Characterization and site-directed mutagenesis of
NifU from *Azotobacter vinelandii*

by

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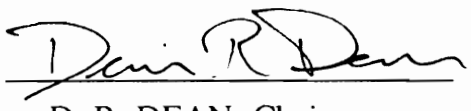
APPROVED



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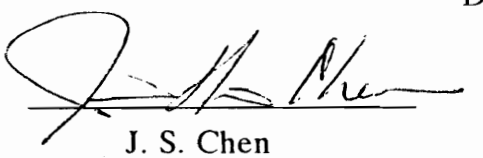

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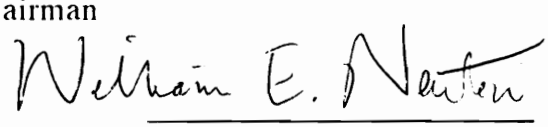

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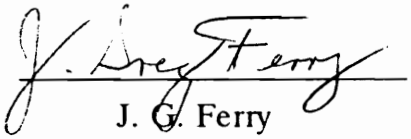

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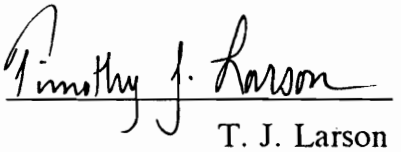

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Biochemistry and Anaerobic Microbiology

(ABSTRACT)

In order to elucidate the function of the *nifU* gene product in nitrogenase maturation in *Azotobacter vinelandii*, the gene product has been hyperexpressed in *Escherichia coli* and characterized by various biophysical techniques. Following the initial characterization, site-directed mutagenesis of conserved cysteinyl residues was performed in order to gain further insight into the structure/function relationship of NifU.

Both the Fe protein and the MoFe protein of nitrogenase require processing by additional *nif* genes including *nifM* (Fe protein), and *nifE, N, B, H, V*, and *Q* (MoFe protein). Two additional genes, *nifU* and *nifS*, are required for the maturation of both nitrogenase component proteins. It has been proposed that they may somehow be involved in metallocluster biosynthesis (Jacobson et al., 1989b). Our laboratory has determined that the *nifS* gene product (NifS) is a pyridoxal-phosphate containing enzyme capable of catalyzing the desulfurization of L-cysteine and can provide the inorganic sulfide necessary for *in vitro* metallocluster biosynthesis of the Fe protein (Zheng, et al., 1993; Zheng, et al., 1994).

Parallel studies involving characterization of NifU have determined that NifU is a [2Fe-2S] cluster containing protein which could be involved in the mobilization of Fe for metallocluster biosynthesis. There are nine conserved cysteinyl residues in NifU from *A. vinelandii* and *Klebsiella pneumoniae* at positions 35, 62, 106, 137, 139, 172, 175, 272, and 275 (numbers refer to the *A. vinelandii* sequence). Mutants with substitutions at seven of these positions result in a Nif slow phenotype while substitutions at two positions had no effect on diazotrophic growth. In contrast, a *nifU* deletion strain is Nif minus. Four of cysteinyl residues function as ligands to the [2Fe-2S] cluster and three have an unknown function. Two lines of evidence indicate that NifU is a fused, multi-functional protein. 1) Site-directed mutagenesis of conserved cysteinyl residues indicate that there are structural as well as functional differences between different groups of residues. 2) Truncated forms of NifU have been found in many different types of bacteria, yeast and humans which do not fix nitrogen. In all these alternate forms of NifU, the cysteinyl residues are also strictly conserved. The different regions within NifU might play a role(s) in the mobilization of Fe for metallocluster biosynthesis.

ACKNOWLEDGEMENTS

I am deeply indebted to my major advisor, Dennis R. Dean, for his guidance during my tenure at the Department of Anaerobic Microbiology. His patience and perseverance will be unforgettable. I am also grateful to my committee members, Dr. Bill Newton, Dr. Jiann-Shin Chen, Dr. Timothy J. Larson, and Dr. J. Greg Ferry, who raised the level of scientific expectation and showed how to meet that expectation. I would like to thank the rest of the Dean lab including fellow students ChulHwan Kim, John Peters, Paul Goodwin and Len Comaratta along with colleagues Limin Zheng and Valerie Cash for making life bearable during my doctoral studies. Also, members of Dr. Newton's laboratory, especially John Cantwell, Joan Shen and Karl Fisher deserve a special thank you for scientific discussions at "Ze Balcony".

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INTRODUCTION

Reduced nitrogen is an essential component of many biomolecules including nucleic acids and proteins. Thus, obtaining usable forms of nitrogen is essential to all living things. Although dinitrogen (N_2) is the predominant element in the Earth's atmosphere, it is inert at room temperature and unusable in this form unless reduced in the presence of a suitable catalyst. Although thermodynamically favorable, the energy required to reduce dinitrogen is very large, as shown in the industrial Haber-Bosch process. This process requires temperatures between 300 and 500°C and pressures of over 300 atmospheres in the presence of an Fe-based catalyst. Nature has overcome this energy intensive problem by reducing dinitrogen to ammonia in a process known as biological nitrogen fixation. This amazing reaction is limited to the procaryotes. These bacteria, known as diazotrophs, supply about 60% of the world's fixed nitrogen compared to 25% from industrial sources and 15% from lightning discharges and UV radiation (Burns and Hardy, 1975).

Diazotrophic bacteria are physiologically diverse and comprise aerobic and anaerobic bacteria from many different genera. In spite of this, the enzyme responsible for nitrogen fixation, nitrogenase, is highly conserved among all of these types of bacteria indicating that the mechanism of nitrogenase is the same. Evidence for a unified mechanism of nitrogen fixation was demonstrated by Emerich and Burris (1978) who surveyed the cross reactivity of the nitrogenase component proteins from eight different bacteria. Nitrogenase activity was detected in 45 of the possible 56 heterologous crosses between obligately aerobic, obligately anaerobic, microaerophilic, facultative anaerobic, photosynthetic and symbiotic bacteria. The stoichiometric reduction of dinitrogen catalyzed by nitrogenase has been established by Simpson and Burris (1984) as:

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$

Another important aspect of nitrogen fixation research involves bacteria that are able to form symbiotic relationships with plants. Members of the genera *Rhizobium* and *Bradyrhizobium* elicit formation of nodules on certain plant roots (legumes) in which they fix nitrogen for the plant. The plant, in turn, provides organic growth substrates for the invading bacterium. Another example of a symbiotic relationship involves the cyanobacterium *Anabaena* and *Azolla* fern. Cavities in the frond of the fern contain nitrogen fixing *Anabaena* which fix nitrogen for the plant. This symbiotic relationship is used as a natural fertilizer for rice plants. Thus, any improvement in the process of nitrogen fixation in these organisms could also improve crop yield.

A major area of focus into the mechanism of nitrogenase concerns the structures, organization and biosynthesis of the nitrogenase metalloclusters which are intimately involved in electron transfer and substrate reduction. These novel iron sulfur structures have been investigated by a number of investigators from many different backgrounds including biochemists, chemists, geneticists, and bioinorganic chemists. This is due to the complex catalytic mechanism which involves protein-protein interaction, electron transport and signal transduction in addition to the unusual substrate reduction properties and novel metal clusters which are unprecedented in biology, features which make nitrogenase an ideal interdisciplinary focal point.

OVERVIEW OF NITROGENASE

Nitrogenase is a two component metalloenzyme composed of the Fe protein and the MoFe protein. Extensive biophysical characterization has revealed that the Fe protein is a γ_2 homodimer ($M_r \approx 60{,}000$; encoded by *nifH*) which contains a [4Fe-4S] cluster bridged between the two subunits. The MoFe

protein is an $\alpha_2\beta_2$ heterotetramer ($M_r \approx 250{,}000$; encoded by *nifDK*) which contains two each of two types of novel metal centers known as the P-cluster (an Fe_8S_8 cluster) and the FeMo-cofactor (Fe_7S_9Mo:homocitrate), see Kim et al., 1992a; Rees et al., 1993a and references therein for some examples. The Fe protein is the sole electron donor to the MoFe protein in a process involving association and dissociation of the two proteins and hydrolysis of two MgATP per electron transferred. In addition to nitrogenase and MgATP, a source of reducing equivalents, protons, and an anaerobic environment are required for nitrogenase turnover.

Figure 1 shows the proposed interaction, according to the Howard-Rees model which is based on the crystal structures of nitrogenase, between the Fe protein homodimer and one $\alpha\beta$ subunit of the MoFe protein from *Azotobacter vinelandii*. The specificity of electron transfer between the Fe protein and the MoFe protein has been suggested to involve a precise docking of the two proteins (Howard and Rees, 1993), such that the [4Fe-4S] cluster of the Fe protein is in closest proximity to the P-cluster of MoFe protein. Biochemical experiments in which the polypeptide environment around the [4Fe-4S] cluster is altered and affect the electron transfer rate during catalytic turnover support this docking model (Peters et al., 1995a). Additional support comes from ADP-ribosylation of the Fe protein from *Rhodospirillum rubrum*. The Fe protein is ADP-ribosylated in this organism by the Transferase (DRAT) and removed by Dinitrogenase Reductase ADP-ribosyl Glycohydrolase (DRAG). Ribosylation of arginine-101 on the Fe protein results in complete inactivation of the nitrogenase enzyme, which occurs under increased NH_3 in the cell (Roberts and Ludden, 1992). This residue is positioned on the surface of the Fe protein in close proximity to the [4Fe-4S] cluster in a region which, according to the Howard-Rees model, is invovled in docking between the Fe protein and the MoFe protein (Georgiadis et al., 1992).

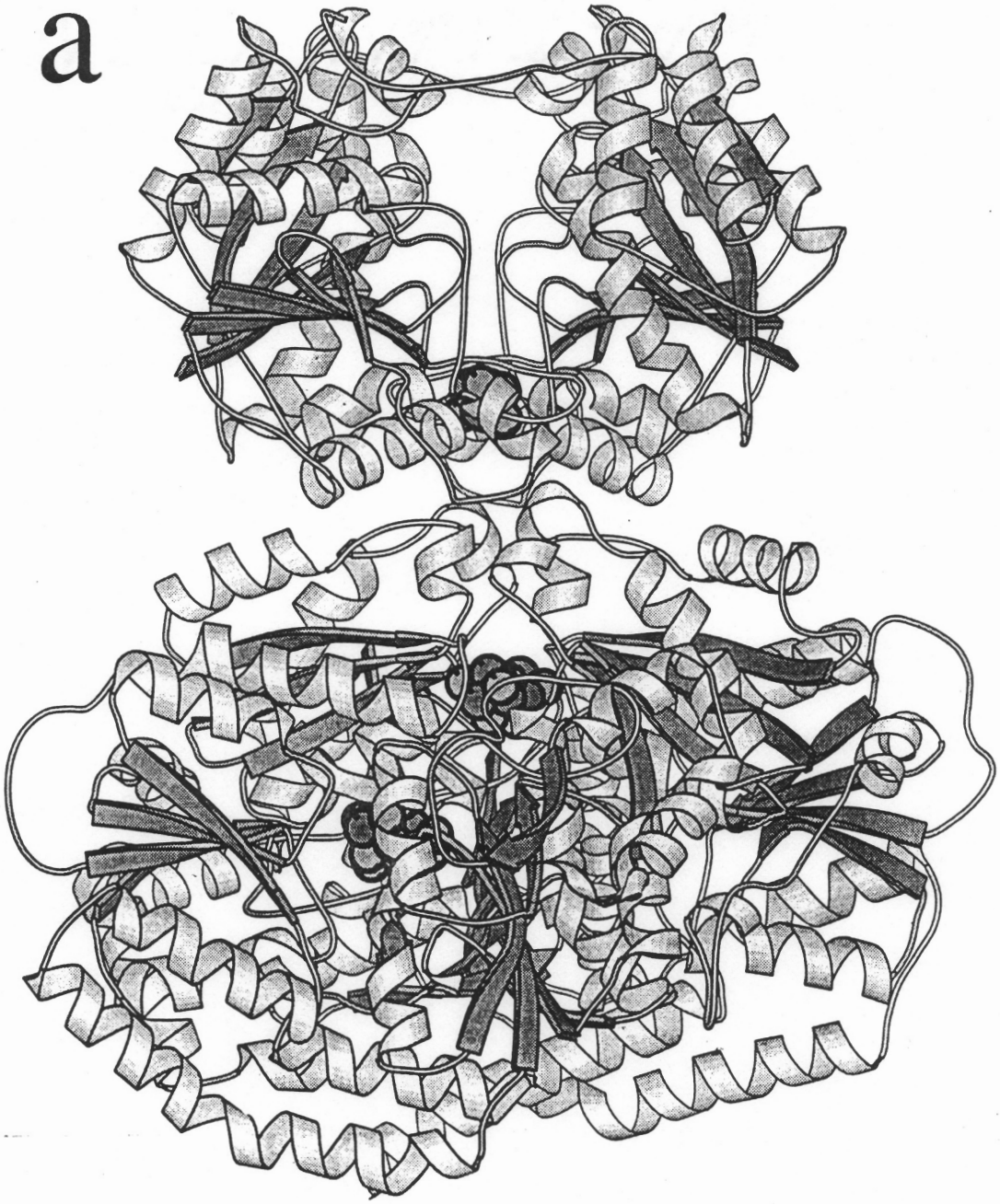
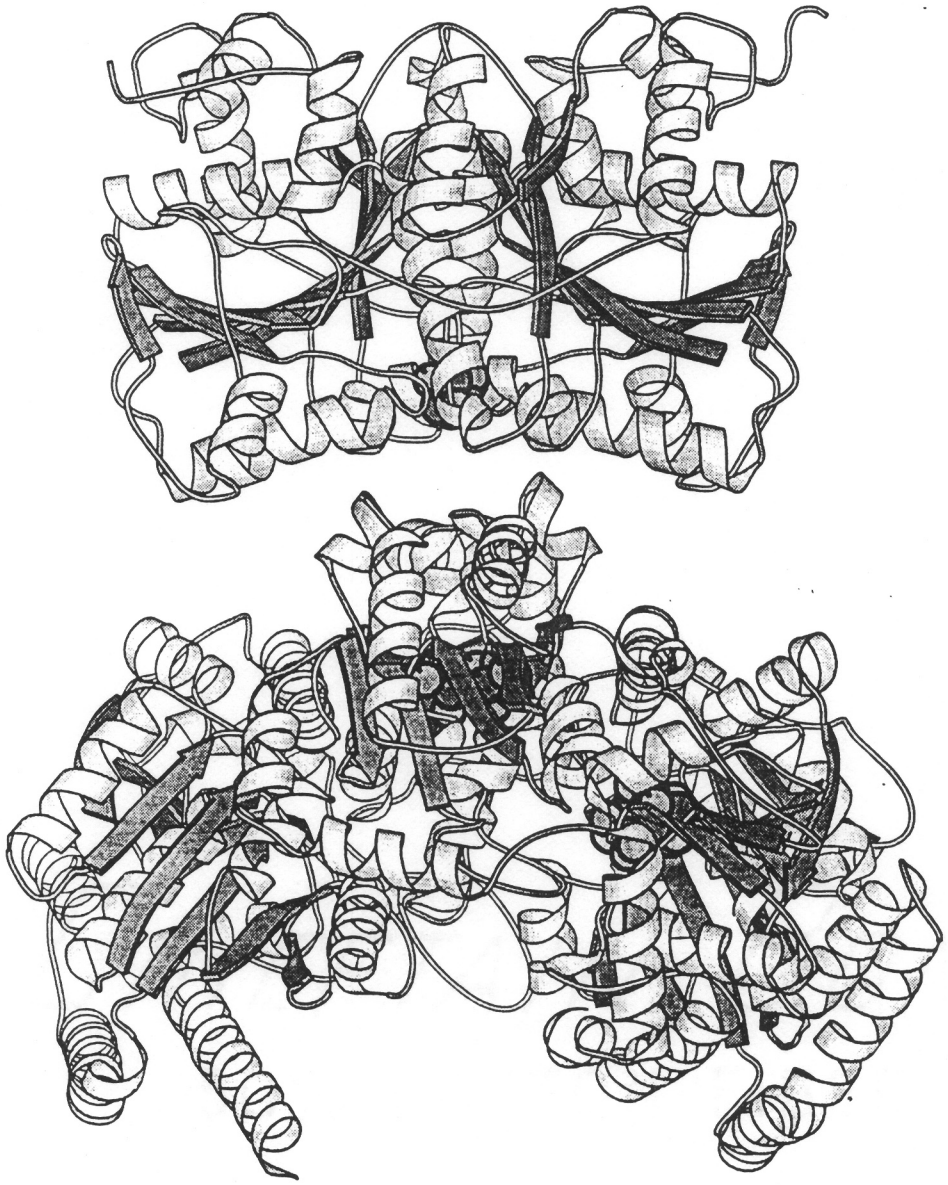


Figure 1. Ribbons diagrams of the *A. vinelandii* Fe protein and the $\alpha\beta$-subunit of the MoFe protein. The two different views, a) and b), represent 90 degree rotations about the y-axis. The metal clusters are represented as space filled models. The view in both a and b shows the Fe protein on top; b shows the two component proteins poised for docking and a shows the docking model proposed by Howard and Rees (1994). Note that the [4Fe-4S] cluster of the Fe protein is in closest proximity to the P-cluster of the MoFe protein. Prepared with the program MOLSCRIPT (Peters, 1995b; Kraulis, 1991).

b



NITROGENASE CATALYSIS

Over the years a consensus model, parts of which have yet to be proven, has evolved for nitrogenase catalysis and is summarized as follows. 1) During catalytic turnover, electrons are delivered one at a time from the Fe protein to the MoFe protein in a process that involves association and dissociation of the component proteins and hydrolysis of at least two MgATP per electron transferred. 2) The Fe protein is the sole electron donor to the MoFe protein, which contains the substrate reduction site. 3) The MgATP binding sites are located on the Fe protein, however, hydrolysis does not occur until formation of a Fe protein-MoFe protein complex. 4) MgATP and the [4Fe-4S] cluster do not come in contact during catalysis. 5) Multiple rounds of electron transfer are required before substrate is reduced. 6) The P-cluster and FeMo-cofactor do not directly interact with one another. 7) The P-cluster can be viewed as an intermediary in electron transfer, acting as an electron acceptor from the Fe and an electron donor to the substrate reduction site, FeMo-cofactor. 8) The MoFe heterotetramer acts as two independent $\alpha\beta$ domains, each containing one one P-clusters and oneFeMo-cofactor. 9) The substrate reduction site(s) is quite flexible, being able to reduce a wide variety of substrates including H_2, N_2 and acetylene.

These catalytic features open up a series of questions which center around the structure/function relationship of the nitrogenase component proteins. Some of these questions are: a) What is the role of MgATP hydrolysis in electron transfer and component protein interaction? b) How does component protein interaction facilitate MgATP hydrolysis? c) What determines the electron transfer event and precise electron path? d) Does the P-cluster store electrons prior to substrate binding and reduction? e) At what redox state(s) do substrates bind to FeMo-cofactor? f) What role(s) does the polypeptide environment around these metal centers play in electron transfer, protein interaction, catalysis and

biosynthesis?

Addressing these questions has involved a multi-disciplinary effort which has focused on biophysical characterization of the metal centers of nitrogenase and biochemical-genetic methods using gene replacement strategies to alter the polypeptide environments surrounding the metal centers, protein-protein interaction sites and metallocluster biosynthesis. Though not all of the above questions have been answered, considerable insights have been gained in all these areas and are summarized in the reviews below. Recent reviews concerning biochemical and kinetic aspects of nitrogenase can be found in Burgess, 1990; Burris, 1991; Newton, 1992; Smith and Eady, 1992; and Yates, 1992; in addition to overviews concerning mechanistic implications, insights into biosynthesis and the x-ray crystal structures can be found in Dean et al., 1993; Mortenson et al., 1993; Rees et al., 1993a; Eady and Leigh, 1994; Howard and Rees, 1994; Kim and Rees, 1994.

Fe PROTEIN STRUCTURE AND FUNCTION

Figure 2 shows the ribbons diagram of the Fe protein structural model. The Fe protein contains the site of MgATP hydrolysis while its [4Fe-4S] cluster is the sole electron donor to the MoFe protein. This cluster is very solvent exposed as determined by x-ray crystallography and spectroscopic studies (Kim and Rees, 1992a; Morgan et. al., 1990). Binding of MgATP results in a reduction in the E_m from $\approx$ -300 to -400 mV (Watt et al., 1986) and, based on the crystal structure, is $\approx$ 20 Å from the cluster (Georgiadis, et al., 1992). Binding of ATP and ADP result in increased exposure of the [4Fe-4S] cluster to iron chelators and changes the line shape of its EPR spectrum from axial to rhombic (reviewed in Mortenson et al., 1993). The polypeptide fold found on the Fe protein, GXXXXGKS known as the Walker A motif, is common for nucleotide binding proteins (Shulz, 1992). This motif has been associated with amino acids 9-16, site-directed mutagenesis of

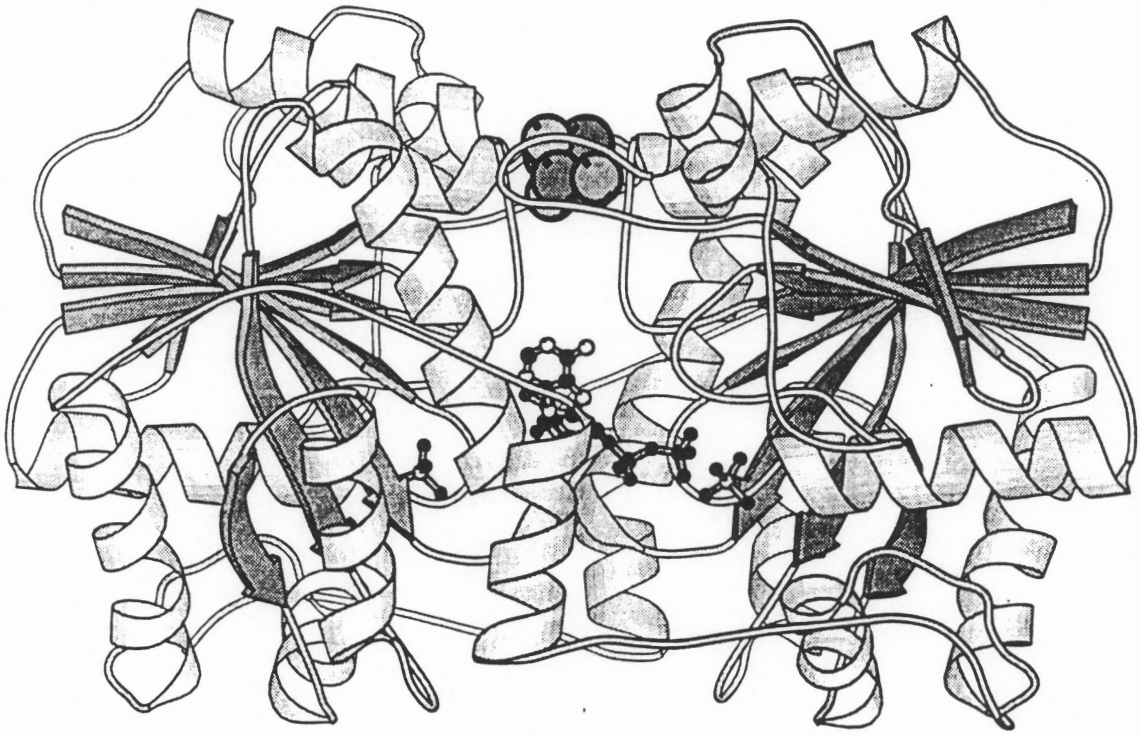


Figure 2. Ribbon diagram of the *A. vinelandii* Fe protein homodimer (Georgiadis, et al., 1992). The [4Fe-4S] cluster is shown at the top of the model bridged between the two subunits. An ADP is shown spanning the two subunits in a ball and stick model. Two molybdate ions (which bind to amino acid residues known to bind terminal phosphate of ATP) are represented in ball and stick form indicate residues which are present in the Walker A motiffs on each subunit. Prepared with the program MOLSCRIPT (Peters, 1995**b**; Kraulis, 1991).

residues in this region support this motif as the nucleotide binding region (Seefeldt et al., 1992; Seefeldt and Mortenson, 1993). Additional residues involved in nucleotide binding include Asp-125 (Wolle et al., 1992) and Ala-157 (Gavini and Burgess, 1992). These residues are located at the interface between the two Fe protein subunits below the [4Fe-4S] cluster (Georgiadis et al., 1992). Increased solvent exposure of the [4Fe-4S] cluster upon binding of MgATP is logical if this event facilitates electron transfer and supports other lines of evidence which indicate that reduction in redox potential results primarily from protein conformational changes as opposed to direct binding to the metal cluster (Yates, 1992). It has been proposed that MgATP binding and hydrolysis results in an allosteric switching behavior between two conformational states of the Fe protein. This phenomenon is also found in cellular regulation (Pai et al., 1993), protein synthesis (Jurnak et al., 1985; Kjeldgaard and Nyborg, 1992), DNA recombination (Story and Steitz, 1992), membrane transport (Karkaria et al., 1990; Riordan et al., 1990), and molecular motors (Rayment et al., 1993).

P-CLUSTER STRUCTURE AND FUNCTION

The P-cluster is a unique Fe_8S_8 cluster which is extruded from the MoFe protein in thiol based solvents as 2[4Fe-4S] clusters (Kurtz et al., 1979). This early result is amazingly accurate when compared to the recent x-ray crystal structure (Figure 3), which shows that the P-cluster consists of 2[4Fe-4S] cluster pairs bridged by two cysteine thiol ligands (α-Cys-88 and β-Cys-95) and a disulfide bond (Kim and Rees, 1994; Chan et al., 1993). The P-cluster is located between the α and β subunits of the MoFe protein such that the remaining ligands (α-Cys-62, α-Cys-154, β-Cys-70 and β-Cys-153) bind to a specific [4Fe-4S] cluster (one half of the P-cluster) from the same subunit. Substitution of these ligands by serine or alanine results in loss of activity and some of these altered proteins do not form the $\alpha_2\beta_2$ tetramer which indicates that P-cluster formation is required for

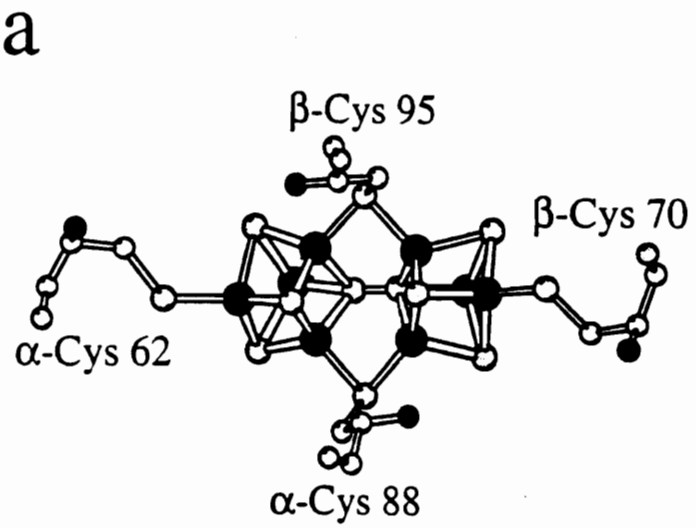


Figure 3. a) Ball and Stick models of the P-cluster and organization of its coordinating cysteinyl residues (Kim and Rees, 1994). For clarity in presentation, all coordinating residues are not shown on each view but the location of which can be imagined by rotating 90 degrees for view b). Fe atoms are represented in black (larger filled circles), S atoms are represented as light gray, carbon atoms are represented as open circles and nitrogen as smaller filled circles. Prepared with the program MOLSCRIPT (Peters, 1995b; Kraulis, 1991).

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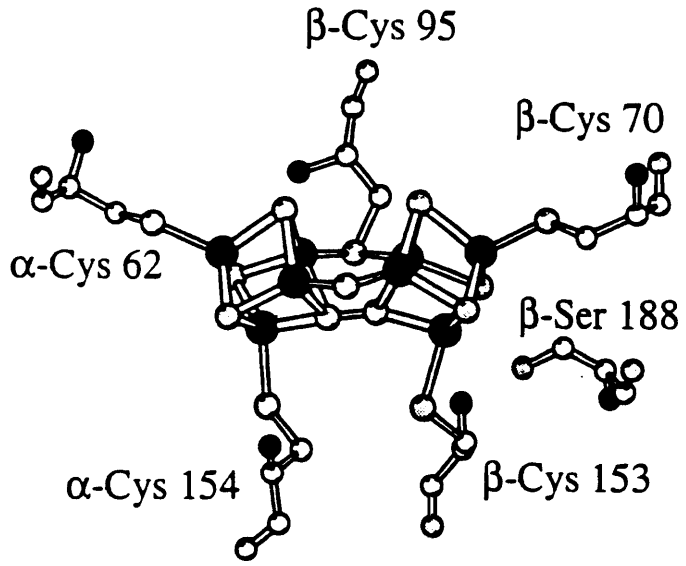

proper MoFe assembly (Govenzensky and Zamir, 1989; Kent et al., 1990). Interestingly, all the Fe atoms in the P-cluster are in the ferrous state, as determined by Mossbauer spectroscopic experiments, which is unprecedented for an iron sulfur protein. The function of this structure is generally believed to be one of electron acceptance and storage with ultimate delivery to the substrate reduction site on FeMo-cofactor (Dean et al., 1990; Lowe et al., 1993). Evidence to support this role comes from biophysical and genetic experiments. The P-cluster is able to undergo a two electron oxidation step at E_m -307 mV and a one electron oxidation at E_m +90 mV suggesting that multiple electron transfer is possible *in vivo* (Pierik, et al., 1993). Site-directed mutagenesis of β-Cys-153 to serine results in a MoFe protein with reduced activity only under high electron flux conditions, ie., with saturating levels of Fe protein compared to MoFe protein (May et al., 1991). Conditions of low electron flux, ie., with limiting amounts of Fe protein compared to MoFe protein, its substrate reduction rates were equal to that of wild type. The substrate reduction site remained undisturbed as determined by MCD and EPR spectroscopies (K. Fisher, W. Newton, M. Johnson, unpublished data). These authors suggest that under high electron flux conditions the electron transfer ability between the P-cluster and FeMo-cofactor is compromised in the altered protein.

The recent x-ray crystallographic data have indicated that β-Tyr-98 is located between the P-cluster and FeMo-cofactor on one of four parallel helices which could form an electron transfer path between these two metal centers (Kim and Rees, 1992b). Substitution of this residue for histidine results in maximum specific activity at a lower Fe protein to MoFe protein component ratio than with wild type MoFe protein (Peters et al., 1995). The steady state activity for this altered protein had 30% of the acetylene reduction activity and 50% proton reduction activity, as determined under Argon, compared to wild type. This result suggested substantial impact on protein turnover and a backup of electrons

between the P-cluster and FeMo-cofactor. These authors caution that this evidence does not necessarily prove that β-Tyr-98 is directly involved in intramolecular electron transfer, merely that it is in a path of possible electron transfer.

FEMO-COFACTOR STRUCTURE AND FUNCTION

FeMo-cofactor was first discovered by Shah and Brill (1977) as a metal containing component stable in N-methyl formamide extracted from acid denatured MoFe protein. The FeMo-cofactor can be imagined as two clusters composed of one 4Fe-3S and one Mo-3Fe-3S bridged by three nonprotein sulfide ligands (see Figure 4; Hoover et al., 1987; Kim and Rees, 1992b). This metal framework contains one molecule of homocitrate which coordinates Mo through one carboxyl and one hydroxyl oxygen. Interestingly, this large cluster is ligated to the MoFe protein with only two ligands, α-Cys-275 (to Fe1) and α-His-442 (to Mo). The tetrahedral coordination of Fe1 and an octahedral coordination of the Mo atom are typical for metalloproteins (Johnson, 1994; Cammack, 1992). An unusual feature of FeMo-cofactor is the trigonal coordination of the six Fe atoms that bind to the bridging sulfides. The unsaturation of these Fe atoms has led to speculation that they may be involved in substrate binding (Kim and Rees, 1994). That FeMo-cofactor is most likely the catalytic, substrate reduction site was shown by several different lines of evidence, two of which are mentioned here. 1) MoFe protein that contains a structurally altered FeMo-cofactor, where citrate is substituted for homocitrate, exhibits altered catalytic properties (Hawkes et al., 1984; Liang et al., 1990). 2) Amino acid substitutions placed in the FeMo-cofactor vicinity have altered catalytic and spectroscopic properties (Scott et al., 1991; Kim et al., 1995).

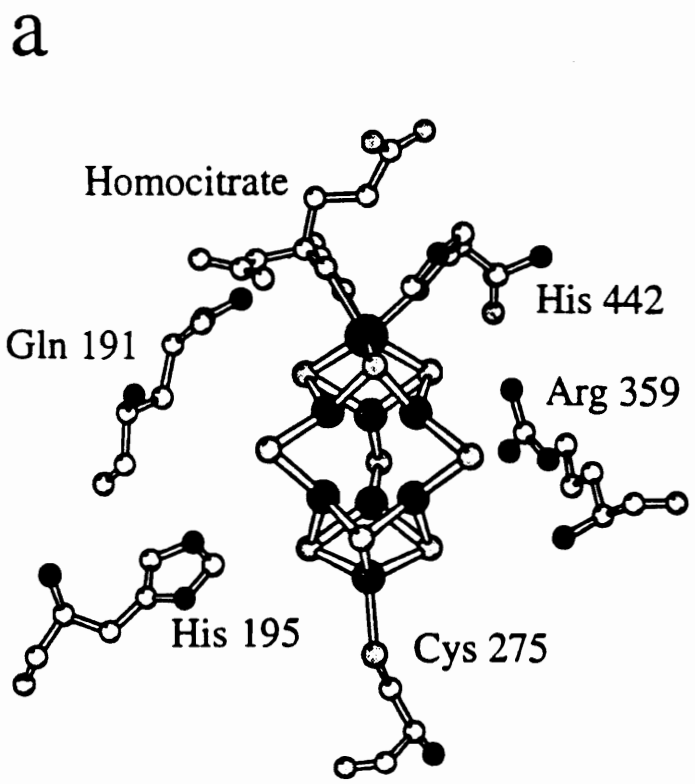

Figure 4. Ball and Stick models of FeMo-cofactor including ligands and select residues in polypeptide environment. The views a), b), and c) are 90° rotations around the y-axis. Mo atom is represented as the large filled circle coordinated by homocitrate. Fe atoms are represented as filled circles within FeMo-cofactor, S atoms are light gray, nitrogen atoms are smallest filled circle and carbon atoms are open circles. Prepared with the program MOLSCRIPT (Peters, 1995c; Kraulis, 1991).

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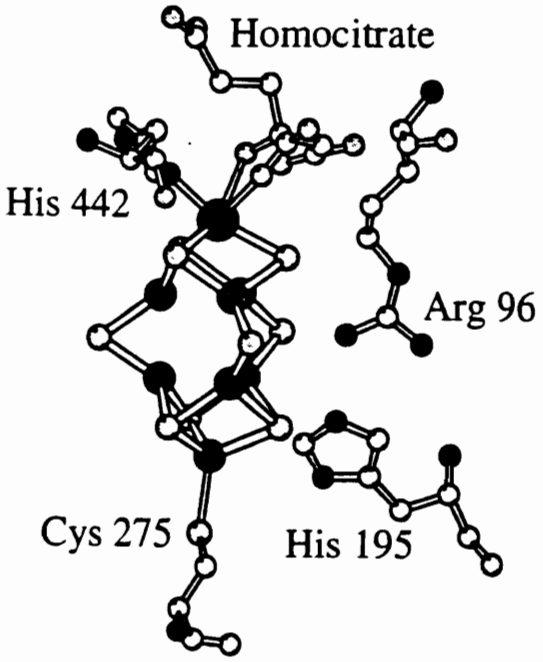

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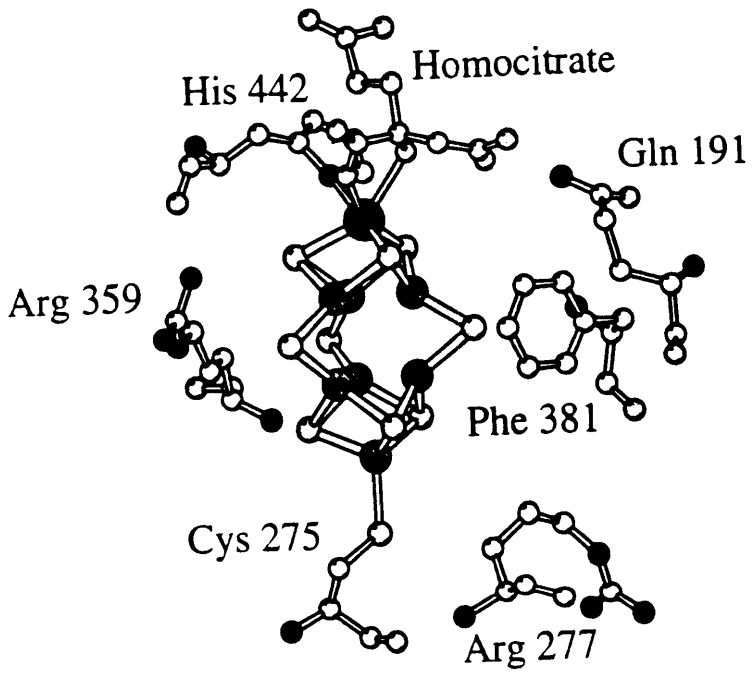

Figure 5 summarizes the currently accepted electron transfer path through nitrogenase along with the steps involving MgATP hydrolysis and substrate reduction.

THE MATURATION OF THE NITROGENASE PROTEINS

The biochemical complexity of nitrogenase is reflected in the genetic organization and in the regulation of accessory genes required for nitrogenase maturation. For example, the primary translation products of the nitrogenase structural genes (*nifH, D,* and *K*) are not active, but require a consortium of *nif*-associated gene products for complete maturation of the nitrogenase structural genes to their active forms. There are 20 *nif* genes in *Klebsiella pneumoniae* and in addition to the structural genes, are genes which are involved in transcriptional regulation, cluster biosynthesis, processing and electron transfer (reviewed in Ludden, 1992; Dean and Jacobson, 1992).

Figure 6 shows the gene organization of the nitrogen fixation *(nif)* genes from *K. pneumoniae* and *A. vinelandii.* The *nif* genes from the type strain *K. pneumoniae* are clustered in a single region of 25kb on the genome. The sequential arrangement of the *nif* genes is very similar (if not exact) in the two genera, although the spatial arrangement varies. Also, because deletion of some of these genes is not lethal, ie., *nifS*, homologs may appear elsewhere in the chromosome (Jacobson et al., 1989). The *nif* cluster from *A. vinelnadii* is interspersed with 11 open reading frames but deletion mutants indicate that none of these are absolutely required for diazotrophic growth (Jacobson et al., 1989). However, their expression is most likely under *nif* regulation since all are preceded by *nif*-specific promoters. This makes sense in light of the fact that their products may facilitate nitrogenase biosynthesis indirectly. For example, orf7 bears sequence homology to o-acetylserine synthase, an enzyme which catalyzes the last step of the cysteine biosynthetic pathway (Evans, et al., 1991). This orf

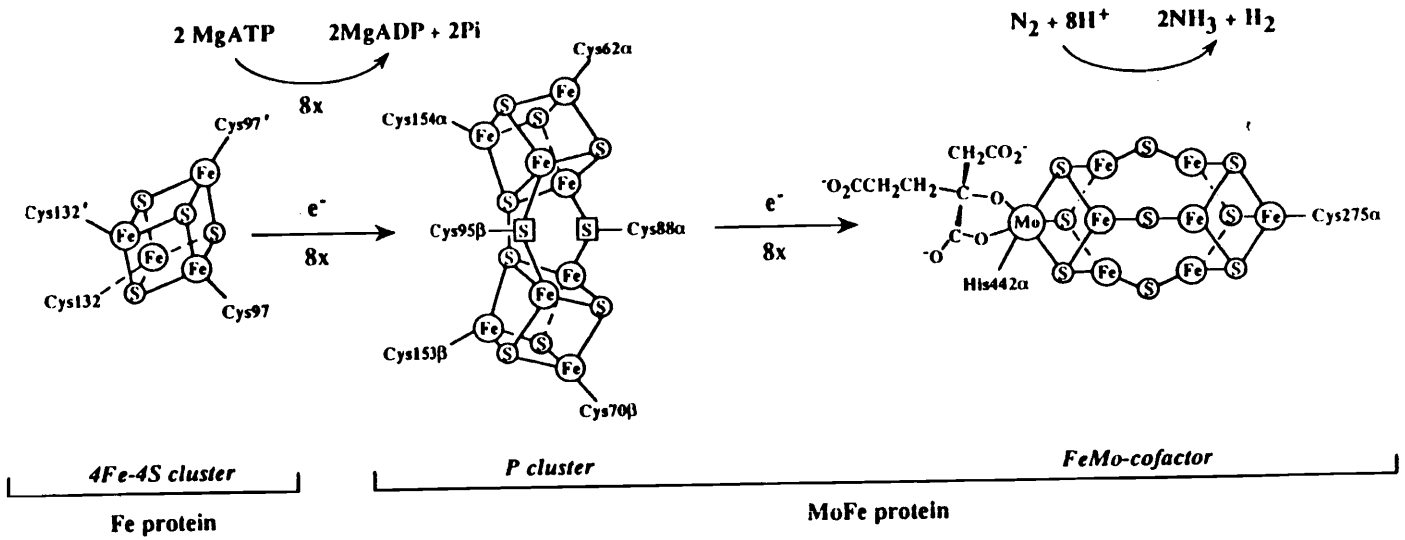

Figure 5. Organization of nitrogenase metal clusters showing electron transfer path, sites of MgATP hydrolysis and substrate reduction. Taken from Dean et al., (1993).

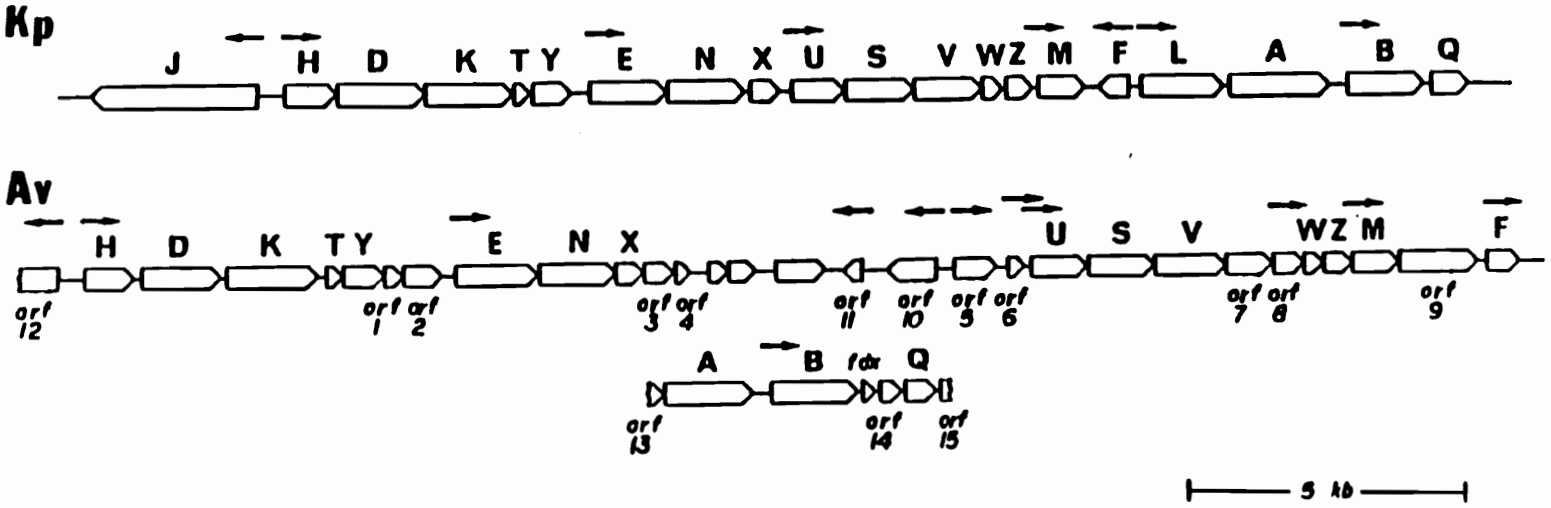

Figure 6. Comparison of the physical organizations of the *nif* genes from *K. pneumoniae* and *A. vinelandii*. Arrows indicate the direction of known or proposed transcription initiation sites. Genes from *A. vinelandii* with no homologs in the *K. pneumoniae nif*-cluster are labelled as orfs with the number designation being arbitrary. Note the similar sequential arrangement of the *nif* genes between the two organisms. Also, note that gene products devoted to regulatory and electron transport roles flank gene products devoted to biosynthesis (see text). Details for development of the *K. pneumoniae* map can be found in Arnold et al., (1988) and development of the *A. vinelandii* map can be found in Jacobson et al., (1989), Bennett et al., (1988), Joerger and Bishop (1988) and references therein (from Dean and Jacobson, 1992).

may work in conjunction with NifS, a pyridoxal-phosphate containing enzyme which catalyzes the desulfurization of L-cysteine. It has been demonstrated that NifS is capable of providing the inorganic sulfur necessary for iron sulfur cluster biosynthesis of apo-Fe protein *in vitro* (Zheng, et al., 1993, see below).

The recognized or proposed functions of the Nif gene products are given in Table 1. In addition to electron transport to nitrogenase and the positive and negative regulatory elements, the majority of the *nif* genes appear to be involved in nitrogenase maturation. The Fe protein specifically requires one *nif* gene, *nifM*, for complete maturation (Jacobson et al., 1989; Howard, et al., 1986). Recently, NifM has been shown to share high sequence homology with Propyl-isomerase from *E. coli*, thus a possible role for NifM is proper folding of the NifH polypeptide to facilitate cluster insertion (Schmid, 1995). As can be seen in Table 1, the majority of the *nif* biosynthetic genes are involved in FeMo-cofactor biosynthesis. A *nif* gene whose exclusive function is dedicated to P-cluster assembly has not been demonstrated. There may be several reasons why a gene has not been found, for example, 1) No gene is specifically required for P-cluster assembly, 2) The apo-MoFe protein generated from P-cluster extraction by thiol based solvents may be unstable, 3) The P-clusters extruded from the polypeptide matrix are no longer intact, thus making reconstitution experiments complicated.

Table 1. Recognized or proposed functions of the *nif* gene products.

Gene	Recognized or proposed function
nifH	Fe protein subunit, FeMo-cofactor biosynthesis
nifD	MoFe protein α subunit
nifK	MoFe protein β subunit
nifT	Function unknown
nifY	FeMo-cofactor biosynthesis in *K. pneumoniae*
nifE	molecular scaffold for FeMo-cofactor biosynthesis; homologous to *nifD* and forms α subunit of $\alpha_2\beta_2$ complex with *nifN* gene product.
nifN	molecular scaffold for FeMo-cofacotr biosynthesis; homologous to *nifK* and forms β subunit of $\alpha_2\beta_2$ complex with *nifE* gene product.
nifX	Function unknown, homologous to *NifY*
nifU	Possible role in sequestering Fe for metallocluster biosynthesis.
NifS	L-cysteine desulfurase, provides the inorganic sulfide for metallocluster biosynthesis.
nifV	Possiblely a homocitrate synthase, organic constituent of FeMo-cofactor
nifW	Function unknown, may be involved in homocitrate incorporation
nifZ	Function unknown, may be involved in homocitrate incorporation
nifM	Fe protein maturation, homologous to cis-trans isomerase of *E. coli*
nifB	Possibly a cofactor precursor (NifB-co)
nifQ	Involved in sequestering Mo
nifA	positive regulatory element; assists σ^{54} binding
nifL	negative regulatory element, homologous to kinases ie., NtrB
nifF	Flavodoxin involved in electron transport to Fe protein.
nifJ	Pyruvate-Flavodoxin oxidoreductase, electron transfer to flavodoxin in *K. pneumoniae*

The majority of the *nif* gene products appear to be involved in FeMo-cofactor biosynthesis and several recent reviews which are dedicated solely to this topic (Dean et al., 1993; Ludden et al., 1993), emphasize the complexity of this metal center.

CHAPTER 2. RECENT DEVELOPMENTS IN THE ANALYSIS OF NITROGENASE FEMO-COFACTOR BIOSYNTHESIS

This is a review chapter written for *Advances in Inorganic Biochemistry*, by Dennis Dean, Steve Muchmore from Purdue University and myself. Figures were provided by Steve Muchmore. This review summarizes the current knowledge of FeMo-cofactor biosynthesis and more recent developments which have general significance to the broader context of metallocluster biosynthesis.

BIOCHEMICAL GENETICS OF NITROGENASE METALLOCLUSTER ASSEMBLY: A BRIEF HISTORICAL PERSPECTIVE.

Considering the structural complexity of FeMo-cofactor biosynthesis and the number of players involved in its assembly, one can reasonably wonder about the sanity of those investigators who first chose to tackle this problem in the late 1970's. Nevertheless, several features worked in favor of this effort. First, at the outset there was no indication that the process would turn out to be so complex. Second, because the physiological need for nitrogenase activity can be circumvented by the addition of a fixed source of nitrogen to the growth medium, it was relatively easy to identify mutants impaired in nitrogenase activity (St. John, et al., 1975). Third, FeMo-cofactor can be chemically extruded intact from the MoFe protein, and it has a biologically unique spectroscopic signature that can be recognized in whole cells or in extracts (Shah and Brill, 1977; Davis, et al., 1972). Fourth, the biochemical assay for nitrogenase activity, the reduction of acetylene to ethylene (Dillworth, 1966; Schollhorn and Burris, 1967), is relatively easy and can be performed using whole cells, crude extracts, or the purified nitrogenase component proteins. Thus, these various features were exploited to initiate what is usually called a "biochemical-genetic" strategy for analysis of the pathway for FeMo-cofactor formation, and this approach has brought us to our present status of understanding of the process. In its simplest form, the biochemical-genetic

strategy involves mixing extracts from two different mutants, both of which are individually impaired in nitrogenase activity, and then asking whether or not nitrogenase activity can be "reconstituted". The power of the biochemical complementation approach is that it provides an assay which permits purification of factors that have complementary biochemical functions. In a related strategy, it was shown that FeMo-cofactor extracted from isolated MoFe protein could be used to reconstitute inactive nitrogenase produced by certain mutant strains (Shah and Brill, 1977). Thus, by using these approaches, it was possible for investigators to screen a large number of mutants and discriminate among those impaired in the structural genes and those impaired in the "maturation" process. Variations on this same theme were also used to identify regulatory genes and those genes whose products provide the physiological electron transport pathway required for nitrogenase activity *in vivo*. Finally, gene-product relationships were established by performing two-dimensional gel electrophoretic analyses of extracts from mutant strains impaired in nitrogen fixation activity (Roberts, et al., 1978).

One problem with the biochemical-genetic strategy is that it cannot necessarily discriminate between two different mutants that are impaired in the same biochemical function but have mutations in different genes. For example, although *nifE* and *nifN* are two distinct genes, mutations in either of them give exactly the same biochemical phenotype. For this reason, it was important that traditional genetic mapping strategies, involving *genetic* complementation, were used in parallel with biochemical complementation experiments (MacNeil, et al., 1978; Merrick, 1980). Because of its ease of genetic manipulation, *K. pneumoniae* was used in the development of the first genetic map of nitrogen fixation genes This choice turned out to be fortuitous because all of the *K. pneumoniae nif* genes are clustered on the chromosome and *K. pneumoniae* does not produce the alternative Mo-independent nitrogenases. The advancement of molecular genetic techniques, in particular, gene cloning, DNA sequencing methods, and

DNA-DNA hybridization techniques, subsequently permitted isolation and elucidation of the respective DNA sequences of all the *K. pneumoniae nif* genes and most genes related to nitrogen fixation from *Azotobacter vinelandii* and *Rhodobacter capsulatus* (see references cited in Dean and Jacobson, 1992). Among the most fascinating and impressive aspects of nitrogenase research is the remarkable accuracy of the insights provided by the application of traditional genetic and biochemical complementation experiments when viewed in the present context of the available DNA sequences and the structural models for the component proteins and their associated metalloclusters.

BIOSYNTHESIS OF FEMO-COFACTOR: A GENERAL OVERVIEW.

Although FeMo-cofactor is the most complex metallocluster associated with nitrogenase, it is also the best studied. As mentioned above, the reason for this is that FeMo-cofactor can be extruded from isolated MoFe protein and can then be used to activate apo-MoFe protein produced by certain mutant strains (apo-MoFe protein refers to MoFe protein that contains intact P-clusters but lacks the FeMo-cofactor). A structural model for FeMo-cofactor, deduced from the crystal structure of the MoFe protein and, which also takes into account its consensus chemical composition and certain biophysical analyses, has been proposed (Chan and Rees, 1993; Kim and Rees, 1992). A review of the FeMo-cofactor structure in Figure 4 shows that the metal-sulfur core of the FeMo-cofactor model is constructed from Mo-3Fe-3S and 4Fe-3S sub-cluster fragments connected by three inorganic sulfides which link opposing pairs of Fe atoms from the sub-cluster fragments. Homocitrate is an organic constituent of FeMo-cofactor and it is coordinated to the Mo atom through its 2-hydroxy and 2-carboxyl groups. FeMo-cofactor is anchored to the MoFe protein by α-Cys-275, which provides a thiolate ligand to an Fe atom at one end of the cofactor, and by α-His-442, which binds the Mo atom through a side-chain nitrogen atom at the

opposite end (residue numbers throughout the text refer to the corresponding *A. vinelandii* primary sequences).

Inspection of the FeMo-cofactor structural model gives rise to a number of questions concerning its biosynthesis. Are the subcluster fragments separately synthesized and then joined? Homocitrate is not a normal metabolite, so where does it come from? How is Mo sequestered for FeMo-cofactor biosynthesis and how does homocitrate become coupled to Mo? Is FeMo-cofactor synthesized stepwise into the apo-MoFe protein or is it separately synthesized and then inserted? How are the Fe and S mobilized and targeted for metallocluster formation? Is the pathway for FeMo-cofactor formation linear or branched? Are there any features common to both FeMo-cofactor biosynthesis and the general process of Fe-S cluster formation? Although detailed answers to all these questions are not yet known, the biochemical-genetic approach has provided considerable insight into the process of FeMo-cofactor formation. For example, it is now known that NifH, NifE, NifN and NifB are absolutely required for FeMo-cofactor formation. Also, although NifY, NifQ and NifV are not absolutely required for FeMo-cofactor formation, they are known, or they are likely, to be directly involved in the process. There is also evidence which at least invites speculation that NifX, NifW and NifZ are involved in some aspect of FeMo-cofactor formation. Finally, there is evidence that NifS and NifU are respectively involved in the general mobilization of the inorganic S and Fe required for nitrogenase metallocluster formation. The known or suspected roles of these various gene products in FeMo-cofactor assembly are described in the following sections.

THE NIFEN COMPLEX: A MOLECULAR SCAFFOLD FOR METALLOCLUSTER ASSEMBLY?

A. Development of the molecular scaffold concept.

During the early stages of the analysis of FeMo-cofactor assembly, it was recognized that NifB, NifE and NifN are absolutely required for FeMo-cofactor formation. The experimental evidence for this conclusion is that extracts prepared from mutants defective in any of these genes have no nitrogenase activity but can be activated by the simple addition of isolated FeMo-cofactor (see, for example Roberts, et al., 1978). This feature permitted investigators to ask whether or not FeMo-cofactor is synthesized separately and then inserted into the apo-MoFe protein or if it is inserted stepwise into the apo-MoFe protein. That the former is what actually occurs was shown by successful reconstitution of nitrogenase activity by mixing a *nifB* mutant extract and a *nifDK* mutant extract, either of which, otherwise, exhibited no activity (Ugalde et al., 1984). Because the source of FeMo-cofactor in such reconstitution experiments must come from the *nifDK* mutant extract, which does not produce the MoFe protein subunits, it is obvious that apo-MoFe protein cannot be required for FeMo-cofactor formation. This observation ultimately led to the hypothesis that FeMo-cofactor is pre-assembled on a "molecular scaffold" provided by some other *nif*-specific gene product(s). Thus, comparisons were made between the primary sequences of the MoFe protein subunits, NifD and NifK, and other *nif*-specific gene products. Such comparisons were prompted by the notion that the molecular scaffold and the MoFe protein must share the ability to recognize some form of the FeMo-cofactor, and consequently, it was suspected they might share some primary sequence conservation in their respective FeMo-cofactor-binding domains. These comparisons revealed that NifE and NifN exhibit striking primary sequence conservation when compared to NifD and NifK, respectively. The extent of the sequence conservation (Brigle, et al., 1987) and the mutual stability requirements

for NifE and NifN (Roberts, et al., 1978) also led to speculation that these gene products form a complex and could contain P-like clusters in addition to providing an FeMo-cofactor assembly site (Brigle, et al., 1987).

At about this same time an *in vitro* assay for FeMo-cofactor biosynthesis was developed where extracts were mixed from various mutant strains known to be defective in FeMo-cofactor biosynthesis in an effort to "reconstitute" nitrogenase activity (Shah, et al., 1986). In these experiments, it was found that NifB and either NifE or NifN deficient extracts could be mixed to achieve FeMo-cofactor formation, providing that Mo and MgATP were also added to the reaction mixture. Because crude extracts were used in these experiments, all other factors necessary for FeMo-cofactor formation were already present. Experiments performed in this way thus provided an assay that permitted the purification of small amounts of the NifEN complex, which was indeed shown to be a heterotetramer, and which probably does contain an Fe-S cluster (Paustian, et al., 1989). It should be emphasized that one of the technical problems in dealing with metallocluster biosynthetic enzymes is that they are probably only required in very low intracellular concentrations. To date, this feature has precluded the purification of the NifEN complex in the quantities necessary for detailed biophysical characterization. Nevertheless, it should be possible to overcome these problems by using gene fusion technologies to increase the expression of the *nifEN* gene products.

B. A homology model for the NifEN complex based on the MoFe protein structure.

If the NifEN complex does indeed provide a molecular scaffold for FeMo-cofactor formation, a number of interesting issues arise, which can now be considered in light of the MoFe protein crystal structure, structural models for FeMo-cofactor and the P cluster, and the primary sequence conservation between

the NifEN complex and the MoFe protein. For example, if the NifEN complex provides a molecular scaffold FeMo-cofactor assembly, how does the scaffold environment compare to the FeMo-cofactor-binding site within the MoFe protein? Also, if Fe-S clusters, analogous to the MoFe protein P clusters are present in the NifEN complex, can any insight be gained into their possible structures by examining their putative polypeptide environments? Finally, primary sequence comparisons and model building could provide some indication as to whether or not homocitrate and Mo are involved in the assembly process at a stage preceding or following the function of the NifEN complex. Based on these considerations, a structural "homology" model of the NifEN complex was developed by comparison to the recently solved MoFe protein crystal structure. The model was assembled using the molecular graphics program LORE (Finzel, submitted). Main chain atoms for equivalent residues were copied from the MoFe protein structure to the NifEN model together with atoms in those residues whose sequence is conserved between the MoFe protein subunits and the NifEN complex (Dean and Jacobson, 1992). For residues whose sequence is not conserved, a table of possible side chain rotomers was checked for conformations that have poor van der Waals contacts with other atoms in the structure, and those exhibiting molecular contacts were rejected. Once the rotomer list was checked, the remaining rotomer that has the highest observed frequency (Ponder and Richards, 1987) was added to the growing model. In this way a model containing one copy of an $\alpha\beta$-unit of NifEN was constructed.

A view of the α-carbon backbone of the Fe protein homodimer and an $\alpha\beta$-unit of the NifEN complex model is shown in Figure 7. As can be seen by comparison of Figure 1 and Figure 7, NifEN was modeled to have the same overall fold as the MoFe protein, which is consistent with sequence comparisons. In Figure 7 the pseudo two-fold axis of the NifEN complex unit is shown paired with the symmetric two fold axis of the Fe protein. This pairing matches the

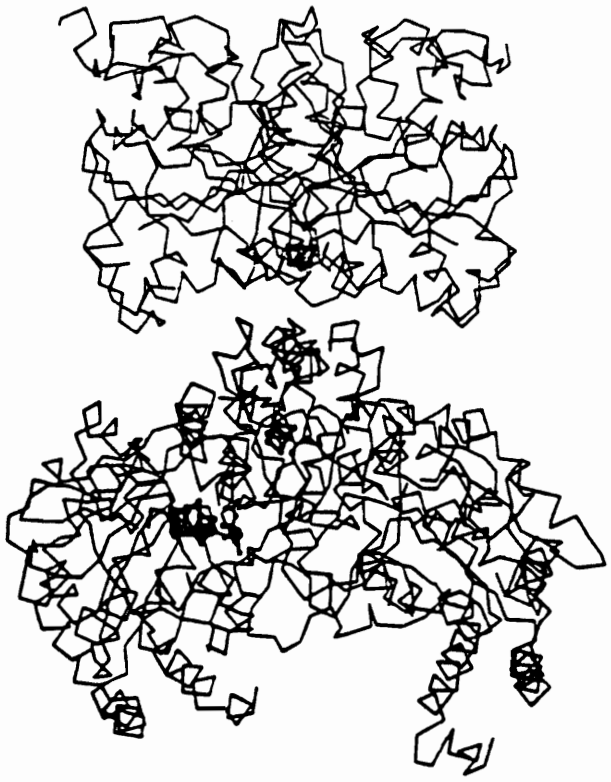

Figure 7. Homology model of the NifEN complex. The model from the α-carbon backbone trace of the Fe protein homodimer (top) and the $\alpha\beta$ unit of the NifEN complex (bottom) poised at a potential interaction site. Metalloclusters are shown in bold. Note the 8-Fe P-cluster in the MoFe protein is replaced by a [4Fe-4S] cluster in the NifEN model. The model pairs the two-fold symmetric surface of the Fe protein homodimer with the exposed surface of the NifEN protein pseudosymmetric $\alpha\beta$-unit interface.

proposed Fe protein and MoFe protein docking model of Rees and Howard (described in detail Howard, 1993) and is presented in this way because Fe protein is also known to be required for FeMo-cofactor biosynthesis (Filler et al., 1986; Robinson et al., 1987) and, therefore, the possibility that the NifEN complex and Fe protein interact during FeMo-cofactor formation has been suggested (Robinson et al., 1987). As far as the possible docking between the Fe protein and the NifEN complex is concerned, we note that, according to the Rees-Howard model, a number of residues predicted to be near this interaction site are conserved in the NifEN primary sequence. These include NifE residues 134-140, and NifN residues 78, 108, 110, and 111. Thus, although not all of the potential interface is conserved, it seems plausible that the overall features of the Fe protein interaction site is conserved in both the MoFe protein and the NifEN complex. The role of Fe protein in FeMo-cofactor biosynthesis is discussed in more detail below.

C. Potential metallocluster environments contained within the NifEN complex.

Two different MoFe protein P cluster structural models have been considered. One of these has each P cluster constructed from paired [Fe-4S] clusters connected by an edge-to-edge disulfide bond (Chan, et al., 1993, figure 3), and the other model, also has the P cluster constructed from paired [4Fe-4S] clusters, but they are connected by a shared sulfur atom rather than by a disulfide bond, Figure 8a (Campobasso, Muchmore, and Bolin, unpublished results). Both models have the P cluster symmetrically bridging the α- and β-subunits of the MoFe through six cysteine mercaptide ligands, three each provided by the α- and β-subunits. This arrangement of protein ligands to the P cluster within the MoFe protein places the P cluster at the pseudosymmetric interface near the proposed Fe protein docking site (see Figure 1). As shown in Figure 7 and Figure 8b, an Fe-S cluster has also been modeled into the NifEN complex structure but as an

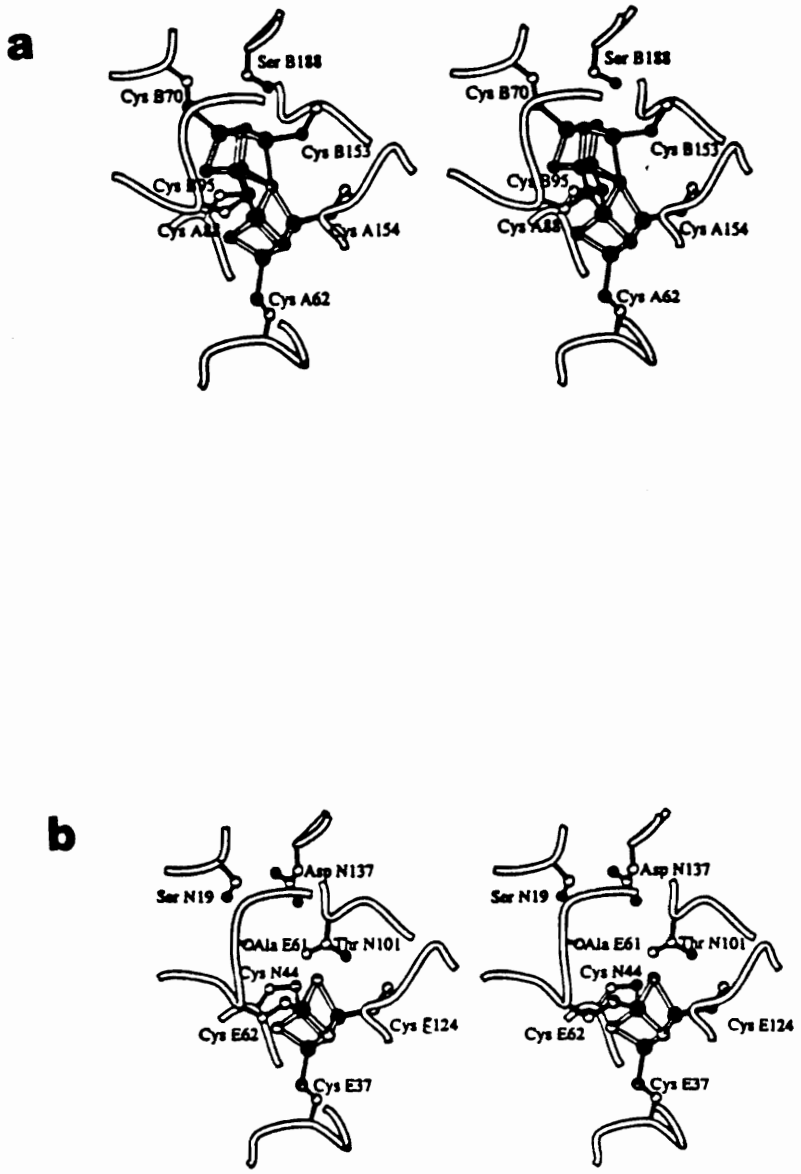

Figure 8. Stereoview of the P-cluster environment of the MoFe Protein (a) and the homologous region contained within the NifEN complex (b). MoFe protein α-subunit residues and β-subunit residues are represented as an A or B, respectively. NifE residues and NifN residues are represented as an E or N respectively. Note that a [4Fe-4S] cluster has been incorporated in the NifEN complex. P-cluster model is based on Campobasso et al. (1993).

[4Fe-4S] cluster rather than an 8Fe-containing P cluster. The reason for this is that sequence comparisons (Dean and Jacobson, 1992) show that only four of the six P cluster cysteines from an $\alpha\beta$ unit of the MoFe protein can be considered conserved in the NifEN primary sequences, and the NifEN homology model places these four cysteines (E-Cys-37, E-Cys-62, E-Cys-124, and N-Cys-44) in an arrangement that can accommodate an [4Fe-4S] cluster. The cavity that would be occupied by the other "half" of the P cluster is possibly filled by residues E-Ala-61, N-Ser-19, N-Thr-101, and N-Asp-137, and, perhaps, water. It is difficult to assign a function to the putative [4Fe-4S] cluster contained within the NifEN complex because the true function of the P clusters contained within the MoFe protein is not yet known. Nevertheless, the consensus view that P clusters are involved in the acceptance, storage, and ultimate transfer of electrons to FeMo-cofactor during catalysis suggests that the putative [4Fe-4S] clusters contained within the NifEN complex could have a function somewhat analogous to the proposed P cluster function, where they broker reducing equivalents that could be used in the formation or release of FeMo-cofactor from the NifEN complex.

FeMo-cofactor is covalently attached to the MoFe protein through D-Cys-275 (Figure 9a) and a corresponding cysteine is conserved in the NifEN model (E-Cys-250, Figure 9b). This conservation indicates that E-Cys-250 might provide a nucleation site for FeMo-cofactor assembly and that the analogous D-Cys-275 could serve as a primary acceptor during the insertion of FeMo-cofactor into the apo-MoFe protein. Site-directed mutagenesis studies have shown that E-Cys-250 is required for FeMo-cofactor biosynthesis (Wilson, et al., 1988) and that D-Cys-275 is required for MoFe protein catalytic activity (Brigle, et al., 1987; Kent, et al., 1989). FeMo-cofactor is also anchored to the MoFe protein by an N ligand provided by D-His-442 to the Mo atom. The corresponding residue in the NifEN model is occupied by asparagine (E-Asn-418), which suggests that FeMo-cofactor is probably not covalently attached to the NifEN complex at this end. This

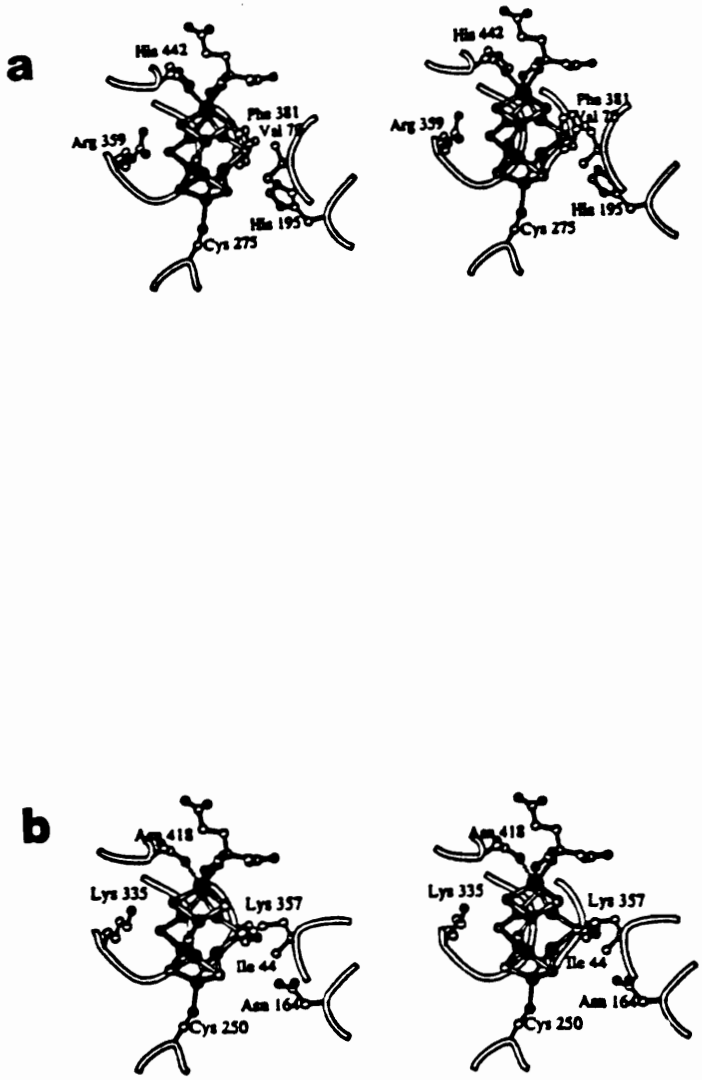

Figure 9. Stereoview of the FeMo-cofactor environment of the MoFe protein (a) and the homologous region contained within the NifEN complex (b). All residues shown in a are from the MoFe protein α-subunit and all residues shown in b are from NifE. Note that the MoFe protein histidine-442 and histidine-195 residues are both replaced by asparagines in the corresponding NifEN complex model. From Muchmore et al., (1995).

feature makes sense in light of the fact that FeMo-cofactor must ultimately escape from the NifEN complex. In this context it is also noteworthy that certain residues contained within MoFe protein's FeMo-cofactor pocket and which approach, or are even hydrogen bonded to the cofactor, are substituted by conservative, but less bulky, amino acids in the NifEN complex model. For example, MoFe protein residues D-His-195 and D-Arg-359 are substituted by E-Asn-164 and E-Lys-335, respectively (Figure 9A and 9B). The importance of these comparisons is that they provide a basis for the design of amino-acid substitution studies that can be used to probe the structural and functional properties of MoFe protein and the NifEN complex. For example, spectroscopic and biochemical studies on an altered MoFe protein where D-His-195 was substituted by the corresponding NifEN complex residue, asparagine, have indicated that D-His-195 has a structural role, which serves to keep FeMo-cofactor attached to the MoFe protein and to correctly position FeMo-cofactor within the polypeptide matrix, such that N_2 binding is possible (Kim et al., 1995). An important experiment that will be done in the future involves substituting the E-Asn-418 residue by histidine and then asking whether or not an intermediate in FeMo-cofactor assembly can be trapped on the NifEN complex. Although not readily apparent in Figure 7, another interesting aspect of the NifEN model is that there appears to be plenty of room for homocitrate within the proposed scaffold environment. This feature hints that both homocitrate and Mo are assembled into the FeMo-cofactor precursor on the NifEN complex, and that the form of FeMo-cofactor that exits the NifEN complex is likely to already have Mo and homocitrate attached to it.

HOMOCITRATE AND THE INVOLVEMENT OF NIFV AND NIFQ, AND THE POSSIBLE INVOLVEMENT OF NIFW AND NIFZ, IN FEMO-COFACTOR ASSEMBLY.

The analysis of mutant strains deficient in the *nifV* gene and the ultimate elucidation of the biochemical function of NifV is one of the more interesting sagas associated with the biochemical genetic characterization of FeMo-cofactor biogenesis, and has been described in detail elsewhere (Ludden et al., 1993, Smith and Eady, 1992). Briefly, the biochemical phenotype associated with the inactivation of *nifV* is the production of an altered MoFe protein, which is able to catalyze the reduction of protons and acetylene at reasonably high levels when compared to the wild type protein, but catalyzes nitrogen reduction at only very low levels. Also, MoFe protein produced by the *nifV* mutant exhibits proton reduction activity that is partially sensitive to CO, whereas wild type MoFe protein-catalyzed proton reduction is insensitive to CO inhibition. The first direct evidence that FeMo-cofactor is associated with the substrate reduction site was shown by experiments where apo-MoFe protein reconstituted by FeMo-cofactor isolated from MoFe protein produced by a *nifV* mutant exhibited the altered catalytic properties associated with the *nifV* phenotype (Hawkes et al., 1984).

During the subsequent development of the *in vitro* biosynthetic assay for FeMo-cofactor biosynthesis it was found that a small molecule, which was not produced by *nifV* mutants of *K. pneumoniae*, is required for *in vitro* FeMo-cofactor formation (Hoover, et al., 1986). It is now known that the product of NifV catalysis is homocitrate (Hoover, et al., 1989), which is the organic constituent that is coupled to the Mo atom of FeMo-cofactor (see Figure 4). Although NifV has not yet been purified in an active form, primary sequence comparisons have shown that it bears considerable identity when compared to isopropylmalate synthase (Wang, et al., 1991). This comparison suggests that NifV is likely to catalyze the condensation of acetyl-CoA and α-ketoglutarate, or

an α-ketoglutarate derivative, to form homocitrate. FeMo-cofactor produced by *K. pneumoniae nifV* mutants has been shown to contain citrate rather than homocitrate (Liang, et al., 1990), whereas *nifV* mutants from *A. vinelandii* might produce an FeMo-cofactor that contains either a mixed population of organic acids (P. Ludden, personal communication) or perhaps no organic acid.

At some stage during FeMo-cofactor formation, homocitrate must become coordinated to Mo. It is not yet known whether or not such coordination requires an enzymatic activity nor is it known if such attachment occurs on the NifEN scaffold. Nevertheless, the structural model of the NifEN complex indicates that there is room for both Mo and homocitrate on the scaffold. Two possible candidates that could be involved in attaching homocitrate to Mo or inserting homocitrate into the NifEN complex are NifW and NifZ. Mutant strains of *A. vinelandii* deleted for *nifW*, *nifZ*, or *nifW* and *nifZ* exhibit lowered diazotrophic growth rates and a modest lowering in MoFe protein activity but no apparent reduction in Fe protein activity (Jacobson et al., 1989; Kim and Burgess, 1994). The similar biochemical and growth rate phenotypes of these mutants, and the fact that both *nifW* and *nifZ* are cotranscribed in *K. pneumoniae* (Paul and Merrick, 1989) and *A. vinelandii* (Jacobson, et al., 1989), is suggestive that NifW and NifZ could form a macromolecular complex. It has been shown that in *Rhodobacter capsulatus* a *nifW-nifV* double mutant has a much more severe reduction in diazotrophic growth than does either a *nifW* or *nifV* mutant (Masepohl, et al., 1989). This *nifW-nifV* phenotype can be partially relieved by the addition of homocitrate to the growth medium, which has led to the suggestion that the NifW could be involved in "processing" homocitrate (Masepohl, et al., 1989). The same phenotype has been observed for a *nifW-nifV* double mutant, as well as a *nifZ-nifV* mutant, from *A. vinelandii,* which lends some credence to the possibility that NifW and NifZ are involved in a common processing step that requires homocitrate (our unpublished results). Along these

lines, it is interesting that no one has yet demonstrated homocitrate synthase activity for isolated NifV, even though several research groups have attempted to do so (our unpublished work; P. W. Ludden, personal communication; W. H. Orme-Johnson, personal communication). Thus, we have wondered if the prior activity of NifW and or NifZ might be required to effect NifV activity. In any event, it appears that NifW and NifZ are both involved in a partially dispensable function related to FeMo-cofactor biosynthesis.

Another unanswered issue concerns how Mo is mobilized for FeMo-cofactor biosynthesis and whether or not an enzymatic activity is required to place Mo in the appropriate oxidation state for FeMo-cofactor formation. The only currently available information is that NifQ appears to be involved in some aspect of Mo metabolism specific to FeMo-cofactor formation (Ugalde et al., 1985). However, NifQ has not yet been purified nor is there any known activity for NifQ. Inspection of NifQ primary sequences reveals the clustering of four conserved cysteines (Dean and Jacobson, 1992). These cysteines might serve as ligands for a redox center, or perhaps they could provide a nucleation site for formation of a Mo-containing precursor that is transferred to the NifEN complex.

FE PROTEIN AND MgATP ARE REQUIRED FOR THE SYNTHESIS OF FEMO-COFACTOR AND ITS INSERTION INTO THE APO-MOFE PROTEIN.

Fe protein is the obligate electron donor to the MoFe protein. During catalysis, reduced Fe protein binds two molecules of MgATP and subsequently delivers a single electron to the MoFe protein in a process that involves hydrolysis of the MgATP and the association-dissociation of the component proteins. Mutant strains bearing deletions or insertions in *nifH*, encoding the Fe protein subunits, also produce an apo-MoFe protein (Filler et al., 1987; Robinson et al., 1987). Thus, one nitrogenase component protein is required for the maturation of the other catalytic component. The *in vitro* reconstitution of apo-MoFe

protein produced by certain *nifH* mutants requires the presence of both MgATP and Fe protein (Robinson et al., 1989; Allen et al., 1993). In contrast, apo-MoFe proteins produced by either *nifE* or *nifB* mutants do not require either MgATP or Fe protein for their reconstitution (Allen et al., 1993; Paustian et al., 1990; Tal et al., 1991). Native gel electrophoretic analyses have also shown that apo-MoFe protein produced by *nifB* or *nifE* mutants are different than apo-MoFe proteins produced by certain *nifH* mutants (Allen et al., 1993; Tal et al., 1991). These results can be explained if it is considered that the Fe protein and MgATP have two distinct functions in FeMo-cofactor biosynthesis. The first function could involve the direct participation of Fe protein and MgATP in FeMo-cofactor biosynthesis, whereas the second function could involve processing the apo-MoFe protein to a form that is amenable to FeMo-cofactor insertion.

The requirement of Fe protein and MgATP for FeMo-cofactor biosynthesis makes sense in light of the apparent structural similarities between the MoFe protein and the NifEN complex. This possibility is particularly attractive because the respective Fe protein docking sites appear to be somewhat conserved when the MoFe protein and the NifEN complex are compared (see Figures 1 and 7). At least three different roles for Fe protein in FeMo-cofactor biosynthesis can be considered. First, the Fe protein and the NifEN complex protein might associate and dissociate in a MgATP dependent process involving an electron transfer reaction required for activation of an intermediate in FeMo-cofactor formation. One possibility is that an FeMo-cofactor precursor contained within the NifEN scaffold could require a reduction step for completion of synthesis of the metallocluster. Second, association of Fe protein and the NifEN complex could result in a conformational change in the NifEN complex resulting in the accessibility of a metallocluster assembly site that is otherwise buried within the NifEN complex. Third, an interaction between Fe protein and the NifEN complex could be required for a conformational change or redox

reaction that results in the release of FeMo-cofactor. In spite of these obvious possibilities, there is little direct evidence to support any of them. In fact, certain strains that produce catalytically inactive Fe protein, for example *nifM* mutants and certain *nifH* point mutants, are still able to produce active MoFe protein (Jacobson et al., 1989; Gavini and Burgess, 1992; Wolle et al., 1992). It is particularly confusing that mutant strains that produce catalytically inactive Fe proteins, which are altered either in their MgATP-binding sites or in their ability to undergo the MgATP-dependent conformational change, also produce MoFe proteins that contain a full complement of FeMo-cofactor (Gavini and Burgess, 1992). The biochemical phenotypes of various mutants altered in Fe protein activity are summarized in Table 2.

The basis for the requirement of Fe protein and MgATP in the insertion of FeMo-cofactor into the apo-MoFe protein is just as perplexing. As described above for FeMo-cofactor biosynthesis, a potential role for Fe protein, and a requirement for MgATP, in the insertion of FeMo-cofactor seems reasonable. For example, it is easy to imagine that it might be necessary for the apo-MoFe protein to be in a particular redox state to accept the FeMo-cofactor. A MgATP-dependent electron transfer provided by the Fe protein is an obvious source for such reducing equivalents. Also, it is reasonable to speculate that interaction of the Fe protein with the MoFe protein could result in a conformational change within the apo-MoFe protein leading to the accessibility of the cofactor binding site, which otherwise might be buried below the protein surface. Nevertheless, the ability of various mutant strains impaired in aspects of Fe protein catalysis and MgATP binding, as described above, to accumulate active MoFe protein does not appear to be consistent with these possibilities.

Table 2. Biochemical phenotypes of strains defective in Fe protein catalytic activity and the effect on FeMo-cofactor biosynthesis and insertion.

Strain	Genotype, biochemical phenotype and effect on FeMo-cofactor biosynthesis
DJ54	Deleted for most of the *nifH* gene, does not produce any Fe protein, exhibits Nif$^-$ phenotype, does not synthesize FeMo-cofactor, will not insert FeMo-cofactor without the addition of isolated Fe protein and MgATP (Robinson et al., 1989).
DJ576	Fe protein residue Asp-125 substituted by Glu-125, Nif$^-$, purified Fe protein from this strain remains able to bind MgATP and undergoes MgATP-dependent conformational change but does not hydrolyze MgATP and does not transfer electrons to the MoFe protein, will undergo MgATP-dependent conformational change, has no effect on FeMo-cofactor biosynthesis (Wolle et al., 1992a).
K15Q	Fe protein residue Lys-15 substituted by Asn-15, exhibits Nif$^-$ phenotype, purified Fe protein from this strain binds MgATP at a lower level than wild type, does not undergo MgATP-dependent conformation change (Seefeldt et al., 1992), has no effect on FeMo-cofactor biosynthesis (L. Seefeldt, personal communication).
DJ230	Fe protein residue Arg-100 substituted by His-100, exhibits Nif$^-$ phenotype, Fe protein altered in its ionic interaction with the MoFe protein, has no effect on FeMo-cofactor biosynthesis (Wolle et al., 1992b).
UW91	Fe protein residue Ala-157 substituted by Ser-157, exhibits Nif$^-$ phenotype, purified Fe protein from this strain does not undergo MgATP-dependent conformational change, does not hydrolyze MgATP, has no effect on FeMo-cofactor biosynthesis (Gavini and Burgess, 1992).
DJ91	Fe protein residue Cys-98 substituted by Ser-98, exhibits Nif$^-$ phenotype, does not contain [4Fe-4S] cluster, does not synthesize FeMo-cofactor (Howard et al., 1989).
DJ136	Deleted for *nifM* gene, does not process Fe protein to an active form, has no effect on FeMo-cofactor biosynthesis (Jacobson et al., 1989).

THE INVOLVEMENT OF A MOLECULAR PROP IN FeMo-COFACTOR INSERTION.

When extracts of mutant strains defective in FeMo-cofactor are electrophoresed under non-denaturing anaerobic conditions, different species of apo-proteins having different electrophoretic mobilities are recognized (Allen et al., 1993; Tal et al., 1991; White et al., 1992; Homer et al., 1993). Individual electrophoretic mobilities are characteristic of a particular mutant strain, where *nifB* and *nifE* extracts produce apo-MoFe proteins having the same electrophoretic mobility, which is different than the electrophoretic mobility of apo-MoFe protein produced by certain *nifH* mutants. Of particular interest is that apo-MoFe proteins produced by *nifB* or *nifE* mutants appear to have two molecules of a small molecular weight protein attached to them. In the case of apo-MoFe protein produced by *K. pneumoniae*, the small molecular weight protein was identified as the *nifY* gene product (Homer et al., 1993; White et al., 1992), whereas, in the case of *A. vinelandii,* the small molecular weight protein (designated as γ) is not the product of a known *nif*-specific gene (Homer et al., 1993). The addition of FeMo-cofactor to extracts that contain apo-MoFe protein having either NifY (*K. pneumoniae*) or γ (*A. vinelandii*) attached, results in the release of NifY or γ, respectively (White et al., 1992; Homer et al., 1993). In contrast, apo-MoFe protein accumulated in extracts of *nifH* deficient strains does not have the associated γ subunits, but the addition of Fe protein and MgATP to such extracts results in the attachment of γ (Allen et al., 1993). The γ subunits attached in this way can subsequently be released from the complex by the addition of FeMo-cofactor (M. Homer, personal communication).

The above findings indicate that apo-MoFe protein must be processed before it becomes fully competent to accept FeMo-cofactor that is preformed on the NifEN complex. This processing step involves the attachment of a small molecular weight polypeptide (NifY or γ) to the apo-MoFe protein and requires

the participation of Fe protein and MgATP. The small molecular weight polypeptide is proposed to serve as a "molecular prop" (White et al., 1992) whose function is to maintain a conformation of the apo-MoFe protein where the FeMo-cofactor-binding site either remains accessible for FeMo-cofactor attachment or is protected from reactivity with some other cellular constituent. Evidence supporting this idea comes from the observation that a *nifB-nifY* double mutant of *K. pneumoniae* accumulates apo-MoFe protein that is only marginally activated by the addition of FeMo-cofactor (Homer et al., 1993). Once FeMo-cofactor becomes inserted it appears that the MoFe protein undergoes a conformational change which results in the dissociation of the molecular prop. There is clear evidence for such a conformational change because activation of apo-MoFe protein by the addition of FeMo-cofactor results in a rather dramatic change in their respective mobilities under conditions of native, non-denaturing electrophoresis.

It is interesting that another *nif*-specific gene product, NifX, bears some sequence identity when compared to NifY (Jacobson et al., 1989). The *nifY* gene is located in the same transcription unit as the nitrogenase structural genes, whereas the *nifX* gene is located in the same transcriptional unit as *nifE* and *nifN* (Figure 6). These similarities naturally lead to the speculation that NifX could be attached to the NifEN complex and act as a molecular prop during the formation of FeMo-cofactor on the NifEN scaffold. Nevertheless direct information concerning this possibility is not yet available.

NifB, A MOLECULAR SCAFFOLD FOR FORMATION OF AN FeMo-COFACTOR PRECURSOR - NifB-COFACTOR ?

NifB is another *nif*-specific gene product that is absolutely required for FeMo-cofactor biosynthesis. Although NifB has not been purified in an active form, the product of NifB activity has been isolated (Shah et al., 1994). This

product is called NifB-cofactor and it is composed of Fe and S. The current working hypothesis is that NifB-cofactor is an FeMo-cofactor precursor, which is formed on NifB, and which ultimately provides the Fe-S core present within the FeMo-cofactor. Thus, like the NifEN complex, NifB can be considered a molecular scaffold upon which a portion of FeMo-cofactor (i.e., NifB-cofactor) is pre-assembled prior to its donation to the NifEN complex. There are several lines of evidence to support this hypothesis. First, there is a cysteine rich region contained within the NifB primary sequence, which could serve as the assembly site for NifB-cofactor. This domain contains a number of hydrophilic residues indicating that NifB-cofactor could be assembled at the NifB-solvent interface (Dean and Jacobson, 1992), a feature that could facilitate the release of NifB-cofactor for following biosynthetic steps. Second, in the case of *Clostridium pasteurianum*, the *nifB* and *nifN* genes are fused (J.-S. Chen, personal communication). This situation indicates that, in organisms such as *K. pneumoniae* and *A. vinelandii*, the NifEN complex and NifB might form a macromolecular unit, or could undergo association and dissociation, where NifB-cofactor formed by NifB activity is efficiently donated to the NifEN complex for further processing. The fact that both NifB activity and NifEN activity copurify in the early stages of NifEN purification supports this possibility (J. Roll, personal communication). Third, it has been shown that, like the MoFe protein, different forms of the NifEN complex, which exhibit different mobilities under conditions of non-denaturing gel electrophoresis, accumulate in different mutant strains. For example, the electrophoretic form of the NifEN complex accumulated in a *nifH*-deficient background is different than that accumulated in a *nifB*-deficient background. Importantly, the form of the NifEN complex accumulated in the *nifB*-deficient background can be converted to the form accumulated by the *nifH*-deficient background by the simple addition of NifB-cofactor (J. Roll, personal communication).

MOBILIZATION OF THE S AND Fe REQUIRED FOR FeMo-COFACTOR FORMATION.

One aspect of the biochemical-genetic analysis of the functions of the various *nif*-specific gene products has involved elimination of the function of each gene using gene-directed mutagenesis, and in turn, asking what the effects are on the activities of the Fe protein and the MoFe protein. In the case of the analysis of *A. vinelandii* genes, this approach has revealed that only mutations in *nifH*, *nifS*, and *nifU* have an effect on *both* Fe protein and MoFe protein catalytic activities (Dean and Jacobson, 1992; Jacobson et al., 1989). For those mutants where Fe protein activity has been eliminated, for example *nifH*-deletion strains, a concomitant loss in MoFe protein can be explained by an inability of the mutant to synthesize or insert FeMo-cofactor. Indeed, MoFe protein can be recovered in extracts of such mutant strains by the simple addition of MgATP, Fe protein and FeMo-cofactor. On the other hand, the MoFe protein activities of *nifU* or *nifS* deletion strains is not stimulated by the addition of FeMo-cofactor. These results suggested that NifS and NifU are involved in one or more steps common to maturation of both the Fe protein and the MoFe protein. The only requirements that are obviously shared by both component proteins is that they need both S and Fe for the formation of their respective metalloclusters.

The approach used to analyze the functions of NifS and NifU involved their hyperproduction in *Escherichia coli* followed by their purification and characterization (Zheng et al., 1993; Fu et al., 1994). The analysis of NifS revealed that it is a homodimer, which contains one molecule of pyridoxal-phosphate per each subunit. The biochemical reaction catalyzed by NifS is the desulfurization of L-cysteine to yield sulfur and L-alanine. An intermediate in the reaction is a NifS bound persulfide, which is proposed to be the activated form of S needed for metallocluster assembly (Zheng et al., 1994). The possibility that NifS can function as an S-donor in Fe-S cluster formation

was tested in an *in vitro* reconstitution experiment where it was shown that, in the presence of ferrous ion, dithiothreitol, and L-cysteine, NifS catalyzes the reactivation of an apo-form of the Fe protein (Zheng and Dean, 1994). These data provide compelling evidence that NifS can activate S for general Fe-S cluster formation but a direct involvement in FeMo-cofactor biosynthesis has not yet been demonstrated. It is also not yet known if NifS functions in the specific delivery of S to the metallocluster assembly site.

NifS-like genes have also recently been discovered in non-nitrogen fixing organisms (Fleischman et al., 1995; Kolman and Soll, 1993; Sun and Setlow, 1993). The presence of NifS-like genes in other organisms indicates that NifS involvement in sequestering the inorganic S needed for nitrogenase metallocluster formation could represent a general mechanism for activating S for Fe-S cluster formation. This possibility is in line with the observation that *nifS* mutants still produce low levels of active Fe protein and MoFe protein (Jacobson et al., 1989). This biochemical phenotype can be explained if it is considered that other NifS-like proteins present in the cell might be able to at least partially supplant the function of NifS in its absence. Another interesting feature concerning the acquisition of S needed for nitrogenase metallocluster assembly is the presence of a *cysE*-like gene within the major *nif*-cluster of *A. vinelandii* (Jacobson et al., 1989; Evans et al., 1991). The *cysE* gene encodes O-acetylserine synthase, which catalyzes the rate-limiting step in cysteine biosynthesis (Denk and Bock, 1987). The presence of such a gene under the control of *nif*-specific regulatory elements indicates that biosynthesis of the nitrogenase metalloclusters places a significant demand on the intracellular cysteine pool.

A final *nif*-specific gene product that could be involved in some aspect of FeMo-cofactor metal core formation is NifU. Loss of NifU function results in a decrease in both Fe protein and MoFe protein activity. Thus, as in the case of *nifS* mutants, this phenotype points to a role for NifU in the mobilization of Fe or

S required for Fe-S core formation. Although its function is not yet known, NifU was recently purified and shown to be a homodimer, which contains two identical [2Fe-2S] clusters (Fu et al., 1994). This feature and the high concentration of cysteine residues contained within the NifU primary sequence (Dean and Jacobson, 1992) have led to speculations concerning its possible role in Fe-S core formation. First, the [2Fe-2S] clusters contained within NifU could have a redox role in releasing sulfide from NifS. In this context NifU is also a potential candidate to serve as an intermediate sulfide carrier. Second, NifU might complement the function of NifS by sequestering the Fe required for nitrogenase Fe-S core formation. In this case, a role for the [2Fe-2S] clusters could be to keep Fe bound at some other site located within NifU in the proper oxidation state necessary for Fe-S cluster formation. Third, an interesting possibility is that nitrogenase Fe-S core precursors could be assembled at one or more sites on NifU, potentially using the available cysteine residues as nucleation sites. In this scenario the [2Fe-2S] clusters could have a redox role in formation, stabilization or release of such precursors during maturation of the nitrogenase component protein. Nevertheless, it is emphasized that a direct role for NifU in FeMo-cofactor formation has not been established. A biochemical assay for NifU, or a biochemical complementation assay, needs to be developed before the true function of NifU will be elucidated.

SUMMARY AND COMMENTS.

Twelve different *nif*-specific gene products (NifH, NifY, NifE, NifN, NifX, NifU, NifS, NifV, NifW, NifZ, NifB and NifQ) have been implicated in some aspect of FeMo-cofactor biosynthesis. Our current understanding and speculations concerning their respective functions and the path for FeMo-cofactor formation and its insertion into the apo-MoFe protein are summarized as follows:

1. FeMo-cofactor is first assembled and then inserted into an apo-MoFe

protein. The apo-MoFe protein probably already has its other metalloclusters, the P-clusters, attached prior to FeMo-cofactor insertion.

2. A molecular prop is attached to the apo-MoFe protein and the prop dissociates from the apo-MoFe protein upon insertion of the FeMo-cofactor. Attachment of the molecular prop to the apo-MoFe protein requires both MgATP and the Fe protein. The molecular prop used by *K. pneumoniae* has been identified as NifY. In the case of *A. vinelandii* the identity of the molecular prop is not known. The function of the molecular prop could be to maintain a conformation of the apo-MoFe protein so that FeMo-cofactor-binding sites remain accessible for FeMo-cofactor attachment, or the prop could serve to protect the FeMo-cofactor site from reaction with some other cellular constituent prior to FeMo-cofactor insertion.

3. Both Fe protein and MgATP are required for insertion of FeMo-cofactor into the apo-MoFe protein but their specific roles are not known. It seems logical that an ATP-dependent association and dissociation of the Fe protein and the apo-MoFe protein/MoFe protein is involved in the process of FeMo-cofactor insertion, although direct evidence for this possibility is not available.

4. NifE and NifN form a heterotetrameric complex that provides a scaffold for FeMo-cofactor biosynthesis. Based on primary sequence comparisons and homology modeling, the NifEN complex is likely to be structurally homologous to the MoFe protein. Also, modeling indicates that the NifEN complex does not contain P-clusters but could contain typical [4Fe-4S] clusters, which bridge the NifE and NifN subunits. The NifEN complex is likely to cycle between a "charged" form, which contains the assembled FeMo-cofactor, and a "discharged" form, which serves as the scaffold upon which FeMo-cofactor is assembled. The first step in the assembly of FeMo-cofactor upon the NifEN complex is likely to involve the acceptance of an Fe-S core, which is separately synthesized through the action of NifB. Subsequent steps probably involve the incorporation of

homocitrate and Mo. Because Fe protein and MgATP are required for FeMo-cofactor formation. it is speculated that an ATP-dependent association of the Fe protein and the NifEN complex could be involved in the catalytic processing of FeMo-cofactor on the NifEN complex or could be involved in the release of completed FeMo-cofactor from the charged NifEN complex. NifX, which has sequence identity when compared to NifY, might be attached to the NifEN complex at some stage of FeMo-cofactor biosynthesis and serve as a molecular prop as described for NifY.

5. Homocitrate is an organic constituent of FeMo-cofactor. Homocitrate is probably formed by the condensation of acetyl-CoA and α-ketoglutarate in a reaction catalyzed by NifV. How homocitrate becomes attached to Mo and how homocitrate and Mo become incorporated into the NifEN scaffold is not known but there is speculation that NifW and NifZ might participate in some aspect of this process. The exact mechanism by which Mo is sequestered and targeted for insertion into FeMo-cofactor and the oxidation state of the activated form of Mo is not known. There is, however, evidence that NifQ is involved in some aspect of processing Mo during FeMo-cofactor biosynthesis.

6. NifB might serve as molecular scaffold for formation of an Fe-S core called NifB-cofactor. NifB-cofactor formed upon NifB is probably incorporated into the discharged form of the NifEN complex. Thus, NifB itself is likely to cycle between a "charged" form, which contains the assembled Fe-S core, and a "discharged" form, upon which the Fe-S core destined for FeMo-cofactor formation is assembled. It does not appear that NifB is directly involved in the incorporation of Mo into the NifEN scaffold because NifB-cofactor does not contain Mo. There is some evidence that the NifEN complex and NifB form a complex or interact with each other at some stage of FeMo-cofactor biosynthesis.

7. NifS is an L-cysteine desulfurase, which probably provides the inorganic sulfur for FeMo-cofactor formation. The reaction catalyzed by NifS involves

formation of a NifS-bound persulfide, which is believed to be the activated form of S destined to become incorporated into Fe-S containing clusters. NifS is able to catalyze Fe-S cluster formation *in vitro*, indicating that it's activity might represent a general mechanism for Fe-S core formation. The identification of *nifS*-like genes in non-nitrogen-fixing organisms supports this idea. However, a direct role for NifS in specifically sequestering S for FeMo-cofactor formation has not been established.

8. NifU is a homodimer that contains two identical [2Fe-2S] clusters. A function for NifU in FeMo-cofactor formation has not been established. There is speculation that NifU could function as an intermediate Fe or S donor during Fe-S core formation.

CHAPTER 3. NIFU AND ITS HOMOLOGOUS FORMS

When the first deletion mutant of *nifU* was made and its effect on diazotrophic growth and the activity of both nitrogenase component proteins was assessed (along with NifS) by Jacobson et al. (1989b), a possible role in metallocluster biosynthesis was suggested. This suggestion was based on the fact that both the Fe protein and the MoFe protein possess Fe-S containing metal centers. Although the metal centers are structurally very different, a unifying feature is their iron and sulfide composition (Kurtz et al., 1979; Shah and Brill, 1977; Kim and Rees, 1994). Following the physical and biochemical characterization of NifS, it is now proposed that NifU may be involved in the Fe side of metallocluster biosynthesis.

The role of NifS in the mobilization of sulfide for nitrogenase metallocluster biosynthesis has been described above. The presence of *nifS* in non-nitrogen fixing organisms, including yeast (see Zheng et al., 1993 and references therein) suggests that NifS activity may be a general mechanism for the mobilization of sulfide for a variety of metalloenzymes. These findings are relevant to NifU because, 1) A similar nitrogenase biochemical phenotype results from either *nifS* or *nifU* deletion strains, 2) NifS, NifU and NifV are required for complete maturation of the alternative nitrogenases as well as for the Mo-nitrogenase in *A. vinelandii* (Kennedy and Dean, 1992), and 3) like *nifS,* homologs to *nifU* have been found in non-nitrogen fixing bacteria.

Complete homologs to NifU are found in aerobic, nitrogen fixing bacteria, and the facultatively anaerobic bacterium, *K. pneumoniae*. The amino acid sequence comparisons, deduced from the gene sequences, show that NifU is found in truncated forms when found outside the realm of nitrogen fixation. These truncated forms of NifU are widespread in the microbial world (Fleischman et al., 1995; Ouzonis, 1993). Figure 10 is a cartoon that represents the three domains of

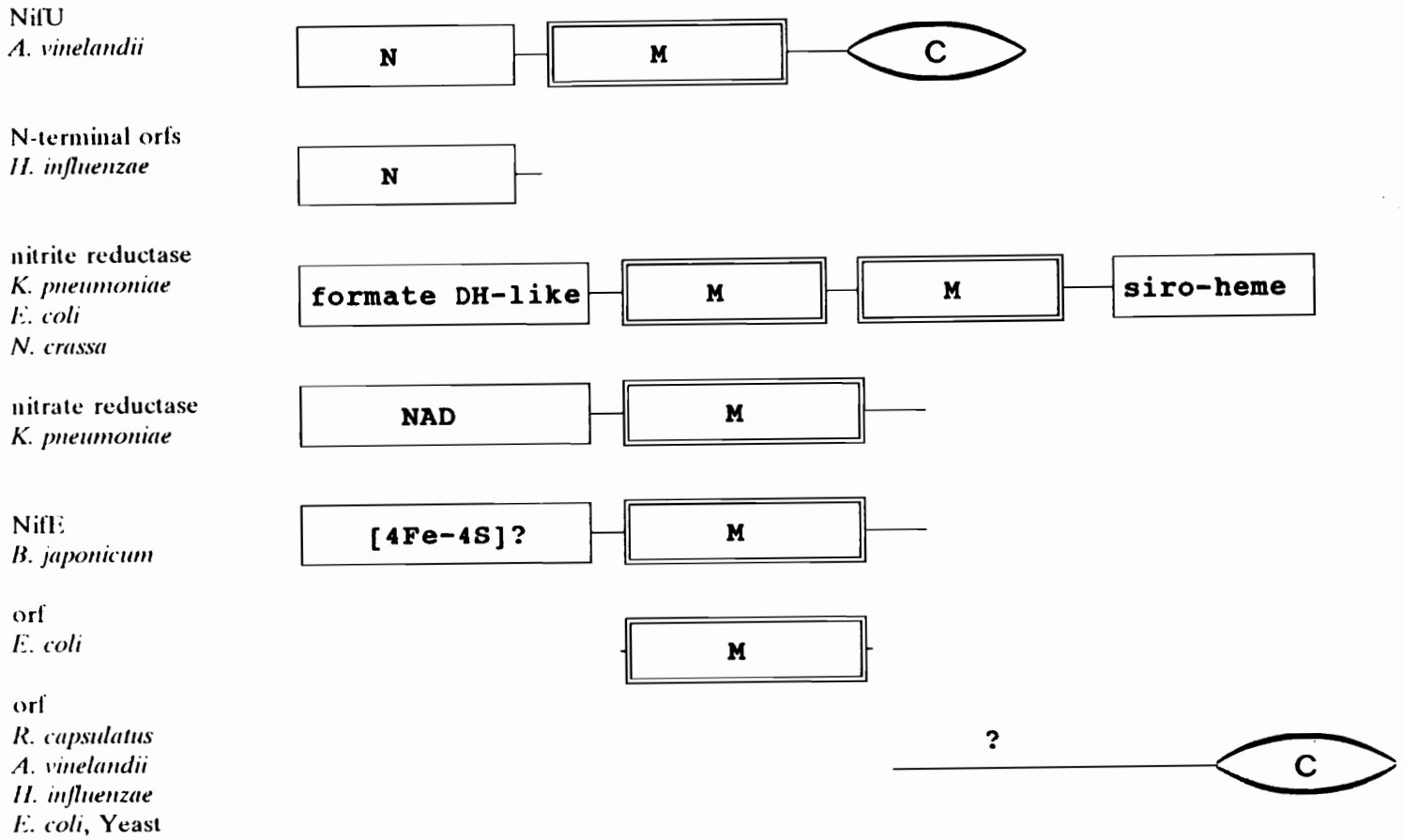

Figure 10. Modular architecture of proteins that contain NifU-like domains (N = N-terminal; M = middle and C = C-terminal. Protein fragments are not drawn to scale, sequence regions for which no homologs are found are indicated by question marks. Nicotinamide Adenine Dinucleotide (NAD), siro-heme and formate dehydrogenase (DH) binding domains are indicated. Adapted from Ouzonis, (1993).

NifU which are located in the many different organisms as N (for N-terminal), M (for middle) and C (for C-terminal).

The N-terminal domain, representing approximately the first 70 amino acid residues of NifU from *A. vinelandii,* have recently been found in *Haemophilus influenzae* and *Mycobacterium leprae* (Fleischman et al., 1995 and GenBank, respectively, see Figure 11). Interestingly, the N-terminal NifU from *H. influenzae* is found directly upstream of NifS, similar to the spatial arrangement found in *nif* clusters thus far.

As can be seen from Figure 10, the M region can be found, 1) fused to other protein domains which contain other electron transfering cofactors, 2) in tandem (as in the nitrite reductases), 3) as an isolated orf, or 4) fused to a gene involved in FeMo-cofactor biosynthesis, ie. *nifE* in *Bradyrhizobium japonicum.* The orf in *E. coli* is located in the bacterioferritin operon and whether it has a role in Fe storage is unknown at this time (Ouzonis et al., 1993)

The C-terminal domain can also be found fused to larger polypeptide domains or in smaller orfs with no obvious difference between the two domains. *A. vinelandii* contains two copies of this domain, one in NifU and the other in orf2, both of which are in the *nif* cluster (Jacobson et al., 1989a). Sequence comparisons of both the middle and C-terminal domains in Figures 12a and -b, show a 30% and 46% sequence conservation, respectively. Note especially that the cysteinyl residues in each domain are highly conserved.

```
      1                                                                                                  100
Azovi ..........  ..........  ....MWDYSE KVKEHFYNPK NAGAVEGAN. .....AIGDV GSLSCGDALR LTLKVDPETD VILDAGFQTF GCGSAIASSS
Klepn ..........  ..........  ....MWNYSE KVKDHFFNPR NARVVDNAN. .....AVGDV GSLSCGDALR LMLRVDPQSE IIEEAGFQTF GCGSAIASSS
Heali VVSSLIFSCH YLTMAFTRIE KRKVKMAYSE KVIDHYENPR NVGSLDKKDS ...NVGTGMV GAPACGDVMQ LQIKCD.DNG IIEDAKFKTY GCGSAIASSS
Mycle ..........  .........M ILRLEQIYQE VILDHYKHPQ HRGLREP... ...FCAQVYH VNPICGDEIY LRVALSDNGA SVADISYEGQ GCSISQASIS

cons  ----------  ----------  -------YSE KV-DH--NP- N---------  ---------V G---CGD---  L----D----  ------F---  GCGSAIASSS


      110
Azovi ALTEMVKGLT LDEALKISNQ DIADYLDGLP PEKMHCSVMG REALQAAVAN YRGETI..ED
Klepn ALTELIIGHT LAEAGQITNQ QIADYLDGLP PEKMHCSVMG QEALRAAIAN FRGESL..EE
Heali LITEWVKGKS LEEAGAIKNS QIAEELE.LP PVKVHCSILA EDAIKAAIAD YKAKQC
Mycle VLAQQVIGQS VPDALNII.S AFTEMCSSRG TIEGDEDVLG DGVAFAGVAK YPARVKCALL

cons  --E-------  L-EA--I-N-  -IA-----LP P-K-HCS--G  --A--AA-A-  Y---------
```

Figure 11. Amino acid sequence comparisons of N-terminal NifU like domains. First column:Swissprot names are as follows: Azovi, *Azotobacter vinelandii*; Klepn, *Klebsiella pneumoniae*; Mycle, *Mycobacterium leprae*. Numbers refer to the *H. influenze* sequence, amino acids which are similar to at least three organisms are shown at the bottom of each sequence as consensus (cons.)

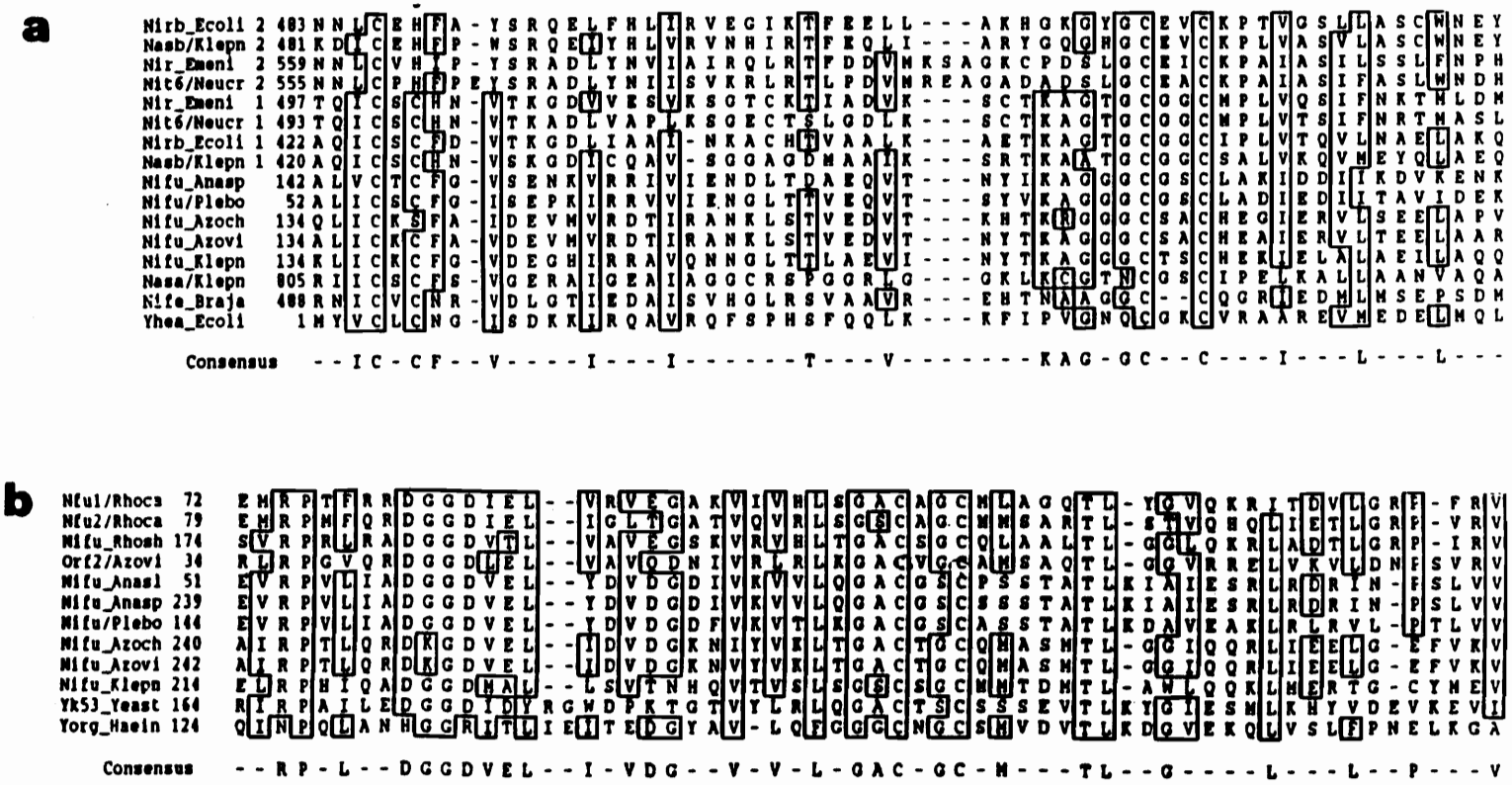

Figure 12. Alignment of the middle (a) and C-terimal (b) domains. First column: SWISSPROT name, second column: position of the domain within the corresponding proteins; last column, EMBL database accession number. Residues conserved in more than half of the sequences and boxed and displayed as consensus (bottom line). Abbreviations are as follows; Nir, Nit6, Nirb, Nasb, nitrite reductase; Nasa, nitrate reductase; Yhea, Yeast; Nfu1 or 2, NifU c-terminal; Yk53, unknown protein in yeast; Klepn, *Klebsiella pneumoniae*; Neucr, *Neurospora crassa*; Azovi, *Azotobacter vinelandii*; Azoch, *Azotobacter chroococcum*; Anasp, *Anabaena* sp.; Braja, *Bradyrhizobium japonicum*; Haein, *Haemophilus influenze*; Rhoca, *Rhodobacter capsulatus*; Rhosh, *Rhodobacter sphaeroides*; From Ouzonis, (1993) and references therein.

CHAPTER 4. SITE-DIRECTED MUTAGENESIS OF CONSERVED CYSTEINYL RESIDUES IN NIFU FROM *Azotobacter vinelandii*

This chapter is the basis for a manuscript to be submitted to the *Journal of Bacteriology* and is authored by Dennis R. Dean and myself.

ABSTRACT

The *nifU* gene product from *Azotobacter vinelandii* is a homodimer containing identical [2Fe-2S] clusters coordinated entirely by cysteinyl ligands (Fu et. al., 1994) and is absolutely required for diazotrophic growth (Jacobson et. al., 1989a). The highly conserved cysteinyl residues in *nifU* genes from *A. vinelandii*, *Klebsiella pneumoniae* and *Anabaena sp.* have an unusual spatial distribution for [2Fe-2S] cluster coordination. Using site-directed mutagenesis, I have substituted alanine for the cysteinyl residues at positions 35, 62, 106, 137 and 139 and have placed multiple substitutions in the two C-X_2-C motifs at positions 172, 175, 272 and 275. These altered proteins were hyperexpressed in a heterologous host and purified. Visible spectroscopy of the isolated altered proteins indicates that proteins having substitutions at positions 137, 139, 172 or 175 no longer contain the [2Fe-2S] cluster. Thus, these are the likely ligands to the metal center. Using gene replacement techniques, we have placed each site-directed mutant into the *A. vinelandii* chromosome and examined the effects on diazotrophic growth. Substitutions at the cluster ligand positions as well as 35, 62 and 106 resulted in a Nif-slow phenotype. In contrast, substitution at positions 272 and 275 had growth rates the same as wild type. The role of the conserved cysteinyl residues and the [2Fe-2S] cluster in relation to nitrogenase metallocluster biosynthesis and stability are discussed.

INTRODUCTION

Biological nitrogen fixation is catalyzed by nitrogenase, a complex, two component metalloenzyme composed of the Fe protein and the MoFe protein (recently reviewed by Kim and Rees, 1994; Dean et al., 1993). Nitrogenase turnover involves the sequential delivery of electrons from the Fe protein to the MoFe protein in a process that involves the hydrolysis of at least two MgATP per electron transfer along with the association and dissociation of the component proteins. The MgATP binding sites are located on the Fe protein (γ_2 homodimer that contains a [4Fe-4S] cluster bridged between the two subunits), while the substrate reduction site is located on the MoFe protein (an $\alpha_2\beta_2$ heterotetramer which contains two Fe_8S_8 centers known as P-clusters and two $MoFe_7S_9$:homocitrate cofactors, Kim and Rees, 1994). The unique reaction carried out by nitrogenase is reflected in the metal centers which take part in the inter- and intra-molecular electron transfer reactions and provide the site for substrate reduction (Liang et al., 1990). The primary translation products from the nitrogenase structural genes are not active but require several *nif*-associated gene products for the assembly and insertion of the appropriate metal centers into their respective component proteins. For example, the product of the Fe protein structural gene, *nifH*, requires the *nifM* gene product while the product of the MoFe protein structural genes, *nifD* and *nifK*, specifically require *nifE, N, H, B, Q,* and *V* (Jacobson, et al., 1989*a*). It is important to note that two *nif* gene products, NifU and NifS, are required for maturation of both the Fe and the MoFe proteins (Jacobson et al., 1989*b*). Because iron and inorganic sulfide are required for both nitrogenase component proteins, we have proposed that *nifU* and *nifS* may be involved in the mobilization of the iron and sulfide necessary for metallocluster formation (Dean et al., 1993; Zheng et al., 1993). Support for this idea comes from our investigations of NifS, a pyridoxal-phosphate containing enzyme, which catalyzes the desulfurization of L-cysteine (Zheng et al., 1994) and

is capable of providing inorganic sulfide for the *in vitro* reconstitution of the Fe protein (Zheng and Dean, 1994). We have previously shown that NifU is a [2Fe-2S] containing protein (Fu et al., 1994). Spectroscopic properties indicated that this metal center was coordinated entirely by cysteinyl ligands with distinct electronic, magnetic and vibrational properties unique for a [2Fe-2S] protein. There are nine conserved cysteinyl residues in the NifU homologs found in *A. vinelandii* (Beynon et al., 1987), *K. pneumoniae* (Beynon et. al., 1987), and *Anabaena sp.* (Mulligan and Haselkorn, 1989). Three questions which I have asked in this investigation of the role of NifU in nitrogenase biosynthesis were, 1) What are the ligands to the [2Fe-2S] cluster? 2) Is the [2Fe-2S] cluster essential for function? and 3) What is the role(s), if any, of the conserved cysteinyl residues which are not cluster ligands? In order to address these questions, I have altered each of the conserved cysteinyl residues by site-directed mutagenesis. The effect of each substitution on nitrogenase activity and on presence or absence of the metal cluster within NifU is described.

EXPERIMENTAL PROCEDURES

Growth of A. vinelandii strains and nitrogenase derepression. The wild-type and mutant strains were grown in modified, liquid Burk nitrogen free media at 30° C. When a fixed nitrogen source was required, ammonium acetate was added to a final concentration of 30 mM. Preparation of inoculum and determination of growth rates were as described previously and monitored with a Summerson-Klett meter using a no. 66 filter (Jacobson et al., 1989*b*). Derepression of nitrogenase synthesis, preparation of crude extracts and assays of Fe and MoFe protein specific activities were performed as described in Jacobson et al. (1989*b*).

Site-directed mutagenesis. Two different methods for site-directed mutagenesis were used. The method of Zoller et al., (1983) was used initially in our laboratory for mutagenesis using degenerate oligonucleotides for substitutions at the two CXXC motifs (172, 175, 272 and 275). Site-directed mutagenesis at amino acid positions 35, 62, 106, 137 and 139 for alanine involved the method of Kunkel et al., (1985) due to its simplicity and greater frequency of mutation. For the alanyl substituions, single stranded template DNA was prepared from plasmid pDB800 which was constructed by insertion of the 1000 bp *Xba* I fragment from pDB525 (Fu et al., 1994) into the pUC119 vector propagated in *Escherichia coli* strain 71-18 (*lac-proAB*) *thi supE* (*F'proAB lacIqZ M15*). The method of Zoller et al. (1983) was used for multiple substitutions using degenerate oligonucleotides. Single-stranded template DNA was prepared from plasmid pDB694 which was constructed as above except the fragment was cloned into M13mp18. Oligonucleotides and primers used are shown in Table 3. Final nucleotide mismatches (underlined) were verified by sequencing (Sanger et al., 1977). Restriction enzymes and T_4 DNA ligase were purchased from Life Technologies Inc. (Bethesda, MD) or New England Biolabs (Beverly, MA). Sequenase was purchased from United States Biochemical Corp. (Cleveland, OH).

Table 3. Construction of site-directed mutants in *nifU* from *Azotobacter vinelandii*

Substitution[a]	Plasmid	Strain	Oligonucleotide[b]
U35CA	pDB801	DJ935	5'-TCGCTGAGTGCCGGTGATGCG-3'
U62CA	pDB802	DJ937	5'-ACCTTCGGCGCCGGTTCCGCC-3'
U106CA	pDB824	DJ961	5'-AAGATGCACGCCTCGGTGATG-3'
U137CA	pDB819	DJ959	5'-GCGCTGATCGCCAAGTGCTTC-3'
U139CA	pDB820	DJ960	5'-ATCTGCAAGGCCTTCGCCGTC-3'
U172CF	pDB632	DJ704	5'-GGCGGTGGCTTTTCCGCCTGC-3'
U172CG	pDB633	DJ706	5'-GGCGGTGGCGGCTCCGCCTGC-3'
U172CM	pDB626	DJ701	5'-GGCGGTGGCATGTCCGCCTGC-3'
U172CP	pDB627	DJ702	5'-GGCGGTGGCCCCTCCGCCTGC-3'
U172CV	pDB631	DJ703	5'-GGCGGTGGCGTCTCCGCCTGC-3'
U172CW	pDB634	DJ707	5'-GGCGGTGGCTGGTCCGCCTGC-3'
U172CY	pDB635	DJ708	5'-GGCGGTGGCTATTCCGCCTGC-3'
U175CL	pDB657	DJ710	5'-TGCTCCGCCTTACACGAGGCT-3'
U175CS	pDB658	DJ711	5'-TGCTCCGCCAGCCACGAGGCT-3'
U175CF	pDB659	DJ713	5'-TGCTCCGCCTTCCACGAGGCT-3'
U175CG	pDB671	DJ714	5'-TGCTCCGCCGGCCACGAGGCT-3'
U272CF	pDB664	DJ873	5'-ACCGGCGCCTTTACCGGCTGC-3'
U272CP	pDB665	DJ874	5'-ACCGGCGCCCCTACCGGCTGC-3'
U272CG	pDB666	DJ875	5'-ACCGGCGCCGGTACCGGCTGC-3'
U272CM	pDB667	DJ876	5'-ACCGGCGCCATGACCGGCTGC-3'
U272CL	pDB669	DJ872	5'-ACCGGCGCCCTCACCGGCTGC-3'

[a] This column contains 1) the mutated gene, 2) the residue position within NifU having the amino acid substitution, and 3) the single letter code for the substituting residue.

[b] Sequence of oligonucleotides used to replace cysteinyl residues, nucleotide mismatches are underlined.

Table 3. continued

Substitution[a]	Plasmid	Strain	Oligonucleotide[b]
U275CE	pDB641	DJ878	5'-TGCACCGGCGAGCAGATGGCC-3'
U275CF	pDB642	DJ879	5'-TGCACCGGCTTTCAGATGGCC-3'
U275CR	pDB643	DJ880	5'-TGCACCGGCCGCCAGATGGCC-3'
U275CA	pDB644	DJ881	5'-TGCACCGGCGCTCAGATGGCC-3'
U275CS	pDB660	DJ882	5'-TGCACCGGCAGTCAGATGGCC-3'
U275CT	pDB661	DJ883	5'-TGCACCGGCACCCAGATGGCC-3
U106CA+ U137CA	pDB836	DJ1045	

[a] This column contains 1) the mutated gene, 2) the residue position within NifU having the amino acid substitution, and 3) the single letter code for the substituting residue.
[b] Sequence of oligonucleotides used to replace cysteinyl residues, nucleotide mismatches are underlined.

A. vinelandii transformations. Transformation of *A. vinelandii* was performed as described by Page and Tigerstrom (1979). Specific mutations within *nifU* corresponding to amino acid positions 35, 62 and 106 which recombined into the chromosome of wild-type *A. vinelandii* were isolated by congression (Robinson et al., 1986). Mutations within *nifU* corresponding to cysteinyl residues at positions 137, 139, 172 and 175 were isolated by marker rescue of the *nifU* deletion strain DJ105, which is Nif-, to prototrophy on Burk nitrogen free agar plates (Jacobsonet al., 1989*b*). After congression experiments failed to yield transformants with an altered Nif phenotype for substitutions of cysteinyl residues 272 and 275, a new strategy was devised in order to insure recombination of mutant DNA into the *A. vinelandii* chromosome. Mutations corresponding to substitutions at 272 and 275 were selected by transforming the Nif-, Kan^r strain DJ852 to prototrophy. Strain DJ852 was constructed with plasmid pDB780 which has a kanamycin resistance cartridge inserted at position 275. Plasmid pDB780 was constructed from the site-directed mutation of cysteine 275 codon TGC to the glycine codon GGT, which created a *Kpn*I restriction site, and designated as plasmid pDB712. This newly created restriction site was used to insert a kanamycin-resistance cartridge at this position 275. Transformed DJ852 was then plated on Burk ammonium acetate-supplemented agar plates, rescued isolates were Nif^+ and Kan^s.

A *nifU* double mutant was constructed by replacing the *Sst*II-*Bam*HI fragment from pDB825 with the corresponding *Sst*II-*Bam*HI fragment from pDB822, Figure 13. This plasmid, pDB836, has two alanyl substitutions at residues 106 and 137. Transformation of pDB836 into DJ105 and plating on Burk nitrogen free agar plates resulted in colonies with two distinct sizes. The larger and less abundant colony type was most likely the result of single point mutations which had recombined into the chromosome. The smaller colonies most likely resulted from both point mutations integrating into the chromosome.

Construction of the NifU double mutant U106CA + U137CA

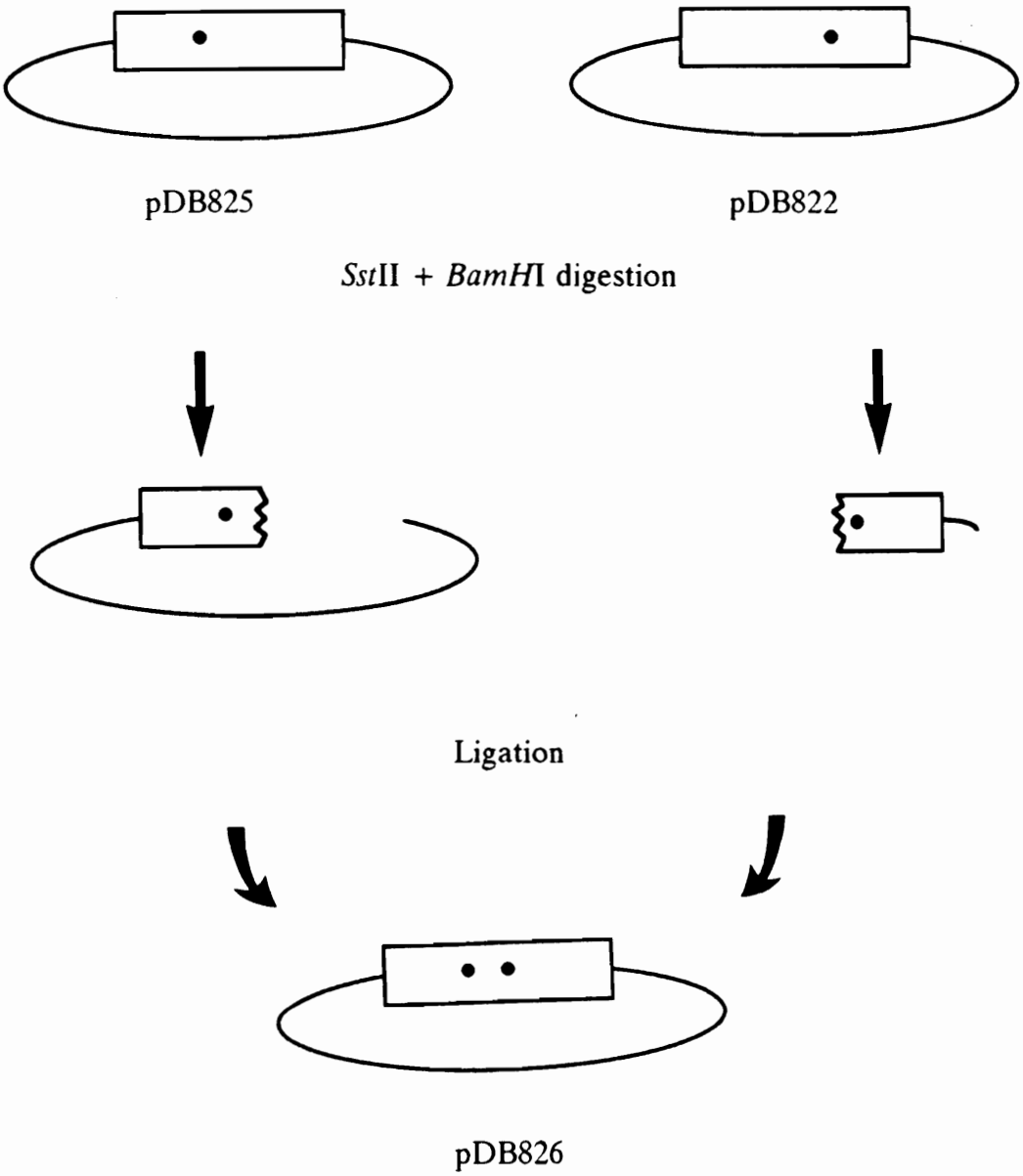

Figure 13. Construction of a double mutant in *nifU*. Positions of cysteinyl codons are indicated by the dots and represent changes from TGC to GCC. Plasmids were separately digested and fragments purified before ligation. Sizes of plasmids and purified DNA fragments are arbitrary.

The *nifF* gene product, NifF, was deleted by transforming *A. vinelandii* strains with plasmid pDB100 and plating on Burk ammonium acetate-supplemented agar plates with kanamycin at 20 μg/ml. Integration of pDB100 into *A. vinelandii* results in a partial deletion of the *nifF* gene and insertion of a kanamycin resistance cartridge (Bennett et al., 1988).

Hyperexpression and purification of wild type and altered fusion proteins. The mutant *nifU* genes from M13mp18 and pUC119 were subcloned into the vector pET-16b which places a polyhistidine fusion to the N-terminus of each protein for rapid purification using metal chelation chromatography (Novagen, Madison, WI). Subcloning of the mutant DNA involved digestions with the restriction enzymes *Nde*I and *Bam*HI and ligating into *Nde*I- *Bam*HI-digested pET-16b vector. Transformation and hyperexpression in the host strain BL21 (DE3) was as described (Fu et. al., 1994). Wild-type and altered proteins were purified anaerobically under an argon atmosphere. Crude extracts were prepared by resuspending approximately 12 g wet cells in 25 mM Tris-HCl buffer (pH 7.4, 2.5 mL of buffer per gram of wet cells) and disrupted by sonication, followed by centrifugation at 20 000 rpm for 20 min. in a Beckmann Type 35 rotor. The supernatant was loaded onto a 1.5 x 10 cm metal chelation column (Novagen, Madison, WI.) charged with 50 mM $NiSO_4$, as described by the manufacturer, using a peristaltic pump at a flow rate of 2 mL/min. The bound NifU was subjected to a step gradient of increasing amounts of imidazole in 20 mM Tris, 500 mM NaCl, pH 7.5 as indicated, 1) 150 mL of 5 mM imidazole (binding buffer), 2) 150 mL of 150 mM imidazole (wash buffer), and 3) 50 mLs of 400 mM imidazole (elution buffer). The wild-type and altered proteins were collected anaerobically and concentrated with an Amicon membrane filter (Beverly, MA.) with a 30 000 MW cutoff. This procedure results in approximately 15 mg of purified protein. Purity was monitored by SDS-PAGE as described by Laemmli

Coomassie Blue. SDS-PAGE molecular weight markers were purchased from Bio-Rad (Melville, NY). The visible spectra of the purified proteins were recorded using a Perkin-Elmer lambda 6 spectrophotometer.

RESULTS

Only four cysteinyl residues are necessary to coordinate a [2Fe-2S] cluster. To verify which of the cysteinyl residues present in NifU are cluster ligands, we chose to purify the altered NifU proteins and spectroscopically characterize each compared to wild-type. The results from the purification using Ni chelation chromatography of wild-type and altered fusion proteins are shown in Figure 14. The electrophoretic mobilities of wild-type and altered proteins were equal on SDS-PAGE and all NifU proteins are approximately 90% homogeneous based intensities of Coomassie Blue-stained bands.

My initial hypothesis was that a substitution of a cysteinyl residue which is not a cluster ligand will not affect the incorporation of the cluster. Though multiple substitutions were introduced at four positions (172, 175, 272 and 275), only one substitution at each position was chosen for hyperexpression and spectral characterization. Figure 15 compares the visible spectra of the wild-type and altered fusion proteins. The visible spectra of altered proteins having substitutions for residues 35, 62 and 106 are the same as the wild-type protein. Altered proteins with substitution of cysteinyl residues at positions 137, 139, 172 or 175 have visible spectra markedly different from wild-type which most likely results from elimination of the [2Fe-2S] cluster, thus these are the likely ligands to the Fe/S cluster. Complete coordination of a [2Fe-2S] cluster by cysteinyl residues in this spatial arrangement, C-X-C-X_n-C-X_2-C, has not been previously shown (Johnson, 1994). Finally, altered proteins with substitution of residues 272 and 275 have a visible spectra the same as wild-type indicating that this C-X_2-C motif is not likely to be involved in coordination of the Fe/S cluster.

How do the cysteinyl substitutions affect the function of NifU? This question was addressed by, 1) transformation of each mutant DNA into *A. vinelandii*, 2) selection for homologous recombination into the chromosome, and 3) comparison of diazotrophic growth rates for each mutant with wild-type and a

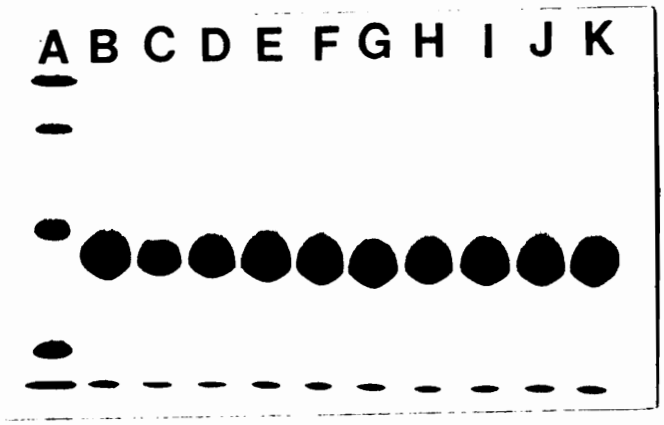

Figure 14. Coomassie-Blue stained SDS-PAGE (12%) of poly-histidine translational fusions of NifU and altered proteins purified by metal chelation chromatography. Lane a, molecular weight standards phosphorylase *b*, $M_r \approx 97$ 400; bovine serum albumin $M_r \approx 66$ 000; ovalbumin, $M_r \approx 42$ 700; carbonic anhydrase, $M_r \approx 31$ 000; and soybean trypsin inhibitor, $M_r \approx 21$ 500. Lanes B-K are wild-type NifU, U35CA, U62CA, U106CA, U137CA, U139CA, U172CV, U175CS, U272CM, and U275CS.

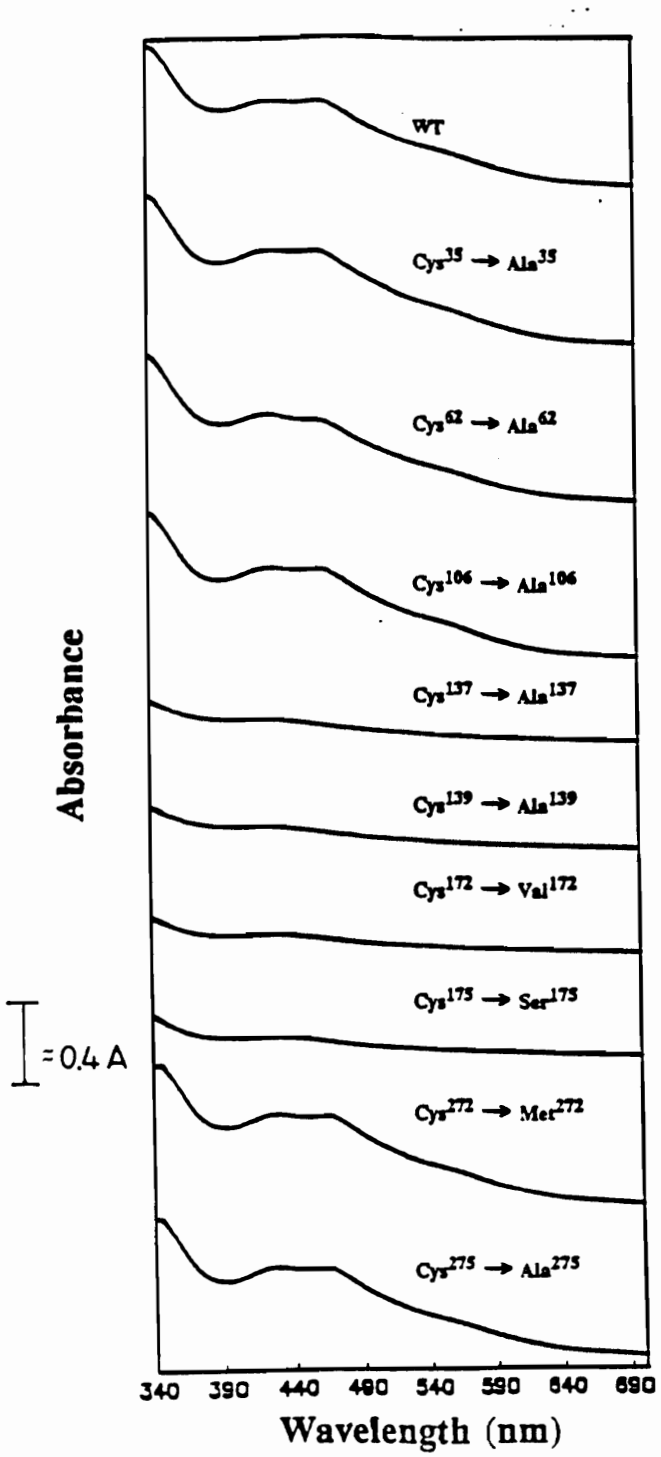

Figure 15. Visible spectra of poly-histidine translational fusions of wild-type NifU and altered proteins. Cysteinyl residue position and substitution are as indicated. Protein concentrations are approximately equal.

nifU deletion strain.

The effect of cysteinyl substitution within NifU on diazotrophic growth is shown in Table 4. Alteration of any one of the seven (out of nine) conserved cysteinyl residues causes a reduction in diazotrophic growth compared to wild-type; Replacing residues 35, 62, 106, 137, 139, 172 and 175 affects diazotrophic growth such that these mutants have a longer doubling time of 5 h compared to 2 h for wild-type. The doubling time for mutants with an altered residue at 35, 62 or 106 is equal to that for mutants with an altered cluster ligand residue at 137, 139, 172, or 175. This feature contrasts with substitutions for 272 and 275 which are in a second C-X_2-C motif; these mutants have a diazotrophic growth rate equal to wild-type.

The effect of two site-directed substitutions in NifU are cumulative as seen in the decreased diazotrophic growth rate of 12 h for the double mutant 106CA + 137CA. These cysteinyl residues are in different domains or regions (see below). One region is towards the N-terminus and the other is in a cluster ligand region. Interestingly, this double mutant is still Nif slow. Mutants with multiple substitutions placed at the cysteinyl residues in the C-X_2-C motifs are similar whether they affect diazotrophic growth or not. No substitution resulted in a complete elimination of diazotrophic growth as with the *nifU* deletion strain DJ105.

How do the cysteinyl substitutions within NifU affect diazotrophic growth? Is electron transfer to the Fe protein incapacitated in some way by elimination of the [2Fe-2S] cluster in NifU? Is the Fe protein function compromised or the MoFe protein or both as in the *nifU* deletion strain (Jacobson, et al., 1989b)? These questions were addressed by comparing the Fe protein and MoFe protein activities in crude extracts from the *nifU* mutant backgrounds with wild-type and the *nifU* deletion strain DJ105. Only one site-directed mutant was chosen as representative of each particular group. For example, cysteinyl residues 35, 62

Table 4. Diazotrophic growth of *Azotobacter vinelandii* mutant strains with substitutions of conserved cysteinyl residues in NifU.

Strain	Cysteinyl Substitution	Diazotrophic growth (doubling time in hrs.)
Wild Type	none	2
DJ105	ΔU[a]	NG[b]
DJ935	U35CA	5
DJ937	U62CA	5
DJ961	U106CA	5
DJ959	U137CA	5
DJ960	U139CA	5
DJ701	U172CM	5
DJ704	U172CF	5
DJ708	U172CY	5
DJ703	U172CV	5
DJ706	U172CG	5
DJ707	U172CW	5
DJ702	U172CP	5
DJ710	U175CL	5
DJ711	U175CS	5
DJ713	U175CF	5
DJ714	U175CG	5
DJ1045[c]	U106CA	
	U137CA	12

[a]indicates a gene deletion.
[b]No growth.
[c]indicates a double mutant.

Table 4. cont.

Strain	Cysteinyl Substitution	Diazotrophic growth (doubling time in hrs.)
DJ873	U272CF	2
DJ874	U272CP	2
DJ875	U272CG	2
DJ876	U272CM	2
DJ872	U272CL	2
DJ878	U275CE	2
DJ879	U275CF	2
DJ880	U275CR	2
DJ881	U275CA	2
DJ882	U275CS	2
DJ883	U275CT	2

and 106 did not result in elimination of the [2Fe-2S] cluster based on the visible spectra of these altered proteins. Thus, 106CA, was chosen to represent this group. Conversely, substitutions for cysteinyl residues 137, 139, 172 and 175 had similar structural phenotypes based on their visible spectra. Thus, 175CS was chosen to represent this group.

The Fe and MoFe protein activities in crude extracts from the various *nifU* and *nifS* mutant backgrounds are shown in Table 5. These results show that, 1) there is a reduction in both the Fe protein and MoFe protein activities with the NifU site-directed mutants (a pattern similar to but overall less dramatic than the *nifU* deletion strain), 2) the Fe protein and MoFe protein activities for both point mutations are approximately equal, which agrees with the diazotrophic growth rates being equal for the two point mutants and, 3) similar to the diazotrophic growth rates above, the effect of two site-directed cysteinyl substitutions within NifU are cumulative. A double mutant having substitutions at 106A and 137A follows a similar pattern with a reduction in both the Fe protein and MoFe protein activities. The component protein activities are intermediate compared to those of the individual point mutations and the *nifU* deletion strain.

The reduction in nitrogenase component protein activities is much more pronounced in the Fe protein than the MoFe protein. The oxygen sensitivity of nitrogenase is well known and the Fe protein is much more sensitive to oxidative inactivation than the MoFe protein (Wang et al., 1985). Thus, the pronounced reduction in Fe protein activity compared to the MoFe protein (in the *nifU* and *nifS* deletion mutants) suggested that these gene products may stabilize nitrogenase under oxidative conditions. Carefully controlled experiments using chemostat cultures of *A. vinelandii* indicated that electron flux to nitrogenase is primarily responsible for prevention of oxidative inactivation (Kuhla and Oelze, 1988). Biochemical-genetic studies have shown that the *nifF* gene product, a flavodoxin, is the sole electron donor to nitrogenase in *K. pneumoniae* and that its

Table 5. Nitrogenase component protein activities.

Strain	Fe protein (Specific activity[a])	MoFe protein (Specific activity[b])
Wild type	42	52
DJ105 (ΔU)[c]	2.5	15.4
DJ116 (ΔS)[c]	3.5	13.3
DJ961 (U106CA)	12	22
DJ711 (U175CS)	15	25
DJ1045 (U106CA + U137CA)	5.0	18

[a]Nanomoles of ethylene formed/min.mg of crude extract in the presence of saturating amounts of purified *A. vinelandii* MoFe protein. All values represent averages of at least four independent determinations

[b]Nanomoles of ethylene formed/min.mg of crude extract in the presence of saturating amounts of purified *A. vinelandii* Fe protein. All values represent averages of at least four independent determinations

[c]Reproduced from Jacobson et al., 1989*b*.

deletion eliminates diazotrophic growth (Shah et al., 1983). Parallel studies in *A. vinelandii* indicate that deletion of *nifF* has no apparent effect on diazotrophic growth (Bennett et al., 1988). It has been proposed that ferredoxin I functions as the electron donor to the Fe protein in the absence of flavodoxin (Morgan et al., 1988). However, the high degree of sequence homology and increased transcription of the *nifF* gene under de-repressing conditions indicates that NifF functions as an electron donor to nitrogenase in *A. vinelandii* (Bennett et al., 1988). Thus, the electron flux which stabilizes nitrogenase towards oxidative damage is likely to be mediated by NifF. What will be the effect on nitrogenase stability if electron flux is disrupted in the nifU and nifS mutant backgrounds?

We chose to place several *nifU* site-directed and *nifS* deletion mutants in *nifF* deletion backgrounds and assess the effect on nitrogenase activity by comparison of their diazotrophic growth rates. Deletion of *nifF* in combination with site-directed mutants from regions I (U62CA) or II (U175CS) resulted in Nif- phenoytpes. In contrast, deletion of *nifF* in combination with site-directed mutants from region III (U275CS) had growth rates equal to wild-type. The results of this experiment are shown in Figure 16. When *nifF* deletion mutations were placed in the various *nifU* mutant backgrounds (which are already Nif slow), diazotrophic growth was eliminated. Also, a *nifS* + *nifF*::kan^r double deletion strain is Nif-. These results can be interpreted as an indication of the importance of electron flux to nitrogenase activity, that when disrupted, nitrogenase stability towards oxidative damage is easily compromised (see discussion).

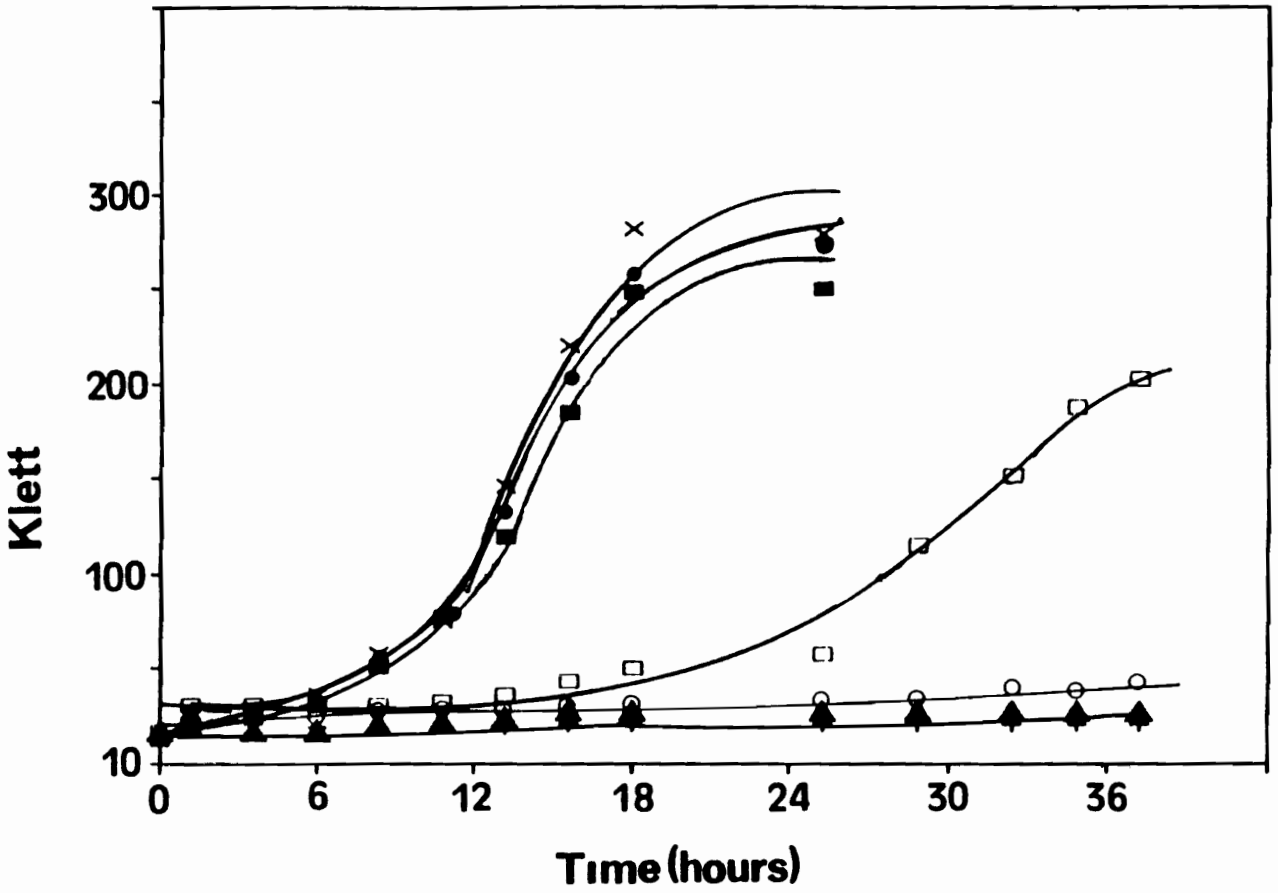

Figure 16. Effect of mutation(s) in electron transport and biosynthetic genes on diazotrophic growth. Wild-type (×), ΔF::Kan^r (■), U175CS (□), U175CS + ΔF::Kan^r (▲), U62CA + ΔF::kan^r (○), U275CS + ΔF::kan^r (●) and ΔS + ΔF::Kan^r (+).

DISCUSSION

The major conclusions from this work are that seven cysteinyl residues in NifU are required for optimal diazotrophic growth, four of which provide ligands to the [2Fe-2S] cluster and three in an as yet undetermined role(s). There are nine conserved cysteinyl residues in NifU at positions 35, 62, 106, 137, 139, 172, 175, 272 and 275 (Beynon et al., 1987). Of these, only 272 and 275, (a C-X_2-C motif towards the C-terminus), are not required for nitrogen fixation in *A. vinelandii*. The structural and growth phenotypes resulting from alteration of the cysteinyl residues follow a pattern along the primary amino acid sequence. With this in mind, NifU can be viewed as a protein with three regions, each of which can be defined (at least in part) by the cysteinyl residues within them. For example, Region I contains residues 35, 62 and 106 and although these residues are required for optimal nitrogenase activity, they are not cluster ligands. Region II contains residues 137, 139, 172 and 175, which provide the ligands to the metal cluster and are also required for optimal nitrogenase activity. Region III contains residues 272 and 275, substitution of these residues affects neither diazotrophic growth nor incorporation of the Fe/S cluster.

The novel magnetic, vibrational, and electronic properties of this [2Fe-2S] cluster are most likely due to the novel spatial arrangement of these coordinating ligands (C-X-C-X_n-C-X_2-C). It is important to note that elimination of the [2Fe-2S] cluster does not result in elimination of function. This suggests that if the cluster functions in an electron transport capacity, this electron transfer role(s) is not absolutely required for NifU function(s). This cluster ligand region (region II, above) has also been found in non-nitrogen fixing bacteria fused to a variety of protein domains in the assimilatory nitrite and nitrate reductases (Exley et al., 1993; Lin et al., 1993; Peakman et al., 1990), NifE in *Bradyrhizobium japonicum* (Aguilar et al., 1990), and as an orf in *E. coli* (Andrews et al., 1989). Site-directed mutagenesis of NifU has confirmed previous suggestions, based on biophysical

evidence, that this domain contains a [2Fe-2S] cluster (Cammack, 1992; Fu et al., 1994).

The second C-X_2-C motif in region III of NifU is not required for diazotrophic growth in *A. vinelandii*. This result supports observations by Masepohl et al., (1993) who have shown that deletion of a similar region does not affect diazotrophic growth in *Rhodobacter capsulatus*. This region is also found in orf2 in *A. vinelandii*, which is also located in the *nif* cluster, and is not required for diazotrophic growth (Jacobson et al., 1989*a*). Other locations include yeast (Purnelle et al., 1992) and *Haemophilus influenzae* (Tomb et al., 1991). Based on these observations, the role of this region in nitrogen fixation is nonessential.

What role do residues 35, 62 and 106 provide in maintaining a fully competent NifU? Recently, this region has also been located in *H. influenzae* (Fleischman, et al., 1995) and in *Mycobacterium leprae* (SwissProt, accession # 15432) as a small orf of unknown function. Interestingly, truncated *nifU* representing region I from *H. influenzae*, is located upstream of *nifS* (which is present in multiple copies in the genome) which is the same spatial arrangement found in *A. vinelandii, K. pneumoniae*, and *Anabaena* sp. The significance of both region I and region III in *H. influenzae* is not known.

A suggested role for NifU in metallocluster biosynthesis is based on the similar biochemical phenotypes of *nifU* and *nifS* deletion strains, namely, both show a reduction in Fe protein and MoFe protein activities (Jacobson et al., 1989*b*) and because NifS has been shown to provide the inorganic sulfide necessary for *in vitro* metallocluster biosynthesis (Zheng and Dean, 1994). Thus, the functions of NifU and NifS are linked by the biochemical phenotypes of the deletion mutants but must be different because of the structural differences between the two proteins. That NifU and NifS play general roles in metallocluster biosynthesis is supported by genetic evidence linking these gene products to maturation of the alternative nitrogenases in *A. vinelandii* as well as

the better characterized Mo-dependent nitrogenase (Kennedy et. al., 1992). Homologs to *nifU* and *nifS* are not found in the alternative *vnf* or *anf* clusters.

We have suggested that NifU plays a role complementary to NifS in metallocluster biosynthesis by the mobilization of Fe (Fu et. al., 1994). How can NifU be involved in the mobilization of Fe based on the evidence determined from site-directed mutagenesis of the conserved cysteinyl residues? A possible scenario is the [2Fe-2S] cluster might play a complementary, redox role by keeping bound, and/or promoting release of, Fe at an alternate site on NifU prior to metallocluster biosynthesis. Support for this idea comes from the mutagenesis of three residues (35, 62 and 106) which are equally required for optimal activity yet are not cluster ligands. Thus, these residues may be involved in loosely binding a transient Fe species, which is stabilized in some way by the [2Fe-2S] cluster of NifU.

Mobilization of Fe by a protein involved in Fe binding and transfer has been demonstrated. Ferrochelatase has been shown to catalyze insertion of ferrous ion in the terminal step of heme biosynthesis (Dailey et al., 1994). Mammalian ferrochelatase contains a [2Fe-2S] cluster and, although its removal eliminates function and the cluster does not undergo reduction during catalysis, the role of this cluster in unknown (Ferreira et al., 1994, Dailey et al., 1994). However, no sequence homology exists between ferrochelatase and NifU, indicating that at least two mechanisms for transient binding and delivery of an Fe species are likely, if this is indeed the role for NifU.

As stated above, electron flux to nitrogenase is the principal mechanism for prevention of oxidative inactivation. Also, NifF is the physiological electron donor in *K. pneumoniae* and a likely electron donor in *A. vinelandii.* Strains containing double mutations with the *nifU* site-directed mutantions in combination with *nifF* were made for the following reasons. Complete homologs to NifU, containing regions I, II and III, have only been found in aerobic, diazotrophic bacteria and

therefore NifU may be essential in conditions where oxygen inactivation of nitrogenase is possible. Deletion of *nifU* in *K. pneumoniae* eliminates the ability to fix nitrogen under micro-aerobic conditions (Robson, 1979 and Christine Kennedy, personal communication). Second, the greater reduction in Fe protein activity in *nifU* and *nifS* deletion mutants, together with its increased sensitivity to oxygen, suggests a role for these gene products in oxidative stabilization of the Fe and MoFe proteins. The effect of *nifU* point mutations which affect diazotrophic growth and of a *nifS* deletion, are more pronounced when placed in conjunction with *nifF* deletion backgrounds. It is likely that the stability of nitrogenase is further compromised in these strains because electron flux and Fe/S cluster assembly have been greatly hindered. The functions of NifU and NifS may therefore be to provide a continuous stream of Fe and inorganic sulfide to the oxidatively damaged metal centers of nitrogenase.

In summary, though the specific function of NifU is not known, site-directed mutagenesis experiments have given insight into the structure/function relationship of NifU. The [2Fe-2S] cluster is necessary for maximal activity of NifU, but its removal does not eliminate NifU function. Three additional residues have been implicated in a complementary role with the cluster which may involve binding of a transient Fe species. The biochemical phenotypes of *nifU* and *nifS* deletion strains link the respective functions of these structurally different proteins. The result of the respective functions is a fully functional nitrogenase, consisting of two component proteins with metal clusters composed of Fe and sulfide cores.

CHAPTER 5. SUMMARY AND OUTLOOK

Like most scientific endeavors, this project has raised as many questions as it has answered. This is further complicated by the discovery of homologs to NifU in a wide variety of organisms. My work has set the stage for elucidating the function of NifU and its homologs. Now that site-directed mutants have been made for all the conserved cysteinyl residues, these mutants and altered proteins can be valuable tools for researchers in other laboratories investigating the role(s) of the *nifU* homologs. The research on Fe/S cluster biosynthesis is open to new discoveries and advancements and, due to the wide spread abundance of metalloenzymes in all types of organisms, is beneficial to almost every facet of biochemistry, and physiology. Now that a truncated homolog has been found in humans, this may also open up many possibilities for health-related research. Some diseases may be caused by a deficiency in a particlular metalloenzyme, or the metallocluster biosynthetic capacity may be compromised in some way, which in turn, leads to a whole cascade of deficient enzymes in the body. Metallocluster biosynthesis and mobilization of the basic components may be more important than we could have ever imagined.

CHAPTER 6. LITERATURE CITED

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APPENDIX I

This section contains two papers describing the characterization of the *nifU* and *nifS* gene products. NifS characterization is described in the paper entitled "Cysteine desulfurase activity indicates a role for NifS in metallocluster biosynthesis" which was published in the *Proceedings of the National Academy of Science USA* by Limin Zheng, Robert White, Valerie Cash, Dennis Dean and myself. My involvement in this project was the initial subcloning of *nifS* using the Polymerase Chain Reaction (PCR) after engineering of an *Nde*I restriction site at the 5' end and a *BamH*I restriction site in the 3' end of the gene. This facilitated subcloning into the T7 expression vector, which could then be used for hyperexpression in *E. coli*. Immediately, we suspected a chromophore was present as the hyperexpressed whole cells had a distinct yellow color compared to uninduced control cells. My involvement ended after early stage purification and visible spectroscopy, which further supported the presence of a chromophore.

Simultaneously, work on the *nifU* gene product was also progressing and to our surprise, whole cells which were induced to hyperexpress NifU had a distinct red color compared to uninduced controls. Following purification, this protein also contained a chromophore and, based on its visible spectra, was entirely different from that in NifS. The characterization of NifU is described in the paper entitled "*nifU* Gene Product from *Azotobacter vinelandii* is a Homodimer That Contains Two Identical [2Fe-2S] Clusters" which was published in *Biochemistry* and authored by Weiguang Fu, Dennis R. Dean, Mike Johnson, Vance Morgan and myself. The collaboration with Johnson, Fu and Morgan from the Center for Metalloenzyme Studies, University of Georgia, Athens, GA., provided the spectroscopic expertise. The midpoint potential of the metal cluster was determined by myself with kind help from Bill Newton.

To our surprise, the two gene products had different chromophores, yet

when either of the respective genes was deleted from the *A. vinelandii* chromosome, the two mutants had the exact same biochemical phenotype. The next steps in probing the functions of NifU and NifS took different routes based on their structural differences. For example, once it was determined that NifS was a pyridoxal-phosphate containing enzyme, and that pyridoxal-phosphate chemistry was well known for de-amination of amino acids, it seemed logical that this enzyme would in some way be involved in amino acid metabolism. Fortunately, NifS only reacted with L-cysteine, and more importantly, did not deaminate but removed elemental S. This immediately suggested that cysteine provides the inorganic S (which is mobilized by NifS) for metallocluster biosynthesis. This information further suggested that NifU may be involved in the Fe side of metallocluster biosynthesis. It is now known that NifU contains a unique spatial arrangement of cysteinyl ligands to its own metal cluster and that additional cysteinyl residues are required for optimal activity *in vivo*. The possible role of the [2Fe-2S] cluster and these cysteinyl residues in Fe transfer is described in this thesis. Evidence to confirm this hypothesis has remained elusive. There is no change in the visible or EPR spectra upon incubation with ferrous or ferric ion. Mössbauer experiments with Vincent Huynh and Steve Lloyd of Emory University indicate a slight interaction of ferrous ion with NifU only when the cluster was in the reduced state. Although no interaction between NifU and ferric ion was detected (whether the cluster was oxidized or reduced) the amount of ferrous ion bound to reduced NifU was too low to be of major significance in these experiments. Most likely, additional factors are required for increased interaction of ferrous ion with NifU.

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Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis

(nitrogenase/FeS cores/pyridoxal phosphate/sulfur mobilization/cysteinyl persulfide)

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ABSTRACT **Biological nitrogen fixation is catalyzed by nitrogenase, a complex metalloenzyme composed of two separately purifiable component proteins encoded by the structural genes *nifH*, *nifD*, and *nifK*. Deletion of the *Azotobacter vinelandii nifS* gene lowers the activities of both nitrogenase component proteins. Because both nitrogenase component proteins have metallocluster prosthetic groups that are composed of iron- and sulfur-containing cores, this result indicated that the *nifS* gene product could be involved in the mobilization of the iron or sulfur required for metallocluster formation. In the present work, it is shown that NIFS is a pyridoxal phosphate-containing homodimer that catalyzes the formation of L-alanine and elemental sulfur by using L-cysteine as substrate. NIFS activity is extremely sensitive to thiol-specific alkylating reagents, which indicates the participation of a cysteinyl thiolate at the active site. Based on these results we propose that an enzyme-bound cysteinyl persulfide that requires the release of the sulfur from the substrate L-cysteine for its formation ultimately provides the inorganic sulfide required for nitrogenase metallocluster formation. The recent discovery of *nifS*-like genes in non-nitrogen-fixing organisms also raises the possibility that the reaction catalyzed by NIFS represents a universal mechanism that involves pyridoxal phosphate chemistry, in the mobilization of the sulfur required for metallocluster formation.**

Many proteins that have important electron transfer properties are known to contain nonheme metalloclusters that are composed mainly of an inorganic iron and sulfide core, but in some cases also contain another metal (e.g., Mo: ref. 1) or an organic constituent (e.g., homocitrate: ref. 2). Included among these is nitrogenase, which catalyzes the reduction of dinitrogen, an essential reaction in the global nitrogen cycle. Although such metalloclusters contained within the nitrogenase component proteins and metalloclusters present in other proteins having redox, catalytic, and regulatory properties are obviously critical to the functions of their respective polypeptides, to our knowledge, there is currently no information concerning the specific steps involved in the mobilization of the inorganic iron and sulfur required for metallocluster assembly.

In a previous study, we reported (3) that the *nifS* gene product (NIFS) from *Azotobacter vinelandii* is required for the full activation of the two metalloproteins that are the catalytic components of nitrogenase. The two component proteins of nitrogenase include an Fe protein [also called dinitrogenase reductase (4), a homodimer that contains an [4Fe-4S] cluster] and MoFe protein [also called dinitrogenase (4), an $\alpha_2\beta_2$ tetramer that contains two eight-Fe centers and two FeMo cofactors] (for a recent review of the composition and functions of the nitrogenase metalloclusters, see ref. 5). The reduction in both *A. vinelandii* nitrogenase component protein activities as a result of *nifS* deletion, which has also been reported for *Klebsiella pneumoniae* (6), could not be attributed to a regulatory effect (3). Thus, because both Fe protein and MoFe protein activities were affected by deletion of *nifS*, and the common feature of both component proteins is that they contain metalloclusters, we have considered that NIFS might be involved in the acquisition or mobilization of the inorganic Fe or sulfur required for metallocluster formation. This possibility is supported by the observation that cell pellets of diazotrophically grown *A. vinelandii nifS* mutants are pale tan rather than the characteristic dark brown of wild type. The dark color of diazotrophically grown wild-type *A. vinelandii* is attributed to the metalloclusters contained within the nitrogenase component proteins that form a substantial portion of the soluble protein fraction.

As a strategy to elucidate the function of the NIFS polypeptide, we chose to produce large amounts of it in *Escherichia coli* to facilitate its purification and biochemical characterization. In the present study, we have purified the NIFS protein, demonstrated that a specific reaction is catalyzed by NIFS, and proposed a biochemical function for NIFS activity in relation to metallocluster formation. In addition, we discuss the possibility that the reaction catalyzed by NIFS represents a universal mechanism for the mobilization of the sulfur required for metallocluster formation.

MATERIALS AND METHODS

Hyperproduction and Purification of NIFS. Hyperproduction of NIFS in *E. coli* was accomplished by constructing a *nifS* gene cartridge *in vitro* and cloning this cartridge into the pT7-7 plasmid such that *nifS* gene expression is controlled by the T7 transcriptional and translational regulatory elements (7). *E. coli* cells [BL21(DE3)] containing the *nifS* expression plasmid were grown in 500-ml batches in Luria broth containing ampicillin (100 mg/liter) at 30°C and NIFS production was induced when cells reached ≈160 Klett units (red filter) by the addition of lactose to 1% final concentration. After the addition of lactose, cells were cultured for an additional 2 h. After the induction period, cells were harvested by centrifugation and stored at −80°C until used.

For purification of NIFS, cell pellets were thawed in 25 mM Tris·HCl (pH 7.4) at 2.5 ml of buffer per g of cell pellet. After disruption by sonication, cells were centrifuged at 15,000 rpm for 20 min in a Beckman Ty35 rotor. Solid streptomycin sulfate was added to the crude extract supernatant, which was then incubated on ice for 15 min. The resulting precipitate was removed by centrifugation at 13,000 rpm in a Sorvall GSA rotor. The supernatant from the streptomycin sulfate-treated sample was then fractionated at ice temperature by using solid ammonium sulfate. NIFS

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Abbreviations: PLP, pyridoxal 5-phosphate; GC/MS, gas chromatography/mass spectrometry; DTT, dithiothreitol.

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precipitated between 25% and 45% ammonium sulfate saturation and this fraction was collected by centrifugation as above and resuspended in buffer (≈20 ml of buffer was added per g of pellet). The sample was then loaded onto a 3 cm × 15 cm Mono Q Sepharose column by using a peristaltic pump and fractionated using a 600-ml 0.1 M–0.6 M NaCl gradient controlled by FPLC pumps. NIFS was eluted between 0.38 M and 0.45 M NaCl. The fractions containing NIFS were pooled and dialyzed at 4°C overnight against 0.5 M ammonium sulfate. The dialyzed sample was then loaded onto a 3 cm × 15 cm Phenyl-Superose column as described above and eluted with a 500-ml decreasing 0.5 M–0.0 M ammonium sulfate gradient. NIFS was eluted at the end of the gradient. All fractions containing NIFS were pooled, frozen, and stored in liquid nitrogen or at −80°C until used. The protein determination was by a biuret reaction (8). The N-terminal sequence analysis of isolated NIFS was performed using an Applied Biosystems model 477A protein sequencer operated by the Virginia Tech sequencing facility. Spectroscopic analysis of NIFS was performed using a Beckman DU-70 spectrophotometer.

Determination of the Molecular Weight of Native and Denatured NIFS. The molecular weight of NIFS monomer was determined by gel electrophoresis of the isolated protein under denaturing conditions as described by Laemmli (9). The native molecular weight was determined by gel-filtration chromatography using a 1 cm × 30 cm Superose 12 FPLC column. The buffer for native molecular weight determination was 50 mM Tris·HCl, pH 7.4/200 mM KCl/10 mM $MgCl_2$. A 0.3 ml/min flow rate was maintained using an FPLC pump.

Isolation and Analysis of the Chromophore Present in NIFS. Purified NIFS protein was dialyzed for 4 h against water at room temperature, and trifluoroacetic acid was added to the resulting solution to 0.2 M. This acidification resulted in the precipitation of the protein as a yellow solid, which changed to a white solid in 10–20 sec as the chromophore protonated and dissociated from the protein. The protein precipitate was removed by centrifugation. Based on the absorbance at 400 nm of a portion of the extracted sample, recorded at neutral pH, the recovery of the cofactor was >90%. The sample was then purified by HPLC using a Whatman Partisil PXS ODS column and a linear gradient of 0–50% (vol/vol) methanol over a 10-min period at a flow rate of 1 ml/min. After its isolation, the chromophore extracted from NIFS was analyzed and identified as pyridoxal 5-phosphate (PLP) by NMR, UV/visible spectroscopy, and gas chromatography/mass spectrometry (GC/MS) analysis as described in *Results*. Phosphate analysis of the acid-hydrolyzed chromophore was conducted using the classical phosphomolybdate method (10). The phosphate released by acid hydrolysis was also identified by GC/MS analysis of the trimethylsilyl derivative. For GC/MS analysis, the HPLC-purified cofactor was hydrolyzed with 6 M HCl for 3 h and, after evaporation of the HCl, the hydrolysis products were converted into trimethylsilyl derivatives by reaction with an equal mixture of *N,O*-bis(trimethylsilyl)acetamide and pyridine for 10 min at 100°C. GC/MS of the resulting derivatized sample was performed using a Durabond column (0.32 mm × 30 mm, J & W Scientific, Rancho Cordova, CA) programmed from 100 to 300°C at 10°C/min. The amount of PLP contained in NIFS was determined using the phenylhydrazine method of Wada and Snell (11). Purified samples of NIFS were assayed directly without deproteinating the samples. Weighted samples of authentic PLP were used as standards.

Assays for NIFS Activity. All NIFS reactions were performed with 0.5 mM L-cysteine in 20 mM Tris·HCl (pH 8.0). Dithiothreitol (DTT, 1 mM) was added to the samples where indicated. For product analysis, NIFS (2 mg/ml) was used and elemental sulfur, hydrogen sulfide, pyruvate, and alanine were quantitated after the reaction mixture was incubated at ambient temperature for 2.5 h (see Table 2).

The elemental sulfur produced was determined by a modification of the cyanolysis method described by Wood (12). Samples (0.50 ml) were mixed with 0.4 ml of 1 M NH_4OH, 3.6 ml of water, and 0.5 ml of 0.5 M KCN, incubated at ambient temperature for 20 min, and then heated at 65°C for 1 min. After the samples were cooled to ambient temperature, 1.0 ml of Goldstein's reagent (12) was added, precipitates were removed by centrifugation, and the absorbance was read at 460 nm.

For pyruvate and alanine assays, the reaction mixtures were loaded onto Amicon Centricons and the products were separated from NIFS by ultrafiltration. Two 0.25-ml aliquots of product were then used for pyruvate assays. The samples were brought to 1.0 ml with water before 0.25 ml of 1.5 M Tris·HCl (pH 9.3), 0.25 ml of 0.58 mM NADH, and 20 units of lactate dehydrogenase were added. Oxidation of NADH was recorded by following the decrease in absorbance at 340 nm compared to a standard curve.

Alanine was quantitated by its deamination to pyruvate, which was then assayed as described above. The NIFS reaction product (0.3 ml) obtained from filtration was incubated at 37°C for 3 h with 30 μl of 100 mM 2-oxoglutarate and 0.3 unit of L-alanine aminotransferase. Two 0.125-ml aliquots were then used for pyruvate assay.

Sulfide formation was quantitated as described by Siegel (13) except that the reaction mixture was prepared in a 1-ml volume by using 2-ml serum vials sealed with Parafilm-wrapped rubber stoppers. The reaction was terminated by injecting 0.1 ml of 0.02 M *N,N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 M HCl and 0.1 ml of 0.03 M $FeCl_3$ in 1.2 M HCl into the vials. After color developed for 20 min and precipitates were removed by centrifugation, the absorbance at 650 nm was recorded and the sulfide concentration was calculated based on a Na_2S standard curve.

Inhibition of NIFS Activity. Purified NIFS [1.7 nmol in 1 ml of 20 mM potassium phosphate (pH 7.0)] was incubated with 0–1.7 nmol of *N*-ethylmaleimide for 3 h at ambient temperature. DTT and substrate cysteine were then added to 1 mM and 0.5 mM, respectively, to initiate the NIFS reaction. NIFS activity was based on the formation of hydrogen sulfide as described above.

RESULTS

Purification of NIFS. Hyperproduction of NIFS was accomplished in *E. coli*. Accumulation of NIFS in crude extracts of BL21(DE3) cells harboring the *nifS* expression plasmid was demonstrated by denaturing gel electrophoresis (Fig. 1, lane B). This analysis showed the accumulation of a soluble polypeptide having a molecular weight of ≈44,000 that was not produced in control cells that harbored the parental plasmid pT7-7 (data not shown). Such results are in good agreement with the predicted molecular weight for

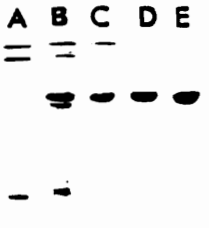

FIG. 1. Polyacrylamide gel electrophoretic analysis of the stages of NIFS purification. Lanes: A, molecular weight standards that include phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme; B, crude extract; C, ammonium sulfate fraction; D, Mono Q Sepharose fraction; E, phenyl-Superose fraction.

NIFS deduced from gene sequence data (14). Cell pellets of the *E. coli* strain that hyperproduced NIFS had a characteristic yellow color, indicating that NIFS contained a chromogenic prosthetic group. This assumption was, in fact, correct and provided a convenient means for visually following the purification of NIFS. NIFS was purified to homogeneity in a four-step procedure that included streptomycin sulfate fractionation, ammonium sulfate fractionation, Mono Q Sepharose column chromatography, and phenyl-Superose column chromatography (Table 1). Purity of the sample was confirmed by gel electrophoresis (Fig. .. lane E) and automated N-terminal sequence analysis. The final yield from the purification, based on the desulfurization of L-cysteine to give hydrogen sulfide, was 39%, and the specific activity of purified NIFS was 89.4 nmol of hydrogen sulfide produced per min per mg of protein (Table 1). The identity of isolated NIFS was confirmed by comparison of the N-terminal sequence determined from the isolated protein (Ala-Asp-Val-Tyr-Leu) to the predicted N-terminal sequence deduced from the gene sequence (14). The N-terminal methionine was not present in the isolated NIFS. Native molecular weight determination of NIFS was accomplished by gel-filtration chromatography using a Superose 12 FPLC column. Based on comparison to the migration of proteins having known molecular weights, native NIFS was shown to be a homodimer having a molecular weight of ≈87,500 (Fig. 2).

Identification of PLP as the Cofactor in NIFS. The isolated chromophore extracted from NIFS was readily retained when a solution of the compound, at neutral pH, was passed through a Q-Sepharose column but was not retained under the same conditions when passed through a CM-Sepharose column, indicating that the compound was anionic. That a portion of the negative charge resulted from the presence of a phosphate ester in the molecule was confirmed by cleavage of the sample by acid phosphatase. Identity of phosphate in the sample was also confirmed by the analysis of phosphate released from the sample by acid hydrolysis. The ultraviolet absorbance spectra of the sample was unaltered after acid hydrolysis, indicating that the phosphate ester was isolated from the chromophore of the molecule.

The purified cofactor dissolved in 0.05 M HCl had absorbance maxima at 230 (s, shoulder), 253, 293, and 324 nm with relative intensities of 0.41, 0.24, 0.52, and 0.51, respectively. The same concentration of cofactor, dissolved in 0.1 M NaOH, had absorbance maxima at 225 (s), 254, 304, and 388 nm with relative intensities of 1.0, 0.34, 0.19, and 0.03, respectively. Changes in the UV spectra of the compound with pH (data not shown) demonstrated that the chromophore showed both acidic and basic pKa values.

^{1}H NMR (400 Mz) of the HPLC-purified cofactor in ^2H_2O acidified with CF_3COO^2H showed resonances [expressed below as ppm (coupling pattern), proton identification] for two compounds in the ratio of ≈4:1. The most abundant compound had resonances at 2.63 (s, singlet), CH_3; 5.23 (d, doublet), CH_2—OP; 6.53 (s), ^{2}HOCHO^{2}H; and 8.21 (s), aromatic H. The minor compound had resonances at 2.69 (s), CH_3; 5.38 (d), CH_2—OP; 8.32 (s), aromatic H; and 10.48 (s), CHO. The chemical shifts of the protons present in the minor compound were similar to those previously reported for PLP recorded at neutral pH (15). The shifts for the major compound were about the same as observed in the minor compound with the exception that the resonance at 10.48 had shifted to 6.53. Thus, we concluded that the major compound was the hydrated form of PLP. The lack of rapid exchange between the pyridoxal aldehyde and its hydrate, required for their detection as separate resonances, has been reported (15).

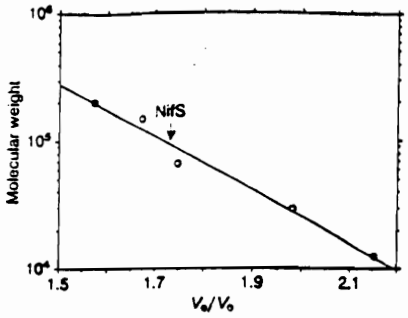

FIG. 2. Native molecular weight determination of NIFS. The void volume (V_o) was identified by blue dextran. Circles are proteins used as molecular weight markers, as follows: cytochrome *c*, M_r 12,400; carbonic anhydrase, M_r 29,000; bovine serum albumin, M_r 66,000; alcohol dehydrogenase, M_r 150,000; and β-amylase, M_r 200,000. The arrow indicates the migration position of native NIFS.

The GC/MS of the trimethylsilyl (TMS) derivative of the products resulting from the acid hydrolysis of the cofactor showed the presence of $(TMS)_3$phosphate and a peak identified as the $(TMS)_2$ derivative of the hemiacetal of pyridoxal (15).

All of these data are consistent with the assignment of the cofactor isolated from NIFS as being PLP. This structural assignment was confirmed by comparison of the extracted cofactor to an authentic sample of PLP. Both compounds had the same retention times on HPLC and TLC. Quantitation of the PLP content in NIFS showed 0.8 mol of PLP per NIFS monomer.

Identification of the Reaction Catalyzed by NIFS. PLP enzymes catalyze a diverse group of elimination and replacement reactions involving amino acids or their derivatives. To test whether or not NIFS catalyzed such a reaction using amino acids as substrate, we individually added each of the 20 L-amino acids to separate samples of NIFS and looked for changes in the visible spectra of the protein. Only L-cysteine was found to effect a change in the visible spectra, with the major absorbance peak at 392 nm changing to 416 nm (Fig. 3). Also, a new peak at 370 nm was formed upon addition of

Table 1. Purification of NIFS

Step	Total protein, mg	Total activity, nmol of H_2S per min	Specific activity, nmol of H_2S per min per mg	Yield, %
Crude extract	3550	40,500	11.4	100
Streptomycin supernatant	2250	37,800	16.8	93
25% ammonium sulfate supernatant	2250	36,000	16.0	89
45% ammonium sulfate precipitate	1110	26,300	23.7	65
Mono Q Sepharose	432	26,000	60.1	64
Phenyl-Superose	176	15,800	89.4	39

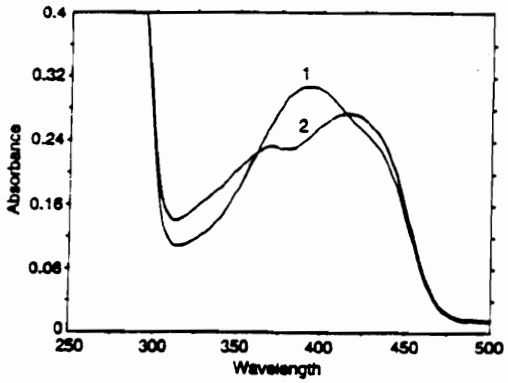

FIG. 3. Spectra of NIFS. Spectroscopic analysis was performed in a 1-ml cell containing purified NIFS at 4 mg/ml. Curves: 1, no addition; 2, in the presence of 0.5 mM L-cysteine.

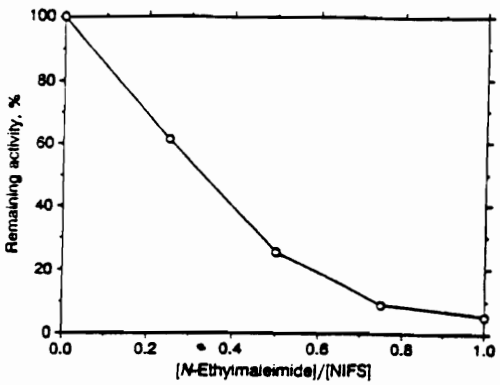

FIG. 4. Effect of *N*-ethylmaleimide on NIFS activity. Circles indicate the remaining NIFS activity, expressed as the percentage of that without inhibitor.

L-cysteine. The addition of D-cysteine had no effect on the spectrum. Incubation of L-cysteine with NIFS for 10 min or longer also resulted in formation of a cloudy precipitate of elemental sulfur. Table 2 shows the result of an analysis of the products formed from L-cysteine by NIFS. These results demonstrate that, in the absence of a reductant, NIFS desulfurates L-cysteine with the concomitant formation of elemental sulfur and L-alanine as the products. In the presence of dithiothreitol, the products were hydrogen sulfide, most likely produced by chemical reduction of the sulfur, and L-alanine.

Effect of Thiol-Specific Alkylating Reagents on NIFS Activity. Based on the consideration of potential mechanisms for desulfurization of L-cysteine catalyzed by NIFS and the possible physiological function for such activity (discussed below), it was of interest to determine whether a cysteinyl residue was involved in catalysis. To test this possibility, NIFS activity was assayed after its preincubation in the presence of thiol-specific alkylating reagents. The activity of NIFS was dramatically inhibited by preincubation of the protein with equimolar concentrations of *p*-chloromercuribenzoic acid, iodoacetamide, or *N*-ethylmaleimide. Fig. 4 shows that NIFS activity could be quantitatively inhibited by equimolar amounts of *N*-ethylmaleimide.

DISCUSSION

Results described in this report show that NIFS is a PLP-containing homodimer that specifically catalyzes the desulfurization of L-cysteine to yield equimolar amounts of L-alanine and elemental sulfur. Our interpretation of these findings is that NIFS catalyzes the release of sulfur from L-cysteine; the sulfur then becomes the sulfide present in the nitrogenase component protein metalloclusters. This suggestion is supported by previous work that showed that the sulfide present in iron–sulfur proteins from *E. coli* likely originates from cysteine sulfur (16). It also provides an explanation for the presence of a *nif*-specific serine acetyltransferase that, in *A. vinelandii*, is located in the same transcriptional unit as the *nifS* gene (14, 17). Formation of *O*-acetyl-L-serine is the step that is feedback-regulated in L-cysteine biosynthesis (18) and, thus, the presence of a serine acetyltransferase under *nif* gene regulation provides the cell a way to meet the increased demand for the L-cysteine that is required for metallocluster cluster formation under nitrogen fixing conditions.

Table 2. Desulfurization of L-cysteine catalyzed by NIFS

Reaction conditions	Products, % cysteine substrate		
	Sulfur	Sulfide	Alanine
L-Cysteine + NIFS	94.7	2.1	80.6
L-Cysteine + DTT + NIFS	8.0	96.5	92.8

Each reaction was performed in 2 or 4 ml of a mixture containing 0.5 mM L-cysteine in 20 mM Tris·HCl (pH 8.0). DTT (1 mM) was added to the reaction mixture where indicated. Reactions were initiated by addition of purified NIFS to 2 mg/ml. The reaction was incubated at ambient temperature for 2.5 h. In the absence of DTT, a small amount of pyruvate was also found as product and was subtracted from the alanine value shown in the table. There was no sulfur, hydrogen sulfide, or alanine produced in control experiments where either the enzyme or L-cysteine was omitted from the reaction mixture.

The route to the formation of sulfur from L-cysteine catalyzed by NIFS may have some similarities to the mechanism proposed for selenocysteine β-lyase, which is also a PLP enzyme and catalyzes the formation of L-alanine and Se by using L-selenocysteine as substrate (19). However, in contrast to the mechanism suggested for the elimination of Se from selenocysteine, we propose a different mechanism for the elimination of sulfur from L-cysteine catalyzed by NIFS. Namely, we suggest that after the cysteine-PLP Schiff's base adduct is formed, a nucleophilic attack occurs by an enzyme thiolate anion at the sulfur of the substrate cysteine. This results in repulsion of the stabilized C-3 anion of alanine and formation of an enzyme-bound cysteinyl persulfide, which in turn is the likely candidate for acting as the sulfur donor during metallocluster formation. In the present experiments, where the suitable acceptors for the activated sulfur are not available, namely, iron or the appropriate apoprotein, the cysteinyl persulfide decomposes to elemental sulfur (20), or, in the presence of a reductant, is reduced to hydrogen sulfide. Evidence for the presence of a highly reactive cysteinyl thiolate group on NIFS that could be involved in the formation of the cysteinyl persulfide during catalysis was obtained by titrating the enzyme with *N*-ethylmaleimide (Fig. 4), which, when present at an equimolar concentration with NIFS, resulted in nearly complete inactivation of NIFS.

Formation of protein-bound cysteinyl persulfides has precedence in thiosulfate sulfurtransferase (rhodanese), which has also been suggested to have a role in metallocluster formation (21). However, rhodanese does not use PLP chemistry and has not been shown to have a specific role in metallocluster formation. Indeed, Sandberg *et al.* (22) have argued against a physiological role for rhodanese in metal-

locluster formation because rhodanese has not been detected in appreciable levels in certain bacteria known to contain nonheme iron sulfur proteins. Moreover, any enzyme that catalyzes formation of sulfide could be expected to assist reconstitution of apoproteins *in vitro* because such reconstitution occurs spontaneously, provided that sufficient sulfide and iron are available (22). In contrast to rhodanese, a specific physiological role for NIFS in mobilizing the sulfur required for nitrogenase metallocluster formation is indicated because NIFS is required for maturation of both the nitrogenase component proteins and because it uses L-cysteine, a known sulfur source for nonheme metalloclusters in *E. coli* (16), as substrate.

Although the deletion of *nifS* lowers the activity of both nitrogenase component proteins, it does not completely eliminate the activity of either one. One explanation for this observation could be that the iron–sulfur cores required for nitrogenase metallocluster assembly could form spontaneously, albeit at a low level, in the absence of NIFS. This possibility is supported by previous work concerning the *in vitro* reconstitution of apoferredoxins using iron and sulfide (23). However, our inability to stimulate the diazotrophic growth of *nifS* mutants by increasing the sulfur and iron supplement to the growth medium does not support the idea of spontaneous iron–sulfur core assembly. An alternative explanation is that NIFS represents a general class of enzymes that use PLP chemistry and L-cysteine as substrate in a universal first step in the mobilization of sulfur for metallocluster formation. Namely, in the absence of NIFS, other NIFS-like enzymes involved in sequestering the sulfur for other metalloproteins might supplant the NIFS function. The identification of genes present in nonnitrogen fixing organisms that encode polypeptides having extraordinary sequence identity when compared to NIFS provides considerable support for this idea. For example, such genes have been identified from *Lactobacillus delbrueckii* (24), *Bacillus subtilis* (25), *Saccharomyces cerevisiae* (24), and *Candida maltosa* (24). Of particular relevance is the high degree of sequence identity among this class of polypeptides concentrated around the lysinyl and cysteinyl residues we have targeted as providing the probable PLP-binding and active-site cysteinyl residues, respectively (Fig. 5). Although the specific functions of the *nifS*-like genes from these organisms are not yet established, Leong-Morgenthaler *et al.* (24) have independently suggested that a common thread among such apparently different metabolic processes could involve redox functions.

In summary, NIFS has been shown to catalyze the desulfurization of L-cysteine to form L-alanine and elemental sulfur. Based on these results, consideration that elimination of NIFS function affects both nitrogenase component protein activities, and the recent discovery of *nifS*-like genes in nonnitrogen fixing organisms, we propose that NIFS activity represents a universal mechanism that uses PLP chemistry in the activation of the sulfur required for metallocluster formation.

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                    202                 325
                    ↓                   ↓
Av  ...IDMLSLCGHKLRAPRGVG...  ...IAASSGSACTSGSLEPS...
An  ...IDMLTISGHKIHAPRGIG...  ...ICASSGSACTSGSLEPS...
Kp  ...IDMLSCSAHKFHGPRGVG...  ...IAASSGSACTSGSLEPS...
Rs  ...IDMLSLSAHKFHGPRGVG...  ...ICVSSGAACASGAMEPS...
Sc  ...IDLLSISSHKIYGPRGIG...  ...IALSSGSACTSASLEPS...
Ld  ...VDMMSFSSHKFHGPRGIG...  ...IYISTTSACASKKADEA...
Bs  ...VDALSMSSHKIHGPRGVG...  ...ICISTGSACSAGYHGPS...
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FIG. 5. Sequence comparison of NIFS with other *nifS*-like gene products. Av, *A. vinelandii* NIFS (26); An, *Anabaena* NIFS (27); Kp, *Klebsiella pneumoniae* NIFS (26); Rs, *Rhodobacter sphaeroides* NIFS (28); Sc, *Saccharomyces cerevisiae nifS*-like YCL17C gene product (24); Ld, *Lactobacillus delbrueckii nifS*-like gene product (24); Bs, *Bacillus subtilus nifS*-like gene product that is involved in NAD biosynthesis (25). Numbers refer to the *A. vinelandii* sequence. Lys-202 is considered to be the probable PLP-binding residue, based on comparison to certain other known sequences of PLP-binding proteins (for example, see ref. 29), and Cys-325 is targeted as the probable active-site cysteinyl because it is the only conserved cysteinyl residue among all known NIFS sequences from diazotrophic organisms. Strictly conserved amino acids are underlined.

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nifU Gene Product from *Azotobacter vinelandii* Is a Homodimer That Contains Two Identical [2Fe-2S] Clusters†

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ABSTRACT: The *nifU* gene product is required for the full activation of the metalloenzyme nitrogenase, the catalytic component of biological nitrogen fixation. In the present work, a hybrid plasmid that contains the *Azotobacter vinelandii nifU* gene was constructed and used to hyperexpress the NIFU protein in *Escherichia coli*. Recombinant NIFU was purified to homogeneity and was found to be a homodimer of 33-kDa subunits with approximately two Fe atoms per subunit. The combination of UV/visible absorption, variable-temperature magnetic circular dichroism, EPR, and resonance Raman spectroscopies shows the presence of a $[2Fe\text{-}2S]^{2+,+}$ center ($E_m = -254$ mV) with complete cysteinyl coordination in each subunit. The electronic, magnetic, and vibrational properties of the $[2Fe\text{-}2S]^{2+,+}$ center do not conform to those established for any of the spectroscopically distinct types of 2Fe ferredoxins. These distinctive properties appear to be a consequence of a novel arrangement of coordinating cysteinyl residues in NIFU, and the residues likely to be involved in cluster coordination are discussed in light of primary sequence comparisons to other putative [2Fe-2S] proteins. The observed physicochemical properties of NIFU and its constituent [2Fe-2S] cluster also provide insight into the role of this protein in nitrogenase metallocluster biosynthesis.

The reduction of dinitrogen to yield ammonia is called nitrogen fixation, and the biological process is catalyzed by nitrogenase, a complex metalloenzyme comprising two separately purifiable component proteins commonly referred to as the Fe protein and the MoFe protein [reviewed in Dean and Jacobson (1992)]. Nitrogenase turnover involves sequential single-electron deliveries from the Fe protein to the MoFe protein by a process that involves hydrolysis of at least two MgATP for each electron transferred and is accompanied by an association and dissociation of the component proteins. Reduction of the Fe protein is accomplished by transfer of electrons from either a flavodoxin or a ferredoxin *in vivo* or by dithionite in *in vitro* assays. The primary sites for MgATP binding and hydrolysis are located on the Fe protein (a γ_2 homodimer that contains a single cubane-type Fe_4S_4 cluster), whereas the sites for substrate binding and reduction are located on the MoFe protein (an $\alpha_2\beta_2$ heterotetramer that contains two Fe_8S_{7-8} clusters and two $MoFe_7S_9$-homocitrate cofactors). The individual metalloclusters contained within the nitrogenase component proteins are very likely to participate in either the inter- or the intramolecular electron transfers required for substrate reduction and/or to provide the site of substrate reduction. See Kim and Rees (1994) or Dean et al. (1993) for brief reviews on the structures of the nitrogenase component proteins and their associated metal clusters.

The biochemical complexity of nitrogenase is reflected in the number and organization of genes required to form the active complex. For example, in addition to those genes that encode the nitrogenase structural components, there is an associated consortium of *nif*-specific genes whose products are required for formation and insertion of the appropriate metalloclusters into their complementary protein partners [see Dean and Jacobson (1992) and Dean et al. (1993) for reviews]. Included among these genes are *nifS* and *nifU*, which in *Azotobacter vinelandii* and *Klebsiella pneumoniae* are cotranscribed and adjacently located on their respective genomes (Beynon et al., 1987). The *A. vinelandii nifU* and *nifS* gene products (hereinafter referred to as NIFU and NIFS, respectively)[1] are distinguished from other *nif* gene products from this organism in that the activities of both are required for the full activation of both nitrogenase component proteins. Thus, because the acquisition of iron and inorganic sulfide for Fe-S core formation is the one feature required for activation of both of the nitrogenase component proteins, we have suggested that NIFS and NIFU might be involved in the mobilization of the iron and sulfide necessary for nitrogenase metallocluster core formation (Zheng et al., 1993; Dean et al., 1993). Indirect support for this idea comes from the pale tan color of crude extracts prepared from *A. vinelandii nifU* or *nifS* mutants (Jacobson et al., 1989b) that contrasts with the deep chocolate brown hue characteristic of crude extracts prepared from diazotrophically grown wild-

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[1] Abbreviations: NIFU, *A. vinelandii nifU* gene product; NIFS, *A. vinelandii nifS* gene product; NHE, normal hydrogen electrode; Fd(s), ferredoxin(s); (VT)MCD, (variable-temperature) magnetic circular dichroism; RR, resonance Raman; S^b, bridging or inorganic S; S^t, terminal or cysteinyl S.

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type cells. The dark color of diazotrophically grown wild-type *A. vinelanaii* is attributed to the metalloclusters contained within the nitrogenase component proteins.

As an approach to determine the respective functions of NIFS and NIFU, we have constructed hybrid plasmids for the purpose of hyperexpressing the *A. vinelandii* proteins in *Escherichia coli* to facilitate their purification and biochemical analyses. Studies of this nature have led to the discovery that NIFS is a pyridoxal phosphate-containing homodimer that catalyzes the desulfurization of L-cysteine (Zheng et al., 1993). An enzyme-bound persulfide is an intermediate in that reaction (Zheng et al., 1994), and we have proposed that this species is the likely sulfur donor in nitrogenase metallocluster core formation. In the present work we describe complementary studies which involve the hyperproduction, isolation, and initial biophysical characterization of NIFU.

MATERIALS AND METHODS

Hyperproduction of NifU in E. coli. Hyperproduction of NIFU in *E. coli* was accomplished by constructing a *nifU* gene cartridge *in vitro* and cloning this cartridge into the pT$_7$-7 plasmid (Tabor & Richardson, 1985) such that *nifU* gene expression was controlled by the T$_7$ phage transcriptional and translational regulatory elements. The *nifU* gene cartridge was constructed by using an isolated 3.4-kb DNA fragment that was generated by *Bam*HI restriction enzyme digestion of the hybrid plasmid pDB133 (Jacobson et al., 1989a) as a template for polymerase chain reaction (PCR) amplification of the *nifU* coding sequence. PCR amplification of *nifU* was performed essentially as recommended by the suppliers of Amplitaq (Perkin-Elmer, Norwalk, CT). Cycling temperatures for PCR amplification of *nifU* were as follows: 95 °C for 1 min, 60 °C for 2 min, and 72 °C for 3 min. The two PCR primers used for *nifU* amplification had the following sequences: 5′ATGCATATGTGGGATTATTCGGAA3′ and 5′ATCGGATCCCGGGTGGTGGCGTTG3′. The amino terminal coding region for NIFU is underlined in the first primer sequence shown above. Following *nifU* amplification, the gene cartridge was digested with the restriction enzymes *Nde*I and *Bam*HI and ligated to *Nde*I- and *Bam*HI-digested pT$_7$-7 DNA. Proper orientation of *nifU* within the resulting hybrid plasmid (pDB525) such that the T$_7$ gene-10 promoter directs *nifU* transcription was confirmed by restriction enzyme digestion of isolated plasmid DNA. The functional integrity of the cloned *nifU* gene contained within pDB525 was demonstrated by transforming the *A. vinelandii nifU* deletion strain DJ105 (Jacobson et al., 1989b) to prototrophy using isolated pDB525 DNA. Transformation of *A. vinelandii* was performed as previously described (Page & von Tigerstrom, 1979). For hyperproduction of NIFU in *E. coli*, pDB525 was transformed into the host strain BL21 (DE3) and the transformed cells were maintained on LB media supplemented with 100 μg/mL ampicillin. Cells were grown in 500-mL batches in LB media at 30 °C and were induced for NIFU production at approximately 160 Klett units (red filter) by the addition of lactose to 1% final concentration. Following the addition of lactose, cells were cultured for an additional 2 h, and after this induction period cells were harvested by centrifugation and frozen at −20 °C.

Purification and Biochemical Analysis of NIFU. NIFU was purified anaerobically under a purified argon atmosphere with 2 mM dithiothreitol added to all buffers. Crude extracts were prepared by resuspending cells in 25 mM Tris buffer (pH 7.4, 2.5 mL of buffer per gram of wet weight cells) and disruption by sonication, followed by centrifugation at 20 000 rpm for 20 min in a Beckman Type 35 rotor. The crude extract was fractionated by addition of solid streptomycin sulfate to 1%, w/v, incubation at ambient temperature for 20 min, and centrifugation as above. NIFU was then precipitated from the streptomycin sulfate-treated crude extract by bringing it to 30% saturation with solid ammonium sulfate. NIFU precipitated in this way was collected by centrifugation as above and resuspended in buffer at a volume equal to the original supernatant volume. The sample was then loaded onto a 2.5 × 20 cm Q Sepharose column (Pharmacia, Piscataway, NJ) using a peristaltic pump and eluted using a 600-mL 0.1 to 0.6 M NaCl gradient controlled by FPLC pumps. NIFU eluted between 0.41 and 0.46 M NaCl and was judged to be greater than 95% pure at this stage on the basis of Coomassie-stained polyacrylamide gels. Further purification was achieved using a 5 × 200 cm Sephacryl-300 gel-filtration column equilibrated with the above buffer containing 0.2 M KCl. The flow rate of the column was 10 mL/min. Metal analyses were performed on highly purified NIFU samples that were passed over the Sephacryl-300 column, whereas other experiments were performed on samples purified by Q Sepharose chromatography. Protein concentrations were determined by the biuret reaction (Gornall et al., 1949). That authentic NIFU was obtained by this scheme was demonstrated by N-terminal sequence analysis performed using an Applied Biosystems Model 477A protein sequenator operated by the Virginia Tech sequencing facility. The M_r of denatured NIFU was determined by gel electrophoresis (Laemmli, 1970), whereas the subunit composition was determined by gel-filtration chromatography using either a 1 × 30 cm Superose-12 FPLC column or a 2.5 × 50 cm Bio Gel A-0.5 m, 100−200 mesh gel-filtration column. The buffer used for column chromatography was 25 mM Tris·HCl (pH 7.4), 0.2 M KCl, and 10 mM $MgCl_2$. The iron content of NIFU was determined using the *o*-phenanthroline method of Fortune and Mellon (1938) using ferrous ammonium sulfate solutions as standards. Attempts to chelate Fe from isolated NIFU were performed using α,α′-dipyridyl as described previously (Walker & Mortenson, 1974). For chelation experiments, α,α′-dipyridyl was added to a final concentration of 4 mM to a 1.5-mL sample of 0.15 μmol of purified NIFU, and the release of Fe was monitored at A_{520}.

Chemical and Electrochemical Redox Titration of NIFU Metal Cluster. Chemical redox titration of the Fe-S center in NIFU was performed using dithionite as the reductant as previously described (Mayhew, 1978). In these experiments dithionite solutions were freshly prepared by adding 0.087 g of dithionite to an anaerobic 5-mL solution of 25 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid to which 0.15 mL of 3 M NaOH had been added. Anaerobiosis of solutions described here and elsewhere was achieved by repeated degassing under purified argon using a Schlenk apparatus. The dithionite solution was diluted 1:10 in the same buffer and then standardized spectrophotometrically using a degassed solution of 0.02 mM methylene blue as described previously (Schultz et al., 1985). Titrations were performed on 1-mL samples containing 0.1 μmol of NIFU in an argon-flushed cuvette sealed with a rubber serum

stopper. Redox titration of NIFU was performed by sequential additions of 1 or 2 μL of the dithionite solution using a gas tight 10-μL Hamilton syringe followed by gentle mixing. The absorbance change at A_{464} following each incremental dithionite addition was recorded using a Beckman DU 7400 diode array spectrophotometer.

The use of redox mediators for determining the midpoint potential of metalloproteins has been described (Watt, 1979). Briefly, 0.12 μmol of NIFU was prepared in a stoppered degassed vessel that contained 25 mM Tris (pH 7.4), 0.2 M KCl, and 2 mM dithiothreitol containing 10 μM safranine O or benzyl viologen as the redox mediator. The working electrode consisted of vitreous reticulated carbon glued to a silver wire that was passed through the rubber stopper. The auxiliary electrode was a platinum wire immersed in 3 M KCl in a Luggin tube fitted with a Vycor tip. The reference electrode was a Ag/AgCl microelectrode (Microelectrodes, Inc., Londonderry, NH). The protein solution was continuously stirred throughout the experiment and maintained under a constant flow of purified argon supplied by a tube fitted through a port in the stopper. Potentials were applied using a Bioanalytical Systems CV-27 voltammograph. Once the potential stabilized, the absorbance was recorded using a Hewlett-Packard 8452A diode array spectrophotometer. Potentials are reported vs the normal hydrogen electrode (NHE) and are therefore corrected by +0.231 V to compensate for the potential of the Ag/AgCl reference electrode vs the NHE. The percentage change in A_{426} was used to monitor the reduction of NIFU. Safranin O does not undergo a redox-associated change in absorption at this wavelength and therefore does not interfere with determination of the NIFU Fe-S cluster midpoint potential. Similar results were obtained using either safranin O or benzyl viologen as redox mediators, although only experimental data obtained with safranin O is presented herein.

Spectroscopic Characterization of NIFU. The sample concentrations given in the figure captions and used in quantifying spectroscopic results are based on protein concentrations and are expressed per monomer (M_r = 33 000). UV/visible absorption spectra were recorded on either a Beckman DU 7400 or a Hewlett-Packard 8452A diode array spectrophotometer. Variable-temperature magnetic circular dichroism (VTMCD) spectra were recorded on samples containing 50% (v/v) glycerol using an Oxford Instruments SM3 split coil superconducting magnet (1.5–300 K and 0–5 T) mated to the Jasco J-500C spectropolarimeter. The experimental protocols used in VTMCD studies for accurate sample temperature and magnetic field measurement, anaerobic sample handling, and assessment of residual strain in frozen samples have been described in detail elsewhere (Johnson, 1988; Thomson et al., 1993). X-band (~9.5 GHz) EPR spectra were recorded on a Bruker ESP-300E EPR spectrometer equipped with an Oxford Instruments ESR-9 flow cryostat. Spin quantitations were carried out under nonsaturating conditions using 1 mM Cu(II)EDTA as the standard and the procedures outlined by Aasa and Vänngård (1975).

Resonance Raman (RR) spectra were recorded using an Instruments SA Ramanor U1000 spectrometer fitted with a cooled RCA 31034 photomultiplier tube with 90° scattering geometry. Spectra were recorded digitally using photon-counting electronics, and improvements in signal-to-noise were achieved by signal averaging multiple scans. Band positions were calibrated using the excitation frequency and CCl_4 and are accurate to ±1 cm^{-1}. Lines from a Coherent Innova 100 10-W argon ion laser or a Coherent Innova 200-K2 krypton ion laser were used for excitation, and plasma lines were removed using a Pellin Broca Prism premonochromator. Using a custom-designed sample cell (Drozdzewski & Johnson, 1988), samples under an Ar atmosphere were placed on the end of a cold finger of an Air Products Displex Model CSA-202E closed-cycle refrigerator. Scattering was collected from the surface of a frozen 10-μL droplet. This enabled the samples to be cooled to 17 K, which facilitated improved spectral resolution and prevented laser-induced sample degradation.

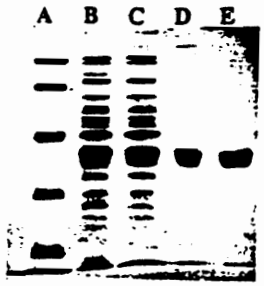

FIGURE 1: Polyacrylamide gel electrophoretic analysis of the stages of NIFU purification. Lane A, molecular weight standards phosporylase *b*, M_r ≈ 97 400; bovine serum albumin, M_r ≈ 66 200; ovalbumin, M_r ≈ 42 700; carbonic anhydrase, M_r ≈ 31 000; and soybean trypsin inhibitor, M_r ≈ 21 500. Lane B, crude extract. Lane C, streptomycin sulfate supernatant fraction. Lane D, ammonium sulfate precipitate fraction. Lane E, Q Sepharose fraction.

RESULTS

Hyperproduction of NIFU from *A. vinelandii* was accomplished in *E. coli* by its controlled heterologous expression using a hybrid plasmid that contains the *nifU* structural gene fused to the cloned transcriptional and translational regulatory elements of the T_7 phage system. Accumulation of NIFU in crude extracts of BL21(DE3) cells harboring the *nifU* expression plasmid was demonstrated by denaturing gel electrophoresis (Figure 1, lane B). This analysis showed the accumulation of a soluble polypeptide having an M_r of ≈33.000 that was not produced in control cells that harbored the parental pT$_7$-7 plasmid. These results are in good agreement with the predicted M_r of 33 274 that was deduced for NIFU from gene sequence data (Beynon et al., 1987). Cell pellets of the *E. coli* strain that hyperproduced NIFU had a characteristic red color. This was the first indication that NIFU is an Fe-S protein and provided a convenient means for visually following NIFU purification. Because of the abundance of NIFU produced in the heterologous expression system, it was readily purified in a simple four-step procedure (Figure 1). The identity of isolated product as NIFU was confirmed by comparison of the N-terminal sequence determined from the isolated protein (Met-Trp-Asp-Tyr) to the predicted N-terminal sequence deduced from the *nifU* gene sequence (Beynon et al., 1987). On the basis of gel-filtration column chromatography, using cytochrome *c* (M_r ≈ 12 400), carbonic anhydrase (M_r ≈ 29 000), bovine serum albumin (M_r ≈ 66 000), and alcohol dehydrogenase (M_r ≈ 150 000) as molecular weight markers, native NIFU was shown to be a homodimer having an apparent M_r ≈ 63 000.

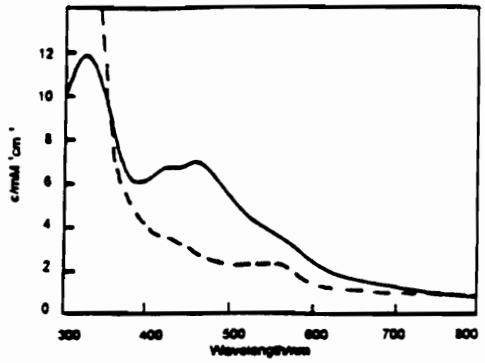

FIGURE 2: Room temperature UV-visible absorption spectra of NIFU. The solid line is the spectrum of NIFU as prepared, and the dashed line is the spectrum of NIFU after anaerobic reduction with a 15-fold stoichiometric excess of sodium dithionite. The protein concentration was 0.20 mM, and the buffering medium was 50 mM Tris·HCl, pH 8.0. The reduced spectrum is dominated by an intense absorption from dithionite centered at 314 nm.

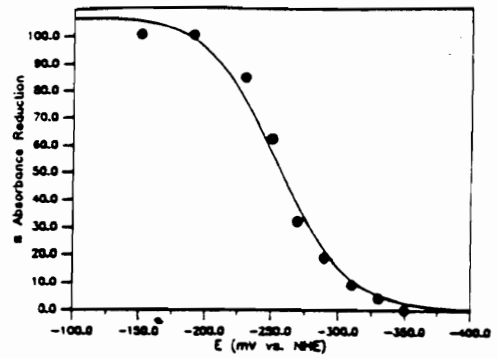

FIGURE 3: Optically monitored electrochemical reduction of NIFU. Conditions are described in Materials and Methods. The solid line is the best fit to a one-electron Nernst equation with $E_m = -254$ mV vs NHE.

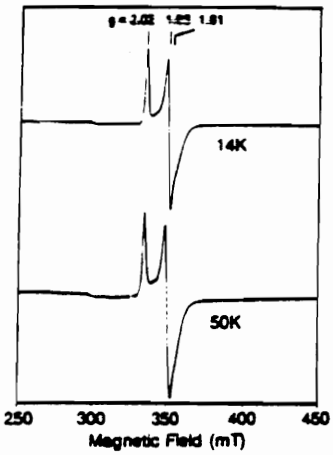

FIGURE 4: X-band EPR spectra of dithionite-reduced NIFU. Conditions of measurement: microwave frequency, 9.46 GHz; modulation amplitude, 1 mT; microwave power, 2 mW; temperatures as indicated.

Purified samples of NIFU exhibited a single band on electrophoresis gels (Figure 1, lane E), and colorimetric iron analyses indicated approximately two irons per subunit (average of 1.8 Fe/subunit from four determinations using three independent NIFU preparations). In accord with the Fe analyses, the UV/visible absorption spectra of NIFU as initially prepared and of dithionite-reduced NIFU (Figure 2) are indicative of a single, dithionite-reducible $[2Fe\text{-}2S]^{2+,+}$ cluster in each subunit. Biological $[2Fe\text{-}2S]^{2+,+}$ centers have characteristic visible absorption spectra. Oxidized clusters have bands centered around 330 nm (ϵ in the range 11 000–16 000 M^{-1} cm^{-1}), 420 nm (ϵ in the range 6 000–11 000 M^{-1} cm^{-1}, not well resolved in some proteins, *e.g.*, Rieske-type proteins), and 460 nm (ϵ in the range 6000–10 000 M^{-1} cm^{-1}), with a broad shoulder centered around 550 nm (ϵ in the range 3000–6000 M^{-1} cm^{-1}) (Dailey et al., 1994). Reduced clusters have relatively featureless spectra that increase in intensity at shorter wavelengths except for a prominent feature centered near 550 nm (ϵ in the range 2000–4000 M^{-1} cm^{-1}) that has been tentatively assigned to an Fe(II) → Fe(III) intervalence band of the localized valence $[2Fe\text{-}2S]^{+}$ core (Fu et al., 1992). The spectra for NIFU conform to this general pattern, with bands at 330 nm (ϵ = 12 000 M^{-1} cm^{-1}) 420 nm (6600 M^{-1} cm^{-1}), and 460 nm (7000 M^{-1} cm^{-1}) and a shoulder at approximately 550 nm ($\epsilon \approx 4000$ M^{-1} cm^{-1}) for the protein as initially prepared and a pronounced feature centered at 570 nm (ϵ = 2500 M^{-1} cm^{-1}) for the reduced protein. Moreover, the extinction coefficients expressed per 33-kDa subunit are consistent with one $[2Fe\text{-}2S]^{2+,+}$ center.

The change in absorption on reduction was used to assess the number of electrons required to effect reduction via quantitative chemical titrations with sodium dithionite and dye-mediated electrochemical redox titrations. Chemical titrations indicated approximately one electron per subunit (average of 0.84 reducing equivalent per subunit from three different experiments using two independent NIFU preparations). Electrochemical redox titrations mediated by safranin O were well fit by one-electron Nernst plots and indicate a midpoint around −254 ± 20 mV vs NHE (Figure 3). The ability of Fe chelators to effect cluster degradation was assessed by experiments in which oxidized and reduced samples of NIFU were treated with a 40-fold excess of α,α′-dipyridyl. The absorption of the $[Fe(\alpha,\alpha'\text{-dipyridyl})_3]^{2+}$ complex at 520 nm was monitored at room temperature as a function of time.[2] Little if any chelation of Fe by α,α′-dipyridyl was observed over 2 h, indicating that the cluster is either inherently stable or inaccessible to solvent.

The magnetic, electronic, and vibrational properties of the Fe-S center in NIFU were investigated by EPR (Figure 4), VTMCD (Figure 5), and RR (Figures 6 and 7) spectroscopies. In addition to providing unambiguous confirmation of the presence of a single $[2Fe\text{-}2S]^{2+,+}$ center, these techniques facilitated assessment of the ligation at the reducible and nonreducible Fe sites and detailed comparison

[2] Although both Fe^{2+} and Fe^{3+} are chelated by α,α′-dipyridyl, the colorimetric assay is only for the ferrous state. However, quantitative removal of Fe as Fe^{2+} has been reported for oxidized Fe-S clusters under denaturing conditions with inorganic sulfide released on cluster degradation as the likely reductant (Anderson & Howard, 1984).

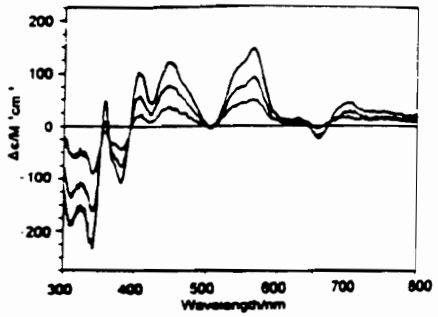

FIGURE 5: VTMCD spectra of dithionite-reduced NIFU. The protein concentration was 0.16 mM, and the buffering medium was 50 mM Tris-HCl, pH 8.0, with 50% (v/v) glycerol. MCD spectra were recorded in 1-mm cuvettes with a magnetic field of 4.5 T, at 1.66, 4.22, and 10.7 K. All MCD bands increase in intensity with decreasing temperature.

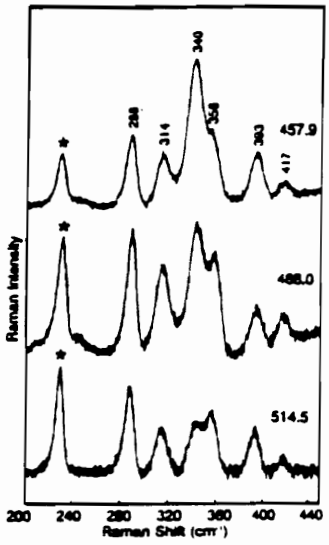

FIGURE 6: Low-temperature resonance Raman spectra of NIFU as prepared. The protein concentration was 1.8 mM, and the buffering medium was 50 mM Tris-HCl, pH 8.0. The spectra were obtained at 17 K using 457.9-, 488.0-, and 514.5-nm laser excitation and are the sum of 36, 28, or 33 scans, respectively. Each scan involved advancing the spectrometer in 0.2-cm^{-1} increments and photon counting for 1 s/point with 6-cm^{-1} spectral resolution. The asterisks indicate lattice modes of ice.

with other well-characterized [2Fe-2S]-containing proteins (Fu et al., 1992; Crouse et al., 1994). In common with other [2Fe-2S] proteins, oxidized NIFU did not exhibit any clearly discernible EPR signals, indicating that the oxidized cluster is diamagnetic at low temperatures as a result of strong antiferromagnetic exchange interaction between the high-spin Fe^{3+} ions. Reduced NIFU exhibited an almost axial EPR signal, $g = 2.02, 1.93, 1.91$, that is observable without significant broadening at 50 K (Figure 4). Spin quantitation of this $S = 1/2$ resonance for two different samples gave values between 0.8 and 0.9 spin/subunit. Taken together, the g-values and the relaxation properties are uniquely indicative of $[2Fe\text{-}2S]^{+}$ centers (Orme-Johnson & Orme-

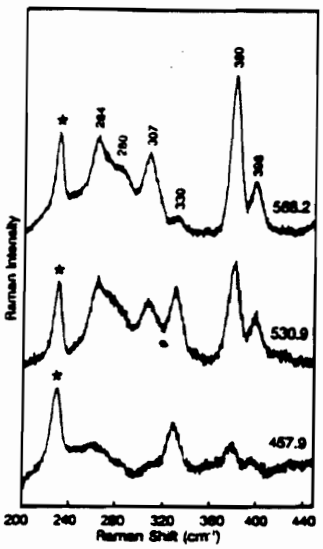

FIGURE 7: Low-temperature resonance Raman spectra of dithionite-reduced NIFU. The sample, with a protein concentration of 2.1 mM in 50 mM Tris-HCl buffer, pH 8.0, was reduced anaerobically with a 20-fold excess of sodium dithionite. The spectra were obtained at 17 K using 568.2-, 530.9-, and 457.9-nm laser excitation and are the sum of 25, 66, or 68 scans, respectively. The data collection parameters are the same as given in Figure 7, and the asterisks indicate lattice modes of ice.

Johnson, 1982) that exhibit $S = 1/2$ ground states as a result of antiferromagnetic exchange interaction between localized valence high-spin Fe^{2+} and Fe^{3+} ions (Bearden & Dunham, 1973).

Biological [2Fe-2S] centers can be divided into two classes termed ferredoxin-type (Fd-type) and Rieske-type that differ in terms of midpoint potentials and the EPR properties of the reduced clusters: Fd-type, $E_m = +70$ to -470 mV, $g_{av} \approx 1.96$; Rieske-type, $E_m = +350$ to -160 mV, $g_{av} \approx 1.90$ (Cammack, 1992; Johnson, 1994). On the basis of the X-ray crystal structures of several highly homologous plant-type Fds (Tsukihara et al., 1990; Jacobson et al., 1993) and/or mutagenesis or chemical modification studies of conserved cysteines (Cupp & Vickery, 1988; Werth et al., 1990; Gerber et al., 1990; Uhlmann et al., 1992; Fujinaga et al., 1993; Cheng et al., 1994), Fd-type clusters are generally considered to have complete cysteinyl coordination, whereas spectroscopic studies of Rieske-type centers have provided strong evidence in favor of coordination by two histidyl residues at the reducible Fe site and two cysteinyl residues at the nonreducible Fe site (Fee et al., 1984; Gurbiel et al., 1989; Kuila et al., 1992). Clearly the EPR and redox properties of the Fe-S center in NIFU, $g_{av} = 1.95$ and $E_m = -260$ mV, are indicative of a Fd-type [2Fe-2S] cluster. Moreover, since the g_{av} value and the g-value anisotropy of the $S = 1/2$ resonance of $[2Fe\text{-}2S]^{+}$ clusters are largely dependent on the ligand field at the localized Fe^{2+} site (Bertrand et al., 1985; Werth et al., 1990), the EPR properties of NIFU are consistent with coordination by two cysteinyl residues at the reducible Fe site. Such arguments must be used with caution, however, since the EPR g-values will depend on both the nature of the ligands and the distortion in the ligand field at

the Fe^{2+} site and require confirmation via techniques such as VTMCD and RR that are sensitive to the nature of the coordinating residues at both Fe sites (Fu et al., 1992).

Three distinct subclasses of Fd-type $[2Fe\text{-}2S]^{2+,+}$ clusters have been identified in simple Fds on the basis of distinctive EPR, VTMCD, and RR spectra (Fu et al., 1992; Han et al., 1989b) and the arrangement of coordinating cysteine residues (Meyer et al., 1986; Crouse et al., 1994). These are plant- or chloroplast-type ($g = 2.05, 1.96, 1.89$), hydroxylase-type such as putidaredoxin and adrenodoxin that donate electrons to cytochrome P450s ($g = 2.02, 1.94, 1.94$), and a small group of Fds of unknown function from nitrogen-fixing bacteria such as *Clostridium pasteurianum* ($g = 2.00, 1.95, 1.92$). The EPR properties of the cluster in NIFU do not correspond to a particular subclass and are intermediate between those of hydroxylase-type Fds and *C. pasteurianum* 2Fe Fd.

Fe-S centers with paramagnetic ($S > 0$) ground states invariably give rise to temperature-dependent MCD spectra, and the pattern of positive and negative bands provides a sensitive probe of the electronic structure and a discriminating method for determining cluster type (Johnson et al., 1982; Johnson, 1988). Comparison of the VTMCD spectra reported for reduced spinach Fd (plant type), putidaredoxin (hydroxylase type), and *C. pasteurianum* 2Fe Fd (Fu et al., 1992) with those of reduced NIFU (Figure 5) unambiguously identifies the Fe-S cluster as a Fd-type $[2Fe\text{-}2S]^{+}$ center, and the intensity of equivalent bands under comparable conditions is consistent with approximately one cluster per subunit. Furthermore, magnetization data (Thomson & Johnson, 1980) collected for each of the prominent bands (data not shown) were well fit by theoretical curves constructed for $g_{\parallel} = 2.02$ and $g_{\perp} = 1.92$. This confirms that the transitions all originate from the $S = 1/2$ ground state responsible for the EPR signal. Each of the three known subclasses of Fd-type centers can be distinguished on the basis of its low-temperature MCD spectra (Fu et al., 1992). Within specific spectral regions, NIFU shows close correspondence to one or the other of these archetypical proteins, but overall the MCD spectrum cannot be identified with any particular subclass.

Despite small variations in the energies of individual bands among Fd-type $[2Fe\text{-}2S]^{+}$ centers, overall they show a one-to-one correspondence in their positive and negative MCD bands in the UV/visible region. The spectra have been tentatively assigned on the basis of MCD studies of oxidized and reduced rubredoxins and resonance Raman excitation profiles (Fu et al., 1992). The MCD bands above 600 nm are attributed to ligand field transitions in view of their low absorption intensities. The positive MCD band attributed to the $Fe^{2+} \rightarrow Fe^{3+}$ intervalence band has shifted to 570 nm in the NIFU spectrum compared to 530 nm in spinach Fd, 540 nm in putidaredoxin, and 550 nm in *C. pasteurianum* 2Fe Fd, and this facilitates clearer observation of the $+-++-+$ pattern of MCD bands in the 350−550-nm region arising from $S \rightarrow Fe^{3+}$ charge transfer at the localized valence Fe^{3+} site. By analogy with the crystallographically defined plant-type Fds, this indicates coordination of the Fe^{3+} site of the $[2Fe\text{-}2S]^{+}$ cluster in NIFU by two cysteine residues. In common with *C. pasteurianum* 2Fe Fd, the negative bands between 300 and 350 nm in the NIFU spectrum that are attributed to $Fe^{2+} \rightarrow S$ charge transfers at the localized valence Fe^{2+} site are more resolved than in plant- or hydroxylase-type Fds. However, the number and

Table 1: Fe−S Stretching Frequencies and Vibrational Assignments for the $[2Fe\text{-}2S]^{2+,+}$ Clusters in NIFU

mode[a]			
D_{2h}	C_{2v}	oxidized (cm^{-1})	reduced (cm^{-1})
$B_{2u}{}^b$	B_1	417	398
$A_g{}^b$	A_1	393	380
$B_{3u}{}^b$	A_1	356	307
$B_{1u}{}^t$	B_2	n.o.[b]	330
$B_{2g}{}^t$	B_2	n.o.[b]	n.o.[b]
$A_g{}^t$	A_1	340	307
$B_{1g}{}^b$	B_1	314	280
$B_{3u}{}^t$	A_1	288	264

[a] Symmetry labels under idealized D_{2h} symmetry for an oxidized $Fe_2S_2{}^bS_4{}^t$ unit and under idealized C_{2v} symmetry for a localized-valence reduced $Fe_2S_2{}^bS_4{}^t$ unit. [b] Not observed with the excitation wavelengths used in this work.

sign of the MCD bands in this region is highly conserved, indicating that the Fe^{2+} site is also coordinated by two cysteine residues. The validity of this interpretation of the MCD spectra and the spectroscopic consequences of non-cysteinyl coordination at either of the localized valence Fe^{3+} and Fe^{2+} sites of the $[2Fe\text{-}2S]^{+}$ center has recently been demonstrated in this laboratory via VTMCD studies of Rieske proteins[3] and of mutants of the plant-type Fd from *Anabaena* in which coordinated cysteines have been individually changed to serines.[4]

Resonance Raman spectroscopy has emerged as a sensitive structural probe of Fe-S centers and a discriminating method for determining Fe-S cluster type (Spiro et al., 1988). It is particularly useful for investigating and identifying Fe-S centers with diamagnetic ground states, such as oxidized $[2Fe\text{-}2S]^{2+}$ clusters, which are not amenable to EPR or VTMCD investigation. All of the eight predicted Fe−S stretching modes of the $Fe_2S_2{}^bS_4{}^t$ unit of $[2Fe\text{-}2S]^{2+}$ centers have been assigned to predominantly Fe−S^b or Fe−S^t modes under idealized D_{2h} symmetry on the basis of extensive isotope shift data for proteins and synthetic analogs and normal mode calculations (Han et al., 1989a,b; Fu et al., 1992). The RR spectra obtained for oxidized NIFU (Figure 6) conform to the general pattern of frequencies established for $[2Fe\text{-}2S]^{2+}$ clusters, and vibrational assignments can be inferred by analogy with the published assignments (Table 1). It was not possible to obtain high-quality spectra with 406.7-nm excitation due to high fluorescence backgrounds. This excitation wavelength is usually required to observe the nearly degenerate $B_{1u}{}^t$ and $B_{2g}{}^t$ modes that differ only in the phases of the Fe−S^t stretches on opposite sides of the $Fe_2S_2{}^b$ core.

Despite the overall similarity in the RR spectra of Fd-type oxidized [2Fe-2S] centers, each subclass is readily distinguishable in terms of the differences in the relative intensities of bands as a function of excitation wavelength and the frequencies of corresponding Fe−S stretching modes (Han et al., 1989b; Crouse et al., 1994). The latter have been interpreted in terms of minor differences in Fe−S bond strengths and variability in the cysteine Fe−S^t−C_β−C_α dihedral angles that govern the extent of mixing of the Fe−S^t vibrations with the internal bending modes of coordinated

[3] T. A. Link, B. R. Crouse, M. G. Finnegan, D. Kuila, J. A. Fee, and M. K. Johnson, manuscript in preparation.

[4] B. R. Crouse, H. Cheng, B. Xia, J. L. Markley, and M. K. Johnson, manuscript in preparation.

cysteines (Han et al., 1989a,b; Fu et al., 1992), *i.e.*, minimal coupling with a dihedral angle of 90° or 270° and maximal coupling with a dihedral angle of 180°. Normal mode calculations for crystallographically defined analog complexes (Han et al., 1989a) indicate that the extent of coupling can be assessed by the frequency separation between the B_{3u}' mode and the A_g', B_{1u}', B_{2g}', and B_{3u}'' modes. For NIFU these frequency separations are intermediate between those of hydroxylase-type and plant-type Fds but similar to those of *C. pasteurianum* 2Fe Fd , suggesting similar cysteine dihedral angles to the [2Fe-2S] in these proteins. Overall, however, the RR spectra for oxidized NIFU, in accord with the EPR and VTMCD spectra of reduced NIFU, are not directly identifiable with any particular subclass of Fd-type centers. Taken together, the spectroscopic results suggest that NIFU constitutes a new subclass of Fd-type [2Fe-2S] centers with a unique arrangement of coordinating cysteines.

Reduced $[2Fe\text{-}2S]^+$ clusters show much greater uniformity in their RR spectra (Fu et al., 1992). Reduced NIFU is no exception, and the spectra are uniquely indicative of a protein-bound $[2Fe\text{-}2S]^+$ center (Figure 7). Assignments (Table 1) can be made by direct analogy with those established for reduced putidaredoxin via $^{34}S^b$-isotope shifts (Fu et al., 1992). Excitation into the putative $Fe^{2+} \rightarrow Fe^{3+}$ intervalence band results in strong enhancement of modes involving substantial contributions from $Fe-S^b$ stretching, 264, 280, 307, 380, and 398 cm^{-1} in reduced NIFU. These modes have similar frequencies and enhancement profiles in all reduced $[2Fe\text{-}2S]^+$ centers investigated thus far, which attests to analogous structures for the mixed valence $Fe_2(\mu_2\text{-}S)_2$ cores. Higher energy excitation (optimally 457.9 nm) is required for enhancement of $Fe-S^t$ stretching at the localized valence Fe^{3+} site, 330 cm^{-1} in reduced NIFU. A band with similar enhancement characteristics is observed at 317 cm^{-1} in adrenodoxin,[3] 319 cm^{-1} in putidaredoxin, 325 cm^{-1} in spinach Fd, and 328 cm^{-1} in *C. pasteurianum* 2Fe Fd (Fu et al., 1992). The appearance of this band is considered to be indicative of coordination by two cysteines at the nonreducible Fe site, and this has recently been confirmed by RR studies of reduced Cys → Ser site-specific mutants of the *Anabaena* plant-type Fd.[4] The frequency of this mode is likely to be critically dependent on $Fe-S^t-C_\beta-C_\alpha$ dihedral angles at the nonreducible Fe site. The higher frequencies, such as those observed for NIFU and *C. pasteurianum* 2Fe Fd, are indicative of stronger coupling with cysteinyl modes and hence of one or both of these dihedral angles approaching 180°.

DISCUSSION

The major conclusions from the present work are that NIFU is a homodimer with identical [2Fe-2S] clusters in each subunit. These [2Fe-2S] clusters have a potential of about −260 mV, and they are coordinated to the polypeptide entirely by cysteinyl thiolate ligands. The coordination of the NIFU [2Fe-2S] clusters by cysteinyl residues is consistent with comparative gene sequence data, which show that among NIFU polypeptide primary sequences deduced from gene sequence data from *A. vinelandii* (Beynon et al., 1987), *K. pneumoniae* (Beynon et al., 1987), and *Anabaena* (Mulligan & Haselkorn, 1989) there are nine conserved cysteinyl residues, and seven of these are contained within domains exhibiting extraordinary sequence conservation (Dean & Jacobson, 1992). NIFU from *A. vinelandii* contains 312 residues, of which residues 35, 62, 106, 137, 139, 172, 175, 272, and 275 are the interspecifically conserved cysteines. Other than the Cys-X_2-Cys motif characteristic of many Fe-S cluster coordinating domains, the spatial organization of the NIFU cysteines does not exhibit a pattern that is typically found for other [2Fe-2S] proteins. Indeed, a novel organization of the cluster coordinating ligands within NIFU is likely to contribute to the unique spectroscopic features when compared to other [2Fe-2S]-containing polypeptides.

Definitive evidence for the specific cysteines involved in the ligation of the [2Fe-2S] cluster in NIFU will require crystallographic and/or mutagenesis studies. However, it is interesting to note, as previously recognized by Ouzounis et al. (1994), that the domain containing the two pairs of closely spaced cysteines in NIFU proteins (Cys137, -139, -172, and -175 in *A. vinelandii* NIFU) bears a striking conservation in primary sequence and one-dimensional spatial organization when compared to tandemly reposed primary sequence domains located in nitrite reductases from *K. pneumoniae* (Lin et al., 1993), *E. coli* (Peakman et al., 1990), *Emericella nidulans* (Johnstone et al., 1990), and *Neurospora crassa* (Exley et al., 1993) and a single C-terminal domain in nitrate reductase from *K. pneumoniae* (Lin et al., 1993). Although it is not firmly established whether or not all these proteins contain [2Fe-2S] clusters, the available biophysical evidence, together with the gene sequence data, clearly indicates that nitrite reductases contain two [2Fe-2S] clusters (Cammack et al., 1982). Hence it is tempting to speculate that NIFU and these other proteins constitute a fourth subclass of Fd-type [2Fe-2S] centers that are coordinated by a C-X-C-X_n-C-X_2-C arrangement of cysteines. This contrasts to C-X_n-C-X_2-C-X_n-C in plant-type Fds (Tsukihara et al., 1990; Jacobson et al. 1993), C-X_3-C-X_2-C-X_n-C in hydroxylase-type Fds (Cupp & Vickery, 1988; Gerber et al., 1990; Uhlmann et al., 1992), and the C-X_2-C-X_n-C-X_3-C arrangement that has been proposed for *C. pasteurianum* Fd (Fujinaga et al., 1993; Crouse et al., 1994). The proposed spacing of coordinating residues in NIFU proteins is also similar to that established by mutagenesis studies for Rieske-type centers, *i.e.*, C-X-H-X_n-C-X_2-H (Johnson, 1994; Davidson et al., 1992), except that histidines replace the second cysteine in each pair. In this connection it is worth noting that the second repeat in each of the homologous nitrite reductase sequences has one histidine in place of a specific cysteine, *i.e.*, C-X-H-X_n-C-X_2-C (Ouzounis et al., 1994).

What is the physiological function of NIFU and what is the specific role of its [2Fe-2S] clusters? Several possible answers to these questions based on the biochemical phenotype of *nifU* mutants and on results of the present work can be considered. Upon first discovering that NIFU contains a redox-active metallocluster, we considered that NIFU might act as a surrogate electron donor to nitrogenase. This possibility seemed particularly attractive because it is known that the normal physiological electron donor to nitrogenase in *A. vinelandii* is a flavodoxin, but the gene encoding this flavodoxin, *nifF*, is not absolutely required for diazotrophic growth (Bennett et al., 1987). However, our results show that the redox potential of NIFU is not sufficiently low to accommodate reduction of the Fe protein. An alternative possibility is that NIFU, together with NIFS,

[3] C. Staples, B. R. Crouse, R. Bernhardt, and M. K. Johnson, unpublished observations.

functions in sequestering the Fe and sulfide required for formation of the Fe-S cores contained within the nitrogenase component proteins. This suggestion is supported by the observations that (i) the biochemical phenotypes of *A. vinelandii nifU* and *nifS* mutants are remarkably similar (Jacobson et al., 1989), (ii) nitrogenase component proteins containing a full complement of metalloclusters are apparently not accumulated in *A. vinelandii nifS* or *nifU* mutants, (iii) the *nifU* and *nifS* genes are cotranscribed (Beynon et al., 1987), and (iv) NIFS appears to have a role in sequestering the inorganic sulfide for nitrogenase Fe-S core formation (Zheng et al., 1993, 1994).

We have shown previously that NIFS is able to catalyze the desulfurization of L-cysteine and that an enzyme-bound persulfide is an intermediate in that reaction. Thus, NIFU might have a redox function which involves catalyzing the release of the sulfide from NIFS. In such a model NIFU might also be considered a candidate to serve as an intermediate sulfide carrier. Another possibility is that NIFU might complement the function of NIFS by sequestering the Fe required for nitrogenase Fe-S core formation. In this case the role of the [2Fe-2S] cluster could be to keep Fe bound at some other site located within NIFU in an oxidation state amenable to nitrogenase Fe-S core formation. As only four cysteines are required for coordination of each [2Fe-2S], it is noteworthy that there are five other cysteinyl residues per each NIFU subunit which have the potential to coordinate additional Fe atoms. If any of these cysteines do indeed coordinate Fe atoms destined for nitrogenase Fe-S core assembly, such Fe is not associated with the isolated protein. Namely, all of the Fe found in isolated NIFU can be accounted for by the two [2Fe-2S] clusters. Nevertheless, this feature is expected if it is considered that any NIFU-bound Fe atoms targeted for nitrogenase Fe-S core formation must be capable of being released during formation of the nitrogenase Fe-S cores and would, therefore, probably only be loosely associated with the polypeptide and readily lost during the purification scheme. Along these same lines we have also considered the possibility that the two [2Fe-2S] clusters contained within NIFU could be intermediates in nitrogenase Fe-S core formation. This consideration was prompted by previous reports that the irons contained within the [4Fe-4S] cluster of the Fe protein can be removed by using chelators (Walker & Mortenson, 1974) and that a [2Fe-2S] species of the Fe protein is an intermediate in that process (Anderson & Howard, 1984). However, because the NIFU [2Fe-2S] clusters are released only very slowly by treatment with the chelator α,α′-dipyridyl, they are unlikely to be precursors of the nitrogenase metalloclusters. In other words, if the [2Fe-2S] clusters from NIFU were capable of being transferred to another protein, facile chelation of their irons by α,α′-dipyridyl would be expected. Nevertheless, it remains an intriguing possibility that nitrogenase Fe-S core precursors could be assembled at one or more sites on NIFU, potentially using the available cysteinyl residues as nucleation sites, and that the [2Fe-2S] clusters could have a redox role in formation, stabilization, or release of such precursors during maturation of the nitrogenase component proteins. The possibility that NIFU might function to sequester the Fe necessary for nitrogenase metallocluster formation has an interesting parallel in the case of human ferrochelatase, which catalyzes the insertion of ferrous iron as the terminal step in heme biosynthesis and which has also recently been shown to be a [2Fe-2S]-containing homodimer (Dailey et al., 1994). However, the role of the [2Fe-2S] center in mammalian ferrochelatase has yet to be determined, and comparison of the primary sequences of NIFU and human ferrochelatase reveals no obvious sequence identity, particularly within regions surrounding cysteinyl residues.

In summary, although the specific function of NIFU is not yet known, its activity is required for the full activation of the nitrogenase component proteins, and the present evidence points to a role in formation of the Fe-S cores of the nitrogenase metalloclusters. Amino acid substitution studies, such as those already used to investigate the coordination of the nitrogenase metalloclusters (Brigle et al., 1987; Dean et al., 1990) as well as a variety of other metalloproteins, including [2Fe-2S] proteins (Werth et al., 1990; Gerber et al., 1990; Uhlmann et al., 1992, Fujinaga et al. 1993; Cheng et al., 1994), should provide insight concerning the organization and physiological function of the [2Fe-2S] clusters contained within NIFU. Also, because large amounts of purified NIFS and NIFU are now available, it is possible to initiate *in vitro* biochemical reconstitution experiments aimed at determining whether or not these proteins directly participate, either separately or in combination, in the mobilization of the inorganic Fe and sulfide required for maturation of the nitrogenase component proteins.

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