BIOSYNTHESIS OF IRON-SULFUR CLUSTERS

by

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Abstract

It is not known whether biosynthesis of [Fe-S] clusters occurs through a spontaneous self-assembly process or an enzymatic process. However, in the *Azotobacter vinelandii* nitrogenase system, it has been proposed that NifS and NifU are involved in the mobilization of sulfur and iron necessary for nitrogenase-specific [Fe-S] cluster assembly. The NifS protein has been shown to have cysteine desulfurase activity and can be used to supply sulfur for the *in vitro* catalytic formation of [Fe-S] clusters. The activity of the NifU protein has not yet been established, but NifU could have functions complementary to NifS by mobilizing iron or serving as an intermediate site necessary for nitrogenase-specific [Fe-S] cluster assembly. A second iron-binding site within NifU was predicted to serve these functions because two identical [2Fe-2S] clusters that had previously been identified within the homodimeric NifU are tightly bound, and the NifU primary sequence is rich in cysteine residues. In this dissertation, I examined the possibility that NifU might mobilize iron or serve as an intermediate site for [Fe-S] cluster assembly, as well as the possibility that NifU could work in concert with NifS.

Primary sequence comparisons, amino acid substitution experiments, and biophysical characterization of recombinantly-produced NifU fragments were used to show that NifU has a modular structure. One module is contained in approximately the C-terminal half of NifU and provides the binding site for the [2Fe-2S] cluster previously identified (the permanent [2Fe-2S] cluster). Cysteine residues Cys^{137}, Cys^{139}, Cys^{172}, and

Cys^{175} serve as ligands to the [2Fe-2S] cluster. Another module (referred to as NifU-1) is contained in approximately the N-terminal third of NifU and provides a second iron-binding site (rubredoxin-like Fe(III)-binding site). Cysteine residues Cys^{35}, Cys^{62}, Cys^{106}, and a putative non-cysteine ligand of unknown origin provide coordination to the iron at this site. The significance of these iron-binding sites was also accessed by showing that cysteine residues involved in providing the rubredoxin-like Fe(III)-binding site and those that provide the [2Fe-2S] cluster binding site are all required for the full physiological function of NifU. The two other cysteine residues contained within NifU, Cys^{272} and Cys^{275}, are neither necessary for binding iron at either site nor are they required for the full physiological function of NifU.

These results provide the basis for a model where iron bound at the rubredoxin-like sites within NifU-1 (one iron per monomer) is proposed to be destined for [Fe-S] cluster formation. It was possible to find *in vitro* evidence supporting this idea. First, it was demonstrated that NifU and NifS are able to form a transient complex. Second, in the presence of NifS as well as L-cysteine and a reducing agent, the Fe(III) contained at the rubredoxin-like sites within the NifU-1 or NifU homodimer can rearrange to form a transient [2Fe-2S] cluster between the two subunits. Finally, a mutant form of NifU-1 was isolated that appears to be trapped in the [2Fe-2S] cluster-containing form, and this [2Fe-2S] cluster (the transient [2Fe-2S] cluster) can be released from the polypeptide matrix upon reduction with dithionite.

Previous work has shown that the permanent [2Fe-2S] clusters of as-isolated NifU are in the oxidized form but can be reduced chemically. The transient [2Fe-2S] cluster formed between rubredoxin-like sites, in contrast, is reductively labile. If the transient

cluster serves as an intermediate [Fe-S] cluster to be destined for [Fe-S] cluster assembly, I propose that the permanent [2Fe-2S] clusters could have redox roles participating in either one or all of the following events. The permanent [2Fe-2S] clusters could have a redox function in the acquisition of iron for initial binding at the mononuclear sites. They could also provide reducing equivalents for releasing the transient [2Fe-2S] cluster. In addition, upon releasing the transient [2Fe-2S] cluster, the permanent [2Fe-2S] clusters could provide the appropriate oxidation state of the irons to be destined to nitrogenase metallocluster core formation.

Finally, because proteins homologous to NifU and NifS are widely distributed in nature, it is suggested that the mechanism for NifU and NifS in the formation of nitrogenase-specific [Fe-S] clusters could represent a general mechanism for [Fe-S] cluster synthesis in other systems.

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ABBREVIATIONS

BCA	Bicinchoninic acid
Bfd	Bacterioferritin-associated ferredoxin
3D-structure	Three dimensional structure
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EPR	Electron paramagnetic resonance
FAD	Flavin adenine dinucleotide
FeMo-cofactor	Iron molybdenum cofactor
HPLC	High performance liquid chromatography
Isc or *isc*	Iron-sulfur cluster
Nif or *nif*	Nitrogen fixation
P-cluster	[8Fe-7S] cluster of the molybdenum-iron protein of nitrogenase
PLP	Pyridoxal phosphate
RR	Resonance Raman
SHE	Standard hydrogen electrode
Tris-HCl	[Tris (hydroxymethyl) aminomethane] hydrochloride
UV	Ultraviolet
VTMCD	Variable-temperature magnetic circular dichroism

CHAPTER I

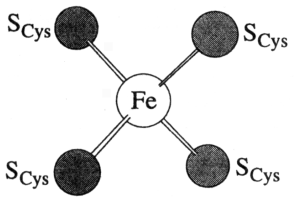
INTRODUCTION

A. Iron-Sulfur Clusters

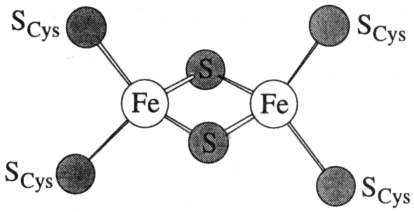
Iron-sulfur [Fe-S] clusters are prosthetic groups contained within a large group of proteins commonly referred to as [Fe-S] proteins. These proteins are found within a variety of life forms ranging from anaerobic to aerobic organisms including prokaryotes, eukaryotes, and archaea (79, 99, 117). [Fe-S] clusters are composed of non-heme iron, inorganic sulfur, and sulfur contributed from cysteine residues, through which the clusters are bound to their protein partners. Inorganic sulfur molecules are often referred to as acid-labile sulfides because treatment of most [Fe-S] proteins with acid results in removing the inorganic sulfurs in the form of H_2S (3, 118). [Fe-S] clusters generally have the structures shown in Figure 1.1 (43). These basic structures are identified as mononuclear, binuclear, trinuclear, and tetranuclear based on the number of iron atoms in the structures. A common feature in these structures is that each iron is approximately tetrahedrally coordinated to the sulfur atoms. The sulfur may come from either cysteine residues as their terminal ligands alone or in combination with inorganic sulfur molecules as their bridging ligands.

Mononuclear type

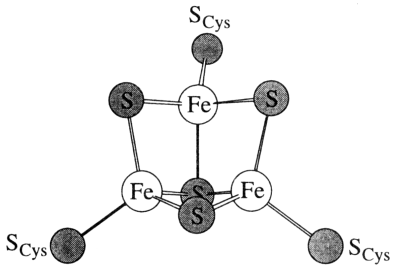
A mononuclear structure, $[Fe(S\text{-}Cys)_4]$ such as that found in rubredoxins, contains a single atom of iron coordinated by four sulfur ligands from cysteine residues without the presence of any acid-labile sulfide. This arrangement is evidenced by the three-dimensional (3D-) structure of the first member of the rubredoxin family isolated from



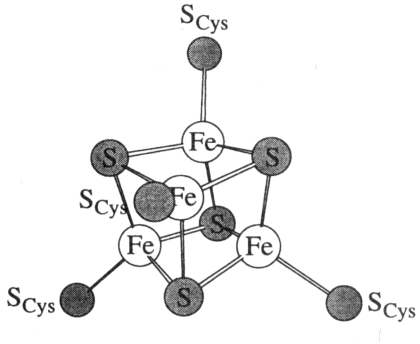
Mononuclear



Binuclear



Trinuclear



Tetranuclear

Figure 1.1: Schematic structures of [Fe-S] clusters generally found in proteins (43).

Clostridium pasteurianum (22, 100 and references therein) and later from other rubredoxins (93 and references therein).

Binuclear type

A binuclear structure, $[Fe_2S_2(S\text{-}Cys)_4]$ or [2Fe-2S] (79), contains two iron atoms and has been observed in a variety of different ferredoxins. The tetrahedral geometry around each iron is provided by two cysteine residues for terminal ligands and two acid-labile sulfide bridges located between the two iron atoms. Thus, [2Fe-2S] clusters are comparable to two rubredoxin structures joined by two labile sulfide atoms. The structure was originally proposed according to spectroscopic and chemical data and was confirmed by X-ray crystallography (7, 63, 78, 100).

Tetranuclear type

A tetranuclear structure, $[Fe_4S_4(S\text{-}Cys)_4]$ or [4Fe-4S] (79), contains four iron atoms. The distorted tetrahedral geometry around each iron is provided by a single cysteine residue as a terminal ligand and four acid-labile sulfides. It is frequently referred to as a cubane cluster (4) of which four irons and four acid-labile sulfides are positioned alternately at each corner of an irregular cube. The 3D-structure of many [4Fe-4S] cubane containing proteins has been determined (12, 101).

Trinuclear type

A trinuclear structure, $[Fe_3S_4(S\text{-}Cys)_3]$ or [3Fe-4S] (79), contains three iron atoms yet still has four acid-labile sulfides. It is sometime called a cuboidal cluster (4) and can be considered structurally as a cubane cluster that is missing one iron. An early report of the crystallographic structure of *Azotobactor vinelandii* ferredoxin I indicated that it contained both [4Fe-4S] and [3Fe-3S] clusters (7, 103). Higher resolution studies have

indicated the original [3Fe-3S] assignment to be in error, and that *A. vinelandii* ferredoxin I contains a [4Fe-4S] and a [3Fe-4S] cluster (101, 102, 104).

B. Functions of iron-sulfur clusters

[Fe-S] proteins are known to be essential for many life-sustaining processes such as photosynthesis, nitrogen fixation, and respiration. However, the function of [Fe-S] proteins is not limited to electron transfer processes. Indeed, [Fe-S] clusters are versatile prosthetic groups that serve as electron transfer, catalytic, structural, biosensor, and iron storage components for their respective proteins (4, 24, 47, 106).

Electron carriers

[Fe-S] clusters can be oxidized and reduced. The broad range of their available redox potentials allows them to serve as intermediate electron carriers for various metabolic reactions. In inter-molecular electron transfer reactions, [Fe-S] clusters contained within rubredoxins and ferredoxins provide a linkage between enzymes (14, 33, 61, 66, 88, 92, 108). Multiple [Fe-S] clusters, as well as other prosthetic groups, are often found within a single polypeptide or multiple subunits of a soluble or membrane-bound protein complex. They conduct intra-molecular electron transfer in a specific pathway from an electron donor to an electron acceptor (64, 72, 80, 113). One example is fumarate reductase, an enzyme involved in anaerobic respiration that uses fumarate as the terminal electron acceptor. Fumarate reductase, a membrane bound protein composed of four subunits (FrdABCD), contains FAD (flavin adenine dinucleotide) and three [Fe-S] clusters, [2Fe-2S], [3Fe-4S], [4Fe-4S]. The optimal pathway for electron flow has been proposed from ubiquinone, an electron donor, to FAD, the [2Fe-2S] cluster, then the

[3Fe-4S] cluster, and finally fumarate without the involvement of the [4Fe-4S] cluster (64, 114).

Catalysis

The ability to store, shift, or delocalize electrons in [Fe-S] clusters makes it possible for the clusters to participate in catalytic redox reactions. One example of this type of process is provided by spinach ferredoxin:thioredoxin reductase (FTR). FTR contains a [4Fe-4S] cluster that is located very close to its active-site disulfide. It couples a single electron donor, namely the photoreduced [2Fe-2S] ferredoxin, to a two-electron acceptor, the substrate thioredoxin disulfide. The reduced thioredoxin then accomplishes thiol-regulation of chloroplast enzymes in the photosynthetic apparatus. The catalytic mechanism in reducing thioredoxin disulfide has been proposed with sequential one-electron steps. During a single electron reduction, the [Fe-S] cluster generates a new disulfide bridge to one of the active-site cysteines to stabilize a thiyl radical intermediate and free thiol from the active site to interact with thioredoxin disulfide. The second electron reduction acts to recycle the active-site disulfide and release the reduced thioredoxin (96, 97).

Certain [Fe-S] clusters are involved in non-redox reactions and function by directly serving as the site of substrate binding and activation. An example of a [Fe-S] protein that has this type of function is the well-characterized aconitase protein, a key enzyme in the Krebs cycle that catalyzes the hydration-dehydration reaction of the transformation between isocitrate and citrate. One Fe atom of the [4Fe-4S] cluster, designated Fe_a, is ligated to an exogenous ligand, a solvent hydroxyl group. It is this iron that binds to its substrates via their carboxyl and hydroxyl groups, and thus the

coordination number of this particular iron changes from four to six during catalysis (24, 49).

Biosensors

[Fe-S] clusters have been found to act as biological sensors of oxygen, iron, and other oxidants, in order to control the associated activity of their cognate regulatory proteins. In other words, these clusters serve as part of a signal transduction mechanism linking a signal and the ultimate regulation effects. For [Fe-S] clusters that participate in regulatory roles, two sensing mechanisms have been observed so far. One is the assembly/disassembly of the [Fe-S] cluster, where the instability of the clusters acts to regulate protein function. The second sensing mechanism involves changes in the oxidation state of the protein-bound [Fe-S] cluster (5, 6, 87).

An example of the first mechanism is the degradation of [4Fe-4S] clusters to [2Fe-2S] clusters regulating the activity of the *Escherichia coli* FNR (fumarate nitrate reduction) protein (50, 51, 82). FNR is a transcriptional regulator involving a metabolic switch from aerobic to anaerobic conditions. Its activity is regulated by the presence or the absence of oxygen. The basis of this regulation is that the oxygen-sensory and DNA-binding motifs are located within different parts of the FNR protein (110). In the absence of oxygen, FNR is a homodimer that contains two [4Fe-4S] clusters and is active in DNA binding (57). Exposure of FNR to oxygen results in the degradation of the [4Fe-4S] clusters to a more stable [2Fe-2S] form, decrease in dimerization, and the inactivation of FNR (50). Reassembly of the [4Fe-4S] clusters from the [2Fe-2S] clusters during oxygen deprivation produces an active FNR both *in vivo* (82) and *in vitro* (35, 50). It has been

suggested that the loss or gain of [4Fe-4S] clusters at the sensory sites of FNR interferes with the dimer interface and in turn with the activity of FNR (56, 57).

For an example of the second sensing mechanism, the reversible oxidation of $[2Fe\text{-}2S]^{1+}$ to $[2Fe\text{-}2S]^{2+}$ with a midpoint potential of E_m = -283 mV regulates the activity of the *E. coli* SoxR (superoxide) protein (21, 29, 30, 41). SoxR is a component of a two-stage transcriptional control system which regulates the oxidative stress response that can be induced by superoxide or nitric oxide. It activates the transcription of a second regulatory protein called SoxS. SoxS then directs transcription of at least 15 genes encoding antioxidant enzymes, DNA repair enzymes, and other proteins under the control of the *soxRS* regulon (19, 20, 59). SoxR is a [2Fe-2S]-containing homodimer. Different forms of SoxR, including the absence (39) or the presence of either oxidized or reduced [Fe-S] clusters, can bind DNA in different ways. However, as revealed *in vitro* (21, 30, 41) and *in vivo* (29, 41), only oxidized SoxR can effectively activate the transcription of its target DNA. It was shown that an unusual distance between the -10 and -35 elements of the *soxS* promoter makes the transcription ineffective unless it is compensated via the conformation induced by oxidized SoxR (39, 40). The isolation of a specific reductase supports this sensing mechanism because this reductase can serve as a pathway for maintaining the reduced form of SoxR (53). It is likely that an excess of superoxide alters the equilibrium toward oxidized SoxR and results in stimulation of the regulation cascade.

Structural components

A structural role of an [Fe-S] cluster in stabilizing the polypeptide chain is seen in *Bacillus subtilis* glutamine phosphoribosylpyrophosphate amidotransferase, an enzyme in

the purine nucleotide biosynthetic pathway. This enzyme is a tetrameric protein containing a [4Fe-4S] cluster per subunit. The [4Fe-4S] clusters (2, 73, 112) are necessary for the enzyme activity but are not involved in a redox reaction (112), catalysis, or allosteric regulation (62). The presence of the [Fe-S] clusters is speculated to be involved in controlling postranslational enzyme processing (62). Also, the 3D-structure of the enzyme shows the involvement of its [Fe-S] clusters with different domains of the mature enzyme (95). Finally, Grandoni *et al*. (34) have shown that the degree of lability for proteolytic degradation of the enzyme *in vivo* is interdependent with the degree in the lability of its [Fe-S] clusters toward oxygen *in vitro*.

A structural role for an [Fe-S] cluster has also been observed for *E. coli* endonuclease III (54, 109) and MutY (32). These enzymes serve as base excision repair agents for oxidative DNA damage. MutY shares sequence and structure homology to endonuclease III although their substrate specificity is different (65, 109). It was shown that a [4Fe-4S] cluster, which is present within the C-terminal region of endonuclease III, is difficult to reduce or oxidize chemically (17). Resonance Raman (RR) spectra did not indicate binding of substrate or inhibitor to the [Fe-S] cluster but did indicate an indirect disturbance of the ligands during substrate binding (27). The 3D-structure of endonuclease III revealed that the [Fe-S] cluster is a part of its DNA binding motifs. Therefore, it is likely that the [Fe-S] cluster of endonuclease III provides a proper orientation of amino acid residues within the region of the expected DNA recognition site (54, 109). Indeed, in the absence of its [Fe-S] cluster, MutY is unable to bind its target DNA (32, 83).

Iron-storage components

In addition to serving as an electron carrier, *C. pasteurianum* ferredoxin is able to serve as an iron-storage protein when exogenous iron supplies become depleted. During iron starvation, flavodoxin, which does not contain iron, substitutes for ferredoxin as an electron transfer agent. Ferredoxin is degraded and the organism continues to grow for a considerable period of time. Iron released from ferredoxin was suggested to provide the endogenous iron necessary for the synthesis of other iron-containing proteins because there was no excretion of iron into the growth medium under these conditions (108). Another attractive candidate for iron storage is the polyferredoxin isolated from a thermophilic archaebacterium, *Methanobacterium thermoautotrophicum* (37, 70). Polyferredoxin contains six tandem repeats of a canonical clostridial ferredoxin sequence. It is thought to contain 12 [4Fe-4S] clusters (37, 85, 98). The relationship between a decrease in polyferredoxin and the growth of *M. thermoautotrophicum* is similar to those of *C. pasteurianum* when the organism undergoes iron deprivation (70).

C. Biosynthesis of iron-sulfur clusters

In spite of the fact that [Fe-S] clusters are essential components of various biological processes, how they are assembled *in vivo* has not yet been established. However, both a spontaneous self-assembly (non-enzymatic) and an enzymatic process have been proposed as possibilities for [Fe-S] cluster formation *in vivo* (25).

A spontaneous self-assembly (non-enzymatic) process

On support of a spontaneous self-assembly hypothesis, chemical synthesis studies have shown that reactions between iron and sulfur are versatile, and synthetic analogs of

biological [Fe-S] clusters can be produced with various types of thiol reagents serving as their terminal ligands (4, 7, 10, 42, 100). The pathways of assembly and intermediates of synthetic analogs together with their ability to readily undergo ligand exchange and conversion have been performed (4) in solvents such as methanol and acetonitrile (42, 124). Nevertheless, chemical synthesis under mild physiologically relevant conditions (aqueous solutions) has only been shown for reconstitution of apoproteins ([Fe-S] proteins without [Fe-S] clusters) using a cocktail of ferrous or ferric ion, sodium sulfide, and reducing agents (10, 38, 71, 91, 119). Moreover, at least one case has been reported of an apoprotein reconstituted with a preformed synthetic analog (15).

An enzymatic process

There are also reasons to believe that [Fe-S] cluster assembly requires assistance from enzymes and occurs through a controlled process. Most organisms have to cope with the poor solubility of iron (Fe^{3+}) under physiological pH (e.g. $[Fe^{3+}] \sim 10^{-18}$ M at pH 7) no matter how abundant iron is in nature (1, 11). To accomplish this, they produce specific receptors and transport systems for iron acquisition (11). Once inside the cells, free iron is able to generate oxidative damage by elevating levels of hydroxyl radical, a highly detrimental oxidant, via Fenton and Haber-Weiss reactions. In these reactions, iron interacts with the by-products of aerobic respiration, O_2^- (superoxide anion) and H_2O_2 (hydrogen peroxide) (1, 60, 105). Thus, organisms must have a specific way to sequester iron to prevent such damage. Ferritins and bacterioferritins are thought to serve this function (1). Although there is no evidence that free sulfide can be detected *in vivo* (71), it is known that free sulfide in amounts less than that required for spontaneous [Fe-S]-cluster self-assembly can inhibit respiratory processes (31, 36, 69, 86, 111).

Therefore, the free form of either iron or sulfide should not be available in favorable quantities for the occurrence of spontaneous [Fe-S] cluster assembly. It is likely that organisms themselves provide specific delivery mechanisms for the supply of iron and sulfide necessary for [Fe-S] cluster formation.

To identify enzymes involved in [Fe-S] cluster synthesis, the *Azotobacter vinelandii* nitrogenase system was used as a model. The reason for this choice is that nitrogenase activity is not essential for growth when a fixed source of nitrogen is added to the growth medium (46) and is, therefore, dispensable under certain conditions. Nitrogenase is a metalloenzyme consisting of two component proteins, the Fe-protein and the MoFe-protein. The Fe-protein is a homodimer encoded by *nifH* and contains a [4Fe-4S] cluster located between the identical subunits. The MoFe-protein is a heterotetramer encoded by *nifD* and *nifK* and contains two P-clusters and two FeMo-cofactors (see Figure 1.2) (13, 18, 80, 81). In addition to the primary products of *nifHDK*, several other *nif*-specific gene products are involved in the production of a functional nitrogenase (18, 45, 46). Nitrogenase is very abundant and represents a significant proportion of the total soluble protein in nitrogen-fixing *A. vinelandii* (120, 123). Therefore, it is possible to use biochemical-genetic analysis of these *nif*-specific genes to determine which ones are involved in the formation of nitrogenase-associated [Fe-S] clusters, which requires a high input of iron and sulfur (46).

Among *nif*-specific genes, deletions of *nifS* and *nifU* diminish the diazotrophic growth properties (the ability to grow by using N_2 as the sole source of nitrogen) of *A. vinelandii* mutants and the activities of both nitrogenase components (46). The loss of the Fe-protein and MoFe-protein activities of nitrogenase is concurrent with the lack of

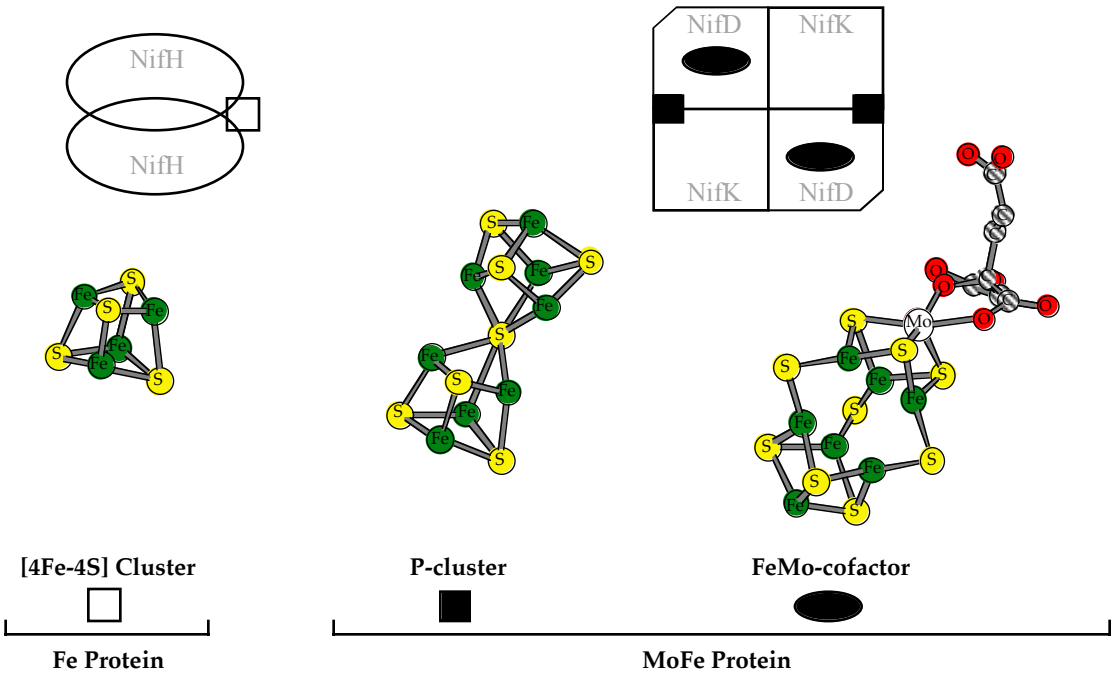


Figure 1.2: Structure and organization of the nitrogenase metalloclusters (described in text). The P-cluster of nitrogenase MoFe-protein is a [8Fe-7S] cluster. The FeMo-cofactor of nitrogenase MoFe-protein has a unique structure that contains a [7FeMo-9S] cluster together with an organic molecule of homocitrate.

their [Fe-S] clusters (123). Therefore, NifS and NifU have been proposed as components required for both Fe-protein and MoFe-protein maturation. Specifically, they are thought to be involved in the mobilization of iron and sulfur for [Fe-S] core formation for the catalytic components of nitrogenase (18, 26, 121-123).

It is now known that NifS provides activated inorganic sulfide for [Fe-S] cluster synthesis by using L-cysteine. NifS is isolated as a pyridoxal phosphate (PLP)-containing homodimer having a molecular weight of ~87,500 (123). By using PLP chemistry, NifS catalyzes L-cysteine desulfurization forming L-alanine as a product and enzyme-bound persulfide at the active site Cys^{325} as an intermediate (122, 123). This intermediate is thought to supply the acid-labile sulfide for [Fe-S] cluster assembly *in vivo* (18, 122). In support of this proposal, an *in vitro* study (121) was used to show that NifS is capable of reconstituting a [4Fe-4S] cluster into apo-Fe protein in the presence of ferrous ion, dithiothreitol (DTT), and the substrate L-cysteine. This reconstitution requires the active site Cys^{325} of NifS because a NifS variant for which the active site Cys^{325} is substituted by alanine is not active in the reconstitution assay. Moreover, cysteine is a significant source of sulfur for [Fe-S] cluster assembly as shown by isotope analysis in intact spinach chloroplasts (107) or in *E. coli* (116). A homolog of *O*-Acetylserine synthase, the enzyme which catalyzes the rate-limiting step in cysteine biosynthesis, is located close to *nifUS* in the *nif*-specific gene cluster. This genetic organization indicates an extra requirement of cysteine for nitrogenase maturation (120).

Since NifS mobilizes sulfur, NifU has been proposed to supply the iron necessary for [Fe-S] core assembly for nitrogenase maturation (18, 26, 121). NifU, as well as NifS, is required for the full activation of nitrogenase. These genes are also cotranscribed (46),

but NifU is totally different from NifS. While NifS contains PLP as its prosthetic group, isolated NifU is a homodimer with two [2Fe-2S] clusters as its prosthetic groups (26). The NifU primary sequence is rich in cysteine residues. Cys^{35}, Cys^{62}, Cys^{106}, Cys^{137}, Cys^{139}, Cys^{172}, Cys^{175}, Cys^{272}, and Cys^{275} are conserved among nitrogen fixation organisms (8, 26). Although Cys^{137}, Cys^{139}, Cys^{172}, Cys^{175} are likely to serve as ligands for the [2Fe-2S] cluster of NifU, there are still five more cysteine residues available to bind more iron (26). Therefore, many possibilities can be considered for the function of NifU (18). The [2Fe-2S] clusters of NifU could serve as a precursor for the formation of nitrogenase-associated [Fe-S] clusters directly. Alternatively, the clusters could provide the proper redox state for acquiring iron from iron-storage proteins and for the stabilization/release of bound iron or its intermediate at another site on NifU.

Homologs to both NifS and NifU have been identified in *A. vinelandii* as well as non-nitrogen-fixing organisms. A segment of DNA containing *iscS*, whose product designated IscS (iron-sulfur cluster) exhibits the same activity as NifS, was isolated from an *A. vinelandii* NifS-deleted mutant (120). This DNA fragment also contains the *iscU* gene, whose product designated IscU is a homolog of NifU. The *iscS* and *iscU* genes are cotranscribed. Primary sequence comparisons indicates that IscS and IscU are conserved and widely distributed in nature. In fact, Ouzounis *et al.* (74) have suggested that NifU is likely to be a modular protein containing separate N-terminal, central, and C-terminal domains. This suggestion is supported by identification of members of the IscU protein family. IscU proteins are highly conserved (~ 63-77% identity) as a group and exhibit sequence identity (~ 40-48%) only to the N-terminal third of NifU including the first three cysteine residues (44). The middle region of NifU that contains the [2Fe-2S]

ligands also shows sequence homology to the internal domain of nitrite reductases (74). Another possible functional homolog to the NifU central domain might be the small *E. coli* protein Bfd (bacterioferritin-associated ferredoxin), whose function is probably associated with iron mobilization or storage. This protein has relatively less sequence similarity when compared to the central domain of NifU, but the same spacing pattern of cysteine residues proposed for the [2Fe-2S] cluster of NifU is conserved. In addition, its [2Fe-2S] cluster has spectroscopic properties close to those of NifU (28, 84).

Because NifS and NifU, which are *nif*-specific gene products, are not present in non-nitrogen fixing organisms and are dispensable under non-nitrogen fixing conditions in *A. vinelandii*, it is likely that an analogous system to NifS and NifU functions in the maturation of other [Fe-S] cluster-containing proteins. An attractive possibility is that the *iscS* and *iscU* gene products serve this function. Supporting a possible "housekeeping" role in [Fe-S] cluster assembly for IscS and IscU is that these gene products are essential for *A. vinelandii* viability under all conditions (120). In addition to *iscSU* genes, *hscBA* (heat-shock cognate) and *fdx* genes are also found in the same transcription unit in *A. vinelandii* and *E. coli* (120). The primary sequences of *hscB* and *hscA* gene products indicate that they belong to molecular chaperone families and are related to *dnaJ* and *dnaK*, respectively (120). However, *hscBA* gene products seem to function under normal growth conditions rather than under heat shock conditions (94). Thus, they were suggested to have a role in "housekeeping" [Fe-S] cluster assembly (120). The *A. vinelandii fdx* gene product was recently shown to be a small [2Fe-2S] cluster-containing ferredoxin (48) and could be functionally homologous to the middle domain of NifU.

Indeed, the *E. coli* gene cluster that contains all of these genes was recently shown to enhance various types of [Fe-S] clusters heterologously synthesized *in vivo* (68).

An analogous system to NifS and NifU for synthesis of [Fe-S] clusters was also reported in *Saccharomyces cerevisiae.* The products of genes designated *ssq1*, *jac1*, *nfs1* were shown to be homologs to the HscA, HscB, and IscS, respectively. They are mitochondrial proteins and are required for [Fe-S] cluster assembly for [Fe-S]-cluster-containing mitochondrial enzymes (105). Kispal *et al*. (52) have recently shown that the *nfs1* gene product not only participates in [Fe-S] cluster assembly of mitochondrial target proteins, but also functions in cluster assembly for [Fe-S] proteins located in the cytosol. NifU homologs were also recently shown to be involved in [Fe-S] cluster synthesis of mitochondrial enzymes (90). The *isu1* and *isu2* gene products are homologous to the N-terminal third of NifU (IscU). The *nfu1* gene product shares sequence homology to the C-terminal domain of NifU, which contains the last two cysteine residues.

In addition to NifS (38, 50) and IscS (23, 120), *in vitro* reconstitution of apoproteins has been achieved using other enzymes. However, the possible physiological significance of [Fe-S] cluster assembly associated with these proteins is uncertain. These enzymes are rhodanese (9, 10, 75, 76), 3-mercaptopyruvate sulfurtransferase (MST) (71), L-cysteine/cystine *C*-*S*-lyase or cysteine desulfhydrase (C-DES) (55, 58), *O*-acetylserine sulfhydrylase A, *O*-acetylserine sulfhydrylase B, and β-cystathionase (25). Rhodanese and MST, which probably share a common ancestor (67), are collectively known as sulfurtransferases and catalyze the transfer of a sulfur atom between a sulfane sulfur donor and a thiophilic acceptor (77). They are widespread in nature (67) and have a broad range of substrates, both as sulfur donors and acceptors (77, 115). For example,

rhodanese can transfer sulfur to both chemical-thiol compounds used in synthetic analog studies of [Fe-S] clusters and clostridial-ferredoxin apoprotein (10). It was suggested that rhodanese provides sulfide for the spontaneous self-assembly of [Fe-S] clusters (89, 123). If rhodanese is necessary for [Fe-S] cluster assembly, deletion of this enzyme should be lethal because [Fe-S] clusters are important in various life-sustaining processes. However, *A. vinelandii* mutant strains containing an impaired rhodanese exhibit the same phenotype as the wild-type strain. In addition, double mutants impaired in rhodanese as well as *nifU* or *nifS* from *A. vinelandii* (*nifUrhdA* or *nifSrhdA*) could not be distinguished from mutants impaired only in *nifU* or *nifS* (16). A double mutant was constructed because it was suggested that incomplete loss of nitrogenase activity in *A. vinelandii nifU* or *nifS* deletion strain (46) might be due to a partial compensation associated with [Fe-S] cluster assembly provided by "housekeeping" components in this organism (18, 123). Thus, the genetic characterization of rhodanese mutants suggests either there are multiple copies of rhodanese in *A. vinelandii* (16) or rhodanese is not a major participant in [Fe-S] cluster synthesis *in vivo*. Several other possibilities for the physiological function of rhodanese have also been proposed (16).

C-DES was isolated from a cyanobacterium strain *Synechocystis* PCC 6714 by monitoring the reconstitution of [2Fe-2S] ferredoxin with ferrous ion, L-cysteine, and glutathione (55, 58). It is a pyridoxal phosphate containing enzyme, but there is only 27% identity when NifS and C-DES are compared (55). Its cysteine desulfhydrase activity results in formation of pyruvate, sulfide, and ammonia when either L-cysteine or cystine is used as a substrate (55, 58). *O*-acetylserine sulfhydrylase B, and β-cystathionase were isolated from *E. coli* by monitoring the reconstitution of dihydroxy-

acid dehydratase, which is a [4Fe-4S]-containing enzyme, in the presence of reactants used for NifS, ferrous ion, DTT, and L-cysteine (25). Characterization of these enzymes indicates that [Fe-S] cluster assembly *in vitro* using these conditions are only side reactions which occur only under certain experimental conditions.

To support the idea that NifS and NifU are involved in [Fe-S] cluster assembly, this dissertation describes work designed to determine how NifU might participate in [Fe-S] cluster assembly. Chapter 2 describes genetic, biochemical and biophysical techniques that were used to identify potential iron-binding sites located within NifU that could sequester iron for [Fe-S] cluster assembly. In addition, it was shown that NifU is a modular protein because polypeptide fragments containing either the N-terminal third or the C-terminal half including the central domain of NifU could separately retain certain individual properties of NifU. Chapter 3 provides evidence that the irons bound at the mononuclear sites, within the N-terminal third of NifU, are destined for [Fe-S] cluster assembly. It also provides evidence that NifU is likely to work in concert with NifS because NifU and NifS can form a transient complex.

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CHAPTER II

MODULAR ORGANIZATION AND IDENTIFICATION OF A LABILE MONONUCLEAR IRON-BINDING SITE WITHIN THE NifU PROTEIN

This chapter was authored by Jeffrey N. Agar[✚], Pramvadee Yuvaniyama[❣], Richard F. Jack[❣], Valerie L. Cash[❣], Archer D. Smith[✚], Dennis R. Dean[❣], and Michael K. Johnson[✚] and was submitted to the Journal of Biological Chemistry in September, 1999. In Dennis Dean's laboratory, my responsibility was the construction of *nifU* fragments (*nifU-1* and *nifU-2*) and all protein purifications. I was also involved in room temperature UV-Visible characterization of wild-type NifU-1 and its variants. Site-directed mutagenesis on *nifU-1* variants and *nifU* $Asp^{37}Ala$ were performed by Valerie Cash. In the laboratory of Michael Johnson, experiments using biophysical characterization techniques, which included low temperature UV-Visible spectroscopy, were conducted by Jeffrey Agar and Archer Smith.

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A. Abstract

The NifS and NifU nitrogen fixation-specific gene products are required for the full activation of both the Fe-protein and MoFe-protein of nitrogenase from *Azotobacter vinelandii*. It has been suggested that NifS and NifU could have complementary functions in the mobilization of sulfur and iron necessary for nitrogenase-specific [Fe-S] cluster assembly because the two nitrogenase component proteins both require the assembly of [Fe-S]-containing clusters for their activation. The NifS protein has been shown to have cysteine desulfurase activity and can be used to supply sulfide for the *in vitro* catalytic formation of [Fe-S] clusters. The NifU protein was previously purified and shown to be a homodimer that contains two identical [2Fe-2S] clusters. In the present work, primary sequence comparisons, amino acid substitution experiments, and optical and resonance Raman spectroscopic characterization of recombinantly-produced NifU and NifU fragments are used to show that NifU has a modular structure. One module is contained in approximately the N-terminal third of NifU and is shown to provide a labile rubredoxin-like Fe(III)-binding site. Cysteine residues Cys^{35}, Cys^{62}, and Cys^{106} are necessary for binding iron in the rubredoxin-like mode and visible extinction coefficients indicate that up to one Fe(III) ion can be bound per NifU monomer. The second module is contained in approximately the C-terminal half of NifU and provides the [2Fe-2S] cluster-binding site. Cysteine residues Cys^{137}, Cys^{139}, Cys^{172}, and Cys^{175} provide ligands to the [2Fe-2S] cluster. The cysteines involved in ligating the mononuclear iron in the rubredoxin-like site and those that provide the [2Fe-2S] cluster ligands are all required for the full physiological function of NifU. The only other two cysteines contained within NifU, Cys^{272} and Cys^{275}, are not necessary for iron binding at either site nor are they

required for the full physiological function of NifU. The results provide the basis for a model where iron bound in labile rubredoxin-like sites within NifU is used for [Fe-S] cluster formation. The [2Fe-2S] clusters contained within NifU are proposed to have a redox function involving the release of iron from bacterioferritin and/or the release of iron or an [Fe-S] cluster precursor from the rubredoxin-like binding site.

B. Introduction

The *A. vinelandii nifU* and *nifS* gene products (NifU and NifS) are proposed to have specific roles in the formation or repair of the [Fe-S] cores of metalloclusters contained within the catalytic components of nitrogenase (7). NifS is a pyridoxal phosphate (PLP)-dependent L-cysteine desulfurase (38, 39) that is able to catalyze the *in vitro* reconstitution of an apo-form of the nitrogenase Fe-protein whose [4Fe-4S] cluster has been removed by chelation (37). The active species in this reaction is an enzyme-bound persulfide that is formed through the nucleophilic attack by an active site cysteine on the PLP-substrate cysteine adduct (38).

Although a specific function for NifU in nitrogenase [Fe-S] cluster formation is not known, the available evidence points to a role either as the iron source necessary for [Fe-S] cluster formation or as an intermediate site for [Fe-S] cluster assembly (7). For NifU to serve either of these functions it must have the ability to transiently bind iron that is destined for [Fe-S] cluster formation. Previous work has shown that isolated NifU is a homodimer that contains two identical [2Fe-2S] clusters (12). It seems unlikely, however, that these [2Fe-2S] clusters represent the source of iron necessary for nitrogenase [Fe-S] cluster formation, since they are tightly bound within the NifU protein and cannot be removed even with strong chelation reagents (12). Thus, we have speculated that the redox-active $[2Fe\text{-}2S]^{2+,+}$ clusters contained within NifU (E_m = -254 mV *vs* SHE), have a redox role involved in the binding or release of iron or an [Fe-S] cluster intermediate at a second site within NifU (12). The NifU protein contains nine cysteine residues, Cys^{35}, Cys^{62}, Cys^{106}, Cys^{137}, Cys^{139}, Cys^{172}, Cys^{175}, Cys^{272}, and Cys^{275} (4, 12). Based on primary sequence comparisons to other [2Fe-2S]-containing proteins,

NifU residues Cys^{137}, Cys^{139}, Cys^{172}, and Cys^{175} are the most likely cluster-coordinating residues (12). Thus, any or all of the remaining cysteine residues could be involved in binding iron at a second site or participate in the assembly of an [Fe-S] cluster intermediate.

Comparison of NifU primary sequences to other proteins contained within the data base has led to speculation that NifU is a modular protein that is organized into distinct structural domains (19, 25). One of these modules includes the central portion of NifU which encompasses the proposed [2Fe-2S] cluster ligands. Another module corresponds to the N-terminal third of NifU and it contains residues Cys^{35}, Cys^{62}, and Cys^{106} (Figure 2.1). Counterparts to each of the cysteine residues contained within the proposed N-terminal module of NifU are also strictly conserved in a family of small proteins designated IscU (36). In the cases of *Escherichia coli*, *Azotobacter vinelandii*, and a variety of other prokaryotic organisms, the *iscU* gene is cotranscribed with another gene designated *iscS* (36). The IscS protein shares considerable primary sequence identity when compared to NifS and it exhibits the same L-cysteine desulfurase activity as NifS (10, 36). Homologs to *iscU* and *iscS* are also widely conserved in nature. For example, a search of the protein database reveals that homologs to these genes are encoded within the yeast, mouse, arabidopsis and human genomes. Indeed, Hwang *et al*. (19) have suggested that the N-terminal domain of NifU represents one of the most highly conserved protein sequence motifs in nature. The wide conservation of *iscS* and *iscU* genes in nature, and their apparent co-transcription in many prokaryotic organisms, indicates that IscU and IscS might have housekeeping roles involved in the general mobilization of iron and sulfur for [Fe-S] cluster formation (36). If this is correct, the

cysteine residues conserved between NifU and the IscU family of proteins (Figure 2.1) are most likely to be involved in providing the proposed second iron-binding site within NifU.

In the present work, the functional significance of each of the nine cysteine residues encoded within the *A. vinelandii nifU* gene was assessed by site-directed mutagenesis and gene-replacement techniques. Purification and spectroscopic characterization of NifU proteins altered in this way were also used to identify the [2Fe-2S] cluster-coordinating cysteine residues. The existence of a labile rubredoxin-like Fe(III)-binding site in NifU and NifU(Asp^{37}Ala) was demonstrated by the combination of optical absorption and resonance Raman spectroscopies. Finally the modular nature of the NifU polypeptide and the ligands to both metal sites were addressed by spectroscopic and redox studies of the purified N-terminal and C-terminal fragments of NifU. These fragments were shown to contain the labile mononuclear iron-binding site and the indigenous [2Fe-2S] cluster, respectively, each with properties very similar to those established in holo NifU.

C. Experimental procedures

DNA Biochemistry, Plasmids, and Strain Constructions

Site-directed mutagenesis and gene-replacement procedures were performed as previously described (5). Isolated pDB800 DNA was used for mutagenesis. This plasmid was constructed by ligating an approximately 1.0 kb *Xba*I DNA fragment isolated from pDB525 (12) into the pUC119 cloning vector. Plasmid pDB800 contains the entire *nifU*-coding sequence. Point mutations generated within pDB800 were

transferred to the *A. vinelandii* chromosome using congression (coincident transfer) as previously described (5). Preparation of competent *A. vinelandii* cells for transformation was performed as previously described by Page and von Tigerstrom (26). The following plasmids were used for the high level, heterologous, expression of *nifU* and *nifU* fragments in the *E. coli* host strain BL21(DE3) (31): pDB525, pDB937, pDB938, pDB822, pDB965, pDB966, pDB967, and pDB1027. The construction of pDB525 was previously described (12). It contains the entire *A. vinelandii nifU* gene cloned into the pT_7-7 vector so that the expression of *nifU* is regulated by the T_7 transcription and translation control elements. Plasmid pDB822 and pDB1027 are the same as pDB525 except that the *nifU* Cys^{137} codon was substituted by an Ala codon (pDB822) or the *nifU* Asp^{37} codon was substituted by Ala (pDB1027). Plasmid pDB937 was generated by polymerase-chain-reaction (PCR) amplification of the first 393 base-pairs of the *nifU*-coding sequence and then subsequently cloning this DNA fragment into the pT_7-7 cloning vector in the appropriate orientation. In this construction the *nifU* Glu^{131} codon was substituted by a termination codon. The NifU polypeptide fragment produced using this construct is referred to as NifU-1 (Figure 2.1). Plasmid pDB938 was similarly generated by PCR amplification of a 564 base-pair fragment that extends from codon 126 within the *nifU*-coding sequence to several base-pairs past the natural *nifU* termination codon. In this construction the *nifU* Ile^{126} codon was substituted by a translation-initiating Met codon. The NifU polypeptide fragment produced in this way is referred to as NifU-2 (Figure 2.1). Oligonucleotides used for PCR-amplification of the gene cartridges encoding NifU-1 and NifU-2 had the following sequences: 5'-ATGCATATGTGGGATTATTCGGAA-3' and 5'-TGTCGGATCCCTCTTAGTGGTCGTCC-3' for

NifU-1; and 5'-CATGCATATGGAGGACGACCACGAAGAG-3' and 5'-ATCGGATCCCGGGTGGTGGCGTTG-3' for NifU-2. Details of the PCR-based strategy for construction of pDB937 and pD938 were the same as previously described in detail for pDB525 (12). Plasmids pDB965, pDB966, and pDB967 are identical to pDB937 except for the following substitutions in the *nifU-1* coding sequence: codon Cys^{35} substituted by an Ala codon (pDB965), codon Cys^{62} substituted by an Ala codon (pDB966), and codon Cys^{106} substituted by an Ala codon (pDB967).

Protein Biochemistry

NifU and altered forms of NifU from *A. vinelandii* that were generated by site-directed mutagenesis were expressed in *E. coli* and purified as previously described (12). NifU-1 and altered forms of NifU-1 were heterologously produced in 500 ml-batch cultures of *E. coli* strain BL21(DE3) as described by Fu *et al*. (12). Protein was typically purified from 30 to 45 grams of wet-weight cells and all manipulations were performed under an argon atmosphere. Crude extracts were prepared by resuspension of cell pellets in a 25 mM Tris-HCl, pH 7.4 buffer, rupturing cells by sonication and centrifugation as previously described (12). Crude extract was fractionated by the addition of solid streptomycin sulfate to 1%, w/v at ambient temperature and centrifugation at 20,000 rpm for 20 min in a Beckman Type 35 rotor. NifU-1 was then precipitated from the streptomycin sulfate-treated crude extract by bringing it to 60% saturation with solid ammonium sulfate. Precipitated NifU-1 was collected by centrifugation as above and resuspended in the same buffer at a volume equal to the original supernatant volume. The sample was then loaded on a 2.5 x 20 cm Q Sepharose column (Pharmacia, Piscataway, NJ) using a peristaltic pump and eluted using a linear 1.5 L (0.1-to-1.0 M) NaCl gradient.

NifU-1 elutes between 0.42 and 0.50 M NaCl. Concentrated sample was then passed over a 2.5 x 20 cm Sephacryl-300 column and eluted using a flow rate of 2.0 ml/min. Purified NifU-1 samples were greater than 95% pure based on denaturing polyacrylamide gel electrophoresis (Figure 2.2). Protein concentrations were determined by the biuret reaction or the bicinchoninic acid (BCA) method (15, 28). Buffer, preparation of crude extracts, streptomycin sulfate treatment and ammonium sulfate fractionation used for NifU-2 purification were the same as for NifU-1 purification except that 2.0 mM dithiothreitol was added to all buffers and NifU-2 was precipitated at 45% ammonium sulfate saturation. The NifU-2 sample obtained after ammonium sulfate fractionation was further purified as described above using a Q sepharose column chromatography except that a 0.6 L (0.1-to-0.6 M) linear NaCl gradient was used. NifU-2 elutes between 0.3 and 0.4 M NaCl. The eluted NifU-2 fraction was concentrated and brought to 0.4 M ammonium sulfate by the addition of an equal volume of buffer (25 mM Tris-HCl pH 7.4) containing 0.8 M ammonium sulfate. The diluted NifU-2 sample was applied to a Pharmacia 1.5 x 15 cm Phenyl Sepharose column and eluted using a 150 ml linearly decreasing (0.4 to 0.0 M) ammonium sulfate gradient. Purified NifU-2 was greater than 95% pure based on polyacrylamide gel electrophoresis (Figure 2.2). The nitrogenase Fe-protein and MoFe-protein were assayed in crude extracts of nitrogenase-derepressed cells by using the same conditions for the acetylene reduction assay as previously described by Jacobson *et al*. (20). Units for nitrogenase component protein activities are expressed as nanomoles acetylene reduced per minute per mg of crude extract protein. *A. vinelandii* cells were cultured in Burk medium (30) and grown at 30°C with (30 mM final concentration) or without the addition of ammonium acetate as a fixed nitrogen source.

Spectroscopic and Electrochemical Characterization of NifU, NifU-1 and NifU-2

The sample concentrations given in the figure captions are based on the quantitation of the purified protein solution by the BCA method (28) or by A_{280}. Spectroscopic results were quantified per NifU, NifU-1, or NifU-2 monomer. UV-Visible absorption spectra were recorded under anaerobic conditions in septum-sealed 1 mm and 1 cm cuvettes, using a Shimadzu 3101PC scanning spectrophotometer fitted with a TCC-260 temperature controller. Variable-temperature magnetic circular dichroism (VTMCD) spectra were recorded using samples containing 55% (v/v) glycerol in 1 mm cuvettes using an Oxford Instruments Spectromag 4000 (0-7 T) split-coil superconducting magnet (1.5-300 K) mated to a Jasco J-715 spectropolarimeter. VTMCD experimental protocols were performed as previously described (21, 33). X-band (~ 9.6 GHz) electron paramagnetic resonance (EPR) spectra were recorded using a Bruker ESP-300E EPR spectrometer equipped with a dual-mode ER-4116 cavity and an Oxford Instruments ESR-9 flow cryostat. Frequencies were measured with either a Systron-Donner 6054B frequency counter or Hewlett Packard 5350B microwave frequency counter, and the magnetic field was calibrated with a Bruker ER035M gaussmeter. Spin quantitations were carried out under non-saturating conditions with 1mM Cu(II)EDTA as the standard, as described by Aasa and Vänngård (1).

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 signal/noise was improved by signal averaging multiple scans. Band positions were calibrated using the excitation frequency and CCl_4 and are accurate to ± 1 cm^{-1}. Laser

excitation lines were generated by Coherent Innova 100 10 W argon ion, and Coherent Innova 200 10 W krypton ion lasers. Plasma lines were removed using a Pellin Broca Prism premonochromator. For RR studies, a 12-μL droplet of sample was placed in a specially designed sample cell (8) attached to the cold finger of an Air Products Displex model CSA-202E closed-cycle refrigerator. The sample temperature was maintained at 18 K during scanning in order to minimizes laser-induced sample degradation. Bands due to the frozen buffer solutions have been subtracted from all the spectra shown in this work after normalization of lattice modes of ice centered at 229 cm^{-1}.

All samples of NifU-1 were pre-treated with 1 mM DTT which was removed by anaerobic gel filtration prior to use in iron-binding studies. NifU-1 and NifU iron titrations were performed at ambient temperature under anaerobic conditions. Ferric ammonium citrate was added to 200 μM NifU-1 in a 50 mM Tris-HCl buffer, pH 7.8, containing 100 mM NaCl, in 0.10 equivalent increments with respect to NifU-1 monomer. The aliquots came from a freshly prepared solution of ferric ammonium citrate and absorption spectra were recorded within 30 seconds of each addition. Iron-binding studies of NifU-1, NifU, and NifU($Asp^{37}Ala$) at 2°C were carried out under strict anaerobic conditions after gel filtration to remove DTT, using a Shimadzu TCC-260 temperature controller and monitoring the absorption changes over a period of 90 min following the addition of a 1.2 or 2.4-fold excess of ferric ammonium citrate. RR samples of iron-bound NifU-1, NifU, and NifU($Asp^{37}Ala$) were prepared by anaerobic incubation of highly concentrated samples (~ 4 mM) with 5 equivalent/monomer of ferric ammonium citrate for 1 hr at 2°C.

Midpoint potentials of $[2Fe\text{-}2S]^{2+,+}$ clusters in NifU and NifU-2 were measured by cyclic voltammetry at a glassy carbon electrode using neomycin (2 mM) as a promoter. The electrochemistry cell used was identical to that described by Hagen (16), with glassy carbon, Pt, and Ag/AgCl as the working, counter, and reference electrodes, respectively. The working electrode was prepared by polishing it with an Al_2O_3 slurry (0.3 μm) and then with diamond spray (1 μm). The scan rate was 10 mV/sec over the potential range -200 to -900 mV (versus the Ag/AgCl electrode).

D. Results

Identification of cysteine residues required for full NifU function

Each of the nine cysteine codons contained within *nifU* were individually substituted by alanine codons and, in some cases, by a variety of other codons. Mutations resulting in such amino acid substitutions were transferred to the *A. vinelandii* chromosome using a gene-replacement procedure (5). Mutant strains constructed in this way were isogenic to the wild-type control strain except for the particular mutation. Because there is no direct enzymatic assay for the effect of amino acid substitutions within NifU, their effect on the maturation of the nitrogenase catalytic components can only be evaluated by indirect physiological effects upon diazotrophic growth rates and by their effect on nitrogenase component protein activities. Typical results for the effects of amino acid substitutions within NifU on diazotrophic growth rates are summarized in Table 2.1. Figure 2.3 compares the diazotrophic growth rates for the wild-type strain, a mutant strain that produces an altered NifU protein having Cys^{106} substituted by alanine, and a strain deleted for *nifU*. The results show that substitution at the Cys^{106} position

results in a lowered diazotrophic growth capability for the mutant strain, but that the effect is not as severe as for a strain having *nifU* deleted (20). Analogous results were obtained for all strains having substitutions for any of the cysteine residues except those substituted at the Cys^{272} or Cys^{275} positions. These latter strains exhibited normal diazotrophic growth that could not be distinguished from the wild-type strain. The low diazotrophic growth rates for the other mutant strains are also reflected in lower activities for both the nitrogenase Fe-protein and MoFe-protein in nitrogenase-derepressed crude extracts. For example, the wild-type strain exhibits an Fe-protein specific activity of 42 units and MoFe-protein specific activity of 52 units, whereas, the strain having Cys^{106} substituted by alanine has an Fe-protein specific activity of 12 units and MoFe-protein a specific activity of 22 units (see Experimental Procedures for activity units). Thus, as in the case of the *nifU* deletion strain (20), substitution for any NifU cysteine residue except Cys^{272} or Cys^{275} results in a mutant strain having substantially lower specific activities for both the Fe-protein and MoFe-protein.

Cysteine residues 137, 139, 172, and 175 provide [2Fe-2S] cluster ligands and are contained within a modular domain.

In order to determine which of the nine cysteine residues provide the [2Fe-2S] cluster ligands, altered NifU was purified from each of nine *E. coli* strains which individually produced an altered NifU protein having one of the cysteines substituted by alanine. On the basis of the UV-Visible spectra of the oxidized proteins and the EPR spectra of the reduced proteins, variant NifU proteins with Cys^{35}, Cys^{62}, Cys^{106}, Cys^{272}, or Cys^{275} individually replaced by Ala, still contained a [2Fe-2S] cluster with properties indistinguishable from that of wild type. In contrast, variant NifU proteins with Cys^{137},

Cys^{139}, Cys^{172}, or Cys^{175} individually replaced by Ala, did not contain a [2Fe-2S] cluster. To further confirm that residues Cys^{137}, Cys^{139}, Cys^{172}, and Cys^{175} are [2Fe-2S] cluster ligands, and that they are contained within a modular domain, a gene cartridge was produced and cloned, which directs the synthesis of a NifU fragment that includes residues 126 to the C-terminus of NifU (designated NifU-2, see Figure 2.1). NifU-2 that was heterologously produced in *E. coli* was isolated (Figure 2.2) and found to contain a $[2Fe\text{-}2S]^{2+,+}$ cluster. Gel exclusion chromatography showed that isolated NifU-2 is a homodimer (data not shown). The midpoint potentials for the $[2Fe\text{-}2S]^{2+,+}$ clusters in NifU and NifU-2 were determined by cyclic voltammetry at a glassy carbon electrode using neomycin as a promoter. Within experimental error, the midpoint potentials were the same for NifU and NifU-2, E_m = -250 ±10 mV (*vs* SHE), and this value is in excellent agreement with that determined by dye-mediated optical redox titrations for NifU, E_m = -254 ±20 mV (12).

The ground and excited properties of the [2Fe-2S] cluster in oxidized and reduced forms of NifU-2 were investigated using the combination of UV-Visible absorption, EPR, VTMCD, and RR spectroscopies. The UV-Visible absorption characteristics of as purified and dithionite-reduced NifU-2 (Figure 2.4) are quantitatively indistinguishable from those of the NifU holoprotein (12). As previously discussed (12), the spectra and extinction coefficients are indicative of one $[2Fe\text{-}2S]^{2+,+}$ cluster per NifU-2 monomer. RR spectra of diamagnetic $[2Fe\text{-}2S]^{2+}$ clusters in the Fe-S stretching region provide a more rigorous assessment of the structural integrity of the cluster in NifU-2 fragment compared to the holoprotein. The spectra are particularly sensitive to the Fe-S-C-C dihedral angles and hydrogen-bonding interactions of coordinating cysteine residues (11,

17, 18). Consequently, different subclasses of [2Fe-2S]-containing ferredoxins, each with complete cysteinyl ligation, but differing in terms of the primary sequence arrangements of coordinating cysteines, are readily distinguishable by RR spectroscopy (11, 17). As shown in Figure 2.5, the RR spectrum of the $[2Fe\text{-}2S]^{2+}$ center in NifU-2 is broader than that obtained for the holoprotein under identical conditions, indicating greater heterogeneity in the cluster environment, but the relative intensities and the frequencies of individual bands are in good agreement. On the basis of the assignments advanced for the all-cysteine-ligated $[2Fe\text{-}2S]^{2+}$ center in NifU under effective D_{2h} symmetry (12), the most significant frequency difference lies in predominantly bridging $B_{3u}{}^{b}$ mode which shifts from 356 cm^{-1} in NifU to 364 cm^{-1} in NifU-2. Since the frequency separation in the predominantly bridging and terminal B_{3u} modes is known to be dependent on the cysteinyl dihedral angles (18), and the $B_{3u}{}^{t}$ modes are assigned to the bands at 288 cm^{-1} and 289 cm^{-1} in NifU and NifU-2, respectively (12), this difference is tentatively attributed to minor changes in the dihedral angles of one or more of the coordinating cysteine residues.

The ground and excited properties of paramagnetic $[2Fe\text{-}2S]^{+}$ cluster in reduced NifU-2 have been investigated using EPR (Figure 2.6) and VTMCD (Figure 2.7) spectroscopies, and the results are compared to those previously obtained and analyzed for NifU (12). Reduced NifU-2 exhibits an $S = 1/2$ EPR signal, $g = 2.022$, 1.928, and 1.890 (based on spectral simulation), that is observed without significant broadening up to 50 K and accounts for 0.9 spin/monomer. These g-values and relaxation properties are characteristic of a $[2Fe\text{-}2S]^{+}$ cluster and indicate that the ground-state properties are essentially identical to those of the completely cysteinyl-ligated $[2Fe\text{-}2S]^{+}$ cluster in the

holoprotein, g = 2.019, 1.927, and 1.892 (based on spectral simulation). The VTMCD spectrum of paramagnetic $[2Fe\text{-}2S]^{+}$ clusters provides a more sensitive monitor of the excited state properties than the absorption spectrum (12). Hence, the near identical VTMCD spectra of reduced NifU-2 and NifU (Figure 2.7) attest to the same excited state structure for the $[2Fe\text{-}2S]^{+}$ clusters. Thus, both NifU and NifU-2 contain one $[2Fe\text{-}2S]^{+}$ cluster per monomer and neither the ground nor the excited state properties are significantly perturbed when the $[2Fe\text{-}2S]^{+}$ clusters from the NifU and NifU-2 proteins are compared.

Identification of a mononuclear iron-binding site in NifU

The initial attempts to monitor Fe(III) ion binding by NifU focused on optically monitored titrations with ferric ammonium citrate at room temperature. However, these experiments did not provide convincing evidence for Fe(III) ion binding to cysteine residues. Anaerobic addition of ferric ammonium citrate (up to 3 equivalents/monomer) to oxidized NifU in 50 mM Tris-HCl buffer, pH 7.8, with 100 mM NaCl and 1 mM DTT, resulted in no significant changes in the UV-Visible absorption spectrum after correction for any possible contributions arising from ferric ammonium citrate and ferric-DTT complexes. In view of the difficulty of optically monitoring Fe(III) ion binding in the presence of the $[2Fe\text{-}2S]^{2+}$ chromophore and the potential of interference from the presence of DTT (required to prevent oligomerization of NifU), similar iron-binding experiments were then pursued with a heterologously expressed N-terminal fragment of NifU, designated NifU-1 (Figure 2.1), that does not contain the [2Fe-2S]-binding domain and remains soluble in the absence of DTT.

NifU residues Cys^{137}, Cys^{139}, Cys^{172}, and Cys^{175} were identified as providing the [2Fe-2S] cluster ligands, and residues Cys^{272} and Cys^{275} were found to be not necessary for the full physiological function of NifU. It therefore seemed logical to expect that, if a second iron-binding site is located on NifU, residues Cys^{35}, Cys^{62}, or Cys^{106} might participate in such binding. This possibility is supported by the strict conservation of residues corresponding to Cys^{35}, Cys^{62}, and Cys^{106} within a class of proteins designated IscU which are proposed to have housekeeping functions related to [Fe-S] cluster formation (36), see Figure 2.1. The IscU proteins are much smaller than NifU and exhibit a high degree of primary sequence identity when compared to the N-terminal region of NifU (36). The primary sequence comparisons between NifU and IscU (Figure 2.1) provided the rationale for the design of a *nifU* gene fragment cartridge which includes Cys^{35}, Cys^{62}, and Cys^{106} and therefore encompasses the region encoding the proposed second iron-binding domain within NifU. This *nifU* fragment encodes NifU residues 1 through 131 and the corresponding protein, designated NifU-1 (Figure 2.1), was heterologously produced in *E. coli* and isolated (Figure 2.2). Gel exclusion chromatography showed that isolated NifU-1 is a homodimer.

The initial evidence for Fe(III) ion binding to the cysteine residues of NifU-1 came from anaerobic room temperature UV-Visible absorption studies as a function of increasing ferric ammonium citrate concentration (Figure 2.8). As the ferric ammonium citrate concentration increases, a chromophore with broad bands centered at 350 and 490 nm appears, reaching a maximum intensity with between 0.7 and 1.0 Fe(III)/NifU-1 monomer. This spectrum is characteristic of a rubredoxin-type ferric center, i.e. a tetrahedral Fe(III) center with predominant or exclusive thiolate ligation. On the basis of

the detailed electronic assignments available for rubredoxin-type FeS_4 centers (3, 9, 14, 24), the bands centered at 360 nm and 490 nm are assigned to multiple overlapping (Cys)Sp$\sigma \rightarrow$Fe(III) charge transfer transitions. In rubredoxins, the iron is coordinated by two pairs of cysteines in C-X-X-C arrangements, and each of these bands is split into two resolvable components largely a result of predominantly axial distortion of the idealized tetrahedral geometry. However, the absence of resolvable splittings in these bands for Fe(III)-bound NifU-1, does not in itself enable assessment of the extent of cysteinyl ligation. For example, both desulforedoxin (absorption bands centered at 370 nm and 507 nm (23)) and a variant of *Clostridium pasteurianum* rubredoxin with Cys^9 replaced by aspartate (absorption bands centered at 365 nm and 515 nm (29)) exhibit unresolved absorption bands very similar to those of Fe(III)-bound NifU-1. In desulforedoxin, the iron center is coordinated by four cysteines in -C-X-X-C- and -C-C- arrangements (2), whereas in the rubredoxin variant, the iron is coordinated by three cysteines and presumably an aspartate in -C-X-X-D- and -C-X-X-C- arrangements (29). The major difference in the absorption characteristics of Fe(III)-bound NifU-1 compared to those of desulforedoxin or the Cys^9Asp variant of *C. pasteurianum* rubredoxin, lies in the 10- to 20-fold lower extinction coefficients for both of the visible charge transfer bands (e.g. ε_{490} = 350 $M^{-1}cm^{-1}$ for Fe(III)-bound NifU-1, compared to ε_{507} = 7000 $M^{-1}cm^{-1}$ for desulforedoxin and ε_{515} = 5200 $M^{-1}cm^{-1}$ for Cys^9Asp *C. pasteurianum* rubredoxin). Since the extinction coefficients are based on protein concentration, this suggests that less than 10% of the NifU-1 protein is in the Fe(III)-bound form, in room-temperature samples that are maximally loaded with iron based on the titration data.

Since the role of a mononuclear iron-binding site in NifU is likely to be transient iron binding, lability of bound iron at room temperature provides an attractive explanation for the low stoichiometry of Fe(III)-bound NifU-1 and our inability to optically detect Fe(III) ion bound to NifU. To test this hypothesis, optically monitored Fe(III) ion binding experiments involving NifU-1 and NifU were carried out under strict anaerobic conditions at 2°C. Incubation of NifU-1 with a 1.2-fold excess of ferric ammonium citrate at 2°C resulted in the gradual appearance of the same rubredoxin-type chromophore that was apparent in the room temperature iron titrations (Figure 2.9A and B, lower panels). The absorption was maximal after 20 min and the extinction coefficient at 490 nm was increased approximately 3-fold compared to the maximum at room temperature. No additional increase in the absorption intensity was apparent for samples treated in the same way with a 2.4-fold excess of ferric ammonium citrate, indicating that this extinction coefficient corresponds to the maximum obtainable at this temperature. Due to the time required to reach equilibrium at 2°C, titrations with ferric ammonium citrate were not practical. Nevertheless, the 3-fold increase in the Fe(III)-bound form of NifU-1 on going from room-temperature to 2°C supports the conclusion that lability of the iron site is responsible for the low stoichiometry of iron binding.

The possibility that lower temperatures might also facilitate identification and characterization of an Fe(III)-bound form of full-length NifU was assessed by optical absorption studies at 2°C of ferric ammonium citrate treated samples of NifU and NifU(Asp^{37}Ala) (Figure 2.9A and B). In view of the similarity in the optical properties of iron-bound NifU-1 and the Cys^{9}Asp variant of *C. pasteurianum* rubredoxin (iron ligated by three cysteines and an aspartate in C-X-X-D and C-X-X-C arrangements), the

Asp^{37}Ala variant of NifU was originally constructed to address the possibility that Asp^{37} is involved with ligating the bound Fe(III) ion. While the results shown below indicate that Asp^{37} is not directly involved with iron ligation, this variant did provide some of the best quality spectroscopic data on the mononuclear Fe(III) site in NifU. In experiments with NifU and NifU(Asp^{37}Ala), DTT was removed by gel-filtration at 2°C, since at this temperature protein aggregation in the absence of DTT was much slower for NifU and not apparent for NifU(Asp^{37}Ala).

In contrast to the experiments conducted at room temperature, the visible absorption of NifU gradually increased at all wavelengths over a period of 40 min on incubation with a 1.2-fold excess of ferric ammonium citrate at 2°C (Figure 2.9A). The absorption changes were monitored for up to 90 min, but the data after 40 min are complicated by the progressive increase of a scattering baseline as a result of protein aggregation. Similar changes in the absorption spectrum were observed in parallel experiments with NifU(Asp^{37}Ala) (Figure 2.9B), but in this case the spectral changes were complete after 55 min, there was no evidence of protein aggregation, and no further changes were apparent after an additional 1 hour at 2°C or after adding an additional 1.2-fold excess of ferric ammonium citrate. The absorption spectrum of the additional chromophore generated in NifU and NifU(Asp^{37}Ala) on incubation with ferric ammonium citrate at 2°C, as assessed by difference spectra (middle panels of Figure 2.9A and B)), are quantitatively very similar except for a more resolved 490 nm band in NifU(Asp^{37}Ala) due to the absence of aggregation problems. Moreover, the optical properties of the mononuclear iron species in the maximally loaded holoproteins are in agreement with those obtained for NifU-1 under analogous conditions (cf. middle and

lower panels of Figure 2.9A and B) except for a 4–to-5-fold increase in extinction coefficients. In both NifU and NifU(Asp^{37}Ala), the additional absorption intensity due to the mononuclear Fe(III) center is lost over a period of 1 hr on warming the sample back to room temperature. This provides a rationalization for the room temperature iron-binding experiments in terms of a kinetically labile iron site. While the lability prevents accurate analytical quantitation of the bound iron, the maximal visible extinction coefficient, ε_{490} = 4200 $M^{-1}cm^{-1}$ (for the Fe(III) center in NifU(Asp^{37}Ala)), is comparable to that of the Cys^{9}Asp variant *C. pasteurianum* rubredoxin, ε_{515} = 5200 $M^{-1}cm^{-1}$, and is therefore consistent with approximately one Fe(III) ion bound per monomer, provided a coordination environment involving three cysteinates is assumed. Evidence for a mode of coordination involving three cysteine residues comes from the RR data presented below.

Strong support for a rubredoxin-type center in Fe(III)-bound forms of NifU-1, NifU and NifU(Asp^{37}Ala) comes from low-temperature RR studies (Figure 2.10A and B). The samples of Fe(III)-bound proteins used in RR experiments were prepared using the same protocol developed for the optical absorption studies by incubating concentrated samples of precooled protein (~ 2 mM) with a 5-fold excess of ferric ammonium citrate and incubating for 1 hr at 2°C under strict anaerobic conditions. As for the absorption studies, the RR spectra of the mononuclear Fe(III) centers in NifU and NifU(Asp^{37}Ala) were obtained by subtracting the spectra associated with the indigenous $[2Fe\text{-}2S]^{2+}$ centers (spectra c in Figure 2.10A and B). The intensities of the dominant 288-cm^{-1} band of the $[2Fe\text{-}2S]^{2+}$ centers were equalized prior to subtraction. The resulting RR spectra for the mononuclear Fe(III) sites in NifU-1 and NifU(Asp^{37}Ala) are in excellent agreement (Figure 2.10B), demonstrating that Asp^{37} is not an iron ligand and that the

coordination environment at this site is not perturbed by the C-terminal truncation. In both cases the spectra comprise two broad bands of comparable intensity centered at 314 and 368 cm^{-1}. Although the same bands are apparent in the difference spectrum corresponding to the mononuclear Fe(III) center in NifU, the spectrum is broader with additional features centered at 325 and 356 cm^{-1} (cf. spectra c and d in Figure 2.10A). On the basis of the absorption results discussed above, this heterogeneity is likely to be a consequence of protein aggregation.

The RR bands at 314 and 368 cm^{-1} associated with the mononuclear Fe(III) site in NifU proteins are readily identified as the symmetric and asymmetric Fe-S stretches of approximately tetrahedral rubredoxin-like center units by analogy with the spectra and assignments made for wild-type and mutant rubredoxins (6, 22, 29, 35) and wild-type desulforedoxin (32). Distorted tetrahedral FeS_4 units in rubredoxins and desulforedoxins have intense symmetric Fe-S stretching bands in the range 312-320 cm^{-1} and weaker, partially-resolved asymmetric Fe-S stretching bands in the range 335-382 cm^{-1}. The splitting in the asymmetric Fe-S stretching modes is most pronounced for the more axially distorted site in desulforedoxin which has well resolved bands at 343 and 381 cm^{-1} (32). Although the Fe-S stretching frequencies of rubredoxin-type site in NifU proteins are in accord with a distorted tetrahedral FeS_4 site, the relative intensities of the symmetric and asymmetric stretching modes and the absence of a resolvable splitting in the asymmetric stretching mode are not. Replacing one of the coordinating cysteines with an oxygenic ligand (serine or aspartate) in mutant rubredoxins has been found to result in major and diverse changes in the RR spectra in the Fe-S stretching region (22, 29, 35). However, there are examples of mutant rubredoxins with one oxygenic ligand, such as the

Cys^{39}Ser variant of *C. pasteurianum* rubredoxin that have RR spectra comprising two bands of comparable intensity centered at 324 cm^{-1} and 377 cm^{-1} (35). The ~10-cm^{-1} downshifts for labile mononuclear Fe(III) site in NifU-1 and NifU(Asp^{37}Ala) are readily rationalized in terms of slightly weaker Fe-S bonds. Hence the overall weakness and relative intensity of the symmetric and asymmetric Fe-S modes of the mononuclear Fe(III) site in NifU are best interpreted in terms of a FeS_3X site with Cys^{35}, Cys^{62}, and Cys^{106} providing the thiolate ligands and X being an unknown O or N ligand.

Attempts to characterize the ground and excited state properties of the mononuclear iron-binding site in NifU-1, NifU, and NifU(Asp^{37}Ala) using EPR and VTMCD spectroscopy were only partially successful. EPR studies of the Fe(III)-bound forms of NifU-1, NifU, and NifU(Asp^{37}Ala) at 4 K and 10 K revealed weak absorption-shaped features centered at g = 9.6 and an intense derivative-shaped feature at g = 4.3. Such a resonance is indicative of a rhombic S = 5/2 species (E/D ~ 0.33). However, these are also the EPR properties of ferric ammonium citrate and adventitiously bound Fe(III) ion, as well as those of tetrahedral Fe(III) centers with complete cysteinyl ligation or three cysteines and one oxygenic ligand (serine or aspartate) (22, 29, 35). Consequently EPR is of little utility for discriminating between different types of high-spin Fe(III) species in NifU proteins. Addition of 50% (v/v) glycerol to Fe(III)-bound NifU-1, NifU, and NifU(Asp^{37}Ala) at room temperature or 2°C resulted in complete and immediate loss of absorption features associated with mononuclear Fe(III) center. Hence the addition of the glycerol enhances the lability of the bound Fe(III) ion, thereby preventing the investigation of the electronic excited state properties via VTMCD studies. The mononuclear iron site also appears to be reductively labile, as evidenced by the complete

absence of charge transitions characteristic of Fe(II)-rubredoxin type centers in the 300-350 nm region (34) in both the 2°C absorption and VTMCD spectra of dithionite reduced samples of Fe(III)-bound NifU-1 (data not show).

Residues Cys^{35}, Cys^{62}, and Cys^{106} are necessary for iron binding to NifU-1.

The results presented above indicate that residues Cys^{35}, Cys^{62}, and Cys^{106} within NifU are involved in the transient binding of iron that is destined for nitrogenase [Fe-S] cluster core formation. If this is correct, substitution of alanine for any of these three cysteine residues would be expected to eliminate the second iron-binding site within NifU. This possibility was tested by producing three different NifU-1 forms that each have one of the respective cysteine residues substituted by alanine. Each of these altered NifU-1 proteins was purified and compared to intact NifU-1 in terms of their ability to bind iron based on optically monitored ferric ammonium citrate titrations. None of the altered NifU-1 proteins exhibited iron-binding capacity that was showed by the intact NifU-1 protein. This general result is illustrated by the ferric ammonium citrate titration data obtained for the Cys^{35}Ala variant of NifU-1 (Figure 2.8). Moreover, none of these three variant NifU-1 proteins exhibited the characteristic absorption spectrum of the Fe(III)-bound form on incubation with stoichiometric amounts of ferric ammonium citrate for 1 hr at 2°C (data not shown).

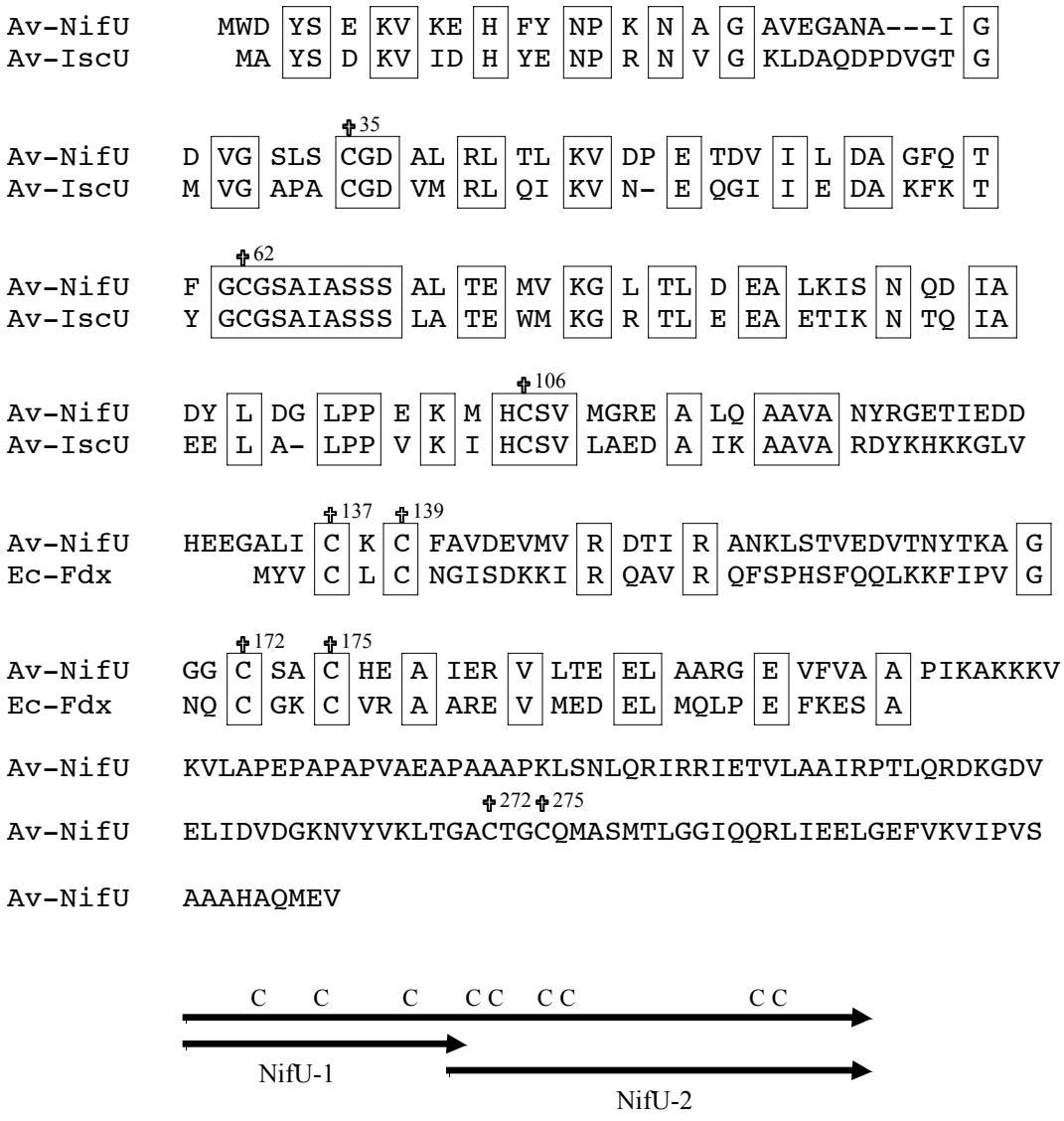

Figure 2.1: Comparison of the primary sequence of NifU from *A. vinelandii* (Av-NifU) to the sequence of IscU from *A. vinelandii* (Av-IscU) and the primary sequence of the bacterioferritin-associated ferredoxin from *E. coli* (Ec-Fdx). Conserved sequences are boxed. The nine cysteines contained within NifU are indicated by a cross above the sequence. A schematic representation of NifU and the nine cysteines contained within NifU is shown below the sequence comparisons. The segments of NifU corresponding to NifU-1 and NifU-2 are also shown.

Table 2.1: Effects of substituting NifU cysteine residues

Residue Substituted[a]	Diazotrophic Growth[b]	S =1/2 EPR Signal[c]
Cys-35	Slow	Yes
Cys-62	Slow	Yes
Cys-106	Slow	Yes
Cys-137	Slow	No
Cys-139	Slow	No
Cys-172	Slow	No
Cys-175	Slow	No
Cys-272	Normal	Yes
Cys-275	Normal	Yes

[a] All residues were substituted by alanine and, in some cases, by a variety of other amino acids. All phenotypes were the same for any substitution at a particular position.

[b] Typical diazotrophic growth rates are shown in Figure 2.3.

[c] Indicates the presence or absence of the characteristic EPR spectrum of the isolated NifU protein. This spectrum is shown in Figure 2.6.

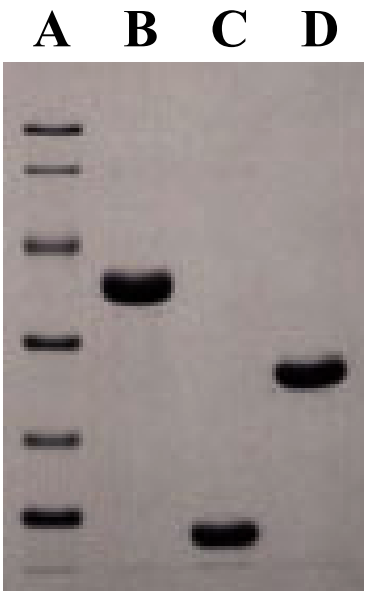

Figure 2.2: Polyacrylamide gel electrophoretic analysis of purified NifU, NifU-1, and NifU-2. **Lanes: A**, molecular weight standards from top to bottom that include phosphorylase *b* (97.4 kD), bovine serum albumin (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD); **B**, purified NifU; **C**, purified NifU-1; **D**, purified NifU-2.

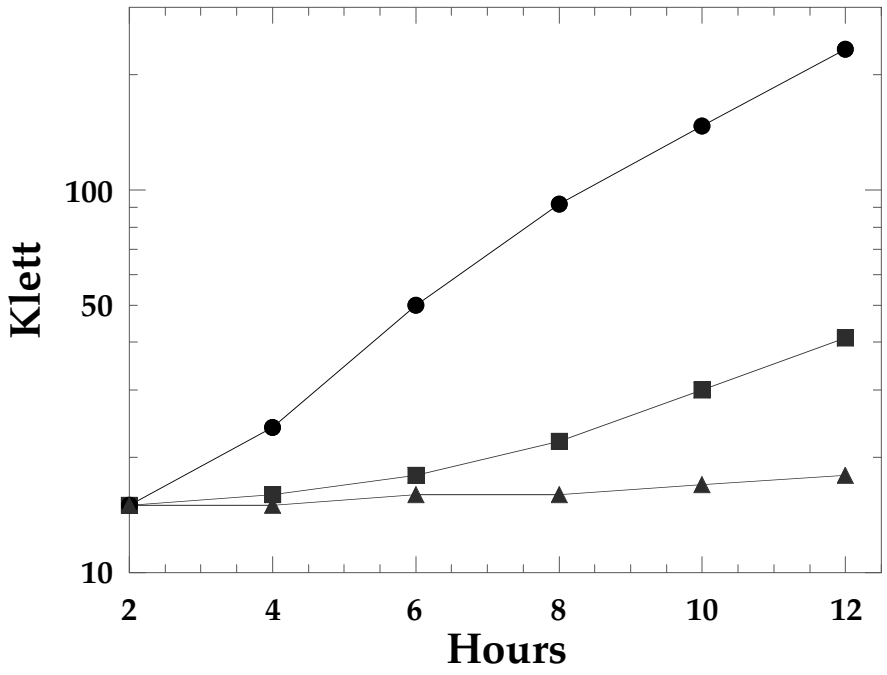

Figure 2.3: Growth of wild-type and *nifU* mutant strains of *A. vinelandii* using N_2 as the sole nitrogen source. Growth was monitored using a Summerson-Klett meter, equipped with a # 66 filter; Wild-type (●), strain DJ961 (■), strain DJ105 (▲). Strain DJ961 has the *nifU* cysteine 106 residue substituted by alanine and strain DJ105 is deleted for *nifU*.

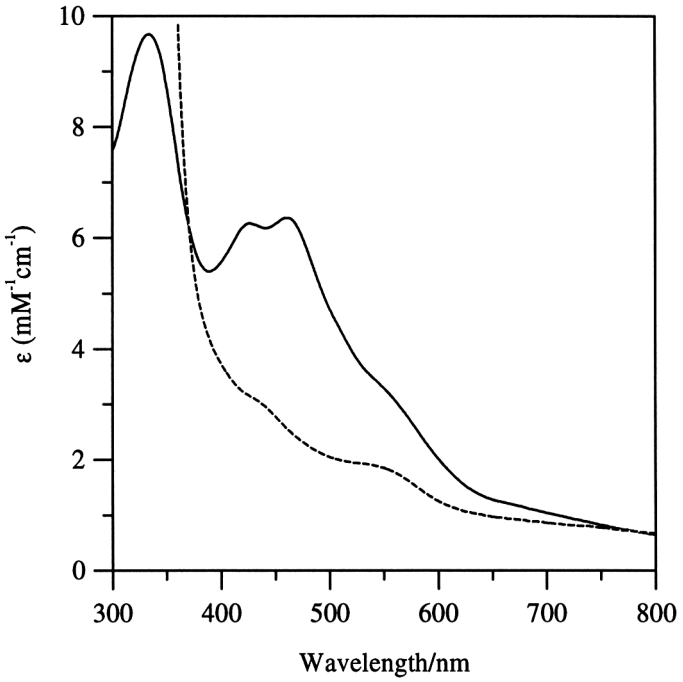

Figure 2.4: Room temperature UV-Visible absorption spectra of NifU-2. The solid line is the spectrum of NifU-2 as isolated, and the dashed line is the spectrum of NifU-2 with a 10-fold stoichiometric excess of sodium dithionite. Protein concentration was 0.21 mM, and the buffering medium was 25 mM Tris-HCl buffer, pH 7.4. The dominant absorption centered at 314 nm in the reduced spectrum results from dithionite.

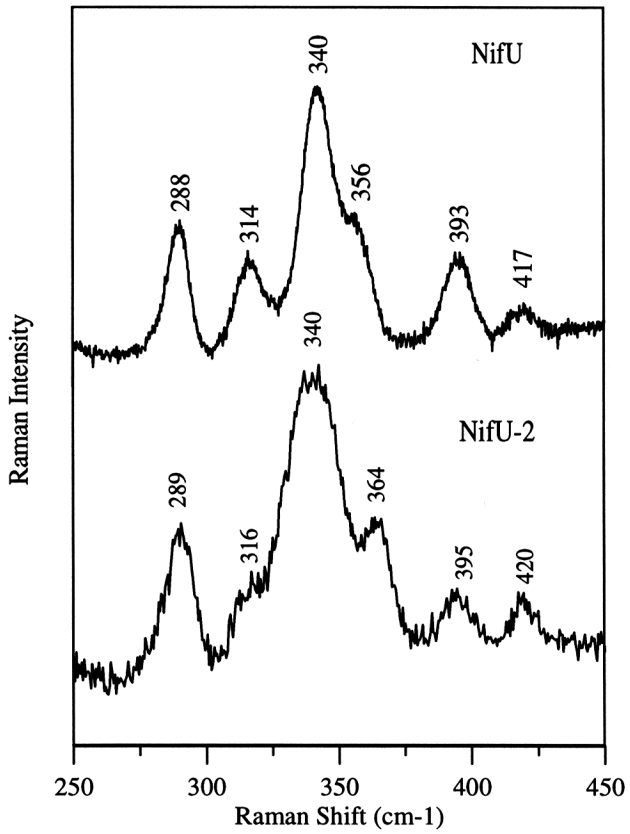

Figure 2.5: Comparison of the low-temperature resonance Raman spectra of NifU and NifU-2. Protein concentrations were ~ 1 mM, and the buffering medium was 25 mM Tris-HCl, pH 7.4. The spectra were obtained at 18 K using 457.9 nm argon laser excitation and each is the sum of 40 scans. Each scan involved advancing the spectrometer in 0.5 cm^{-1} increments and photon counting for 1 s/point with 6 cm^{-1} resolution.

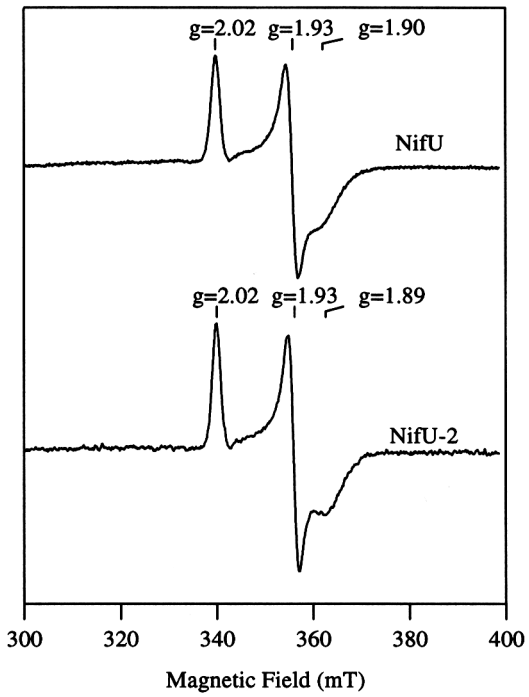

Figure 2.6: Comparison of the X-band EPR spectra of dithionite-reduced NifU and NifU-2. Conditions of measurement**:** microwave frequency, 9.60 GHz; modulation amplitude, 6.43 G; microwave power, 0.51 mW; temperature, 20 K. Protein concentrations were 0.3 mM, and the buffering medium was 25 mM Tris-HCl, pH 7.4. Simulated spectra are offset directly under each of the experimental spectra. The spectra were simulated with the following parameters**:** NifU, $g_{1,2,3} = 2.019, 1.927, 1.892$ and linewidths $l_{1,2,3} = 1.12, 1.10, 3.34$ mT; NifU-2, $g_{1,2,3} = 2.022, 1.928, 1.890$ and linewidths $l_{1,2,3} = 0.90, 0.93, 3.00$ mT.

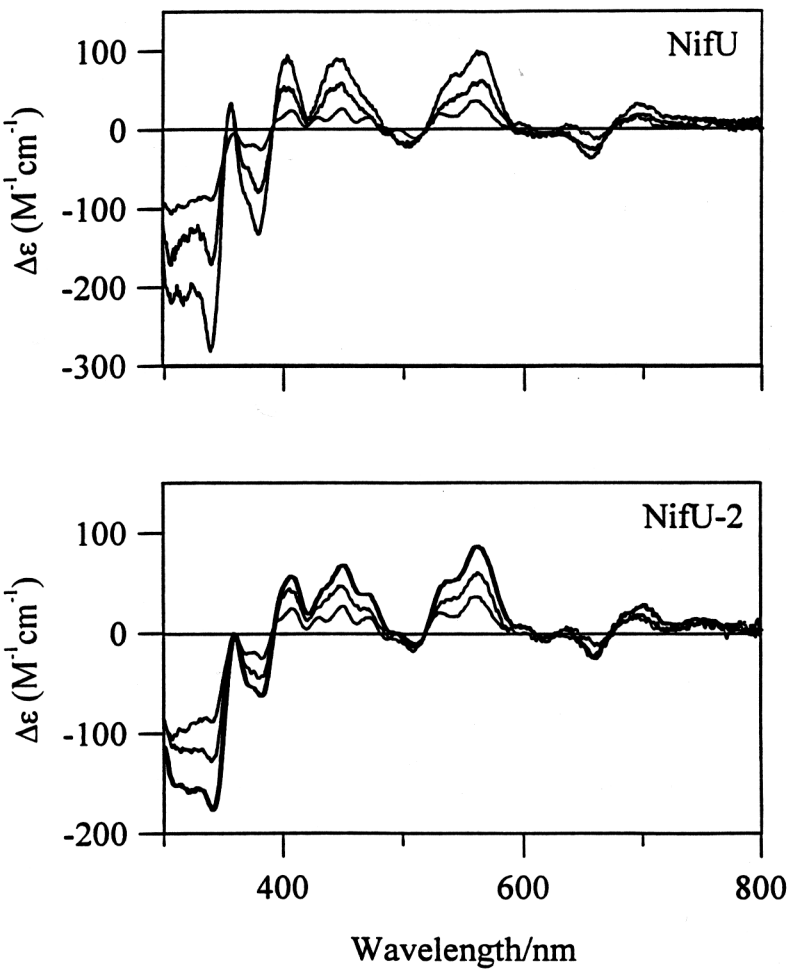

Figure 2.7: Comparison of the VTMCD spectra of dithionite-reduced NifU and NifU-2. Protein concentrations were 0.11 mM, and the buffering medium was 25 mM Tris-HCl, pH 7.4, with 55% (v/v) ethylene glycol. MCD spectra were recorded in 1-mm cuvettes with a magnetic field of 6.0 T, at 1.70, 4.22, and 10.0 K. All bands increase in intensity with decreasing temperature.

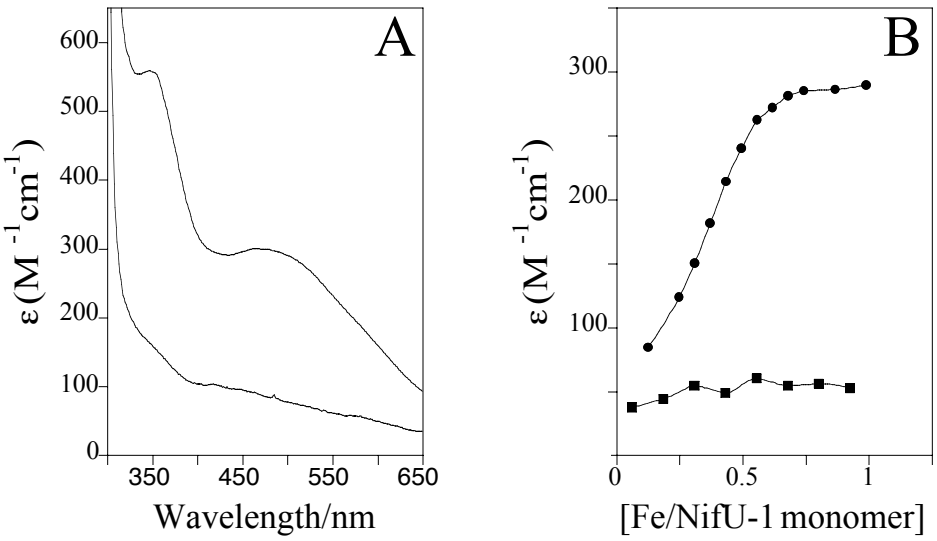

Figure 2.8: Anaerobic titrations of native NifU-1 (●) and a variant of NifU-1 in which cysteine 35 is replaced by alanine (■) versus ferric ammonium citrate concentration monitored by UV-Visible absorption spectroscopy at room temperature. The pathlength was 1 cm and the protein concentrations were 0.16 mM, with 50 mM Tris-HCl buffer, pH 7.8, containing 100 mM NaCl as the buffering medium. The left panel shows the spectra after addition of 1.0 equivalent of ferric ammonium citrate/NifU-1 monomer and the right panel shows the absorbance at 490 nm as a function the number of equivalents of ferric ammonium citrate/NifU-1 monomer.

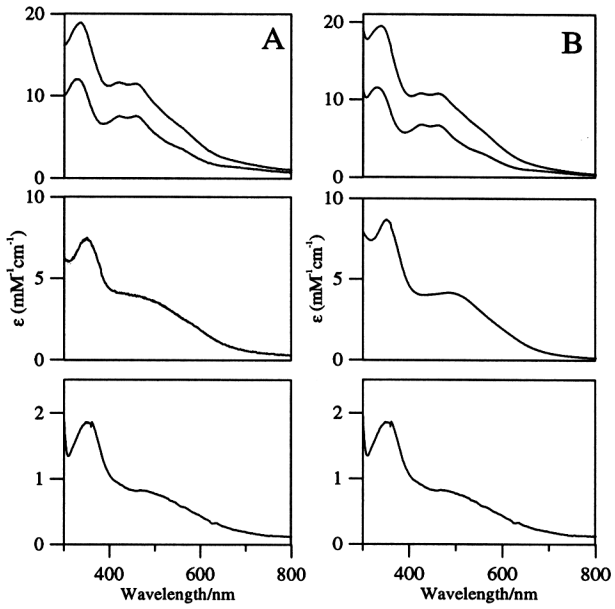

Figure 2.9: (A) Low temperature (2°C) absorption spectra of Fe(III)-bound forms of NifU and NifU-1. **Upper panel:** NifU (0.04 mM in 100 mM Tris-HCl buffer, pH 8) before (lower spectrum) and after (upper spectrum) incubation with a 1.2-fold stoichiometric excess of ferric ammonium citrate for 40 min at 2°C in a 1-cm pathlength cell. **Middle panel:** Difference spectrum of the spectra shown in the upper panel. **Lower panel:** NifU-1 (0.10 mM in 100 mM Tris-HCl buffer, pH 8) after incubation with a 1.2-fold stoichiometric excess of ferric ammonium citrate for 40 min at 2°C in a 1-cm pathlength cell. **(B)** Low temperature (2°C) absorption spectra of Fe(III)-bound forms of NifU($Asp^{37}Ala$) and NifU-1. **Upper panel:** NifU($Asp^{37}Ala$) (0.57 mM in 100 mM Tris-HCl buffer, pH 8) before (lower spectrum) and after (upper spectrum) incubation with a 1.2-fold stoichiometric excess of ferric ammonium citrate for 55 min at 2°C in a 1-mm pathlength cell. **Middle panel:** Difference spectrum of the spectra shown in the upper panel. **Lower panel:** As lower panel in A.

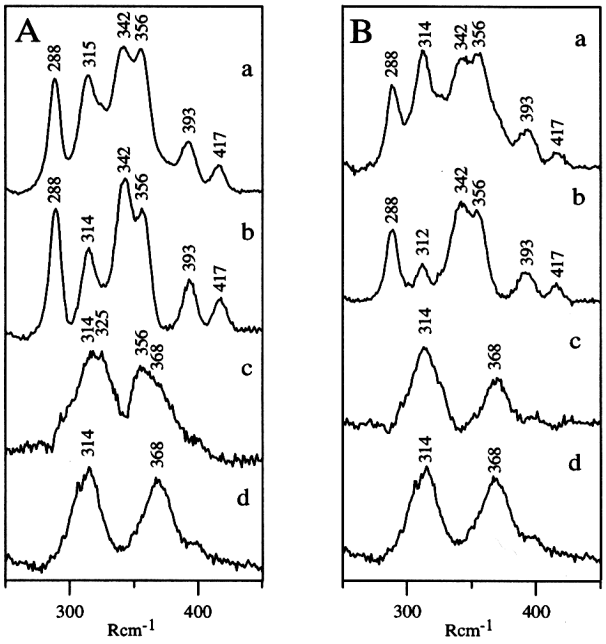

Figure 2.10: (A) Low temperature (18 K) resonance Raman spectra of Fe(III)-bound forms of NifU and NifU-1. (a) NifU incubated with a 5-fold excess of ferric ammonium citrate at 2°C for 60 min. (b) NifU as isolated. (c) Difference spectrum (a) minus (b). (d) NifU-1 incubated with a 5-fold excess of ferric ammonium citrate at 2°C for 60 min. **(B)** Low temperature resonance Raman spectra of Fe(III)-bound forms of NifU(Asp^{37}Ala) and NifU-1. (a) NifU(Asp^{37}Ala) incubated with a 5-fold excess of ferric ammonium citrate at 2°C for 60 min. (b) NifU(Asp^{37}Ala) as isolated. (c) Difference spectrum (a) minus (b). (d) NifU-1 incubated with a 5-fold excess of ferric ammonium citrate at 2°C for 60 min. The spectra in panels A and B were collected on samples (~4 mM in 100 mM Tris-HCl buffer at pH 8) maintained at 18 K. Each spectrum was recorded using 496-nm excitation with 70-mW of laser power at the sample and is the sum of 40-50 scans. Each scan involved advancing the spectrometer in 1 cm^{-1} increments and photon counting for 2 s/point with 6 cm^{-1} resolution.

E. Discussion

Spectroscopic and functional characterization of site-directed variants of NifU in which each of the nine cysteine residues have been targeted individually, and of heterologously expressed N-terminal and C-terminal fragments of NifU containing the first three and last six cysteine residues, respectively, have shown that NifU is a modular protein with at least two distinct domains. The C-terminal half, NifU-2, contains a [2Fe-2S]$^{2+,+}$ cluster coordinated by residues Cys^{137}, Cys^{139}, Cys^{172}, and Cys^{175}, as evidenced by the observation that the cluster is not assembled in variants in which any one of these cysteines residues is substituted by alanine. The important role of the NifU [2Fe-2S]$^{2+,+}$ cluster in nitrogenase [Fe-S] cluster assembly is demonstrated by the low diazotrophic growth rates and specific activities of the nitrogenase Fe-protein and MoFe-proteins in strains grown with variant NifU proteins that are deficient in this cluster. Substitutions for either of the cysteines closest to the C-terminus, Cys^{272} or Cys^{275}, did not alter the spectroscopic properties of the indigenous [2Fe-2S]$^{2+,+}$ cluster, and produced no effect on diazotrophic growth or the ability of the NifU/NifS system to assemble [Fe-S] clusters in the nitrogenase Fe-protein and MoFe-protein. A specific role for these two cysteines in NifU remains to be determined.

The ability of NifU-2 to assemble a [2Fe-2S]$^{2+,+}$ cluster with spectroscopic and redox properties almost identical to that of the holoprotein, coupled with the evidence that NifU-1 and NifU are capable of binding Fe(III) ion at a similar rubredoxin-like site, is consistent with NifU having a modular nature in which the C-terminal (NifU-2) domain can function separately from the N-terminal (NifU-1) domain, as previously suggested by Hwang *et al*. (19). Moreover, there are examples of NifU-2-like proteins,

such as the bacterioferritin-associated ferredoxin from *E. coli* (13, 27), which have the same arrangement of cluster-ligating cysteines as NifU, but lack the three N-terminal and two C-terminal cysteines (Figure 2.1). Bacterioferritin-associated ferredoxin contains a $[2Fe\text{-}2S]^{2+,+}$ cluster with UV-Visible absorption, RR, VTMCD, EPR, and redox properties very similar to those of the equivalent cluster in NifU and NifU-2 (13, 27). It has been suggested, although not yet proven, that bacterioferritin-associated ferredoxin plays a crucial redox role in mediating the release or uptake of iron from bacterioferritin (27). In light of the nearly identical properties of NifU-2 and bacterioferritin-associated ferredoxin, their respective functions are likely to be similar. Hence a possible role for the indigenous [2Fe-2S] cluster in NifU lies in redox chemistry related to the acquisition of iron for [Fe-S] cluster formation. Alternatively, the [2Fe-2S] cluster in NifU may play a redox role in the release of bound Fe(III) ion from the NifU-1 domain and/or the formation/release of an [Fe-S] cluster intermediate from the NifU-1 domain. Defining more precisely the role of the [2Fe-2S] cluster in NifU is a major goal of our ongoing research into [Fe-S] cluster assembly.

By working at low temperatures in order to decrease the lability of bound iron and by utilizing NifU($Asp^{37}Ala$) that is less susceptible to aggregation in the absence of DTT, both NifU-1 and NifU have been shown to be capable of binding a mononuclear Fe(III) ion in an analogous rubredoxin-like environment by using the combination of optical absorption and RR spectroscopies. The lability of the iron bound at this site inhibits accurate assessment of the binding stoichiometry. Nevertheless, the RR spectra are best interpreted in terms of an approximately tetrahedral site involving three rather than four cysteinate ligands, and the maximal visible extinction coefficients are comparable to

those observed for rubredoxin variants with three cysteine and one oxygenic ligand. Hence we conclude that up to one Fe(III) ion can be bound per monomer in a site with three cysteine ligands. The nature of the fourth ligand is undetermined at present, and water or a protein based oxygenic or nitrogenous ligands are all viable candidates. Compelling evidence that all three of the cysteines in the NifU-1 domain are involved in iron binding and [Fe-S] cluster assembly of NifU is provided by amino acid substitution results. Individual Cys-to-Ala substitutions involving each of the three N-terminal cysteines in NifU, Cys^{35}, Cys^{62}, and Cys^{106}, show that all three are essential for optimal diazotrophic growth rates, producing nitrogenase component proteins with optimal activities, and binding Fe(III) ion in a labile rubredoxin-type site in the NifU-1 domain. Moreover, the NifU-1 domain is very similar in size and shows a high degree of primary sequence identity when compared to the IscU proteins that are widespread in prokaryotic and eukaryotic organisms (19), and are proposed to have housekeeping roles in the general mobilization of iron for [Fe-S] cluster formation (36), see Figure 2.1.

The ability of NifU to transiently bind Fe(III) ion provides the first direct evidence in support of the hypothesis that NifU is involved with the mobilization of iron for [Fe-S] cluster assembly. However, many questions still remain concerning the function of NifU. Of paramount importance is the question of whether NifU provides an iron or [Fe-S] cluster delivery system. Binding iron to NifU may be the precursor to assembly of a transient [Fe-S] cluster in the presence of NifS which is then transferred intact to an apoprotein. Indeed we have recently been successful in obtaining optical absorption and RR evidence for the assembly of a labile $[2Fe\text{-}2S]^{2+}$ in NifU-1, NifU-1($Asp^{37}Ala$), and NifU($Asp^{37}Ala$) in the presence of Fe(III) ion, L-cysteine, and catalytic amounts of NifS

(see Chapter III). Hence the notion of NifU as a scaffold for assembly of a [2Fe-2S] unit that can be inserted intact into an apoprotein is a viable hypothesis. The possibility that it is capable of performing either iron or [Fe-S] delivery roles as the need dictates, must also be considered. For both processes the question of the mechanism of release of the transiently bound iron or [Fe-S] cluster fragment has yet to be addressed. Reductive release mediated by the indigenous $[2Fe\text{-}2S]^{2+,+}$ cluster is an attractive hypothesis in light of complete absence of spectroscopic evidence for a cysteinyl-ligated Fe(II) site in the reduced NifU-1.

In summary, site-directed mutagenesis and gene replacement techniques have been used to determine that NifU cysteine residues Cys^{35}, Cys^{62}, Cys^{106}, Cys^{137}, Cys^{139}, Cys^{172}, and Cys^{175} are all necessary for full physiological function. Residues Cys^{137}, Cys^{139}, Cys^{172}, and Cys^{175} have been identified as the [2Fe-2S] cluster ligands and residues Cys^{35}, Cys^{62}, and Cys^{106} have been shown to be necessary for binding iron at a second site within NifU. These results, and the lability of iron binding at the second site, are consistent with a model where iron destined for [Fe-S] cluster formation is transiently bound at this site.

F. Acknowledgment

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CHAPTER III

NifS-DIRECTED ASSEMBLY OF A TRANSIENT [2Fe-2S] CLUSTER WITHIN THE NifU PROTEIN

This chapter was authored by Pramvadee Yuvaniyama[♥], Jeffrey N. Agar[✚], Valerie L. Cash[♥], Michael K. Johnson[✚], and Dennis R. Dean[♥]. It has been accepted for publication in the Proceedings of the National Academy of Sciences USA (PNAS) in November, 1999. In Dennis Dean's laboratory, I was involved in all protein purification and UV-Visible characterization in the experiments. I also determined the interaction between NifS and NifU. Site-directed mutagenesis on *nifU-1* variants was performed by Valerie Cash. In the laboratory of Michael Johnson, experiments using RR, EPR, and MCD spectroscopy were investigated by Jeffrey Agar.

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A. Abstract

The NifS and NifU proteins from *Azotobacter vinelandii* are required for the full activation of nitrogenase. NifS is a homodimeric cysteine desulfurase that supplies the inorganic sulfide necessary for formation of the [Fe-S] clusters contained within the nitrogenase component proteins. NifU has been suggested to have a complementary role to NifS either by mobilizing the iron necessary for nitrogenase [Fe-S] cluster formation or by providing an intermediate [Fe-S] cluster assembly site. As isolated, the homodimeric NifU protein contains one $[2Fe\text{-}2S]^{2+,+}$ cluster per subunit, that is referred to as the "permanent" cluster. In the present work it is shown that NifU is able to interact with NifS and that a second, "transient" [2Fe-2S] cluster can be assembled within NifU *in vitro* when incubated in the presence of ferric ion, L-cysteine, and catalytic amounts of NifS. Approximately one transient [2Fe-2S] cluster is assembled per homodimer. The transient [2Fe-2S] cluster species is labile and rapidly released upon reduction. It is proposed that transient [2Fe-2S] cluster units are formed on NifU and then released to supply the inorganic iron and sulfur necessary for maturation of the nitrogenase component proteins. The role of the permanent [2Fe-2S] clusters contained within NifU is not yet known but they could have a redox function involving either the formation or release of transient [2Fe-2S] cluster units assembled on NifU. Because homologs to both NifU and NifS, respectively designated IscU and IscS, are found in non-nitrogen fixing organisms, it is possible that the function of NifU proposed here could represent a general mechanism for the maturation of [Fe-S] cluster-containing proteins.

B. Introduction

Iron-sulfur clusters are found in numerous proteins that have important redox, catalytic, or regulatory properties (for a recent review see (2)). Moreover, [Fe-S] clusters are intimately involved in the respective functions of these proteins. For example, [Fe-S] clusters are known to act as electron carriers, environmental sensors, or to be involved in substrate binding and activation. Advances in our understanding of the structures, organization, and reactivity of certain biologically relevant [Fe-S] clusters have involved determination of the spectroscopic and electronic properties of protein-bound [Fe-S] clusters, characterization of clusters chemically extruded from their polypeptide matrices, and preparation of synthetic [Fe-S] clusters. Until recently, however, the biological mechanism by which [Fe-S] clusters are formed has received scant attention. It was shown many years ago that [Fe-S] clusters could be spontaneously incorporated into apo-forms of certain ferredoxins by simply incubating them *in vitro* in a solution that contains iron and sulfide (15). However, considering the toxicity of free iron and sulfide, it is unlikely that protein-bound [Fe-S] clusters are spontaneously formed *in vivo* from free iron and sulfide. It is more likely that the iron and sulfur necessary for [Fe-S] cluster formation is delivered to the cluster assembly site by intermediate carrier proteins.

Previous work has led to the proposal that the NifU and NifS nitrogen fixation-specific gene products are involved in the acquisition of iron and sulfur necessary for the maturation of the two nitrogenase component proteins, both of which contain [Fe-S] clusters (5). It was shown that NifS is a pyridoxal phosphate-dependent L-cysteine desulfurase and that an enzyme-bound persulfide is an intermediate in that reaction (23, 24). Thus, NifS has been targeted as the source of inorganic sulfide necessary for

nitrogenase [Fe-S] cluster formation. We have recently found that NifU is a modular protein having two distinct types of iron-binding sites (see Figure 3.1). One of these binding site types is located within the central third of the NifU primary sequence (6), (see also Chapter II) and binds a $[2Fe\text{-}2S]^{2+,+}$ cluster (one cluster binding site per subunit). The second type of site is a labile mononuclear iron-binding site (see Chapter II) located within the N-terminal third of the NifU primary sequence (one mononuclear site per subunit). Because the $[2Fe\text{-}2S]^{2+,+}$ clusters present in as-isolated NifU are tightly bound to the protein (6), the labile mononuclear site is the likely source of iron for nitrogenase [Fe-S] cluster assembly. If NifS and NifU do have complementary functions in the mobilization of sulfur and iron for nitrogenase [Fe-S] cluster assembly, several different pathways for this process can be considered. For example, NifS and NifU could either operate independently from each other during [Fe-S] cluster formation or they could function together. If NifU and NifS function together, then iron and sulfur could be separately released from each of them during cluster assembly or an [Fe-S] cluster precursor could be preformed and then released. In the present work we have addressed these issues by asking if NifU and NifS are able to form a macromolecular complex and whether or not a labile [Fe-S] cluster species can be formed on NifU.

C. Experimental Procedures

Plasmids and Strains

Construction of plasmids used for the heterologous expression of altered forms of NifU in *Escherichia coli* were previously described (see Chapter II). Plasmid pDB822 was used to express a full-length version of NifU for which the Cys^{137} residue is

substituted by Ala. This form of NifU is designated NifU(Cys^{137}Ala). Plasmid pDB1041 was used to express a full-length version of NifU for which the Asp^{37} residue was substituted by Ala (designated NifU(Asp^{37}Ala). Plasmid pDB937 was used to express a truncated form of NifU that includes the first 131 residues of NifU. This truncated form of NifU is designated NifU-1. Plasmid pDB1044 was obtained by the oligonucleotide-directed mutagenesis of pDB937. This plasmid respectively expresses a form of NifU-1 that has the Asp^{37} residue substituted by Ala and is designated NifU-1(Asp^{37}Ala). A schematic representation of NifU and different forms of NifU used in the present work is shown in Figure 3.1.

Biochemical manipulations

The purification of NifS and altered forms of NifU was performed as previously described (24); (see also Chapter II). The optimized *in vitro* [Fe-S] cluster biosynthetic system includes the following: 0.05 mM NifU-1 dimer (or other form of NifU-1 or NifU), 0.1 mM ferric ammonium citrate, 5 mM β-mercaptoethanol, 1 mM L-cysteine, 1 μM NifS, and 0.1 M NaCl in a 25 mM Tris-HCl pH 7.4 buffer. All volumes were 1.0 ml and reactions were carried out anoxically in a septum-sealed cuvette under an argon atmosphere. Protein samples were purified under argon and buffers used were extensively degassed and sparged with argon prior to use. Anoxic conditions were maintained using a Schlenk apparatus and/or an anaerobic glove box. Reactions were initiated by the addition of L-cysteine and were monitored by UV-Visible spectroscopy as described below.

NifU and NifS complex formation was monitored by gel-exclusion HPLC (Beckman System Gold) column chromatography using a Zorbax GF-250 column.

Volumes of 100 μl containing 6 nmoles of NifU or NifS (or 6 nmoles of NifU plus 6 nmoles of NifS) in a 25 mM Tris-HCl pH 7.4 buffer containing 20 mM NaCl and 1.0 mM dithiothreitol were injected onto the column. Samples that contained a mixture of NifU and NifS were pre-incubated at room temperature for 8 min prior to loading the column. For sample mixtures where a molar excess of NifU and NifS was used, the sample contained approximately 6 nmoles of one protein and 12 nmoles of the other. Elution of the protein samples was monitored by visible absorbance at 405 nm. Results obtained using the Beckman System Gold HPLC were also independently confirmed using a Pharmacia FPLC chromatography system fitted with a Superose 12 column.

Spectroscopic methods

All sample concentrations are based on protein determinations and are expressed per NifU or NifU-1 monomer. UV-Visible absorption spectra were recorded under anoxic conditions in septum-sealed 1 mm and 1 cm cuvettes, using a Shimadzu 3101PC scanning spectrophotometer or a Cary diode array spectrophotometer. X-band (~ 9.6 GHz) EPR spectra were recorded using a Bruker ESP-300E EPR spectrometer equipped with a dual-mode ER-4116 cavity and an Oxford Instruments ESR-9 flow cryostat.

Resonance Raman spectra were recorded using an Instruments SA Ramanor U1000 spectrometer fitted with a cooled RCA 31034 photomultiplier tube using 90° scattering geometry and lines from a Coherent Sabre10-W argon ion laser. Spectra were recorded digitally using photon-counting electronics, and signal/noise was improved by signal averaging multiple scans. Band positions were calibrated using the excitation frequency and CCl_4 and are accurate to +/- 1 cm^{-1}. Samples consisted of a 12-μl droplet of concentrated protein (2-4 mM) that was placed in a custom designed sample cell

attached to the cold finger of an Air Products Displex model CSA-202E closed-cycle refrigerator. The sample temperature was maintained at 18 K during scanning in order to minimize laser-induced sample degradation. Bands due to the frozen buffer solutions have been subtracted from all the spectra shown in this work after normalization of lattice modes of ice centered at 229 cm^{-1}.

D. Results

NifU and NifS complex formation

That NifU and NifS do not form a tight complex was determined in two different ways. First, NifU was not found to copurify with NifS when NifS was isolated from crude extracts prepared from nitrogen-fixing *A. vinelandii* cells. Second, specific immunoprecipitation of either NifU or NifS from *A. vinelandii* crude extracts did not result in the coprecipitation of the complementary protein. However, NifU and NifS are able to form a transient complex because an equimolar mixture of NifU and NifS results in the appearance of a new peak during size-exclusion column chromatography when compared to individually chromatographed samples of either NifU or NifS (Figure 3.2). Calibration of the column and denaturing gel electrophoresis of the peak fraction of the NifU-NifS complex indicates formation of a heterotetrameric complex. When a 2-fold molar excess of NifU was mixed with NifS, a peak corresponding to the NifU-NifS complex and a peak corresponding to uncomplexed NifU could be resolved by gel-exclusion chromatography. The converse experiment involving the addition of a 2-fold molar excess of NifS also resulted in the appearance of two peaks during chromatography of the sample, one corresponding to NifS and the other corresponding to the NifU-NifS

complex. The shoulder recognized in the NifU-NifS complex fraction shown in Figure 3.2 is reproducible and probably reflects that dissociation of the complex occurs during chromatography.

Experimental rationale

In previous work we found that seven cysteines contained within NifU are required for its full *in vivo* function (see Chapter II). Four of these cysteines (Cys^{137}, Cys^{139}, Cys^{172}, and Cys^{175}) are contained within a central segment of NifU and provide the ligands for the [2Fe-2S] cluster present in each monomer of the dimeric as-isolated NifU protein. Because the [2Fe-2S] clusters contained within as-isolated NifU protein are tightly bound and cannot be released by chelating reagents, we refer to them as the "permanent" clusters and consider it unlikely that they represent precursors destined for assembly of the nitrogenase metalloclusters. We therefore became interested in asking if a second, more labile cluster might be assembled elsewhere on NifU. The three other cysteines (Cys^{35}, Cys^{62}, and Cys^{106}) required for the full *in vivo* NifU function were targeted as the most likely residues to participate in providing such an assembly site for formation of a "transient" cluster. The possibility for assembly of a second [Fe-S] cluster on NifU was also considered because we recently obtained evidence for the presence of one mononuclear iron-binding site within each subunit of the as-isolated NifU protein (see Chapter II). Thus, we considered the possibility that binding of iron at the mononuclear site could represent an intermediate stage in the assembly of a transient [Fe-S] cluster on NifU. Binding of iron at the mononuclear site(s) requires residues Cys^{35}, Cys^{62}, and Cys^{106}.

Our ability to test whether or not a transient [Fe-S] cluster could be assembled on NifU was complicated by the presence of one permanent [2Fe-2S] cluster in each monomer of intact as-isolated NifU. This problem was circumvented in two different ways. First, a portion of NifU that corresponds only to the N-terminal third of the NifU coding sequence was recombinantly expressed and then isolated. This truncated form of NifU is referred to as NifU-1 and it does not include those cysteines that provide the coordinating ligands to the permanent [2Fe-2S] cluster contained within full-length NifU. Thus, the as-isolated form of NifU-1, which is a homodimer, does not contain the permanent [2Fe-2S] clusters contained within full-length NifU (see Figure 3.1). Second, a full-length form of altered NifU that carries an alanine substitution for one of the [2Fe-2S] cluster coordinating residues (residue Cys^{137}) was also recombinantly produced and isolated. This altered NifU protein is referred to as NifU(Cys^{137}Ala) and also does not contain the permanent [2Fe-2S] cluster in its as-isolated form (Figure 3.1). Purified samples of NifU-1 and NifU(Cys^{137}Ala) were then used in experiments described below which demonstrate that they can serve as scaffolds for NifS-catalyzed formation of [2Fe-2S]$^{2+}$ clusters.

NifU-1 and NifU(Ala^{137}) can serve as scaffolds for assembly of a [2Fe-2S]$^{2+}$ cluster.

It was possible to develop an optimized *in vitro* [Fe-S] cluster biosynthetic system that includes 0.05 mM NifU-1 dimer or NifU(Cys^{137}Ala), 0.1 mM ferric ammonium citrate, 5 mM β-mercaptoethanol, 1 mM L-cysteine, and 1 μM NifS. In this biosynthetic system, NifS is present at only very low levels when compared to NifU-1 or NifU(Cys^{137}Ala). One reason for performing the biosynthetic assay in this way was to ensure that the pyridoxal-phosphate chromophore present in NifS would not interfere

with the ability to detect [Fe-S] cluster formation using UV-Visible absorption and resonance Raman spectroscopies. Also, by using only catalytic amounts of NifS it was possible to monitor the time-dependent formation of the [Fe-S] clusters. A typical experiment in which NifU-1 was used as a scaffold for [Fe-S] cluster assembly is shown in Panels A & B of Figure 3.3. These data show that there is a time-dependent assembly of a chromophoric species in NifU-1 that exhibits absorbance inflections at 325, 420, 465, and 550 nm (Figure 3.3, Panel A). This UV-Visible absorption spectrum is characteristic of $[2Fe\text{-}2S]^{2+}$ cluster-containing proteins (4) although the absorption peaks are less well defined in our sample when compared to similar spectra from typical $[2Fe\text{-}2S]^{2+}$ cluster-containing proteins. The most likely explanation for the relatively featureless nature of the absorption spectrum is that, once formed, the $[2Fe\text{-}2S]^{2+}$ cluster is relatively unstable at ambient temperature. This instability is manifested by a concomitant accumulation of a colloidal precipitate of iron sulfide that is responsible for the spectral inflections becoming increasingly less well defined upon incubation of the sample beyond about 200 minutes. A colloidal iron-sulfide precipitate also accumulates when NifU-1 is omitted from the reaction mixture, but this occurs at a much slower rate than the rate observed for [Fe-S] cluster biosynthesis in the complete system.

Panel B in Figure 3.3 (curves a & b) shows the time-dependence of [2Fe-2S] cluster assembly on the NifU-1 scaffold. These data also show that cluster formation is dependent on the concentration of NifS with an approximate doubling in the rate of cluster formation when the amount of NifS in the reaction mixture is doubled. The results of control experiments presented in Panel B of Figure 3.3 also show that an altered form of NifS, for which the active site Cys^{325} residue was substituted by alanine (23), is

not active in cluster formation. Altered forms of NifU-1, in which any one of the three cysteine residues (Cys^{35}, Cys^{62}, and Cys^{106}) has been substituted by alanine, were also ineffective in cluster assembly. Finally, no [2Fe-2S] cluster formation occurred if any of the above-mentioned components of the biosynthetic cocktail were omitted. When NifU(Cys^{137}Ala) was substituted for NifU-1 in the assembly mixture, a spectrum having a very similar lineshape and intensity and the same absorbance maxima were observed (data not shown).

Isolation of a NifU-1 variant that contains a stabilized $[2Fe\text{-}2S]^{2+}$ cluster

The $[2Fe\text{-}2S]^{2+}$ cluster assembled onto NifU-1 or NifU(Cys^{137}Ala) is labile *in vitro*. For example, as judged by A_{420}/A_{280} ratios, more than 50% of the cluster was lost when gel-filtration was used to remove excess reagents. Such lability (also see below) was not unexpected considering that the proposed physiological function of the cluster is to supply the iron and sulfur necessary for nitrogenase metallocluster assembly. In other words, the transient [2Fe-2S] cluster must have a mechanism to escape from the NifU scaffold during the maturation of the nitrogenase component proteins and therefore should not be tightly bound to NifU. During the course of our studies using altered forms of NifU-1 as an approach to examine the nature of the mononuclear iron-binding site, we fortuitously identified an altered form of NifU-1 that contains some $[2Fe\text{-}2S]^{2+}$ cluster in its as-isolated state (Panel C, Figure 3.3). This altered form of NifU-1 has the Asp^{37} residue substituted by alanine and is referred to as NifU-1(Asp^{37}Ala). The as-isolated form of NifU-1(Asp^{37}Ala) contained approximately 0.15 Fe per monomer. Moreover, the $[2Fe\text{-}2S]^{2+}$ cluster contained within as-isolated NifU-1(Asp^{37}Ala) is stable with no loss or change in the UV-Visible spectrum even after incubation at ambient temperature for 12

hours. Stabilization of an [Fe-S] cluster resulting from an amino acid substitution has precedence in the case of the FNR protein from *E. coli*. In this case a variant form was identified that contains a more stable [4Fe-4S] cluster and is affected in signal transduction events dependent upon cluster assembly and disassembly (12).

When NifU-1($Asp^{37}Ala$) was used as a scaffold for cluster assembly (Panel C, Figure 3.3) a three-fold increase in visible absorption intensity over a period of 200 mins was observed. The resulting spectrum is characteristic of a biological $[2Fe\text{-}2S]^{2+}$ center and, in accord with the iron analyses of the as-isolated sample, the extinction coefficients (e.g. $\varepsilon_{420} = 3.0\ mM^{-1}cm^{-1}$) are indicative of approximately 0.5 clusters per monomer or 1.0 cluster per homodimer. This conclusion is based on the range of extinction coefficients for typical $[2Fe\text{-}2S]^{2+}$ proteins ($\varepsilon_{420} = 6\text{-}11\ mM^{-1}cm^{-1}$ (4)). Analogous biosynthetic cluster reconstitution experiments were also carried out with a full-length form of NifU that has the Asp^{37} residue substituted by alanine (designated NifU($Asp^{37}Ala$)). The resulting UV-Visible absorption spectrum was indistinguishable from that of the starting spectrum originating from the permanent $[2Fe\text{-}2S]^{2+}$ clusters, except for a uniform 30% increase in absorption intensity in the 300-800 nm region. This result is consistent with the formation of about one additional $[2Fe\text{-}2S]^{2+}$ cluster per homodimer (data not shown). It should be noted that in the biosynthetic system described here, only one ferric ion is added per protein monomer. Consequently, no more than one $[2Fe\text{-}2S]^{2+}$ cluster could be formed per homodimer. Nevertheless, during the development of the synthetic system, we found that the addition of higher levels of ferric ion did not increase the amount of transient cluster accumulated when any of the various forms of NifU were used in the assembly cocktail. The main consequence of a 2-fold or

5-fold increase in the ferric ammonium citrate concentration was to decrease the quality of the absorption data due to the increase in the accumulation of colloidal iron sulfide.

Resonance Raman evidence for assembly of the transient [2Fe-2S]$^{2+}$ cluster

Resonance Raman spectroscopy was used to identify and further characterize the transient [2Fe-2S]$^{2+}$ clusters present in the various forms of NifU proteins investigated in this work. A comparison of the low-temperature resonance Raman spectra of the permanent [2Fe-2S]$^{2+}$ clusters in NifU and of the transient [2Fe-2S]$^{2+}$ assembled in NifU-1 using 488-nm excitation is shown in Figure 3.4 A & B. In both spectra, the pattern and the frequency of bands in the Fe-S stretching region are uniquely indicative of [2Fe-2S]$^{2+}$ clusters (3, 6, 8, 9). The relative intensities of equivalent bands are remarkably similar for both clusters, but the frequencies are all up-shifted by 6-8 cm^{-1} for the transient [2Fe-2S]$^{2+}$ cluster in NifU-1 when compared to the permanent clusters in NifU. Hence, the vibrational assignments made for the permanent clusters in NifU (6) can be transferred directly to the transient cluster. Also, the relative intensities and frequencies of the bands in NifU-1 are almost identical to those of the [2Fe-2S]$^{2+}$ cluster in human ferrochelatase (3) (Figure 3.4), which has recently shown to have complete cysteinyl ligation on the basis of amino acid substitution (18) and crystallographic studies (H. A. Dailey, personal communication). This result strongly suggests that the cluster assembled on NifU-1 has complete cysteinyl ligation. Because there are only three cysteines available in each monomer, this situation indicates that the transient cluster is most likely bridged between the subunits. The higher Fe-S stretching frequencies for the transient cluster in NifU-1 compared to the permanent clusters in NifU indicate stronger Fe-S bonds for the transient structure. Thus, the lability of the transient cluster is more likely to be a consequence of

enhanced solvent accessibility resulting from its location at the subunit interface, rather than from an intrinsic thermodynamic instability.

Although the lability of the transient $[2Fe\text{-}2S]^{2+}$ cluster has thus far impeded our attempts to obtain resonance Raman spectra from reconstituted full-length NifU, the decreased lability in NifU($Asp^{37}Ala$) has provided an opportunity to assess the resonance Raman spectrum of the cluster in a full-length version of NifU (Figure 3.5). The spectrum of the reconstituted form of NifU($Asp^{37}Ala$) [Figure 3.5 (a)] is clearly dominated by the permanent clusters of as-isolated NifU [Figure 3.5 (b)]. Nevertheless, the difference between the reconstituted and as-isolated data sets [Figure 3.5 (c)] reveals a spectrum very similar to that of the reconstituted $[2Fe\text{-}2S]^{2+}$ cluster in NifU-1($Asp^{37}Ala$) [Figure 3.5 (d)] and having approximately half the intensity of the permanent clusters. Because Fe-S stretching frequencies are very sensitive to minor perturbations in the cluster environment, this result indicates a negligible change in the transient cluster environment on removal of the C-terminal domain. Moreover, there is a close similarity in the resonance Raman spectra of the transient clusters reconstituted in NifU-1 [Figure 3.4 (b)], NifU-1($Asp^{37}Ala$) [Figure 3.5 (d)], and NifU($Asp^{37}Ala$) [Figure 3.5 (c)]. The main differences among these species lie in the frequencies of the total symmetric predominantly Fe-S(Cys) modes assigned at 349, 337, and 340 cm^{-1} in NifU-1, NifU-1($Asp^{37}Ala$), and NifU($Asp^{37}Ala$), respectively. This variability is likely to originate from differences between the wild-type and $Asp^{37}Ala$ variant in terms of cysteinyl Fe-S-C-C dihedral angles and/or hydrogen bonding interactions involving cysteinyl-S atoms. An attractive possibility is that substitution of the Asp^{37} residue by alanine reduces cluster lability by decreasing solvent accessibility and that this feature is reflected in the

resonance Raman as a result of perturbed hydrogen-bonding interactions involving the ligating cysteine S atoms. In summary, all of the resonance Raman spectra clearly demonstrate NifS-mediated assembly of a similar transient [2Fe-2S] cluster in a variety of different forms of NifU.

Release of the [2Fe-2S] cluster upon reduction

UV-Visible spectra and/or resonance Raman spectra of the [Fe-S] clusters formed in the biosynthetic system described here provide evidence for the catalytic formation of a labile $[2Fe\text{-}2S]^{2+}$ cluster within the NifU protein. Complementary spectroscopic evidence for this conclusion could not be obtained by using either electron paramagnetic resonance or variable-temperature magnetic circular dichroism spectroscopy due to the extreme reductive lability of the cluster. For example, dithionite-mediated reduction of the transient $[2Fe\text{-}2S]^{2+}$ cluster resulted in an immediate, complete, and irreversible bleaching of the visible spectrum. Also, no paramagnetic form of the transient cluster could be trapped even by freezing the sample within 10 seconds after addition of a 2-fold excess of dithionite. The dithionite-reduced samples exhibited no $S = 1/2$ EPR signals over the temperature range 10-100 K and no temperature-dependent MCD bands, suggesting that the $[2Fe\text{-}2S]^{2+}$ form is unstable and degraded immediately upon reduction. The lability of the transient [2Fe-2S] cluster upon reduction was also shown by quantitative capture of the Fe(II) ion released upon dithionite reduction by using the Fe(II)-chelating reagent α,α' dipyridyl (see Appendix I).

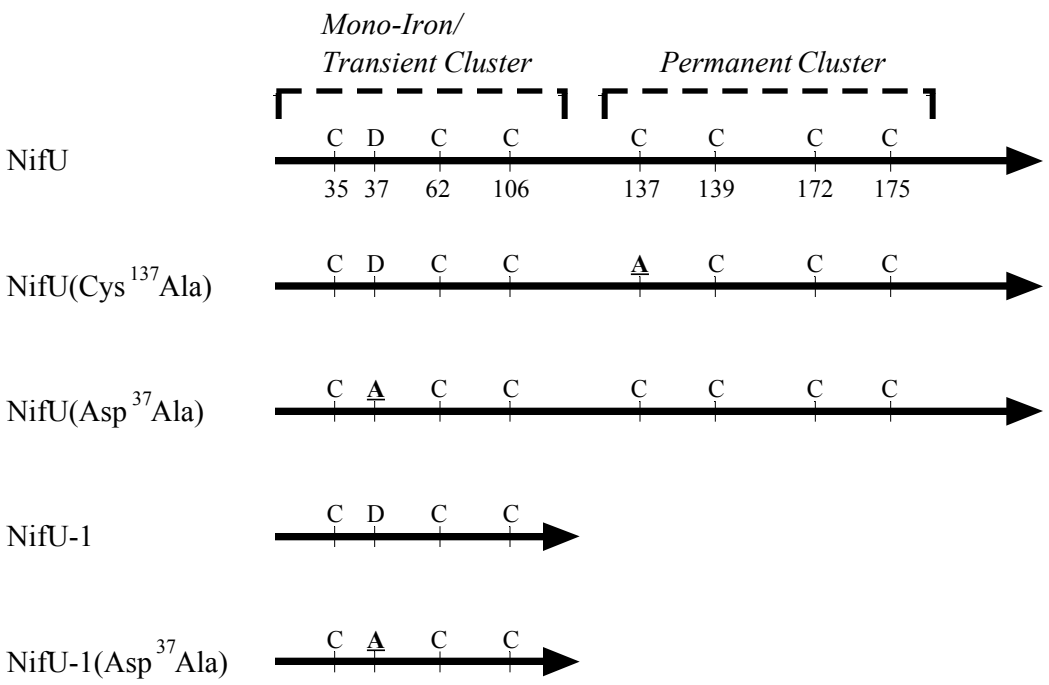

Figure 3.1: Schematic representation of NifU and different forms of NifU. The uppermost line represents full-length NifU. Above the line are indicated the cysteine (C) and aspartate (D) residues relevant to the present work. The numerical residue positions are indicated below the line. Substituting residues present in altered forms of NifU or NifU-1 are indicated by a bold and underlined letter at the appropriate position. Dashed brackets at the top of the figure indicate the respective mononuclear iron/transient [2Fe-2S] cluster domain and permanent [2Fe-2S] cluster domain within the NifU primary sequence.

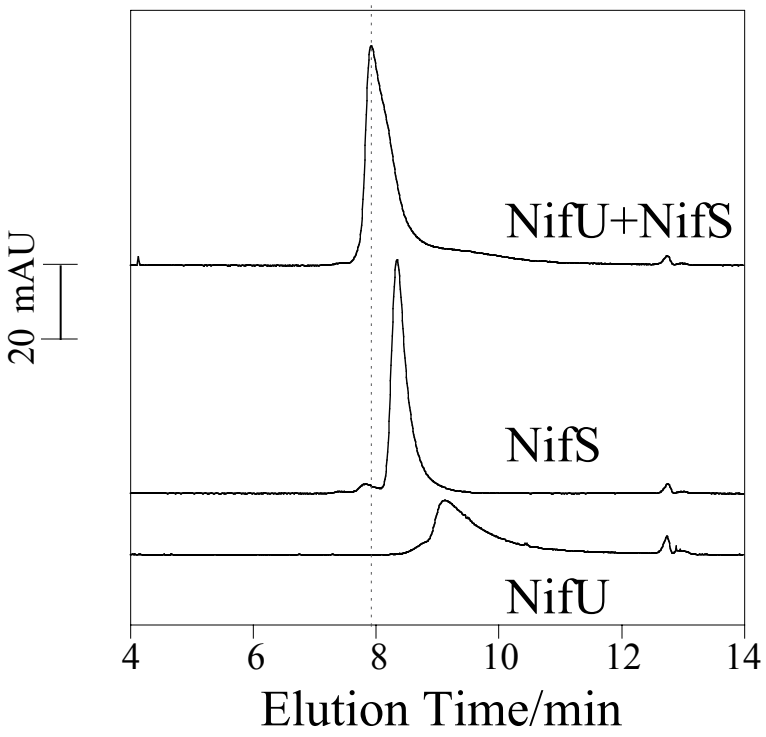

Figure 3.2: Complex formation between NifU and NifS. The figure shows the elution profiles of NifS, NifU, or an equimolar mixture of NifU and NifS using size-exclusion chromatography. Conditions used are described in Experimental Procedures.

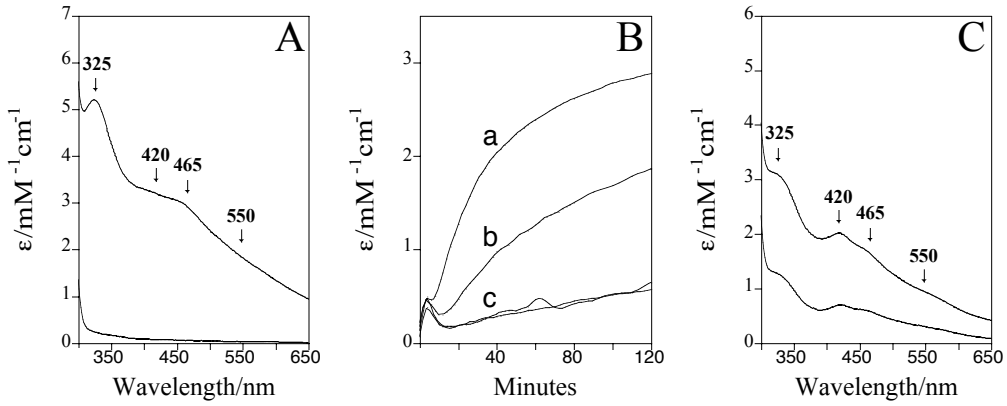

Figure 3.3: NifS-dependent *in vitro* [Fe-S] cluster assembly. **Panel A** represents the UV-Visible spectrum of NifU-1 prior to *in vitro* [Fe-S] cluster assembly (before L-cysteine was added to initiate assembly, lower spectrum) and after 140 min of *in vitro* cluster assembly (upper spectrum). The post-assembly spectrum shown in Panel A is the maximum that could be obtained. **Panel C** represents the UV-Visible spectrum of NifU-1(Asp^{37}Ala) prior to *in vitro* [Fe-S] cluster assembly (lower spectrum) and after 80 min of *in vitro* [Fe-S] cluster assembly (upper spectrum). The post-assembly spectrum shown in Panel C represents approximately 60% of the maximum that could be obtained. **Panel B** shows the time-dependence of [Fe-S] cluster assembly as monitored by the change in extinction coefficient at 465 nm versus time following initiation of the [Fe-S] cluster assembly reaction. The time-dependence for cluster assembly shown in line (a) of Panel B corresponds to the same sample shown in Panel A. Line (b) of Panel B shows cluster assembly using conditions the same as for line (a) except that half the amount of NifS was added to the assembly cocktail. Data shown in the lines labeled (c) are controls. One data set corresponds to conditions that are the same as used for line (a) except an altered form of NifS having the active site Cys^{325} residue substituted by alanine was used. The other data set in (c) corresponds to conditions that are the same as used for line (a) except an altered form of NifU-1 having the Cys^{62} residue substituted by alanine was used. Conditions for [Fe-S] cluster assembly are described in Experimental Procedures.

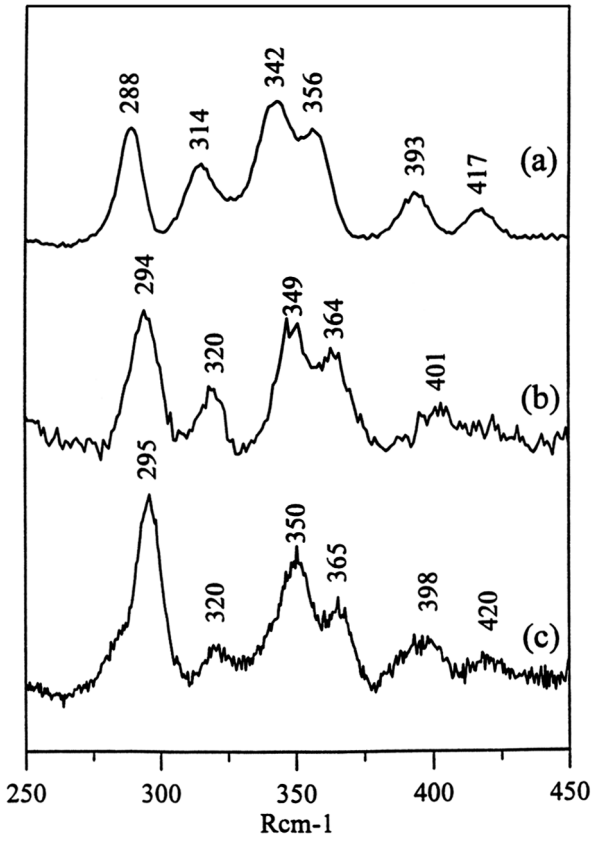

Figure 3.4: Comparison of the low-temperature resonance Raman spectra of the [2Fe-2S]$^{2+}$ clusters in as-isolated NifU (a), reconstituted NifU-1 (b), and human ferrochelatase (c). All samples (2-4 mM in 100 mM Tris-HCl, pH 7.8 buffer) were in the form of concentrated frozen droplets maintained at 18 K. The spectra were recorded using 488-nm excitation with 70-mW of laser power at the sample and are the sum of 19, 90, and 80 scans for (a), (b), and (c), respectively. Each scan involved advancing the spectrometer in 1 cm^{-1} increments (0.5 cm^{-1} for (c)) and photon counting for 1s/point with 6 cm^{-1} resolution.

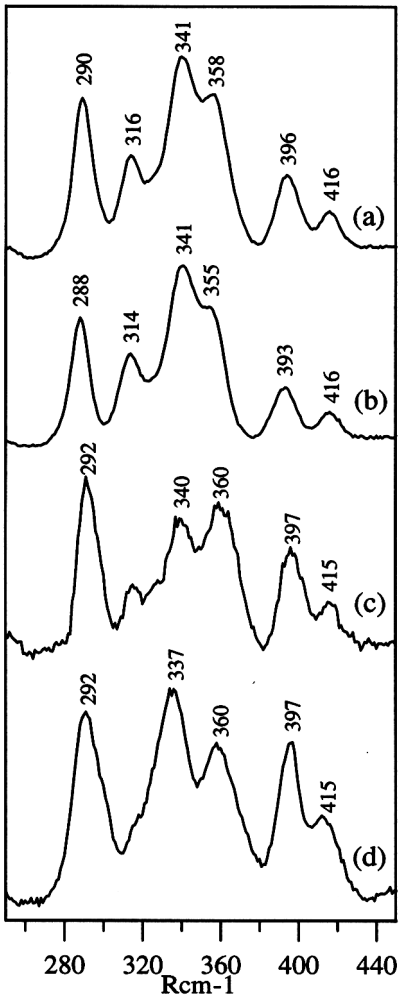

Figure 3.5: Low-temperature resonance Raman spectra of the transient $[2Fe\text{-}2S]^{2+}$ clusters. (a) NifU(Asp^{37}Ala) after treatment using the cluster biosynthetic system; (b) as-isolated NifU(Asp^{37}Ala); (c) difference spectrum [spectrum (a) minus spectrum (b)]; (d) NifU-1(Asp^{37}Ala) after treatment using the cluster biosynthetic system. The conditions used are the same as described in Figure 3.4 and each spectrum is the sum of 33 scans.

E. Discussion

NifU contains two distinct types of iron-binding sites. In as-isolated NifU one of these types of sites is occupied by a $[2Fe\text{-}2S]^{2+,+}$ cluster that we refer to as the permanent cluster (6). The other type of iron-binding site is a mononuclear site that is predominantly unoccupied in the as-isolated protein but can be filled *in vitro* by the addition of ferric ion (see Chapter II). In the present work it is shown that L-cysteine and catalytic amounts of NifS can be used to assemble an additional labile $[2Fe\text{-}2S]^{2+}$ cluster within a variety of different forms of NifU protein. The lability of this cluster and the presence of the permanent clusters have so far prevented definitive identification of this transient cluster in full-length wild-type NifU. However, a combination of UV-Visible absorption and resonance Raman studies have provided abundant evidence for the *in vitro* assembly of this cluster in samples of NifU-1 and NifU($Cys^{137}Ala$) variant, neither of which contain the permanent clusters. Other evidence has been obtained using the NifU-1($Asp^{37}Ala$) and NifU($Asp^{37}Ala$) variants in which the transient cluster is less labile. The same three cysteine residues necessary for iron binding at the mononuclear site are also required for NifS-directed assembly of the transient [2Fe-2S] cluster (Figure 3.1). Although we cannot yet rule out the possibility that a set of conditions exist whereby one transient $[2Fe\text{-}2S]^{2+}$ cluster can be assembled within each subunit, the available data are consistent with a model in which ferric ions bound at the individual mononuclear sites are rearranged upon donation of sulfur by NifS to form a $[2Fe\text{-}2S]^{2+}$ cluster that is bridged between the two NifU subunits. Evidence supporting this mode of binding include: (i) the lability of the transient [2Fe-2S] cluster; (ii) the iron-binding stoichiometry at the mononuclear sites; (iii) a maximal UV-Visible absorption intensity consistent with no

more than one transient [2Fe-2S] cluster per NifU homodimer and; (iv) a resonance Raman spectrum that is interpreted in terms of four cysteine ligands due to the close correspondence to that of human ferrochelatase.

The lability of the transient cluster and its release from the polypeptide matrix upon reduction is consistent with the hypothesis that the function of the transient cluster is to provide iron and sulfide necessary for the formation of the [Fe-S] cores of the nitrogenase metalloclusters. The role of the permanent [2Fe-2S] clusters contained within NifU is not yet known. However, the observed release of the transient cluster upon reduction indicates that the role of the permanent clusters could be to provide reducing equivalents for that process. In this context we note that both irons contained in the NifU-bound transient cluster are in the ferric oxidation state and that previous work has shown that chemical reconstitution of [Fe-S] cluster-containing proteins is most effective when ferrous ions are used in the reconstitution system (15). Thus, reduction of the transient cluster might be important not only for its release but also for placing irons destined for nitrogenase metallocluster core formation in the appropriate oxidation state. It is also possible that the permanent $[2Fe\text{-}2S]^{2+,+}$ clusters could have a redox function in the acquisition of iron for initial binding at the mononuclear sites. There is no *a priori* reason why the permanent clusters could not participate in all of these functions.

Genes encoding homologs to NifU and NifS are also located within the genomes of a wide variety of non-nitrogen fixing organisms (22). We have designated these as "*isc*" (iron-sulfur cluster) genes in order to indicate the proposed role of their products in the housekeeping function of general [Fe-S] cluster assembly. In line with this proposal the *isc* genes have been found to be essential for *A. vinelandii* viability (22). There is also

mounting biochemical and genetic evidence from other laboratories that the *isc* gene products are involved in [Fe-S] cluster assembly in both prokaryotic (16) and eukaryotic (13, 14, 17, 20) organisms. A comparison of the organization of the NifU protein and its proposed housekeeping counterpart designated IscU is relevant to the work described here. For example, the IscU protein is considerably truncated when compared to NifU, bearing sequence identity only to the N-terminal third of NifU. This portion of NifU corresponds to the NifU-1 fragment described in the present work. The NifU Cys^{35}, Cys^{62}, and Cys^{106} residues contained within this segment are also strictly conserved in all *iscU* gene products identified so far. In fact, the IscU primary sequence is among the most conserved sequence motifs in nature (10). The IscU protein does not contain a sequence corresponding to the permanent $[2Fe\text{-}2S]^{2+,+}$ cluster-binding domain present in NifU. However, there is another gene contained within the *isc* gene cluster whose product does harbor weak primary sequence identity when compared to the $[2Fe\text{-}2S]^{2+,+}$ cluster-binding region of NifU. This small ferredoxin has been purified and shown to contain a $[2Fe\text{-}2S]^{2+,+}$ cluster that is nearly identical in its spectroscopic and electronic properties when compared to the [2Fe-2S] clusters contained within as-isolated NifU (11, 21). Thus, a function analogous to that provided by the NifU permanent cluster might also be duplicated by this ferredoxin. It is interesting that a bacterial ferritin-associated $[2Fe\text{-}2S]^{2+,+}$ ferredoxin having the same spatial arrangement of cluster-coordinating cysteines, as well as the same spectroscopic and electronic properties as the *isc*-specific ferredoxin, has also been identified in *E. coli* (1, 7). This observation has led to speculation that a function of the bacterial ferritin-associated ferredoxin could involve the

release of iron from ferritin for [Fe-S] cluster assembly, a suggestion also in line with a possible role for the permanent clusters contained within NifU.

Although it is not yet known whether or not a [2Fe-2S] cluster can be assembled on IscU, both IscU and IscS from *A. vinelandii* have been recombinantly produced and isolated. IscS exhibits an L-cysteine desulfurase activity (22) similar to that demonstrated for NifS (24). Also, in preliminary work, IscS and IscU have been found to form a macromolecular complex similar to that described for NifS and NifU (our unpublished results). In spite of these similarities the assembly of [Fe-S] clusters catalyzed by the Isc system might be considerably more complex than we have found so far for the Nif system. For example, there are heat-shock-cognate (Hsc) proteins encoded within bacterial genomes that have been suggested to have chaperone functions involving either the formation of [Fe-S] clusters or their insertion into various target proteins (19). Also, in the case of *Saccharomyces cerevisiae*, certain Hsc proteins have already been implicated in the physiological assembly of [Fe-S] clusters (13, 19, 20). In contrast, there are no known *nif*-specific gene products homologous to the Hsc family of proteins.

Finally, it is noted that there remain a number of important gaps in our understanding of the mobilization of the iron and sulfur required for maturation of [Fe-S] cluster-containing proteins. Although the present work demonstrates the *in vitro* ability of NifU to assemble a transient [2Fe-2S] cluster in the presence of NifS, whether or not this system functions by directly donating a [2Fe-2S] unit for *in vivo* cluster assembly still needs to be determined. If [Fe-S] clusters are assembled in this way, then there are fundamental issues concerning how the transient cluster is delivered to the target protein and how the [2Fe-2S] units might be assembled into higher order clusters. In addition, it

is not yet known how the NifS-bound persulfide is physiologically released for assembly of the transient cluster. We believe that the Nif system will continue to provide a model for the biochemical-genetic approach to address these issues.

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CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

The *Azotobacter vinelandii* NifU and NifS are required for the maturation of both the Fe-protein and MoFe-protein of nitrogenase (1, 2, 4, 10). NifU and NifS, therefore, have been used as a model for the study of a possible *in vivo* mechanism for [Fe-S] cluster assembly. In this mechanism, it is believed that iron and sulfide are provided by specific delivery systems instead of free iron and sulfide. Previous work has shown that the NifS is a homodimeric enzyme that catalyzes a pyridoxal phosphate (PLP)-dependent L-cysteine desulfurization (10). An intermediate of this reaction is NifS-bound persulfide that can be used to supply sulfur for *in vitro* catalytic formation of [Fe-S] clusters (8, 9). NifU was isolated as a homodimeric protein containing one chemically reducible [2Fe-2S]$^{2+}$ cluster per subunit (referred to as the "permanent" [2Fe-2S] cluster) (2). However, NifU activity has not yet been identified. This dissertation describes work designed to determine how NifU might participate in [Fe-S] cluster assembly. The results are summarized by the following.

1) Among the nine cysteine residues contained within NifU (Cys^{35}, Cys^{62}, Cys^{106}, Cys^{137}, Cys^{139}, Cys^{172}, Cys^{175}, Cys^{272}, and Cys^{275}), only Cys^{272} and Cys^{275} are not required for the full physiological function of NifU. This conclusion is based on genetic evidence using site-directed mutagenesis of the individual cysteine residues and gene replacement techniques. The resulting *A. vinelandii* mutant strains were compared with the wild type for their diazotrophic growth properties and the activity of the two component proteins of nitrogenase.

2) Cys^{137}, Cys^{139}, Cys^{172}, and Cys^{175}, located within the C-terminal half of NifU, serve as ligands for the permanent [2Fe-2S] cluster. The C-terminal half of NifU is referred to as NifU-2.

3) Cys^{35}, Cys^{62}, and Cys^{106}, located within the N-terminal third of NifU, are highly conserved among NifU and NifU-like proteins in nature. The N-terminal third of NifU is referred to as NifU-1. Cys^{35}, Cys^{62}, and Cys^{106} together with a non-cysteine residue, which is still unknown, provide ligands for Fe(III) binding as in the rubredoxin-like mononuclear type. NifU could accommodate up to one iron per subunit for rubredoxin-like binding, and the mononuclear centers at these sites are labile.

4) Gel-exclusion chromatography showed that NifU and NifS can form a transient complex *in vitro*.

5) In the presence of catalytic amounts of NifS as well as L-cysteine and a reducing agent, the Fe(III) ions contained at the rubredoxin-like sites of NifU can rearrange to form a transient [2Fe-2S] cluster. The transient [2Fe-2S] cluster is located between the homodimeric subunits of NifU. Ligands to the transient [2Fe-2S] cluster are exclusively provided by cysteine residues.

6) NifU variants, generated using site-directed mutagenesis, can trap the [2Fe-S] cluster that was discussed in 5). The [2Fe-2S] cluster in one of the NifU variants can be released readily upon dithionite reduction.

From these results and previous work, a model for [Fe-S] cluster assembly mediated by the *A. vinelandii* NifU and NifS can be drawn as follows:

A) NifU mobilizes Fe(III) ions to form mononuclear-type centers at its N-terminal domain (one iron per monomer). This could represent an intermediate stage of NifU.

B) NifS mobilizes sulfur from L-cysteine, and NifS-bound persulfide is formed. Therefore, NifS-bound persulfide serves as a sulfur donor, or NifS serves as a sulfur delivery protein.

C) Upon donation of sulfur from NifS to NifU, both Fe(III) ions bound at the mononuclear sites rearrange to form a transient [2Fe-2S] cluster between the NifU subunits.

D) The transient [2Fe-2S] cluster formed on NifU is then released and is used for the formation of nitrogenase-associated [Fe-S] clusters. Therefore, NifU not only mobilizes iron but also serves as an intermediate [Fe-S] assembly site.

E) NifU and NifS could undergo a new cycle and form more transient [2Fe-2S] clusters.

F) Although the function of the permanent [2Fe-2S] clusters within NifU was not determined, it is speculated that their ability to undergo redox reaction might make them contribute to either step A) or D), or both steps.

The predictions in F) are based on the characteristics of *E. coli* bacterioferritin-associated ferredoxin (Bfd) (3, 6). Bfd contains a [2Fe-2S] cluster, whose coordinating cysteine residues have the same spatial arrangement as those of the permanent [2Fe-2S] clusters of NifU. The spectroscopic and redox properties of the [2Fe-2S] cluster of Bfd and those of the permanent [2Fe-2S] clusters of NifU are very similar. For these reasons, Bfd and the domain of NifU that contains the permanent [2Fe-2S] clusters are likely to have similar functions. Bfd has been suggested to mediate the release or uptake of iron from bacterioferritin; therefore, in step A) of the model, reducing equivalents provided by the permanent [2Fe-2S] clusters could be used for release of iron from bacterioferritin. Because the transient [2Fe-2S] cluster formed between the subunits of NifU is labile and

can be released upon reduction, as in step D), the permanent [2Fe-2S] clusters could have a redox role in releasing the transient [2Fe-2S] cluster and/or to place the irons being released in the appropriate oxidation state.

This model highlights the involvement of NifU in [Fe-S] cluster assembly from what has been predicted previously. However, many aspects are not completely understood in this model. It is not known how sulfur is released from NifS and used for the formation of the transient [2Fe-2S] cluster within NifU. Even if the permanent [2Fe-2S] clusters of NifU have redox roles in either step A) or D), what reductant provides reducing equivalents for NifU is still not known. In the case where the transient [2Fe-2S] clusters released from NifU are destined for the maturation of nitrogenase component proteins, how they will be delivered and used to achieve higher order clusters remains to be answered. In addition, how NifU and NifS are regulated in order to provide the appropriate amount of iron and sulfur necessary for [Fe-S] cluster assembly is an open question. Besides, this model is based on many experiments designed to analyze NifU properties *in vitro*. Therefore, it is necessary to find more *in vivo* evidence to strengthen the model.

NifU is likely a modular protein because polypeptide fragments from either the N-terminal third (NifU-1) or the C-terminal half (NifU-2) of NifU could separately retain certain individual properties of NifU. In experiments described in this dissertation, NifU-1 contains the first three cysteine residues of NifU, and NifU-2 contains the last six cysteine residues of NifU. They were recombinantly produced with four amino acid residues (Glu-Asp-Asp-His) overlapping between them (see Experimental procedures of Chapter II). In order to understand the function of NifU as a modular protein *in vivo*, one

could construct *A. vinelandii* mutant strains similar to the experiments used *in vitro*. Then, diazotrophic growth properties and the specific activities of nitrogenase component proteins of these mutant strains could provide a phenotype of the mutants. In one strain, *nifU* could be replaced by genes encoding NifU-1 and NifU-2 proteins. Thus, this strain will contain completely disconnected NifU-1 and NifU-2 domains. In other strains, *nifU* could be modified in such a way that their products would have multiple copies of the overlapping amino acid residues (Glu-Asp-Asp-His) connected between NifU-1 and NifU-2 domains. Such connection might serve as a solvent exposed loop fused between NifU-1 and NifU-2 domains with variation in length in different strains. If NifU-1 and NifU-2 domains can work independently *in vivo*, the mutant strains should be able to grow under nitrogen fixing conditions.

A ferredoxin, which is encoded by a gene within the *iscS* and *iscU* gene cluster of *E. coli* and *A. vinelandii* (7), and Bfd are thought to provide a function homologous to the permanent [2Fe-2S] cluster-binding region of NifU. This idea could also be tested in line with the above experiment by substitution of this region of NifU with its functional homologs. However, this experiment might be more difficult because the ferredoxin and Bfd lack the last two cysteine region when their sequences are compared to NifU sequence. In other words, the region to be used in the substitution experiment is in the middle of the first three and the last two cysteine residues within NifU. Nevertheless, if the last two cysteine residues are dispensable, control strains in this experiment could be isogenic strains to the ones that was suggested in the above experiment except for the last two cysteine region deleted. Therefore, only homologs to only the permanent [2Fe-2S]

cluster-binding region of NifU are compared, either completely disconnected from the NifU-1 domain or with some variation in length between them.

Although good evidence was shown *in vitro* that NifU and NifS can form a transient complex, it is not known that association and disassociation of NifU and NifS actually are required *in vivo*. To further investigate for *in vivo* evidence, NifU and NifS might be modified so that they will be fused to each other. Provided that all altered proteins are still active, the NifU and NifS fusion (NifU-NifS) could be varied by using a short connection and a long connection for comparison. Rationale to perform this experiment comes from the fact that *nifU* and *nifS* are adjacently cotranscribed and from my preliminary experiments. These earlier experiments were involved in characterization of NifU-1(Asp^{37}Ala) and NifU(Asp^{37}Ala), the NifU variants that were shown to stabilize the transient [2Fe-2S] cluster. These experiments indicated that NifU-1(Asp^{37}Ala) is able to form a complex with NifS, whereas NifU-1 is not. A complex between NifU(Asp^{37}Ala) and NifS appears to be tighter and have a different configuration than that of NifU and NifS. In addition, the *A. vinelandii* mutant strain carrying NifU(Asp^{37}Ala) exhibits slow growth under nitrogen fixation (data not shown). Because of these results, it is possible that the equilibrium between association and disassociation of NifU and NifS is shifted to the association side in this mutant strain. The new equilibrium toward association of the NifS and NifU(Asp^{37}Ala) then affects the maturation of the nitrogenase component proteins, and in turn this mutant could not fix N_2 properly. Therefore, the NifU-NifS fusion proteins could alter the equilibrium through the association of NifU and NifS. However, care must be taken in interpretation of the results of this experiment because there is no guarantee that an interaction between

different NifU-NifS molecules will not occur. In other words, it is not known where the site or the position of the interaction would be.

An alternative experiment to test the *in vivo* association and disassociation of NifU and NifS might be to replace *nifS* with *iscS* providing that their expression patterns are the same. Along with the gene replacement *in vivo* is testing an *in vitro* interaction between the NifU and IscS. The basis for these experiments is that either *A. vinelandii* or *E. coli* IscS has the same activity as NifS. Also, it is expected that the interaction of NifU and NifS would be more specific than that of NifU and IscS (e.g. the affinity between NifU and NifS is higher than that between NifU and IscS). If this is true, the presence of IscS instead of NifS could affect the equilibrium towards the dissociation side. Then, the rate of maturation of the nitrogenase [Fe-S] cluster assembly might be decreased. As a result, such a mutant strain should be significantly impaired in diazotrophic growth.

NifU as isolated contains the permanent [2Fe-2S] clusters in their oxidized form. The $[2Fe\text{-}2S]^{2+}$ clusters can be reduced chemically to $[2Fe\text{-}2S]^{+}$ clusters with midpoint potential E_m = -254 mV. With this feature, NifU permanent clusters have been proposed to have redox activity participating in the mobilization of iron and/or release of the iron or an intermediate at the other iron-binding site. To investigate whether or not the permanent [2Fe-2S] clusters of NifU could have redox roles *in vivo*, *A. vinelandii* mutant strains should be constructed. Such mutants might contain NifU variants that maintain the permanent [2Fe-2S] clusters but are altered in redox potential (e.g. lower or higher than that of wild type NifU). It is known that alteration in either H-bonds or hydrophobic interaction could affect the redox potential of the [Fe-S] cluster (5). Therefore, site-directed mutagenesis at amino acid residue(s) that can provide H-bonds to the permanent

[2Fe-2S] clusters is a likely way to achieve such mutations. Because a protein crystal of NifU has recently been accomplished (M. K. Chan, Ohio State University, personal communication), when the 3D-structure of NifU is available, selection of which amino acids to alter should be easier.

It would be interesting to see if the transient [2Fe-2S] cluster of NifU can be used for the reconstitution of the [4Fe-4S] cluster of Fe-protein *in vitro*. In the present work, the destination of the transient [2Fe-2S] cluster formed on NifU was not determined. It has only been suggested that if NifU is involved in mobilization of iron, the iron or an intermediate assembled on NifU should be able to escape from NifU to form the [Fe-S] clusters associated with nitrogenase component proteins. In previous work, it was shown *in vitro* that a [4Fe-4S] cluster can be reconstituted into the apo-Fe protein, and the resulting protein is nearly identical to the native Fe-protein (8). In that experiment, apo-Fe protein was incubated with NifS, L-cysteine, Fe(II) ion, and DTT. However, it is also possible that the formation of the [4Fe-4S] cluster of the Fe-protein occurs through two [2Fe-2S] cluster-building blocks and the addition of acid-labile sulfides to complete the process. Therefore, the transient [2Fe-2S] clusters formed on NifU could serve as such building blocks, and NifS could deliver more sulfur as it is needed. To investigate the *in vitro* reconstitution with the system used in the present work (see Chapter III), omitting NifU from the reaction mixture will be used as a control to ensure that the reconstitution is mediated by NifU. The reaction mixture used in this system is quite different when compared to the previous system; β-mercaptoethanol is used instead of DTT; Fe(III) ion is used instead of Fe(II) ion and the iron concentration is twenty time less.

As previously mentioned, it is possible that NifU and NifS deliver the transient [2Fe-2S] cluster and sulfur directly to the apo-Fe protein. Therefore, whether or not the reconstitution will succeed under the conditions examined, the protein-protein interaction should be investigated. The series of the protein-protein interactions to be determined could be the interaction between apo-Fe protein and NifU, between apo-Fe protein and NifS, and with all these components. Nevertheless, it is known that the function of several *nif*-specific gene products have not yet been identified. Thus, the model cannot rule out the possibility that other *nif*-specific gene products are involved in the [Fe-S] cluster assembly.

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APPENDIX I

RELEASE OF THE TRANSIENT [2Fe-2S] CLUSTER

In order to show the lability of the transient [2Fe-2S] cluster upon reduction, the Fe(II)-chelating reagent α,α' dipyridyl was used to quantitatively capture the Fe(II) ions released upon dithionite reduction. The as-isolated form of NifU-1(Asp^{37}Ala) was used for the experiment because this protein already contains some [2Fe-2S] cluster. Thus, the experiment was not complicated by the need to add exogenous iron to the sample.

As-isolated NifU-1(Asp^{37}Ala) was pre-incubated in the presence of the Fe(II)-chelating reagent α,α' dipyridyl (Arrow A) with no apparent change in the UV-Visible spectrum. Upon the addition of dithionite (Arrow B) there is an immediate change in the absorption peak at 520 nm corresponding to an Fe(II)-α,α' dipyridyl complex. Based on the known extinction coefficient of the Fe(II)-α,α' dipyridyl complex, and iron analysis of the original protein sample, it was calculated that 97% of the protein-bound iron could be removed by chelation following dithionite reduction.

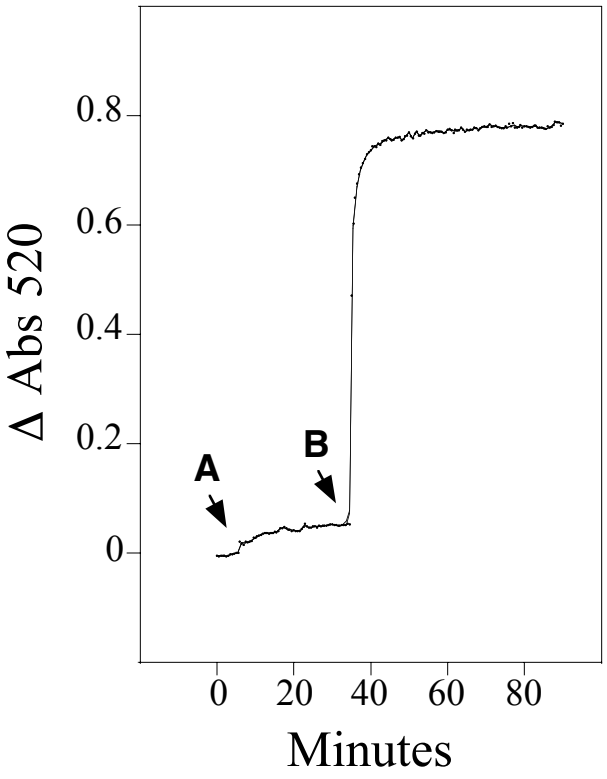

Figure AP1: Release of the transient [2Fe-2S] cluster. Spectroscopic method was performed as described in Chapter III. The concentration of as-isolated NifU-1(Asp^{37}Ala) was started at 0.8 mM (A_{520} = ~ 0.4) with a total volume of 0.6 ml. α,α' dipyridyl was added to 4 mM final concentration (Arrow A) with a total volume of 0.75 ml, and the mixture was incubated for 30 min. After dithionite was added to 2 mM final concentration (Arrow B), the mixture was incubated for 45 min more. Because the addition of the Fe(II)-chelating reagent α,α' dipyridyl and dithionite caused overall dilution, and the addition of dithionite resulted in the cluster bleaching, the absorption at 520 nm was reduced in each of these steps. For this reason, the base-line absorption in this experiment came from a control. The control was prepared the same way as that of the experiment except the Fe(II)-chelating reagent was replaced with the same volume of distilled water.

VITA

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