MUTAGENESIS OF NIFE AND NIFN FROM AZOTOBACTER VINELANDII

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(ABSTRACT)

The products of *nifE* and *nifN* from *Azotobacter vinelandii*, which are involved in the biosynthesis of the iron-molybdenum cofactor (FeMo-co) from nitrogenase, have been analyzed using a variety of mutagenic techniques.

NifE was the object of several site-specific amino acid substitutions that were designed to elicit information regarding metal cluster ligands, subunit-subunit interactions, and the proposed transfer of FeMo-co from a *nifEN*-products complex to the apo-MoFe protein. A model of metal cluster binding regions within the *nifEN*-products is discussed insofar as it relates to the rationale for the targeting of particular amino acids for substitution.

A translational fusion between *nifN* and *lacZ* was constructed and used to study the regulation of *nifEN*. This gene fusion was regulated in the same manner as wild type *nifN* and produced a fusion protein which was enzymatically active with respect to substrates of β-galactosidase. Results from mutant strains which carry lesions in *nifH* or *nifA* in addition to the *nifN*::*lacZ* fusion are presented and discussed.

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INTRODUCTION

Nitrogen is a major component of proteins and nucleic acids and is a major constituent (approximately 79%) of the earth's atmosphere. Paradoxically, atmospheric nitrogen occurs in a gaseous triple-bonded form that is unusable by most types of life, and nitrogen availability is therefore a significant limiting factor in most ecosystems. Atmospheric nitrogen, N_2, must first be converted to ammonia or nitrate in order to be usable by most forms of life.

Nitrogen fixation is the conversion of nitrogen in its gaseous state to ammonia or nitrate. Nitrate is produced by high energy radiation such as lightning or occasionally by meteorite trails or cosmic radiation. Ammonia is produced biologically by symbiotic bacteria living in association with leguminous and root-nodulated nonleguminous plants, by free-living aerobic and anaerobic soil bacteria, and by blue-green algae. Ammonia is also produced from N_2 industrially for use as fertilizer, but biological nitrogen fixation accounts for most of the nitrogen inputs to the biosphere.

THE EVOLUTION OF BIOLOGICAL NITROGEN FIXATION

Since the ability to fix nitrogen would not have evolved without selective pressure favoring its existence, it can be argued that biological systems for N_2 reduction did not arise until the reserves of fixed nitrogen in the biosphere were depleted. There is still considerable uncertainty as to when the earth's biosphere became an N-limited system, however, with estimates ranging from an ancient 3.5

billion years ago to the more recent 1.5 billion years ago (67). This range of dates is too broad for satisfactory inquiry into the evolutionary age of biological N_2 fixation. It also does not account for the possibility that such systems arose in an isolated area which was depleted of fixed nitrogen earlier than the rest of the biosphere, but it does seem to indicate that nitrogen fixation evolved at least 1.5 billion years ago.

More information can be obtained from looking at the organisms that are capable of fixing nitrogen and at the differences among the particular systems in those organisms. Nif genes are present in a wide variety of diversified eubacteria and have even recently been found in methanogenic archebacteria (86,87). This scattered distribution can be explained as evidence that an early common ancestor of these various genera had the capability of nitrogen fixation, but can be explained also as having arisen through lateral spread of the nif genes. These two possibilities have been distinguished by determining similarity coefficients for eight different nitrogenase Fe proteins and comparing the values to similarity coefficients of 16S-rRNA from the same or closely related species used in the Fe protein similarity calculations (35). This study concluded that the Fe protein and 16S-rRNA had evolved concurrently, that is, that the nif genes did not arise in these organisms through lateral transfer but had in fact been present since the genera had diverged. This places the origins of biological nitrogen fixation closer to the older estimates suggested above than to the more recent estimates.

A recent discovery is helpful in imagining potential precursor

enzymes from which nitrogenase may have evolved. It has been shown that a Rhizobium in chemostat culture can assimilate nitrogen from cyanide in a nitrogenase-dependent reduction of cyanide (92). Thus, perhaps nitrogenase has evolved from some ancestral enzyme system which utilized cyanide as a source of nitrogen.

THE DISCOVERY OF NITROGEN FIXATION

Nitrogen fixation was first discovered one hundred years ago by Hermann Hellriegel and Herman Wilfarth, agricultural researchers studying sugar beet nutrition at the agricultural experiment station in Bernberg, Germany (see references 68 and 76). They performed studies where a variety of plants were grown in sterilized soils with or without an added fixed source of nitrogen, and found that for several plants a direct relationship between concentration of soil nitrogen and total yield of the plant could be observed. For pea plants, a legume, erratic results were observed which included high yields of plant tissue in the absence of an added nitrogen source. Hellriegel and Wilfarth ultimately did experiments where different bacterial suspensions were added to the soil along with the pea seeds. They thereby showed that atmospheric nitrogen was being incorporated into the plant by some mechanism which involved the formation of root nodules, and that this nodulation was dependent on inoculation with specific bacterial types. This work was controversial at the time because it had already been shown that carbon is incorporated into plants from an atmospheric reservoir, during photosynthesis, and it was thought that all plants were capable of using atmospheric nitrogen as well.

Soon after these experiments had described symbiotic nitrogen fixation by bacteria, free-living microorganisms were also proposed to be capable of fixing nitrogen. In 1893 Clostridium pasteurianum was reported to be an anaerobic free-living nitrogen fixer and eight years later Azotobacter chroococcum was identified as a free-living nitrogen fixer which lived aerobically.

NITROGENASE

The biological reduction of dinitrogen to ammonia is catalyzed by the enzyme nitrogenase. Different types of nitrogenase exist (9,30,31,74), but one type, **conventional** nitrogenase (hereinafter referred to as nitrogenase) has been more thoroughly characterized than the others. Nitrogenase is composed of two separately-purifiable components, one which contains iron (the Fe protein), and one which contains molybdenum and iron (the MoFe protein). The iron protein acts as a single-electron reductant of the MoFe protein in a reaction in which MgATP is hydrolyzed to MgADP. Eight electron transfer steps occur in the reaction:

$$N_2 + 8H^+ + 8e^- \text{---> } 2NH_3 + H_2$$

At least two MgATP molecules are hydrolyzed per electron transferred to nitrogen, or 16 MgATP per reduced dinitrogen (29), and reducing equivalents, protons and an anaerobic environment are required in addition to MgATP for nitrogenase turnover.

In Azotobacter vinelandii the Fe protein is a dimer of 60,500 daltons which is composed of two identical subunits of 30,000 daltons each (93). These subunits are encoded by nifH, and five cysteine

residues are conserved in all known nifH product sequences (32). The Fe protein contains a single Fe_4S_4 cluster which cysteine-thiol reactivity data indicate is probably symmetrically bridged between the subunits, with cys97 and cys132 (A. vinelandii, reference 11) providing ligands to the metal center (32). Upon binding MgATP, the Fe protein undergoes a conformational change, indicated by the change in reactivity of its Fe-S center with chelators (99). The Fe protein is the only known reductant of the MoFe protein which results in catalytic activity.

The A. vinelandii MoFe protein is a 220,000 dalton tetramer of two pairs of nonidentical subunits each with an M_r of about 61,000 (95). The α- and β-subunits of the MoFe protein are encoded by nifD and nifK, respectively, and the sequences of these polypeptides from a number of organisms are available. Mössbauer and quantitative extrusion studies indicate that the MoFe protein contains four Fe_4S_4 clusters and two iron-molybdenum cofactors (FeMo-co) of stoichiometry $MoFe_{6-8}S_{4-10}$ (16). The metal centers of the MoFe protein are likely to function as redox centers participating in the catalytic reduction of nitrogen, but little information is available on their structures, functions, and individual redox properties.

ALTERNATIVE NITROGENASES

In 1980 it was reported that A. vinelandii strains which had lesions in the genes which encoded the structural components of conventional nitrogenase were still capable of fixing nitrogen (7). It was suggested that nitrogen fixation was occurring in these strains by an alternative nitrogenase which was only expressed in the absence of

both a fixed nitrogen source and molybdenum.

It has since been shown that A. vinelandii has at least two alternative nitrogenases (8,20) and that A. chroococcum and some other species have at least one alternative nitrogen fixing system (74). Both of the known alternative nitrogenases are also two-component enzymes with components analogous to the Fe protein and the MoFe protein.

One of the alternative nitrogenases, called V nitrogenase or nitrogenase-2, is found in both A. vinelandii and A. chroococcum. V nitrogenase has a VFe protein which X-ray absorption data and extrusion studies indicate contains a vanadium and iron cofactor analogous to FeMo-co which has been termed FeVa-co (2,15). The VFe protein has been isolated from both organisms (30,74) and was in fact partially purified and characterized before it was proposed to be different from conventional nitrogenase's MoFe protein (17).

It was shown that the VFe protein is composed of three pairs of nonidentical subunits and the genes encoding the subunits of the structural components of V nitrogenase have been isolated and sequenced (25). This vanadium nitrogenase exhibits different physical, chemical and catalytic properties than conventional nitrogenase, and its expression is regulated by the presence or absence of NH_3^+ and Mo. One of the catalytic differences between the VFe protein and the MoFe protein is that the former is able to catalyze the reduction of C_2H_2 to C_2H_6 (23) and it has been shown that this catalytic action is also a property of apo-MoFe protein which has been activated by purified FeVa-co (89). The transfer of this activity is a strong indication that FeVa-co is the active catalytic center of the VFe protein, as

conventional nitrogenase does not catalyze the reduction of acetylene to ethane. The MoFe protein which has been activated by FeVa-co does not catalyze the reduction of N_2, however, which indicates that this process involves specific interactions between the MoFe or VFe proteins and their respective cofactors.

Using the reduction of C_2H_2 to C_2H_6 in Mo-deficient media as an assay for the presence of alternative systems in various genera, a V nitrogenase system has been shown to probably be operating in Clostridium pasteurianum (23), Azorhizobium caulinodans, and six pseudomonads, but not in Klebsiella pneumoniae or Azospirillum brasilense (19). When the medium was supplemented with Mo, this reduction was not seen for any of the species.

A second alternative nitrogenase, so far identified only in A. vinelandii, does not appear to contain either molybdenum or vanadium (20). This second alternative system has been isolated and characterized and the genes encoding the structural components have been cloned and sequenced (10). The component that corresponds to the MoFe protein of conventional nitrogenase is called dinitrogenase-3, and its two subunits appear to assemble into at least two active configurations, $\alpha_2\beta_2$ and $\alpha_2\beta_1$. The expression of this alternative nitrogenase is regulated by the presence or absence of NH_3^+, Mo and V (10).

This third system, the vanadium nitrogenase, and conventional nitrogenase all appear to share some enzymes required for the maturation of their structural components. The product of nifB has been implicated in the biosynthesis of cofactors for all three systems, and is thought to have a role in binding and donating a cofactor precursor (46). The

product of nifM is known to have a role in the maturation of the Fe protein of the conventional system (73), and it seems to be necessary for the maturation of the alternative Fe proteins as well (48). The regulation of expression for these genes involved in all three systems must necessarily be more complex than other nif genes, and it will be interesting to see how this regulation is accomplished.

THE IRON-MOLYBDENUM COFACTOR

A variety of data indicate that the FeMo-co plays an important role in substrate binding and reduction. Klebsiella pneumoniae and A. vinelandii strains which lack FeMo-co also lack catalytic activity, and the addition of purified FeMo-co to extracts of such strains results in the activation of MoFe protein, and significantly, this activation follows saturation kinetics (11,79). In addition, K. pneumoniae mutants which carry a lesion in nifV synthesize an altered FeMo-co, and MoFe protein from these strains or apo-MoFe protein which has been reactivated by addition of the altered cofactor exhibits dramatic differences in substrate recognition and reactivity (34). Further indications that FeMo-co comprises the active site of nitrogenase are the observations that FeMo-co gives nitrogenase its characteristic electron paramagnetic resonance spectrum of s=3/2, which changes during enzyme turnover (69) and that FeMo-co is capable of catalyzing the reduction of acetylene to ethylene (81).

Several enzymes have been shown to contain molybdenum as a component of their metal centers, but FeMo-co is unique in its structure and properties. For example, a molybdenum cofactor from xanthine

oxidase will activate nitrate reductase in an extract from a Neurospora crassa mutant, but this cofactor will not activate apo-MoFe protein, and isolated FeMo-co will not reactivate xanthine oxidase or nitrate reductase (65). Like these other molybdenum cofactors, FeMo-co is extremely sensitive to oxygen (79).

ORGANIZATION OF THE NIF GENES

In the facultative anaerobe K. pneumoniae, 21 nif-specific genes spanning 23kb comprise the nif cluster (54) and in A. vinelandii a 28,791 base pair region has been isolated and sequenced which contains thirty proposed nif-specific genes (44). These genes either share sequence identity with K. pneumoniae nif genes, appear to be cotranscribed with nif genes, or are preceded by potential nif-specific promoter sequences. For the function of these individual gene products in nitrogenase, see Table 1. A comparison of the nif-cluster from A. vinelandii and from K. pneumoniae is presented in Figure 1.

The extensive sequence analysis of nif genes from several diverse organisms has indicated a significant role for gene duplication or amplification in the evolution of nitrogenase. Sequence identity is apparent in nifD,K,E and N (22,97) as well as in inter-system comparisons of the structural components of the alternative systems (10,25), which suggests multiple duplications of common ancestor genes. In A. chroococcum, two regions of DNA, one outside the main nif cluster, hybridize to a nifEN probe (27), which indicates that homologues (probably derived from a gene duplication event) of niFE and nifN also exist in that organism.

In the freeliving anaerobic nitrogen-fixer C. pasteurianum, gene duplication events have produced at least six nifH or nifH-like sequences, and it has been suggested that at least some of the encoded products might have evolved functions other than as the normal reductant of the MoFe protein (102). These possible separate functions include involvement in an alternative nitrogenase system or in modulating nitrogenase activity under particular physiological conditions, as well as involvement in FeMo-co biosynthesis (which is a function of the normal Fe protein in A. vinelandii (73) and K. pneumoniae (28)). A multi-gene family for the molybdenum-pterin binding protein has also been identified in C. pasteurianum (36).

Some methanogenic bacteria archebacteria have been shown to be capable of fixing nitrogen, and in some of these methanogens more than one copy of a nifH-like gene appears to be present (87). One very interesting result was reported which showed that several nitrogen-fixing methanogens had regions of DNA which would hybridize to heterologous nifH, nifD, or nifK probes but not to nifA, nifJ, or nifNE probes (86). This latter result suggests that some unique mechanisms for nitrogen regulation and for the assembly of nitrogenase may have evolved in these organisms.

NIF GENES INVOLVED IN FEMO-CO BIOSYNTHESIS

Six genes are known to be involved in the biosynthesis of FeMo-co: nifB, nifQ, nifV, nifE, nifN, and nifH. Mutant studies have elicited a great deal of information about the specific function of these genes

Table 1. A listing of the nif genes whose product or function is known, and applicable references.

GENE	PRODUCT/FUNCTION	REFERENCE
nifA	positive transcriptional regulatory element	5,24,61
nifB	FeMo-co, FeVa-co biosynthesis	15,46
nifD	MoFe protein alpha-subunit	24,70
nifE	FeMo-co biosynthesis	12,64
nifF	flavodoxin	4,59
nifH	Fe protein subunit	24,28,70
nifJ	pyruvate:flavodoxin oxidoreductase	82
nifK	MoFe protein beta-subunit	24,70
nifL	negative transcriptional regulatory element	3
nifM	Fe protein maturase	41,73,
nifN	FeMo-co biosynthesis	12,64
nifQ	molybdenum processing	42
nifV	homocitrate synthase/ FeMo-co biosynthesis	34,39,40

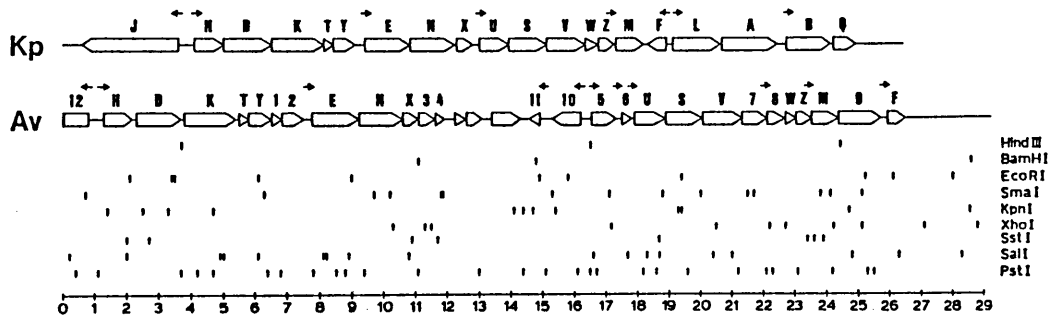

Figure 1. A comparison of the nif clusters of Azotobacter vinelandii and Klebsiella pneumoniae. From (44).

with regard to FeMo-co biosynthesis.

NifQ

NifB and nifQ form a single transcriptional unit in K. pneumoniae. Strains carrying point mutations, Mu insertions, or deletions in nifQ exhibit a leaky nif- phenotype, and activity can be generated in the extracts of such strains by the addition of purified FeMo-co (42). The nif- phenotype can also be rescued by the addition of millimolar concentrations of molybdenum to the growth media (42). These mutants are not impaired in the uptake of molybdenum, and thus nifQ has a role in molybdenum accumulation and processing into nitrogenase. Molybdenum accumulation is inhibited by O_2 and NH_4^+ (66).

NifB

Strains carrying mutations in nifB are unable to grow diazotrophically and MoFe protein from such strains has been found to be lacking FeMo-co. In addition, there are some indications that this MoFe protein may not carry its full complement of Fe_4S_4 centers, and yet, it can be reactivated by the addition of purified FeMo-co (33). In an in vitro FeMo-co biosynthesis system (82,discussion below) nifB was found to be required in stoichiometric rather than catalytic quantities, and it has been suggested that nifB is involved in donating a precursor for FeMo-co biosynthesis (83). In studies utilizing this assay system, most of the nifB activity was found to be membrane associated in K. pneumoniae but in the soluble fraction of A. vinelandii extracts (83).

The nifB gene has been cloned and its DNA sequence determined for A. vinelandii (46), K. pneumoniae (15), Rhizobium meliloti(15), and Rhizobium leguminosarum (75). Several clustered cysteine residues are

present in all of these nifB products, and it has been noted that these cysteines are present in hydrophilic regions, which suggests that these residues are solvent exposed (15). The nifB product is thought to bind a precursor to FeMo-co on the outside of the protein or within a hydrophilic pocket.

In addition to its role in FeMo-co synthesis, the nifB product has recently been implicated in FeVa-co biosynthesis and in the biosynthesis of the cofactor of nitrogenase-3 (46). NifB- strains were found to be defective in all three nitrogenase systems of A. vinelandii.

NifV

Nitrogenase in K. pneumoniae strains carrying a lesion in nifV effectively reduce acetylene but not N_2, and H_2 evolution is inhibited by CO (51,52). When FeMo-co is extracted from the MoFe protein of these strains and used to reconstitute activity of apo-MoFe protein, the resultant MoFe protein exhibits the nifV phenotype, that is, it exhibits the same alterations of substrate recognition and reactivity (34). Thus, the defect in nifV mutants is in an altered cofactor.

An in vitro system for the synthesis of FeMo-co has been described (82), and one of the requirements for this system is a low molecular weight factor which has been called the V-factor, which is produced only in the presence of the nifV gene product (38). V-factor accumulates in the media of all derepressed cultures, including mutant strains so long as the mutation is not within nifV, which indicates that the nifV product is involved in the synthesis of the V-factor and not merely its uptake (39). The structure of the V-factor has been determined using NMR spectroscopy and mass spectrometry, which have identified the nifV

factor as (R)-2-hydroxy-1,2,4-butanetricarboxylic acid (homocitrate) (39). The nifV thus appears to encode a homocitrate synthase, and homocitrate plays a role in the synthesis of FeMo-co. This conclusion is supported by studies using the in vitro FeMo-co synthesis system and by the observation that the addition of homocitrate to the derepression media of nifV mutants cures the nifV- phenotype (83). Since homocitrate can complex with both Fe^{3+} and MoO_4^{2-}, it is possible that it functions in the uptake, initial processing, or targeting of metal ions for FeMo-co synthesis, but these possibilities have yet to be distinguished from the possibility that homocitrate is incorporated into FeMo-co (39).

The in vitro FeMo-co synthesis assay requires molybdate, ATP, at least the nifENBH gene products, and homocitrate (83). In order to study the role of homocitrate in FeMo-co synthesis, several organic acids which are analogues of homocitrate have been tested for their ability to function in the in vitro synthesis system (40). Only citrate showed activity in the assay, and it was required at concentrations 100-fold higher than homocitrate (39). When FeMo-co synthesized in the presence of citrate was used to reactivate apo-MoFe protein, the resulting MoFe protein exhibited the alterations in substrate reactivity and recognition of the nifV- mutants, and thus it has been proposed that in vivo these mutant strains are utilizing citrate for FeMo-co biosynthesis (40).

NifE and nifN

Strains which carry lesions in nifE or nifN exhibit the same phenotype as nifB- mutants, that is, they fail to synthesize a FeMo-co and are therefore incapable of diazotrophic growth, but MoFe protein

activity can be seen upon the addition of purified FeMo-co to cell extracts (11). It was recognized during two dimensional gel analyses of K. pneumoniae extracts that the nifE and nifN gene products had a similar relationship in charge and size when compared to the nifD (MoFe protein α-subunit) and nifK (MoFe protein β-subunit) gene products, respectively (70). In this same study, a mutual stability relationship was noted between the nifE- and nifN-encoded polypeptides, a characteristic which could indicate that these gene products form a complex (70). This data led to the proposal that a nifE and nifN gene products complex might be formed which was related to the MoFe protein and which would be capable of binding a molybdenum species (22).

This model of a nifEN complex led to the isolation and sequence determination of nifE and nifN from A. vinelandii (11) and K. pneumoniae (78). When the primary sequences of the nifD and nifE, and the nifK and nifN gene products are compared, there is a high degree of sequence identity, which provides strong evidence for the above model and indicates an ancestral relationship between the genes(11). This sequence determination also revealed that nifE and nifN are separated by only 12 nucleotides, and that the termination signal for nifE and the apparent ribosome binding site for nifN overlap, an arrangement which could facilitate translational coupling. Such coupling is frequently found for gene whose products form complexes in equimolar amounts, thus providing further evidence that the nifE- and nifN- encoded polypeptides might form a complex related to the MoFe protein.

The MoFe protein is not required for the biosynthesis of FeMo-co (72,97), and active FeMo-co accumulates in a protein bound form in

strains which lack MoFe protein (43,97). Significantly, this accumulation does not occur in strains which also carry lesions in nifB, nifE, or nifN (97), and this observation led to the proposal that the nifEN complex might act as a scaffold onto which the FeMo-co is assembled prior to transfer to the apo-MoFe protein (11). This proposal was supported by the recent purification and characterization of the nifE and nifN gene products, as the polypeptides co-purified in equimolar amounts as a protein of M_r 210,000 (64).

NifH

Although strains which lack the MoFe protein produce appreciable amounts of active FeMo-co, it has been observed that strains which lack the Fe protein fail to synthesize FeMo-co (72,73). A strain which carries a deletion in nifH was constructed and it was shown that the apo-MoFe protein from such a strain could be activated simply by the addition of purified FeMo-co, which suggested that the Fe protein's role may be one of stabilization or insertion of the FeMo-co into the MoFe protein (73). Furthermore, extracts of these mutant strains could carry out in vitro synthesis of FeMo-co provided that purified Fe protein was added, which indicates that the Fe protein is required physically, not for expression of some other nif gene(s).

It is known that the Fe protein, in a reaction that is coupled to MgATP hydrolysis, binds and transfers one electron to the MoFe protein during nitrogenase turnover. Since the nifEN complex is known to be structurally homologous to the MoFe protein and since FeMo-co biosynthesis also requires ATP, it would be easy to imagine the Fe protein acting in an analogous manner during both nitrogenase turnover

and FeMo-co biosynthesis. Intriguingly, strains of both K. pneumoniae (28) and A. vinelandii (79) have been described which encode an altered Fe protein that won't function catalytically in nitrogenase turnover but functions normally in FeMo-co biosynthesis.

Strains which carry mutations in nifM synthesize an Fe protein which is inactive with regards to reducing the MoFe protein, but these strains have normal amounts of MoFe protein activity and therefore normal amounts of FeMo-co (71). Thus, the Fe protein doesn't interact identically with both the MoFe protein and the nifEN complex, although it may behave similarly and play a redox role in both reactions. However, it has been suggested that the nifM gene product might act as a nif-specific sulfur transferase (62) and therefore that Fe protein from nifM$^-$ strains lacks its Fe_4S_4 center. Thus, the fact that Fe protein from nifM mutants is still active with respect to FeMo-co biosynthesis may indicate that stabilization is more important than electron transfer in this reaction.

SITE-DIRECTED MUTAGENESIS OF NIFE

INTRODUCTION

The nifEN complex is thought to be very similar to the more thoroughly studied MoFe protein. These similarities include such characteristics as shared structural features, the ability to bind FeMo-co, the presence of Fe and S, significant amino acid identity and probably evolutionary origin (11,26,64). Although some important differences must exist between the two complexes, since FeMo-co is synthesized on the one and then transferred to the other, it is reasonable to assume that studies of the MoFe protein reveal information concerning the less well characterized nifEN gene products complex.

Mossbauer spectroscopy and quantitative extrusion studies indicate that the metal atoms within the MoFe protein are organized into six metal-containing prosthetic groups of at least two types, four Fe_4S_4 clusters and two FeMo-co clusters (88,104). These studies and those utilizing electron paramagnetic resonance (58,60,88,89,104), as well as genetic studies (34) implicate the metal centers' involvement with substrate binding and reduction. Very little is currently known about the individual structures, redox properties, protein ligands to, or role in substrate reduction of these metal centers, besides the likelihood that FeMo-co is the substrate-reducing site of the MoFe protein. However, work in the laboratory of D. Dean has led to a proposed model of the structural domains of the MoFe protein with regard to metallocluster binding (13).

The nifE and nifN gene products have only recently been purified (64) and no exhaustive characterization has yet taken place. Sequence

comparisons between the MoFe protein subunits and the subunits of the proposed complex involved in FeMo-co biosynthesis point to several conserved domains for potential Fe_4S_4 cluster- and FeMo-co-binding ligands (13), and it has been shown that the nifEN complex does contain Fe and S (64). Thus it is possible that the nifEN complex also binds two FeMo-co clusters and contains four Fe_4S_4 clusters.

The similarities in structure and differences in function of the MoFe protein and the nifEN complex can be used to make predictions concerning the nifEN protein. In the MoFe protein the Fe_4S_4 clusters are likely to play a role in substrate reduction, but the nifEN complex has never been shown to be capable of catalyzing nitrogen fixation and thus any Fe_4S_4 clusters in this complex are probably involved either in a redox role in the formation of active FeMo-co or in stabilizing the complex. It is likely that the ligands provided by the nifEN complex to FeMo-co are similar to but different from the corresponding ligands in the MoFe protein, in light of the probability that FeMo-co is first synthesized on the nifEN complex and then transferred to the MoFe protein where it becomes irreversibly bound. Since there is no evidence that a novel type of subunit interaction has evolved in the nifEN complex, it can be predicted that in this case the substrate-binding complex and the proposed FeMo-co-synthesizing complex are identical or very similar.

In light of the above information, a site-directed mutagenesis study of the nifE gene product was undertaken. Such studies can yield several different kinds of information about the pathway of FeMo-co biosynthesis and the structure and function of both the MoFe protein and

the nifEN complex. For example, if a specific amino acid alteration were to result in the disruption of a set of Fe_4S_4 clusters, such as altering the nature of the protein ligand to the metals, the resultant complex might behave differently than the wildtype complex. Such an alteration would provide data on the nature of ligation to the metal clusters and could also provide information on the redox role which these centers play in FeMo-co biosynthesis. It is possible that an altered cofactor might be produced by one of these mutant strains which could prove useful in the biophysical characterization of wildtype FeMo-co. Another interesting phenotype which might result from such an alteration would be the interruption of FeMo-co biosynthesis at a particular intermediate in the pathway, which would help determine the steps involved in biosynthesis. Site-specific alterations could also uncover information on the nature of subunit-subunit interactions in the nifEN complex.

Another important phenomenon is the transfer of mature FeMo-co from the nifEN gene-products complex to the apo-MoFe protein. Site-directed mutagenesis can be used to study this process by making the potential ligands to FeMo-co on the biosynthetic complex more like those on the MoFe protein. FeMo-co might thus be trapped on the nifEN protein, and therefore be incapable of activating apo-nitrogenase except after in vitro extraction of the FeMo-co with suitable solvents. Radioactive Mo studies could also be used to follow the transfer reaction.

Five site-specific alterations were made and characterized within nifEN in this study. These strains represented all possible nif phenotypes, that is, no, slow, and normal diazotrophic growth capabilities.

MATERIALS AND METHODS

Materials. All restriction endonucleases and the large fragment of DNA polymerase I were purchased from either Bethesda Research Laboratories (Gaithersburg, MD), New England Biolabs (Beverly, MA), or Boehringer Mannheim Biochemicals (Indianapolis, IN). Deoxynucleotide triphosphates and dideoxynucleotide triphosphates were purchased from Pharmacia (Piscataway, NJ). [α-^{35}S]dATP was purchased from New England Nuclear (Wilmington, DE). Ultrapure acrylamide and DNA grade formamide were obtained from Bethesda Research Laboratories. Ultrapure urea was purchased from Boehringer Mannheim Biochemicals. Cesium chloride, ethidium bromide and rifampicin were purchased from Sigma Chemical Co (St. Louis, MO). All other chemicals were reagent grade available commercially.

Oligonucleotide-directed mutagenesis. Oligonucleotides used for mutagenesis were synthesized on an Applied Biosystems 381A DNA Synthesizer system and purified by thin-layer chromatography. Oligonucleotide-directed mutagenesis was performed by the method of Zoller and Smith (55). Templates used for mutagenesis were a 3.4-kb SmaI fragment from pDB7 (11) cloned into the HincII site of bacteriophage M13mp18 (55) and a 1.8-kb SphI fragment from pDB7 cloned into the SphI site of M13mp18. These clones are depicted in Figure 2. The SmaI fragment (used for constructing strains DJ118 and DJ119) contains a carboxy-terminal segment of nifY, all of nifE, and an amino terminal portion of nifN. The SphI fragment (used for constructing

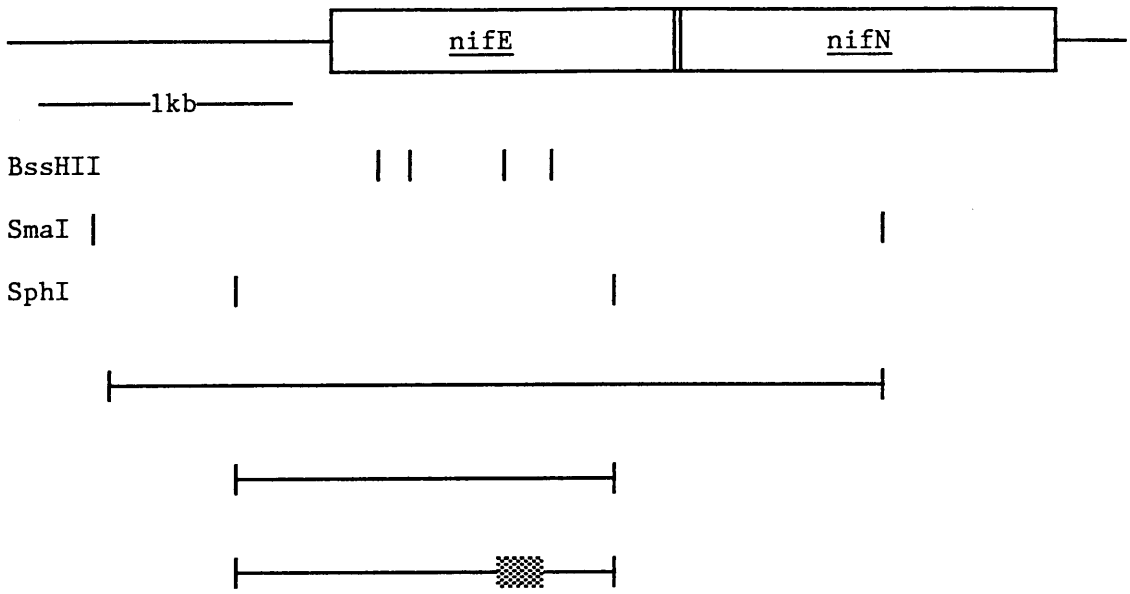

Figure 2. Clones used for oligonucleotide-directed mutagenesis of the nifE product. ▒ represents region which has been deleted.

strains DJ192, DJ202, and DJ234) contains a carboxy-terminal segment of nifY and most of nifE. Single-lane dideoxy sequencing was used to screen for the desired mutations, and identified clones were confirmed by using all four reactions to insure that no other mutations were present in the 400 nucleotides surrounding the desired mutation. Double-stranded replicative form DNA carrying the known base-pair(s) substitution(s) (henceforth referred to as mutation-vector DNA) in the nifE coding sequence was prepared for each mutation construction and used for transformation of A. vinelandii cells. Mutation-vector DNA for strain DJ234 was constructed using template prepared from mutation-vector DNA for DJ192, and mutation-vector DNA for DJ202 was constructed using template prepared from mutation-vector DNA for DJ234. Mutant strains which were constructed are presented in table 2.

Isolation of mutant strains. Transversion mutations carried on mutation-vector DNA were transferred to the A. vinelandii chromosome in either one or two steps, depending on the resultant nif phenotype of that particular mutant strain (see reference 12). Strains which have specific alterations in the nifE gene product will exhibit one of three possible nif phenotypes, that is, no growth, slow growth, or normal growth in media lacking a fixed nitrogen source. Those mutations which do not destroy the strain's capability to grow diazotrophically (slow or normal growth) can be introduced into the A. vinelandii chromosome by a marker-rescue procedure whereby mutation-vector DNA is used to transform a strain deleted for nifE to prototrophy. For this purpose a 200-bp BssHII deletion was made in the 1.6-kb SphI clone which is described above, and via a homologous double recombination event this deletion was

Table 2. Strains constructed by site-directed mutagenesis of nifE.

Strain	Alteration	Phenotype
DJ118	nifE124CS	Nif^+
DJ119	nifE250CS	Nif^-
DJ192	nifE249VH	Nif^+
DJ204	nifE249VH/251SY/252KR	Nif^+
DJ234	nifE249VH/251SY	Nif^+

introduced into the A. vinelandii chromosome. The resultant strain, DJ186, was incapable of diazotrophic growth and was used in the construction of strains DJ192, DJ204, and DJ234, all of which are capable of fixing nitrogen. Those mutations which result in the loss of the capability to grow diazotrophically can be introduced into the chromosome by the coincident transfer (congression) of an uncharacterized rifampicin-resistance marker. A. vinelandii wild type cells (Rif^S) are simultaneously transformed with mutation-vector DNA and purified A. vinelandii chromosomal DNA which carries the marker. Rif^r transformants are identified on rifampicin-containing medium and transformants are scored in the absence and presence of a fixed nitrogen source to identify strains having the Nif^- phenotype.

Cell growth and nitrogenase derepression. Wildtype and mutant strains of A. vinelandii were cultured on a modified Burk medium (93) that was supplemented with filter-sterilized urea to a final concentration of 10mM when a fixed nitrogen source was required. For derepression of nitrogenase synthesis, 11 liters of urea-supplemented Burk medium was inoculated with a 500 ml culture (approximately 200 Klett units, no. 54 filter) in a New Brunswick Microgen SF116 fermentor of 12 liter working capacity. All cultures were stirred at 300rpm at 30°C with an aeration rate of 12 liters/min. When the culture density reached approximately 110 Klett units, cells were concentrated to 1 liter with a Millipore tangential-flow ultrafiltration apparatus. Two liters of sterile nitrogen-free Burk medium were then added to the fermentor, and the culture was concentrated again. A similar second wash was taken to 500 ml final volume. Eleven liters of sterile,

nitrogen-free Burk medium was used to transfer cells back to the fermentor and incubated as above for 2.5h. Derepressed cells were harvested as above except that the cells were washed with chilled 0.05 M Tris-Cl (pH 8.0), then centrifuged at 10,000 x g for 10 min and stored at -80°C until needed.

RESULTS AND DISCUSSION

Assignment of metal cluster binding domains within the MoFe protein.

As previously noted, the MoFe protein contains two iron-molybdenum cofactors and four Fe_4S_4 clusters. A variety of methods have been used to identify specific domains within the MoFe protein as probable binding regions to these metal clusters. These assignments have guided the choice of amino acid residues within the nifEN protein targeted for specific alterations in this study.

Biochemical data.

Specific amino acids can be identified as potentially providing ligands to metal centers by assuming that the solvents used for extraction of those clusters are likely to duplicate the functional groups of the residues which make up the coordination environment of the clusters within the native protein. The Fe_4S_4 clusters of the MoFe protein can be extracted with excess thiols in a denaturing organic solvent, which indicates that the main ligand mode to these clusters is cysteine thiol linkages. This is a typical manner of Fe-S cluster attachment to proteins, found for example in ferredoxins. However, the redox and spectroscopic properties of the Fe-S clusters within the MoFe protein are unusual and indicate that there are probably some other ligands of an unknown nature binding these centers to the MoFe

protein.

While the Fe_4S_4 clusters of the MoFe protein can be extruded by treatment with thiols in a denaturing organic solvent, isolation of the FeMo-co requires anaerobic acid/base treatment (which destroys the Fe_4S_4 centers) followed by extraction with an additional solvent, typically N-methylformamide (NMF) (79). Extraction into NMF was initially taken as evidence that a dissociable proton was a necessary characteristic of a suitable FeMo-co extraction solvent, but recent extractions into dimethylformamide and acetonitrile seem to contradict this proposal (49). These other solvents, however, may be less than ideal.

The electron spin echo modulation spectrum indicates that nitrogen, presumably provided by an amino acid side chain, makes up at least one of the MoFe protein's ligands to FeMo-co (95). However, it has also been suggested that the primary coordination of the cofactor is via deprotonated backbone amide ligands (99). The potential for at least one cysteinyl mercaptide ligand to FeMo-co is indicated by the observation that isolated FeMo-co can react with a single thiolate per Mo atom, and by X-ray absorption spectroscopy studies which indicate that the coordination environment of Mo in isolated FeMo-co differs by one S atom from that environment present in intact MoFe protein (77).

Sequence analysis.

There is now available a large amount of sequence data for nifD (MoFe protein α-subunit), nifK (MoFe protein β-subunit), nifE and nifN from several phylogenetically diverse species (11,56,78,96 and references therein). Analysis of this sequence data reveals patterns of sequence conservation which are useful for targeting specific residues

as potential ligands to the metal centers and for the assignment of potential metal cluster binding domains. These patterns of conservation occur in interspecies comparisons of MoFe protein subunits, in interspecies comparisons of nifEN protein subunits, in intersubunit comparisons of the MoFe protein subunits, in intersubunit comparisons of the nifEN protein subunits, and in intersubunit comparisons between the MoFe and the nifEN protein subunits.

Comparison of amino acid sequences encoded by nifD reveals that there are five cysteine residues conserved in this polypeptide across six diverse species, and that across five Gram-negative species there is an overall sequence identity of 56-59% (100). Using the A. vinelandii α-subunit for a numbering system, these residues are cys62, cys88, cys154, cys183, and cys275 (11). Also significant is the fact that the residues surrounding these cysteines are highly conserved, with cys275 occurring in a stretch of nine amino acids strictly conserved across the five Gram-negative organisms, cys88 within a stretch of ten conserved amino acids, and cys62 and cys183 occurring in these five organisms within stretches of nineteen and twenty strictly conserved amino acids, respectively.

Comparison of the amino acid sequences encoded by nifK reveals that there are three cysteine residues conserved in this polypeptide across five diverse species (100). Using the A. vinelandii β-subunit as a reference for numbering (11) these are cys70, cys95, and cys153. Unlike the conserved cysteines of the α-subunit, these cysteines do not occur in highly conserved stretches of amino acids, except for cys153 which is in a stretch of twelve residues conserved across four Gram-negative

species.

These comparisons lead to the conclusion that sixteen conserved cysteines are available for binding the six metal clusters within the MoFe protein tetramer. Thus, the coordination of these clusters within the MoFe protein must involve some other type of ligand as well.

Since Mössbauer spectroscopy has indicated that the Fe_4S_4 clusters of the MoFe protein are in nearly identical environments, it is significant that there are no apparent structural repeats within either subunit of the MoFe protein. This suggests that the Fe_4S_4 centers are located within both subunits or are at least bridged between the two subunits. This model of Fe_4S_4 cluster binding is supported by intersubunit sequence comparisons which reveal that there are structural units common to both the α- and β-subunits of the MoFe protein (97,100). In this model, cys62, cys88, and cys154 of the nifD product correspond to cys70, cys95, and cys153 of the nifK product. These cysteine residues have a conserved spacing pattern and several residues in this region other than these cysteines are conserved between the subunits. This suggests that these residues are involved in the coordination of the Fe_4S_4 centers of the MoFe protein, and makes conserved residues present in these regions a logical target of site-directed mutagenesis.

If the proposal that cys62, cys88, and cys154 of the α-subunit and cys70, cys95, and cys153 of the β-subunit from the MoFe protein are involved in binding Fe_4S_4 clusters is correct, then cys183 and cys275 of the α-subunit remain as possible candidates for ligands to FeMo-co. This assignment is useful in identifying potential N-donor ligands to FeMo-co since, at least on the primary sequence level, there are a

number of conserved histidine, asparagine, and glutamine residues in the regions surrounding these two cysteines. These include gln191, his195, his196 and asn199 in the region surrounding cys154, and his274 and asn279, in the region surrounding cys275. Another consequence of the assignment of potential FeMo-co binding domains and Fe-S cluster binding domains is the proposal that FeMo-co is bound entirely within the α-subunit of the MoFe protein.

This distinction between Fe_4S_4 cluster binding domains and FeMo-co binding domains is important in deciding on the types of site-specific alterations to make in these regions, since the goals of alterations within FeMo-co binding domains and Fe_4S_4 cluster domains can be different. This is discussed in more detail below.

Targeting of amino acids of nifE for specific alterations.

Due to the number of similarities between the nifEN complex and the MoFe protein, the reasoning applied in the previous section can be useful in making predictions concerning the nature of metal cluster binding domains within the nifEN complex. It was stated earlier that the nifD- and nifE-, and the nifK- and nifN-encoded polypeptides share sequence identity (12). Here that identity is discussed in relation to the proposed binding domains of the MoFe protein.

In Figure 3, a comparison of A. vinelandii nifE (12), K. pneumoniae nifE (78), A. vinelandii nifD (11) sequences is presented. Of the five cysteines that are considered conserved within the nifD sequence, only four are conserved in the nifE product. In Figure 4, a comparison of the deduced amino acid sequences of the A. vinelandii and K. pneumoniae nifN gene products and the A. vinelandii nifK product is presented.

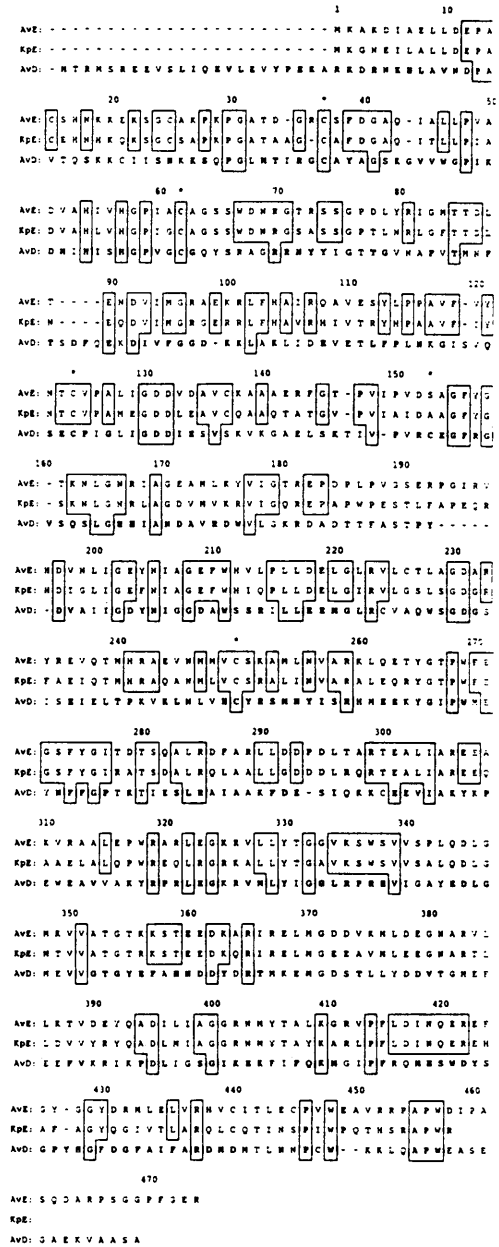

Figure 3. A comparison of the products encoded by A. vinelandii nifE (44), K. pneumoniae nifE (78), and A. vinelandii nifD (10). Those areas of identity which are conserved are boxed. Bold letters of the A. vinelandii nifD product sequence represent amino acids which are conserved across all known nifD polypeptides.

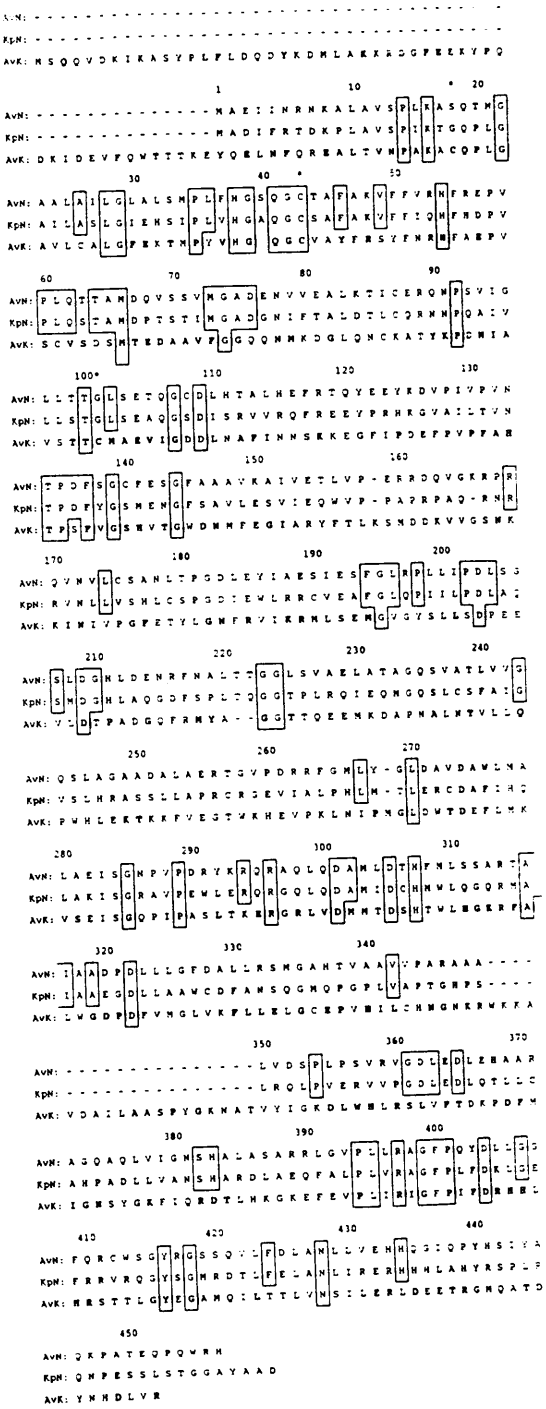

Figure 4. A comparison of the products encoded by A. vinelandii nifN (44), K. pneumoniae nifN (78), and A. vinelandii nifK (10). Those areas of identity which are conserved across all known sequences are boxed. Bold letters of the A. vinelandii nifK product sequence represent amino acids which are conserved across all known nifK polypeptides.

subunit, only one is conserved in the nifN polypeptides. These conservation patterns are also found in unpublished preliminary data from Rhodobacter capsulatus (57) and Rhizobium meliloti (1) presented at the Seventh International Conference on Nitrogen Fixation in March of 1988.

It is interesting that an additional two cysteines that are not conserved within the nifD product may be considered conserved within all of the nifE sequences. These cys residues (cys15 and cys25 of A. vinelandii nifE product) are also flanked by conserved regions, which may indicate that they have a functional role in the nifE product and are not merely the result of alignment manipulations.

It should also be noted that in all of the gram-negative MoFe protein α-subunit sequences available, a cysteine residue is present at either -4 or -5 residues from the amino acid which corresponds to nifE cys25. Since this is approximately midway between nifE cys15 and cys25 and since there is no additional nifE-nifD product identity in this region, it may be more accurate to describe the nifD cysteines as being located at +5 or +6 residues from nifE cys15.

This analysis reveals that there are only 10-12 conserved cysteines in the nifEN protein which are available as ligands to metal centers. Thus, if the nifEN protein and the MoFe protein carry the same complement of metal centers, the ligands to corresponding centers might be slightly different in the two complexes. In the case of FeMo-co, the coordination is thought to be different in the two complexes since the cofactor is only transiently bound to the nifEN protein but irreversibly bound by the MoFe protein. Alternative explanations for the different

numbers of conserved cysteines also exist. For example, all of the cysteines conserved in the MoFe protein may not have a role as a metal ligand, or conversely the nifEN protein may have fewer metal centers than the MoFe protein. This second possibility is discussed in more detail later.

FeMo-co binding domains.

Cys183 and cys275 of the α-subunit of the MoFe protein were earlier proposed to have a role in binding FeMo-co. The nifE-nifD product comparison reveals that no cysteine residue corresponding to cys183 is conserved within the nifE product while cysteines analogous to cys275 are present in all nifE polypeptide sequences available (in A. vinelandii nifE product, cys250). Five amino acids of the fully conserved twenty residue stretch surrounding cys183 of the MoFe protein were also conserved in all of the nifE product sequences, while only the cysteine in the fully conserved nine residue stretch surrounding cys275 of the MoFe protein was also conserved in the nifE polypeptide. In terms of metal-cluster binding, however, three of the five residues which are conserved in the nifE stretch which corresponds to the nifD cys183 region are glycines, and these residues are generally important only in a structural sense, for example in reverse turns of the polypeptide. The other two amino acids in this region conserved across all nifE- and nifD- sequences are α-subunit residues phe186 and ala198. It is possible that the aromatic portion of the phenylalanine plays a role in stabilizing FeMo-co binding.

As the coordination of FeMo-co is likely to be different in the MoFe and the nifEN proteins, lack of sequence identity between nifE-

and nifD- products may be as informative as the presence of sequence identity. In this regard it is important to note that not only is cys183 of the MoFe protein not conserved in the nifE products, it is not found in any one of these products. Of the six residues which were identified as potentially providing N-ligands to FeMo-co on the basis of their conservation in nifD product sequences and their adjacent location to cys183 and cys275 within the primary sequence of the α-subunit, none are conserved within the nifE product. Furthermore, the absence of these six residues is conserved, i.e a histidine is not found in even one of the nifE polypeptides in positions corresponding to interspecifically conserved nifD his195 and his196. It may be significant that in every nifE sequence available, a lysine residue is in the position which corresponds to interspecifically conserved α-subunit gln191.

It is not known whether the coordination of FeMo-co in the nifEN protein involves one, both, or neither of the domains which correspond to the cys183 and cys275 domains of the MoFe protein. This sequence analysis suggests two types of experiments to test the possible involvement of these regions. The first type of experiment involves altering a residue which is conserved in both the nifD- and the nifE-products and seeing whether this residue is essential to normal FeMo-co biosynthesis. The second type of study which this sequence analysis suggests is to alter specific residues of the nifE product which are not conserved in relation to conserved residues of the nifD product. Strains constructed in this way would have an nifEN protein which more closely resembled the MoFe protein than does wildtype nifEN protein. It

might be possible to thereby trap the cofactor or an intermediate on the nifEN complex, which should prove useful in characterizing the ligands to FeMo-co and the steps involved in its biosynthesis.

Fe_4S_4-cluster binding domains.

When sequence comparisons are made between the nifE-, nifN-, nifD-, and nifK- encoded polypeptides from a number of diverse organisms (see Figure 5), an interesting conserved pattern emerges which seems to indicate a structural unit common to all of these subunits. This structural unit represents the domains which are proposed to be involved in binding Fe_4S_4 clusters in the MoFe protein (see discussion above).

Interspecifically conserved cys70 and cys153 of the MoFe protein β-subunit are not conserved within the nifN product. These cys residues are part of the conserved structural unit which is thought to play a role in Fe_4S_4 cluster binding. This may indicate that the nifEN protein has fewer Fe-S clusters to coordinate than the MoFe protein does or that the coordination of corresponding clusters is different in the two complexes. If the nifEN protein contains fewer Fe-S clusters than the MoFe protein, then the fact that the structural unit is fully conserved within the nifE product leads to interesting speculation on the location of individual Fe_4S_4 centers within the MoFe protein. Specifically, this pattern of conservation may be an indication that the individual Fe_4S_4 clusters of the MoFe protein are bridged between identical subunits or fully contained by individual subunits, as opposed to the other possibility that they are bridged between non-identical subunits. This suggestion is attractive because it accommodates the recent observation that there are two slightly different environments for the individual

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CpD   KKTR-29aa-RxCxxxG-6aa-G-9aa-HGxxGC-7aa-RR-23aa-ExxxVFGG-25aa-CxxxxIGDDI
RjD   AKRR-29aa-RxCxxxG-6aa-G-9aa-HGxxGC-7aa-RR-22aa-ExxxVFGG-26aa-CxxxxIGDDI
AvD   RKDR-31aa-RxCxxxG-6aa-G-9aa-HGxxGC-7aa-RR-22aa-ExxxVFGG-26aa-CxxxxIGDDI

AvE   KKEK-11aa-xxCxxxG-6aa-P-8aa-HGxxAC-6aa-NR-19aa-ExxxIMGR-26aa-CxxxxxGDDx
KpE   HKQK-12aa-xxCxxxG-6aa-P-8aa-HGxxGC-6aa-NR-19aa-ExxxIMGR-26aa-CxxxxxGDDx

CpK             KxCxxxG-6aa-G-8aa-HGxxGC-9aa-RH-13aa-ExxxVFGG-25aa-CxxxxxGDDL
RjK    KKK-38aa-KxCxxxG-6aa-G-8aa-HGxxGC-9aa-RH-13aa-ExxxVFGG-25aa-CxxxxxGDDL
AvK    KKR-39aa-KxCxxxG-6aa-G-8aa-HGxxGC-9aa-RH-13aa-ExxxVFGG-25aa-CxxxxxGDDL

AvN             KxxxxxG-6aa-G-8aa-HGxxGC-9aa-RH-13aa-xxxxxMGA-25aa-xxxxxxGxDx
KpN             KxxxxxG-6aa-G-8aa-HGxxGC-9aa-QH-13aa-xxxxxMGA-25aa-xxxxxxGxDx
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Figure 5. An intersubunit comparison of the proposed Fe-S cluster binding domains of the nifEN- products complex and of the MoFe protein from a variety of organisms. Adapted from reference 100.

Fe_4S_4 clusters within the MoFe protein. However, other explanations are possible.

The fact that this structural unit is conserved within the nifE and nifN polypeptides seems to indicate that Fe_4S_4 clusters are probably present in the nifEN protein. These metal centers might have a redox role in FeMo-co synthesis or they might function in stabilizing the nifEN protein. Site-directed mutagenesis studies of these regions would probably be most effective if they altered residues which were conserved across the four polypeptides or at least between the nifE- and nifD- or the nifK- and nifN- encoded polypeptides.

Specific amino acid substitutions within the nifE product.

When the nifE product cys124 residue, which corresponds to the interspecifically conserved α-subunit residue cys154, is substituted by serine, the resulting strain is dramatically altered in its ability to grow diazotrophically (Figure 6, strain DJ118). This residue has been proposed to be involved in Fe-S center binding. The slow diazotrophic growth rate of this strain may be due to an impaired ability to synthesize normal FeMo-co or to the production of an altered FeMo-co which has a lowered specific activity. An additional possibility is that the strain is affected in the transfer of FeMo-co to the MoFe protein in such a way that this transfer has become rate-limiting on nitrogen fixation. These possibilities could not be distinguished on the basis of MoFe protein reconstitution experiments, where it was found that an increase in MoFe protein activity occurred upon addition of purified FeMo-co to cell extracts of the mutant (Table 2). Such an increase seems to lower the possibility that this strain's defect is due

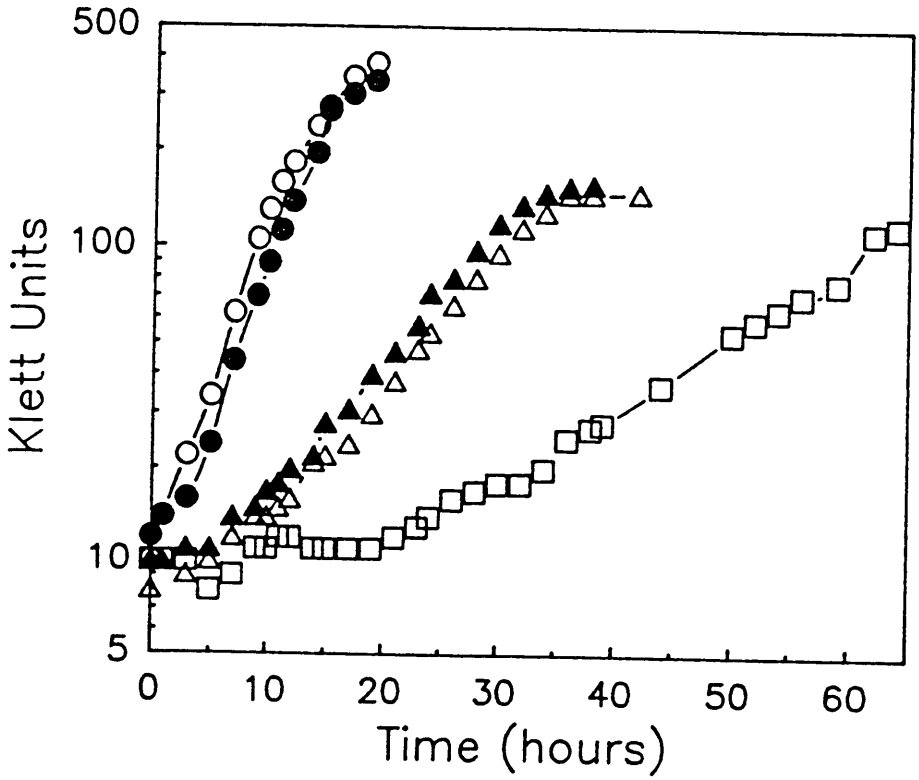

Figure 6. Growth rates of Nif$^+$ strains constructed during this study. Open circle = wildtype, closed circle = DJ192, open triangle = DJ204, closed triangle=DJ234, open box = DJ118.

Table 3. Complementation with isolated FeMo-co of extracts of *A. vinelandii* *nifE* mutant strains.

Strain	Genotype	Activity[a] -FeMo-co	Activity[a] +FeMo-co
DJ35[b]	Δ *nifE*	0.1	15.1
DJ118	*nifE*124CS	0.2	6.7
DJ119	*nifE*250CS	0.2	12.5

[a]Nanomoles of ethylene formed per minute per milligram of crude extract protein in the presence or absence of added FeMo-co. All values represent the average of three determinations. All activity measurements were performed in the presence of excess purified *A. vinelandii* Fe protein.

[b]Data from reference 12.

to an altered cofactor, but this is the result that would be observed if such a cofactor were held only loosely by the MoFe protein and could be displaced by the wild type cofactor.

These possibilities can be distinguished with the use of the in vitro FeMo-co biosynthesis system. If an altered cofactor is being produced then addition of excess amounts of this altered cofactor to purified apo-MoFe protein would not be able to reconstitute normal levels of MoFe protein activity. Conversely, if normal levels of MoFe protein activity were reconstituted, a slow rate of transfer might be distinguished from a slow biosynthesis of FeMo-co by Mo binding studies. Serine was chosen for this substitution because it is not likely that this alteration will result in a structural distortion of the polypeptide. Additionally, it is possible that the sulfur ligands to a cluster might be replaced by oxygen ligands, and that this replacement might have some distinguishable effect.

It is interesting that this strain is capable of any diazotrophic growth, since the corresponding alteration of MoFe protein α-subunit cys154 to serine resulted in a completely Nif^-_strain (13). One possible explanation for this difference is that in the nifE product serine can donate an oxygen ligand to a metal cluster while in the nifD product this donation is not sufficient for MoFe protein activity.

An additional cys-to-ser strain was constructed by altering nifE cys250, which corresponds to the interspecifically conserved α-subunit residue cys275. This residue is proposed to play a role in FeMo-co binding, and the strain carrying this alteration (DJ119) is incapable of diazotrophic growth. MoFe protein activity can be reconstituted by

the addition of purified FeMo-co (see Table 2), and it is likely that this strain is simply not capable of synthesizing the cofactor. The corresponding alteration in the MoFe protein α-subunit, nifD275cys-to-ser, was also incapable of diazotrophic growth (13).

Another set of experiments focused on altering the nifE product in such a way that its proposed FeMo-co binding region progressively more closely resembled the proposed FeMo-co binding region of the α-subunit of the MoFe protein. The purpose of these studies was to attempt to trap the cofactor or an intermediate on the nifEN complex. Using this rationale, the nifE residues val249, ser251, and lys252 were sequentially substituted with histidine, tyrosine, and arginine, respectively. These substitutions make the nifE product cys250 region progressively more like the interspecifically conserved cys275 region of the α-subunit.

The substitution of histidine for valine at position 249 in the nifE product did not significantly affect diazotrophic growth capability (Figure 6, strain DJ192). This seems to rule out the possibility that this histidine (α-subunit his276) provides an N donor ligand to the FeMo-co in the MoFe protein and that this difference is responsible for the irreversible binding of the cofactor to the MoFe protein. When the additional substitution of a tyrosine for the serine at position 251 was made, the diazotrophic growth rate of the resulting strain was noticeably slowed (Figure 6, strain DJ234). This result is puzzling since an alteration in the α-subunit which is designed to make it mimic the nifE product (nifD276tyr-to-ser) does not seem to be affected in terms of diazotrophic growth rate (12). Whether or not this aromatic

residue is involved in the transfer of FeMo-co to the MoFe protein could be studied by Mo trapping studies or by introducing both substitutions (nifE251ser-to-tyr and nifD276tyr-to-ser) into the same strain and seeing whether this strain is capable of growing without a fixed N source.

The additional substitution of an arginine for nifE252lys in the double mutant strain (resulting in a nifE249his/251tyr/252arg mutant) did not further affect the diazotrophic growth rate (Figure 6, strain DJ204).

NIFN::LACZ STUDIES

INTRODUCTION

A great deal is known about the transcriptional regulation of nitrogen fixation genes in both Klebsiella and Azotobacter by levels of intracellular nitrogen. There is a two-tiered cascade of regulation in which three genes, ntrABC, regulate expression of the operon nifLA, whose gene products are responsible for control of the individual nif operons. Nif promoters have a consensus sequence at -24, -12, which was first recognized in K. pneumoniae (6) and has since been extended to Azotobacter, Rhizobium, Azorhizobium, Bradyrhizobium, Thiobacillus, and Desulfovibrio (55, and references therein). The ntrA gene product is an RNA polymerase sigma factor which recognizes the -24, -12 consensus sequence of nif promoters (37). In Escherichia coli the ntrB product catalyzes phosphorylation of the ntrC product under N-limiting conditions and dephosphorylation when nitrogen is in excess. The nifLA operon has two weak ntrC binding sites at -142 and -163 and this operon is positively regulated by ntrC-product binding there (103).

NifA-product is a positive regulator of transcription from nif-promoters, and nifL acts as a negative regulatory molecule. The nifL- and nifA-products share sequence identity with the ntrB- and ntrC-products, respectively; however, nifL in-frame deletion experiments have shown that nifL-product can only inactivate the nifA-product, which it does in response to increased O_2 or N levels (3).

Transcriptional regulation is also taking place in response to intracellular Mo and V concentrations, but the mechanism of this regulation is not yet understood.

In addition to transcriptional regulation, some of the nif gene products are unstable except in the presence of other nif products. For example in K. pneumoniae it has been found that the nifD and the nifK products, and the nifE and the nifN products, respectively, are unstable except in the presence of their complement protein (70). However, this phenomenon has not been observed with respect to the A. vinelandii nifD and nifK gene products (72).

DNA sequence analysis of the nif region from A. vinelandii indicates that translational coupling of adjacent nif genes occurs frequently (44). Gene groups which possess overlapping translation initiation and termination signals include nifT-nifY, nifE-nifN-nifX, nifU-nifS, nifV-ORF7, ORF8-nifW, and nifZ-nifM-ORF9 (44).

In order to study regulation at a translational level it is necessary to have some way of determining the presence and quantity of an individual gene product in mutant strains relative to the wildtype strain. One method of making such assays rapid and simple is to construct a gene fusion in which a marker gene such as lacZ is fused in-frame with the gene coding for the product of interest. In this way a fusion protein will be produced which is assumed to be regulated in the same manner as the gene being studied but which has the additional characteristic of being simple to assay for. Another type of fusion involves using the promoter region of lacZ along with the gene itself so that one is able to assay the transcriptional regulation of a particular gene by the production of β-galactosidase.

Translational lac-fusions have been used by many groups to study regulation of the nif operons. Such fusions have been introduced

using Mulac phages to study the ammonia and oxygen regulation of all of the nif operons in K. pneumoniae (24,50), as well as cloned onto broad host range plasmids and used to study regulation in A. vinelandii by K. pneumoniae nif regulatory elements (47).

In this study the gene encoding β-galactosidase, lacZ, was fused in frame to the carboxy-terminal end of nifN to produce a gene whose expression was under the same translational control as nifN but which encoded a hybrid protein whose amino-terminal end was composed of approximately 70% of the nifN gene product and whose carboxy-terminal end was composed of β-galactosidase. The resultant hybrid protein was enzymatically active with respect to synthetic chromogenic substrates of the β-galactosidase and thus could be quantitatively assayed.

The new hybrid gene was introduced into the chromosome of A. vinelandii via a double recombination event. It is important that the gene fusion be introduced chromosomally in a single copy rather than on an extra-chromosomal plasmid as the elevation of copy number of the nifH promoter has been shown to unbalance nif gene expression, presumably by sequestering the available nifA-product (positive activator) molecules (14).

The nifN::lacZ fusion was introduced into the wildtype strain and a variety of strains carrying specific deletions or deletions and insertions in other of the nif genes. Comparisons of the β-galactosidase activity in these strains allowed comparisons of rates of nifN translation and thus the determination of any effects of the deletion of specific genes on nifN translation.

MATERIALS AND METHODS

Materials. All restriction endonucleases and the large fragment of DNA polymerase I used in this study were purchased from Bethesda Research Laboratories (Gaithersburg, MD)or New England Biolabs (Beverly, MA). 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) was purchased from Bethesda Research Laboratories and cesium chloride, p-nitrophenyl-β-D-galactopyranoside (PNPG), ethidium bromide, toluene, and rifampicin were purchased from Sigma Chemical Company (St. Louis,MO).

Construction of *nifN*::*lacZ* fusion. Plasmid pDB54 (11), which has a 6.3-kb BglII insert spanning the *nifENX* region of *Azotobacter vinelandii* cloned into the BamHI site of pUC8, was digested with XhoI and the resultant 7.2-kb band electroeluted from an agarose gel. The 6.2-kb *lacZ*-containing SalI fragment was eluted from a SalI-digest of pSKS105 (85) and ligated into the XhoI site of the pDB54 derivative to produce pDB130, which was amplified and purified in *E. coli* as described (11).

Transformation of *A. vinelandii* and construction of mutant strains. Wildtype and various strains of *A. vinelandii* carrying specific deletions within *nif* genes were transformed in liquid cultures as described by Page and von Tigerstrom (63) with pDB130. Mutants which had taken up the plasmid and recombined the gene fusion into their chromosome were initially selected by their ability to produce blue colonies on modified Burk's medium plates (93) with a limiting N source (ammonium acetate to 0.027 mg/ml) that had been overlayed with X-gal. Colonies from this initial selection were picked and streaked again until all progeny colonies were blue, indicating that all copies of the chromosome which were expressed carried the *lac* fusion. One strain,

DJ139, was constructed in the opposite order, by transforming DJ107 (*nifN*::*lacZ*) with pDB167 (deletion and insertion in *nifA*, see reference 4), outgrowing for 17h in Burk medium to allow for chromosome segregation, and then selecting for colonies which had lost the ability to turn bright blue on Burk medium plates with limiting N and X-gal.

Cell growth and nitrogenase derepression. The strains used in this study were grown in modified Burk's medium (93), which was supplemented to a final concentration of 2.2 mg/ml ammonium acetate when a fixed source of nitrogen was included. For derepression of nitrogenase synthesis and β-galactosidase assays, cells were grown to mid-logarithmic phase and harvested by centrifugation. Harvested cells were resuspended in Burk's nitrogen-free medium, repelleted, and then resuspended in the original volume of Burk nitrogen-free medium and incubated for an additional 3h. All cultures were 100 ml volumes grown at 30°C with vigorous shaking in 500 ml Klett flasks.

β-galactosidase activity assays. Assays were carried out essentially as described in (56). After 3h of derepression, 0.1 ml of cells (OD_{600} noted) were added to 0.9 ml Z-buffer, vortexed briefly, and 0.02 ml toluene was added. Toluene was allowed to evaporate for 45 min at 30°C and 0.8 mg PNPG in 0.2 ml dH_2O was added (time noted). 0.5 ml 1M sodium carbonate was added after a faint color had developed (time noted), and the A_{420} of the supernatant immediately checked with a spectrophotometer. Activity was determined by the following formula:

$$\frac{OD_{420} \times 1000}{OD_{600} \times \text{volume of cells (ml)} \times \text{elapsed time(min)}}$$

RESULTS AND DISCUSSION

A nifN::lacZ gene fusion has been constructed and introduced into the A. vinelandii chromosome by homologous recombination. The derepression of the transcription and translation of this fusion protein is presented in Figure 7.

This gene fusion is regulated in the same manner as wildtype nifN with respect to NH_3^+ and encodes an enzymatically active fusion protein which can be assayed. Thus, this gene fusion is useful for assaying the regulation of nifN under different conditions. This gene fusion has been introduced chromosomally to several strains of A. vinelandii which carry deletions in other nif-encoding genes.

The β-galactosidase activities of three strains which carry the nifN::lacZ gene fusion are presented in Table 3. The values shown are for cultures derepressed for nitrogen fixation. As expected, the strain possessing a nifA deletion and insertion showed significantly less activity than the other strains. This effect is due to the lack of positive transcriptional regulation by the nifA gene product. Unexpected was the result that any activity was present at all. Similar β-galactosidase activities were observed in all of the nifN::lacZ strains when they were assayed during growth in media containing a fixed nitrogen source, and this seems to indicate that low-level transcription and translation of nifEN may be taking place even when A. vinelandii is not growing diazotrophically. No activity was observed when wildtype cells were assayed, in agreement with the fact that A. vinelandii doesn't produce a β-galactosidase (21), and it is therefore unlikely that the observed activity is due to non-enzymatic cleavage of the

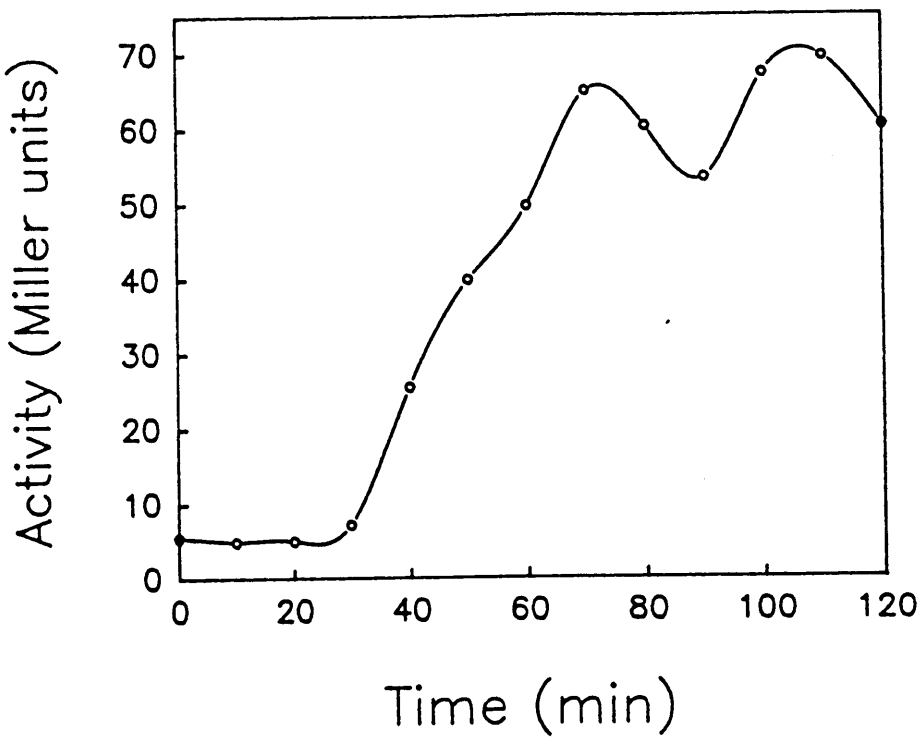

Figure 7. Derepression of strain DJ107 (nifN::lacZ).

Table 4. β-galactosidase activities of various strains carrying the nifN::lacZ gene fusion.

Strain	Genotype	Activity[a]
DJ107	nifN::lacZ	51.6
DJ109	ΔnifH, nifN::lacZ	66.6
DJ139	ΔnifA::kan^r, nifN::lacZ	3.8

[a]Activity is expressed in Miller units (reference 56)

chromophore used in the assay.

It has been observed that some strains which do not produce an active Fe protein do not produce FeMo-co either. It has been proposed that the Fe protein may bind a nifEN products complex and carry out a reductive role analogous to the role it plays in N_2 reduction, but strains have been described which lack Fe protein activity and still accumulate normal amounts of nitrogenase (see literature review, under the role of nifH in FeMo-co biosynthesis).

Results obtained with the nifN::lacZ gene fusion indicate that nifH or its encoded polypeptide do not regulate the transcription or translation of nifEN. No major difference was observed between the fusion protein activities of a strain which carried a disrupted nifH gene and one in which nifH was intact. This result is not surprising considering the known redox role of the Fe protein in nitrogenase turnover and the relationship between the nifEN products complex and the MoFe protein, as the simplest explanation of the Fe protein's role in FeMo-co biosynthesis is that of a reductive agent of the nifEN products complex.

Several other strains were constructed which underwent only initial screening as they appeared to exhibit the same fusion protein activity as did DJ107. Such strains include DJ108 (nifN::lacZ, deletion in nifK), DJ110 (nifN::lacZ, deletion in nifH), DJ111 (nifN::lacZ, deletion in nifD), and DJ145 (nifN::lacZ, deletion in nifKY).

The range of fusion protein activities in these and in other gene fusions our lab has constructed in A. vinelandii differs strikingly from the range of activities observed in other nif-lac fusion studies

utilizing K. pneumoniae. While β-galactosidase activities of derepressed strains in this study were all below 100 Miller units, results in other labs have found activities from 220-3,500, including 950 for a nifN::lacZ fusion (24), from 70-2,400, including 550 for a fusion with nifN (50), and from 800-2,400 Miller units (47). It is not thought that this discrepancy is significant due to the fact that our studies have involved chromosomal integration of the gene fusions while the other studies have involved the use of fusions on extrachromosomal plasmids. Also, a fusion protein is likely to have a different specific activity than wildtype β-galactosidase, so that comparisons of specific activities of different fusion proteins or between one fusion protein and wildtype β-galactosidase are meaningless. One study which isolated three different nifJ::lacZ fusions noted that under derepressing conditions, β-galactosidase activities varied up to 40-fold (50).

The nifN::lacZ fusion protein should be easily purified by exploiting both its large size and its β-galactosidase activity. β-galactosidase can be purified on a preparitive scale in a two step procedure which utilizes a substrate analogue inhibitor, p-aminophenyl-β-D-thiogalactopyranoside (92). The substrate inhibitor is immobilized on a agarose matrix and the enzyme will bind tightly to this ligand at a neutral pH, whereas elution can be achieved at a pH of 10. Once purified, the fusion protein will be useful for the generation of antibodies to the wildtype nifN product, and these antibodies should be useful for additional study of the role of nifN and nifE in the biosynthesis of FeMo-co.

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