into apodinitrogenase (10, 15).

We overexpressed NafY in *Escherichia coli* cells and found that the purified protein has an N-terminal domain and a C-terminal domain. The C-terminal domain of NafY is conserved among the NifX, NifY, VnfX, and NifB family of proteins and has been referred to as the “core domain” of NafY. The core domain is able to bind to FeMo-co but unable to bind to apodinitrogenase (16). The crystal structure of the core domain has been determined and shown to consist of a five-stranded β -sheet flanked by five α -helices (16). Here we report on the biochemical properties of the purified NafY protein and the NafY-FeMo-co complex. We also present data suggesting that the His¹²¹ amino acid residue of NafY is involved in FeMo-co binding.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—*A. vinelandii* strain DJ (wild type) was obtained from D. R. Dean (Department of Biochemistry, Virginia Technical Institute, Blacksburg, VA). Strain UW146 (*nifB-nafY*) has been described previously (10). Growth in the presence of molybdate, *nif* derepression, and cell breakage has been described (17). *E. coli* DH5 α and BL21 (pREP-4) strains were grown in Luria-Bertani medium at 37 °C with shaking (250 rpm). For growth of *E. coli* on

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This paper is dedicated to the memory of Luisa Alvarez.

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¹ The abbreviations used are: FeMo-co, iron-molybdenum cofactor; NifB-co, NifB cofactor; GST, glutathione S-transferase; PBS, phosphate-buffered saline; FPLC, fast protein liquid chromatography.

plates, medium solidified with 1.5% agar was used. Antibiotics were used at standard concentrations (18).

Expression and Purification of NafY—Overproduction of NafY in *E. coli* was accomplished by fusing a *nafY* gene cartridge to the glutathione *S*-transferase (GST) gene, whose expression is controlled by a *tac* promoter. Plasmid pRHB62 contains the *nafY* gene cartridge ligated into the pGEX-4T-3 plasmid (Amersham Biosciences), which incorporates a thrombin cleavage site to remove GST from the fusion partner. The overproduction of GST-NafY, purification of the NafY fusion protein, and separation of the GST moiety from NafY using thrombin were performed as described previously (16). A typical purification procedure yielded 15 mg of NafY from 500 ml of cell culture. The preparations of purified NafY were supplemented with glycerol to 10% (v/v), made anaerobic through degassing with alternate cycles of vacuum and flushing with argon gas, and frozen as droplets into liquid nitrogen until used.

When pure preparations of the GST-NafY fusion protein were required, the purification protocol was identical to that of NafY, but the incubation with thrombin protease was omitted, and GST-NafY was eluted from the glutathione-Sepharose column with 5 column volumes of 10 mM reduced glutathione in PBS (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 140 mM NaCl, 2.7 mM KCl, pH 7.4). The eluted GST-NafY protein had some minor protein contaminants at this point, and the preparation was loaded into a 1 cm × 10 cm Mono-Q column (Amersham Biosciences) equilibrated in PBS for further purification. The column was washed with 1 column volume of PBS, and proteins were eluted from the column using a linear 150–500 mM NaCl gradient in PBS. GST-NafY eluted at about 350 mM NaCl and was estimated to be >95% pure based on SDS-PAGE analysis.

Site-directed Mutagenesis of *nafY*—Generation of site-directed mutations in *nafY* was carried out using plasmid pRHB62 as template and the QuikChange kit (Stratagene) according to the manufacturer's recommendations. The presence of the introduced mutations was confirmed by sequencing. Plasmids pRHB68 (C166S), pRHB69 (C166A), pRHB70 (H121L), pRHB71 (C125A), and pRHB72 (C125A,H188L) were generated. Plasmids encoding NafY variants were transferred to *E. coli* strain BL21 (pREP-4) by transformation. The overexpression and purification of NafY variants was carried out as described above for the wild-type NafY.

Purification of Other Components—Apodinitrogenase was purified in its NafY-deficient $\alpha_2\beta_2$ form by affinity chromatography utilizing the GST-NafY fusion protein bound to glutathione-Sepharose 4B resin (>95% purity).² The resultant protein was stored anaerobically in liquid nitrogen until used. Methods for the purification of FeMo-co (19) and NifB-co (20) have been described previously.

Determination of the Native Molecular Mass of NafY—The native molecular mass of NafY was determined by equilibrium sedimentation of pure NafY in 0.2 M sodium phosphate buffer, 0.1 M NaCl, pH 7.5. Equilibrium sedimentation experiments were performed in a Beckman XL-A analytical ultracentrifuge using 12-mm double sector charcoal-filled Epon centerpieces. Three 100- μ l samples were prepared by dilution of a dialyzed stock solution with its dialysate to give concentrations of 0.25, 0.5, and 0.75 mg of NafY/ml. 105 μ l of dialysate were in the reference sector of each cell. Concentration gradients were recorded at 280 nm every 3–4 h until the gradients were superimposable. Equilibrium data were attained at 10,000 rpm and 17,300 rpm at 20 °C.

Gel filtration chromatography experiments were performed by applying 100 μ l of sample (containing 80 μ g of NafY) onto a Superose 6, Superdex 200, or a Sephacryl S-200 column connected to an FPLC apparatus (Amersham Biosciences). Columns were 1 cm diameter × 30 cm long. PBS buffer and a 0.5 ml/min flow rate were used. When FeMo-co was included in the analysis, PBS was made anaerobic by sparging with nitrogen for 2 h and then adding 0.1 mM sodium dithionite.

Quantitation of the Affinity of NafY for FeMo-co and Interaction between NafY and NifB-co—The K_d of the NafY-FeMo-co complex was estimated using the equation developed by Lundblad *et al.* (21), after measuring the changes in the intrinsic fluorescence of NafY upon FeMo-co binding. Fluorescence changes in NafY were recorded at room temperature with a PerkinElmer Life Sciences LS 50 B spectrofluorometer using pure NafY protein in PBS supplemented with 10% glycerol. The instrument settings were: λ_{ex} = 295 nm; excitation slit, 2.5 nm; λ_{em} = 354 nm; and emission slit, 7.5 nm. The values were corrected for dilution and for inner filter effects. The interaction between NafY and NifB-co was studied measuring the changes in the intrinsic fluorescence of NafY because of the presence of increasing concentrations of

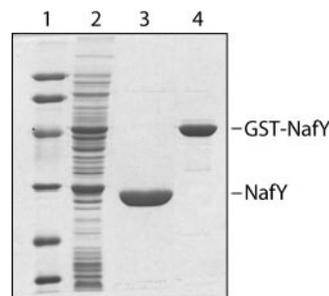


FIG. 1. SDS-PAGE analysis of purified NafY and GST-NafY proteins. NafY and the GST-NafY fusion protein were purified from recombinant *E. coli* cells by affinity chromatography to glutathione-Sepharose (see “Experimental Procedures” for details). Lane 1, molecular mass markers (Bio-Rad low range markers: lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; ovalbumin, 45 kDa; serum albumin, 66.2 kDa; and phosphorylase b, 97.4 kDa); lane 2, cell-free extract of *E. coli* cells overexpressing NafY; lane 3, purified NafY; lane 4, purified GST-NafY fusion protein.

NifB-co. Fluorescence changes in NafY were recorded as above. The values were corrected for dilution and for inner filter effects.

Determination of the Number of FeMo-co-binding Sites in NafY—Purified NafY and isolated FeMo-co were used to determine the stoichiometry of the binding. All glassware was rinsed with 4 N HCl overnight to remove traces of contaminating molybdenum and iron and then rinsed thoroughly with deionized water. An amount of FeMo-co equivalent to 10 nmol of molybdenum was added to 3 nmol of NafY in 1.35 ml of anaerobic 25 mM Tris-HCl buffer, 1 mM sodium dithionite, pH 7.5. The mixture was incubated for 5 min at room temperature and then applied to a 1 cm × 30 cm Superdex 200 column equilibrated in the same buffer to separate unbound FeMo-co from the NafY-FeMo-co complex. The protein sample was eluted in the same buffer and then subjected to protein and metal analyses. The iron and molybdenum contents of the protein samples were determined at the University of Georgia by inductively coupled plasma mass spectrometry.

SDS-PAGE and Immunoblot Analysis—The procedure for SDS-PAGE has been described (22). Immunoblot analysis was performed as described by Brandner *et al.* (23). Purified preparations of NafY described in this work were used to raise anti-NafY antibodies at the Polyclonal Antibody Service of the University of Wisconsin-Madison.

Anoxic Native Gel Electrophoresis and Iron Staining—The proteins were separated on anoxic native gels with a 7–16% acrylamide and a 0–20% sucrose gradient as described (11). When needed, native gels were stained for iron as described previously (24).

Protein Assays—The protein concentrations were determined by the bicinchoninic acid method using bovine serum albumin as standard (25).

EPR Analysis—We performed EPR analysis of the NafY-FeMo-co complex. The complex was generated by mixing an excess of NafY (3.5 mg, 130 nmol) and 50 nmol of FeMo-co in a total volume of 36.5 ml of anaerobic 25 mM Tris-HCl, 1 mM sodium dithionite, pH 7.5 buffer. The complex was then anaerobically concentrated in an Amicon cell, diluted, and reconcentrated with the same buffer until the concentration of *N*-methylformamide was under 1% (v/v). EPR was performed at a microwave frequency of 9.44 GHz and a modulation amplitude of 10 millitesla using a Bruker ESP 300E EPR spectrometer equipped with a Bruker ER0815 frequency converter, a Bruker ER041 XG microwave bridge, and a Oxford ITC temperature controller.

RESULTS AND DISCUSSION

Overexpression and Purification of NafY and GST-NafY—The NafY protein from *A. vinelandii* was produced in *E. coli* BL21 (pREP-4) cells carrying pRHB62 plasmid as a GST-NafY fusion protein of about 50 kDa. Accumulation of soluble GST-NafY in crude extracts of BL21 cells was achieved in highly aerated cultures after incubation at 30 °C in the presence of 0.2 mM isopropyl- β -D-thiogalactopyranoside (Fig. 1, lane 2). (Incubation of BL21 cells at 37 °C and 1 mM isopropyl- β -D-thiogalactopyranoside resulted in the accumulation of insoluble GST-NafY.) The protocol for purification of NafY involved three steps: glutathione-Sepharose affinity chromatography, *in situ* digestion with thrombin to separate NafY from the GST moiety, and benzamidine-Sepharose affinity chromatography to

² L. M. Rubio, and P. W. Ludden, unpublished results.

remove the thrombin (see “Experimental Procedures” for details). The purity of NafY at the end of the process was determined to be >99% based on SDS-PAGE analysis (Fig. 1, lane 3). The UV-visible absorption spectrum of the as-isolated NafY protein has a single peak with a maximum at 281 nm and is featureless in the 310–900 nm range (data not shown). The as-isolated NafY protein does not contain significant levels of iron or molybdenum.

Purification of the GST-NafY fusion protein was performed in two steps: a glutathione-Sepharose affinity chromatography and a Mono-Q anion-exchange chromatography (see “Experimental Procedures”). The purity of GST-NafY at the end of the purification protocol was determined to be >95% based on SDS-PAGE analysis (Fig. 1, lane 4).

Unassociated and FeMo-co-Bound Forms of NafY Are Monomeric—We began characterizing the purified NafY by determining its molecular mass and quaternary structure. Homer *et al.* (13) had reported that, in cell-free extracts of *A. vinelandii*, NafY is a dimer of 46 kDa that monomerizes into 23-kDa subunits upon FeMo-co binding. The result was based on gel filtration chromatography using a Sephacryl resin. In the process of determining the molecular masses of the unassociated and the FeMo-co-bound forms of purified NafY, we found that both forms are monomeric under the conditions employed here.

Equilibrium centrifugation experiments were performed because they are less sensitive to protein shape-induced errors than gel filtration. The native molecular mass of NafY was determined in three different samples containing 0.25, 0.5, and 0.75 mg of NafY/ml. Analysis of the equilibrium centrifugation data yielded an average mass of $28,012 \pm 134$ Da for the unassociated NafY (Fig. 2A). This number is in good agreement with the predicted molecular mass of a NafY monomer as deduced from the *nafY* gene sequence (26,141 Da) and also with the $26,152 \pm 3$ Da number obtained by electrospray ionization mass spectrometry on several preparations of purified NafY (data not shown). Importantly, the analysis of the equilibrium centrifugation experiments showed no significant levels of NafY dimer formation in any of the three different concentrations of NafY tested (K_{eq} for the dimer/monomer pair = 0.042 ± 0.008). These results clearly indicate that the unassociated form of NafY is a monomer.

We were not able to determine the molecular mass of the FeMo-co-bound form of NafY by equilibrium centrifugation because of technical problems with the O₂ lability of FeMo-co. Therefore, for this analysis we performed gel filtration chromatography on Sephacryl S-200, Superose 6, or Superdex 200 FPLC columns and followed the elution profile of FeMo-co by measuring the absorbance at 436 nm. The NafY-FeMo-co complex is easily generated by mixing pure NafY and FeMo-co in anoxic buffer immediately before the chromatography and is stable during the gel filtration. Surprisingly, the 436-nm signal was detected as a 46-kDa species (Fig. 2B). This 46-kDa species corresponds to NafY-bound FeMo-co as demonstrated by the coelution of NafY and FeMo-co at the same fraction (shown by SDS-PAGE analysis in the inset of Fig. 2B). We also subjected the unassociated form of NafY to gel filtration chromatography under the same conditions and followed the elution profile by absorbance at 280 nm (Fig. 2B). Unassociated NafY behaves as a 44-kDa species. Therefore, both NafY and the NafY-FeMo-co complex exhibit the same retention volume, indicating that NafY does not change its association state upon FeMo-co binding.

The gel filtration results presented here differ from those reported previously. One explanation would be that the form of NafY present in crude extracts of *A. vinelandii* (studied in Ref. 13) behaves differently from the NafY purified from *E. coli* cells (studied here). However, this is unlikely because both proteins

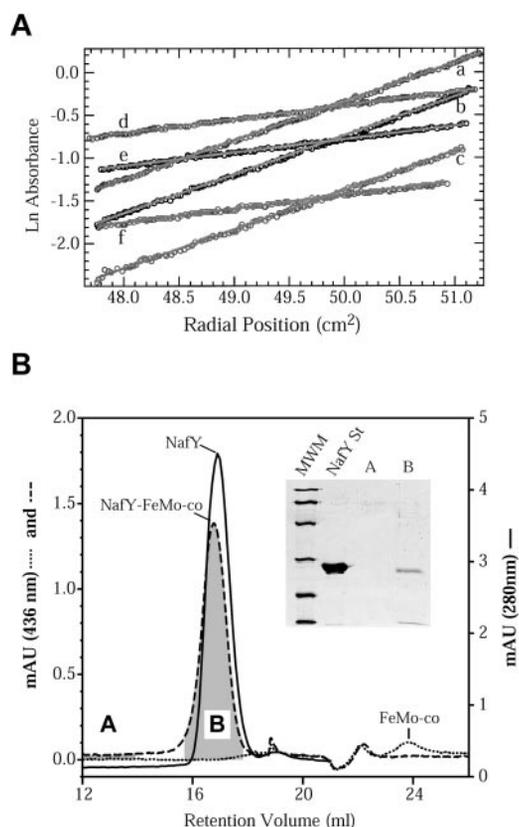


FIG. 2. Molecular mass determination of NafY and the NafY-FeMo-co complex. A, equilibrium radial absorbance profiles (circles) and linear fits for analytical ultracentrifugation measured at 10,000 rpm (curves d–f) and 17,300 rpm (curves a–c). NafY concentrations were 0.25 mg/ml (curves c and f), 0.5 mg/ml (curves b and e), and 0.75 mg/ml (curves a and d). B, gel filtration analysis of NafY and the NafY-FeMo-co complex. A Superose 6 FPLC column was used. The elution profile of NafY (solid line) was determined by absorbance at 280 nm. The elution profiles of unbound FeMo-co (dotted line) and NafY-bound FeMo-co (dashed line) were determined by absorbance at 436 nm. Aliquots from fractions A and B were subjected to SDS-PAGE to screen for the presence of NafY. MWM, Bio-Rad low-range molecular mass markers; NafY St, pure NafY loaded as a size standard. The same results were obtained when using a Superdex 200 or Sephacryl S-200 column.

have identical migration patterns on anoxic native gel electrophoresis (data not shown). Our gel filtration and equilibrium centrifugation results also differ from each other. Although NafY behaves almost as a dimer in gel filtration, it clearly behaves as a monomer during equilibrium centrifugation. Equilibrium centrifugation is far less sensitive than gel filtration to errors because of anomalies in protein shape, and the simplest explanation is that the size of NafY is overestimated when analyzed by gel filtration chromatography because the shape of NafY would be very different from spherical. In any event, the results presented here suggest that: 1) NafY is a monomeric 26-kDa protein and 2) FeMo-co binding to NafY does not alter this monomeric state.

Purified NafY Is Able to Bind FeMo-co—The capability of purified NafY to bind FeMo-co was assayed by means of anoxic native gel electrophoresis. It has previously been shown that the FeMo-co-bound form of NafY migrates faster than the unassociated NafY in native gel electrophoresis of crude extracts of *A. vinelandii* (13). Here we show that purified NafY also shifts to a more quickly migrating species in native gels upon FeMo-co binding (compare lanes 1 and 2 in Fig. 3A). The presence of FeMo-co in the more quickly migrating species is supported by iron staining of the gel, where only the more quickly migrating species gives a positive iron stain (Fig. 3B,

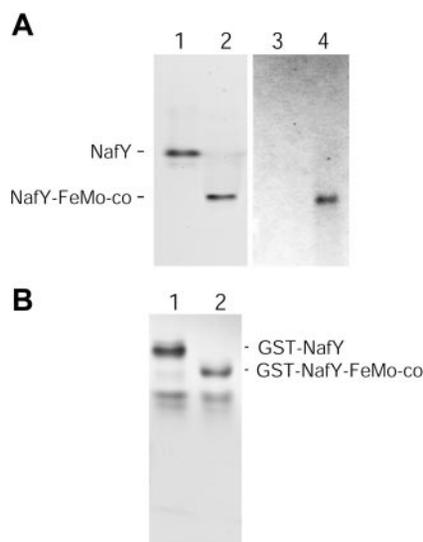


FIG. 3. Gel shift analysis demonstrating binding of purified NafY to FeMo-co. The samples were prepared in anaerobic 9-ml serum vials and contained 10 μg of NafY (or GST-NafY) in 150 μl of anaerobic buffer (25 mM Tris, 10% glycerol, pH 7.5). When indicated NafY was incubated with 1.25 nmol of purified FeMo-co for 5 min on ice to allow the binding of FeMo-co to NafY (A, lanes 2 and 4; B, lane 2). 50- μl samples were subsequently electrophoresed for 20 h in an anoxic native gel. A, anoxic native gel illustrating the shift in NafY mobility upon FeMo-co binding. Lanes 1 and 2 were stained with Coomassie, and lanes 3 and 4 were stained for iron. B, anoxic native gel stained with Coomassie, illustrating the shift in GST-NafY mobility upon FeMo-co binding.

lane 2). Because the oligomerization state of NafY does not change upon FeMo-co binding, the migration shift of NafY in native gels suggests that the protein is either more compact in the presence of FeMo-co or that the net charge on the protein has changed. In addition, NafY mobility shift does not require the presence of any additional protein; therefore NafY alone is sufficient for FeMo-co binding. The GST-NafY fusion protein is also capable of binding FeMo-co and shifting to a more quickly migrating species in anoxic native gel electrophoresis (compare lanes 1 and 2 in Fig. 3B). It seems that the GST moiety of the GST-NafY fusion protein does not obstruct the FeMo-co-binding site present in NafY.

We have also performed EPR analysis of the NafY-FeMo-co complex. The EPR spectrum of NafY-bound FeMo-co at 10 K retains the characteristic $S = 3/2$ signal ($g = 4.42, 3.70,$ and 1.98) of the dithionite-reduced M center of dinitrogenase and *N*-methylformamide-extracted free FeMo-co (Fig. 4). Although the EPR spectral components of NafY-FeMo-co are broader than the dinitrogenase M center, the overall spectrum is better defined than free FeMo-co; the sharpening of the spectrum upon complexation of FeMo-co to NafY is similar to the observed effect of thiophenol addition to free FeMo-co in *N*-methylformamide (26). The sharp feature at $g = 2.03$ in the NafY-FeMo-co spectrum is observed in variable amounts in different samples and is not present in the NafY spectrum (data not shown); therefore, it may arise from protic or oxidic decomposition of FeMo-co. The small feature at $g = 6.0$ arises from an electronic excited state also present in the spectra of the M center and free FeMo-co (27). The EPR spectrum of NafY-FeMo-co establishes that no fundamental alteration of the cofactor occurs upon binding to NafY. It is proposed that the sharpness of the $S = 3/2$ signal from the M center is due to the coordination environment of FeMo-co in dinitrogenase. The broadness of the signal shown in Fig. 4 (curve b) implies that there is structural flexibility for FeMo-co within the complex.

NafY Binds One Molecule of FeMo-co with High Affinity—To

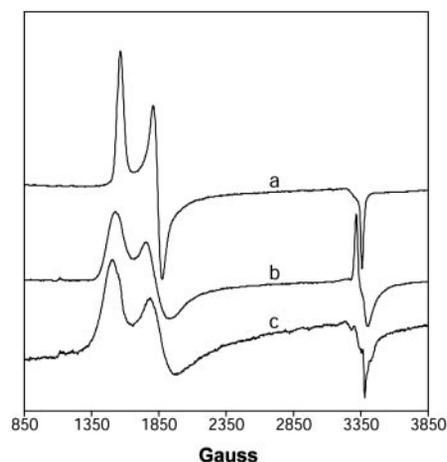


FIG. 4. EPR analysis of the NafY-FeMo-co complex. The complex was generated and concentrated as described under "Experimental Procedures." EPR of the NafY-FeMo-co complex (curve b) was recorded at a microwave frequency of 9.44 GHz, a power of 10 mW, a temperature of 10 K, and a modulation amplitude of 10 mT. The EPR spectra of pure dinitrogenase (curve a) and FeMo-co extracted in *N*-methylformamide (curve c) were obtained under the same conditions and included for comparison purposes.

calculate the dissociation constant of the NafY-FeMo-co complex, we needed to know the number of FeMo-co-binding sites in NafY. To address this question, the following experiment was performed. First, pure NafY was incubated with saturating amounts of FeMo-co to generate a NafY-FeMo-co complex; second, unbound FeMo-co was separated from the NafY-FeMo-co complex by anaerobic gel filtration in a Superdex 200 FPLC column; and third, protein, iron, and molybdenum contents of the fraction containing NafY (and bound FeMo-co) were determined, and their molar ratios were calculated. The result presented in Table I, where the protein:molybdenum:iron molar ratio obtained is close to 1:1:7, strongly suggests that NafY has one FeMo-co-binding site per monomer. In a parallel experiment with no FeMo-co added, the as-purified NafY shows no significant levels of iron and molybdenum.

The fluorescence spectrum of NafY shows an excitation maximum at 283 nm and an emission maximum at 354 nm, indicating that the primary fluorophores are solvent-exposed tryptophanyl residues. The addition of FeMo-co to NafY partially quenched the fluorescence emission of the protein (Fig. 5A). The addition of saturating amounts of FeMo-co to NafY quenches up to 80% of the total fluorescence emission at 354 nm (represented in Fig. 5A by curve c obtained after addition of 4 nmol of FeMo-co to 1.5 nmol of NafY). Fig. 5B shows the titration of NafY fluorescence quenching by FeMo-co after being corrected for nonspecific quenching (see below). The quenching follows a hyperbolic response, and these data were used to determine a K_d of 62 nM for the NafY-FeMo-co complex. For this analysis, we used an equation (21) that takes into account the existence of one FeMo-co-binding site/NafY molecule and a situation of ligand depletion because a very important fraction of the total FeMo-co present in the assay is in the NafY-bound form. We performed parallel control reactions in which tryptophan or ovalbumin was substituting for NafY to subtract the effect of nonspecific quenching from the total quenching observed. The nonspecific quenching is probably due to interfering substances present in our preparations of FeMo-co (such as sodium dithionite) and correlates linearly to the FeMo-co added. A similar nonspecific fluorescence quenching is obtained when O_2 -denatured FeMo-co is added to NafY, suggesting that only intact FeMo-co binds to NafY (data not shown).

In conclusion, these experiments demonstrate that the as-

TABLE I
Metal contents of as-purified NafY and the *in vitro* generated NafY-FeMo-co complex

Protein sample	Molybdenum	Iron
	<i>mol/mol NafY</i>	<i>mol/mol NafY</i>
NafY	ND ^a	ND
NafY-FeMo-co complex ^b	1.2 ± 0.1	7.3 ± 1.3

^a ND, there were not detectable metal levels above the blank.

^b 10 nmol of FeMo-co were added to 3 nmol of NafY in 1.35 ml of anaerobic Tris-HCl buffer and incubated for 5 min at room temperature. The mixture was subjected to gel filtration chromatography to remove unbound FeMo-co, and the NafY-FeMo-co complex was collected and subjected to protein and metal analyses. The values are the averages of two assays performed separately.

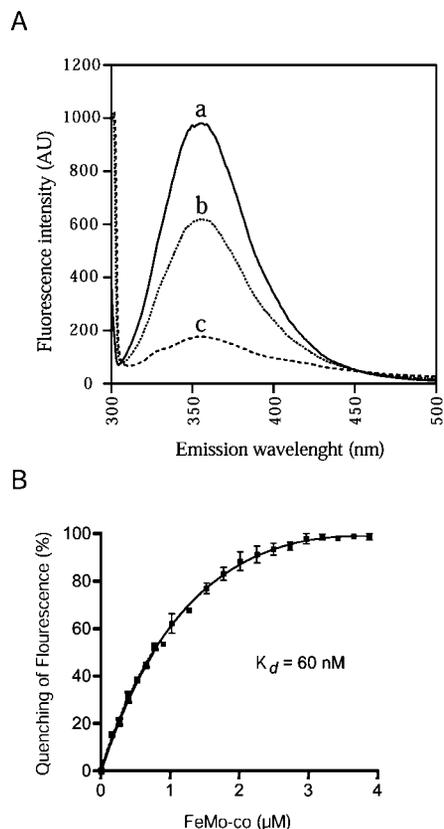


FIG. 5. Quenching of the intrinsic fluorescence of NafY upon FeMo-co binding. A, fluorescence emission spectra of NafY in the absence and the presence of FeMo-co. The reaction mixtures (1 ml of anoxic PBS, 10% glycerol) include: 1.5 nmol of NafY (curve a), 1.5 nmol of NafY plus 1 nmol FeMo-co (curve b), and 1.5 nmol of NafY plus 4 nmol FeMo-co (curve c). The instrument settings were: $\lambda_{\text{ex}} = 295 \text{ nm}$; excitation slit, 2.5 nm, $\lambda_{\text{em}} = 300\text{--}500 \text{ nm}$; and emission slit, 7.5 nm. Fluorescence intensity is given in arbitrary units. B, titration of the NafY fluorescence quenching with FeMo-co. The reaction mixtures (1 ml of anoxic PBS, 10% glycerol) include 1.5 nmol of purified NafY and increasing amounts of isolated FeMo-co. The K_d of the NafY-FeMo-co complex was estimated using the equation developed by (21). The instrument settings were: $\lambda_{\text{ex}} = 295 \text{ nm}$; excitation slit, 2.5 nm; $\lambda_{\text{em}} = 354 \text{ nm}$; and emission slit, 7.5 nm.

purified NafY protein retains the capability of binding FeMo-co with high affinity ($K_d = 62 \text{ nM}$). FeMo-co is labile in an aqueous environment, even if it is kept strictly anaerobic (19). Because of this lability, it is thought to exist in protein-bound forms inside the cells. In extracts of *A. vinelandii*, FeMo-co accumulates into mature dinitrogenase or in NafY when dinitrogenase is absent. The high affinity of NafY for FeMo-co determined here is therefore not surprising. If the physiological concentration of FeMo-co in the cell lies into the nM range tested here, our results would be consistent with the idea of NafY working as a reservoir that stabilizes FeMo-co before insertion into apodini-

trogenase. That being the case, one could expect little accumulation of FeMo-co in a mutant strain lacking both NafY and dinitrogenase. This hypothesis is currently under study.

The His¹²¹ Residue of NafY Is Involved in FeMo-co Binding—The α subunit of dinitrogenase binds FeMo-co covalently through the side chains of residues Cys²⁷⁵ (to the distal iron atom) and His⁴⁴² (to the molybdenum atom). Although NafY and dinitrogenase have different foldings for FeMo-co binding, Cys and His residues on NafY are still good candidates to coordinate the cofactor. NafY has three cysteine residues (Cys¹²⁵, Cys¹⁶⁶, and Cys¹⁹⁶) and three histidine residues (His³⁵, His¹²¹, and His¹⁸⁸). We have substituted for those Cys or His residues in NafY that are conserved (or lie in a conserved region) among NafY homologs (Fig. 6). H121L, C125A, C166A, C166S, and a double C125A,H188L variant of NafY have been generated. It is interesting to note that all the selected residues lie within the core domain of NafY, which has been shown to be sufficient for FeMo-co binding (16). The NafY variants have been overexpressed and purified from *E. coli* cells following the same protocol used for the wild type. The purity of all NafY-variant preparations was >95% as estimated by SDS-PAGE (Fig. 7).

The effect of these changes on FeMo-co binding by NafY was assayed by means of anoxic native gel electrophoresis. Each purified NafY variant was incubated with FeMo-co anaerobically for 5 min on ice, and the reaction mix was subjected to electrophoresis. The positions on the native gels of the different unassociated NafY variants (control reactions with no FeMo-co added to NafY) and their corresponding NafY-FeMo-co complexes were visualized by Coomassie staining (Fig. 8). The C125A variant and the C125A,H188L double variant of NafY show the same migration pattern as the wild-type NafY. These mutations have no apparent effect on FeMo-co binding. On the other hand, the H121L variant is clearly affected in its capability to bind FeMo-co (compare *second* and *fourth* lanes). Residue His¹²¹ is located on the surface of the core domain of NafY (16) and is conserved among NafY homologs with the exception of *A. vinelandii* NafY (Fig. 6). Our results suggest that His¹²¹ of NafY is involved in the coordination of FeMo-co. The analysis of the mutations in residue Cys¹⁶⁶ is more complex because they have a partial inability to bind FeMo-co. The substitution of Cys by Ser in position 166 has a more profound effect than the substitution of Cys by Ala, which is difficult to understand if the side chain of Cys¹⁶⁶ was coordinating the cofactor. Moreover, residue Cys¹⁶⁶ is not solvent-exposed but is somewhat buried inside the core domain of NafY (16), and thus it is unlikely to be a direct ligand to FeMo-co. It is not clear whether the substitutions at position 166 are affecting FeMo-co binding directly or indirectly because of a change on the overall folding of NafY.

All NafY variants were tested for apodinitrogenase binding as well. The migration pattern of the variants was identical to that of the wild-type NafY (see below), and it is concluded that none of these mutations affect the binding of NafY to apodinitrogenase (Fig. 9C). This result is not surprising considering that all of these changed residues are within the core domain of NafY, which does not bind to apodinitrogenase in the absence of the first domain. In conclusion, our site-directed mutagenesis data, in combination with the structural data obtained previously (16), suggest that residue His¹²¹ of NafY is involved in the coordination of FeMo-co.

Purified NafY and GST-NafY Proteins Are Able to Bind to $\alpha_2\beta_2$ Apodinitrogenase from the Mutant Strain UW146 (nifB-nafY)—It is well known that in cell extracts of *A. vinelandii* NafY (γ) not only binds to FeMo-co but also binds to apodinitrogenase. In fact, cell extracts of the FeMo-co-deficient strain

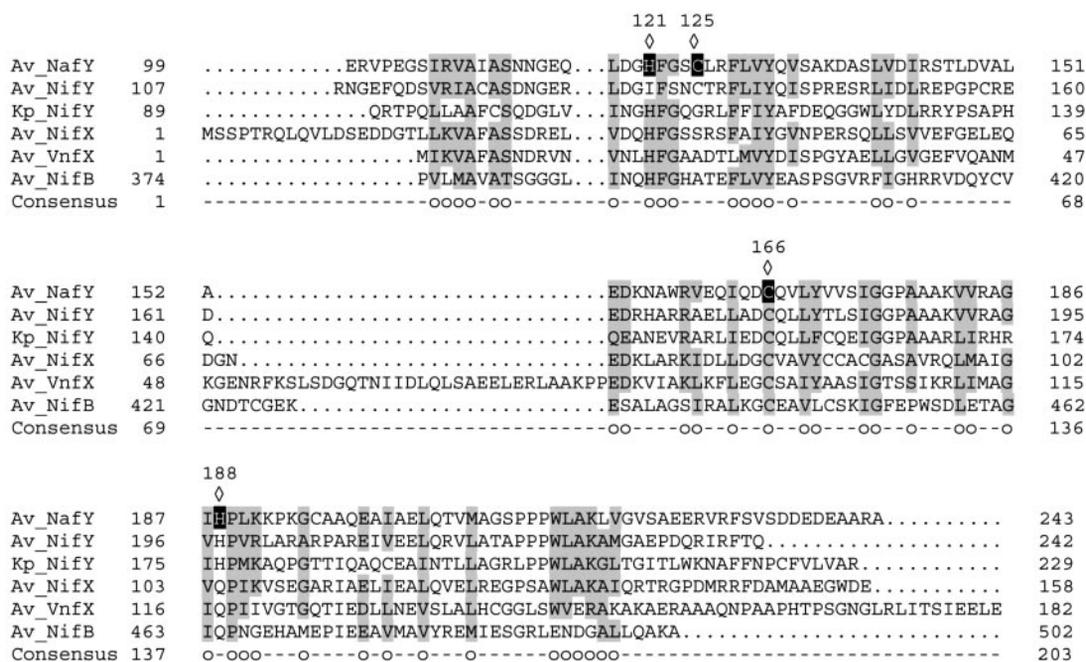


FIG. 6. Amino acid sequence alignment of members of the NafY family of proteins illustrating the position of the mutated His and Cys residues within NafY. The entire sequence is shown for Av_NifX and Av_VnfX. Alignments of the conserved core domain (16) are shown for Av_NafY, Av_NifY, Kp_NifY, and Av_NifB. The amino acid sequences are indicated by the single-letter code. Gaps were introduced for optimal alignments. The diamonds point to amino acid residues changed by site-directed mutagenesis. The circles indicate amino acid residues identical or similar in five or more of the aligned proteins. Av, *A. vinelandii*; Kp, *K. pneumoniae*.

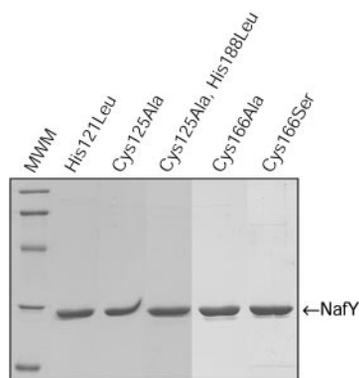


FIG. 7. SDS-PAGE analysis of purified mutant NafY variants. Samples of NafY mutant variants purified from recombinant *E. coli* cells (see "Experimental Procedures" for details) were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. The molecular mass standards (MWM) are: 97, 66, 45, 31, and 21 kDa.

UW45 (*nifB*) present a stable hexameric apodinitrogenase with an $\alpha_2\beta_2\gamma_2$ composition (8). We wanted to know whether our preparations of purified NafY retain this capability. Purified NafY was added to the cell-free extract of the FeMo-co-deficient, NafY-deficient *A. vinelandii* UW146 strain (*nifBnafY*) and incubated for 5 min on ice. The mix was electrophoresed on an anoxic native gel, and proteins were transferred to a nitrocellulose membrane where an immunoblot was developed with antibodies to NafY (Fig. 9A). As expected, there was no reacting material in extracts of mutant UW146 (data not shown). When purified NafY was added to the extract of UW146, NafY shifted from its original nonassociated position to the apodinitrogenase-associated migrating position in the anoxic native gel. This is interpreted as a stable binding between NafY and apodinitrogenase. Interestingly, the GST-NafY fusion protein was also capable of binding apodinitrogenase when added to the extract of UW146, and this property has been used to purify $\alpha_2\beta_2$ apodinitrogenase from UW146 cells.² This demonstrates that both NafY and the GST-NafY fusion protein can be ex-

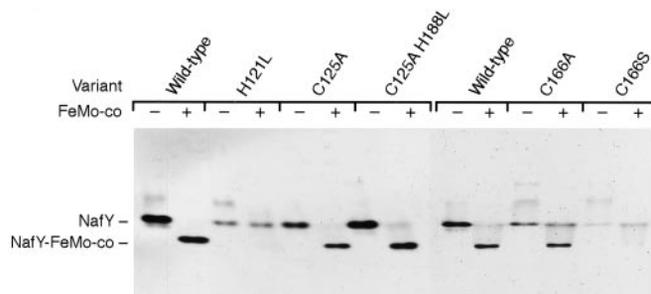


FIG. 8. Gel shift analysis of the interaction of purified NafY variants with FeMo-co. The samples were prepared in anaerobic 9-ml serum vials and contained 10 μ g of the purified NafY variant in 150 μ l of anaerobic buffer (25 mM Tris, 10% glycerol, pH 7.5). When indicated NafY was incubated with 1.25 nmol of purified FeMo-co for 5 min on ice to allow the binding of FeMo-co to NafY. 50- μ l samples were subsequently electrophoresed for 20 h in an anoxic native gel that was finally stained with Coomassie Brilliant Blue.

pressed and purified from *E. coli* cells and retain the capability to bind apodinitrogenase. Moreover, the interaction of NafY with $\alpha_2\beta_2$ apodinitrogenase does not require the presence of any additional protein, as illustrated by the comigration of both proteins when a sample containing only pure $\alpha_2\beta_2$ apodinitrogenase and NafY is subjected to anoxic native gel electrophoresis (Fig. 9B).

Interaction of NafY with NifB-co—NifB-co, the product of NifB, is an iron-sulfur cluster of unidentified structure that serves as the specific precursor for the biosynthesis of FeMo-co of Mo-nitrogenase, FeV-co of V-nitrogenase, and FeFe-co of Fe-only-nitrogenase (20, 28, 29). It has been previously shown that NifB-co binds to proteins involved in the biosynthesis of FeMo-co, such as NifX (30) and NifNE (31), or the biosynthesis of FeV-co, such as VnfX (32). Because NafY, NifX, and VnfX are homologs (10) and are proposed to have similar structures, the prediction is that NafY would also be able to bind NifB-co. However, no iron or sulfur has been found associated to NafY in the form of NifB-co or other intermediates when studied by anoxic native gel electrophoresis. Labeled iron and sulfur from

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