Characterization of the γ Protein and Its Involvement in the Metallocluster Assembly and Maturation of Dinitrogenase from Azotobacter vinelandii*

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Dinitrogenase, the enzyme capable of catalyzing the reduction of N2, is a heterotetramer (α2β2) and contains the iron-molybdenum cofactor (FeMo-co) at the active site of the enzyme. Mutant strains unable to synthesize FeMo-co accumulate an apo form of dinitrogenase, which is enzymatically inactive but can be activated in vitro by the addition of purified FeMo-co. Apodinitrogenase from certain mutant strains of Azotobacter vinelandii has a subunit composition of αβ2γ2. The γ subunit has been implicated as necessary for the efficient activation of apodinitrogenase in vitro.

Characterization of γ protein in crude extracts and partially pure fractions has suggested that it is a chaperone-insertase required by apodinitrogenase for the insertion of FeMo-co. There are three major forms of γ protein detectable by Western analysis of native gels. An apodinitrogenase-associated form is found in extracts of nifB or nifNE strains and dissociates from the apocomplex upon addition of purified FeMo-co. A second form of γ protein is unassociated with other proteins and exists as a homodimer. Both of these forms of γ protein can be converted to a third form by the addition of purified FeMo-co. This conversion requires the addition of active FeMo-co and correlates with the incorporation of iron into γ protein. Crude extracts that contain this form of γ protein are capable of donating FeMo-co to apodinitrogenase, thereby activating the apodinitrogenase.

These data support a model in which γ protein is able to interact with both FeMo-co and apodinitrogenase, facilitate FeMo-co insertion into apodinitrogenase, and then dissociate from the activated dinitrogenase complex.

Nitrogenase is comprised of two components: dinitrogenase (also known as component I or the MoFe protein) and dinitrogenase reductase (also known as component II, NifH, or the Fe protein). Nitrogenase catalyzes the ATP- and reductant-dependent reduction of N2 and other substrates. Dinitrogenase is a 240-kDa αβ2 tetramer encoded by nifD (1). Dinitrogenase contains two types of metal centers: the P-cluster (8Fe-8S) (2) and the FeMo-co site of the enzyme. Mutant strains unable to synthesize FeMo-co accumulate an apo form of dinitrogenase, and then dissociate from the activated dinitrogenase (reviewed in Ref. 1).

In vivo biosynthesis of FeMo-co depends on the activities of several nif genes, including nifQ, -B, -V, -N, -E, and -H. Mutations in some of these genes result in strains that are unable to fix N2 and accumulate an apo form of dinitrogenase lacking the active site (reviewed in Ref. 1). When isolated from nifB or nifNE strains of either Klebsiella pneumoniae or Azotobacter vinelandii, apodinitrogenase has an additional associated subunit (7, 8). This complex has an αβ2γ2 subunit composition. In K. pneumoniae, the third subunit is encoded by nifY (8, 9), and the addition of purified FeMo-co to pure apodinitrogenase is sufficient to yield catalytically active dinitrogenase (7, 8). Addition of FeMo-co effects the dissociation of NifY from the Apo-I complex upon formation of the holoenzyme (8). In A. vinelandii, the identity of the gene encoding the third subunit, termed γ, is not known (7) although it is known not to be the product of the gene designated nifY in A. vinelandii (10). Further, the third subunits from the K. pneumoniae and A. vinelandii share no antigenicity and have shown different dissociation requirements in vitro (7, 8).

Apodinitrogenase from a nifH strain is biochemically distinct from the hexameric apodinitrogenase forms described above (11), requiring dinitrogenase reductase and ATP for in vitro activation by FeMo-co (12, 13). Further analysis revealed that γ protein was absent from the apodinitrogenase complex in crude extracts of nifH strains and that the presence of dinitrogenase reductase and ATP promoted association of the γ subunit, forming the hexameric apodinitrogenase similar to that found in the nifB and nifNE strains (13).

An implication of this result is that γ protein is essential for the proper in vitro activation of apodinitrogenase by FeMo-co. In this work, we have further characterized γ protein using both in vivo and in vitro techniques. The results suggest a role for γ protein in which it interacts with both apodinitrogenase and FeMo-co; it holds the apodinitrogenase in a conformation that allows access to the FeMo-co ligand site, and it is also capable of binding FeMo-co and subsequently donating it to apodinitrogenase.

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† The abbreviations used are: FeMo-co, iron-molybdenum cofactor; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.
Because γ protein performs both of these functions, we have chosen to describe it as a chaperone-insertase.

**EXPERIMENTAL PROCEDURES**

**Strains**—A. vinelandii and K. pneumoniae strains used are described in Table I. Growth, derepression of the strains for nif proteins, and preparation of cell-free extracts have been described previously (17). Strain DJ 1030 contains a deletion/insertion mutation within the nifB gene and a deletion in the nifH gene. It was constructed by transforming A. vinelandii strain DJ 54, which contains a deletion within nifH (16), with pDB218 DNA followed by selection for kanamycin-resistant transformants and scoring these for ampicillin sensitivity; mutants were further verified as described earlier (10). Plasmid pDB218 was constructed by deleting an approximately 700-base pair SphI restriction enzyme fragment internal to the nifB coding sequence (15) from a plasmid, containing a 1.7-kilobase pair SalI fragment with most of nifAB, and replacing it with an SphI restriction enzyme fragment that contains a gene cartridge encoding the kanamycin-resistance gene from pUC-K135 (Pharmacia Biotech Inc.).

**Materials/Chemicals**—Sodium dithionite was from Fluka Chemicals. Leupeptin, phenylmethylsulfonyl fluoride, Trizma base, MOPS, phosphocreatine, creatine phosphokinase, all nucleotides, and Reactive Red 120 resin were from Sigma. Hydroxyapatite gel, ammonium persulfate, and acrylamide/bis solution were from Bio-Rad. Phosphocreatine, creatine phosphokinase, all nucleotides, and Reactive Red 120 resin were from Sigma. Hydroxyapatite gel, ammonium persulfate, and acrylamide/bis solution were from Bio-Rad. M-Nephylformamide and 3,5-diaminobenzoic acid were from Aldrich. Immobilon-P membrane was from Millipore. Q-Sepharose and the Sephacryl S-200 column, and fractions were collected and assayed. Fractions were analyzed for proteins by Western blots of anoxic native gels and of SDS-PAGE to identify the gamma-containing fractions and to ensure that γ protein was present in the expected form (described under “Results”). All fractions were collected and analyzed for FeMo-co activity by adding the fractions to crude extract from A. vinelandii strain UW45 (nifB) or DJ 1030 (nifBnifH). Two peaks of activity were detected: one eluted with the fraction containing γ protein and represents protein-bound FeMo-co, and the other eluted in the included volume, presumably as unbound FeMo-co. Dinitrogenase reductase (200 μg) and MgATP (1.5 mM final concentration) were added to the fractions being assayed to promote association of γ protein with apodinitrogenase. Activated apodinitrogenase was assayed for acetylene reduction as described (7). As a control FeMo-co alone was also applied to a Sephacryl S-200 column, and fractions were collected and assayed for FeMo-co; only one peak was detected at the included volume, similar in position to the peak of unbound FeMo-co.

**Partial Purification of Apodinitrogenase and γ Protein**—All buffers contained 0.025 M MOPS, pH 7.4, 20% glycerol, 1.7 mM sodium dithionite, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM leupeptin unless otherwise indicated. Partially purified fractions containing apodinitrogenase and unassociated γ protein were obtained as described (7) through the hydroxyapatite column step. FeMo-co-activatable fractions were then applied to a Q-Sepharose column. Protein fractions were eluted with a 0.1-0.6 M NaCl gradient, with the apodinitrogenase/γ protein fraction eluting at approximately 0.25 M NaCl. Apodinitrogenase and γ protein constitute approximately 30% of the protein in the sample as based on a densitometry scan of a Coomassie-stained gel.

**Iron Stain**—Native gels were stained for iron as described previously (24).

**Molecular Weight Determination**—Protein fractions were analyzed by Sephacryl S-200 gel exclusion chromatography. Column fractions were collected in an anaerobic chamber. Column fractions were precipitated with trichloroacetic acid and run on SDS-PAGE for further analysis. SDS-PAGE followed by Western blotting with antibody to γ protein were also performed on all the fractions.

**RESULTS AND DISCUSSION**

Several Electrophoretically Distinct Species of γ Protein Are Accumulated in Vivo—To clarify subsequent presentation of data, three species of γ protein will be defined in this section. These species are electrophoretically distinct on anoxic native gels and differ in at least one of two properties: association with other proteins or FeMo-co (Fig. 1, lanes 1-3). The B form will be shown to be associated with apodinitrogenase, either purified or in crude extracts (Fig. 1, lane 1). The B form will be shown to occur in all strains and under all growth conditions examined thus far. The C form of γ protein will be shown to be

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**Table I**

| Strain Number | Genotype | Reference |
|---------------|----------|-----------|
| UW 14        | Wild type       | (14)     |
| UW45 15      | nifB45         | (15)     |
| DJ 33 10     | ΔnifDK        | (10)     |
| DJ 40 10     | Δnif           | (10)     |
| DJ 54 16     | ΔnifH          | (16)     |
| DJ 1030 17   | ΔnifH ΔnifB   | This paper |
Fig. 2. Dissociation of γ protein from apodinitrogenase. Western blots of identical anoxic native gels. Panel A was developed with antibody to γ protein, and panel B was developed with antibody to dinitrogenase. Arrows denote the forms of γ protein in panel A and apodinitrogenase (a) and holodinitrogenase (h) in panel B. Lane 1, extract from nif-derepressed UW (wild type) (−150 ng); lane 2, extract from UW45 (nifB) (~150 ng); lane 3, extract from UW45 (nifB) + 5 µl FeMo-co; lane 4, partially purified apodinitrogenase; protein (~0.75 ng); lane 5, partially purified apodinitrogenase; protein + 5 µl FeMo-co; lane 6, partially purified apodinitrogenase; protein + 5 µl FeMo-co + extract from ammonium-grown UW (wild type) (~50 ng).

A monomer that is associated with FeMo-co (Fig. 1, lane 3). It is visible in samples containing either the A form or the B form of γ protein that have been incubated with purified FeMo-co.

Requirements for the Dissociation of γ Protein from Apodinitrogenase—In K. pneumoniae, the dissociation of NiFe from the apodinitrogenase hexamer is effected by the addition of FeMo-co regardless of the purity of the apodinitrogenase (8). In A. vinelandii, however, dissociation of γ protein was not observed when FeMo-co was added to highly purified apodinitrogenase hexamer (7). In an effort to reconcile and explain these apparent differences in the in vitro requirements for dissociation, we examined A. vinelandii apodinitrogenase in various stages of purification using Western blots of native gels. Fig. 2 shows Western blots of an anoxic native gel, one half (panel A) developed with antibody to γ protein and the other (panel B) developed with antibody to dinitrogenase.

To study the dissociation of γ protein from apodinitrogenase in crude extract from A. vinelandii, crude extract from UW45 (nifB) was used. It contains apodinitrogenase (Fig. 2B, lane 2) and both the A form (apodinitrogenase-associated) and the B form (unassociated) of γ protein (Fig. 2A, lane 2). The faint band detected above the B form and below the A form is thought to be a proteolytic product due to high protease contamination in the DNase added during cell breakage (data not shown) and the faint band detected immediately below the B form is unidentified and does not appear reproducibly. The addition of FeMo-co completely activated the apodinitrogenase, as demonstrated by an acetylene reduction assay (Table I, lines 1 and 2), and caused the dinitrogenase to migrate at the position of holodinitrogenase (Fig. 2B, lane 3), both indicating that the apodinitrogenase had been activated to the holoform. There was no longer any γ protein associated with nitrogenase (Fig. 2A, lane 3) indicating that, at least in crude extract, activation of apodinitrogenase by FeMo-co was sufficient to cause dissociation of γ protein.

Purified apodinitrogenase from UW45 (nifB) is associated with γ protein (Fig. 3, lane 1), and in contrast to the above result, addition of a saturating amount of FeMo-co (as defined by apodinitrogenase activation measured by an acetylene reduction assay) promoted incomplete dissociation of γ protein (Fig. 3, lane 2). For unknown reasons, when twice as much FeMo-co was added to an identical sample, nearly all of the γ protein dissociated (Fig. 3, lane 3). The observation that a saturating amount of FeMo-co (saturating for activation of apodinitrogenase as determined by acetylene reduction) causes less dissociation of γ protein from the purified apodinitrogenase than in crude extracts suggests the involvement of other proteins or factors in this process in crude extract. A partially purified fraction containing apodinitrogenase and γ protein (see “Experimental Procedures”) from UW45 (nifB) was examined and showed dissociation properties similar to that of the highly purified sample above. There are two major species of γ protein in the partially purified sample: the A form and the B form (Fig. 2A and B, lane 4). The band detected immediately below the A form is the proteolyzed form of γ protein associated with apodinitrogenase (data not shown). When saturating levels of FeMo-co (as defined by apodinitrogenase activation measured by an acetylene reduction assay) were added to this sample, the apodinitrogenase was activated (Table II, lines 3 and 4) and showed an appropriate shift in gel migration (Fig. 2B, lane 5), but not all of the γ protein dissociated (Fig. 2A, lane 5). This distinction between the activation of apodinitrogenase and the dissociation of γ protein is consistent with previous results (7) where the purified apodinitrogenase could be activated without dissociation of γ protein.

To determine if some factor is missing from the partially purified apodinitrogenase fraction that might assist in dissociation of γ protein, crude extracts from various strains were added to this preparation. Addition of crude extract from ammonium-grown UW (wild type, Fig. 2A, lane 6) or from DJ 40 (a deletion of most of the main cluster of nif genes, data not shown) was sufficient to promote complete dissociation of γ protein from apodinitrogenase upon activation with the same FeMo-co levels without affecting dinitrogenase activity (Table II, line 5), suggesting that another protein may be involved in dissociation. The presence of the putative dissociation factor in these particular extracts suggests it is neither nif-encoded nor nif-co-regulated. Further, the enhancement of dissociation of γ protein from apodinitrogenase was probably due to a specific protein in A. vinelandii, since the addition of either bovine serum albumin or crude extract from K. pneumoniae nif deletion strain (UN1978, Ref. 25) showed no effect on activity or dissociation (data not shown). Appropriate solvent controls for FeMo-co addition showed that addition of N-methylformamide alone had no effect on the dissociation of γ protein (data not shown). It seems likely that the putative “dissociation factor” is a specific A. vinelandii protein that increases the efficiency of the dissociation process but is not required for activation.

Accumulation of the C Form of γ Protein (FeMo-co-bound) Is Dependent upon FeMo-co Addition—As noted above, there are different forms of γ protein that are not associated with apodinitrogenase. While the addition of FeMo-co promotes the dissociation of the A form of γ protein from the apodinitrogenase complex, it also causes a mobility shift of the B form; both forms now migrate as the C form (Fig. 2A, lane 3). The faint band just above the C form is unidentified but does not appear reproducibly. As shown below, form C of γ protein is probably bound to FeMo-co.

B and C Forms of γ Protein Are Unassociated with Other Proteins—To determine whether the B and C forms represent a complex of γ protein with other proteins or distinct forms of γ protein itself, the partially purified apodinitrogenase/γ protein fraction (see “Experimental Procedures”) was examined on two-dimensional native/SDS-PAGE (see “Experimental Procedures”) before and after FeMo-co addition. The first dimension allows protein complexes to remain intact, whereas the second dimension denatures them, allowing resolution of individual protein subunits. Western analysis (Fig. 4, C and D) allowed the identification of γ protein on the Coomassie-stained gels.
Characterization of γ Protein from A. vinelandii

Table II

| Source of apodinitrogenase | FeMo-co source | Other additions | Specific activity |
|---------------------------|---------------|----------------|------------------|
| 1. UW45 (nifB) extract    | None          |                | <0.001           |
| 2. UW45 extract           | 5 µl purified FeMo-co |                | 19.8             |
| 3. Partially purified apol/γ | None          |                | <0.001           |
| 4. Partially purified apol/γ | 5 µl purified FeMo-co |                | 168.0            |
| 5. Partially purified apol/γ | 5 µl purified FeMo-co | UW-NH₄-grown, extract (~100 ng) | 158.0 |

*FeMo-co was added at levels saturating (5 µl) for activation of apodinitrogenase.

All samples contained 20 ml of purified dinitrogen reductase (~400 µg) and 800 µl of ATP-mix.

Nitrogenase specific activity is expressed as nmol of ethylene produced/min/mg of sample tested.

Fig. 3. Dissociation of γ protein from purified apodinitrogenase. Western blot of anoxic native gel developed with antibody to γ protein. Lane 1, purified apodinitrogenase (~2 ng); lane 2, purified apodinitrogenase + 5 µl FeMo-co; lane 3, purified apodinitrogenase + 10 µl FeMo-co.

Fig. 4. Two-dimensional electrophoresis of partially purified apodinitrogenase/γ protein. Panels A and B are Coomassie-stained, while panels C and D are Western blots developed with antibody to γ protein. Panels A and D display samples of partially purified apodinitrogenase/γ protein (~125 ng). Panels B and D are the identical samples to which FeMo-co has been added. A, B, and C forms of γ protein are circled in the panels. The first dimension (native gel) was run left to right, and the second dimension (SDS-PAGE) was run from top to bottom as pictured.

(Fig. 4, A and B), which were then examined for the change in migration of any other proteins concomitant with the mobility change of γ protein upon FeMo-co addition.

Should either the B or C forms of γ protein form a complex with another protein, that protein should either shift with γ protein in the horizontal dimension or shift to a different position upon its dissociation from γ protein. The failure to see such a shift in the horizontal position of any other protein argues that neither the B nor C forms of γ protein form such a complex (Fig. 4, panels A and B). As demonstrated below, the B and C forms appear to be dimeric and monomeric, respectively, so that any putative associated protein would necessarily be at a 1:1 or 1:2 molar ratio and therefore be easily detected in this analysis.

Form B of γ Protein (Unassociated) Is a Dimer—To determine the molecular weight/oligomeric state of the B form of γ protein, partially pure fractions of apodinitrogenase/γ protein (see "Experimental Procedures") were analyzed by anoxic Sephacryl S-200 gel filtration chromatography (Fig. 5), and the fractions from the sizing column were then analyzed for γ protein by Western blots of SDS-PAGE and native gels. The B form reproducibly showed an elution profile that predicts a molecular weight (M_r) of ~46,000, which is twice the monomeric weight predicted from SDS-PAGE (23,000–26,000). These data indicate that the B form of γ protein is probably a homodimer.

C Form (FeMo-co-bound) of γ Protein Is a Monomer—Because the B and C forms of γ protein migrate so differently on native gels, it seemed possible that they differed in their oligomerization. Partially purified B form of γ protein was treated with FeMo-co as described under "Experimental Procedures," and Western blot analysis of anoxic native gels verified that the shift to the C form had occurred quantitatively. This sample was then analyzed on an anoxic Sephacryl S-200 column (Fig. 5, peak B) and fractions were assayed by Western blots of SDS-PAGE and anoxic native gels. The C form of γ protein elutes at a point that corresponds to a molecular weight (M_r) of approximately 23,000 and is, therefore, apparently a monomer. Analysis on native gels confirmed that γ protein remained in the C form after elution from the S-200 column.

C Form Is Bound to FeMo-co—Because the B form of γ protein shifts to the C form when FeMo-co is added, it was important to establish whether or not the effect is specific to intact FeMo-co. Fig. 6A shows that oxidized FeMo-co (Fig. 6A, lane 3), N-methylformamide (NMF, the solvent FeMo-co is solubilized in; lane 4), MoS₄ (a known inhibitor of FeMo-co insertion; lane 5), NifB-co (a metal species involved in FeMo-co synthesis; lane 6), and MoO₃²⁻ (lane 7) elicit no mobility change in γ protein, nor do they inhibit the mobility change when they are added prior to FeMo-co addition (Fig. 6, lanes 8 and 9, and data not shown). Thus, the mobility shift response of γ protein is specific to active FeMo-co. γ protein responds specifically to FeMo-co regardless of whether the γ protein is from nif-derepressed cells (Fig. 2, lanes 2 and 3) or ammonium-grown cells (Fig. 6A, lanes 1 and 2).

The shift from B form to C form strongly suggests that γ protein interacts with FeMo-co. The following experiment also demonstrates that γ protein becomes associated with iron upon FeMo-co addition. Samples of the partially purified apodinitrogenase/γ protein fraction (see "Experimental Procedures") were analyzed on a native gel with and without FeMo-co addition and then stained for iron. Fig. 7 shows that the apodinitrogenase is the only major iron-containing protein present in the sample before FeMo-co addition; form B of γ protein contains no detectable iron. Following FeMo-co addition, there is a major iron-containing band that co-migrates with the C form of γ.
Characterization of γ Protein from A. vinelandii

FeMo-co can be synthesized in vivo. It was demonstrated in Fig. 4 that no other protein in this sample responds detectably to the addition of FeMo-co (except apodinitrogenase), and it is therefore highly probable that γ protein has incorporated the iron. Negative controls utilizing either oxidized FeMo-co or NifB-co did not cause incorporation of detectable iron in the gel (data not shown). This result is consistent with the hypothesis that the electrophoretic shift of γ protein is due to its binding of FeMo-co.

Crude extract containing the C Form of γ Protein (FeMo-co-bound) Can Donate FeMo-co to Apodinitrogenase—To test whether or not the iron-containing compound bound to form C of γ protein is actually FeMo-co, the complex was tested for its ability to donate FeMo-co to apodinitrogenase as described under "Experimental Procedures." Crude extract containing the B form of γ protein was incubated with FeMo-co to convert γ protein to the C form, desalted using a Sephadex G-25 column to remove unbound FeMo-co, and then added to apodinitrogenase-containing crude extracts. Extracts from two strains that accumulate distinct apodinitrogenases of differing subunit composition were tested. The first was from DJ 1030 (nifBnifH), and the absence of dinitrogenase reductase (the nifH product) prevents the association of γ protein with apodinitrogenase (12, 13) so that only an αβαβ tetrameric form of apodinitrogenase is present (13). The other extract was from UW45 (nifB) and has the apodinitrogenase hexamer that has already been described (7). We reasoned that the relative ability of the C form to activate these two forms of apodinitrogenase could provide insight into the order of events in the insertion process and the nature of the γ-FeMo-co species.

Addition of the Sephadex G-25 eluate containing the presumed γ-FeMo-co complex to the extract from DJ 1030 (Table III, lines 4 and 7) yielded a specific activity similar to that of adding purified FeMo-co (Table III, lines 5 and 6). The Sephadex G-25 eluate that contains the C form is therefore capable of activating the tetrameric apodinitrogenase. To be sure that association of the C form of γ protein with apodinitrogenase could occur, the assays were performed in the presence of dinitrogenase reductase and nucleotides.

The Sephadex G-25 eluate containing the C form was also added to the extract from strain UW45, which contains the apodinitrogenase hexamer. The Sephadex G-25 eluate that contains the C form is capable of activating the hexameric apodinitrogenase (Table III, lines 1 and 3) to levels comparable with adding purified FeMo-co directly (Table III, line 2). The Sephadex G-25 eluates containing the C form of γ protein are therefore capable of activating the apodinitrogenase hexamer as well.

This result can be explained by either of two possibilities; either the C form donates the FeMo-co to γ protein already associated with the apodinitrogenase complex, or the C form replaces the associated γ protein in the apodinitrogenase complex. Because both forms of apodinitrogenase were activable by the Sephadex G-25 eluate containing the C form, a distinction between the two possibilities cannot be made, and this will be discussed below.

The volume of Sephadex G-25 eluate required to generate the same levels of nitrogenase activity is much greater than that necessary with purified FeMo-co because the Sephadex G-25 column dilutes the protein eluate. We cannot exclude the possibility of other proteins in the crude extracts binding and donating FeMo-co; however, the results described above suggest that this is unlikely. Purification of γ protein and subsequent activation assays with the purified protein will be essential to demonstrate specificity of the insertion process.

Although γ protein responds specifically to and most likely binds FeMo-co in vitro, it was of particular interest to see if such a FeMo-co-bound γ protein species could accumulate in vivo. FeMo-co can be synthesized in vivo in the absence of the structural components of dinitrogenase (26, 27), and it would be under these conditions that one might expect a γ-bound FeMo-co species to accumulate. When crude extracts from A. vinelandii strains UW6 (nifK) and UW10 (nifD) were examined by a Western blot of a native gel (data not shown), ~50% of the
detectable γ protein was in the C form, consistent with the idea that the FeMo-co that is synthesized in these strains accumulates on γ protein. When crude extract from DJ33 extract was examined, however, the detectable levels of FeMo-co were consistently much lower than those seen in UW6 and UW10, and none of the C form was detected; it is possible that the levels of the C form are lower than the detection limit of our Western blots of native gels. While the reason for these differences is unresolved, it may be dependent on the observation that UW6 and UW10 are capable of accumulating inactive NifKD peptides, whereas DJ33 is a complete nifKD deletion. Regardless, this data indicates that the C form of γ protein is of physiological relevance and that this response to FeMo-co does occur in vivo.

These results are reminiscent of previous observations of a protein estimated to be 65 kDa in strains with mutations in the structural genes for nitrogenase (26, 27); this protein accumulates significant amounts of molybdenum and is capable of activating apodinitrogenase-containing extracts (26). The presence of the C form in UW6 and UW10 (the two strains used in the analysis of the 65-kDa protein) is consistent with the possibility that γ protein and the 65-kDa protein are the same.

The C Form Can Be Converted to the B Form by Exposure to Air—As shown below, when the C form of γ protein was exposed to air, it was converted to the B form, and this shift appeared to happen in two steps during treatment. Crude extract from NH₄-grown strain UW (wild type) cells contained the B form of γ protein (Fig. 8, lane 1) that shifted upon FeMo-co addition (Fig. 8, lane 2). When the mixture of FeMo-co and crude extract was then exposed to air for 60 min or 30 min, all of the γ protein migrated as the B form (lanes 3 and 4). Our hypothesis is that bound FeMo-co is destroyed upon oxygen exposure and dissociates from γ protein, allowing γ protein to dimerize to the B form. Lane 6 shows that reincubation of the air-treated sample in lane 4 with FeMo-co promoted the recovery of the C form. This result suggests that binding of FeMo-co to γ protein is reversible and that γ protein itself is not damaged by the air treatment.

When the mixture of FeMo-co and crude extract was only exposed for 15 min, γ protein migrated at a position between the B and C forms (lane 5). This intermediate form might represent either the binding of a single FeMo-co to the γ dimer or a γ monomer free of any metals. When FeMo-co was titrated against the B form of γ protein, no such intermediate was observed; γ protein migrated at the B position, the C position, or a combination of both (data not shown). This result supports the latter model, where the intermediate is monomerized γ protein having no metals bound.

It is interesting to note that FeMo-co in aqueous solution is destroyed upon exposure to air in less than 1 min (4), whereas the C form of γ protein is still present after 5 min of exposure to air (data not shown). While it is not known whether the metal species remaining bound to γ protein after 5 min is still capable of activating dinitrogenase, this result suggests that γ protein might provide some degree of oxygen stability or protection to FeMo-co.

The Gene Encoding γ Protein Is Unknown but Is Not a nif Gene—When apodinitrogenase was first purified from A. vinelandii and γ protein was found to be tightly associated with the complex, the N terminus of this protein was sequenced and found to have no similarity to any known sequence in the gene banks (7). Further analysis has shown that the protein is not antigenic with its K. pneumoniae counterpart, NifY (8), and the very acidic pl of γ protein precludes its being encoded by A. vinelandii nifY (10).

In an effort to identify the gene encoding γ protein and gain some insight into its regulation, crude extracts from various strains grown under a range of conditions were examined by Western blots of native gels and SDS-PAGE to see if any of the resulting extracts lacked γ protein. γ Protein was present in similar amounts in all of the strains and under all of the conditions we examined: ammonium-grown, urea-grown, and nitrate-grown, derepressed and heat-shocked UW (wild type), and ammonium-grown and derepressed DJ40 (a deletion of the entire major nif gene cluster; Ref. 10). The similar amounts of γ protein present indicate that γ protein is not encoded by a nif gene nor is it co-regulated. The presence of γ protein under a range of physiological conditions suggests it has other roles in the cell besides maturation of dinitrogenase. It is possible, for example, that it is a processing protein for some other metal-containing proteins.

Models for the Role of γ Protein in Dinitrogenase Maturation—Our working models for the FeMo-co insertion process are illustrated in Fig. 9. The difference between the two schemes is whether or not γ protein binds FeMo-co before (scheme 1) or after (scheme 2) its association with the apodini-
trogenase complex. It is clear from the data presented in the present work that both of these possibilities might take place in vitro. For example, it is possible to activate the hexameric form of apodinitrogenase whether it accumulates in vivo (as in a nifB strain) or whether it is synthesized in vitro (following the association reaction with extract from a nifH strain). Conversely, samples containing the C form of γ protein can donate FeMo-co to the tetrameric forms present in nifH strains when supplied with dinitrogenase reductase and nucleotides to allow association of γ protein with apodinitrogenase. The ability of samples containing the C form to donate FeMo-co to the hexameric form of apodinitrogenase is somewhat puzzling but can be rationalized by arguing that 1) γ protein that has bound FeMo-co has a higher affinity for apodinitrogenase and displaces the associated γ protein already present on the apodinitrogenase or 2) γ protein that has associated with apodinitrogenase has a higher affinity for FeMo-co, and FeMo-co can be directly passed from unassociated γ protein to associated γ protein. A purified association/insertion system using pure tetrameric or hexameric apodinitrogenase, γ protein, dinitrogenase reductase, nucleotides, and purified FeMo-co is required to properly distinguish among these possibilities. While it is unclear which of the models is more likely to occur in vivo, it is possible that the cell utilizes a combination of the two schemes depending on the relative rates of synthesis of apodinitrogenase and FeMo-co.

A remaining issue in these studies is where γ protein binds the apodinitrogenase complex. γ Protein's role in promoting insertion and actually binding FeMo-co necessitates its binding to the α subunit of nitrogenase, proximal to the FeMo-co binding site. Analysis of crystallographic data of dinitrogenase (5) and chemical modification data of the apodinitrogenase hexamer2 indicate that the hexameric apodinitrogenase, in contrast to the holodinitrogenase, has an exposed cysteine (Cys-275α) involved in FeMo-co binding and an exposed non-ligand cysteine (Cys-45α).2 This implies a structural change in domain III (5) during the maturation of dinitrogenase, the proposed binding region for γ protein is a potential “hinge” region in domain III of the α subunit that potentially has a degree of flexibility and could expose the FeMo-co site if held in the right conformation.2

It has been suggested that γ protein and its K. pneumoniae counterpart NifY are chaperone-like proteins, whose function is to hold the dinitrogenase subunits in a conformation that is competent for FeMo-co insertion (8). The tight association of γ protein with the apodinitrogenase complex and its dissociation upon addition of required metals are reminiscent of proteins in two other metalloenzyme systems, urease (Ni) and tyrosinase (Cu) (28–30). These proteins are also believed to be chaperone/Accessory proteins required to stabilize the apoproteins; however, neither has been shown to bind metals themselves, so it is unclear if these have the insertase activity that γ protein appears to have.

In conclusion, data presented here show that the A. vinelandii γ protein is involved in the maturation of dinitrogenase. Its association with the dinitrogenase complex is required for proper insertion of FeMo-co, perhaps by binding to the α subunit such that the FeMo-co binding site remains accessible. Further, γ protein binds FeMo-co itself and probably inserts the co-factor into its proper position within the α subunit. γ protein therefore may have properties of both metal co-factor insertases and chaperone proteins.

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REFERENCES

1. Dean, D. R., Bolin, J. T., and Zheng, L. (1993) J. Bacteriol. 175, 6737–6744
2. Bolin, J. T., Campobasso, N., Muchmore, S. W., Morgan, T. V., and Mortenson.
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1. E. L. (1993) in Molybdenum Enzymes, Coenzymes and Model Systems (Stiefel, E. I., Coucouvanis, D., and Newton, W. E., eds) pp. 186–195, American Chemical Society, Washington, D. C.
2. Chan, M. K., Kim, J., and Rees, D. C. (1993) Science 260, 792–794
3. Shah, V. K., and Brill, W. J. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3249–3253
4. Kim, J., and Rees, D. C. (1992) Science 360, 553–560
5. Hawkes, T. R., McLean, P. A., and Smith, B. E. (1984) Biochem. J. 217, 317–321
6. Paustian, T. D., Shah, V. K., and Roberts, G. P. (1990) Biochemistry 29, 3515–3522
7. Shah, V. K., and Brill, W. J. (1973) Biochim. Biophys. Acta 256, 498–511
8. Blake, M. S., Johnson, K. H., Settler, R. A., Wilson, M. S., Cash, V. L., Beynon, J., Newton, W. E., and Dean, D. R. (1989) J. Bacteriol. 171, 1017–1027
9. Tal, S., Chun, T. W., Gavini, N., and Burgess, B. K. (1991) J. Biol. Chem. 266, 10654–10657
10. Robinson, A. C., Chun, T. W., Li, J. G., and Burgess, B. K. (1989) J. Biol. Chem. 264, 10088–10095
11. Allen, R. M., Homier, M. J., Chatterjee, R., Ludden, P. W., Roberts, G. P., and Shah, V. K. (1993) J. Biol. Chem. 268, 23670–23674
12. Bush, R. A., and Wilson, P. W. (1959) Nature 184, 381–382
13. J. R. D., and Bishop, P. E. (1988) J. Bacteriol. 170, 1475–1487
14. Robinson, A. C., Dean, D. R., and Burgess, B. K. (1987) J. Biol. Chem. 262, 14327–14332
15. Shah, V. K., Davis, L. C., and Brill, W. J. (1972) Biochim. Biophys. Acta 256, 498–511
16. Blake, M. S., Johnson, K. H., Russell-Jones, G. J., and Gotchlich, E. C. (1984) Anal. Biochem. 136, 175–179
17. Brandner, J. P., McEwan, A. G., Kaplan, S., and Donohue, T. J. (1989) J. Bacteriol. 171, 360–368
18. O’Farrell, P. H. (1975) J. Biol. Chem. 250, 4007–4021
19. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76–85
20. Shah, V. K., and Brill, W. J. (1973) Biochim. Biophys. Acta 256, 445–454
21. Shah, V. K., Imperial, J., Ugalde, R. A., Ludden, P. W., and Brill, W. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1636–1640
22. Kuo, C. F., and Fridovich, I. (1988) Anal. Biochem. 170, 183–185
23. Brandner, J. P., McEwan, A. G., Kaplan, S., and Donohue, T. J. (1989) J. Bacteriol. 171, 253–266
24. Imperial, J., Shah, V. K., Ugalde, R. A., Ludden, P. W., and Brill, W. J. (1987) J. Bacteriol. 169, 1784–1786
25. Ugalde, R. A., Imperial, J., Shah, V. K., and Brill, W. J. (1984) J. Bacteriol. 159, 888–893
26. Park, I.-S., Carr, M. B., and Hausinger, R. P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3233–3237
27. Chen, L.-Y., Leu, W.-M., Wang, K.-T., and Lee, Y.-H. W. (1992) J. Biol. Chem. 267, 20100–20107
28. Chen, L.-Y., Chen, M.-Y., Leu, W.-M., Tsai, T.-Y., and Lee, Y.-H. W. (1993) J. Biol. Chem. 268, 18710–18716
Characterization of the γ Protein and Its Involvement in the Metallocluster Assembly and Maturation of Dinitrogenase from *Azotobacter vinelandii*

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