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Activity, Reconstitution, and Accumulation of Nitrogenase Components in *Azotobacter vinelandii* Mutant Strains Containing Defined Deletions within the Nitrogenase Structural Gene Cluster

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The *Azotobacter vinelandii* genes encoding the nitrogenase structural components are clustered and ordered: *nifH* (Fe protein)-*nifD* (MoFe protein α subunit)-*nifK* (MoFe protein β subunit). In this study various *A. vinelandii* mutant strains which contain defined deletions within the nitrogenase structural genes were isolated and studied. Mutants deleted for the *nifD* or *nifK* genes were still able to accumulate significant amounts of the unaltered MoFe protein subunit as well as active Fe protein. Extracts of such *nifD* or *nifK* deletion strains had no MoFe protein activity. However, active MoFe protein could be reconstituted by mixing extracts of the mutant strains. These results establish an approach for the purification of the individual MoFe protein subunits. Mutants lacking either or both of the MoFe protein subunits were still able to synthesize the iron-molybdenum cofactor (FeMo-cofactor), indicating that in *A. vinelandii* the FeMo-cofactor is preassembled and inserted into the MoFe protein. In contrast, a mutant strain lacking both the Fe protein and the MoFe protein failed to accumulate any detectable FeMo-cofactor. The further utility of specifically altered *A. vinelandii* strains for the study of the assembly, structure, and reactivity of nitrogenase is discussed.

Nitrogenase is under intense study as the catalytic component of the biological nitrogen fixation system (for recent reviews, see references 6 and 15). It is composed of two separately purified proteins called the iron protein (Fe protein) and the molybdenum-iron protein (MoFe protein). The Fe protein has two identical subunits, a native molecular weight of ca. 60,000, and contains a single [4Fe-4S] cluster (6, 15). The MoFe protein is a two α-two β tetramer with a native molecular weight of ca. 220,000 and contains two Mo atoms, 32 ± 3 Fe atoms, and a similar number of S^{2-} atoms per molecule (6, 15). These metal atoms are believed to be arranged in four highly unusual [4Fe-4S] clusters, two iron-molybdenum cofactor (FeMo-cofactor) centers, and two poorly understood iron clusters called the S centers (15). During catalysis the MoFe protein and the Fe protein associate and dissociate. The Fe protein serves as a specific electron donor for the MoFe protein (10) which contains the site for substrate binding and reduction (11). In addition to nitrogenase, nitrogen fixation requires a source of reducing equivalents, magnesium ATP, protons, and an anaerobic environment (5).

Recent efforts in our laboratories have focused on the development of a genetic system for the further analysis of nitrogenase from the diazotroph *Azotobacter vinelandii*. Toward this end the nitrogenase structural gene cluster has been isolated and its complete nucleotide sequence has been determined (4). These genes include *nifH* (Fe protein), *nifD* (MoFe protein α subunit), and *nifK* (MoFe protein β subunit). In the present study we used various purified nitrogenase structural gene fragments to create defined deletions within the *A. vinelandii* genomic nitrogenase structural gene cluster. The effect of these mutations on nitrogenase component protein accumulation and activity and the utility of such specifically altered strains for the study of the assembly, structure, and reactivity of nitrogenase are presented and discussed.

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MATERIALS AND METHODS

Cell growth and nitrogenase derepression. The wild-type and various mutant strains of *A. vinelandii* were cultured in a modified Burk medium (20). This medium was supplemented to a final concentration of 30 mM with filter-sterilized ammonium acetate when a fixed source of nitrogen was included in the medium. Cells used to prepare extracts for two-dimensional gel electrophoresis were grown in 100-ml aliquots in 500-ml flasks at 30°C. For reactivity studies, cells were grown in 10-liter batches in a New Brunswick microfermentor at 30°C. The cultures were mixed at 400 rpm and sparged with 8,000 cm^3 of air per min. For derepression of nitrogenase synthesis, all cultures were initially grown in Burk ammonium acetate-supplemented medium to the mid-logarithmic phase (Klett units, ca. 110 with a no. 54 filter) and were harvested by centrifugation. Harvested cells were immediately resuspended in the original volume of Burk nitrogen-free medium and incubated as described above for an additional 3 h. Derepressed cells were harvested, washed with 1.4 volumes of 0.05 M Tris hydrochloride (pH 8.0), repelleted by centrifugation at 10,000 × *g* for 10 min, and stored at −80°C until needed.

***Escherichia coli* growth and plasmid preparation.** Growth of *E. coli* strains carrying hybrid *nif*-containing plasmids and the preparation, restriction enzyme digestion, and ligation of hybrid plasmid DNAs were all performed as described previously (4). Details of plasmid constructions are described in the legend to Fig. 1.

***A. vinelandii* transformations and construction of *nif* deletion strains.** Transformations of *A. vinelandii* wild-type cells with purified *A. vinelandii* Rif^r DNA and hybrid *nif*-containing plasmid DNA were performed in liquid cultures as described by Page and von Tigerstrom (16). Specific *nif* deletions contained within various hybrid plasmids (Fig. 1)

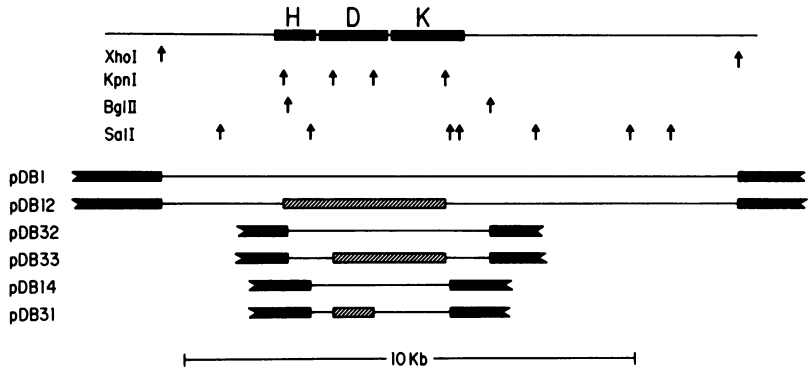


FIG. 1. Physical map of *A. vinelandii nif* structural gene cluster and plasmids used for deletion strain constructions. pDB1 is a *nif*-pKT230 (1) hybrid. The indicated *nif-Xho*I fragment was cloned into the *Xho*I site of pKT230. pDB12 is a deletion derivative of pDB1. All of the internal *Kpn*I fragments contained within pDB1 are deleted in pDB12. pDB32 is a *nif*-pUC8 (13) hybrid. The indicated *nif-Bgl*II fragment was cloned into the *Bam*HI site of pUC8. pDB33 is a deletion derivative of pDB32. The internal *Kpn*I fragments contained within pDB32 are deleted in pDB33. pDB13 is a *nif*-pUC8 (13) hybrid. The indicated *nif-Sal*I fragment was cloned into the *Sal*I site of pUC8. The internal *Kpn*I fragment contained within pDB14 is deleted in pDB31. Other plasmids used for deletion strain constructions have already been described (3). Thin lines represent *A. vinelandii* DNA, and thick lines represent vector DNA. Crosshatches indicate deleted regions. Kb, kilobases.

were transferred to the *A. vinelandii* chromosome by using congression. Congression of the *nif* deletion was accomplished by adding equal amounts (1.0 μg each) of genomic Rif^r DNA and a particular deletion plasmid DNA preparation to competent cultures. After allowing time for phenotypic lag, the transformed cultures were spread on Burk ammonium acetate-supplemented medium plates containing 5.0 μg of rifampin per ml. Rif^r transformants were scored on Burk nitrogen-free and Burk ammonium acetate-supplemented medium plates to identify Nif$^-$ cotransformants. The further analysis of the Nif$^-$ transformants is described in the Results and Discussion section.

Two-dimensional gel electrophoresis. Preparation of extracts for two-dimensional gel electrophoresis (hereafter referred to as gel analysis) was performed as described by Bishop et al. (2). Gel analysis was performed as described by O'Farrel (14), using modifications suggested by Bishop et al. (2).

Enzyme assay. Frozen cells were suspended in 1.4 volumes of cold 0.05 M Tris hydrochloride (pH 8.0) and ruptured by two passes through a chilled French pressure cell at 12,000 lb/in^2. The crude extract was obtained by spinning the lysate at 14,000 × *g* for 20 min at 4°C in a Sorvall RC2 centrifuge. The extract was then degassed, pelleted, and stored in liquid nitrogen until needed. C_2H_2 reduction assays were performed at 30°C in 9.5-ml calibrated vials fitted with butyl rubber serum caps. The reaction mixture used contained (in 1.0 ml): 38 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]-KOH (pH 7.4), 2.5 mM ATP, 5.0 mM $MgCl_2$, 30 mM creatine phosphate, 20 mM $Na_2S_2O_4$, and 2.5 U of creatine phosphokinase. The vessel containing the reaction mixture was degassed and filled with 0.1 atm (10.1 kPa) of C_2H_2 and 0.9 atm of Ar as described previously (7). $Na_2S_2O_4$ was added, and the mixture was preincubated with shaking at 30°C for 5 min. For MoFe protein activity determinations, either the reaction was started by adding 0.2 ml of the appropriate extract or various amounts of the purified *A. vinelandii* Fe protein were added first and then the reaction was started by adding 0.2 ml of the appropriate extract. For assays with extracts prepared from the *A. vinelandii* mutant strain UW45, equal volumes of extracts prepared from UW45 and the appropriate wild-type or deletion strain (or various amounts of the isolated FeMo-cofactor) were mixed and preincubated at 30°C for 45 min. Then 0.2 ml of the mixture was used to initiate the C_2H_2 reduction assay. For assays involving mixtures of CA13 and DJ100 extracts, 0.1 ml of DJ100 extract was mixed for 10 min with 0.025 to 0.4 ml of CA13 extract, and the entire mixture was used to initiate the C_2H_2 reduction assay. Unless otherwise indicated, the reactions were terminated after shaking for 8 min at 30°C by adding 0.1 ml of 2.5 M H_2SO_4. Separate experiments revealed the reaction to be linear for the period studied.

Gas samples, 200 μl at bottle pressure, were taken with a pressure-lock syringe (Precision Sampling, Baton Rouge, La.) and were analyzed with a Varion 3700 gas chromatograph with a Poropack N column (He) and a flame ionization detector. Calibration gas was obtained from Applied Science Laboratories (State College, Pa.). Calculations of the total amounts of C_2H_4 formed were based on the calibrated vial volume minus 1.1 ml of the liquid phase and then expressed as nanomoles of C_2H_4 formed per minute per milligram of protein. The numbers reported represent the means and standard deviations of typically six determinations. All experiments were performed on at least two independently grown batches of each mutant. *A. vinelandii* Fe protein, MoFe protein, and FeMo-cofactor were purified as described previously (7). The purified MoFe protein and Fe protein used in this study had specific activities of 2,605 and 2,103 nmol of H_2 evolved per min per mg of protein, respectively. The isolated FeMo-cofactor was 1.3 mM in Mo and had a specific activity of 242 nmol of C_2H_4 formed per min per ng · atom of Mo. Protein was determined by the Biuret method (9). Protein concentrations for cell extracts were (milligrams per milliliter): 56.0 (wild type); 36.4 (DJ33); 31.4 (CA12); 29.2 (CA13); 32.0 (DJ100); and 31.6 (UW45).

Chemicals. ATP, creatine phosphate, creatine phosphokinase, and TES were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

Construction of *nif* structural gene deletion strains. The *A. vinelandii* nitrogenase structural genes have been previously isolated, and their complete nucleotide sequence has been determined (4). These genes are clustered and arranged as follows: promoter-*nifH* (Fe protein)-*nifD* (MoFe protein α subunit)-*nifK* (MoFe protein β subunit). During those stud-

ies, numerous hybrid plasmids were constructed which contain various portions of the *nif* structural gene cluster and in some cases contain additional sequences flanking the structural genes. Hybrid plasmids relevant to the current study are shown or referenced in Fig. 1. Because we know the sequence of the *A. vinelandii nif* structural gene region we were able to construct hybrid plasmid derivatives which carry defined deletions within the various cloned *nif* structural gene-coding regions (Fig. 1). Deletion plasmid derivative DNAs were purified and used to transform competent *A. vinelandii* wild-type cells. Two different recombination events resulted in the transformation of recipient cells to the Nif^- phenotype. A single-crossover event resulted in the incorporation of the entire hybrid plasmid into the host chromosome and the consequent interruption and duplication of the *A. vinelandii nif* regions carried on the plasmid. Double-crossover events (schematically shown in Fig. 2) resulted in the transfer of the *nif* deletion contained within the plasmid to the homologous region on the host chromosome with the subsequent loss of the plasmid vector. These different recombination events were easily distinguishable since single-crossover-event recombinants became endowed with the antibiotic resistance phenotype carried on the plasmid vector. These strains were also unstable (reverting to the Nif^+ and antibiotic-sensitive phenotypes at high frequency), probably owing to the reciprocal deletion of the integrated plasmid by recombination between *nif* duplicated regions. Double-crossover-event recombinants which carried the nonrevertable Nif^- phenotype (Fig. 3) were retained for further study.

The locations of the endpoints of each deletion carried on the deletion plasmids are known, and thus the exact nature of each deletion contained within the various mutant strains is also known. For the deletion strains used in this study, each deletion is in frame with regard to the coding sequences of fused genes. Such in-frame deletions are important for the interpretation of physiological studies (discussed later), since potential polar affects upon downstream transcription or translation or both owing to early polypeptide chain termination are avoided. The exact endpoints of each deletion are indicated in the legend to Fig. 3.

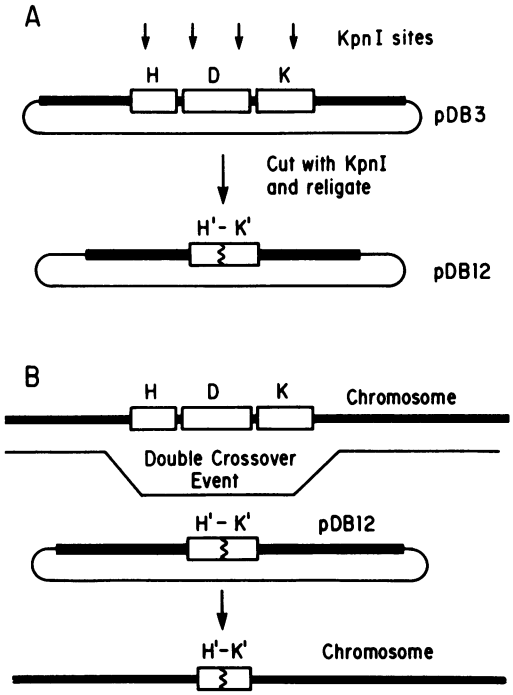


FIG. 2. Strategy for construction of deletion strains. (A) Internal *nif* structural gene sequences are deleted from a hybrid plasmid by restriction enzyme digestion and religation. (B) Purified deletion plasmid DNA is used in transformation of wild-type *A. vinelandii* cells. A double-crossover event results in transfer of the deletion from the plasmid to the chromosome.

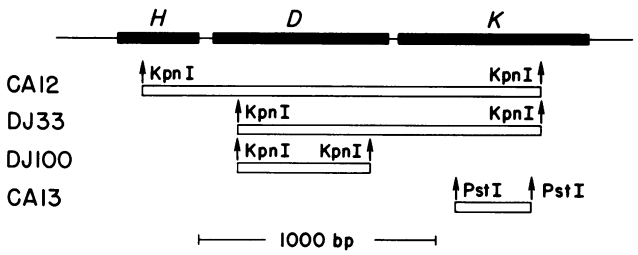
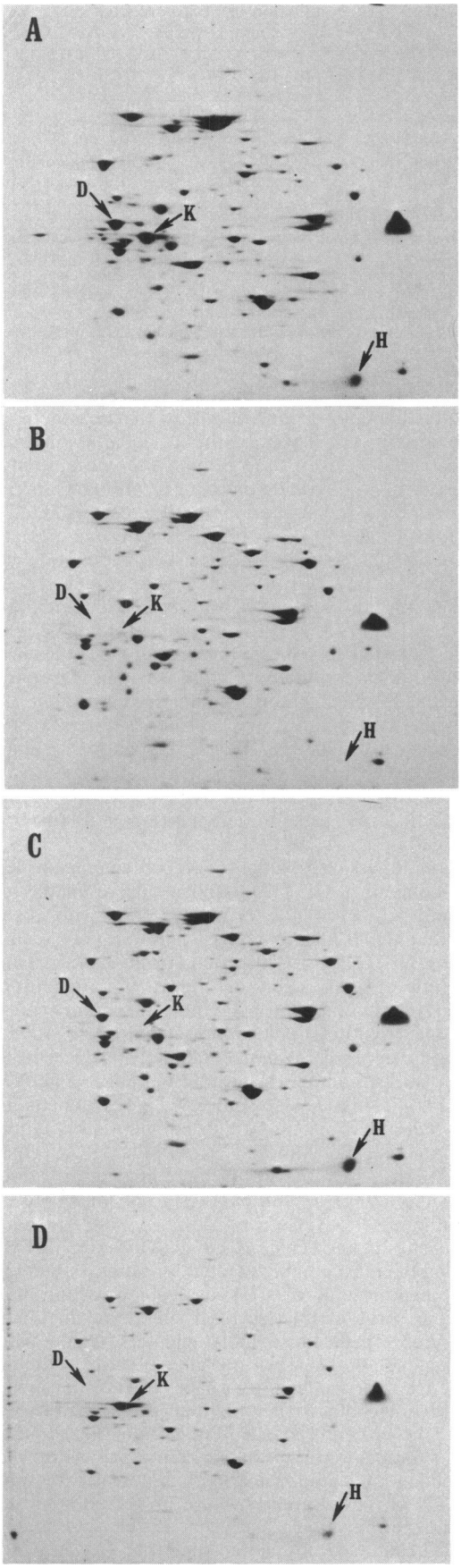


FIG. 3. Location of deletions in strains used in this study. The various gene fusions result in in-phase fusion of the following gene products: CA12 (Fe protein amino acid 66 to MoFe protein β subunit amino acid 313); DJ33 (MoFe protein α subunit amino acid 103 to MoFe protein β subunit amino acid 311); DJ100 (MoFe protein α subunit amino acid 103 to MoFe protein α subunit amino acid 377); CA13 (MoFe protein β subunit amino acid 135 to MoFe protein β subunit amino acid 294). The complete sequence of these genes can be found in reference 4. Strain CA13 was constructed previously (3) and kindly provided to us. Strain CA12 was constructed in collaboration with P. Bishop. bp, Base pairs.

Identification of *nif* structural gene-encoded products. Comparisons of gel analyses of extracts prepared from nitrogenase-derepressed wild-type *A. vinelandii* and the various *nif* structural gene deletion strains were used to identify the *nif* structural gene-encoded products (Fig. 4). In Fig. 4A the gel analysis of nitrogenase-derepressed wild-type extract is shown and the identified *nifHDK*-encoded products are indicated. The *nifH* gene product was first identified by comparing the migration pattern of purified Fe protein on gels (data not shown) with the wild-type crude extract pattern. The disappearance of the identified *nifH*-encoded product in strain CA12 (Δ*nifHDK*) but not in strains DJ33 (Δ*nifDK*), DJ100 (Δ*nifD*), and CA13 (Δ*nifK*) confirms the identity of the *nifH*-encoded gene product (Fig. 4).

Comparison of gel analyses of purified MoFe protein (data not shown) with gels of crude extracts of nitrogenase-derepressed wild-type *A. vinelandii* also allowed identification of the MoFe protein subunits but did not indicate which spots corresponded to the respective α and β subunits. Comparison of gels of extracts of nitrogenase-derepressed cultures of DJ100 (Δ*nifD*) and CA13 (Δ*nifK*) did permit the assignment of the individual MoFe protein subunits to particular spots on the gels (Fig. 4C and D). The relative migration pattern of the individual MoFe protein subunits was somewhat surprising because the M_r of the α subunit is 55,379 and the M_r of the β subunit is 59,467 based on the amino acid sequences deduced from the nucleotide sequence data, yet the α subunit migrated more slowly than the β subunit in the second dimension. This finding must reflect a processing mechanism or an anomalous migration pattern due to the conformation or charge or both of the respective subunits.

Accumulation and activities of *nif* structural gene products. Previous work with *Klebsiella pneumoniae* mutants has established that some strains mutated in one of the *nif*



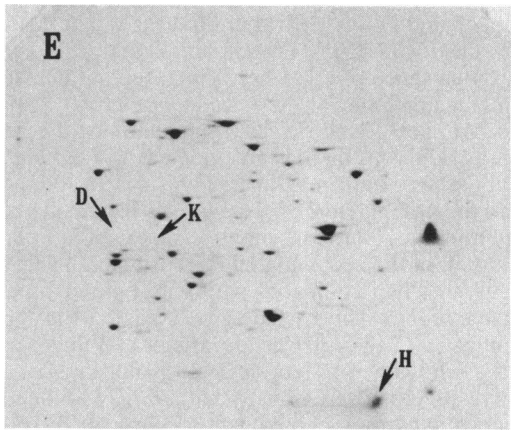


FIG. 4. Two-dimensional gel electrophoresis of protein extracts of wild-type and *A. vinelandii* deletion mutant strains. For each gel the first dimension is the isoelectric-focusing dimension (left to right, pH 6.7 to 4.5) and the second dimension is the size dimension (up to down, large to small). (A) Wild type; (B) CA12; (C) CA13; (D) DJ100; (E) DJ33.

structural genes often result in a lowered accumulation of other identifiable *nif*-specific products (17). For example, insertion mutations located in *nifD* or *nifK* result in lowered accumulation of the Fe protein, indicating that in *K. pneumoniae* intact MoFe protein is required for the stability of the Fe protein or the full expression of the *nifH* gene. Similarly, mutants altered in the *nifD* or *nifK* product often fail to accumulate both MoFe protein subunits (17). Radioactive-pulse experiments indicated that such failure to accumulate one subunit in the absence of the other was due primarily to a mutual stability requirement rather than a synthetic effect (17).

In the present study we examined the effect of deleting various *A. vinelandii nif* structural gene regions upon the accumulation and activity of the unaltered components. In Fig. 4C, D, and E it is obvious that significant amounts of Fe protein accumulate in the absence of the individual MoFe protein subunits. This result is also confirmed by Fe protein activity measurements (Table 1). In these experiments we did notice a small but reproducible overproduction of Fe protein activity in strain CA13 (Δ*nifK*) when compared with the wild-type or other mutant strains.

TABLE 1. Fe protein and MoFe protein activities in *A. vinelandii* wild-type and mutant strains

Strain	Sp act	
	Fe protein[a]	MoFe protein[b]
Wild type	48.0 ± 1.0	55.4 ± 0.03
CA12 (Δ*nifHDK*)	0.0	0.0
DJ33 (Δ*nifDK*)	28.4 ± 1.6	0.0
CA13 (Δ*nifK*)	57.0 ± 3.4	0.0
DJ100 (Δ*nifD*)	30.0 ± 2.0	0.0
UW45	34.3 ± 2.6	0.19 ± 0.016

[a] Nanomoles of C_2H_4 formed per minute per milligram of crude extract protein after addition of saturating levels of purified *A. vinelandii* MoFe protein. For CA12 the Fe protein assay was run for 30 min.

[b] Nanomoles of C_2H_4 formed per minute per milligram of crude extract protein. Wild-type and CA12 extract after the addition of saturating purified Fe protein. Others already had saturating Fe protein present. For CA12, DJ33, CA13, and DJ100 extracts, MoFe protein assays were run for 30 min. The assay is sensitive enough to detect easily 2 orders of magnitude less activity than was found for the UW45 extract.

In Fig. 4C and D it is clear that both the α and β subunits of the *A. vinelandii* MoFe protein are significantly accumulated in the absence of the other. Thus, the mutual stability requirements apparent for the *K. pneumoniae* MoFe protein subunits are not found for *A. vinelandii* MoFe protein subunits. In addition, there is no evidence for degradation of either subunit as would be indicated by smearing on the gels. Although the comparison of gel patterns cannot be considered quantitative, we consistently observed a relatively lower amount of the accumulated *nifD* product in extracts of CA13 (Δ*nifK*) when compared with wild-type or other mutant strain extracts. The physiological basis for the apparent overproduction of the *nifH* gene product and underproduction of the *nifD* gene product in CA13 is not known.

We have suggested previously that a *nifEN* product complex might function as a surrogate MoFe protein (8). This suggestion was based on the striking homology of the *nifEN* gene products when compared with the *nifDK* gene products (8) and on an earlier report that some *A. vinelandii* mutants lacking MoFe protein cross-reacting material still retained low levels of MoFe protein activity (19). The data in Table 1 show that none of the mutants which are deleted for *nifD* or *nifK* show any detectable MoFe protein activity. Thus, both subunits are required for MoFe protein activity, and no other *nif*-specific component can provide MoFe proteinlike activity under the conditions used in this study.

FeMo-cofactor biosynthesis. The Mo site of the MoFe protein appears to be a small inorganic cluster composed of Mo, Fe, and S^{2-} (6, 15, 18). This species (FeMo-cofactor) can be isolated free of the MoFe protein (18) and can be used to reconstitute MoFe protein activity in extracts of *K. pneumoniae* and *A. vinelandii* which accumulate an inactive, FeMo-cofactor-deficient MoFe protein species (17, 18). In the case of *K. pneumoniae*, mutation in any of three alleles (*nifE*, *nifN*, or *nifB*) can lead to a nonleaky FeMo-cofactorless MoFe protein phenotype (17). Mutation in another allele, *nifV*, can result in accumulation of an altered FeMo-cofactor containing MoFe protein which has dramatically altered nitrogenase substrate reactivity (12). Thus, FeMo-cofactor occupies an important role in substrate reduction, and its structure and synthesis are of considerable interest.

Regarding potential biosynthetic pathways, FeMo-cofactor could be assembled stepwise into the MoFe protein or it could be preassembled and inserted into an immature MoFe protein. Two observations regarding such potential pathways have been reported. First, cell extracts of *K. pneumoniae* mutants completely lacking the MoFe protein polypeptides are able to provide small amounts of FeMo-cofactor for the reconstitution of FeMo-cofactor-deficient mutants (i.e., in *nifE*, *nifN*, or *nifB* mutants) (21). Thus, the data in that report strongly suggest that in *K. pneumoniae*, FeMo-cofactor can be assembled in the complete absence of MoFe protein polypeptides (21). Second, it has been found that the *nifE* (8)- and *nifN* (unpublished data)-encoded polypeptides from *A. vinelandii* bear striking sequence homology when compared with the *nifD*- and *nifK*-encoded polypeptides, respectively. Thus, the requirement for *nifEN*-encoded products for FeMo-cofactor biosynthesis (17) and the *nifEN-nifDK*-encoded product homologies suggest that FeMo-cofactor resides on a *nifEN* product complex sometime before its donation to the immature MoFe protein (8).

The availability of characterized *A. vinelandii nif* structural gene deletion strains has now permitted parallel studies to those reported for *K. pneumoniae* (21). Extracts of DJ33 (Δ*nifDK*), DJ100 (Δ*nifD*), and CA13 (Δ*nifK*) can all supply FeMo-cofactor for reconstitution of the FeMo-cofactorless MoFe protein synthesized by the *A. vinelandii* mutant strain UW45 (Table 2). As in the *K. pneumoniae* mutant extract reconstitutions, *A. vinelandii* reconstitutions were low when compared with reconstitutions with excess isolated FeMo-cofactor (Table 2). This result is not unexpected since FeMo-cofactor assembly and insertion should be a catalytic activity upon the MoFe protein, and consequently only small amounts of the FeMo-cofactor might accumulate in the absence of the MoFe protein polypeptides. However, it should be noted that these experiments (21) (Table 2) only measure the amount of FeMo-cofactor that is free to reconstitute the FeMo-cofactor-deficient MoFe protein. They do not necessarily measure the total FeMo-cofactor present in these cells. For example, if FeMo-cofactor is bound to the accumulated α or β subunits in their respective *A. vinelandii* mutant extracts it might not be available for reconstitution of the UW45 MoFe protein. This possibility is under further investigation.

TABLE 2. Accumulation of FeMo-cofactor in *A. vinelandii* mutant strains

Sample plus UW45 extract	Sp act of FeMo-cofactor[a]
FeMo-cofactor[b]	16.4 ± 1.8
CA12 (Δ*nifHDK*)	0.0
DJ33 (Δ*nifDK*)	0.47 ± 0.02
CA13 (Δ*nifK*)	1.50 ± 0.01
DJ100 (Δ*nifD*)	0.78 ± 0.03

[a] Nanomoles of C_2H_4 formed per minute per milligram of *A. vinelandii* UW45 extract protein. Background activity of 0.19 for UW45 extract alone has been subtracted from all values. No activity was obtained when isolated FeMo-cofactor was added to CA12, DJ33, CA13, or DJ100 extracts in the absence of UW45 extract.

[b] Excess isolated FeMo-cofactor was added to the UW45 extract.

Although we were able to detect significant levels of FeMo-cofactor in DJ33 (Δ*nifDK*), we were unable to detect any FeMo-cofactor in CA12 (Δ*nifHDK*). This result indicates that the Fe protein is somehow involved in FeMo-cofactor biosynthesis or insertion or that it is involved in the regulation of expression of FeMo-cofactor biosynthetic genes. A requirement for Fe protein activity for FeMo-cofactor biosynthesis or insertion is not likely since at least one strain of *A. vinelandii* completely lacking Fe protein activity still accumulates normal amounts of active MoFe protein (19). There is also some evidence from studies of *K. pneumoniae* that the Fe protein polypeptide is required for FeMo-cofactor biosynthesis (W. A. Filler, R. A. Dixon, and B. E. Smith, Abstr. 6th Int. Symp. Nitrogen Fixation, 1985, abstr. no. 3-09) or for full expression of the FeMo-cofactor biosynthetic genes (17).

Reconstitution of MoFe protein activity. The MoFe protein is among the most complex metalloproteins, containing from six to eight metal clusters assembled within an α2-β2 tetrameric protein (1). Currently there is no information concerning where those metal clusters reside within the individual subunits or their possible location at the interface between subunits. One approach toward unraveling the spatial arrangement of the individual metal clusters within or among the subunits is the purification of the individual, native subunits and their subsequent analysis by the many biochemical and biophysical techniques already applied to the study of the tetrameric MoFe protein. The observation that the gel migration patterns of the individual MoFe protein subunits accumulated in DJ100 (Δ*nifD*) and CA13

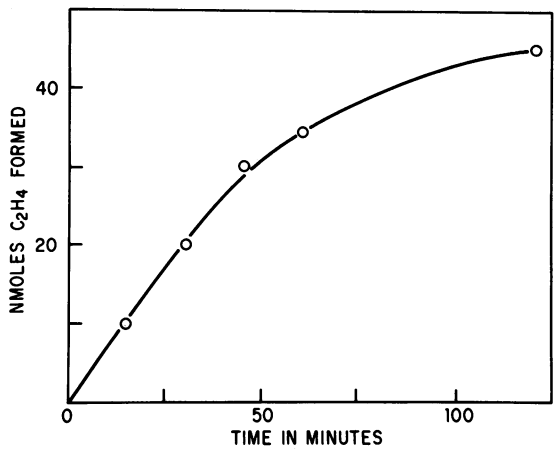


FIG. 5. Reconstitution of MoFe protein activity. Equal amounts of CA13 and DJ100 cells were ruptured to make a crude extract, and 0.2 ml (2.9 mg of protein) was assayed as described in Materials and Methods. Activity is linear with time for about 50 min. Nonlinearity at longer times is normal for nitrogenase assays owing to the instability of creatine phosphokinase.

(Δ*nifK*) are identical to the respective wild-type MoFe protein patterns indicated that the individual subunits accumulated in these mutant strains are likely to be in a nondenatured form. Further, these mutants should contain all other *nif* gene products essential for the synthesis and insertion of FeMo-cofactor and the [Fe-S] centers. We therefore tested for the reconstitution of MoFe protein activity by mixing crude extracts prepared from nitrogenase-derepressed DJ100 and CA13.

Neither CA13 nor DJ100 extracts contained any detectable MoFe protein activity (Table 1). Data shown in Fig. 5 demonstrate that when an extract prepared from a mixture of CA13 and DJ100 cells was immediately assayed, significant nitrogenase activity was observed. Although the reconstituted MoFe protein activity was low when compared with similar amounts of crude extracts prepared and assayed from wild-type cells, the reconstituted activities were reproducible and far above background levels. The observation that the activity was not stimulated with time indicates that an active MoFe protein was already formed at the onset of the assay. Thus, the presence of ATP and reductant in the assay system did not enhance the formation of the active MoFe protein from its component parts. Similar results were obtained when cell extracts of CA13 and DJ100 were prepared separately, mixed, and assayed within 10 min of mixing. When these separately prepared extracts were mixed and assayed it was found that activity was greatly enhanced by proportionately increasing the amount of CA13 extract relative to DJ100 extract. The maximum activity (1.2 nmol of C_2H_4 formed per min per mg of DJ100 protein) was obtained at a 3.5-to-1 ratio of CA13 to DJ100 extract protein. These results indicate that more β subunit was available for reconstitution than was α subunit in the respective extracts, a finding consistent with our conclusion based on gel analyses of extracts from these mutant strains.

The above data demonstrate that α and β subunits accumulated in the absence of each other can rapidly assemble into an active MoFe protein species. Whether the reconstituted protein represents a species identical to the native MoFe protein and contains all the ancillary metal clusters in their proper spatial arrangements is not yet known. It should be emphasized that many variables (e.g., ionic strength, temperature) could affect the proper reconstitution of the MoFe protein, and it is probable that much higher reconstituted activities will be obtained under more optimum conditions. Nevertheless, data presented here establish an assay which can now be used to purify the individual MoFe protein subunits in a nondenatured form.

In summary, a collection of *nif* structural gene deletion strains from *A. vinelandii* were isolated and characterized. These strains should prove useful for further strategies regarding the biochemical and biophysical characterization of nitrogenase from this organism. Such studies include the site-directed mutagenesis of the nitrogenase structural genes, purification and characterization of the individual MoFe protein subunits, and the elucidation of the pathway for FeMo-cofactor biosynthesis. Finally, the procedures described here and elsewhere (3) for the directed deletion of characterized *A. vinelandii* genomic regions should find general application toward the study of other *nif*-specific genes from this organism.

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