CHAPTER 4

Iron-Sulfur Cluster Assembly: NifU-Directed Activation of the Nitrogenase Fe Protein

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This manuscript describes biochemical and genetic experiments that establish the involvement of NifU in the maturation of nitrogenase Fe protein. Genetic experiments involving amino acid substitutions within the N-terminal and C-terminal domains of NifU indicate that both domains can separately participate in nitrogenase-specific [Fe-S] cluster formation, although the N-terminal domain appears to have the dominant function. These *in vivo* experiments were supported by *in vitro* [Fe-S] cluster assembly and transfer experiments involving the activation of an apo-form of the nitrogenase Fe protein.

I was involved in performing experiments pertaining to plasmid construction, bacterial strain construction, protein purification, and cluster assembly and activation of apo-Fe protein assays. This chapter was written and submitted for publication with the intention for its use to satisfy a portion of the research completed for this dissertation. As the leading author, I performed most of the experimental work and participated in writing the document at all stages of its preparation, and prepared the figures that are included in this chapter.

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4.1 - Introduction

The *Azotobacter vinelandii* NifU protein is an approximately 60 kDa homodimer proposed to provide a molecular scaffold for formation of [Fe-S] clusters or Fe-S cluster precursors required for full activation of the nitrogenase catalytic components (Fu et al., 1994; Yuvaniyama et al., 2000). Primary sequence comparisons among NifU homologs indicate that it is a modular protein having three distinct domains (Agar et al., 2000b; Hwang et al., 1996). The N-terminal domain includes three cysteine residues conserved among all known NifU and NifU-like proteins. Incubation of the recombinantly expressed NifU N-terminal domain with NifS (a cysteine desulfurase), L-cysteine, and Fe^{2+} results in formation of labile [2Fe-2S] clusters on the NifU fragment, providing evidence that this domain could provide a scaffold for assembly of "transient" [Fe-S] clusters destined for nitrogenase activation (Yuvaniyama et al., 2000). In support of this hypothesis biochemical and genetic studies established that individual substitution of any of the three cysteine residues contained in the N-terminal domain of NifU impaired, but did not eliminate, the physiological maturation of the nitrogenase component proteins (Agar et al., 2000b).

It was subsequently shown that the N-terminal NifU domain is highly homologous to a small protein, designated IscU (Zheng et al., 1998). IscU also contains three conserved cysteines and can provide a scaffold for the sequential *in vitro* assembly of [2Fe-2S] and [4Fe-4S] clusters when incubated with IscS (a NifS cysteine desulfurase homolog), L-cysteine and Fe^{2+} (Agar et al., 2000a; Agar et al., 2000c). The IscS and IscU proteins, together with a suite of other proteins, IscA, HscB, HscA, and Fdx, whose corresponding genes are clustered on both the *A. vinelandii* and *Escherichia coli* genomes, have been shown by genetic experiments using *E. coli* to be involved in the maturation of a variety of Fe-S cluster-containing proteins involved in intermediary metabolism, such as aconitase and glutamate synthase (Nakamura et al., 1999; Schwartz et al., 2000; Takahashi & Nakamura, 1999; Tokumoto & Takahashi, 2001). In the case of *A. vinelandii*, the *iscSUAhscBAfdx* gene cluster cannot be deleted because this gene cluster is essential under growth conditions for which the *nif*-specific genes are either

expressed or not expressed (Zheng et al., 1998). Thus, under nitrogen fixing conditions, NifU is apparently unable to effectively substitute for IscU function. Similarly, NifU deletion strains are severely impaired in their capacity for diazotrophic growth, indicating that IscU cannot effectively replace the function of NifU (Jacobson et al., 1989b). In contrast, the *iscSUAhscBAfdx* genes are not essential in *E. coli* because there is some redundant function provided by another [Fe-S] cluster assembly apparatus encoded by *sufABCDSE*. Elegant genetic studies have demonstrated that the *E. coli iscSUAhscBAfdx* genes are essential in the absence of *sufABCDS* and *vice versa* (Takahashi & Tokumoto, 2002). Although the *A. vinelandii* genome encodes proteins homologous to SufSE, recently shown to encode a two-component cysteine desulfurase (Loiseau et al., 2003; Ollagnier-de-Choudens et al., 2003; Outten et al., 2003), it clearly does not contain homologs to *sufBCD*, which probably explains why the "*isc*" gene cluster is essential in this organism.

The central domain of NifU contains four conserved cysteines that have been shown to coordinate one redox-active $[2Fe\text{-}2S]^{2+/1+}$ cluster per each NifU subunit (Agar et al., 2000b). These clusters have been provisionally designated "permanent" clusters because they do not appear to be precursors destined for nitrogenase maturation. Rather, it has been proposed that these clusters could have a redox function or some other role related to the physiological formation or release of "transient" clusters assembled on the N-terminal domain (Agar et al., 2000b; Yuvaniyama et al., 2000). In line with this hypothesis substitution of any of these cysteine residues also results in a decreased capacity of *A. vinelandii* for diazotrophic growth.

The C-terminal domain of NifU contains two conserved cysteine residues and previous mutagenesis studies demonstrated that they could be substituted by alanine, with no apparent affect on the physiological maturation of nitrogenase (Agar et al., 2000b). Nevertheless, small proteins exhibiting significant sequence similarity to the C-terminal domain of NifU, including conservation of the two cysteine residues, have been shown capable of providing *in vitro* templates for the assembly of labile [2Fe-2S] or [4Fe-4S] clusters (Leon et al., 2003; Nishio & Nakai, 2000; Schilke et al., 1999; Tong et al., 2003).

These results suggest that the C-terminal NifU domain might provide a second transient cluster assembly site and could explain why strains deleted for *nifU* are severely impaired in their capacity for diazotrophic growth, whereas strains individually substituted for cysteines contained in the N-terminal module remain capable of significant diazotrophic growth.

In the present work, complementary biochemical and genetic experiments were performed to examine the functional significance of the N-terminal and C-terminal domains of NifU in nitrogenase maturation. Towards this end, the capacity for diazotrophic growth of mutant strains having various combinations of alanine substitutions for conserved cysteines located within the N-terminal and C-terminal domains was examined. Furthermore, an effective method for NifU-directed *in vitro* activation of an apo-form of the nitrogenase Fe protein was developed and the capacity for *in vitro* activation of apo-Fe protein using altered forms of NifU having various combinations of alanine substitutions for conserved cysteines located within the N-terminal and C-terminal domains was also examined.

4.2 - Experimental Methods

Plasmids and strains

Recombinant plasmids (pDB42 and pDB133) that contain *A. vinelandii nifU* and *nifS* genes (Jacobson et al., 1989a) were used to construct plasmids for heterologous expression in *E. coli*, or for mutagenesis of genomically expressed NifU or NifS. Mutagenesis was performed using a commercial kit (GeneEditor-Promega) and nucleotide sequences were confirmed by DNA sequence analyses performed by the Virginia Tech sequencing service. For heterologous expression of *nifU* and *nifS* an approximately 6.5 kb *Pci*I-*Bam*HI restriction enzyme generated DNA fragment was cloned into the pAra13 expression vector (Cagnon et al., 1991) digested with *Nco*I and *Bgl*II to create pDB1284. This procedure places expression of *nifU* and downstream genes under the control of the *ara* transcriptional and translational elements. In addition to *nifU* and *nifS*, pDB1284 contains *nifUSVWZM,* which were excised by *Xho*I digestion

and religation to create pDB1289. Plasmid pDB1289 and its derivatives, created by site-directed mutagenesis, were used for the heterologous expression of *nifU* and *nifS* for biochemical experiments. Plasmid pDB551 (Zheng et al., 1993) was used for heterologous expression of *nifS* in the absence of *nifU*. A version of a *nifUS* expression plasmid for which eight histidine codons were placed between *nifS* codons 396 and 397 was constructed by inserting a synthetic DNA fragment into the unique *Stu*I restriction site of pDB1289. For the construction of mutant *A. vinelandii* strains, the appropriate plasmids were used in DNA transformation experiments resulting in double reciprocal recombination between the genome and the plasmid vector as previously described in detail (Jacobson et al., 1989a; Jacobson et al., 1989b). Plasmids used in this work are not capable of autonomous replication in *A. vinelandii* and they are listed in Table 1. *A. vinelandii* cells were cultured in a defined minimal medium without the addition of a fixed nitrogen source (Strandberg & Wilson, 1968).

NifU and NifS expression

E. coli strain TB1 was used as the host for heterologous expression of *nifU*, co-expression of *nifU* and *nifS*, or co-expression of various mutant forms of *nifU* and *nifS*. The various recombinant plasmids used for heterologous expression were derived from pAra-13, as described above. For the individual expression of *nifS*, a previously described plasmid (pDB551, (Zheng et al., 1993)) was used, which places the control of *nifS* expression under control of the T_7 promoter. For these experiments pT_7-7 was the vector and *E. coli* strain BL21(DE3) was used as the host. For heterologous expression, cells harboring the appropriate plasmid were cultured in 2 l flasks containing 500 ml of LB medium supplemented with 50 mg of ampicillin in a reciprocal shaker at 30 ºC/300 rpm until they reached 160-180 Klett (Red filter). Expression of cloned *nif* genes was induced by addition of 1.5 g of arabinose or 5 g lactose, depending on the expression system used. After induction cells were cultured for 3 hours, harvested by centrifugation, and the cell pellets were frozen at –20ºC until used.

Protein biochemistry

Table 1

Plasmids Used in This Work

Plasmid	Vector	Genotype	Purpose
pDB133	pUC7	*orf5'-iscA^{nif}-nifU-nifS-nifV'*	plasmid construction
pDB551	pT_7-7	*nifS*	recombinant expression
pDB1289	pAra 13	*nifU-nifS*	recombinant expression
pDB1305	pAra 13	*nifU*	recombinant expression
PDB1306	pAra 13	*nifU-nifS* (poly-his)	recombinant expression
pDB1311	pAra 13	*nifU-nifS(325^{A})*	recombinant expression
pDB1312	pUC7	*orf5'-iscA^{nif}-nifU(35^{A})-nifS-nifV'*	construct DJ1415
pDB1313	pUC7	*orf5'-iscA^{nif}-nifU(35^{A},62^{A})-nifS-nifV'*	construct DJ1416
pDB1315	pUC7	*orf5'-iscA^{nif}-ΔnifU::Kan^{R}-nifS-nifV'*	construct DJ1412
pDB1317	pUC7	*orf5'-iscA^{nif}-ΔnifU(codons 23-270)-nifS-nifV'*	construct DJ1413
pDB1318	pUC7	*orf5'-iscA^{nif}-nifU(35^{A}, 62^{A}, 106^{A})-nifS-nifV'*	construct DJ1417
pDB1324	pAra 13	*nifU(35^{A})-nifS*	recombinant expression
pDB1330	pAra 13	*nifU(35^{A}, 62^{A})-nifS*	recombinant expression
pDB1341	pAra 13	*nifU(35^{A}, 62^{A}, 106^{A})-nifS*	recombinant expression
pDB1346	pUC7	*orf5'-iscA^{nif}-nifU(35^{A}, 62^{A}, 106^{A}, 275^{A})-nifS-nifV'*	construct DJ1431
pDB1355	pAra 13	*nifU(35^{A}, 62^{A}, 106^{A}, 275^{A})-nifS*	recombinant expression

NifU and NifS purification. All protein manipulations were performed under anoxic conditions maintained using either Schlenk lines or a Coy anaerobic chamber containing 3% hydrogen gas balanced with nitrogen gas. Cell pellets were resuspended in 4 ml of 25 mM Tris-HCl (pH 7.4), 5 mM β-mercaptoethanol for each gram of cells (wet weight) and crude extracts prepared by sonication followed by centrifugation at 15,000 x *g* for 20 min at 4 ºC. The supernatant was applied to a 1.5 x 15 cm Q-sepharose column (Pharmacia) equilibrated with 25 mM Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl and 5 mM β-mercaptoethanol. The loaded column was washed with 100 ml of the same buffer and a linear gradient of 150 ml (0.1-0.7 M NaCl) was then applied. Partially purified NifU or NifU and NifS were eluted around 0.5 M NaCl and stored in liquid nitrogen until used. NifS purification was performed as previously described (Zheng et al., 1993). Individually purified NifU or NifS samples were quantified using the biuret method (Chromy et al., 1974) whereas the amount of NifU and NifS in partially purified samples was estimated by polyacrylamide gel electrophoresis (Laemmli, 1970) using highly purified NifU and NifS as standards.

[Fe-S] cluster assembly. Each five ml [Fe-S] cluster assembly cocktail included a 25 mM Tris-HCl buffer (pH 7.4) that contained 50 μM NifU, 50 μM NifS, 0.5 mM L-cysteine, 2 mM $Fe(NH_4)_2(SO_4)_2$, 3 mM β-mercaptoethanol, and ~200 mM NaCl. For control experiments, described in the text and in Table 2, NifU, NifS, Fe^{2+} or L-cysteine was omitted from the assembly cocktail or an altered form of NifS having the active site cysteine-325 residue substituted by alanine was used. Other experiments, also described in the text and in Figure 3, involved using various altered forms of NifU having one or more cysteine residues substituted by alanine. All assembly reactions were incubated on ice for 1 h and then applied to a mixed bed chromatography column containing 1 x 1.5 cm uncharged chelating Sepharose (Phamacia) and 1 x 3 cm Q-Sepharose (Pharmacia) previously equilibrated with 100 mM NaCl, Tris-HCl (pH7.4) buffer. After loading, the sample was washed with 10 ml of the same buffer, which removes unreacted L-cysteine. Proteins were then eluted using 400 mM NaCl in 25 mM Tris-HCl (pH 7.4) buffer. Free Fe^{2+}, free S^{2-} and polymeric iron-sulfides remain bound to the column. NifU and NifS concentrations were estimated as described above and Fe analysis was performed as

described by Fortune and Mellow (Fortune & Mellow, 1938). In order to prepare a sample of [Fe-S] cluster-loaded NifU that was separated from NifS the same procedures described above were followed, except for the use of a NifS that carries a His-tag located near the C-terminus. Expression of *nifU* and His-tagged *nifS* for this experiment was controlled using pDB1306 (Table1). After completion of the assembly reaction, and after mixed bed chromatography as described above, the sample was applied to a 1.5 x 3.0 cm chelating Sepharose column charged with $NiSO_4$. Under these conditions [Fe-S] cluster-loaded NifU passes through the column while His-tagged NifS remains bound to the column.

Apo-Fe protein preparation. Diazotrophically grown *A. vinelandii* cells were broken using osmotic shock followed by centrifugation at 30,000 x *g* for 1 hour at 4 °C as previously described (Christiansen et al., 1998). The crude extract was then applied to a Q-Sepharose chromatography column (5 x 20 cm) equilibrated with 25 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl and 1 mM dithionite. The column was then washed with 400 ml of the same buffer. The fraction containing Fe protein was eluted with a linear NaCl gradient (0.1-0.7 M NaCl) with the Fe protein peak appearing between 0.58-0.62 M NaCl. This fraction was concentrated under an Ar atmosphere to 15 ml using an amicon YM30 filter (Millipore). To remove the [4Fe-4S] cluster from the Fe protein a 5 ml of solution containing 25 mM ATP, 75 mM $MgCl_2$, 20 mM dithionite and 20 mM α-α'-dipyridyl was added and the sample incubated on ice for 1.5 h. This treatment resulted in a color change in the sample from deep brown to dark cherry red. Ten mls of this sample were loaded on a Pharmacia S-300 column (2.6 x 35 cm) equilibrated with 25 mM Tris-HCl buffer (pH 7.4), 5 mM ATP, 15 mM $MgCl_2$, 0.15 M NaCl (no dithionite) and eluted with the same buffer. The colorless, dithionite-free fraction containing apo-Fe protein was collected between 110-120 ml of the elution volume.

Apo-Fe protein activation. Apo-Fe protein activation cocktails contained 5.6 μM apo-Fe protein, 5.6 μM cluster-loaded NifU, 5.6 μM NifS, 4 mM dithiothreitol, and 4 mM MgATP in a 25 mM Tris-HCl buffer (pH 7.4). For titration experiments the concentration of NifU and NifS were varied as described in the text and shown in Figure

3. Fractions containing NifU and NifS were added last and Fe protein was assayed immediately. Prolonged incubation did not increase the level of reconstituted activity indicating that [Fe-S] cluster transfer from NifU to apo-Fe protein is rapid.

Fe protein assay. Fe protein activity was measured by the nitrogenase proton reduction assay described previously (Peters et al., 1994). All assays were carried out under an Ar atmosphere in sealed 9.2 ml vials. Each assay sample contained 500 μl of activation mix, corresponding to 2.8 nmol of Fe protein (0.19 mg), and 1.4 nmol MoFe protein (0.31 mg) in a final volume of 1 ml. Control experiments were used to show that total hydrogen evolution catalyzed under these conditions was linear with respect to Fe protein concentration. Assay vials were incubated for 20 min in a 30 ºC shaking water bath and the reaction terminated by the addition of 250 μl 0.25 M EDTA. Hydrogen gas produced was measured using a Shimadzu GC-14 gas chromatograph equipped with a molecular sieve 5A column and a thermal conductivity detector. Under these assay conditions Fe protein purified in this laboratory has a specific activity of 800 nmol H_2 produced per minute per mg of Fe protein. Levels of activation of apo-Fe protein were calculated as a percentage of this value. Under the assay conditions described here the apo-Fe protein is unable to support any H_2 evolution.

4.3 - Results

Functional analysis of the N-terminal and C-terminal domains of NifU

In previous work it was shown that deletion of the *A. vinelandii nifU* gene nearly eliminates diazotrophic growth capacity (Jacobson et al., 1989b). In contrast, substitution of alanine for any of the three N-terminal cysteines proposed to be involved in providing ligands for [Fe-S] cluster assembly lowers diazotrophic capacity, but not as severely as for the *nifU* deletion strain (Agar et al., 2000b). These findings were also confirmed in the present work (Figure 1). There are three plausible explanations for these results: (i) substitution of only one cysteine within the proposed N-terminal assembly scaffold does not entirely eliminate [Fe-S] cluster assembly within the N-terminal domain; (ii) a second

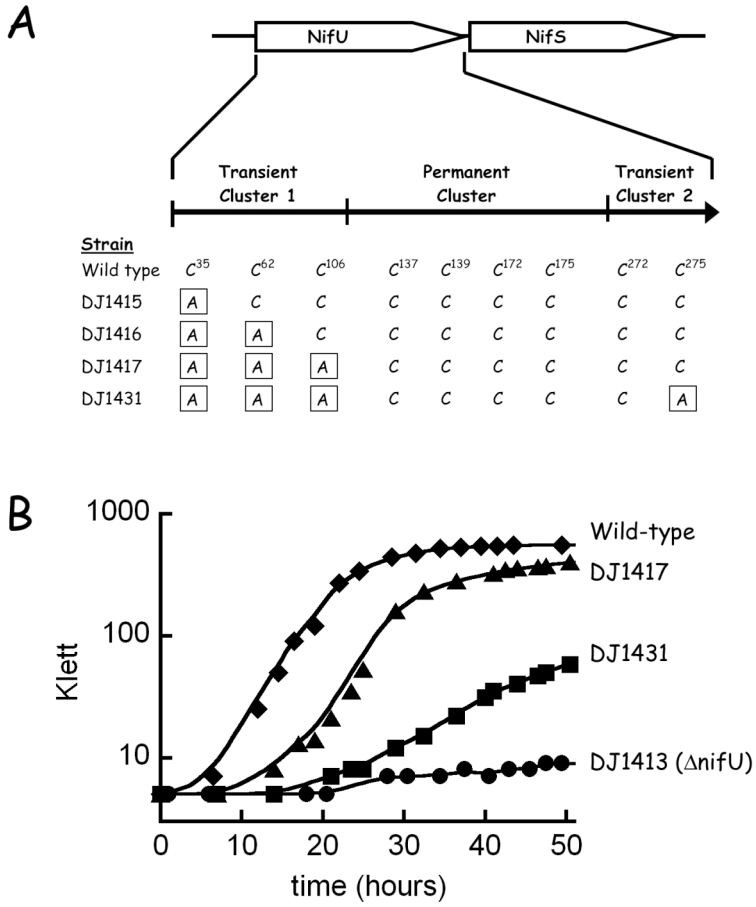

Figure 1. Effect of alanine substitutions for conserved cysteines located within NifU on diazotrophic growth. **(A)** Schematic representation of the *nifU* and *nifS* genes, domain organization of NifU, and location of conserved cysteine residues. For individual mutant strains the locations of alanine substitutions within the primary NifU sequence are boxed. **(B)** *A. vinelandii* strains having the indicated alanine substitutions, or having a large in-frame deletion within *nifU* were grown in liquid minimal medium with no fixed nitrogen source and growth monitored by observing an increase in turbidity using a Klett meter (Red filter). All strains showed the same growth rate (doubling time approximately 2.2 h) when cultured in the same medium supplemented with ammonium acetate or urea as the nitrogen source. Strains DJ1415, DJ1416 exhibited the same diazotrophic growth pattern as DJ1417.

[Fe-S] cluster assembly site is located within NifU; or (iii) NifU has some other function involving nitrogenase activation. These possibilities were examined by determining the effect on diazotrophic growth capacity as a result of substitution of one, two or all three cysteines located within the N-terminal domain. The different combinations of alanine for cysteine substitutions are shown in Figure 1A and the effect of these substitutions on diazotrophic growth is shown in Figure 1B. These and previous results show that the same diazotrophic growth phenotype results as a consequence of alanine substitution for any or all three of the N-terminal cysteines, indicating that cluster assembly necessary for nitrogenase maturation does not absolutely require a functional N-terminal NifU domain.

Recent studies have shown that a class of small proteins, sometimes designated Nfu, exhibit modest primary sequence identity when compared with the C-terminal domain of NifU, including conservation of the two cysteines located within this domain, and can be used as *in vitro* scaffolds for the assembly of [2Fe-2S] or [4Fe-4S] clusters (Nishio & Nakai, 2000; Tong et al., 2003). Previous work has also shown that substitution of either of these cysteines within NifU has no apparent affect on the capacity for diazotrophic growth for the respective mutant strains (Agar et al., 2000b). In the present work a quadruple mutant strain was constructed where all three cysteines located within the N-terminal domain plus one cysteine located within the C-terminal domain are substituted by alanine. This mutant strain (Figure 1) has a much lower capacity for diazotrophic growth than either the wild type strain or strains having combinations of substitutions within the N-terminal domain.

Although a mutant strain having substitutions within both the N-terminal and C-terminal domains is severely impaired in diazotrophic growth it is still able to grow at a faster rate than a mutant strain that carries a large deletion in *nifU* (DJ1413, Table 1). These results are the same as previously reported for a different strain (DJ105, (Jacobson et al., 1989b)) that carries a relatively small deletion spanning a region of *nifU* that encodes portions of the N-terminal and central domains. In the current work, the strain having a much larger deletion was constructed so that it could be certain all the functional properties of NifU were eliminated. Both DJ1413 and DJ105 carry in-frame deletions so

the phenotype cannot be assigned to a polar affect on downstream gene expression. Our interpretation of these results is that the central domain within NifU, which includes one $[2Fe\text{-}2S]^{2+/1+}$ per NifU monomer, must have some function that contributes to the physiological formation, stability, or delivery of transient [Fe-S] clusters that are formed on one or both of the proposed [Fe-S] cluster assembly scaffolds. Although the precise function of the central domain has yet to be determined, previous studies have already established that alanine substitution for any of the four cluster-coordinating cysteine residues eliminates assembly of the permanent $[2Fe\text{-}2S]^{2+/1+}$ cluster but does not have a severe impact on the capacity for diazotrophic growth (Agar et al., 2000b). In summary, the available genetic information indicates a redundant function for the N-terminal and C-terminal domains in the assembly of [Fe-S] clusters for nitrogenase maturation, and that the central domain could have a non-essential function that contributes to the effective formation or delivery of [Fe-S] clusters during nitrogenase maturation.

Development of an in vitro system for activation of the nitrogenase Fe protein

Examination of the NifU primary sequence, as well as genetic studies, have long been interpreted to indicate that NifU could have some function in the maturation of nitrogenase proteins related to assembly of their associated [Fe-S] clusters (Beynon et al., 1987; Jacobson et al., 1989b). The formation of labile [Fe-S] species on NifU when incubated with NifS (a pyridoxal phosphate-dependent cysteine desulfurase), Fe^{2+} and L-cysteine subsequently led to the hypothesis that NifU and the related IscU family of proteins provide molecular scaffolds for the assembly of transient [Fe-S] clusters (Agar et al., 2000c; Yuvaniyama et al., 2000). However, prior to the present work, this hypothesis had not been tested in the case of NifU by using *in vitro* [Fe-S] cluster assembly and transfer experiments. There is a report that transient [2Fe-2S] clusters assembled on the IscU scaffold can be transferred to an apo-ferredoxin (Wu et al., 2002), but this process is slow and requires a large molar excess of cluster-loaded IscU relative to the target protein. Such inefficiency could be related to a possible requirement for the participation of molecular chaperones in the process of Isc-directed [Fe-S] cluster assembly and transfer. Moreover, there are no published reports for IscU-directed *in vitro* activation for any apo-forms of proteins that have catalytic activities and have also been shown by

complementary biochemical and genetic experiments to be genuine physiological targets. There are several attractive aspects about the Nif system, which circumvents some of the complexities involved in the development of an Isc-based system for *in vitro* [Fe-S] protein maturation. First, there are only two known protein components – NifU and NifS – that are required for nitrogenase-specific [Fe-S] cluster formation. Second, complementary genetic and biochemical experiments have established that the nitrogenase Fe protein is a physiological target for NifU-directed [Fe-S] assembly (Jacobson et al., 1989b). Third, there is a well-established assay for Fe protein activity. Fourth, Fe protein can be used to prepare a target for *in vitro* [Fe-S] cluster transfer because its [4Fe-4S] cluster can be removed by chelation in the presence of MgATP and reducing agents (Walker & Mortenson, 1974).

To develop a method for the *in vitro* NifU-directed activation of the nitrogenase apo-Fe protein, a plasmid was constructed that enables the high-level heterologous expression of *A. vinelandii* NifU and NifS in *E. coli* (Table 1). Because NifU and NifS are cotranscribed, and their expression appears to be translationally coupled, this system permits the accumulation of NifU and NifS in the same molar ratios normally produced in *A. vinelandii* cells. To prepare samples for *in vitro* [Fe-S] cluster assembly, NifU and NifS were partially purified from crude extracts using Q-sepharose anion exchange chromatography (Figure 2). Samples prepared in this way were then incubated with Fe^{2+} and L-cysteine to achieve *in vitro* [Fe-S] cluster assembly. Details of the assembly conditions are presented in the *Experimental Methods*. After assembly was complete the sample was applied to a mixed-bed column that includes Q-Sepharose and uncharged metal-chelate resins and the sample washed with low-salt (100 mM NaCl) buffer. NifS and cluster-charged NifU were then co-eluted with high-salt (400 mM NaCl) buffer. Control experiments established that any free S^{2-} liberated by NifS, or polymeric iron-sulfides, remained tightly bound to the Q-sepharose resin, and free Fe^{2+} not incorporated into NifU remained tightly bound to the metal-chelate resin. Samples from the eluted fraction were then mixed with inactive apo-Fe protein (apo-Fe protein and NifU were present in approximately equimolar ratios in the activation reaction) and then immediately measured for Fe protein activity. During the development of the NifU-

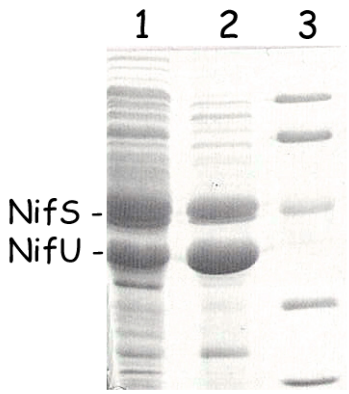

Figure 2. Gel electrophoretic analysis of NifUS samples. Proteins were separated by 15% SDS-PAGE and stained with Coomassie brillant blue. Lane 1: crude extract of proteins expressed using plasmid pDB1289; Lane 2, same sample as in Lane 1 after Q-Sepharose column chromatography; Lane 3, M_r standards (phosphorylase b, bovine serum albumin, ovoalbuminn, carbonic anhydrase and soybean trypsin inhibitor). Crude extracts from other strains having amino acid substitutions in NifU or NifS showed almost identical patterns of expression and purification behavior.

activation protocol different parameters were varied to establish optimum conditions for [Fe-S] cluster assembly and Fe protein activation. For example, various concentrations of Fe^{2+} and L-cysteine were added to the assembly mix, which revealed that activation was approximately linear with respect to these reagents with maximum activation occurring at 2.0 mM Fe^{2+} and 0.5 mM L-cysteine, respectively. As shown in Table 2, almost no activation occurred if NifU, NifS, L-cysteine, or Fe^{2+} were individually omitted from the assembly mix but a high level of activation was consistently achieved with the complete system. Also, there was almost no activation if an altered form of NifS, having the active-site cysteine-325 residue substituted by alanine, was used in place of wild-type NifS in the complete [Fe-S] cluster assembly cocktail (Table 2). Activation greater than 90% was rarely achieved under any condition, which could be the result of some irreversible damage that might occur during preparation of the apo-Fe protein. In separate experiments it was found that inclusion of dithiotheitol in the activation mix was not absolutely required, although, if omitted, there was a large variation in activation from experiment to experiment. From these results we conclude that dithiothreitol is not necessarily required for cluster transfer from NifU to the apo-Fe protein, but a reducing agent could be required for the stabilization of apo-Fe protein in a form amenable to *in vitro* apo-Fe protein activation. Although MgATP is included in the [Fe-S] cluster biosynthetic cocktail there is no evidence to indicate that nucleotides are required for effective [Fe-S] cluster transfer. MgATP is included in the activation cocktail because it is required for chelation of the Fe protein [4Fe-4S] cluster using α-α'dipyridyl and it is also required for assay of nitrogenase activity. It seems unlikely that MgATP is required for *in vivo* [Fe-S] cluster insertion during Fe protein maturation because altered forms of Fe protein deficient in nucleotide binding or hydrolysis have a full complement of the [4Fe-4S] cluster (Wolle et al., 1992).

The total Fe bound to NifU was measured by Fe analysis of the assembly mix after the mixed-bed chromatography step, which measures Fe contained within any [Fe-S] clusters assembled within NifU, as well as the two permanent [2Fe-2S] clusters. Such Fe analyses indicated that each NifU contained 4.3 Fe per NifU dimer prior to cluster assembly and 13.5 Fe per NifU dimer after cluster assembly. These values represent the

Table 2

Activation of the Nitrogenase Apo-Fe protein

Components were mixed to achieve [Fe-S] cluster assembly, processed as described in *Experimental Methods*, added to apo-Fe protein, and assayed for Fe protein activity.

Component	Included in Assembly Mix						
NifU	+	+	+	-	+	+	+
NifS	+	+	+	+	-	+ [a]	+ [b]
cysteine	+	-	+	+	+	+	+
Fe^{+2}	-	+	+	+	+	+	+
%activation	<1	<1	85[c]	<1	<1	<1	67

[a]For this sample the NifS used in the assembly mix carried an alanine substitution for the active-site cysteine-325 residue. NifS-325^{A} has no cysteine desulfurase activity. [b]For this sample the NifS used in the assembly mix carried a polyhistidine-tag near the C-terminus. The His-tagged NifS was removed from the assembly mix using a Ni^{+}-charged chelating Sepharose prior to activation of the apo-Fe protein as described in the *experimental methods* section. [c]Represents the average of ten different activation experiments. All other activation experiments were independently reproduced at least twice.

average of three independent measurements for both sample types with respective standard errors of 0.5 and 3.0 Fe per NifU dimer. Given the lability of the transient clusters and the handling required for sample preparation, it is likely that the ~8 irons assembled into each NifU homodimer represents a low estimate of the total Fe that can be incorporated into transient clusters. Namely, experiments described here provide no information about the composition and organization of the transient clusters assembled into NifU. Biophysical analyses will be necessary to address these issues and will require considerable scale-up of an [Fe-S] assembly system similar to the one described here. These experiments are currently in progress and will be reported elsewhere.

Activation of apo-Fe protein was also measured by the simple addition of 80 μM free S^{2-} and Fe^{2+}, which represents the estimated concentration of S^{2-} and Fe that could be contributed by NifU in the complete activation mixture as described above. The addition of 80 μM free S^{2-} and Fe^{2+} resulted in only 3% activation. Similarly, the addition of a much higher level of free S^{2-} and Fe^{2+} (400 μM) resulted in only 11% activation, far below the level routinely observed for NifU-directed activation. These results indicate that NifU-directed activation probably occurs by the direct transfer of an [Fe-S] cluster intermediate from the NifU scaffold, perhaps by a simple ligand exchange mechanism, rather than by the release of free Fe^{2+} and S^{2-} into solution with subsequent reassembly within the apo-Fe protein.

A final experiment was used to determine whether or not the observed activation requires the participation of NifU and NifS, both of which are included in the routine activation mix, or if cluster-loaded NifU alone is sufficient to achieve activation. These experiments were performed as already described except that the recombinantly expressed NifS contained a polyhistidine tag located near the C-terminus. Genetic experiments were used to show that incorporation of a His-tag at this location has no adverse affect on the capacity for diazotrophic growth, indicating that the His-tagged form of NifS retains its full physiological function. Following [Fe-S] cluster assembly and removal of free $Fe^{2+}$and S^{2-} using the mixed-bed column as described in *Experimental Methods*, NifS was separated from NifU using a metal-chelate column charged with Ni^{2+}.

Complete separation of NifS and the [Fe-S] cluster-loaded NifU fraction was confirmed by gel electrophoresis of the flow-through fraction. When this fraction was mixed with apo-Fe protein, 67% activation was observed (Table 2). Although this level is not as high as the 85% level of activation consistently observed in other experiments, we attribute the modestly lower level of activation as arising from the lability of clusters assembled on NifU. Namely, there is some loss of [Fe-S] clusters from the NifU scaffold during the extra sample handling required for separation of NifU and NifS. From these results, we conclude NifS is not required to achieve effective transfer once transient [Fe-S] clusters have been assembled on the NifU scaffold.

In vitro activation of the apo-Fe protein by altered forms of NifU

Once it was established that a high level of *in vitro* activation of the apo-Fe protein could be achieved, the efficiency of this process was examined by titration experiments where the molar ratio of cluster-loaded NifU and apo-Fe protein was varied. The results (Figure 3) reveal a sigmoidal, NifU concentration-dependent activation where maximum activity is obtained at an approximately equi-molar concentration of NifU and apo-Fe protein. It is not yet known if the sigmoidal activation pattern recognized at low NifU concentrations is mechanistically relevant, although this pattern is always observed.

In separate experiments the same type of titration was performed using altered forms of NifU for which the three N-terminal cysteines are substituted by alanine, or for which these residues and a cysteine located in the C-terminal domain are all substituted by alanine. The results of these experiments show that functional inactivation of the proposed N-terminal scaffold diminishes, but does not eliminate, the capacity for *in vitro* activation of apo-Fe protein (Figure 3). A sigmoidal pattern of activation was also observed when the molar ratio of the altered NifU and apo-Fe protein was varied. The same results were obtained when altered forms of NifU for which any of the three conserved cysteines within the N-terminal domain have been individually substituted by alanine were used (data not shown). In contrast, inactivation of potential assembly sites within both the N-terminal and C-terminal domains by amino acid substitution eliminates *in vitro* apo-Fe protein activation (Figure 3). The difference between the *in vitro* effects

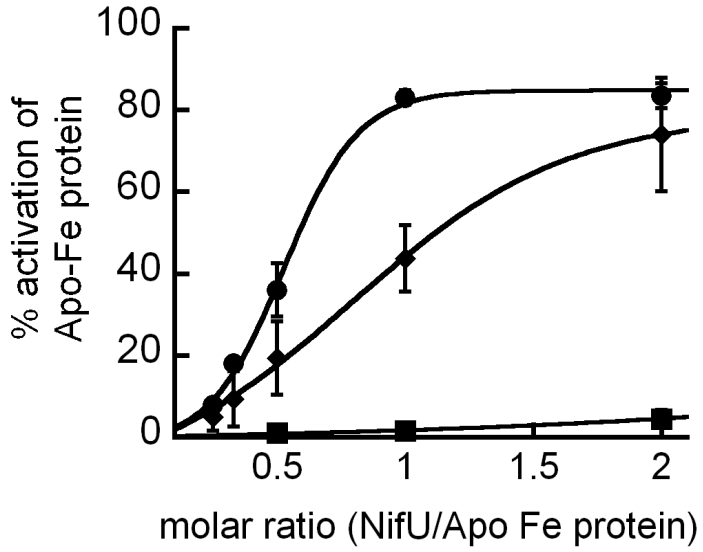

Figure 3. Titration of apo-Fe protein activation with increasing concentrations of NifU or altered forms of NifU used as scaffolds for in vitro [Fe-S] clusters biosynthetic reactions. The apo-Fe protein concentration was kept constant at 5.6 μM and the concentration of the [Fe-S] cluster biosynthetic reaction containing was varied to give the NifU/apoFe protein indicated on the X-axis. Details of the components contained in the [Fe-S] cluster assembly cocktail, apo-Fe protein activation, and assay conditions for measurement of apo-Fe protein activation are described in *Experimental Methods*. Samples used for activation of apo-Fe protein had the following forms of NifU: Closed circles, wild type NifU (produced by pDB1289); Diamonds, NifU having three N-terminal domain cysteines substituted by alanine (residues 35, 62, 106, produced by pDB1341); Squares, NifU having three N-terminal domain cysteines substituted by alanine and one C-terminal domain cysteine substituted alanine (residues 35, 62, 106, and 275, produced by pDB1355).

on inactivation of both proposed [Fe-S] cluster biosynthetic sites when compared to the *in vivo* effects are noteworthy. Our interpretation of these results is that the more severe effect recognized in *in vitro* experiments reflects that an alternative biosynthetic pathway, which operates *in vivo*, is not duplicated in the *in vitro* system.

4.4 – Discussion

The results of genetic, physiological and biochemical experiments described here provide evidence that the NifU family of proteins have the capacity to provide two separate [Fe-S] cluster assembly scaffold functions. Biochemical activation experiments indicate that the proposed assembly scaffold located within the N-terminal domain is a more efficient donor for *in vitro* apo-Fe protein activation and this result is consistent with genetic experiments that establish a minor role for the C-terminal domain under the physiological conditions used in the present work. In fact, this and other work revealed that a physiological phenotype associated with inactivation of the C-terminal domain function is only manifested when the N-terminal domain is also inactivated. The results with the bacterial system are in agreement with previous work with yeast, where it was shown that a combination of *isu1* and *nfu1* deletions is required to elicit a phenotype in that organism (Schilke et al., 1999). The yeast *isu1* gene encodes a protein analogous to the N-terminal domain within NifU and the separate *nfu1* gene encodes a protein analogous to the C-terminal domain of NifU. Genetic experiments described here also indicate there is at least one other scaffold, in addition to those contained within NifU, that can be used at a very low level for nitrogenase-associated [Fe-S] cluster assembly. Namely, inactivation of both the N-terminal and C-terminal domains of NifU does not eliminate nitrogenase maturation. One possibility we have considered is that the product of a gene located immediately upstream from *nifU*, designated ^{Nif}IscA, which has also been shown to be capable of serving as an [Fe-S] cluster assembly scaffold *in vitro* (Krebs et al., 2001), could serve this function. To test this possibility a strain was constructed where a 93 base-pair deletion within ^{Nif}*iscA* (12) was placed in combination with substitutions that inactive both the N-terminal and C-terminal domains of NifU. This strain is not further impaired in diazotrophic growth so it seems unlikely that ^{Nif}IscA

significantly contributes to nitrogenase maturation under the conditions used in the present work (our unpublished data). Another possibility is that, as previously suggested, the *isc*-encoded system could make some very minor contribution to nitrogenase maturation, but this possibility has not been tested because deletion of the *A. vinelandii isc* operon is lethal (Zheng et al., 1998). Yet another possibility is that an *A. vinelandii* gene, we designate *nfuA*, which encodes a product bearing primary sequence homology to the C-terminal domain of NifU, could contribute to nitrogenase-specific Fe-S cluster biogenesis in the absence of intact NifU function. It is interesting that the *A. vinelandii nfuA* gene product, and certain other bacterial *nfuA* gene products, also exhibit a significant level of primary sequence identity within their N-terminal coding regions when compared to IscA. It therefore appears that the bacterial *nfuA* gene product, like NifU, is a modular protein, although its function is not yet known. It should be noted that the C-terminal region of the bacterial *nfuA* gene product is also similar to the human Nfu1 protein, which has already been shown to provide a scaffold for Fe-S cluster biosynthesis *in vitro* (17).

A physiological explanation for why *A. vinelandii* has a specific pathway for nitrogenase [Fe-S] cluster assembly is most likely related to the fact that the nitrogenase catalytic components – Fe protein and MoFe protein - represent a high fraction of the total cellular protein under nitrogen-fixing conditions and both have [Fe-S] clusters. Thus, it would appear that diazotrophic growth conditions place a high demand on the mobilization of Fe and S required for the activation of the nitrogenase component proteins. The probable channeling of S towards the assembly of [Fe-S] proteins involved in intermediary metabolism, for example, aconitase, versus S targeted for maturation of the nitrogenase component proteins is further highlighted by the fact *A. vinelandii* encodes *nif*-specific and *isc*-specific O-acetyl serine synthases (Zheng et al., 1998), which are genetically distinct from the *cysE* encoded O-acetyl serine synthase required for general cysteine biosynthesis. O-acetyl serine synthase catalyzes the rate-limiting step in cysteine biosynthesis (Denk & Bock, 1987).

Why there are multiple scaffolds for nitrogenase-specific [Fe-S] cluster assembly, two contained within NifU, is much less clear. There are a number of possible explanations that bear on this question. One possibility is that one scaffold operates primarily in the *de novo* synthesis of nitrogenase [Fe-S] clusters, whereas another might operate primarily in the reassembly of [Fe-S] clusters that are damaged during metabolism. A second possibility is that different scaffolds could be more efficient under different metabolic conditions, for example Fe or S limitation. Third, there are many *nif*-specific proteins, other than the catalytic components, that contain [Fe-S] clusters. Among these are included NifEN, NifB, NifQ, and a variety of ferredoxins (Dos Santos et al., 2004a). Thus, separate [Fe-S] cluster scaffolds present within NifU could be related to target specificity during the maturation of the various [Fe-S] proteins that participate in nitrogen fixation. If there is such target specificity related to the N-terminal and C-terminal domains of NifU, the present work indicates there must also be some functional "cross-talk" between them. Finally, there could be some functional relationship between cluster assembly that occurs on the N-terminal and C-terminal domains. For example, [Fe-S] clusters assembled at one site might be required for the effective assembly at a second site, or, perhaps cluster transfer could occur between sites to achieve the most efficient assembly pathway. Experiments described here do not distinguish among these possibilities but the same combination of genetic and biochemical strategies, together with biophysical analyses, can now be used to address these issues.

Finally, a method for the rapid and efficient *in vitro* transfer of [Fe-S] clusters assembled on the NifU scaffold to a known physiological target protein has been developed. The results indicate that, in the case of nitrogenase maturation, accessory molecular chaperones or other protein components do not appear to be required. This situation can be compared to a report of *in vitro* IscU-directed [Fe-S] cluster transfer, which is comparatively slow and inefficient, requiring a large molar excess of cluster-loaded IscU relative to the target protein (Wu et al., 2002). While such comparisons do not necessarily indicate that previous IscU-directed [Fe-S] cluster transfer experiments are physiologically irrelevant, it does suggest that molecular chaperones and nucleotide

hydrolysis associated with the Isc-specific [Fe-S] cluster biosynthetic machinery are likely to be required for effective transfer of [Fe-S] clusters to the appropriate target proteins and/or assembly of the correct [Fe-S] cluster type. This suggestion is supported by genetic experiments that have shown an important physiological role for HscB and HscA in the maturation of [Fe-S] proteins such as aconitase and glutamate synthase (Takahashi & Nakamura, 1999; Tokumoto & Takahashi, 2001).

CHAPTER 5

NifU and NifS are required for the maturation of nitrogenase and cannot replace the function of *isc*-gene products in *Azotobacter vinelandii*

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This manuscript describes experiments that demonstrate that the *nif*-specific [Fe-S] cluster biosynthetic system from *Azotobacter vinelandii*, which is required for nitrogenase maturation, cannot functionally replace the *isc* [Fe-S] cluster system used for the maturation of other [Fe-S] proteins. This chapter was written and submitted for publication with the intention for its use to satisfy a portion of the research completed for this dissertation. As a co-author, I constructed the strains that contain a copy of *nifU* and *nifS* under control of sucrose promoter. Additionally, I was responsible for the construction of the strain containing amino acid substitution at the potential chaperone-binding motif. I also was involved in verifying the ability of NifU replace the function of IscU. I had participated in the writing the document at all stages of its preparation, and preparing the figures included in this chapter.

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5.1 - Introduction

Simple complexes of iron and inorganic sulphide ([Fe-S] clusters) are contained in a diverse group of proteins, called [Fe-S] proteins, which participate in a wide variety of cellular processes, including electron transfer, catalysis, and regulation of gene expression. Such functional versatility of [Fe-S] proteins is related to the structural and electronic plasticity of their cognate [Fe-S] clusters. The most familiar [Fe-S] clusters include [2Fe-2S] and [4Fe-4S] clusters, which are usually covalently attached to their protein partners through cysteine mercaptide ligands. In spite of their structural simplicity, the formation and insertion of [Fe-S] clusters into their protein partners is a complicated process.

Initial insights about the pathway for [Fe-S] cluster assembly were gained through analysis of *Azotobacter vinelandii* genes required for activation of nitrogenase, the catalytic component of biological nitrogen fixation. Nitrogenase comprises two catalytic partners, called the Fe protein and the MoFe protein, and both of these are [Fe-S] proteins (Christiansen et al., 2001). A biochemical-genetic analysis of nitrogen-fixation-specific (*nif*) genes required for nitrogenase maturation revealed that two of them, *nifU* and *nifS*, are uniquely required for the activation of both the Fe protein and the MoFe protein (Dos Santos et al., 2004a; Jacobson et al., 1989). Subsequent studies suggested that NifS is a cysteine desulphurase that uses pyridoxal-phosphate chemistry to activate S in the form of an enzyme-bound persulphide (Zheng et al., 1993) and that NifU provides a molecular scaffold for assembly of "transient" [Fe-S] cluster units destined for nitrogenase maturation (Agar et al., 2000). Key observations used to validate this model include: (*i*) NifU and NifS are able to form a transient macromolecular complex (Yuvaniyama et al., 2000), (*ii*) co-incubation of NifU and NifS in the presence of L-cysteine and Fe^{+} results in the formation of labile [Fe-S] clusters on NifU (Yuvaniyama et al., 2000), (*iii*) [Fe-S] cluster-loaded NifU can be used for the effective *in vitro* activation of apo-Fe protein (Dos Santos et al., 2004b), and (*iv*) placement of certain amino acid substitutions within NifU results in trapping of the transient [Fe-S] cluster on the NifU scaffold (Yuvaniyama

et al., 2000), thereby compromising the capacity for both *in vivo* and *in vitro* nitrogenase activation (Dos Santos et al., 2004b).

5.2 - The *isc* and *suf* systems also have [Fe-S] cluster biosynthetic functions

Although genetic inactivation of either NifU or NifS results in a dramatically lowered capacity for the *in vivo* maturation of nitrogenase, loss of NifU or NifS function does not completely eliminate the capacity for nitrogen fixation (Jacobson et al., 1989). This result indicated that NifU or NifS activities could be replaced at low levels by some other cellular activities. A search for other cellular components having NifU-like and NifS-like activities resulted in the identification of a group of genes proposed to be required for the maturation of other [Fe-S] proteins, for example, aconitase, that are not related to nitrogen fixation. This gene cluster (Figure 1), designated "*isc*" (*i*ron-*s*ulphur-*c*luster), encodes proteins having functions analogous to NifU (IscU) and NifS (IscS), as well as several other proteins including an alternative scaffold (IscA), molecular chaperones (HscB and HscA), a ferredoxin (Fdx), and a negative regulator (IscR) (Zheng et al., 1998). Genes encoding homologs to IscS, IscU, IscA, HscB, HscA and Fdx are widely distributed in nature and a variety of genetic studies have clearly implicated all of them in some aspect of the maturation of [Fe-S] proteins (Muhlenhoff & Lill, 2000; Tokumoto & Takahashi, 2001). A third type of [Fe-S] protein maturation machinery was identified in *Escherichia coli*, which has been designated "*suf*" (Takahashi & Tokumoto, 2002). In the case of *E. coli*, which also has an intact *isc* gene cluster, genetic and physiological studies have established that the *isc* system operates under "normal" growth conditions whereas the *suf* system operates under conditions of Fe limitation or oxygen stress (Outten et al., 2004). Although there appears to be specialized components that differentiate the three identified [Fe-S] cluster biosynthetic machineries they are

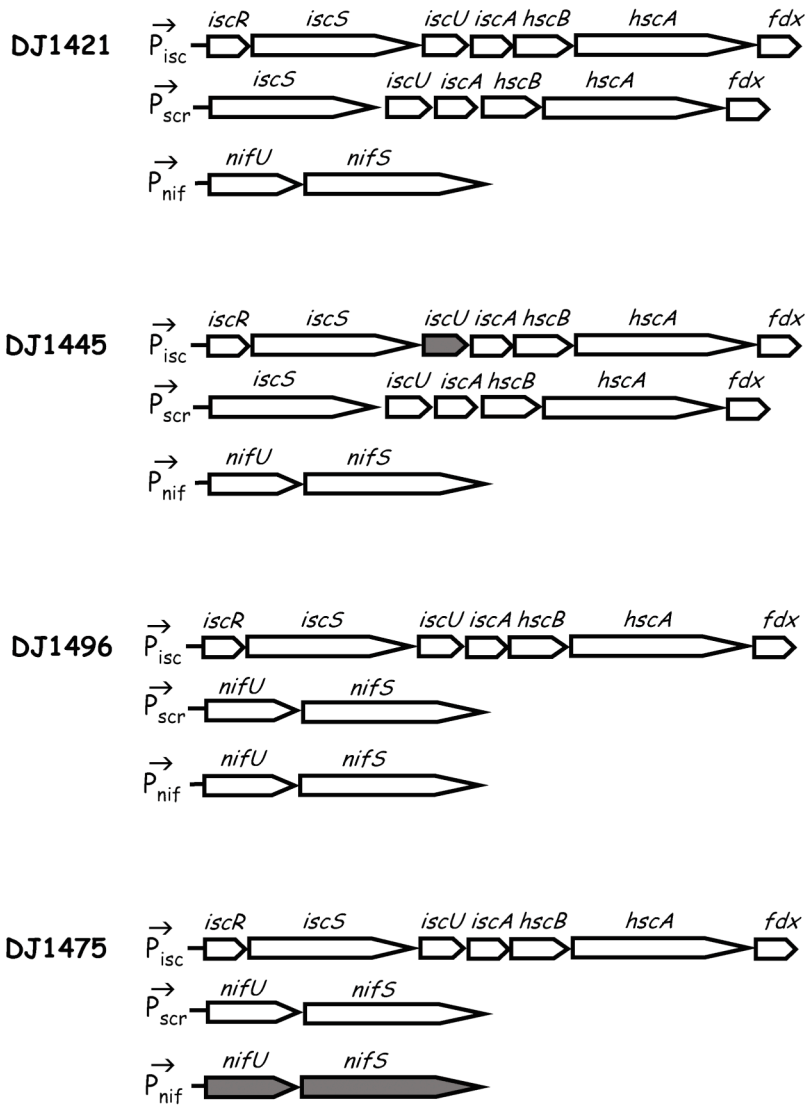

Figure 1. Organization of the *isc* gene cluster and *nifUS* genes in *A. vinelandii* strains used in this work. Individual strains are designated DJ1421, DJ1445, DJ1496 and DJ1475. The promoters designated P*isc* and P*nif* control expression of the normal *isc* and *nif* gene clusters. P*scr* is a sucrose-inducible promoter and it controls expression of the duplicated version of the genes indicated. In frame deletions are showing in shaded colors.

unified by an apparent requirement for a cysteine desulphurase and [Fe-S] cluster assembly scaffold.

The picture that has emerged concerning [Fe-S] protein maturation is that some organisms have generalized "housekeeping" [Fe-S] cluster biosynthetic machinery as well as other more "specialized" [Fe-S] cluster biosynthetic machinery. However, whether or not a particular type of [Fe-S] cluster biosynthetic machinery, *isc*, *suf*, or *nif*, operates in a "housekeeping" capacity or in a "specialized" capacity appears to depend on a particular organism. Indeed, for *Helicobacter pylori* and *Thermatoga maritima* the *nif*-like and *suf*-like systems, respectively, appear to be the only intact [Fe-S] cluster biosynthetic systems available to these organisms (Johnson et al., 2004b). Interesting questions attached to the role of various [Fe-S] cluster biosynthetic machineries involve their evolutionary and functional relationships.

5.3 - Controlled expression of *isc* and *nif* genes

As mentioned above the capacity for *nifU* or *nifS* deletion strains to fix nitrogen at a very low level indicated their functions could be partially supplanted by some other [Fe-S] cluster biosynthetic system (Jacobson et al., 1989). Although this hypothesis led to the discovery of the *isc*-gene cluster, the possibility that *isc* gene products can participate in the maturation of nitrogenase could not be directly tested because genetic experiments indicated that inactivation of the *isc* genes is lethal (Zheng et al., 1998). This situation also precluded the opportunity to examine whether or not a *nif*-specific [Fe-S] cluster biosynthetic component can functionally replace an *isc*-specific component. In order to overcome this problem and to develop an opportunity to examine the specific functions of different components of the *isc*-specific [Fe-S] cluster biosynthetic machinery we developed a method for the controlled expression of individual *isc*-specific and *nif*-specific components uncoupled from their normal regulatory components.

In *A. vinelandii* and many other bacteria, the *isc* genes are controlled by a negative feedback mechanism where the holo-form of an [Fe-S] cluster-containing regulatory protein (encoded by *iscR*, see Figure 1) represses transcription of the *isc* gene cluster (Frazzon & Dean, 2001; Schwartz et al., 2001). The *nif* genes are controlled by a complex regulatory cascade involving regulatory proteins encoded by *nifA* and *nifL* and are only expressed under conditions that required nitrogen fixation (Little et al., 2000). In order to uncouple *isc*- or *nif*-regulated components from their normal regulatory elements, the isolated genes were placed under the control of a sucrose-inducible (*scr*) promoter *in vitro* by using recombinant techniques and subsequently reintegrated into the chromosome in single copy by using reciprocal recombination (Figure 1). Details of the genetic constructions will be reported elsewhere. The *scr* promoter is negatively regulated by the availability of sucrose in the same way that the *lac* promoter is controlled by the availability of lactose. These constructions resulted in duplication of the genes of choice (Figure 1, strain DJ1421 and strain DJ1496), where expression of one copy is controlled by the normal regulatory elements and the *scr* promoter controls expression of the second copy.

5.4 - NifU and NifS cannot functionally replace IscU and IscS

Control experiments established that genes regulated by *scr* are expressed at a relatively high level when sucrose is present in the growth medium but are not expressed at detectable levels in the absence of sucrose. This situation permitted the placement of deletions within the normal copy of a particular gene (see Figure 1, strain DJ1445 and strain DJ1475) whose function is replaced by the second copy, providing that cells are grown in the presence of sucrose under conditions where the function of the deleted gene is required. When sucrose is removed from the growth medium, in this case replaced by glucose, products of genes controlled by the *scr* promoter are gradually depleted from the cell. In this way the physiological and biochemical consequences of the loss of function of a particular gene product can be unambiguously evaluated. The results of controlled

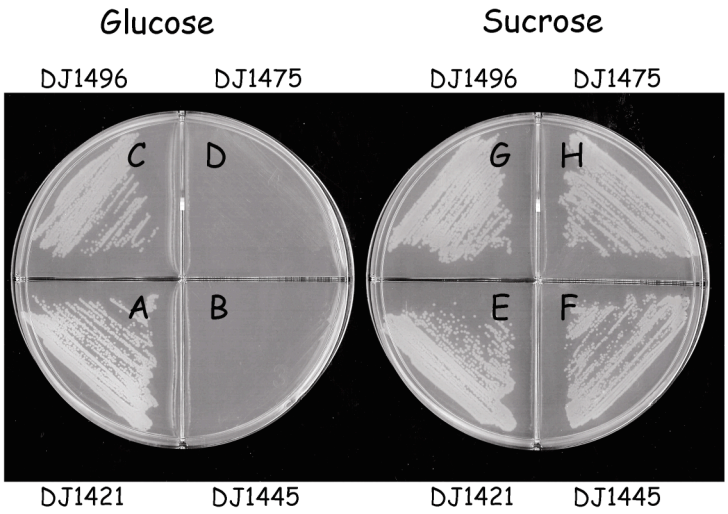

Figure 2. Growth of *A. vinelandii* strains cultured under different conditions. Details of the growth conditions are described in the text.

expression experiments are shown in Figure 2. For the experiments shown in Figure 2, all cells were cultured in a medium that does not contain any nitrogen source so the cells must be capable of performing nitrogen fixation in order to grow. All strains show normal growth when cultured in the presence of sucrose (Figure 2, panels E, F, G and H). However, in the case of DJ1445 there is no growth when cells are depleted for IscU (Figure 2, panel B). In the case of strain DJ1445, depletion of IscU eliminates the capacity for growth under nitrogen-fixing conditions (Figure 2 panel B) or when a fixed nitrogen source is added to the growth medium (data not shown). These results suggest that IscU is essential under both growth conditions and that NifU cannot functionally replace IscU.

A possible explanation for the inability of NifU to functionally replace IscU is that NifU is sequestered into a macromolecular *nif*-specific complex so that it is not available for other cellular processes. To test this possibility strain DJ1496 was constructed, which contains two copies of the *nifU* and *nifS* genes, one copy whose expression is under *nif* control and the other copy under *scr* control. This strain was then used as a recipient in genetic transformation experiments where we attempted to separately delete the *iscU* gene and the *iscS* gene. In these experiments, neither the *iscU* gene nor the *iscS* gene could be deleted, even under conditions where *nifU* and *nifS* are expressed independently from the *nif*-specific components and in the absence of other *nif*-specific components. In order to confirm that *nifU* and *nifS* are actually expressed when regulated by the *scr* promoter, a derivative of DJ1496 was constructed, where the *nif*-regulated copies of *nifU* and *nifS* are deleted (Figure 1, strain DJ1475). This strain is capable of growing under nitrogen-fixing conditions when cultured in the presence sucrose (Figure 2, panel H), but not when cultured in the absence of sucrose (Figure 2, panel D), establishing that the *scr*-regulated *nifU* and *nifS* gene products have functional activity.

Although the specific function of the molecular chaperones, HscB and HscA (Figure 1) is not understood, they are required for *isc*-directed [Fe-S] cluster assembly and IscU is known to specifically interact with an HscBA complex (Hoff et al., 2000).

This interaction is dependent upon an oligopeptide sequence within IscU (LPPVK) which is necessary and sufficient to stimulate intrinsic HscA-directed ATPase activity (Hoff et al., 2002). Comparison of IscU and NifU primary sequences shows that the LPPVK signature sequence within IscU is replaced by LPPEK in NifU (Hoff et al., 2002; Johnson et al., 2004a). We therefore considered a second possible explanation for the inability of NifU to functionally replace IscU. Namely, that NifU does not productively interact with the molecular chaperones HscBA, and that such a specific interaction might be required for maturation of [Fe-S] proteins other than the nitrogenase components. To test this possibility, the LPPEK sequence in NifU was converted to the canonical LPPVK sequence within IscU, and experiments similar to those already described were repeated. However, this modification did not endow NifU with an ability to functionally replace IscU. In aggregate these experiments establish that, under the conditions used here, there is a high degree of specificity for [Fe-S] cluster assembly components in *A. vinelandii*. In particular, the *nif*-specific [Fe-S] cluster assembly components are required to maintain an active nitrogenase and cannot be effectively used to replace the *isc*-specific [Fe-S] cluster assembly components required for the maturation of other [Fe-S] proteins, such as aconitase.

Our results are relevant to a recent report by Takahashi and co-workers where it was shown that heterologous expression of a "*nif*-like" [Fe-S] cluster biosynthetic system from *Entamoeba histolytica* could replace the function of the *suf*- or *isc*-type of [Fe-S] cluster biosynthetic systems, but only under anaerobic conditions (Ali et al., 2004). This finding is in line with the suggestion that *nif*-like [Fe-S] cluster biosynthetic systems from non-nitrogen-fixing organisms, such as *E. histolytica* and *H. pylori,* do not have a specialized function but instead, are utilized for "housekeeping" [Fe-S] protein maturation in these organisms. It therefore appears that, in spite of considerable primary sequence identity among members of *nif*-like and genuine *nif*-specific [Fe-S] cluster biosynthetic systems, the *nif*-specific system (at least for *A. vinelandii*) has evolved an exquisite function specialized for nitrogenase maturation. We believe it should be possible to exploit primary sequence differences between components of *nif*-like and *nif*-specific [Fe-S] cluster biosynthetic systems, differences in their corresponding three-

dimensional structures (when they become available), as well as genetic strategies, to determine the basis for target specificity, a feature that is not yet understood for any [Fe-S] protein maturation process.