Assembly of Iron-Sulfur Clusters *in Vivo*

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ABSTRACT

Iron-sulfur [Fe-S] clusters are protein cofactors that facilitate various life-sustaining biological processes. Their *in vivo* assembly is accomplished by three different systems known to date. These are: the NIF system which provides [Fe-S] clusters for nitrogenase and other nitrogen-fixing proteins, the SUF system which is induced during conditions of oxidative stress and iron starvation in *E. coli,* and the ISC system which serves as the housekeeping assembly apparatus. The latter is the focus of this dissertation and includes the proteins IscR, IscS, IscU, IscA, HscB, HscA, Fdx, and IscX. IscU is purified in its cluster-less (apo) form, but can serve as a scaffold to assemble [Fe-S] clusters *in vitro* in the presence of excess iron and sulfide. To test the scaffold hypothesis and gain insight into the events that occur during [Fe-S] cluster assembly and delivery, we developed two methods that allow the isolation of IscU and other ISC proteins *in vivo*. In the first method, *Azotobacter vinelandii* IscU is isolated from its native host, whereas in the second, it is isolated recombinantly from *E. coli* using a vector that allows expression of the entire *isc* operon. We found that IscU exists *in vivo* in two forms: apo-IscU and [2Fe-2S]$^{2+}$ cluster-loaded IscU which are believed to be conformationally distinct. Both transient and stable IscU-IscS complexes were identified, indicating that the two proteins interact *in vivo* in a manner that involves their association and dissociation. The [2Fe-2S]$^{2+}$-IscU species was present as a single entity, whereas significant amounts of apo-IscU were found associated with IscS, suggesting that IscU-IscS dissociation is triggered by the completion of [2Fe-2S] clusters. Both apo and [2Fe-2S]$^{2+}$-IscU were predominantly monomeric whereas IscU-IscS complexes were determined to have an $\alpha_2\beta_2$ composition. IscU was purified in the absence of the chaperones HscA and HscB and was also shown to accommodate a [2Fe-2S]$^{2+}$ cluster similar to the one bound to IscU isolated from wild type cells. The findings suggest that [2Fe-2S]$^{2+}$-IscU exists in one conformation *in vivo* and that any conformational changes on IscU are exerted after

[2Fe-2S] cluster formation. *In silico* studies showed that a flexible loop containing the conserved LPPVK motif, which is responsible for interactions with HscA, may facilitate cluster exposure to either mediate its delivery to acceptor proteins or participation in the construction of [4Fe-4S] clusters. Experiments with NfuA, a protein similar to the C-terminal domain of NifU, demonstrated that NfuA and similar proteins might serve as [Fe-S] cluster carriers to accomplish the efficient delivery of nascent cofactors to the various recipient proteins.

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ABBREVIATIONS

BSA	Bovine serum albumin
CD	Circular dichroism
DTT	Dithiothreitol
EPR	Electro-paramagnetic resonance
IMAC	Immobilized metal affinity chromatography
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonylfluoride
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
VTMCD	Variable-temperature magnetic circular dichroism

CHAPTER 1. Introduction

Nature employs a number of protein prosthetic groups to facilitate chemical reactions. Inorganic cofactors known as iron-sulfur ([Fe-S]) clusters are structurally and functionally diverse prosthetic groups. They have been proposed to be descendants of ancient FeS and FeS_2 catalytic surfaces that evolved structurally with time and became incorporated into proteins to facilitate biochemical processes (Wachtershauser, 2000). Although a number of [Fe-S] cluster structures have been identified in nature, the most common ones utilized by proteins are the [2Fe-2S] and the [4Fe-4S] clusters. These endow proteins with several abilities including electron transfer, catalysis, substrate modification and activation, environmental sensing (oxygen and iron), sulfur donation, and structural stabilization (Johnson *et al.*, 2005a). Because of their widespread presence in nature, [Fe-S] clusters are involved in vital biological processes including respiration, nitrogen fixation, photosynthesis, and DNA repair amongst others.

Despite their paramount significance in nature, the biological synthesis of [Fe-S] clusters is a relatively new area of study that has only received significant attention in the past decade. It has been well established that [Fe-S] clusters can assemble on proteins spontaneously from the addition of Fe^{2+} and S^{2-} ions *in vitro* (Malkin & Rabinowitz, 1966). This phenomenon is unlikely to occur *in vivo*, however, due to the toxicity associated with free iron and sulfur in the cell. Thus, the concept arose that cluster synthesis is a protein-mediated process that limits the poisonous effects of inorganic iron and sulfur. Multiple proteins have been demonstrated or proposed to be involved in the biogenesis of iron-sulfur clusters (Figure 1.1). This dissertation describes the development of two methods that allow the study of [Fe-S] cluster formation *in vivo*. The model organism of choice is the obligate aerobe, nitrogen-fixing bacterium *Azotobacter vinelandii*, known for its genetic malleability, facile culturing, and convenient growth rates.

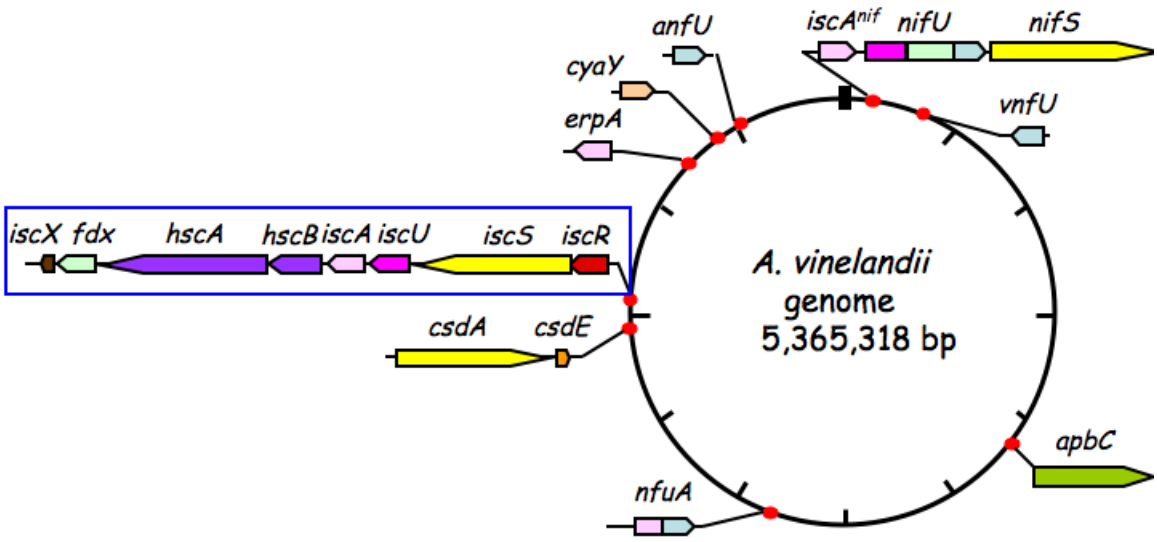

Figure 1.1. *Azotobacter vinelandii* genes involved or proposed to be involved in [Fe-S] cluster biosynthesis. Black circle represents *A. vinelandii* chromosome. Genes are represented by thick arrows and have been located relative to the origin of replication (solid black rectangle at top of circle). Gene locations were identified thanks to genome sequencing and annotation studies (unpublished, manuscript in preparation). Genes or gene domains with the same color indicate sequence conservation between the genes. Orientation of the arrow represents orientation of gene transcription and translation. The *isc* operon is surrounded by a blue rectangle.

There are three systems or "machineries" known to date that synthesize [Fe-S] clusters. These are the NIF (nitrogen fixation), ISC (iron-sulfur cluster), and SUF (sulfur mobilization) machineries and have been mainly studied in *Azotobacter vinelandii*, *Escherichia coli*, and *Saccharomyces cerevisiae*. It can be generally said that: 1) the NIF system is expressed during nitrogen fixing conditions and provides [Fe-S] clusters for nitrogenase and other proteins involved in nitrogen fixation; 2) the ISC system assembles [Fe-S] clusters for general "housekeeping" target proteins; and 3) the SUF system is utilized by the cell during stressful conditions such as oxidative damage or iron limitation. Although these three systems differ in composition, gene organization, and conditions of expression, they all utilize the following pair of proteins that comprise the minimum set required for [Fe-S] assembly. With the exception of some archaea, all known living organisms produce a pyridoxyl-5'-phosphate (PLP)-dependent cysteine desulfurase protein, which removes S from the amino acid cysteine and donates it to a proposed scaffold protein upon which nascent [Fe-S] clusters are built (Boyd *et al.*, 2008a). The *Azotobacter vinelandii* NIF proteins (NifS cysteine desulfurase and NifU scaffold) exemplify the basic two-component apparatus required for [Fe-S] cluster biogenesis.

The ISC and SUF systems are more complicated in that they require additional components to assemble and deliver clusters. IscS and IscU proteins act *in vitro* as a cysteine desulfurase and assembly scaffold respectively. In *A. vinelandii* and other bacteria, they are encoded by genes that are part of a larger operon, which consists of eight total genes: *iscR, iscS, iscU, iscA, hscB, hscA, fdx,* and *iscX* (Figure 1.1). The roles of the rest of the ISC proteins in [Fe-S] cluster assembly or delivery are either unknown or are debated among experts in the field. For instance, IscA has been proposed to act as an iron donor, alternative assembly scaffold, and [Fe-S] cluster transporter. HscA and HscB resemble the *E. coli* DnaK and DnaJ chaperones in primary and tertiary structure and have thus been suggested to also have a molecular chaperone function. Recent evidence has linked the chaperones with a role in [Fe-S] cluster transfer from IscU to target proteins, although the mechanism has not been established. The role of the ferredoxin Fdx is also not understood, but it has been proposed to serve as an electron source during [Fe-S] cluster assembly. The ISC system is the most widespread [Fe-S]

cluster biosynthetic system and its components are strongly conserved among prokaryotes and eukaryotes. A more extensive description of the literature associated with the ISC system is provided in Chapter 2.

The SUF system of *E. coli* is also comprised of multiple components. The *sufABCDSE* operon also produces the pair of a proposed scaffold (SufA or SufB) and a cysteine desulfurase (SufS). Unlike the ISC system, which is the most widespread in nature, the SUF system is employed by fewer species, mostly bacteria and photosynthetic organisms. In *E. coli*, the SUF proteins are utilized during stressful conditions (Outten *et al.*, 2004). In other organisms, the SUF system appears to be the sole machinery that generates [Fe-S] clusters for its proteins.

Why are there so many different systems to perform the same work? It has not been demonstrated which system arose first in evolution or whether they developed independently from one another. Nevertheless, the strong conservation of the systems among all organisms is indicative of the indispensable nature of [Fe-S] clusters and the need to assemble them such that the poisonous effects by their free inorganic parts are avoided.

Despite the wealth of findings on bacterial and eukaryotic [Fe-S] cluster production during the past decade, the specific mechanism by which the ISC members work together to form and deliver intact [Fe-S] clusters is still unclear. All biochemical and biophysical studies performed so far have utilized ISC proteins that have been recombinantly expressed independently from the rest of the *isc* operon. IscU has been shown *in vitro* to assemble both $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ clusters using Fe^{+2}, L-cysteine, and catalytic amounts of IscS under anaerobic conditions (Agar *et al.*, 2000a, Chandramouli, 2007). However, the observation that almost all the proteins produced by the ISC system affect the maturation of [Fe-S] proteins and the strong conservation of the *isc* genes in most organisms suggest that the ISC system works cooperatively and interdependently. Moreover, recent reports have demonstrated that additional non-ISC components are also involved or proposed to be involved in [Fe-S] cluster assembly or transfer (Bandyopadhyay *et al.*, 2008c, Boyd *et al.*, 2008c).

There are several deficiencies associated with using *in vitro* methods to study the mechanism of [Fe-S] cluster assembly. The major concern is the use of heterologous

systems to produce proteins, which results in decoupled and disproportionate expression compared to the rest of the *isc* operon. This has resulted in the isolation of proteins in their cluster-less or apo form. The holo, i.e. cluster-loaded, form of a protein is accomplished with *in vitro* reconstitution. Knowing that [Fe-S] clusters can form spontaneously in the presence of excess Fe^{+2} and S^{-2}, the possibility of dealing with artifacts needs always to be considered. Heterologous expression may affect the protein fold and oligomeric state which may play significant roles in a process that undergoes a series of mechanistic stages. Moreover, the protein is not purified from its natural host which may result in failure to identify other components, unique to that organism, that might be involved in the final fold of the protein or the cofactor associated with it.

In contrast, *in vivo* studies allow the identification of intermediate ISC species that might be involved in the mechanism of [Fe-S] cluster assembly. They also allow the detection of physiological interactions between the ISC players or other unidentified, cellular components such as non-ISC proteins or small molecules such as glutathione. The ISC machinery employs multiple components and undergoes a series of stages to accomplish the maturation of cellular [Fe-S] proteins, very much like a factory that utilizes multiple people and equipment to develop a product from raw materials and ensure its intact delivery to the appropriate clientele. Therefore, when studying the process of [Fe-S] cluster assembly, it is critical to take into account effects that might be exerted by all the components involved in the process. Thus far, no studies have been conducted to biochemically characterize ISC proteins that have been isolated *in vivo*, from a background wherein all factors that may be involved in [Fe-S] cluster assembly are present.

The major theme of this dissertation is the study of formation of iron-sulfur clusters on the proposed assembly scaffold IscU *in vivo.* Specifically, this work describes the development of two *in vivo* methods to isolate and perform biochemical studies of ISC proteins. In addition to the characterization of clusters formed on IscU, the identification of intermediate species and the interplay between IscU and the other ISC components are discussed. The aspect of cluster transfer from IscU to apo-target proteins is also investigated.

The remainder of this introductory chapter provides a brief overview of the chapters included in this dissertation. Chapter 2 provides a literature review, in which structural and functional features of [Fe-S] clusters are described followed by a history of the discovery of the NIF and ISC iron-sulfur cluster biosynthetic systems. An extensive review of previous studies associated with IscU and other ISC proteins is provided. The roles of non-ISC proteins that have been shown or proposed to be involved in [Fe-S] cluster biosynthesis are also described. This is followed by a brief discussion about cross-talk between the three known [Fe-S] cluster biosynthetic machineries. Although the majority of the literature review refers to studies performed with prokaryotic organisms, findings from work with eukaryotic organisms as well as human diseases associated with defects in iron-sulfur cluster assembly are also discussed.

Chapter 3 describes the isolation of IscU *in vivo* from *Azotobacter vinelandii* using a genetic setting that allows the elevated production of the ISC proteins at levels that exceed physiological demand. IscU is isolated bound with a labile $[2Fe\text{-}2S]^{2+}$ cluster, thus providing *in vivo* evidence for the role of IscU in the cell as a scaffold. Intermediate species of IscU are identified including an IscU variant, which is isolated in a tight complex with IscS, the cysteine desulfurase. The findings of this work demonstrate that *in vivo* IscU acts as a scaffold, assumes distinct forms, and maintains a dynamic relationship with IscS that involves their association and dissociation during cluster synthesis.

Chapter 4 describes the development of a method that allows the isolation of ISC proteins *in vivo* by using an *E. coli* recombinant system. The plasmids used to express the ISC proteins contain all the *isc* genes under control of the arabinose regulatory elements. This method results in protein yields that are 12-fold higher compared to yields obtained with the method used in Chapter 3. In addition to characterizing IscU from a wild type genetic background, IscU is also isolated from a background where HscA and HscB are inactivated in order to gain insight into the role of the chaperones in [Fe-S] cluster assembly and/or delivery.

In Chapter 5, the focus switches to the aspect of cluster transfer from scaffold proteins to apo-target proteins. The main body of the chapter describes the biochemical characterization of a $[4Fe\text{-}4S]^{2+}$ cluster formed on NfuA *in vitro* and its transfer to apo-

aconitase, a [4Fe-4S] cluster-containing enzyme. NfuA is a modular protein, the C-terminus of which exhibits similarities with the C-terminal domain of NifU. In this work, it is demonstrated that a [4Fe-4S] cluster formed on NfuA can be transferred to apo-aconitase at a rate that is 3-fold higher compared to the rate of transfer from IscU to apo-aconitase. These findings led to the proposed role of NfuA as an intermediate cluster carrier.

The last chapter, Chapter 6, summarizes the findings obtained through the experiments conducted for this dissertation and the major conclusions drawn from their results. A comparison between current *in vivo* findings and previous *in vitro* work outlines the differences and similarities between the two methods.

Several appendices follow the main body of this dissertation. Appendix I provides preliminary findings associated with *in vivo* isolation and characterization of IscA and an IscA variant containing a substitution of a conserved serine residue for a cysteine. The presented findings demonstrate that [Fe-S] clusters are formed on IscA *in vivo,* which supports two of the current models suggesting that IscA functions as an alternative scaffold or an [Fe-S] cluster transporter. Appendix II describes work associated with the development of two systems that can be used to evaluate the maturation of [2Fe-2S] target proteins. In one system, a strain was constructed that allows isolation of the oxygenase component of benzoate-1,2-dioxygenase, a protein that contains a [2Fe-2S] cluster. In the other system, another [2Fe-2S] protein, called FeSII or Shethna protein was cloned, expressed, and purified. The holo-protein was treated with chelating agents to remove the cluster and re-purified. [2Fe-2S]-FeSII differs spectroscopically from [2Fe-2S]-IscU, a feature that can be exploited to monitor cluster transfer from one protein to the other. Appendix III describes findings related to studies performed with the ISC ferredoxin whereas appendix IV summarizes findings related to two paralogs of NADPH-dependent ferredoxin reductases. In the latter, *A. vinelandii* strains were constructed in which each of the ferredoxin reductase genes is inactivated, one of which was found to be required for viability. Appendix V describes the author's contribution to a project in which the genome of *Azotobacter vinelandii* was sequenced and annotated.

CHAPTER 2. Literature Review

2.1 Iron-sulfur clusters are ancient inorganic prosthetic groups

Iron-sulfide species, mainly in the form of FeS and FeS_2 are proposed to have composed ancient surfaces upon which prebiotic chemical reactions took place (Wachtershauser, 1988, Osterberg, 1997). Formation of FeS_2 from iron and gaseous H_2S, both of which were abundant in the hot reducing conditions of ancient Earth, provided the electrons and energy required for the various primitive chemical processes (Wachtershauser, 1988). The ubiquitous nature of Fe/S chemistry in early biological processes can be seen in some archaeal organisms that utilize [Fe-S] cluster-containing ferredoxins in place of cofactors such as NAD(P)/NAD(P)H that are used in the analogous pathways in higher eubacteria (Daniel, 1995). Today, these FeS species have been incorporated into protein prosthetic groups and carry out biological reactions in a variety of life-sustaining processes. Commonly referred to as iron-sulfur ([Fe-S]) clusters, these cofactors typically assume the rhombic [2Fe-2S] and the cubane [4Fe-4S] forms. In addition, [Fe-S] clusters have been found to be part of such complex compounds as the P-cluster or the Fe-Mo cofactor of the enzyme nitrogenase (Figure 2.1). They are typically ligated to their host proteins via cysteine residues, although histidine, aspartate, or H_2O ligands have also been observed. [Fe-S] clusters also have extremely versatile oxidation-reduction properties that are influenced by the host protein environment. The span of reduction potential for [Fe-S] cluster-containing proteins ([Fe-S] proteins) covers an amazingly large range, from below -600 mV to over 400 mV (Beinert, 2000). The versatile structural and electronic properties of [Fe-S] clusters, which confer on their host proteins the ability to participate in a diverse range of biochemical reactions, arise mainly from the unique chemical properties of sulfur. Sulfur can occupy 3d orbitals and is multivalent, with oxidation states from -2 to +6, meaning that it is as prone to donate electrons as it is to accept them. Therefore, S can assume the role of an electrophile (as in a disulfide) or that of a nucleophile (as in a thiol). Iron, though not as versatile as sulfur, also exists in multiple oxidation states, with the two most common ones being the Fe^{+3} and Fe^{+2} forms.

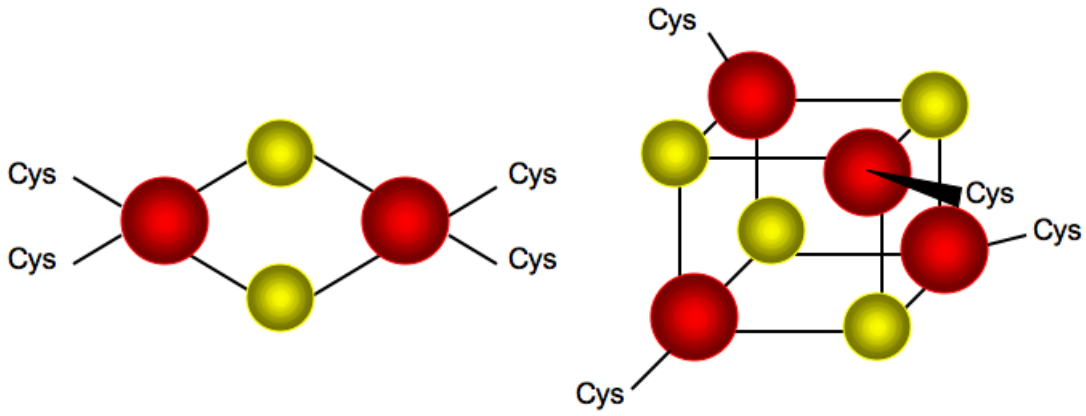

Figure 2.1. Illustration of [2Fe-2S] and [4Fe-4S] clusters. Iron atoms are illustrated in red and sulfur atoms are illustrated in yellow.

2.2 Iron-sulfur clusters perform diverse functions

Nature employs its iron-sulfur clusters in a variety of ways, an observation that might reflect the ubiquitous role of ancient iron-sulfide species as mediators of prebiotic and protobiotic reactions. The most common function that [Fe-S] clusters perform in biological systems is electron transfer. Of the 110 different [Fe-S] proteins known to exist in *E. coli*, nearly half are utilized for electron transfer (Fontecave, 2006). Vital processes such as photosynthesis and nitrogen fixation also utilize [Fe-S] clusters for electron transfer. Other functions performed by [Fe-S] clusters include: catalysis, exemplified by the TCA cycle enzyme aconitase, in which the [4Fe-4S] cluster interacts with the substrate and acts as a Lewis acid (Emptage *et al.*, 1983); oxygen and iron sensing, as performed by FNR and iron responsive elements respectively (Jordan *et al.*, 1997, Rouault & Klausner, 1996); sulfur donation, as in the case of the biotin and lipoic acid synthases (Ugulava *et al.*, 2001, Douglas *et al.*, 2006); tRNA modification, as in the case of MiaB (Pierrel *et al.*, 2002); and structural stabilization as exemplified by several DNA repair enzymes (Cunningham *et al.*, 1989, Porello *et al.*, 1998). [Fe-S] clusters not only endow proteins with a particular ability, they also inactivate a protein under specific cellular conditions. For example, in human mitochondrial glutaredoxin 2, a [2Fe-2S] cluster bridged between two subunits occupies the active site and renders the protein inactive. The protein becomes activated during oxidative stress conditions, when the cluster becomes degraded (Johansson *et al.*, 2007). In aggregate, [Fe-S] clusters have evolved to endow proteins with various abilities and thus their biosynthesis is expected to affect multiple biological processes within a cell.

2.3 NifS and NifU form [Fe-S] clusters for nitrogenase: the "scaffold hypothesis"

Although the focus of this dissertation is the study of the ISC machinery, the pioneering studies that led to the discovery of the ISC system were performed on the NIF proteins, a brief overview of which is provided in this section. In the presence of ferrous and sulfide salts and absence of oxygen, [Fe-S] clusters can be spontaneously formed on apo [Fe-S] proteins. This process, commonly referred to as cluster "reconstitution", was

first demonstrated on the clostridial ferredoxin in 1966 by Malkin and Rabinowitz (Malkin & Rabinowitz, 1966). However, spontaneous assembly of [Fe-S] clusters from unaccompanied inorganic reagents cannot mirror *in vivo* events because of the cellular toxicity that is caused by free iron and sulfur. Almost two decades later, cysteine was identified as the source of sulfur in *E. coli* [Fe-S] proteins (White, 1983). Subsequently, Dean and colleagues identified a new function for the *A. vinelandii* NifS as a cysteine desulfurase. They showed that NifS was a PLP-containing enzyme that catalyzes the removal of sulfur from cysteine to yield alanine and elemental sulfur (Zheng *et al.*, 1993). This work opened the door to the discovery of an entirely new family of enzymes that has attracted the interest of many scientists (Mihara & Esaki, 2002, Kessler, 2006, Mueller, 2006)

The mechanism of cysteine desulfurization has been studied extensively (Zheng *et al.*, 1994, Kaiser *et al.*, 2000, Tirupati *et al.*, 2004). Briefly, the substrate L-cysteine binds to the PLP cofactor in the enzyme's active site to displace the internal diamine between PLP and the active site lysine residue to form the external diamine (Figure 2.2). The external diamine species is commonly referred to as the PLP-cysteine adduct. The thiolate anion of a conserved cysteine residue in the active site then performs a nucleophilic attack on the thiol of the substrate molecule to yield a persulfurated form of the active site cysteine. Subsequently, the alanine product is released and the internal diamine is reformed (Zheng *et al.*, 1994, Kaiser *et al.*, 2000). Depending on the cysteine desulfurase, the sulfur atom that is bound to the active site cysteine in the form of a persulfide can be removed to be used in either [Fe-S] cluster assembly or for the biogenesis of other cellular sulfur-containing cofactors (Kessler, 2006).

The *nifS* gene in *A. vinelandii* and other organisms is co-transcribed with *nifU*, the product of which is a modular protein that consists of three distinct domains: the N-terminal, central, and C-terminal domains. The N-terminal, central, and C-terminal domains contain three, four, and two conserved cysteine residues respectively. These nine conserved cysteines suggest the ligation of multiple [Fe-S] clusters (Smith *et al.*, 2005b). Heterologously expressed NifU is isolated as a homodimer containing one stable [2Fe-2S] cluster per subunit (Fu *et al.*, 1994). This cluster is known as the permanent

cluster and its role in the function of NifU is unknown. In a landmark paper, Yuvaniyama *et al.* showed that in the presence of Fe^{+3}, L-cysteine, and NifS, an additional, transient $[2Fe\text{-}2S]^{2+}$ cluster can be formed on NifU (Yuvaniyama *et al.*, 2000). This cluster is reductively labile because it is released immediately upon treatment with dithionite. That work led to the idea that NifU might act as a "scaffold" upon which clusters are built and then transferred for the maturation of nitrogenase and other [Fe-S] proteins involved in nitrogen fixation. Dos Santos *et al.* later showed that NifU and the conserved cysteine residues on both the N- and C-terminal domains are required for diazotrophic growth and for the activation of the nitrogenase Fe protein *in vivo* (Dos Santos *et al.*, 2004). An additional study helped identify formation of [2Fe-2S] and [4Fe-4S] clusters on NifU *in vitro* through advanced biophysical techniques (Smith et al., 2005b).

2.4 Discovery of the "housekeeping" ISC machinery

Elimination of NifU or NifS resulted in a ~97% decrease of Fe protein activity and ~86% decrease of nitrogenase activity in *Azotobacter vinelandii (Jacobson et al., 1989b).* The remainder of the activity was hypothesized to be due to the presence of an additional [Fe-S] cluster assembly system. *A. vinelandii* cells carrying a deletion in the *nifS* gene were used to purify a protein that also exhibited cysteine desulfurase activity (Zheng *et al.*, 1998). The protein was digested and sequenced and oligonucleotide primers were used to amplify the relevant *A. vinelandii* genomic region. The study revealed six genes, namely *iscSUA-hscBA-fdx*, which were found to be strikingly conserved as more genome sequences become available. With the exception of some archaea, all organisms with available sequence information contain proteins similar in sequence to IscU/NifU and IscS/NifS (Boyd *et al.*, 2008a). In fact, the sequence of IscU seems to be one of the most strongly conserved among all organisms (Hwang *et al.*, 1996), making the significance of this protein for the cell even more appreciable.

Soon after the discovery of the *isc* gene cluster by Dean's group, Takahashi *et al.* conducted a study in which a plasmid containing the *isc* operon was coexpressed with plasmids containing various ferredoxins as reporter proteins. They found that the absence of the genes *iscS, iscA, hscA*, and *fdx* resulted in a decrease of holo reporter

ferredoxins (Takahashi & Nakamura, 1999). In another study, *E. coli* strains lacking IscS, IscU, HscB, HscA, and Fdx were constructed, which exhibited retarded growth rates and reduced activities of the [4Fe-4S] cluster-containing enzymes glutamate synthase and succinate dehydrogenase (Tokumoto & Takahashi, 2001). Inactivation of *iscA* had a small but distinguishable effect on the [Fe-S] protein activities (Tokumoto & Takahashi, 2001). Johnson *et al.* later developed a system for controlled expression of the *isc* genes to show that the *A. vinelandii* genes *iscS, iscU, hscBA*, and *fdx* are essential (Johnson *et al.*, 2006). In the same study, it was also shown that deletion of *iscA* resulted in a null-growth phenotype when the strain was cultured under conditions of oxidative stress.

2.5 IscU acts as a [Fe-S] cluster scaffold *in vitro*

The significant similarity between the sequences of IscU and the N-terminal domain of NifU (46% identity) led to the hypothesis that IscU could also act as a scaffold for the formation of [Fe-S] clusters required by cellular [Fe-S] proteins not involved in nitrogen fixation. *In vitro* studies by members of Dean's group demonstrated that a [Fe-S] cluster can be assembled on heterologously expressed *A. vinelandii* IscU upon anaerobic incubation of excess L-cysteine, Fe^{+3}, and catalytic amounts of IscS (Agar *et al.*, 2000c). The UV-visible absorption spectrum of this sample exhibited distinct peaks at 320, 410, 456, and a shoulder at 510 nm, which are characteristic features of a $[2Fe\text{-}2S]^{+2}$ protein (Dailey *et al.*, 1994). The identity of the cluster was further supported in that work by Raman resonance spectroscopy. This cluster is both reductively and oxidatively labile as it is destroyed in the presence of 1 mM dithionite as well as upon exposure to air. While the mechanism by which a [2Fe-2S] cluster is formed from its components is still not understood, it has been established that transfer of S^o from IscS to IscU is the first step in the assembly (Smith *et al.*, 2001) and this transfer occurs without an intermediate carrier (Urbina *et al.*, 2001). However, sulfur needs to be converted to S^{2-} before it can be recruited for the formation of a [Fe-S] cluster. It has been proposed that all or some of the electrons needed to reduce S^o originate from the ferrous atoms which need to undergo oxidation in order to be incorporated into the iron-sulfur cluster structure (Krebs *et al.*, 2001). This mechanism would prevent futile production of toxic S^{2-} into the cytosol (Krebs et al., 2001).

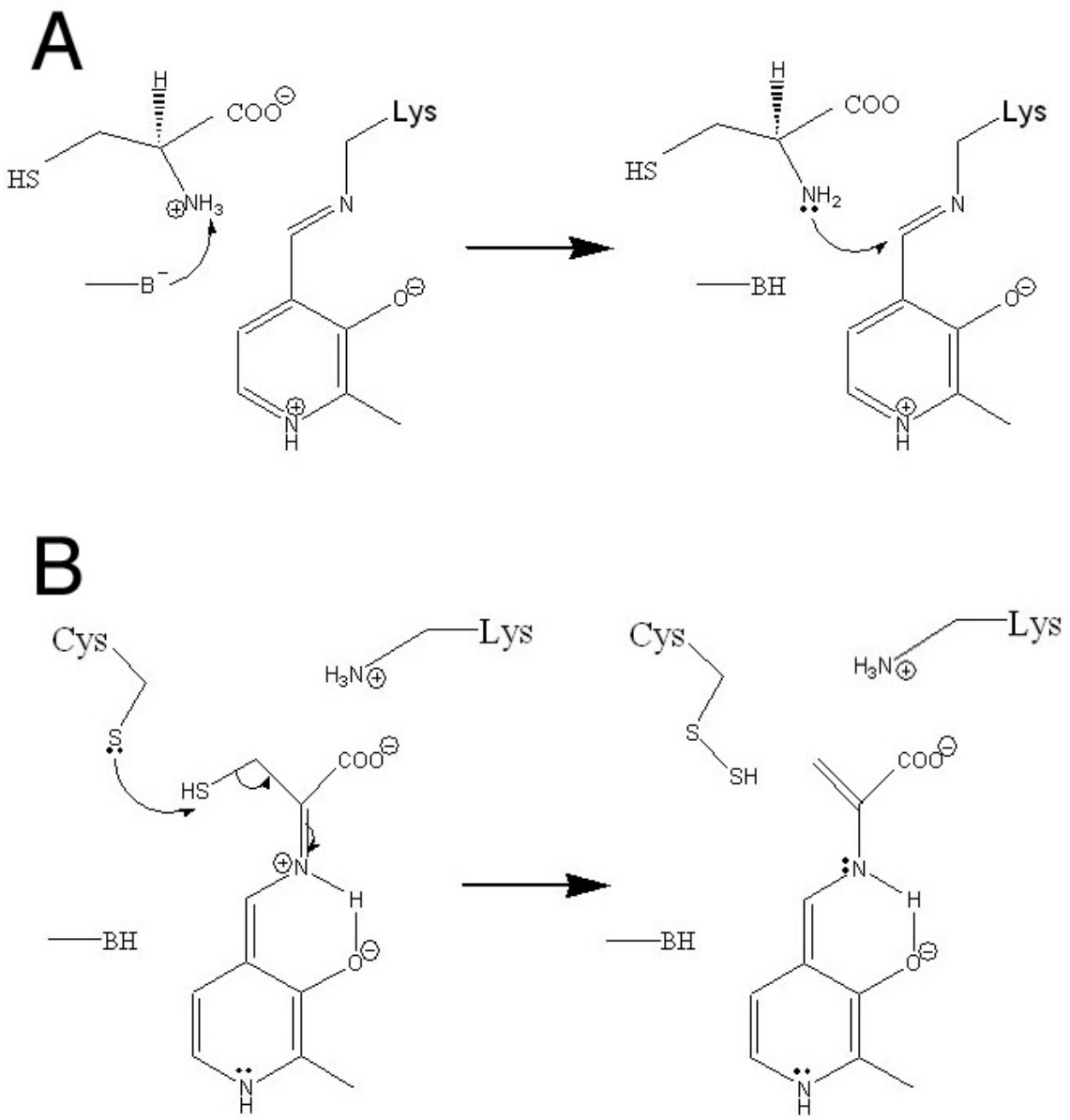

Figure 2.2. Selected steps in the cysteine desulfurase mechanism. (A) Formation of external diamine and (B) formation of persulfide on the active site cysteine.

The nature of interaction between IscS and IscU is a controversial topic that needs further investigation. Several studies have shown that IscS and IscU form a physiologically relevant complex *in vitro.* Whether this complex is covalently or non-covalently bound has been a matter of debate (Smith et al., 2001, Smith *et al.*, 2005a, Urbina et al., 2001, Kato *et al.*, 2002, Agar et al., 2000c). Using surface plasmon resonance (SPR) methods, Urbina *et al.* demonstrated that IscS and IscU bind tightly *in vitro* (K_d ~ 2μM) and this interaction is weakened when the C-terminal region of IscS is deleted, suggesting a role for this region in mediating binding to the scaffold protein (Urbina et al., 2001). Covalently bound IscS-IscU complex involving heterodisulfide interactions has been observed – though an agreement has not been reached over the residues involved in complex formation (Kato et al., 2002, Smith et al., 2005a). It is important to mention that these studies were performed in the absence of an [Fe-S] cluster, so it is possible that the covalent interactions observed are a result of liberated thiols, unoccupied by an [Fe-S] cluster.

Formation of [Fe-S] clusters on IscU *in vitro* was first demonstrated almost a decade ago (Agar et al., 2000c). In an experiment where cluster formation on IscU was monitored over time, it was shown that a [2Fe-2S] cluster per IscU dimer is first formed, followed by two [2Fe-2S] clusters per dimer, followed by one [4Fe-4S] cluster per dimer (Agar et al., 2000a). This information supported the hypothesis that IscU functions as a scaffold for synthesis of both [2Fe-2S] and [4Fe-4S] clusters. It also provided evidence that IscU utilizes a mechanism for forming a [4Fe-4S] cluster by combining two [2Fe-2S]-cluster building blocks as opposed to the sequential addition of Fe and S to an existing [2Fe-2S] cluster or a mechanism that bypasses [2Fe-2S] cluster formation (Figure 2.3). Chandramouli *et al.* showed that ~1 reducing equivalent per [2Fe-2$S^{]2+}$ is required to couple two [2Fe-2S] clusters on an IscU dimer to form a [4Fe-4S] cluster (Chandramouli, 2007). They also observed that reduced ferredoxin, the protein product of the *fdx* gene in the *isc* operon, can competently perform the reductive coupling of two [2Fe-2S] clusters to form a [4Fe-4S] cluster. It is important to note that [4Fe-4S] cluster formation has so far only been observed on *A. vinelandii* IscU, suggesting possible scaffold specificity in this organism.

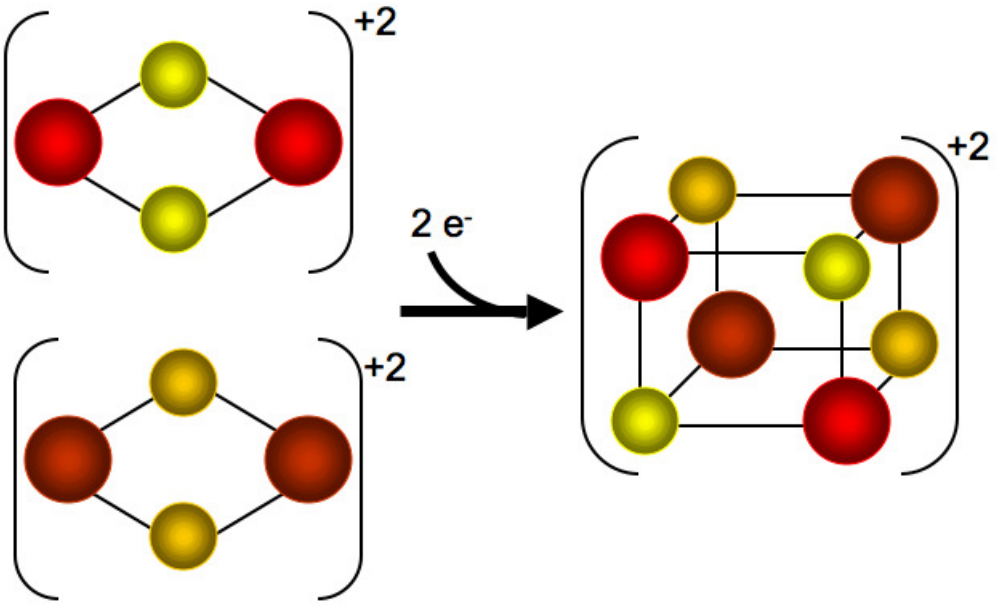

Figure 2.3. Formation of a [4Fe-4S] cluster through the reductive coupling of two [2Fe-2S] clusters. Iron atoms are illustrated in bright and dull red and sulfur atoms are illustrated in bright and dull yellow. The iron in the $[2Fe\text{-}2S]^{2+}$ cluster exists in the ferric (Fe^{3+}) state whereas $[4Fe\text{-}4S]^{2+}$ clusters contain a mixture of two Fe^{3+} and two Fe^{2+} ions.

The ability of IscU to transfer its [2Fe-2S] cluster to apo-target proteins has been demonstrated in several organisms (Wu *et al.*, 2002b, Bonomi *et al.*, 2005, Wu *et al.*, 2002c). Various methods have been employed to monitor [2Fe-2S]-cluster transfer although in all cases the recipient protein of choice was the *isc* ferredoxin Fdx. This may be problematic since Fdx which is a [2Fe-2S] protein has also been proposed to be involved in cluster synthesis (Muhlenhoff *et al.*, 2003). Experiments using labeled ^{57}Fe and Mossbauer spectroscopy showed that the cluster formed on the apo-target protein contained only labeled iron demonstrating that the cluster is transferred to the recipient protein intact, without degradation of the cluster to its inorganic parts and re-assembly on the apo-target (Wu et al., 2002c). Bonomi's group demonstrated that multiple turnover of cluster transfer can be observed from IscU to apo-ferredoxin, thereby indicating that IscU transfers its clusters catalytically (Bonomi et al., 2005). IscU carrying a [4Fe-4S] cluster is also able to directly transfer its cluster to apo-aconitase, a [4Fe-4S] TCA cycle enzyme, without the requirement of an intermediate carrier (Unciuleac *et al.*, 2007). In all experiments described to date, cluster transfer has been monitored using cluster-loaded IscU and apo-target protein. The impact of IscS on cluster transfer rates has not been investigated, which may be of significance considering that IscU and IscS are potential physiological partners *in vivo*. In all cases, the cluster transfer process from IscU to apo-target protein was relatively slow (sometimes slower than the doubling time of the organism) or required superstoichiometric amounts of scaffold. These observations support the possibility that IscU acts as a central scaffold and accessory proteins are used to relay nascent clusters to the various recipient proteins in the cell.

To get a sense of the mechanistic details in the [Fe-S] cluster assembly process, several groups have attempted to gain structural insight by solving the NMR and crystallographic structures of IscU from different organisms. Ramelot *et al.* solved the NMR structure of *H. influenzae* IscU with zinc bound at the active site (Ramelot *et al.*, 2004). They found that the three conserved cysteine residues as well as the conserved His105 were positioned on the surface of the protein bound to a Zn atom, thus suggesting that these residues may be involved in cluster ligation. In *A. vinelandii*, the alanine substitutions of these residues disable [Fe-S] cluster assembly on IscU *in vitro* and have fatal consequences *in vivo* (Johnson et al., 2006, Smith et al., 2005a). Two other

important residues, Lys103 and Asp39 were also positioned near the proposed active site. The roles of these residues in cluster assembly are not understood, although both are required for growth in *A. vinelandii* (Johnson et al., 2006). A peptide containing a C-terminal region of IscU including residue Lys103 was found to be required for interactions with the molecular chaperone HscA (Hoff *et al.*, 2002). In addition, substitution of Asp39 to alanine results in a dominant-negative phenotype (Johnson et al., 2006). The latter suggests that the Asp39Ala substitution may impede appropriate associations/dissociations of IscU subunits with each other or with other [Fe-S] cluster biosynthetic components.

The Asp39 residue of IscU has received much attention due the stability conferred to the cluster bound to the protein when this residue is substituted by an alanine (Foster *et al.*, 2000, Smith et al., 2005b, Wu et al., 2002c, Wu et al., 2002b). Asp39 was initially thought to be a cluster ligand but its substitution to a cysteine, which is typically a stronger ligand, did not result in a stable cluster whereas its substitution to an alanine did. These observations led to the hypothesis that Asp39 may participate in catalyzing cluster transfer from IscU to an apo-target protein. One mechanism by which cluster exchange may be accomplished is by deprotonation of the target protein thiol groups by the aspartate carboxylate group. These can then perform nucleophilic attacks on the cysteine residues involved in cluster ligation (Wu et al., 2002b). Cluster transfer experiments showed that IscU carrying an Asp to Ala substitution of residue 39 is much less efficient in directing maturation of apo-ferredoxin compared to wild type IscU, reinforcing the hypothesis that Asp39 is involved in cluster transfer (Wu et al., 2002c).

During the course of this work, Takahashi's group was able to solve the crystal structure of a [2Fe-2S] cluster-containing IscU variant carrying an Asp39Ala substitution from the thermophilic bacterium *Aquifex aeolicus* at a 2.3 Å resolution (Shimomura *et al.*, 2008). The overall quaternary structure of the protein was reported to be trimeric and only one of the subunits had a bound [2Fe-2S] cluster. This finding is inconsistent with previous studies from several organisms that have reported IscU to exist as a monomer or a homodimer. Heterologously expressed *A. vinelandii* IscU was reported as a dimer (Agar et al., 2000c), although monomeric forms of the recombinant protein have also been detected recently (O'Carroll, unpublished data). On the other hand, *E. coli* and *H.*

influenzae IscU have been shown to exist as monomers (Adinolfi *et al.*, 2004, Ramelot et al., 2004). In all cases, IscU was heterologously expressed at levels unnaturally higher than the rest of the ISC machinery, which raises concerns and questions about whether the multimeric states of IscU reflect physiological or artifactual phenomena. Isolation of IscU from an *in vivo* system where all iron-sulfur cluster biosynthetic components are expressed is crucial for the elucidation of the oligomeric state of IscU.

Nevertheless, the architectural details revealed by the crystal structure of IscU provide significant insight. The three subunits that comprise the asymmetric trimer appear to have different protein folds, suggesting that IscU may undergo a series of conformational changes during the process of [Fe-S] cluster assembly or delivery to recipient proteins. As predicted by the NMR structure from *H. influenzae*, the residues involved in cluster ligation in *A. aeolicus* IscU are the three conserved cysteines: Cys36, Cys63, and Cys107, as well as His106 (Shimomura *et al*., 2008). Cluster ligation by three cysteines and one histidine is atypical, though it has been observed in one other case: the mitochondrial outer membrane protein mitoNEET, the function of which has not been determined (Hou *et al.*, 2007). In the crystal structure of *A. aeolicus* IscU, the conserved Lys103 residue is positioned almost 10 Å away from the [2Fe-2S] cluster whereas the same residue in the NMR structure of *H. influenzae* IscU is very close to the proposed [2Fe-2S] cluster site (Ramelot et al., 2004). It is possible that the loop containing this residue acts as an active site "gate" that opens when the appropriate conditions are met for release of the cluster to recipient proteins. This hypothesis seems plausible when considering the previously reported significance of this residue in interactions with the molecular chaperone HscA (Hoff *et al*., 2002, Hoff *et al.*, 2003) and the proposed role of HscA in mediating cluster release (see section 2.8) (Chandramouli & Johnson, 2006).

In summary, IscU has been shown to serve as a scaffold for both [2Fe-2S] and [4Fe-4S] clusters *in vitro* in the presence of iron, cysteine, and IscS. However, it has not been established whether this observation represents a physiological process. *In vivo* studies are necessary to test the scaffold hypothesis as well as to determine any physiological protein-protein interactions that may occur between IscU and other cellular components.

Table 2.I. List of proteins involved in [Fe-S] cluster biosynthesis and their proposed functions.

Protein	Conditions in which protein is essential	Established or proposed function
IscR	None	[2Fe-2S] negative regulator of *isc* operon
IscS (Nfs1)*	All	Cysteine desulfurase: provides S for [Fe-S] cluster assembly
Isd11	All	Cysteine desulfurase accessory protein, only in eukaryotes
IscU (Isu1/2)*	All	Proposed [Fe-S] cluster assembly scaffold
IscA (Isa1/2)*	O_2 stress, Lys/Glu auxotrophy in yeast	Proposed alternative scaffold, [Fe-S] cluster transporter, iron donor
HscB (Jac1)*	All	J-type co-chaperone, similar to DnaJ
HscA (Ssq1)*	All	K-type (Hsp70) chaperone, similar to DnaK
Mge1p	All	Nucleotide exchange factor, works with Ssq1 in yeast
Fdx (Yah1)*	All	[2Fe-2S] ferredoxin, proposed to provide electrons during [Fe-S] cluster assembly
IscX	None	Protein of unknown function
NifU	Diazotrophy	Proposed [Fe-S] assembly scaffold during nitrogen fixation, modular protein
NifS	Diazotrophy	Nitrogen fixation cysteine desulfurase
$IscA^{Nif}$	O_2 stress	Proposed alternative scaffold
NfuA	O_2 stress	Modular protein, proposed [Fe-S] cluster scaffold
AnfU	nd**	Proposed alternative [Fe-S] cluster assembly scaffold for Fe nitrogenase
VnfU	nd**	Proposed alternative [Fe-S] cluster assembly scaffold for V nitrogenase
SufA	O_2, Fe stress	Proposed [Fe-S] cluster assembly scaffold, similar to IscA
SufB	O_2, Fe stress	Proposed [Fe-S] cluster assembly scaffold, no similarity to IscA or IscU
SufC	O_2, Fe stress	ATPase, function unknown
SufD	O_2, Fe stress	Function unknown
SufS	O_2, Fe stress	Cysteine desulfurase
SufE	O_2, Fe stress	Cysteine desulfurase accessory protein, accepts S from SufS
ApbC	tricarballylate	P-loop NTPase, specialized scaffold in S. enterica
Nbp35	Yeast cytosol	P-loop NTPase, proposed cytosolic scaffold in yeast
Cfd11	Yeast cytosol	P-loop NTPase, proposed cytosolic scaffold in yeast
CyaY (Yfh1)*	None	Proposed iron donor, similar to eukaryotic frataxin
ErpA	All	Specialized scaffold, involved in isoprenoid biosynthesis
Iba57	Lys/Glu auxotrophy in yeast	A-type scaffold required for biotin synthase and SAM enzymes
Grx5	None	Proposed [Fe-S] cluster carrier in yeast

* Eukaryotic names are in parentheses

**nd = not determined

2.6 Specialized iron-sulfur cluster scaffolds and carriers

Since the introduction of the scaffold hypothesis by Dean's group (Dean *et al.*, 1993), a number of proteins have been reported to assemble [Fe-S] clusters *in vitro* and sometimes transfer them to cellular targets. Most of the genes that encode these proteins are either not required for viability or are only required under specific environmental or nutritional conditions. Because the presence of IscU is strongly conserved in all known organisms, a popular model emerged that IscU acts as a central scaffold while other proteins serve as alternative or specialized scaffolds. Some of these proteins have been described to carry out scaffolding roles for specialized acceptor proteins during specific environmental conditions. In the case of eukaryotes, scaffolds may serve in specific cellular compartments. Table 2.I provides a list of the proteins involved in [Fe-S] cluster assembly and their proposed functions.

The first alternative scaffolds to be described were IscA from *E. coli* and $IscA^{Nif}$ from *A. vinelandii* (Ollagnier-de-Choudens *et al.*, 2001, Krebs et al., 2001). IscA proteins belong to what has been referred to as the A-type family of scaffolds. An *iscA* gene is almost always cotranscribed with the *iscSU* or *nifSU* genes, suggesting a significant role for this component in the formation of [Fe-S] clusters and perhaps a collaborative function with IscU and IscS. However, unlike the *iscSU* and *nifSU* pairs, neither IscA nor $IscA^{Nif}$ are required for viability or nitrogen fixation respectively. Nevertheless, the presence of IscA is not completely dispensable since its impairment has a small but reproducible effect on the maturation of recipient [Fe-S] cluster enzymes (Takahashi & Nakamura, 1999, Johnson et al., 2006, Jensen & Culotta, 2000). It wasn't until recently that a null-growth phenotype related to the deletion of *iscA* was elicited under conditions of oxidative stress (Johnson et al., 2006). In *Saccharomyces cerevisiae*, deletion of the *iscA* homologs *isa1* and *isa2* resulted in a requirement for lysine and glutamate and an overall increase in mitochondrial iron levels (Jensen & Culotta, 2000). These observations suggest that IscA either has a redundant role, specializes in serving specific recipient proteins, or functions under specific growth conditions.

Similarly to NifU and IscU, the formation of labile $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{+2}$ clusters were observed on homodimeric molecules of IscA and $IscA^{Nif}$ (Ollagnier-de-Choudens et al., 2001, Krebs et al., 2001). Subsequent studies have shown that [2Fe-2S] and [4Fe-4S]

clusters can be formed on IscA from other organisms as well such as cyanobacteria (Wollenberg *et al.*, 2003), *Schizosaccharomyces pombe* (Wu *et al.*, 2002a), and the extremophile *Acidithiobacillus ferrooxidans* (Zeng *et al.*, 2007a). Like IscU, IscA has three highly conserved cysteines that are proposed to serve as [Fe-S] cluster ligands. A conserved aspartate residue was recently demonstrated to be the fourth ligand in IscA from *A. ferrooxidans* (Jiang *et al.*, 2008). Both in *A. vinelandii* and in yeast, substitutions of these cysteines to alanines result in similar phenotypes as the strains that carry deletions (Jensen & Culotta, 2000, Johnson et al., 2006). However, not all residues of one molecule are necessarily involved in cluster ligation since most IscA proteins have been demonstrated to be homodimers (Wollenberg et al., 2003, Krebs et al., 2001, Ollagnier-De Choudens *et al.*, 2003). This allows a situation in which the cluster is shared between two protomers. In *Schizosaccharomyces pombe,* elimination of only two of the cysteines renders IscA unable to form clusters (Wu et al., 2002a). The three crystal structures available for IscA provide concrete details about the structural relationship between IscA and its [Fe-S] cluster (Cupp-Vickery *et al.*, 2004b, Bilder *et al.*, 2004, Morimoto *et al.*, 2006). In all structures, IscA exists in the tetrameric form even though they are dimeric in solution. This difference may be due to the crystallization conditions used. The active site containing a [2Fe-2S] cluster is shared by two subunits in the dimeric species. Two cysteine ligands from each protomer coordinate the cluster, which is positioned at the interface of the two subunits. The cluster is solvent-exposed, a finding that is consistent with a scaffolding role for IscA.

IscA-bound clusters have also been shown to activate apo iron-sulfur proteins. *E. coli* IscA loaded with a $[2Fe\text{-}2S]^{2+}$ cluster was able to produce holo ferredoxin after anaerobic incubation with the apo protein (Ollagnier-de-Choudens et al., 2001). In that case, a specific interaction was observed between IscA and the ISC ferredoxin but not the rest of the ISC proteins, which suggests a functional association between these two components *in vivo* (Ollagnier-de-Choudens *et al*., 2001). In another case, IscA from cyanobacteria was able to transfer [2Fe-2S] and [4Fe-4S] clusters to apo ferredoxin and apo adenosine 5'-phosphosulfate reductase respectively (Wollenberg *et al*., 2003).

SufA is another A-type protein that is produced as part of the SUF machinery described in section 2.10 of this review. The SUF machinery is not produced in *A.*

vinelandii, but in *E. coli* it has been demonstrated to function during conditions of oxidative stress and iron starvation (Outten *et al.*, 2004). As with IscA, inactivation of *sufA* in *E. coli* does not confer a noticeable growth phenotype. However, a double deletion in *sufA* and *iscA* renders *E. coli* cells unable to grow aerobically (Lu *et al.*, 2008). Like IscA, SufA was demonstrated to assemble [2Fe-2S] and [4Fe-4S] clusters and transfer them to apo-ferredoxin and apo-BioB (biotin synthase) respectively (Ollagnier-De Choudens et al., 2003). This occurs in a biphasic reaction that involves a fast scaffold-target complex formation followed by a slower (~60 min) transfer of the cluster (Ollagnier-De-Choudens *et al.*, 2004). SufA could activate apo-BioB from different organisms suggesting little species specificity (Ollagnier-De-Choudens et al., 2004). The crystal structure of SufA, obtained in its apo, homodimeric form (Wada *et al.*, 2005), revealed a globular protein largely covered in acidic residues, a feature that is also conserved in the IscA crystal structure. The two conserved cysteines (CxC) in the C-terminal region of the protein are positioned such that they could ligate an [Fe-S] cluster positioned at the interface of two subunits. The conserved Cys50 is located too far away from the active site to be involved in cluster binding. Therefore, it could be involved in reactions that would facilitate *de novo* cluster assembly, release, or acceptance from other proteins if not assembled on SufA (Wada et al., 2005).

Recently, findings on the properties of another A-type iron-sulfur protein, called ErpA, were reported (Loiseau *et al.*, 2007). This protein was found to be required for cellular viability in *E. coli* during aerobiosis and fermentative anaerobiosis. It was also found that inactivation of *erpA* is deleterious for the biosynthesis of isoprenoid compounds, which serve as precursors for the biogenesis of ubiquinone and menaquinone. The *in vitro* assembly of [2Fe-2S] and [4Fe-4S] clusters was accomplished on ErpA as was noticed for IscA and SufA (Ollagnier-De-Choudens et al., 2004). ErpA could assemble a [4Fe-4S] cluster *in vitro* and transfer it to apo-IspG, a [4Fe-4S] enzyme involved in the isoprenoid biosynthesis pathway. An ErpA homolog is also present in *A. vinelandii* where inactivation of the gene results in a null-growth phenotype during typical growth conditions (Johnson DC, unpublished).

Around the same time, a protein referred to as Iba57p located in the mitochondrial matrix of yeast, was identified to be required for optimal respiration and mitochondrial

DNA maintenance (Gelling *et al.*, 2008). This is not an A-type protein but its inactivation resulted in the same phenotypes that were observed for the Isa1 and Isa2 yeast proteins, the eukaryotic homologs of IscA. In addition, Iba57p was shown to interact with Isa1 and Isa2, suggesting that *in vivo* the trio may form a functional complex. Iba57p was demonstrated to be essential for biotin synthase and radical SAM enzymes although they were not involved in the *de novo* synthesis of [Fe-S] clusters on those enzymes. It is possible that Iba57 and Isa1/2 are involved in a step after [Fe-S] cluster insertion. The role of [Fe-S] cluster repair is also possible considering that biotin synthase and lipoyl synthase use the sulfur from their [Fe-S] cluster to donate to their substrates (Jameson *et al.*, 2004, Douglas et al., 2006). The findings on ErpA and Iba57p introduced the first evidence supporting the hypothesis that A-type proteins or other non-IscU scaffolds serve as specialized scaffolds that are responsible for the activation of a specific group of proteins.

Glutaredoxins have also been implicated in [Fe-S] cluster biosynthesis (Achebach *et al.*, 2004, Vilella *et al.*, 2004, Molina-Navarro *et al.*, 2006, Bandyopadhyay *et al.*, 2008b). Glutaredoxins are thiol-disulfide oxidoreductases, which in conjunction with glutathione, NADPH and a glutathione reductase, provide a reduced cellular environment to protect it from oxidative damage (Lillig *et al.*, 2008). Most organisms contain multiple glutaredoxins which are of two types: monothiol glutaredoxins characterized by a CXXS motif and dithiol glutaredoxins which are characterized by a CXXC motif. Monothiol glutaredoxins containing [2Fe-2S] clusters bridged between two subunits, ligated by one cysteine from each subunit as well as two glutathione molecules have recently been observed (Lillig *et al.*, 2005, Bandyopadhyay et al., 2008b). In human mitochondrial glutaredoxin 2, the presence of a [2Fe-2S] cluster inactivates the protein, which becomes active only during oxidative stress conditions when the cluster is degraded and the active site cysteines are liberated (Johansson et al., 2007). In yeast, depletion of the glutaredoxin Grx5 affects maturation of [Fe-S] enzymes, but has no effect on the ability of the IscU scaffold to bind [Fe-S] clusters (Muhlenhoff et al., 2003). It was therefore proposed that Grx5 is involved at a step after [Fe-S] cluster assembly. Bandyopadhyay *et al.* demonstrated that glutaredoxins are not only able to assemble [Fe-S] clusters *in vitro*, but they also efficiently transfer their cluster to an apo protein at rates 25 times higher

than IscU (Bandyopadhyay et al., 2008b). These findings have led to the proposed role of glutaredoxins as storage or transporter [Fe-S] proteins. It should be noted that, only monothiol glutaredoxins containing a CXXS sequence motif, as opposed to dithiol proteins characterized by a CXXC motif, were able to assemble and transfer clusters suggesting functional specificity between the two protein subfamilies (Bandyopadhyay et al., 2008b). The CXXS sequence motif also happens to be conserved in the IscA family of proteins. It remains to be seen whether IscA proteins can also participate in efficient cluster transfer to target proteins either promiscuously or to a specific group of proteins.

In addition to A-type (IscA, SufA, ErpA) and U-type (IscU, NifU) scaffolds, another family of proteins commonly referred to as Nfu-type scaffolds has also emerged. This distinct group of proteins was first recognized as a C-terminal domain in the modular protein NifU of *Azotobacter vinelandii* (Dos Santos et al., 2004). The Nfu domain of NifU contains two conserved cysteines in the form of the CXXC motif. Neither cysteine is required for diazotrophy (Agar *et al.*, 2000b), but are proposed to contribute to the ligation of a labile [4Fe-4S] cluster which can be readily assembled *in vitro*. Unlike the N-terminal domain of NifU, which can sequentially form [2Fe-2S] and [4Fe-4S] clusters, only [4Fe-4S] clusters are observed on the Nfu domain of NifU. This suggests that, *in vivo,* clusters may not be assembled *de novo* on the Nfu scaffold domain, but rather transferred from the N-terminal scaffold. Proteins with homology to the Nfu domain of NifU have also been identified in other organisms where Nfu can assemble both [2Fe-2S] and [4Fe-4S] clusters *in vitro* and transfer them to apo target proteins (Touraine *et al.*, 2004, Tong *et al.*, 2003). In some organisms, the presence of Nfu proteins seems to be redundant, but in others like cyanobacteria for example, Nfu is unique and required for viability, suggesting that Nfu may function as the general scaffold that builds clusters *de novo* (Yabe *et al.*, 2004, Nishio & Nakai, 2000).

A distinct family of proteins capable of ATPase and scaffolding activity has emerged recently. The family is exemplified by the yeast Nbp35 protein (Stehling *et al.*, 2008, Hausmann *et al.*, 2005, Netz *et al.*, 2007) and its *S. enterica* homolog ApbC (Boyd *et al.*, 2008c, Boyd *et al.*, 2008d, Boyd *et al.*, 2008b). Nbp35 and ApbC are P-loop NTPases characterized by conserved cysteine residues at the N-terminal domain. Nbp35 is essential in yeast and affects maturation of the cytosolic [Fe-S] cluster containing enzyme

isopropylmalate isomerase Leu1, but has no effect in the biogenesis of mitochondrial [Fe-S] enzymes (Hausmann et al., 2005). This finding led to the hypothesis that Nbp35 acts as a scaffold for the maturation of cytosolic proteins. Nbp35 appears to form a functional complex with Cfd1, another yeast cytosolic P-loop NTPase proposed to play a role in cytosolic [Fe-S] cluster assembly (Netz et al., 2007). However, the *A. thaliana* Nbp35 and the prokaryotic homolog ApbC seem to function on their own (Kohbushi *et al.*, 2008, Boyd et al., 2008c, Bych *et al.*, 2008). *Salmonella enterica* ApbC is not essential for viability but is required for growth on the sugar tricarballylate, the utilization of which employs a [4Fe-4S] enzyme (Boyd et al., 2008b). A recent report demonstrated that some archaeal organisms, which do not produce the ISC or any of the other known [Fe-S] cluster biosynthetic organisms, utilize ApbC homologs as their [Fe-S] cluster scaffold (Boyd et al., 2008a). It should be noted that the ATPase activity of ApbC is crucial for function but ATP hydrolysis does not seem to be required for [Fe-S] cluster assembly (Boyd et al., 2008d). Whether the two functions are related *in vivo* remains to be established. Nevertheless, it is important to recognize that both the ISC and the SUF machineries produce scaffold and energy-generating proteins, suggesting that ATPase activity is likely required at some step in [Fe-S] cluster synthesis or transfer.

Overall, the above findings suggest that cells utilize several proteins to accommodate their needs for [Fe-S] clusters and that the [Fe-S] cluster biosynthetic process *in vivo* is more complex than their *in vitro* assembly. The idea has emerged that some proteins (such as IscU, NifU, and in some cases Nfu) act as central scaffolds upon which clusters are built *de novo,* whereas other proteins assume specialized scaffolding, storage, or carrier roles and function during specific growth conditions or for specific target proteins.

2.7 IscA and CyaY as candidates for the role of iron delivery agent in [Fe-S] cluster assembly

The mechanism by which Fe is delivered for [Fe-S] cluster assembly is not understood. The *iscA* gene is almost always co-transcribed with the *iscUS* pair, which makes the product of this gene an attractive candidate for the role of iron delivery. However, unlike the universal S donor IscS, the *iscA* gene is not essential for growth and its inactivation results in slightly, albeit reproducibly, lowered activity of [Fe-S] proteins

(Johnson et al., 2006, Tokumoto & Takahashi, 2001). Thus, it is possible that the cell utilizes multiple iron carriers. Several articles from Ding and colleagues describe the ability of *E. coli* IscA and its homolog SufA to bind iron with high affinity (K_d = 3.0 x 10^{-19} M^{-1}) and deliver it to IscU *in vitro* (Ding *et al.*, 2004, Ding *et al.*, 2005b, Lu et al., 2008, Ding *et al.*, 2005a, Yang, 2006, Ding *et al.*, 2007). This has been supported by the observation that Fe preferentially binds to IscA but not IscU (Yang, 2006) and that IscA from *Synechococcus spp.* is purified with iron bound to it (Wollenberg et al., 2003). IscA is proposed to mobilize iron to IscU via cysteine. One proposed model involves the interaction of free cysteine with IscA resulting in iron-bound cysteine which then undergoes desulfurization, leading to the Fe/S species that is subsequently donated to IscU (Ding et al., 2005a).

IscA is not the only protein that has been proposed to serve as an iron donor. Frataxin, a eukaryotic protein that has implications in the neurodegenerative disease, Friedrich's ataxia, was also shown to bind iron with high affinity (Yoon & Cowan, 2003). Iron-bound frataxin can donate the iron for assembly of [Fe-S] clusters on IscU (Layer *et al.*, 2006, Yoon & Cowan, 2003). Neither frataxin nor its prokaryotic homolog CyaY, binds iron with high affinity during typical growth conditions. However, under conditions of oxidative stress, CyaY binds iron with high affinity thus lowering the production of free radicals from the Fenton reaction (Ding et al., 2007). It is possible that frataxin/CyaY proteins act as cell detoxifiers by reducing iron levels during conditions of oxidative stress.

2.8 The molecular chaperones HscA and HscB stimulate cluster delivery

HscA and HscB are heat-shock cognate proteins that belong to the Hsp70 family of chaperones and their Hsp20 cochaperones, respectively. The 70kDa-heat shock protein family is widely distributed in eukaryotes, bacteria, and archaea where they mediate protein folding processes by helping proteins acquire their native fold, preventing their aggregation, or resolubilizing and refolding aggregated proteins (Mayer & Bukau, 2005). They also facilitate protein trafficking across cell membranes and assembly/disassembly of protein complexes (Young *et al.*, 2003). All Hsp70 chaperones have an N-terminal

ATP-binding domain and a C-terminal substrate-binding domain. The nucleotide binding domain is flexible and undergoes conformational changes that allow it to open and close to bind ATP and release ADP and P_i (Zhang & Zuiderweg, 2004). In a canonical ATPase, ATP hydrolysis is the rate-limiting step in the ATPase cycle and essential for chaperone activity. ATP binding is followed by a conformational change of the chaperone that is described as the "tense" (T) state, which has low affinity for substrates. ATP hydrolysis to ADP and phosphate facilitates the closing of the substrate binding domain, thus "locking-in" the substrate. The latter is referred to as the "relaxed" (R) state and is characterized by high substrate affinity. This is followed by release of ADP and P_i and subsequent discharge of substrate. Binding of ATP and re-conversion to the T-state begins the next cycle. The ADP/ATP exchange rate is stimulated by the presence of a nucleotide exchange factor such as GrpE, which stimulates nucleotide exchange by up to 5,000 fold in the case of *E. coli* DnaK (Packschies *et al.*, 1997). The basal ATPase activity of Hsp70s is relatively low, and it is slightly stimulated by the presence of substrate and highly stimulated (over three orders of magnitude) when both substrate and the Hsp20 cochaperone are present (Laufen *et al.*, 1999). In addition to promoting ATP hydrolysis, the role of the Hsp20 chaperone is to target specific substrates to Hsp70 (Mayer & Bukau, 2005). This mechanism prevents wasteful hydrolysis of ATP and ensures that ATP is only used in the presence of specific substrates.

HscA exhibits several properties characteristic of Hsp70 proteins such as ATP hydrolysis and the requirement of a Hsp20 chaperone (HscB). Like other Hsp70s, HscA has an N-terminal ATP-binding domain and a C-terminal substrate-binding domain. Primary and tertiary structure comparisons of HscA with other Hsp70s, including the bacterial DnaK, reveal a strongly conserved general architecture suggesting similarities in function. The intrinsic activity of HscA is also very low (~0.6 min^{-1}) and is stimulated up to ~4 fold by the presence of the co-chaperone HscB (Vickery *et al.*, 1997). Mechanistic studies by Silberg and Vickery revealed several differences between *E. coli* HscA and its homolog DnaK (Silberg & Vickery, 2000). The overall affinity of HscA for ATP and ADP is much lower than that for DnaK. The rate of ATP association to HscA is the same as that to DnaK, however, the rate of ADP dissociation is much faster (>1,000 fold) for HscA compared to DnaK. This finding explains the lack of requirement for a nucleotide

exchange factor by HscA. As a side note, the HscA homolog in yeast does utilize a nucleotide release factor, Mge1p, suggesting possible mechanistic differences between the two systems. Although ATP hydrolysis is also the rate-limiting step during steady-state conditions, ADP release and subsequent substrate release is much faster (>1,000 fold) than the rate of ATP hydrolysis. The situation is different in the analogous DnaK cycle where nucleotide exchange occurs at the same rate as ATP hydrolysis. Based on this finding, the ADP/substrate-bound state would be expected to be short-lived in the case of HscA, suggesting that regulation might occur at the ATP hydrolysis step rather than the ADP dissociation step.

HscA is unique from other Hsp70s in that it does not serve a wide range of peptide substrates. Rather, the only "client" protein with which it has been shown to interact is IscU, the proposed [Fe-S] cluster assembly protein (Silberg *et al.*, 2001). Genomic analyses place *hscB* and *hscA* closely associated with the *isc* genes in most organisms, including eukaryotes, in which their homologs are referred to as *ssq1* (*hscA*) and *jac1* (*hscB*). The expression of these proteins is constitutive and independent of heat shock, hence their designation as "heat-shock cognate" proteins (Vickery et al., 1997). The genes *hscA* and *hscB* appear to only be associated with the ISC system as analogous genes and are not conserved in NIF and SUF systems studied so far. This specialized relationship with the iron-sulfur cluster assembly machinery has initiated several studies to attempt to elucidate the role of the HscA and HscB chaperones in the biogenesis of [Fe-S] clusters.

Several genetic studies have confirmed the involvement of the chaperones in [Fe-S] cluster assembly. Takahashi and coworkers reported reduced levels in production of several ferredoxins in the absence of *hscA* (Takahashi & Nakamura, 1999). A null-growth phenotype was conferred by deletion of *hscA* in *E. coli* (Tokumoto & Takahashi, 2001) and *A. vinelandii* (Johnson et al., 2006) accompanied by reduced activities of [4Fe-4S]-cluster containing enzymes when cells were depleted for the chaperone proteins.

A significant portion of what is known about the interplay between HscA, HscB and IscU and their involvement in the ATPase cycle is owed to the pioneering work of Larry Vickery and colleagues. Recombinantly produced IscU from *E. coli* increases the ATPase activity at similar levels as HscB alone (Hoff *et al.*, 2000). However, IscU and

HscB synergistically stimulate the rate of hydrolysis (k_{hyd}) by over 400 fold (Hoff et al., 2000, Silberg *et al.*, 2004). The rate of ATP binding is not affected by the presence of HscB, but HscB interacts strongly with HscA in a 1:1 ratio only when ATP is present. Negligible association occurs between HscB and HscA in the ADP-bound state indicating that HscB is discharged after ATP is hydrolyzed. ATP hydrolysis is accompanied by a conversion of HscA from the T state to the R state which is stimulated ~60 fold in the presence of HscB and IscU together (Silberg *et al*., 2004). Collectively, the kinetic studies of the HscA ATPase cycle by Vickery and his team have resulted in the following model (Figure 2.4). HscA in the ATP-bound T-state interacts with HscB and IscU for a short time to convert HscA to the "relaxed", ADP-bound state with high affinity for IscU accompanied by release of HscB. This is followed by release of ADP and P_i and re-binding of ATP. IscU is then released before HscA is re-converted to the T-state for another cycle (Figure 2.4). It is important to highlight that these studies were performed with apo-IscU and that the current model might be different *in vivo* where IscU is cluster-loaded.

The nature of association between HscB and IscU has been investigated using several techniques. Surface plasmon resonance experiments performed by Hoff *et al.* showed that both apo and cluster-bound IscU bind very tightly to HscB, though the [2Fe-2S] cluster-containing form binds with a 2-fold higher affinity (Hoff *et al.*, 2000). Unlike *E. coli* DnaJ, HscB does not exhibit any chaperone activity and its C-terminal domain differs from that of DnaJ, which interacts with Hsp40 proteins. The uniqueness of the C-terminal domain of HscB suggests its involvement in substrate specificity, a hypothesis that was investigated by the following structural studies. The crystal structure of HscB revealed an L-shaped, asymmetric structure composed of three distinct domains. The C-terminal domain of HscB has a solvent-exposed, aspartate-rich region, multiple mutations of which in the yeast HscB homolog markedly reduced the affinity of HscB for IscU *in vitro* (Andrew *et al.*, 2006, Cupp-Vickery & Vickery, 2000). Recent NMR studies further localized the specific sites on HscB responsible for interaction with IscU. The solvent exposed region of HscB is characterized by a hydrophobic region that possibly stabilizes an HscB-IscU association. This hydrophobic patch is flanked by acidic and

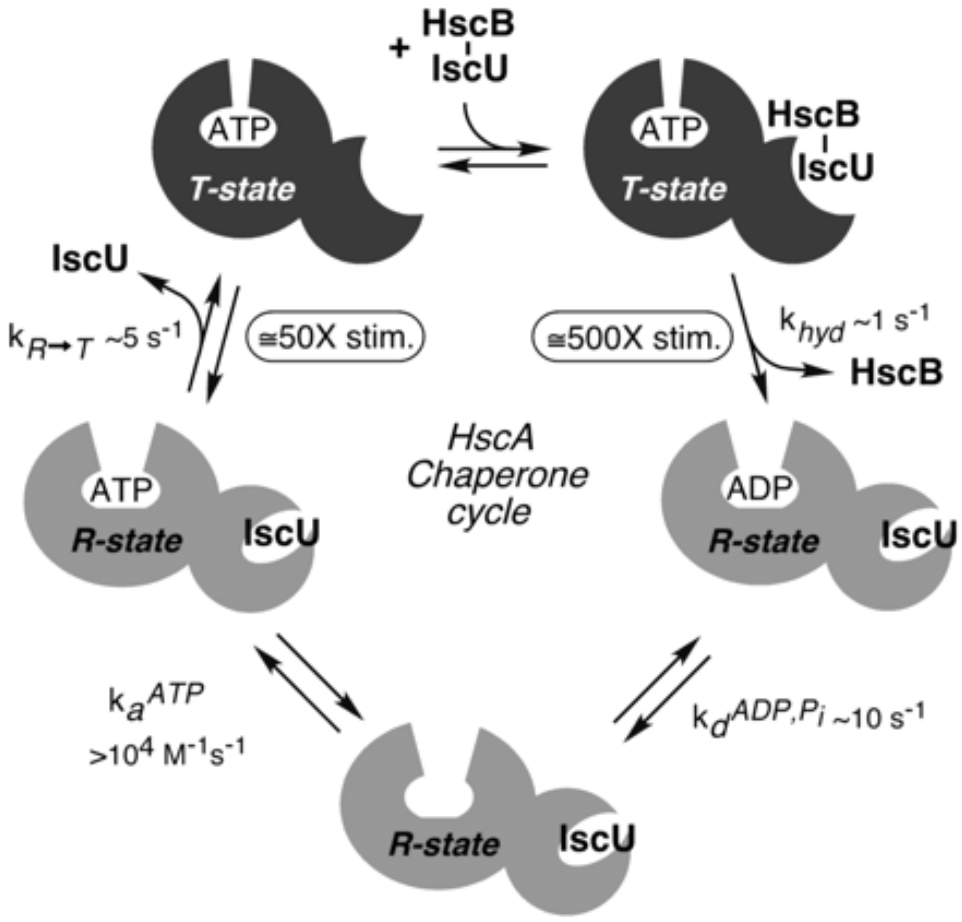

Figure 2.4. A model of the HscA chaperone cycle. Dark and light gray shapes represent HscA protein alternating between the T and R states. Figure reprinted with permission from the Journal of Biological Chemistry (Silberg *et al*., 2004).

basic regions, which are expected to direct the binding of the substrate to HscB (Fuzery *et al.*, 2008).

IscU also binds to HscA, specifically to the C-terminal substrate-binding domain, an association that is intensified in the presence of HscB (Hoff et al., 2000, Silberg et al., 2001). This finding combined with the synergistic stimulation of HscA ATPase activity by HscB and IscU helped corroborate a model in which HscB binds a [2Fe-2S] cluster-containing IscU and delivers it to HscA. IscU in turn binds to HscA through interactions with a specific motif of its C-terminal region (Hoff et al., 2002). This motif is strongly conserved among most IscU proteins and is characterized by an LPPVK motif. This motif is unique to IscU proteins, it exists as LPPEK in NifU proteins, and it is absent in the SUF system. The NIF and the SUF systems do not seem to utilize chaperones in the assembly or transfer of [Fe-S] clusters and so the presence of the motif in the NifU protein may be an evolutionary inheritance. Affinity studies with a synthetic ELPPVKIHC peptide revealed high affinity of this region for HscA but not HscB. Also, the presence of HscB and the oligopeptide did not synergistically stimulate ATPase activity of HscA, meaning that the entire IscU protein or a different region is responsible for stimulating ATP hydrolysis (Hoff et al., 2002). In summary, these findings demonstrated that IscU interacts with HscA in a specific manner that is different from that with HscB.

An investigation into the contribution of each of the residues in the LPPVK motif in interactions with HscA revealed that substitution of the second proline by alanine has the largest effect on ATPase activity and binding affinity, followed by the lysine and the valine residue. In *A. vinelandii,* substitution of the Lys103 residue for an alanine resulted in a null growth phenotype, demonstrating the significance of the role of this amino acid in cellular viability (Johnson DC, unpublished data). The crystal structure of HscA bound with the ELPPVKIHC peptide at a 1.95 Å resolution revealed that the peptide is positioned at a hydrophobic cleft within HscA at a relatively narrow "mouth-like" opening, though large enough that major conformational changes of the substrate-binding domain may not be necessary (Cupp-Vickery *et al.*, 2004a). The HscA residues that are involved in contact with the peptide are mostly hydrophobic ones – with only one water molecule present – and are proposed to stabilize the peptide-chaperone complex. The

orientation of the peptide is the opposite of that observed for peptides bound the analogous region of *E. coli* DnaK and other Hsp70 chaperones (Tapley & Vickery, 2004). This difference in orientation suggests that specific interactions may have to occur for IscU to bind to the substrate-binding domain, the nature of which may have resulted in the specialized function of HscA in [Fe-S] cluster assembly.

The actual role of the chaperones in [Fe-S] cluster biogenesis is still uncertain. Some insight was provided on this issue by groups working on the eukaryotic HscBA homologs who demonstrated, using ^{55}Fe immunoprecipitation analyses and *in vitro* experiments, that the chaperones are not required for the formation of clusters on the scaffold protein and that they are involved at a subsequent step (Dutkiewicz *et al.*, 2006, Muhlenhoff et al., 2003). Recent studies involving yeast depleted for Ssq1, the HscA homolog, established that IscU protein levels are increased in the absence of Ssq1, a finding that was interpreted as a need for higher amounts of scaffold proteins in the cell in the absence of an efficient cluster transfer machinery (Andrew *et al.*, 2008). The idea of the chaperones being involved in cluster transfer was tested by Chandramouli and Johnson who showed that *A. vinelandii* HscA and HscB can stimulate the transfer of a [2Fe-2S] cluster from IscU to apo-ferredoxin (Chandramouli & Johnson, 2006). Another group found that a subtle conformational change occurs on [2Fe-2S] cluster-loaded IscU that is short-lived and therefore difficult to characterize (Bonomi *et al.*, 2008). The role of HscBA in cluster transfer must be specialized since DnaK and DnaJ homologs in the organism *Thermotoga maritima* (which lacks HscBA homologs) were able to increase cluster stability but inhibited its transfer to an apo protein (Wu *et al.*, 2005). Additional studies regarding possible structural changes that may be imposed upon IscU by HscA need to be carried out to gain further insight into the mechanism employed by the chaperones in [Fe-S] cluster transfer.

The question regarding how the chaperones affect formation or transfer of [4Fe-4S] clusters remains unanswered. In a recent study, HscA and HscB did not appear to have an effect in the transfer of [4Fe-4S] cluster from IscU to aconitase *in vitro* (Unciuleac et al., 2007). However, the activities of [4Fe-4S] cluster-containing enzymes are debilitated by the absence of the chaperones (Johnson et al., 2006, Tokumoto & Takahashi, 2001) indicating an essential function of these proteins in [4Fe-4S] cluster formation/transfer

that remains to be characterized. Moreover, the HscBA proteins have only been demonstrated to associate with IscU and not any of the other alternative or specialized scaffolds. Therefore, it remains to be established how essential non-IscU scaffolds, such as NifU and cyanobacterial NfU, transfer their clusters without the help of chaperones. Future studies will hopefully provide answers to this and many other unanswered questions regarding the role and mechanism of the HscA and HscB chaperones in [Fe-S] cluster biogenesis.

2.9 The roles of ferredoxin (Fdx) and IscX in [Fe-S] cluster assembly remain unidentified

The *isc fdx* is co-expressed with *hscBA* in a polycistronic message that might be independently regulated suggesting that their protein products might work together *in vivo* (Seaton & Vickery, 1994). Inactivation of the *fdx* gene prevents cellular viability and affects the maturation of [Fe-S] cluster-containing enzymes in *A. vinelandii* and other organisms (Tokumoto & Takahashi, 2001, Johnson et al., 2006, Barros & Nobrega, 1999). The precise function of Fdx in [Fe-S] biogenesis is unknown. Its low redox potential (-380 mV) (Knoell & Knappe, 1974) and the popular participation of ferredoxins in electron transfer have led to hypotheses that Fdx provides electrons to convert sulfane (S^o) sulfur or ferric (Fe^{+3}) iron to the sulfide (S^{-2}) and ferrous (Fe^{+2}) forms that are appropriate for cluster synthesis. Another step in which ferredoxin could be involved is the reaction witnessed *in vitro* wherein two [2Fe-2S] cluster-containing IscU species are reductively coupled to form a homodimeric [4Fe-4S] cluster (see Figure 2.3) (Agar et al., 2000a, Chandramouli, 2007). Neither of these processes have been tested so far *in vivo*. However, in *S. cerevisiae*, it was shown that depletion of Yah1, the Fdx homolog, renders IscU unable to bind iron and, consequently, form [Fe-S] clusters (Muhlenhoff et al., 2003). The latter is controversial because Fdx requires a [2Fe-2S] cluster itself (Jung *et al.*, 1999a, Kakuta *et al.*, 2001, Zeng *et al.*, 2007b).

Interactions between IscU and Fdx have been established on the basis of their ability to form cross-linked complexes, suggesting they may be redox partners *in vivo* (Wu et al., 2002b). Holo-IscU was able to form a complex with both apo and holo-Fdx but a complex between apo-IscU and either apo- or holo-Fdx could not be observed (Wu et al.,

2002b). This finding suggests two possible *in vivo* scenarios: 1) IscU acts as a scaffold and transfers its cluster to apo-ferredoxin, or 2) ferredoxin is specialized to only play a role during a step after [2Fe-2S] cluster formation, such as [4Fe-4S] cluster formation. Complex formation between Fdx and IscA has also been observed (Ollagnier-de-Choudens et al., 2001). However, cluster transfer from IscA to apo-Fdx was much less efficient than transfer from IscU suggesting that IscA is unlikely to be the physiological cluster donor to ferredoxin (Bonomi *et al*., 2005). Taken together, these findings open up the possibility of a multi-protein complex involving IscU, IscA, and Fdx in which the latter may play a redox role to catalyze cluster formation or transfer on IscA.

The crystal structure of Fdx reveals a monomeric entity bound to a solvent-exposed [2Fe-2S] cluster via four conserved cysteines (Kakuta et al., 2001). The molecule is highly acidic and could interact with basic regions of other proteins, such as the basic N-terminal domain of IscA. A fifth cysteine, which is conserved only among *isc* ferredoxins, is not ligated to the cluster but is solvent exposed and could play a significant part in the role of Fdx (Kakuta et al., 2001).

The last gene in the *isc* operon, *iscX*, codes for a small (7.9 kDa), acidic protein with an uncharacterized function. The presence of this gene is conserved in the *isc* operon and several experiments have demonstrated interactions of this protein with IscS (Pastore *et al.*, 2006, Tokumoto *et al.*, 2002). It is therefore presumed to have a function in [Fe-S] cluster biosynthesis. However, there is minimal genetic and biochemical insight into the character of this protein. Mutations in *iscX,* also known as *yfhJ*, do not have distinct phenotypic effects on *E. coli* cells (Tokumoto & Takahashi, 2001). A crystal structure of IscX has been resolved and it portrays an asymmetric monomeric molecule that is covered with negatively charged patches (Shimomura *et al.*, 2005). This observation is reminiscent of the frataxin and CyaY molecules, which have been proposed to bind iron and deliver it for [Fe-S] cluster synthesis (Cho *et al.*, 2000, Dhe-Paganon *et al.*, 2000). One study showed that IscX is able to bind iron ions (Pastore *et al.*, 2006), however, further studies are required to pinpoint the role of IscX in [Fe-S] cluster biosynthesis.

2.10 The SUF machinery synthesizes clusters during stressful conditions

The SUF system is another [Fe-S] cluster biosynthetic factory that is mainly found in some photosynthetic organisms such as plants and in some bacteria including pathogenic ones (Kessler & Papenbrock, 2005). Specifically, the SUF system appears to be the only [Fe-S] cluster biosynthetic system in chloroplasts. In *E. coli,* the SUF system operates during stressful conditions such as oxidative stress and iron starvation (Takahashi & Tokumoto, 2002). The SUF proteins are encoded by a set of six genes clustered in the operon *sufABCDSE*. SufS is also a PLP-dependent cysteine desulfurase that provides S for [Fe-S] cluster synthesis. Unlike IscS and NifS, which do not require an accessory factor, SufS activity is stimulated by the protein SufE. The two proteins constitute a separate two-component class of cysteine desulfurases that is conserved in several organisms (Loiseau *et al.*, 2003). SufS removes the sulfur from free cysteine, relays it to SufE, which can subsequently donate it to a scaffold protein (Layer *et al.*, 2007). It should be noted that SufE exhibits structural similarity with IscU but no sequence similarity (Goldsmith-Fischman *et al.*, 2004). Moreover, SufE can bind S but no Fe or [Fe-S] clusters. In contrast, SufB can receive sulfur from the SufSE duo and can assemble a [4Fe-4S] cluster in the presence of ferrous salts, making this protein a candidate for the scaffolding role in the SUF apparatus (Layer et al., 2007). However, unlike all clusters formed on IscU or other characterized scaffold proteins, the [4Fe-4S] cluster formed on SufB is stable upon reduction. This finding is consistent with a redox rather than a scaffold role for the cluster formed on SufB. The SufC protein exhibits ATPase activity and forms a complex with the SufBD proteins to stimulate SufSE activity. However, ATP hydrolysis is not required for this stimulation and, therefore, it is probable that additional functions remain to be assigned to the SufBCD complex. The coexistence of a scaffold and ATPase protein is a recurring pattern as it has been observed in the ISC system and the ApbC/Nbp35 proteins suggesting a link between the two functions. SufA has been demonstrated to act as a scaffold *in vitro* based on its ability to assemble [2Fe-2S] clusters and unstable [4Fe-4S] clusters. How all the SUF proteins work together to assemble [Fe-S] clusters and deliver them to target proteins remains subject to further research.

2.11 Cross-talk between bacterial [Fe-S] cluster biosynthetic systems

The two bacterial organisms in which [Fe-S] cluster biosynthesis has been studied the most are *E. coli* and *A. vinelandii*. Both microbes utilize the ISC apparatus for construction of [Fe-S] clusters for "housekeeping" purposes, whereas *E. coli* utilizes the SUF machinery during iron starvation and oxidative stress and *A. vinelandii* uses its NIF system during nitrogen-fixing conditions. The similarities and distinct features of the three [Fe-S] cluster biosynthetic systems have sparked an interest in investigating the roles of the biosynthetic components by studying functional complementation and ways in which expression between the different machineries is coordinated.

The *E. coli* SUF machinery employs several proteins that exhibit sequence, structural, and/or functional similarities with the ISC proteins. For example, SufA and IscA are 40% identical in amino acid sequence and both can assemble [2Fe-2S] and [4Fe-4S] clusters *in vitro*, a characteristic that earned them the proposed role of "alternative scaffolds". Neither the presence of SufA nor IscA by itself is vital for the cell, but a double $IscA^-/SufA^-$ mutant results in a null-growth phenotype during aerobiosis indicating functional redundancy between the two proteins (Lu et al., 2008). There is no IscU homolog that is produced by the *suf* operon. SufE presents structural similarity with IscU but no amino acid sequence similarity (Goldsmith-Fischman et al., 2004). Like IscU, SufE can accept sulfur from SufS but it is unable to assemble clusters. In contrast, SufB has no sequence or structural similarity with IscU but it appears to be able to assemble [4Fe-4S] clusters (Layer et al., 2007). SufB and SufD form a complex with SufC, an ATPase that shares no similarity with the HscBA chaperones other than their ability to hydrolyze ATP. Other than the functional redundancy between IscA and SufA, it is not known whether individual SUF proteins are able to functionally complement ISC proteins and vice versa. What has been shown is that the *suf* operon can complement inactivation of the entire *isc* operon but only when expressed at high levels (Takahashi & Tokumoto, 2002). One protein that links the two systems is the regulatory protein, IscR, which can exist in the cluster-less, "apo" form or loaded with a labile [2Fe-2S] cluster. The cluster-bound form has been shown to repress expression of the *isc* operon as part of a negative feedback loop (Schwartz *et al.*, 2001). During oxidative stress conditions, the apo form of the protein activates the *suf* operon (Yeo *et al.*, 2006). It would appear that activation of the SUF system requires the occurrence of three additional events:

activation by OxyR, activation by the integration host factor IHF, and de-repression by iron-bound Fur (Lee *et al.*, 2004, Xu & Moller, 2008).

Some functional crosstalk has also been observed between the *Azotobacter vinelandii* NIF and ISC systems (Dos Santos *et al.*, 2007). While NifU expressed at physiological levels cannot rescue the null-growth phenotype associated with deletion of *iscU* (Johnson *et al.*, 2005b), Dos Santos *et al.* found that NifU produced at elevated levels can accommodate the cell with the needed [Fe-S] clusters. In particular, NifU by itself (without co-expression of NifS), can functionally replace IscU, suggesting that the scaffold-cysteine desulfurase interactions are somewhat conserved in the two systems. NifS however cannot rescue the null-growth phenotype associated with depletion of IscS, even when produced at elevated levels. This result is in accordance with the overwhelming evidence indicating that IscS is a universal sulfur donor not only for [Fe-S] clusters, but for other S-containing cofactors as well. In contrast, both IscU and IscS expressed at elevated levels are able to functionally replace NifU and NifS to provide clusters for nitrogenase and its associated [Fe-S] proteins (Dos Santos et al., 2007).

2.12 [Fe-S] cluster assembly in eukaryotes

The mechanism of cluster assembly is conserved among bacteria and higher organisms as evidenced by the ability of bacteria and yeast to assemble [Fe-S] clusters onto human proteins (Coghlan & Vickery, 1989). Much of the current understanding about eukaryotic assembly of [Fe-S] clusters is owed to studies performed on *Saccharomyces cerevisiae* (Lill & Muhlenhoff, 2006, Lill & Muhlenhoff, 2008b). Eukaryotes utilize a mitochondrial [Fe-S] cluster assembly system that is similar to the ISC machinery in bacteria. Proteins involved in cytosolic [Fe-S] cluster assembly have also been identified, although that field is still new and interplay between mitochondrial, cytosolic, and nuclear [Fe-S] assembly is being debated among experts. Homologs of many of the components involved in the prokaryotic ISC-dependent cluster assembly have also been identified in yeast. IscU, referred to as Isu1 in yeast, is well conserved among prokaryotes and eukaryotic organisms with over 70% amino acid sequence identity between the human and *E. coli* proteins (Schilke *et al.*, 1999). Yeast studies using radiolabelled ^{55}Fe have demonstrated that Isu1 binds iron *in vivo* and that this

binding is dependent on the presence of Nfs1, the yeast IscS homolog. Unlike IscS, Nfs1 requires an essential accessory protein factor, Isd11, which is absent in prokaryotes and is proposed to transfer sulfur from Nfs1 to Isu1 (Wiedemann *et al.*, 2006, Adam *et al.*, 2006). The iron donor is unknown although frataxin has been suggested as a candidate (discussed above). Two membrane proteins Mrs3 and Mrs4 have been shown to be important for iron insertion into the mitochondria (Zhang *et al.*, 2006). The eukaryotic HscA and HscB homologs, known as Ssq1 and Jac1 respectively, are required at a step after [Fe-S] cluster assembly on Isu1 (Muhlenhoff et al., 2003, Dutkiewicz et al., 2006). The mechanism of cluster transfer in eukaryotes may be different since the Ssq1 chaperone, unlike its prokaryotic homolog, requires the nucleotide exchange factor Mge1 (Lutz *et al.*, 2001, Schmidt *et al.*, 2001). In addition to ATP, GTP was also found to be a requirement for [Fe-S] cluster biogenesis (Amutha *et al.*, 2008). The glutaredoxin Grx5 has also been implicated at a step after [Fe-S] cluster assembly, but its exact role is still under investigation (Rodriguez-Manzaneque *et al.*, 2002, Muhlenhoff et al., 2003).

The maturation of [Fe-S] proteins in the cytosol is a relatively undeveloped area of study and the role of the mitochondrial cluster assembly machinery in this process is debated. There are no Isu cytosolic paralogs, which initially led to the proposal that [Fe-S] clusters are synthesized in the mitochondria and subsequently transferred to the cytosol. In line with this hypothesis, the mitochondrial membrane ABC transporter Atm1 has been suggested to facilitate the inter-membrane transfer of a sulfur-containing compound from the matrix to the cytosolic space (Kuhnke *et al.*, 2006, Kispal *et al.*, 1999). Outside the mitochondria, a scaffold protein complex has been identified that involves a P-loop NTPase Cfd1 and a scaffold-type protein Nbp35 that can assemble [Fe-S] clusters *in vitro.* The function of this complex is dependent on the mitochondrial cluster assembly export system, suggesting that the exported moiety is utilized by the Cfd1-Nbp35 complex to form [Fe-S] clusters.

2.13 Assembly of [Fe-S] clusters and significance for human diseases

Because [Fe-S] clusters serve a crucial role in many essential biochemical processes, defects in the assembly of these cofactors can result in diseases with diverse symptoms. Thus far, defects in at least three proteins involved in [Fe-S] cluster assembly have been

shown to result in various diseases: Friedreich's ataxia, sideroblastic anemia, and cardiomyopathy with exercise intolerance. Friedreich's ataxia, a neurodegenerative disease, is a debilitating disorder that is characterized by gradual spinocerebellar degeneration and is associated with muscle weakness, speech and limb ataxia, and cardiomyopathy (Rouault & Tong, 2008). The source of this disorder was pinpointed to defects in frataxin, a mitochondrial iron-binding protein, homologs of which have been described as iron chaperones for [Fe-S] cluster assembly on the scaffold protein in various eukaryotic and bacterial organisms (Chen *et al.*, 2002, Kondapalli *et al.*, 2008, Wang & Craig, 2008, Layer et al., 2006). Sideroblastic anemia is characterized by mitochondrial iron overload in red cell precursors. The cause for this disease was traced to mutations in Grx5 (Camaschella *et al.*, 2007), a monothiol glutaredoxin has been proposed to assemble and/or deliver [Fe-S] clusters to target proteins (Bandyopadhyay et al., 2008b, Wingert *et al.*, 2005). In addition, Swedish patients suffering from cardiomyopathy with severe exercise intolerance were shown to have a single mutation in the assembly scaffold *ISU* that causes a splicing defect and subsequent lower levels of ISU in mitochondria. Impairment of [Fe-S] cluster biogenesis leads to a number of other disorders in humans that are either associated with unbalanced iron homeostasis or proteins that require [Fe-S] clusters to function properly (Lill & Muhlenhoff, 2008b).

2.14 Summary

Despite the rapid growth of the understanding of [Fe-S] cluster biogenesis during the past decade, the exact mechanistic events involved in the synthesis of [Fe-S] clusters are unclear. The majority of the studies have employed *in vitro* methods, which have provided significant insight, but have led to controversy on some aspects of the pathway. IscU can assemble both [2Fe-2S] and [4Fe-4S] clusters *in vitro* in the presence of excess starting materials, however, it is not known whether this observation represents a physiological process. Thus far, no studies have been conducted with ISC components produced *in vivo*. A major portion of this dissertation describes the development of two methods that allow the isolation of the ISC proteins produced *in vivo*, provides evidence for the scaffold hypothesis and insight into the mechanism entailed in the formation of iron-sulfur clusters.

CHAPTER 3. *In vivo* iron-sulfur cluster formation

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This article describes the *in vivo* isolation of IscU and IscU^{39DA} from *Azotobacter vinelandii*. I performed all the experiments and data analysis described in this section related to the isolation of wild type IscU. I was involved at all stages of the manuscript preparation including: writing my part of "Materials and Methods" and "Results" sections and participating in preparations of the abstract, figures and the "Introduction" and "Discussion" sections.

3.1 Abstract

It has been proposed that [Fe-S] clusters destined for the maturation of [Fe-S] proteins can be preassembled on a molecular scaffold designated IscU. In the present work, it is shown that production of the intact *Azotobacter vinelandii* [Fe-S] cluster biosynthetic machinery at levels exceeding the amount required for cellular maturation of [Fe-S] proteins results in the accumulation of: (*i*) apo-IscU, (*ii*) an oxygen-labile [2Fe-2S] cluster-loaded form of IscU, and (*iii*) IscU complexed with the S delivery protein, IscS. It is suggested these species represent different stages of the [Fe-S] cluster assembly process. Substitution of the IscU Asp^{39} residue by Ala results in the *in vivo* trapping of a stoichiometric, non-covalent, non-dissociating IscU-IscS complex that contains an oxygen-resistant [Fe-S] species. In aggregate, these results validate the scaffold hypothesis for [Fe-S] cluster assembly and indicate that *in vivo* [Fe-S] cluster formation is a dynamic process that involves the reversible interaction of IscU and IscS.

3.2 Introduction

Iron-sulfur [Fe-S] clusters are small inorganic prosthetic groups that participate in a variety of biochemical processes, including electron transfer, substrate binding and activation, redox catalysis, DNA replication and repair, regulation of gene expression, and tRNA modification (Johnson *et al.*, 2005, Ayala-Castro *et al.*, 2008). The most abundant [Fe-S] clusters fall into two structural types, rhombic [2Fe-2S]- and cubane [4Fe-4S]-clusters, and these are typically attached to their protein partners, called [Fe-S] proteins, through cysteine thiolate ligands. In spite of their simple composition and structure, biological formation of [Fe-S] clusters is remarkably complex, a feature that could be related to the physiological toxicity of Fe^{2+} and S^{2-} in their free forms. The canonical [Fe-S] protein maturation machinery found in many bacteria and most, if not all, eukaryotes is called the ISC (iron-sulfur cluster) system (Johnson et al., 2005a, Lill & Muhlenhoff, 2008a). In *A. vinelandii*, this system includes a proposed [Fe-S] cluster assembly scaffold (IscU), a sulfur trafficking pyridoxal-5'-phosphate (PLP)–dependent cysteine desulfurase (IscS), molecular chaperones (HscB and HscA), a ferredoxin (Fdx),

a possible Fe donor or auxiliary [Fe-S] cluster assembly/carrier protein (IscA), a protein of unknown function (IscX) and a regulatory protein (IscR) (Zheng *et al.*, 1998). Although details of the assembly process are not known, the current model proposes that [Fe-S] clusters are formed on IscU, through the delivery of S by IscS, and delivery of Fe by an unknown mechanism. HscB and HscA are proposed to be involved in the subsequent delivery of [Fe-S] clusters from IscU to various target proteins (Muhlenhoff *et al.*, 2003, Chandramouli & Johnson, 2006). The ISC associated ferredoxin has been proposed to be involved in the formation of different cluster types on IscU and/or the redox-dependent release of [Fe-S] clusters from IscU. It has also been suggested that auxiliary [Fe-S] cluster carrier proteins could be involved in the distribution of [Fe-S] clusters assembled on IscU to other target proteins (Bandyopadhyay *et al.*, 2008d, Angelini *et al.*, 2008b).

Although there is a substantial body of evidence that [Fe-S] clusters can be assembled on the proposed IscU scaffold *in vitro* (Agar *et al.*, 2000a, Unciuleac *et al.*, 2007), validation of the "scaffold hypothesis" for formation of simple [Fe-S] clusters has not been clearly established by *in vivo* methods. Furthermore, nothing is known about the dynamic aspects of *in vivo* [Fe-S] cluster assembly. The *in vivo* analysis of [Fe-S] cluster assembly presents a number of technical challenges. For example, because [Fe-S] clusters are destined for delivery to other proteins, they are expected to be readily detached from the assembly scaffold after *in vivo* assembly is complete. This possibility is supported by the observation that [Fe-S] clusters assembled on IscU *in vitro* are both reductively and oxidatively labile (Agar et al., 2000a). Also, the physiological accumulation of ISC components is subject to a negative feedback regulatory mechanism in response to a demand for [Fe-S] protein maturation. This situation is expected to result in a very low physiological accumulation of [Fe-S] cluster assembly intermediates. In the present work, these problems were circumvented in several ways. First, an *A. vinelandii* strain was constructed that has expression of ISC components decoupled from IscR regulation such that the expression of ISC components exceeds the physiological demand for [Fe-S] protein maturation. Using this genetic background, a second strain was constructed that produces a physiologically active form of IscU that contains an affinity tag, which permits the rapid and gentle purification of IscU. Finally, a third strain that

carries an amino acid substitution within IscU, which results in a dominant-negative phenotype, was constructed. Isolation of IscU produced by these different strains permitted a direct test of the scaffold hypothesis, and also provided insights about the mechanistic features of the *in vivo* [Fe-S] cluster assembly process.

3.3 Materials and Methods

Strain and plasmid construction. The technical procedures involved in plasmid and strain constructions, including construction of strain DJ1421 (Johnson *et al.*, 2006), which served as the parental strain used in the present work have been previously described in detail. Strain DJ1601 carries an in-frame 120 bp deletion within the *iscR* gene, which removes residues 21 through 60 within IscR. Strain DJ1697 is isogenic with DJ1601 except that it contains an 8-polyhistidine coding sequence (5′-GGGCCATCACCATCACCACCATCACCATAT-3′) inserted at the *Stu*I site of *iscU*, which is located at the C-terminal coding region of *iscU*. Introduction of the histidine tag changed the C-terminal region of the protein from KGLV to KGGHHHHHHHHHILV. Strain DJ1766 carries the same poly-histidine encoding tag as DJ1697 and also has the Asp^{39} codon (GAC) of *iscU* substituted by an Ala^{39} codon (GCC). DJ1766 also carries an insertion element within the *recA* gene to ensure strain stability (Johnson et al., 2006). The relevant genomic regions of strains DJ1421, DJ1601, DJ1697 and DJ1766 are shown in Figure 3.1, all of which were confirmed by DNA sequence analysis of PCR-amplified genomic DNA. Two strains were used to assess the effect of an *iscR* deletion on ISC gene expression. The first of these, DJ1525 is isogenic with DJ1421 except that it contains a *Φ*(*hscA'-lacZ*) fusion within the endogenous ISC gene cluster. The other strain, DJ1580, carries the same *Φ*(*hscA'-lacZ*) fusion as DJ1525, and it also carries a 186 bp in-frame deletion within *iscR* removing codons 57 through 119. Construction of similar strains that carry the same *Φ*(*hscA'-lacZ*) fusion placed within DJ1525 and DJ1580 have been previously described in detail (Johnson et al., 2006). Heterologous production of the *A. vinelandii* IscS and his-tagged IscU Ala^{39} protein in *E. coli* was accomplished using a derivative of the plasmid vector pAra13 (Dos Santos *et al.*, 2004), which has the expression of these genes placed under control of the *ara* regulatory elements. For

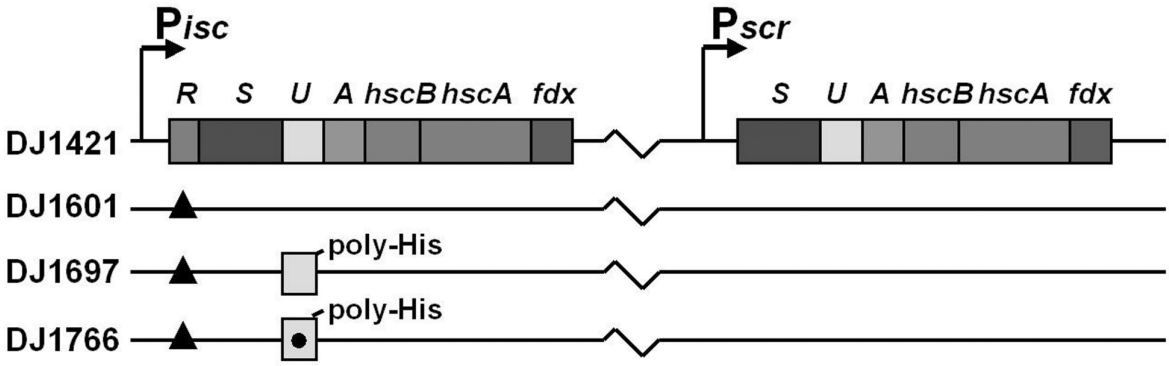

Figure 3.1. Schematic representation of strains used in this work. Strains DJ1601, DJ1697 and DJ1766 each carry a 120 bp in-frame deletion within the *iscR* gene (indicated by black triangles). Strains DJ1697 and DJ1766 produce an IscU that carries a polyhistidine tag located at the C-terminus. The his-tagged version of IscU produced by DJ1766 also has the Asp^{39} residue substituted by Ala (indicated by a black dot). Straight lines indicate genetic identity to DJ1421.

construction of this plasmid, pDB1712, a *Bsp*HI-*Hind*III fragment that contains *iscS*, *iscU* and a portion of *iscA* was used. Cell growth and arabinose-stimulated gene expression was performed as previously described (Dos Santos et al., 2004).

Growth and purification of IscU and the IscU Ala^{39}-IscS complex produced by strains DJ1697 and DJ1766. *A. vinelandii* cells were grown at 30^{o}C in Burk's medium (Strandberg & Wilson, 1968) supplemented with 10 mM urea in a 150-liter fermenter. Harvested cells (100 to 200 g wet weight) were resuspended in 1.5 volumes of degassed, argon-sparged, 50 mM Tris-HCl, pH 8.0 (Buffer A) containing 0.5 M NaCl, pepstatin A (0.14 mg/l) and phenylmethanesulfonyl fluoride (0.5 mM) and lysed by one or two passages through a Nano DeBee homogenizer at 25,000 psi (B.E.E International Inc., South Easton, MA). Crude extracts were obtained by centrifuging cell lysates at 235,000 x *g* for 1 hour at 4^{o}C in a Beckman Coulter Optima LE-80K ultracentrifuge using a TI 45 rotor. All subsequent IscU purification steps using DJ1697 extracts were performed in a Coy anaerobic chamber containing a gas mixture of 4% H_2 and 96% N_2, whereas purification of the IscU Ala^{39}-IscS complex produced by DJ1766 was performed on the bench-top under anoxic conditions maintained using a Schlenk line and degassed argon-sparged buffers. Cell extracts were applied to XK-16 or XK-26 Pharmacia Biotech columns respectively packed with either 5 ml or 15 ml of Affiland iminodiacetic acid-Sepharose (IMAC) resin. IMAC columns were charged with 50 mM $NiSO_4$, and equilibrated with 5 volumes of the above buffer prior to loading crude extracts. In the case of DJ1697 extracts, bound protein was sequentially washed with three column volumes of Buffer A containing 0.5 M NaCl, three column volumes of Buffer A containing 0.1 M NaCl and 20 mM imidazole, and then eluted using Buffer A containing 0.1 M NaCl and 200 mM imidazole. In the case of DJ1766 extracts, bound protein was washed with three column volumes of Buffer A containing 0.5 M NaCl and 20 mM imidazole, 3 column volumes containing 0.5 M NaCl and 40 mM imidazole and then eluted with Buffer A containing 0.5 M NaCl and 100 mM imidazole.

For further separation of the IMAC fraction that contains IscU produced by DJ1697, the sample was diluted 10 fold in Buffer A containing 1 mM DTT and applied to a 1-ml or 5-ml HiTrap Q HP column (GE Healthcare) and washed with 10 volumes of the same

buffer. IscU was subsequently eluted using a 0-200 mM NaCl gradient of 10 bed volumes in Buffer A containing 1 mM DTT. The remaining bound protein, identified as IscS, was subsequently eluted from the column using Buffer A that contains 1 M NaCl and 1 mM DTT. A representative elution profile is shown in Figure 3.2.

For further purification of the IMAC fraction that contains the IscU Ala^{39}-IscS complex produced by strain DJ1766 the sample was diluted 5 fold with Buffer A (containing 0.5 mM Tris (2-carboxyethyl) phosphine and applied to a 5 ml 1.5 x 3.0 cm Q-sepharose anion exchange column (GE Healthcare). The loaded column was washed with approximately 5 volumes of Buffer A containing 150 mM NaCl and subsequently eluted with Buffer A containing 400 mM NaCl.

Purification of recombinantly expressed IscS in *E. coli* was performed essentially as previously reported (Zheng *et al.*, 1993) with the exception that 10% glycerol was added to the buffers and the protein was stored in 0.6 M NaCl.

Gel exclusion chromatography. A pre-packed Superose 12 gel filtration column (GE Healthcare) was used to estimate the M_r of IscU purified from DJ1697 and the IscU Ala^{39}-IscS complex prepared from DJ1766. The column was pre-equilibrated with a degassed and argon-sparged 20 mM Tris-HCl buffer (pH 7.8) containing 0.2 M NaCl. Approximately 0.5 mg were applied to the column and subsequently eluted using a flow rate of 0.5 ml/min.

Analytical and spectroscopic analyses. Protein concentration was determined using either the biuret method or the Bradford protein assay (Bio-Rad). All analytical analyses were performed in triplicate. Iron was quantified using the commercial Quantichrom™ iron assay kit (DIFE-250) purchased from Bioassay Systems. Sulfide and PLP quantifications were performed as previously described (Chen & Mortenson, 1977, Wada & Snell, 1962). UV-visible absorption spectroscopy analyses were performed at room temperature using a Cary 50 Bio Spectrophotometer. For amino acid analysis, 25 µg of the IscU Ala^{39}-IscS complex were separated by SDS-PAGE. Stained bands corresponding to IscU and IscS were excised and delivered to the Keck Biotechnology

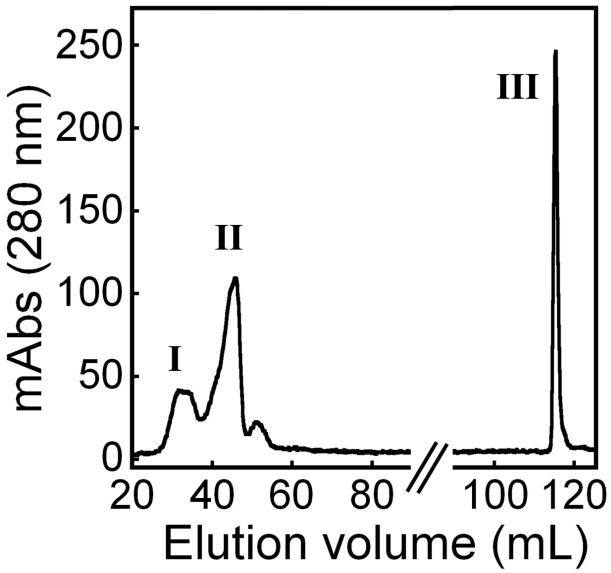

Figure 3.2. Anion-exchange chromatography elution profile of the IscU-containing IMAC fraction prepared from DJ1697. Apo-IscU (fraction I), [Fe-S] cluster-loaded IscU (fraction II), IscS (fraction III). Fractions I and II were separated by a 50-ml 0-200 mM NaCl gradient, whereas fraction III was eluted with 1 M NaCl.

Resource Laboratory (Yale University) for quantitative amino acid analysis using a Hitachi L-8900 amino acid analyzer.

Enzyme assays and kinetic analyses. All assays were performed at room temperature. Aconitase and isocitrate dehydrogenase activities were measured using the same procedures previously described (Johnson et al., 2006). Substrate affinity was evaluated by monitoring the immediate ΔAbs_{400} elicited by the addition of increasing concentrations of L-cysteine to 50 nmoles of IscS or IscU Ala^{39}-IscS complex. The ΔAbs_{400} values were standardized to the amount of PLP present in each protein sample. The time-dependent relaxation of the ΔAbs_{400} induced by the addition of a 10 fold molar excess of L-cysteine to either IscS or the IscU Ala^{39}-IscS complex was obtained by recording the UV-visible spectrum every 20 sec after substrate addition.

Mass spectrometry. Mass spectra were obtained through the Virginia Tech proteomics research incubator service. Collision induced LC/MS/MS spectra were obtained from tryptic digested samples and data were processed by Xcalibur version 1.2 software.

3.4 Results

Genetic Constructions and Experimental Rationale. In the case of *A. vinelandii*, the genetic manipulation of ISC components is complicated by the fact that *iscS*, *iscU*, *hscB*, *hscA* and *fdx* are essential (Johnson et al., 2006). We therefore, previously constructed a strain (DJ1421) that has a duplicate copy of *iscS*, *iscU*, *iscA*, *hscB*, *hscA* and *fdx* placed under control of the inducible sucrose (SCR) catabolic regulatory elements (Figure 3.1). Thus, the phenotypic consequences of substitutions placed in any of the endogenous ISC components can be assessed by growing cells in the presence of sucrose, a condition under which the duplicated ISC region is expressed, or in the absence of sucrose, a condition under which the duplicated ISC region is not expressed. DJ1421 was used for all other strain constructions in this work (Johnson et al., 2006).

Previous attempts to isolate an [Fe-S] cluster-loaded form of IscU from a wild-type strain of *A. vinelandii* were not successful. Among possible explanations that could

contribute to this failure is that ISC expression is subjected to a negative feedback loop (Schwartz *et al.*, 2001). Namely, once the demand for [Fe-S] cluster biosynthetic capacity is satisfied, IscR represses further expression of the ISC transcriptional unit. We, therefore, reasoned that expression of ISC components at a level that exceeds the demand for maturation of [Fe-S] proteins could result in the accumulation of an [Fe-S] cluster-loaded form of IscU. In order to accomplish the elevated expression of ISC components, a strain (DJ1601, Figure 3.1) was constructed that has an in-frame deletion within the *iscR* gene. An important aspect of this experimental strategy is that it permits the elevated expression of ISC components but not at the extreme non-physiological levels commonly associated with heterologous recombinant expression. This strategy also has the advantage that the coordinate expression of all the ISC components remains intact. Thus, artifacts that might be anticipated to result from the hyperexpression or unbalanced expression of ISC components are avoided. The consequence of placing a deletion within *iscR* was assessed by comparing the β-galactosidase activity in a strain that has an *hscA::lacZ* fusion and an intact *iscR* gene (DJ1525) to an isogenic strain that also carries an *iscR* deletion (DJ1580). This analysis revealed that loss of IscR function results in a 5 to 7 fold increase in ISC expression, a result that was also confirmed by western analysis (data not shown).

A second anticipated problem associated with isolation of [Fe-S] cluster-loaded forms of IscU produced *in vivo* is the expected lability of the associated [Fe-S] clusters (Agar et al., 2000a). Thus, it was necessary to develop a method for the rapid and gentle purification of IscU from cell extracts. To accomplish this goal, strain DJ1697 was constructed (Figure 3.1) that encodes a polyhistidine tag within the C-terminal coding region of *iscU* in combination with an in-frame deletion in *iscR*. This construction permitted the application of immobilized metal affinity chromatography (IMAC) for rapid purification of IscU as described in the next section. It was also necessary to establish that incorporation of the polyhistidine tag within the C-terminal coding region of IscU does not adversely affect the *in vivo* function of IscU. This was accomplished by showing that strain DJ1697 has the same growth rate either when cultured using sucrose as the carbon source, a condition under which the SCR-regulated untagged version of IscU is also expressed, or using glucose as the carbon source, a condition under which

only the IscR-regulated, tagged, copy of IscU is expressed. We also found that incorporation of the polyhistidine tag at the C-terminal region of IscU has no adverse effect on the accumulation of active aconitase, a [4Fe-4S] cluster-dependent enzyme, indicating that the tag does not impair the *in vivo* capacity for maturation of [Fe-S] proteins.

A final genetic construction involved substitution of the Asp^{39} residue by Ala in the polyhistidine tagged version of IscU in a genetic background for which *iscR* is also deleted (DJ1766, Figure 3.1). This strain is unable to grow in the absence of sucrose indicating that the Ala^{39} substitution eliminates the function of IscU. Previous work with a similar strain revealed that substitution of the IscU Asp^{39} residue by Ala^{39} results in a dominant-negative phenotype (very slow growth) when the wild type SCR-regulated allele of *iscU* is also expressed (Johnson et al., 2006). The dominant-negative effect of the IscU Ala^{39} substitution is also manifested by a 70% loss in the activity of aconitase. In previous studies, it was shown that *in vitro* assembly of [2Fe-2S] clusters using the Ala^{39}-substituted form of IscU from *A. vinelandii* (Unciuleac et al., 2007), or the analogous substitution within IscU from other organisms (Foster *et al.*, 2000, Shimomura *et al.*, 2007), result in stabilization of the associated [2Fe-2S] cluster when compared to wild type IscU. Thus, one explanation for the dominant-negative effect exhibited by DJ1766 is that stable [Fe-S] cluster species become trapped on the proposed IscU scaffold *in vivo* and are, therefore, unavailable for [Fe-S] protein maturation. Another possibility is that the IscU Ala^{39} substitution could result in sequestering some other component of the ISC biosynthetic machinery within a non-productive complex. Construction of DJ1766 was designed to elucidate the basis for the dominant-negative phenotype associated with the IscU Ala^{39} substitution.

Labile [Fe-S] clusters are assembled on IscU *in vivo.* The histidine-tagged version of IscU was isolated in two steps that involved IMAC and anion-exchange chromatography. Fractions eluted from the IMAC column containing IscU were analyzed by SDS-PAGE and were found to also contain substoichiometric amounts of IscS (data not shown). Pooled fractions that contained IscU were then applied to an anion-exchange column and eluted using a salt gradient. Anion exchange chromatography resolved the sample into

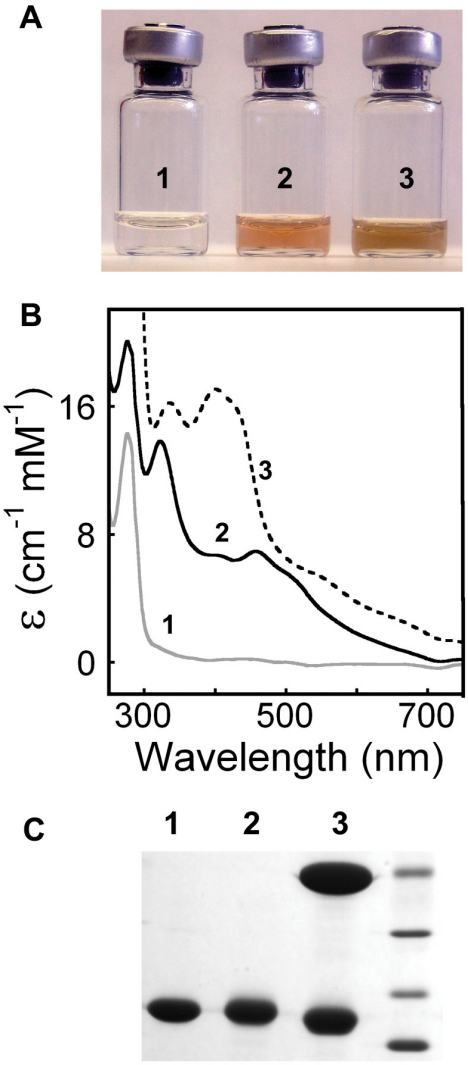

Figure 3.3. Comparison of samples that contain IscU or IscU Ala^{39}-IscS complex prepared by anion-exchange chromatography. In each panel, (1) indicates apo-IscU prepared from DJ1697 (see fraction I in Figure 3.2), (2) indicates [Fe-S] cluster-loaded IscU prepared from DJ1697 (see fraction II in Figure 3.2), and (3), indicates the IscU Ala^{39}-IscS complex prepared from DJ1766. Each sample contained 67 μM IscU. (**A**) Photograph of samples. (**B**) UV-visible absorption spectra. (**C**) SDS-PAGE (lanes 1, 2 and 3). IscU in lane 3 has a faster electrophoretic mobility when compared to lanes 1 and 2 because it carries the Ala^{39} substitution. Unlabeled lane: M_r standards (ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400).

three major fractions designated I, II, and III (Figure 3.2). Fraction I is colorless (Figure 3.3A), exhibits no distinctive absorbance in the visible spectrum (Figure 3.3B), and contains only IscU (Figure 3.3C) (Agar et al., 2000c). When fraction I was further analyzed by gel filtration chromatography it was resolved into two fractions that correspond to monomeric and dimeric forms of IscU. The monomeric form of IscU was usually the predominant species but the relative amount of monomer and dimer varied from experiment to experiment (data not shown). Chemical analysis of fraction I indicated that it contains approximately 0.21 (+/- .01) Fe and 0.18 (+/- .01) acid labile sulfide per IscU. Fraction II is red (Figure 3.3A), only contains IscU and exhibits a UV-visible absorption spectrum (Figure 3.3B) that has identical features when compared to the [2Fe-2S]-cluster loaded IscU produced by *in vitro* reconstitution methods (Figure 3.3C). The maximum extinction coefficients for this sample were $\varepsilon_{456} = 6.0\ mM^{-1}cm^{-1}$ and $\varepsilon_{410} = 6.6\ mM^{-1}cm^{-1}$ and these values are similar to those reported for [2Fe-2S] cluster-containing ferredoxins (Dailey *et al.*, 1994). When fraction II was analyzed by gel filtration, it could also be resolved into apparent monomers and dimers, although the monomeric species was predominant. Chemical analysis of fraction II indicated that it contains approximately 1.29 (+/- 0.05) Fe and 1.14 (+/- 0.29) acid labile sulfide per IscU. The [2Fe-2S] species contained within IscU prepared *in vivo* is rapidly destroyed when exposed to air (Figure 3.4), and this same feature has been reported for [2Fe-2S] species assembled on IscU by *in vitro* methods (Agar et al., 2000a). Fraction III was identified as IscS by western analysis and because it has the same UV-visible absorption spectrum and the same electrophoretic mobility as previously reported for IscS, it contains PLP, and it exhibits cysteine desulfurase activity.

Isolation of a stable [Fe-S] cluster-loaded non-covalent $\alpha_2\beta_2$ IscU-IscS complex. The Ala^{39}-substituted form of IscU produced by DJ1766 was subjected to a very similar purification procedure to the one described above. In this case, anion exchange chromatography yielded a single brown fraction, in contrast to the colorless and red fractions described above (see Figure 3.3A). SDS-PAGE and quantitative amino acid analysis revealed that IscU-Ala^{39} and IscS are isolated as a nearly exact stoichiometric complex (Figure 3.3C). The identity of IscU $Ala^{39}$and IscS purified from DJ1766

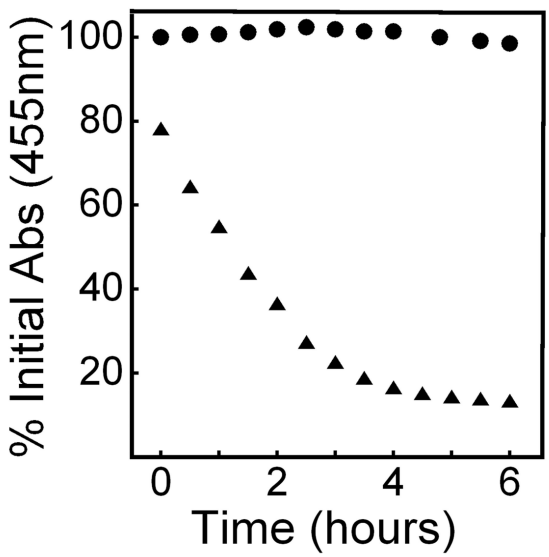

Figure 3.4. Time-dependent effect on the Abs_{455nm} for [Fe-S] cluster-loaded IscU (▲) and [Fe-S] cluster-containing IscU Ala^{39}-IscS complex (●) upon exposure to air. Each sample contained 130 μM of IscU.

extracts was further established by mass spectrometry. Size exclusion chromatography indicated that the IscU Ala^{39}-IscS complex has an approximate M_r of 127,000, which is consistent with the complex being comprised of an $\alpha_2\beta_2$ heterotetramer. Chemical analysis of the complex showed that it contains approximately 1.2 (+/- 0.2) Fe and 0.95 (+/- 0.1) acid labile sulfide per IscU and approximately 0.9 (+/- 0.2) PLP per IscS. Subtraction of the UV-visible spectrum of the [2Fe-2S] cluster-loaded form of IscU (Figure 3.3B, spectrum 2) from the IscU Ala^{39}-IscS complex (Figure 3.3B, spectrum 3) yields the same characteristic spectrum associated with isolated IscS. Furthermore, subtraction of an equivalent IscS spectrum from the IscU Ala^{39}-IscS complex spectrum (Figure 3.3B, spectrum 3) yields a spectrum that has the same features as the [2Fe-2S] cluster-loaded form of IscU shown in Figure 3.3B. Thus, it can be concluded that the IscU Ala^{39}-IscS complex is likely to be loaded with predominantly a [2Fe-2S] cluster, although the presence of other [Fe-S] species has not been excluded. This question will be addressed by future biophysical analyses. In contrast to the oxidative lability of the [2Fe-2S] cluster present in isolated IscU, the [Fe-S] species contained within the IscU Ala^{39}-IscS complex is not degraded upon prolonged exposure to oxygen (Figure 3.4). Three lines of evidence indicate that the stable $\alpha_2\beta_2$ IscU Ala^{39}-IscS complex is not the result of a covalent disulfide linkage between IscU and IscS. First, IscU and IscS are not separated by anion exchange or size exclusion chromatographies when DTT is included in the running buffer; second, IscU and IscS are not separated by native PAGE under reducing conditions; and third, the complex is resolved into two major bands, corresponding to IscU and IscS when subjected to SDS-PAGE under non-reducing conditions.

In separate experiments, we also tested if an [Fe-S] cluster-loaded form of the *A. vinelandii* IscU Ala^{39}-IscS complex is produced when these proteins are heterologously expressed at high levels by using recombinant plasmid gene expression in *E. coli*. In this case, IscU Ala^{39} and IscS are also isolated as a tight complex but it does not contain an [Fe-S] species. These results underscore the difficulty in obtaining meaningful physiological insight when using either high level expression or unbalanced expression of [Fe-S] cluster assembly components. Nevertheless, the result obtained here using heterologously produced IscU Ala^{39} and IscS indicates that an [Fe-S] cluster is not

required to stabilize the non-dissociating complex. It was also found that an [Fe-S] cluster could not be assembled *in vitro* within the heterologously produced apo-form of the IscU Ala^{39}-IscS complex by the addition of L-cysteine and Fe^{++}. These results indicate that IscU and IscS must be dissociated to permit the initiation of [Fe-S] cluster assembly.

The IscU Ala^{39}-IscS complex retains cysteine desulfurase activity. Addition of L-cysteine to resting state IscS results in a rapid and characteristic shift in the visible absorption spectrum (Figure 3.5A) (Zheng et al., 1998, Kaiser *et al.*, 2000). This spectral shift most likely represents a stage in the initial formation of the external PLP-substrate aldimine or reformation of the internal PLP aldimine upon the release of alanine (Kaiser et al., 2000). This feature was used to estimate the affinity of L-cysteine for IscS by titrating the spectral shift with increasing concentrations of L-cysteine (Figure 3.5, Panel C). It should be noted that an accurate K_d value (estimated K_d ~ 0.6 mM) could not be determined by this method because substrate binding is inhibited at high concentrations of L-cysteine (Figure 3.5, Panel C) (Zheng et al., 1998). Addition of L-cysteine to the IscU Ala^{39}-IscS complex also results in the same spectral shift recognized for isolated IscS (compare spectra in Figure 3.5, Panels A & B) and titration with L-cysteine (Figure 3.5, Panel C) reveals that both IscS and the IscU Ala^{39}-IscS complex have approximately the same affinity for L-cysteine. After elicitation of the spectral shift as a result of L-cysteine addition, the resting state spectrum is regenerated in a time-dependent manner, as shown in Figure 3.5, Panel D, corresponding to the desulfurization of L-cysteine. Although the specific rate-limiting step in the overall cysteine desulfurization reaction for class I IscS-type enzymes is not known (for example, substrate C-S bond cleavage, persulfide cleavage, H abstraction, or L-alanine release) it is clear that formation of the enzyme-substrate complex is not rate limiting. Data presented in Figure 3.5, Panel D, also show that regeneration of the as isolated spectrum after L-cysteine addition occurs approximately 12 times slower for the IscU Ala^{39}-IscS complex ($k = 0.0035\ s^{-1}$) when compared to IscS ($k = 0.03\ s^{-1}$). Whether or not this feature represents a change in the same rate-limiting step for the complex when compared to IscS is not known. However, it can be concluded that, although L-cysteine has approximately the same affinity for IscS

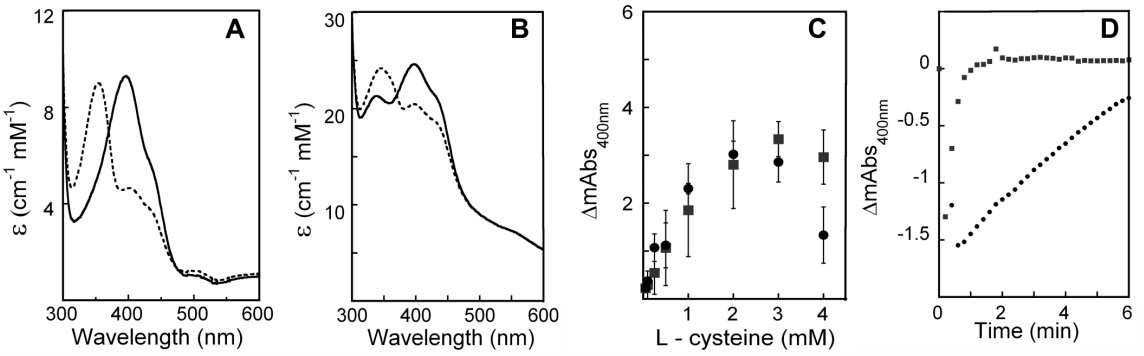

Figure 3.5. Spectroscopic and kinetic features associated with IscS-catalyzed L-cysteine desulfurization. (**A**) UV-visible absorption spectra of IscS (34 μM) in the as-isolated resting state (solid line) and immediately after the addition of a 30 molar excess of L-cysteine (dashed line). (**B**) UV-visible spectra of the IscU Ala^{39}-IscS complex (34 μM) in the as-isolated resting state (solid line) and immediately after the addition of a 30 molar excess of L-cysteine (dashed line). (**C**) $\Delta mAbs_{400nm}$ for 50 μM IscS (■) and 50 μM of the IscU Ala^{39}-IscS complex (●) upon the addition of increasing concentrations of L-cysteine. Each spectral change was recorded immediately after the addition of the indicated concentration of L-cysteine to the resting state as-isolated sample. Note that apparent substrate binding is inhibited at high L-cysteine concentrations. (**D**) Time-dependent relaxation of the $\Delta mAbs_{400nm}$ for IscS (■) and the IscU Ala^{39}-IscS complex (●) after the addition of a 10 fold molar excess of L-cysteine.

as for the IscU Ala^{39}-IscS complex, a subsequent step in the desulfurization reaction is slowed when IscS is captured in a complex with IscU.

3.5 Discussion

Our first experimental strategy was to ask if an [Fe-S] cluster-loaded form of IscU accumulates on the proposed IscU scaffold *in vivo* under conditions for which the intact [Fe-S] cluster biosynthetic machinery exceeds the demand for [Fe-S] protein maturation. The answer to this question is yes and, importantly, the [Fe-S] species that accumulates on IscU under these conditions exhibits a UV-visible absorption spectrum lineshape that is identical when compared to the [2Fe-2S] cluster-loaded form of IscU produced by *in vitro* reconstitution experiments (Agar et al., 2000c). Thus, the present work validates the physiological relevance of previous *in vitro* studies on the assembly of [Fe-S] clusters on the proposed IscU scaffold. Previous work has also reported on the isolation of a cluster-loaded form of an IscU-like protein produced *in vivo*, but this study involved an extremely high level of heterologous expression of the recombinant IscU-like protein in the absence of other assembly components and a 20 h incubation after induction of recombinant expression (Shimomura et al., 2007). The present work is different because: (*i*) the [2Fe-2S] cluster-loaded form of IscU was isolated from samples that did not involve the hyperproduction of IscU; (*ii*) the balanced expression of the other IscR-regulated [Fe-S] protein maturation components remained intact and (*iii*) cells used for IscU purification were harvested during mid-logarithmic growth. Thus, potential artifacts that could be associated with hyperexpression of IscU or unbalanced expression of ISC components have been avoided. The work described herein also shows that different forms of IscU representing different stages in the process of [Fe-S] cluster assembly can accumulate *in vivo*. These forms include apo-IscU, [2Fe-2S] cluster-loaded IscU, and IscU complexed with IscS. An ability to produce a dimeric [4Fe-4S] cluster-loaded form of IscU by using *in vitro* reconstitution methods has been previously reported (Unciuleac et al., 2007, Agar et al., 2000a). However, no significant accumulation of a [4Fe-4S] cluster-loaded IscU species produced *in vivo* was recognized in the current work. It is possible that a [4Fe-4S] cluster-loaded form of IscU, if present, existed in concentrations

too low to be observed by the methods used, is too labile for purification by the methods used here, or is very short lived when produced *in vivo*. Nevertheless, our ability to detect dimeric forms of apo and [2Fe-2S] cluster-loaded IscU in the present work, supports the possibility that a [4Fe-4S] cluster-loaded species of IscU could be produced *in vivo* by using the same reductive coupling mechanism demonstrated by *in vitro* experiments (Unciuleac et al., 2007). Another aspect worth noting is that apo and [2Fe-2S] cluster-loaded forms of IscU can be separated by anion-exchange chromatography, indicating they are likely to have different conformations (Figure 3.2). This feature could be relevant to other aspects of [Fe-S] protein maturation, such as the selective interaction of [Fe-S] cluster-loaded forms of IscU with specific [Fe-S] cluster receiving proteins or for effective [Fe-S] cluster release.

Our second experimental strategy was aimed at determining the biochemical basis for the dominant-negative phenotype associated with the IscU Asp^{39}-substituted protein (Johnson et al., 2006). Isolation of the substituted form of IscU revealed that it is purified as a non-covalent, non-dissociating, stoichiometric complex with IscS, and that it contains an [Fe-S] species that is resistant to oxidative degradation. The dominant-negative phenotype associated with the IscU Ala^{39} substitution can, therefore, be attributed to an inability for the release of [Fe-S] clusters assembled on IscU, as well as the sequestering of IscS, such that it is not available for other intracellular S-trafficking functions. The nearly exact stoichiometry of the IscU Ala^{39}-IscS complex suggests there is only one IscU monomer associated with each of the two, separate cysteine desulfurase active sites contained within the IscS homodimer. Crystallographically determined structures of IscS have revealed that the active sites are located on opposite sides of the IscS homodimer and are separated by ~30 angstroms (Cupp-Vickery *et al.*, 2003). This indicates that [Fe-S] species are likely to be initially assembled on monomeric forms of IscU. Another mechanistic insight to emerge is related to the observation that the non-dissociating IscU Ala^{39}-IscS complex is isolated in a form that apparently contains [2Fe-2S] clusters. This suggests that completion of [2Fe-2S] cluster assembly on the IscU scaffold does not necessarily occur through the sequential delivery of S atoms from IscS in a process that involves the association and dissociation of IscS and IscU. Rather, it would appear that [2Fe-2S] cluster assembly occurs either in a single step involving

polysulfurated IscS, or that sequential desulfurization steps occur within the IscU-IscS complex to achieve completion of [2Fe-2S] cluster assembly. With respect to the latter possibility, it is interesting that the overall activity of IscS is much lower when trapped in a complex with IscU. This feature could be related to restricted accessibility of the active site cysteine, which is contained on a flexible loop (Cupp-Vickery et al., 2003), for attack on the substrate cysteine-PLP adduct. The sequential events that occur during [Fe-S] cluster assembly have not been revealed by the present work. Nevertheless, our ability to isolate a [2Fe-2S] cluster form of IscU that is not associated with IscS indicates that IscU has the capacity to dissociate from IscS *in vivo* upon completion of [2Fe-2S] cluster assembly.

In summary, we make the following conclusions: (1) IscU serves as an *in vivo* scaffold for formation of [Fe-S] clusters that are destined for the maturation of [Fe-S] proteins, (2) both apo and [Fe-S] cluster-loaded forms of IscU can accumulate *in vivo* and these species are likely to be conformationally distinct, and (3) [Fe-S] cluster assembly is a dynamic process that involves the association and dissociation of IscU and IscS.

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CHAPTER 4. *In vivo* iron-sulfur cluster formation using an *E. coli* recombinant system: role of the chaperones HscA and HscB

I was the major contributor in the work described in this chapter. I designed and performed most of the experiments and analyzed the data. Valerie Cash constructed plasmid pDB1720 and performed 2D-gel electrophoresis of *E. coli* TB1 cells transformed with pDB1720. Justin Lemkul from Dr. David Bevan's lab performed the molecular dynamics simulations on IscU. Dr. Keith Ray from Dr. Richard Helm's lab performed sequencing of protein spots and bands from SDS-PAGE gels using mass-spectrometry. Strains DJ1752, DJ1764, and DJ1807 were constructed by Callie Raulfs.

4.1 Abstract

In vivo iron-sulfur cluster formation is a complex process that involves the participation of at least eight proteins that are part of the iron-sulfur cluster (ISC) biosynthetic machinery. We have developed a method for isolating ISC proteins produced *in vivo* by expressing the entire *Azotobacter vinelandii isc* operon heterologously in *E. coli*. Using this method, IscU was isolated at 13 times higher protein yields and bound to a $[2Fe\text{-}2S]^{2+}$ cluster that has similar characteristics with IscU isolated from its native host (Raulfs *et al.*, 2008). The role of the chaperones HscA and HscB in [Fe-S] cluster assembly was examined by isolating IscU from a strain with a plasmid that expresses all the ISC proteins except for HscA and HscB. IscU isolated from cells lacking the chaperones contained a $[2Fe\text{-}2S]^{2+}$ cluster identical to the one isolated from a wild type background. The $[2Fe\text{-}2S]^{2+}$ cluster bound to IscU produced in the absence of the chaperones was: *i*) present at higher occupancy, *ii*) exhibited the same lability, and *iii*) did not confer distinct conformational differences compared to the cluster bound to IscU produced in the presence of an intact ISC machinery. Moreover, IscU was present at 5-fold higher levels when isolated from an *A. vinelandii* strain that lacks HscA. The results indicate that, *in vivo*, the chaperones are involved after the formation of $[2Fe\text{-}2S]^{2+}$

clusters on IscU. Molecular dynamics simulations demonstrated that the loop containing the LLPVK region on IscU that is responsible for interactions with HscA is flexible and could act as a protective "gate" for the nascent iron-sulfur clusters. Analysis of the proteins that co-elute with IscU indicated that the ISC machinery may form multi-protein complexes during [Fe-S] cluster assembly.

4.2 Introduction

Cells employ inorganic protein prosthetic groups called iron-sulfur ([Fe-S]) clusters to carry out such biochemical processes as electron transfer, substrate activation and reaction catalysis, environmental sensing and structural stabilization (Johnson *et al.*, 2005a, Beinert *et al.*, 1997, Ayala-Castro *et al.*, 2008, Bandyopadhyay *et al.*, 2008a). Due to the inherent toxicity of free iron and sulfur ions in the cellular environment, the process of [Fe-S] cluster biosynthesis *in vivo* is complex and protein-mediated to ensure maturation of [Fe-S] proteins while limiting cellular harm. Most organisms use the services of the so-called ISC (iron-sulfur cluster) machinery to build [Fe-S] clusters, a multi-protein system that is strongly conserved throughout all kingdoms. In *Azotobacter vinelandii*, a nitrogen-fixing bacterium, the ISC machinery consists of a total of eight proteins, namely IscR, IscS, IscU, IscA, HscB, HscA, Fdx, and IscX. Most of these are essential for cell survival and their impairment has debilitating effects on [Fe-S] enzymes (Johnson *et al.*, 2006, Takahashi & Nakamura, 1999). Thus, the ISC components are expected to work in concert and each one to play a significant part in [Fe-S] cluster assembly.

Though the iron source for [Fe-S] cluster assembly is still being debated, it has been established that sulfur atoms are derived from free cysteine via a PLP-dependent desulfurization reaction catalyzed by the cysteine desulfurase IscS. The "activated" sulfur is in the form of a persulfide on a conserved cysteine residue of IscS. Sulfur atoms can be donated to IscU, a scaffold protein upon which clusters are built (Agar *et al.*, 2000c, Agar *et al.*, 2000a). The most common [Fe-S] clusters are the [2Fe-2S] and the [4Fe-4S] clusters. The formation of both structures has been observed on IscU *in vitro* but only [2Fe-2S] clusters have been isolated bound to IscU *in vivo* (Agar *et al.*, 2000a,

Raulfs *et al*., 2008). The role of IscA in the pathway of [Fe-S] cluster formation is debated as some studies suggest that IscA acts as an iron donor and others show that it can bind [Fe-S] clusters and transfer them to apo-proteins, thus acting as an alternative scaffold or cluster transporter (Krebs *et al.*, 2001, Ding *et al.*, 2004, Bonomi *et al.*, 2005, Ollagnier-De-Choudens *et al.*, 2004). The role of ferredoxin is also controversial because different reports suggest it might provide electrons for the *de novo* assembly of [2Fe-2S] clusters (Muhlenhoff *et al.*, 2003) or for the reductive coupling of two $[2Fe\text{-}2S]^{2+}$ clusters to form one $[4Fe\text{-}4S]^{2+}$ cluster on IscU (Chandramouli, 2007).

Once [Fe-S] clusters are formed, they need to be delivered to their appropriate target proteins. The molecular chaperones HscA and HscB, which share sequence and structural similarities with the *E. coli* DnaK and DnaJ proteins respectively, have been implicated in this process. Previous studies in *Saccharomyces cerevisiae* showed that the chaperones are involved at a step after [Fe-S] cluster assembly on IscU (Muhlenhoff *et al.*, 2003, Dutkiewicz *et al.*, 2006). It was later demonstrated that the chaperones facilitate cluster transfer from IscU to apo-ferredoxin (Chandramouli & Johnson, 2006, Bonomi *et al.*, 2008). However, the latter experiments were performed *in vitro* with individually overexpressed and purified ISC components. Therefore, the *in vivo* effect of the chaperones on the nature of [Fe-S] clusters formed on IscU and its ability to interact with other ISC components is not known.

Previous attempts to purify recombinantly expressed *A. vinelandii* IscU resulted only in apo forms of the protein, which raised concerns regarding the validity of the results obtained by *in vitro* reconstitution. Subsequently, we developed a method that allows biochemical studies on iron-sulfur cluster biosynthetic proteins *in vivo* (Raulfs *et al*., 2008). In that work, the chromosome of the genetically malleable *A. vinelandii* was altered such that expression of the ISC components is uncoupled from the endogenous regulator and IscU is produced carrying a poly-histidine tag that allows efficient isolation from its native organism. The study demonstrated that IscU serves as a scaffold *in vivo,* exists in two distinct apo and $[2Fe\text{-}2S]^{2+}$ cluster-loaded forms, and interacts with IscS in a transient manner to form at least a [2Fe-2S] cluster. The current work presents the development of a new method for studying iron-sulfur cluster formation *in vivo* that results in 12 times higher protein yields compared to our previous design. This is

accomplished by the heterologous expression of all ISC proteins concomitantly in *E. coli*. We have used both strategies to study the biochemical and physiological effects of the chaperones HscA and HscB on [Fe-S] cluster assembly.

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4.3 Materials and Methods

Plasmid and strain constructions. Plasmid pDB1720 was constructed using pDB1282 as a parent, a plasmid that contains the entire *isc* operon (*iscSUAhscBAfdxiscX*) under control of the arabinose promoter, and introduction of an octa-histidine cartridge at the StuI site of IscU. Plasmid pDB1722 was constructed by removal of a 1.7 kb region at the SbfI sites of *hscBA*. This deletion maintains the reading frame. The *E. coli hscA* strain was a generous gift from Prof. Wayne Outten at University of South Carolina. The *hscA* gene is inactivated with insertion of a chloramphenicol resistance cassette in the *E. coli* strain W3110 (unpublished strain). To construct *A. vinelandii* strain DJ1788, an intermediate strain was first constructed, DJ1765, which has the *hscBAfdxiscX* region placed under control of the sucrose regulatory elements. Subsequently, DJ1788 was constructed by transforming DJ1765 with pDB1687, which contains a 70bp in-frame deletion in *hscA,* and pDB528, a plasmid that has the *recA* gene inactivated by a kanamycin resistance cassette. Construction of DJ1766 was described previously (Raulfs *et al.*, 2008). Strains DJ1752 and DJ1764 are isogenic to DJ1697 with the exception that they produce the IscU variants $IscU^{63CA}$ and $IscU^{106CA}$ respectively. Both strains were constructed by transforming DJ1697 with pDB1658 or pDB1659 (TGC to GCC codon changes at positions 63 and 106 respectively). Strain DJ1807 was constructed by transforming DJ1697 with pDB1210, a plasmid in which Cys328 of IscS has been substituted for an alanine (TGC to GCC codon change). In all strains producing variant proteins, the *recA* gene was inactivated by insertion of a kanamycin resistance cassette to prevent recombination between the endogenous *isc* copy and the P*scr* regulated *isc* copy.

Depletion growths for DJ1788 were accomplished by growing cells in Burk's medium containing 13 mM ammonium acetate and 2% sucrose as a primary carbon source (BN) (Johnson *et al.*, 2006) to their late log phase, collecting them by centrifugation, washing once with media free of sucrose and resuspending in sterile BN

media containing 1% glucose instead of sucrose. The resuspended cells were used to inoculate BN media containing 1% glucose at OD_{600} = 0.15 and were allowed to grow to OD_{600} = ~1.0, at which point they were collected by centrifugation.

Cell growth and IscU purification and characterization. *E. coli* cells transformed with the various plasmids were cultured at 30°C in 1-liter LB media containing 100 µg/ml ampicillin to OD_{600} = ~0.5, at which point they were induced with 0.3% w/v L-arabinose (Spectrum), 1 mM L-cysteine, 0.75 mM ammonium iron(III) citrate, and 1 mM pyridoxine-HCl. Cells were allowed to continue shaking and 300 rpm for 4 hours, after which they were collected by centrifugation at 4225 x g for 5 min. Cells were lysed and IscU was purified using the procedures published in (Raulfs *et al.*, 2008). UV-visible absorption spectra, size exclusion experiments, and elemental Fe and S analyses were also performed as published previously (Raulfs *et al.*, 2008). Protein concentration was measured using the bicinchoninic acid method with BSA as a standard. Circular dichroism spectra were measured using a Jasco J-720 spectropolarimeter. Samples were measured in 1-cm Quartz cuvettes from 200-800 nm at 200 nm/min.

Western blot and aconitase assays. Strains DJ1697, DJ1766, DJ1752, DJ1764, DJ1807, and DJ1788 were grown in BN media to OD_{600} = ~1.5, and collected by centrifugation at 4225 x *g* for 5 min. Cells were resuspended in 125 mM Tris-HCl, pH 8.0 and lysed with a French Pressure cell at 12,000 psi. The lysate was centrifuged at 235,000 *g* for 30 min and the cell extracts were used for Western analyses and aconitase/isocitrate dehydrogenase assays. For the western analyses, 50 µg of each crude extract (measured with Biuret protein assay) were loaded onto a SDS-PAGE gel and bands were transferred to a nitrocellulose membrane (Johnson *et al.*, 2006). The procedure described in (Johnson *et al.*, 2006) was used to detect the presence of IscU in each cell extract. As an internal control, a duplicate membrane was treated with anti-GroEL antibodies. Aconitase and isocitrate dehydrogenase activities in *A. vinelandii* cell extracts were measured as described previously (Bandyopadhyay et al., 2008d).

2D-gel electrophoresis. Cell extracts were prepared as in the "Western blot and aconitase assays" section above with the exception that cells were lysed using a NanoDeBee homogenizer at 27,000 psi (B.E.E International, Inc.). For each sample, 150 µg of total protein (measured with Biuret assay) were treated with reagents present in the 2-D Clean-Up Kit by Ettan™ as recommended by supplier. Samples were loaded onto a 11-cm, pH 3-11 IPG strip for the first dimension (GE Healthcare, Immobiline™ Dry Strip). The strip was rehydrated and allowed to run in the first dimension using an Ettan IPGPhor 11/3 Isoelectric Focusing Unit as recommended by the supplier. A non-linear pH gradient was applied to achieve best resolution in the pH 5-7 range. Criterion™ precast gels (12.5% Tris-HCl, 1.0 mm with 11-cm IPG+1 well comb) were used for the second dimension. Gels were stained with Coomassie dye and protein spots of interest were cut out, trypsin-digested, and sequenced using mass spectrometery as described previously (Raulfs *et al*., 2008).

Molecular dynamics simulations. MD simulations were performed using the crystal structure of IscU from *A. aeolicus* (PDB code 2Z7E). The protomer containing the [Fe-S] cluster was removed from the trimeric structure and treated as a single entity. The Fe and S atoms were also removed and the cluster-ligating cysteines were protonated. Distance restraints were placed at the active site to mimic the presence of the [Fe-S] cluster. Once the protein was solvated in an octahedral box of SPC water, it was assigned parameters from the Gromos96 53a6 force field. Eight Na^+ counterions were added to give an electrically net neutral system. Equilibration was conducted for 100 ps at constant volume (NVT), and then for 100 ps at constant pressure (NPT). During equilibration, position restraints were placed on all heavy atoms of the protein. Following equilibration, 50 ns of unrestrained MD were conducted using an NPT ensemble. Temperature was regulated at 300 K using a Nose'-Hoover chain method, with separate coupling groups for the protein and solvent, including ions. Pressure was maintained at 1 bar isotropically using the Parrinello-Rahman method. Short-range, non-bonded interactions were cut off at 1.2 nm, and the Particle Mesh Ewald (PME) method was used for long-range electrostatic calculations. Dispersion correction was applied to

long-range van der Waals terms. Analyses were performed using the Gromacs software. IscU crystal structure figures were prepared using the program VMD 1.8.6.

4.4 Results

Development of an *E. coli* recombinant system to study [Fe-S] cluster formation *in vivo*. Previously we developed a method that allowed the isolation of *A. vinelandii* IscU from its native organism (Raulfs *et al.*, 2008). In that study, expression of the ISC proteins beyond the physiological requirements was accomplished by preventing expression of IscR, a regulator that represses *isc* expression via a negative feedback loop. A polyhistidine tag at the C-terminus of IscU allowed the efficient isolation of the protein from a genetic background wherein all ISC components are expressed. Although the previous design proved to be successful in isolating intermediate species in [Fe-S] cluster assembly, those species did not accumulate at high enough levels to allow convenient characterizations. The present work reports the development of a method that allows isolation of the ISC proteins *in vivo* while using a recombinant system in *E. coli*. Two criteria needed to be satisfied: 1) intact expression of all ISC proteins and, 2) expression of the ISC proteins beyond the physiological requirement and independently of negative feedback regulation. To accomplish this, the entire *isc* operon from *A. vinelandii* (*iscSUA-hscBA-fdx-iscX)* was placed in a plasmid under control of the *araBAD* regulatory elements thus allowing controlled expression of the operon in the presence of arabinose. Next, a histidine tag was inserted at the C-terminal region of IscU (Figure 4.1, plasmid pDB1720) for efficient isolation of the protein. One- and two-dimensional SDS-polyacrylamide gel electrophoreses of *E. coli* cells transformed with pDB1720 were performed before and after induction with arabinose to ensure expression of all ISC proteins (Figure 4.2 and data not shown). IscS and IscU were identified at distinct locations in the 2D-gel along with some streaking, a phenomenon that is likely associated with non-specific oxidation of thiol groups (Figure 4.2).

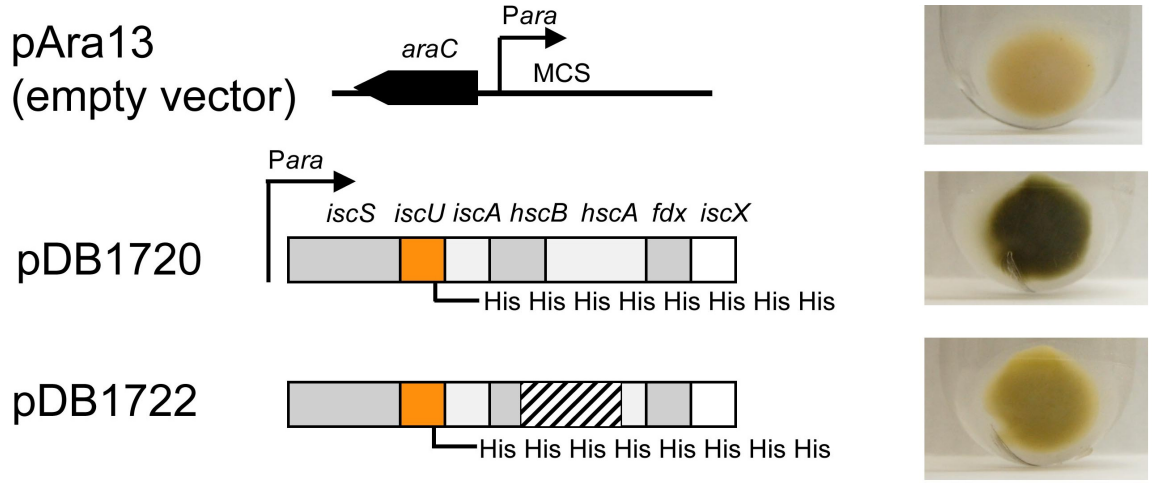

Figure 4.1. Plasmids constructed for the isolation of IscU *in vivo* using an *E. coli* recombinant system. Box with slanted lines indicates in-frame deletion in *hscBA*. Cell pellets were photographed after induction for 4 hours at 30°C with 0.3 % w/v arabinose, 1 mM L-cysteine, 0.75 mM ammonium iron(III) citrate, and 1 mM pyridoxine-HCl.

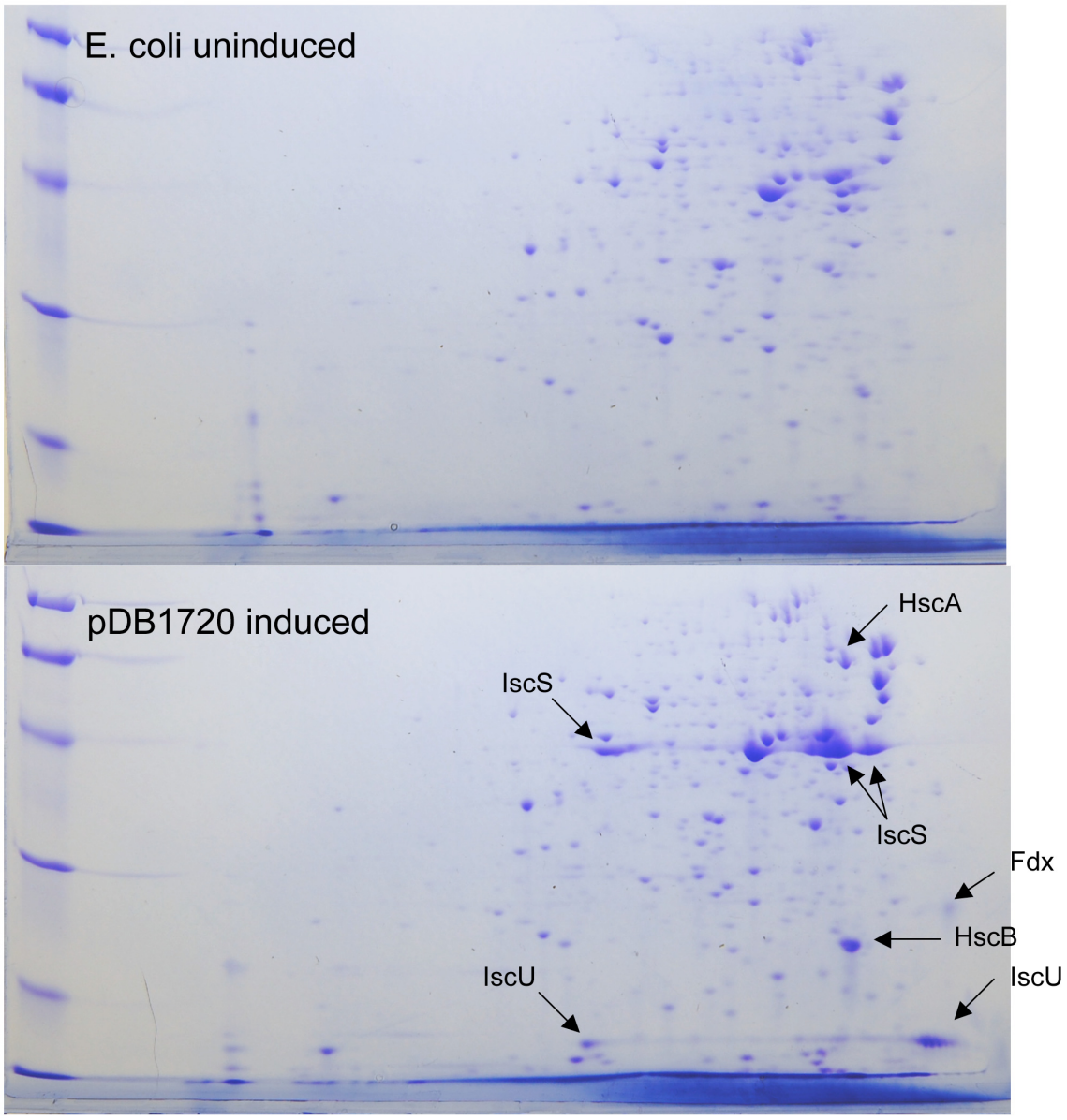

Figure 4.2. 2D-gel electrophoresis of *E. coli* TB1 cells transformed with pDB1720 before and after induction. Induction was performed as described in the legend of Figure 4.1. Cell extract preparation and 2D-gel electrophoresis procedures are described in the "Materials and Methods" section. A non-linear pH gradient was applied to achieve best resolution in the pH 5-7 range.Molecular weight lane was loaded with a mixture of phosphorylase b (97,400), bovine serum albumin (M*r* 66,200), ovalbumin (M*r* 45,000), carbonic anhydrase (M*r* 31,000), soybean trypsin inhibitor (M*r* 21,500), and lysozyme (M*r* 14,400).

Use of the *E. coli* recombinant system provides physiologically relevant results. It was necessary to establish that the *E. coli* recombinant system could be used to provide physiologically relevant results. This was accomplished by comparing the results obtained in this work with those obtained using *A. vinelandii* as the source of protein expression. IscU from pDB1720 was isolated using the same procedure that was used to isolate IscU from *A. vinelandii*, namely a two-step method involving immobilized metal affinity chromatography (IMAC) followed by anion-exchange chromatography (Raulfs *et al.*, 2008). The additon of 1 mM L-cysteine, 0.75 mM ferric ammonium citrate, and 1 mM pyridoxine-HCl in the induction media resulted in cluster occupancy similar to what was observed for IscU isolated from *A. vinelandii*. The pyridoxine was added to increase occupancy of the PLP cofactor on IscS. The yield of cluster-loaded IscU using this system was ~320 μg/g cell paste compared to 24 μg/g cell paste that was obtained from *A. vinelandii*.

The fraction resulting from the IMAC column contained mainly IscU and IscS, exhibited a dark red color, and its UV-visible absorption spectrum supported the presence of an [Fe-S] cluster (data not shown). The IMAC eluate was passed over an anion-exchange column and a NaCl gradient was applied. Several distinct forms eluted during this step: a) small amounts of apo-IscU, b) $[2Fe\text{-}2S]^{2+}$ cluster-loaded IscU, and c) yellow colored fractions containing IscS and IscU (Figure 4.3). A comparison between cluster-loaded IscU isolated from *A. vinelandii* and IscU recombinantly expressed and purified from *E. coli* revealed many similarities. Both species bind a $[2Fe\text{-}2S]^{2+}$ cluster as judged from their UV-visible absorption spectra (data not shown). Iron and sulfide analysis of IscU isolated in this work revealed 1.26 ± 0.15 Fe and 1.28 ± 0.19 S per IscU polypeptide, which are comparable to the values obtained for IscU isolated from *A. vinelandii*. Both species are isolated as a mixture of monomer and dimer with the monomeric form dominating (data not shown). Also, both species are labile when exposed to air or treated with dithionite, a suitable characteristic for a transient cofactor. The results demonstrate that the method presented in this work to isolate ISC proteins can be used to study the iron-sulfur cluster biosynthetic pathway *in vivo*.

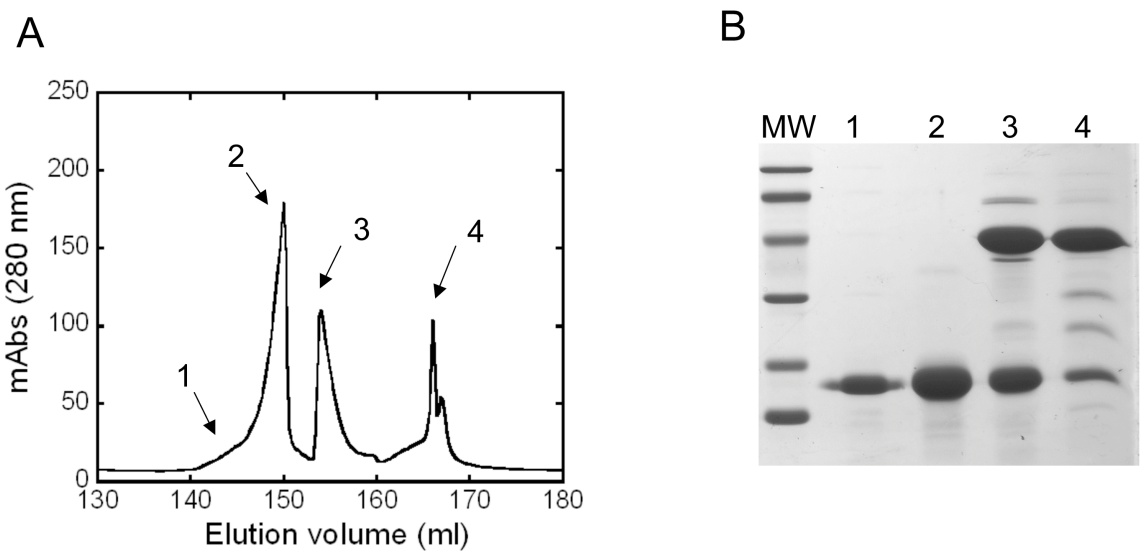

Figure 4.3. Anion-exchange elution profile (A) and 15% SDS-PAGE of elution fractions (B). Fraction 1 contains apo-IscU, fraction 2 contains $[2Fe\text{-}2S]^{2+}$ cluster-loaded IscU, fractions 3 and 4 contain IscS and IscU. Molecular weight (MW) lane contains the same standards as in Figure 4.2.

HscA and HscB do not affect formation of $[2Fe\text{-}2S]^{2+}$ clusters on IscU. One of the advantages of isolating IscU using the method described above is that the effects of the other ISC components can be assessed by deleting one or more components at a time and comparing the IscU protein molecules isolated from different genetic backgrounds. To gain insight into the role of the chaperones in the assembly of [Fe-S] clusters, IscU was isolated from a plasmid that is isogenic to pDB1720 except that *hscB* and *hscA* were inactivated by the introduction of an in-frame deletion (Figure 4.1, pDB1722). To avoid any effects on IscU by the endogenous *E. coli hscA*, both pDB1720 and pDB1722 were introduced into a strain of *E. coli* cells in which *hscA* was impaired by a deletion/insertion inactivation. Figure 4.1 shows that cells transformed with pDB1720 exhibit a black color compared to cells transformed with empty vector. The phenotype may be a result of FeS precipitants formed by excess Fe and S atoms resulting from the degradation of clusters that are synthesized beyond the physiological needs. In contrast, cells transformed with pDB1722 had a much lighter color suggesting either that cluster synthesis is defective or nascent clusters are prevented from being released to the cytosol. Nevertheless, the pellet of cells transformed with pDB1722 exhibit a darker red color compared to the empty vector, suggesting that perhaps clusters are still being built but are protein-bound (Figure 4.1). The effect of the plasmids on the pellet color was the same whether plasmids were introduced into the cloning host strain TB1 or the *hscA* strain (data not shown).

To examine whether deletion of *hscBA* has an effect on IscU or the cluster bound to it, IscU was isolated from *E. coli hscA* strain transformed with pDB1722 and grown as described above. Similar IMAC and anion-exchange elution profiles were observed for this purification as for cells transformed with pDB1720 (data not shown). Cluster-containing IscU exhibited a bright red color and its UV-visible absorption spectrum was similar to that of IscU isolated from pDB1720 (Figure 4.4). The result demonstrates that HscB and HscA do not affect the synthesis of [Fe-S] clusters, specifically $[2Fe\text{-}2S]^{2+}$ clusters, on IscU. Whether HscA and HscB play a role in [4Fe-4S] cluster formation could not be assessed because the latter were not detected during any of the purifications with the techniques used in this work.

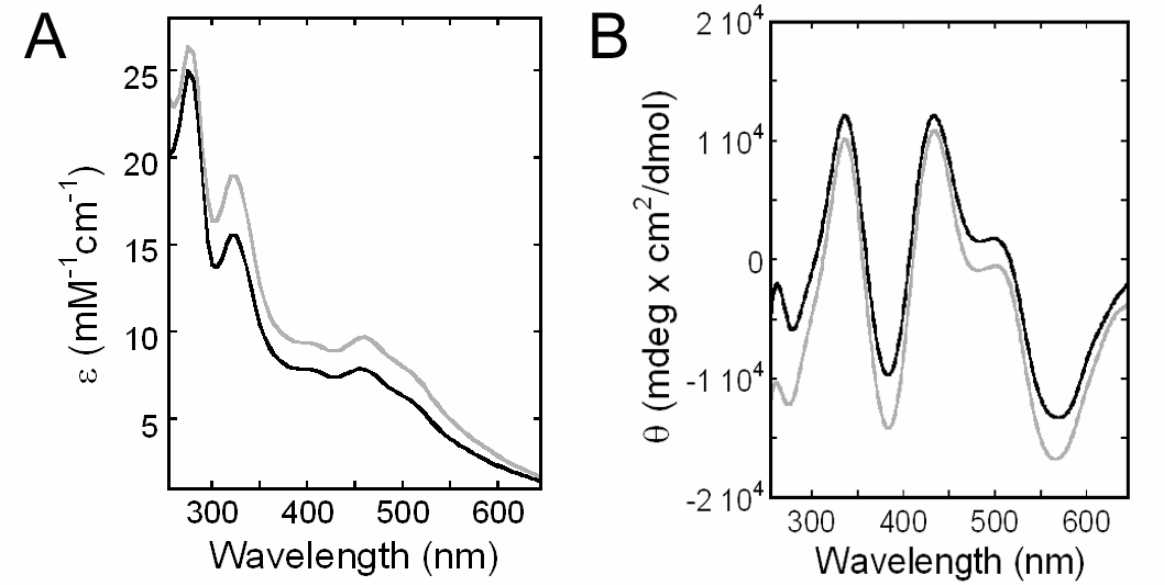

Figure 4.4. UV-visible absorption (A) and circular dichroism (B) spectra of IscU isolated from pDB1720 (black) and pDB1722 (gray).

When [2Fe-2S]-IscU from pDB1722 was analyzed for its Fe and S content, we found that it was comprised of 2.1 Fe and 1.9 S per IscU. These numbers are higher than what was observed for wild type IscU, which was isolated containing 1.3 Fe and 1.3 S per IscU polypeptide. Both IscU from pDB1720 and IscU from pDB1722 were predominantly monomeric with low amounts of dimeric species present (data not shown). If assumed that all species are monomeric, the Fe and S analyses indicate that ~100% of IscU molecules are occupied with a $[2Fe\text{-}2S]^{2+}$ cluster in the case of pDB1722 compared to ~65% occupancy in the case of wild type pDB1720. The results suggest that the cluster bound to the IscU species isolated from a wild type background may be more labile compared to clusters formed on IscU in the absence of chaperones.

HscA and HscB do not affect the nature of [2Fe-2S] clusters bound to IscU. A series of analyses were performed to compare the cluster lability and the protein conformation between the $[2Fe\text{-}2S]^{2+}$-IscU species isolated using pDB1720 and pDB1722. Both species were exposed to air and treated with 1 mM dithionite in order to gauge their lability to oxidative and reducing agents. In both cases, the cluster was labile and was degraded at similar rates (data not shown). Both species were also treated with varying concentrations (0.1-2.0 mM) of the ferrous chelator 2,2'-dipyridyl and 1 mM of two ferric chelators, namely EDTA and 1,2-dihydroxybenzene-3,5-disulfonic acid (commonly known as Tiron). In either case, a significant difference in the lability of the cluster on the two IscU species could not be distinguished (data not shown). Circular dichroism (CD) spectra of both $[2Fe\text{-}2S]^{2+}$-IscU species were measured to probe possible structural differences between the two molecules. Unlike most proteins, [2Fe-2S] proteins have distinctive CD features in the visible region. Both IscU species that were isolated had similar CD spectral features that resembled previously published spectra (Figure 4.4 and (Bonomi *et al.*, 2008, Bonomi *et al.*, 2005)). In aggregate, these results demonstrate that the chaperones do not impair formation of $[2Fe\text{-}2S]^{2+}$ clusters on IscU and those clusters exhibit similar lability to the ones formed on IscU isolated from a wild type background. They also demonstrate that the two IscU intermediates do not have discernable conformational differences that can be elicited with the methods used in this study.

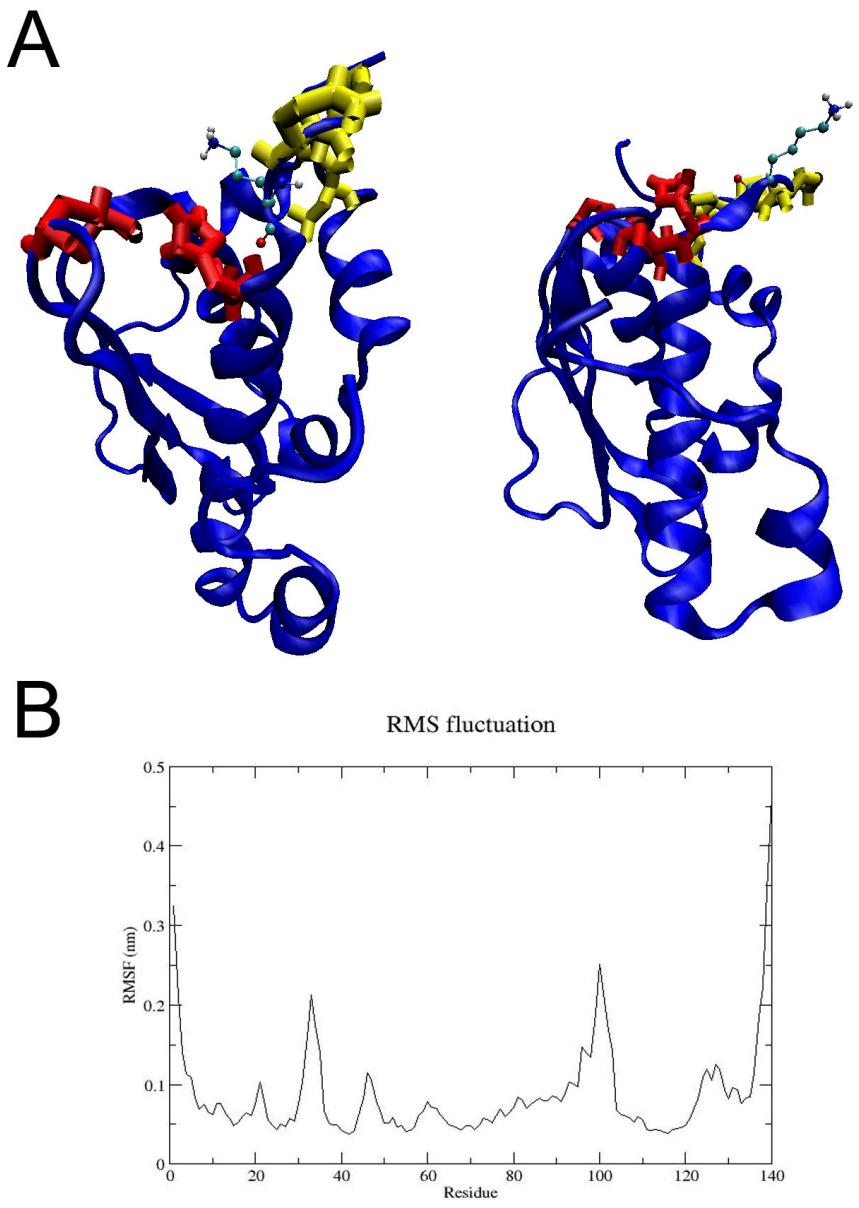

Figure 4.5. LPPVK loop acts as an active site "gate". (A) Snapshots from 50 nsec molecular dynamics simulations showing two different positions assumed by the LLPQK region of *A. aeolicus* IscU (note that it is LPPVK in *A. vinelandii*). IscU molecule is represented in blue NewCartoon; residues Cys34, Cys61, His104, and Cys105 are represented in red stick model; residues Leu98, Pro99, Pro100, and Gln101 are represented in yellow stick model; and residue Lys102 is represented in CPK model. (B) Root mean square (RMS) fluctuation of IscU residues throughout the simulation.

LLPVK region on IscU is a flexible loop that may act as a cluster "gate". The C-terminal region of IscU that bears a conserved LLPVK motif has been implicated in interactions with HscBA. In the crystal structure of trimeric IscU from *A. aeolicus*, the LPPQK region is part of a loop, the position of which varies among the three protomers. This observation led to the hypothesis that the region may act as a "gate" that protects nascent clusters and moves away when they are ready to be released. *In silico* experiments were performed to monitor the behavior of the LPPQK loop. The [2Fe-2S] cluster-loaded subunit in the trimeric crystal structure of IscU was removed from its tertiary structure and was used to perform 50 nsec molecular dynamics simulations. The results demonstrated that the LPPQK region was flexible with the lysine side chain assuming positions close to (4-5 Angstroms) and far away from (10-12 Angstroms) the active site (Figure 4.5). The Cys35 residue and both the N- and C-termini were flexible during the simulation as well (Figure 4.5B). The Cys35 residue also frequently converted between positions close to the active site (as in cluster ligation) and away from it (more solvent exposed).

Depletion of HscBA impairs [4Fe-4S] protein maturation and results in higher [2Fe-2S]-IscU levels. Because expression of the *isc* genes is controlled by the external P*ara* promoter, the *E. coli* recombinant system described in this work does not provide any information about effects on physiological protein levels that may be exerted when different assembly components are impaired. An *A. vinelandii* strain was constructed that allows isolation of IscU from a genetic background in which *hscA* is inactivated (DJ1788, Figure 4.6). DJ1788 is similar to DJ1697, the strain that was used to isolate IscU from *A. vinelandii*, in that it also carries an in-frame deletion in *iscR* and a poly-histidine tag at the C-terminus of IscU (Figure 4.6). In contrast to DJ1697, DJ1788 carries an in-frame deletion in *hscA*. In addition, only the *hscBAfdxiscX* region (as opposed to the entire *isc* operon) is controlled by the sucrose promoter. Removal of the *iscSUA* region from the sucrose-regulated copy prevents uncontrolled expression of the *hscBAfdxiscX* region from a weaker promoter that lies upstream of *hscB*.

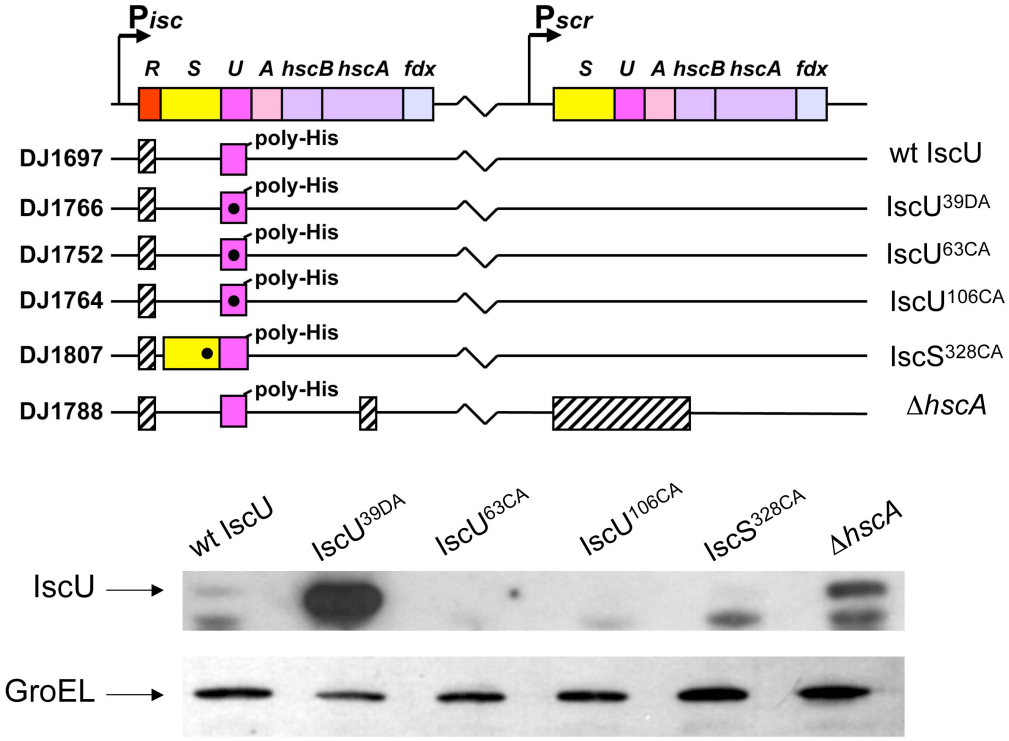

Figure 4.6. Genetic alterations in [Fe-S] cluster biosynthetic genes affect IscU protein levels. (Top) Diagrammatic representation of the *A. vinelandii* strains used for detection of IscU levels. All strains carry an in-frame deletion in *iscR* (represented by slanted lines) and a poly-histidine tag at the C-terminus of IscU. Black dots represent amino acid substitutions described on the right. DJ1788 also carries an in-frame deletion in the P*isc*-regulated copy of *hscA* and the *iscSUA* genes are removed from the P*scr*-regulated region. (Bottom) Western blots of crude extracts from each strain treated with anti-IscU antibody (top blot) or anti-GroEL antibody as an internal control (bottom blot). Strains were grown in sucrose-containing media to OD_{600} = 1.5 and crude extracts were prepared as described in "Materials and Methods".

DJ1788 does not grow in the absence of the second, wild type *hscBAfdxiscX* region (when sucrose is absent from the growth media) indicating that HscA is essential for cell survival. The activity of aconitase, a [4Fe-4S] TCA cycle enzyme, in DJ1788 cells was decreased to ~8% of the activity observed in DJ1697 cells (data not shown). In contrast, the activity of isocitrate dehydrogenase, a TCA enzyme that does not require a [Fe-S] cluster remained unaffected, indicating that HscA is required for the maturation of at least one [4Fe-4S] enzyme.

When IscU is purified from HscA-depleted DJ1788 cells, the [2Fe-2S] cluster-loaded form is isolated at 5 times higher yields compared to its DJ1697 counterpart. To gauge the overall IscU levels within the cell, we performed Western analyses of crude extracts from strains containing various genetic alterations in the *isc* operon. Densitometry measurements of the western blot shown in Figure 4.6 indicated that IscU is expressed at 5 times higher levels in DJ1788 crude extracts compared to DJ1697 extracts, consistent with the finding described above. On the other hand, cells from a strain that produces an IscU variant that is bound to an unusually stable cluster ($IscU^{D39A}$) demonstrated the highest IscU levels (25-fold increase compared to IscU from DJ1697). In contrast, amino acid substitutions on IscU or IscS that are required for the assembly of [Fe-S] clusters demonstrated negligible levels of IscU. Since all strains carry an in-frame deletion in IscR, the difference in protein levels is independent of IscR regulation. The results suggest that there may be a correlation between cluster stability and protein levels of IscU.

[Fe-S] cluster assembly involves formation of multi-protein complexes. The method developed in this work allows the isolation of ISC proteins as well as the identification of physiological interactions between the ISC components. The small histidine tag at the flexible C-terminal region of IscU is expected to have little interference with the detection of protein-protein interactions compared to other methods used for the same purpose. It is also expected to be less artifactual than cross-linking experiments which are not reliable for detecting cluster-dependent interactions (due to lability of the cluster). Several IscU-containing fractions were collected during both purification steps that varied in their protein content. Protein bands were cut from SDS-PAGE gels and

sequenced using mass-spectrometry. The low imidazole fraction from the IMAC step was comprised of a number of proteins including IscU, IscS, HscB, and IscA suggesting that the ISC proteins may form transient multi-protein complexes during [Fe-S] cluster assembly (Figure 4.7). The high imidazole fraction contained mainly cluster-bound IscU and IscS. This fraction is a mixture of several species because upon passage through an anion-exchange column it resolves into $[2Fe\text{-}2S]^{2+}$-IscU and apo-IscUS complexes of varying stoichiometry.

4.5 Discussion

The present study reports the development of a method that allows isolation of the ISC proteins produced *in vivo* at high yields. This was accomplished by placing the entire *isc* operon in the arabinose expression vector to allow the concomitant production of all the ISC proteins in *E. coli*. IscU isolated using this method was bound to a [2Fe-2S] cluster, a result that is different from previous attempts to purify recombinantly expressed IscU, which was always isolated in its apo form. The finding demonstrates that the all ISC proteins need to be present at proportionate levels for [Fe-S] clusters to be assembled on IscU. $[2Fe\text{-}2S]^{2+}$-IscU isolated using this system exhibited similar characteristics to the analogous species isolated from *A. vinelandii* as the native host, rendering our results physiologically relevant. The recombinant method reported in this study exhibits several advantages: 1) it does not require elaborate strain constructions, thus providing a convenient way to study the [Fe-S] cluster biosynthetic pathway by isolating ISC proteins encoded by plasmids that carry various genetic alterations in the *isc* operon, 2) it allows the identification and isolation of protein complexes involved in the pathway, 3) it results in higher protein yields, thus requiring less starting material and reducing the time and cost associated with each purification, and 4) it increases the chances of isolating short-lived or unstable intermediates or ones that are present at relatively small amounts. Notably, it can be used to study any biological process that involves collaborative protein systems encoded by polycistronic messages.

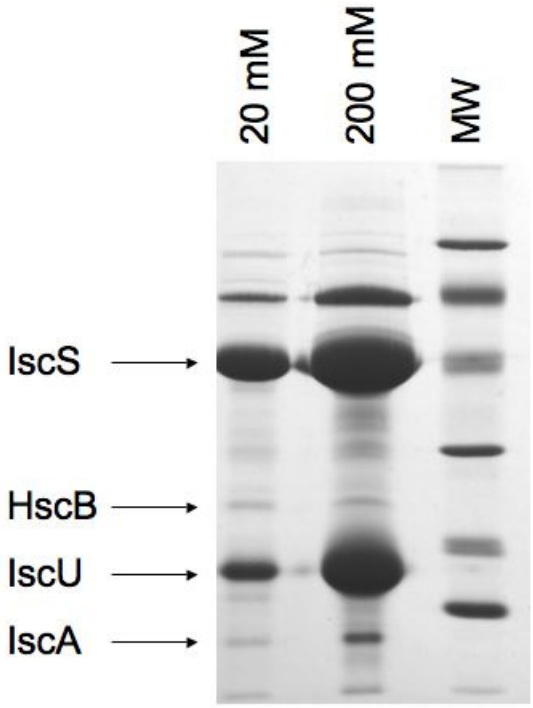

Figure 4.7. Protein-protein interactions between [Fe-S] cluster biosynthetic components. 15% SDS-PAGE gels of 20 mM and 200 mM imidazole fractions from the IMAC purification step of *E. coli* cells transformed with pDB1720. Bands pointed by arrows were cut out from the 20 mM wash fraction and sequenced by mass spectrometry. Lane labeled "MW" contains molecular weight ladder (see Figure 4.2)

The *E. coli* recombinant method was employed to study the role of the chaperones in the biogenesis of [Fe-S] clusters. *E. coli* cells were transformed with either a wild type plasmid containing the entire *isc* operon or one lacking the molecular chaperones HscA and HscB. The results provide *in vivo* evidence that the chaperones are not required for the formation of [Fe-S] clusters on IscU, a finding that is consistent with previous studies (Dutkiewicz *et al*., 2006, Muhlenhoff *et al*., 2003). We specifically found that the chaperones do not impede the formation of $[2Fe\text{-}2S]^{2+}$ clusters on IscU. The possibility of the chaperones being involved in the synthesis of [4Fe-4S] clusters could not be assessed, nor excluded, since the presence of the latter could not be detected on IscU isolated from a wild type background. The finding that IscU isolated from a wild type background has lower cluster occupancy than IscU isolated from cells lacking the chaperones supports the hypothesis and previous *in vitro* evidence that the chaperones mediate the release of [Fe-S] clusters from the scaffold (Chandramouli & Johnson, 2006, Bonomi *et al*., 2008). However, a series of tests showed that the clusters from both species exhibit similar lability and confer similar protein conformation. The findings suggest that the $[2Fe\text{-}2S]^{2+}$ cluster-loaded intermediate of IscU exists in one conformation *in vivo*, which may be subject to structural changes only when certain criteria are met, such as the presence of a recipient protein, in order to avoid futile hydrolysis of ATP. The observation that apo-IscU might exist in a different conformation suggests that the changes are exerted concomitantly with or after cluster release.

Molecular dynamics simulations showed that Lys103 is a highly flexible residue and, as part of the LPPVK region, may act as a "gate" that converts from a "closed" position that protects the nascent cluster to an "open" position that exposes the cluster. In the "open" position the loop is positioned away from the active site and stretched such that it is possible for it to interact with the substrate-binding domain of HscA. Preliminary results from the purification of *A. vinelandii* $IscU^{103KA}$ demonstrate that apo-$IscU^{103KA}$ is present at higher amounts in the cell compared to wild type apo-IscU. The finding is consistent with a role for Lys103 as an active site "gate". The residue Cys35 also exhibited pronounced flexibility. This may suggest some significance for this residue in interactions with other IscU subunits or other proteins. The latter hypothesis is supported

by the observation that an *A. vinelandii* strain carrying a Cys35Ala substitution could not be obtained even in the presence of a second, intact *isc* copy suggesting that this substitution has a dominant-negative phenotype (Johnson *et al.*, 2006).

Because Hsp70 proteins have been implicated in mediating assembly/disassembly of multi-protein complexes (Mayer & Bukau, 2005), the effect of the chaperones in complex formation and dissociation between IscS and IscU was also investigated. IscS and IscU co-elute in both cases demonstrating that HscA does not affect complex formation. HscA also does not appear to have an apparent effect on complex dissociation since monomeric [2Fe-2S]-IscU species were isolated in both cases. The anion-exchange chromatography fractions containing IscS-IscU complex species do not contain any clusters (judged from UV-visible absorption spectra and iron analyses, data not shown). The observation that cluster-loaded IscU is isolated alone, whereas the IscU-IscS complexes are apo, suggests that apo-IscU may have a higher affinity for IscS than holo-IscU. Since only the *hscA* gene is inactivated in the endogenous *E. coli isc* operon, it cannot be assessed whether the *E. coli* HscB protein has an effect on any of the above steps.

A significant observation that could not be explained from the findings presented here pertains to the much darker color associated with cells transformed with the plasmid that expresses the entire *isc* operon compared to cells transformed with plasmids that lack *hscBA*. One hypothesis is that the black color may be due to the formation of FeS precipitants, which result from degraded [Fe-S] clusters. Another possibility is that the chaperones affect the desulfurization process by IscS. A defective desulfurization would result in less sulfide being produced and, therefore, fewer FeS precipitants.

Another piece of information that emerged from this study is the observation that impairment of HscA results in higher protein levels of IscU. This phenomenon was also recently observed by Craig and her team who showed that chaperone-depleted yeast cells produce 15-20 fold higher levels of IscU (Andrew *et al.*, 2008). In that study, the group hypothesized that higher levels of IscU are produced to compensate for the loss of the chaperones. That is a valid scenario, but it should be noted that this phenomenon is observed even when IscR, the negative regulator of the *isc* operon, is removed indicating that either IscU expression might be regulated by another cellular component or the presence of [Fe-S] clusters on IscU affects protein stability. The latter is supported by the

observation that strains which lack the ability to assemble [Fe-S] clusters produce IscU at negligible levels, whereas a strain that produces IscU bound to a stable cluster is present at much higher levels compared to wild type IscU (Figure 4.6). It is reasonable to speculate that apo-IscU may be degraded in the cell whereas the holo form is more stable. Another possibility is that the apo-conformation of IscU tends to be more membrane-associated and could, therefore, be present at higher levels in the pellet, compared to the cluster-loaded conformation. Further studies need to be performed to explain these observations.

Another clue provided by this study is the observation that IscA co-elutes in a complex with IscU, IscS, and HscB. This finding suggests that the ISC components are likely to work together in a multi-protein complex to form and deliver clusters. This result is reminiscent of studies performed on the SUF proteins, which were also found to form a multi-protein complex (Layer *et al.*, 2007). In that work, it was suggested that this formation may be a mechanism for protecting sulfur transfer during cluster assembly. A similar situation may the case in the ISC system. A multiprotein complex, in addition to other functions, may protect clusters during their assembly or it may limit free iron and sulfur from exerting their toxic effects in the cell. It is also possible that IscA is a recipient protein and the chaperones facilitate delivery of the nascent clusters from the IscSU complex to IscA. Alternatively, IscA may serve as a template for [4Fe-4S] cluster formation and the chaperones may be involved in facilitating this process. Both hypotheses are supported by *in vitro* studies that have demonstrated efficient transfer of [2Fe-2S] clusters from IscU to IscA but not vice versa and that IscA can accommodate [4Fe-4S] clusters (Ollagnier-De-Choudens et al., 2004, Zeng et al., 2007a, Krebs et al., 2001).

In conclusion, we have developed a method for isolating ISC proteins produced *in vivo* by exploiting the convenience of heterologous expression in *E. coli*. The entire *isc* operon was placed under control of the arabinose promoter and *A. vinelandii* IscU was found to have the same characteristics as IscU isolated from its native host. We used this method to study the role of the chaperones HscA and HscB in [Fe-S] cluster assembly and found that 1) the chaperones are not involved in the formation of $[2Fe\text{-}2S]^{2+}$ clusters on IscU, 2) the chaperones do not affect assembly/disassembly of the IscS-IscU complex,

3) $[2Fe\text{-}2S]^{2+}$-IscU is present at higher levels in the absence of the chaperones, and 4) the LPPVK region of IscU that is responsible for interactions with HscA is a flexible loop that may act as a protective "gate" during [Fe-S] cluster assembly. Finally, IscS, IscU, HscB, and IscA co-elute suggesting that the iron-sulfur cluster biosynthetic pathway employs multi-protein complexes.

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CHAPTER 5. A proposed role for the *Azotobacter vinelandii* NfuA protein as an intermediate iron-sulfur cluster carrier

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The work described in this section provides evidence for the formation of [4Fe-4S] clusters on the protein NfuA and its ability to transfer the nascent cluster efficiently to apo-aconitase. I was involved in the purification of NfuA variants containing cysteine to alanine substitutions, size exclusion chromatography of wild type and variant NfuA proteins, monitoring of aconitase activity in the cell extracts of NfuA inactivated strain and strains carrying NfuA variants, purification and preparation of apo-aconitase to be used for activation assays, activation of aconitase by NfuA variants, and phenotypic analysis of strains reported in the published article as well as construction of strains that were related to the project but were not included in the publication. I also wrote the first draft of the Experimental Procedures and Results sections and was involved in revisions of the entire manuscript.

5.1 Abstract

Iron-sulfur clusters ([Fe-S] clusters) are assembled on molecular scaffolds and subsequently used for maturation of proteins that require [Fe-S] clusters for their functions. Previous studies have shown that *Azotobacter vinelandii* produces at least two [Fe-S] cluster assembly scaffolds: NifU, required for the maturation of nitrogenase, and IscU, required for the general maturation of other [Fe-S] proteins. *A. vinelandii* also encodes a protein designated NfuA, which shares amino acid sequence similarity with the C-terminal region of NifU. The activity of aconitase, a [4Fe-4S] cluster-containing enzyme, is markedly diminished in a strain containing an inactivated *nfuA* gene. This inactivation also results in a null-growth phenotype when the strain is cultivated under elevated oxygen concentrations. NifU has a limited ability to serve the function of NfuA, as its expression at high-levels corrects the defect of the *nfuA*-disrupted strain. Spectroscopic and analytical studies indicate that one [4Fe-4S] cluster can be assembled *in vitro* within a dimeric form of NfuA. The resultant [4Fe-4S] cluster-loaded form of NfuA is competent for rapid *in vitro* activation of apo-aconitase. Based on these results a model is proposed where NfuA could represent a class of intermediate [Fe-S] cluster carriers involved in [Fe-S] protein maturation.

5.2 Introduction

The *in vivo* maturation of simple [Fe-S] proteins is proposed to require preassembly of [Fe-S] species on molecular scaffolds. The first [Fe-S] cluster assembly system to be described is the NIF system from *Azotobacter vinelandii*. This system consists of a cysteine desulfurase, encoded by *nifS,* which supplies the S for [Fe-S] cluster formation, and a proposed scaffold protein, encoded by *nifU* (Johnson *et al.*, 2005a). The NIF system is specialized for the maturation of [Fe-S] proteins involved in nitrogen fixation.

A. vinelandii also contains a second [Fe-S] protein maturation system designated ISC. The ISC system is required for the general maturation of cellular [Fe-S] proteins involved in intermediary metabolism, such as aconitase (Zheng *et al.*, 1998). The ISC system is more complicated than the NIF system as it includes the products of eight contiguous

genes, *iscR, iscS, iscU, iscA, hscB, hscA, fdx,* and *iscX* (Johnson *et al.*, 2006). Although the NIF and ISC systems exhibit physiological target specificity, each can partially replace the function of the other, when expressed at high level (Johnson *et al.*, 2005b, Dos Santos *et al.*, 2007).

Even though the NIF and ISC systems are differentiated by their apparent target specificities, they share a number of common structural and functional features. For example, NifS and IscS have similar sequences, and they both exhibit cysteine desulfurase activity (Zheng *et al.*, 1998). IscU also shares considerable sequence identity when compared to the N-terminal domain of NifU, including conservation of three cysteine residues that are likely to provide the nucleation site(s) for [Fe-S] cluster assembly (Zheng *et al*., 1998, Agar *et al.*, 2000a).

NifU is a modular protein that contains three distinct domains (Figure 5.1). The central domain contains a stable redox-active [2Fe-2S] cluster with an as-yet-unknown function (Agar *et al.*, 2000b). *In vitro* and *in vivo* experiments have established that labile [Fe-S] clusters can be assembled on both the N-terminal and C-terminal domains of NifU, and such cluster-loaded forms of NifU can be used for activation of the nitrogenase Fe-protein (Dos Santos *et al.*, 2004, Smith *et al*., 2005b). Thus, NifU contains two different sites upon which labile [Fe-S] clusters can be assembled *in vitro,* but the functional relationship between these sites is not yet known.

There are no genes within the ISC transcriptional unit that encode proteins with sequence similarity to the C-terminal domain of NifU. However, located elsewhere on the *A. vinelandii* genome is a gene, designated *nfuA*, whose product encodes a protein having a C-terminal sequence similar to the C-terminal domain of NifU (Figure 5.1). The sequence conservation between NifU and NfuA includes two cysteine residues that are required for the *in vitro* assembly of [Fe-S] clusters within the NifU C-terminal domain. Like NifU, NfuA also appears to be a modular protein because the amino acid sequence within its N-terminal region shares some sequence similarity with another protein involved in [Fe-S] protein maturation designated IscA (Figure 5.1). IscA is a non-essential protein encoded within the ISC transcriptional unit and it has been proposed to serve as alternative [Fe-S] cluster assembly scaffold (Ollagnier-de-Choudens *et al.*, 2001, Krebs *et al.*, 2001, Abdel-Ghany *et al.*, 2005) or as an Fe donor during the [Fe-S] cluster

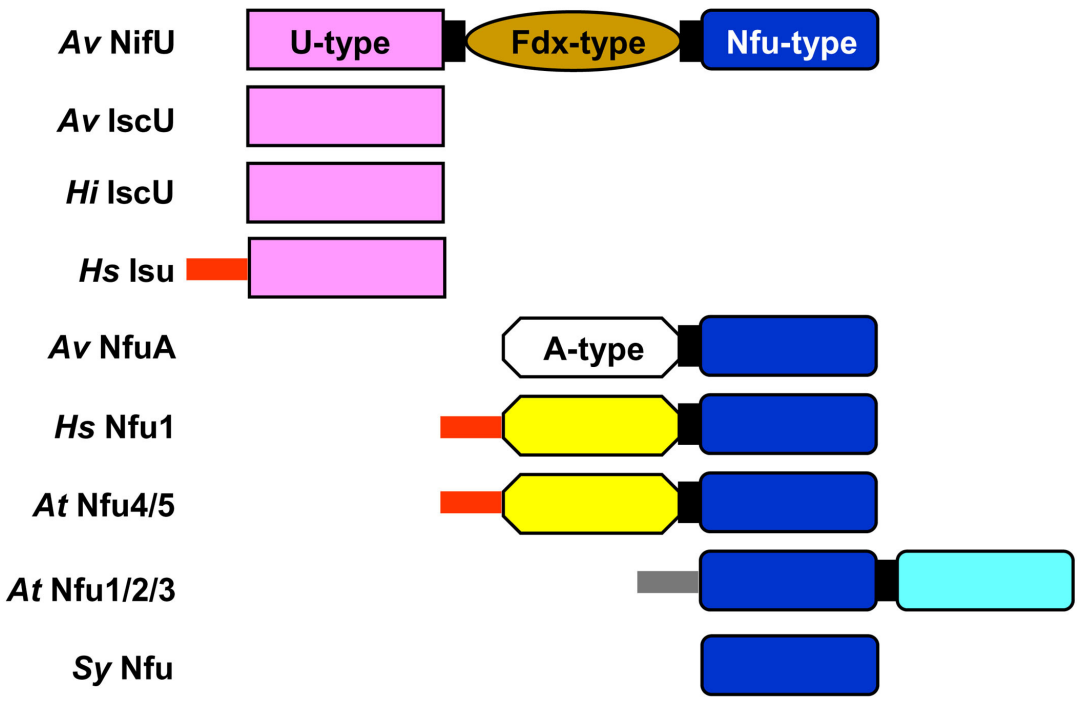

Figure 5.1. Schematic representation of domain structures of IscU/NifU/Nfu proteins from bacteria (*A. vinelandii Av* IscU, *Av* NifU, *Av* NfuA and *Haemophilus influenzae Hi* IscU), mitochondria (human *Hs* Isu, *Hs* Nfu1 and *Arabidopsis thaliana At* Nfu4/5), plant chloroplast (*Arabidopsis thaliana At* Nfu1/2/3) and cyanobacteria (*Synechocystis* sp. *Sy* Nfu). The pink boxes represent U-type domains and the brown box represents a Fdx-like domain. Nfu-type domains with a CXXC motif are colored deep blue and those without CXXC motif are colored light blue. The white box represents the N-terminal A-type domain (without the conserved cysteine residues) that is present in bacterial Nfu-type proteins. The yellow boxes represent the N-terminal domain of mitochondrial Nfu-type proteins. The red and grey sticks represent mitochondrial and plastid targeting sequences, respectively.

assembly process (Ding *et al.*, 2004). IscA contains three cysteine residues that are essential for its physiological function (Johnson *et al*., 2006). However, sequence conservation between IscA and the N-terminal domain of NfuA does not include the three cysteine residues contained within IscA.

Sequence conservation between the N-terminal region of NfuA and the IscA family of proteins, as well as the conservation between the C-terminal domains of NfuA and NifU suggest that NfuA could play a role in [Fe-S] protein maturation. This possibility is supported by evidence that [Fe-S] clusters can be assembled on NfuA-like proteins from other organisms (Tong *et al.*, 2003, Nishio & Nakai, 2000, Leon *et al.*, 2003, Yabe *et al.*, 2004). In some cases, deletion of genes encoding NfuA-like proteins impairs [Fe-S] protein maturation (Touraine *et al.*, 2004, Yabe *et al.,* 2004, Schilke *et al.*, 1999, Balasubramanian *et al.*, 2006). In the present work we have performed a genetic, biochemical and spectroscopic analysis of NfuA from *A. vinelandii* in order to gain insight into its cellular function in this organism.

5.3 Materials and Methods

Materials. Materials used in this work were purchased from Sigma, Fisher Scientific, New England Biolabs, Invitrogen, or from other sources as indicated throughout the text. *Bioinformatics and DNA analysis*—Sequence alignments were performed using the SDSC Biology Workbench program. The Lasergene 7 software was used to analyze DNA and protein sequences. When necessary, *A. vinelandii* genomic DNA was extracted, amplified, and sequenced as described previously (Dos Santos *et al.*, 2007).

Plasmid and strain construction. Construction of *A. vinelandii* strains via transformation with appropriate plasmids has been described previously (Jacobson *et al.*, 1989a). In this work, DJ1707 was obtained by transformation of our wild type laboratory strain designated DJ (ATCC BAA-1303) with pDB1598, a plasmid that includes a kanamycin resistance cartridge inserted into the *Xho*I sites of *nfuA*. This construct results in deletion of a 290 bp fragment from the *nfuA* gene. DJ1759 and DJ1769 respectively produce the $NfuA^{C152A}$ - and $NfuA^{C155A}$- substituted forms of NfuA and were constructed

by using plasmids pDB1610 and pDB1614, which contain site-directed cysteine to alanine substitutions (codon change of UGC to GCC) at residue positions 152 and 155, respectively. Site-directed mutagenesis procedures were conducted using the GeneEditor™ system by Promega. The parent plasmid used for deletion, insertion or amino acid substitutions was pDB1577, which contains the intact *nfuA* gene, as well as 570 bp downstream of the gene, in the pUC-7 vector.

Abundant production and controlled expression of NifUS and their variants was achieved by placing the desired genes under the control of the inducible arabinose regulatory elements as described previously (Dos Santos *et al*., 2007). Plasmid pDB1598 was used to inactivate *nfuA* in DJ1626, a strain where *nifUS* are placed under the control of the *araBAD* promoter with concomitant in-frame deletion of the *nif*-regulated *nifUS* (Dos Santos *et al.*, 2007). This inactivation yielded strain DJ1772. Similarly, strains DJ1773 and DJ1791 were constructed, which contain *nifU* variants with cysteine to alanine substitutions at residues 35 and 275 respectively and are otherwise isogenic to DJ1772.

Plasmid pDB1417 was designed by insertion of *nfuA,* flanked by NdeI and BamHI restriction sites, into the NdeI and BamHI sites of pT7-7 for recombinant expression. Similarly, plasmids pDB1582 and pDB1583 were constructed for recombinant expression of $NfuA^{152CA}$ and $NfuA^{155CA}$, respectively.

Cell growth at ambient and elevated oxygen levels. All strains used in this study were cultured in Burk's medium supplemented with 13 mM ammonium acetate (Strandberg & Wilson, 1968). When appropriate, media were also supplemented with 20 mM L-arabinose. To expose cells to elevated oxygen concentrations, Petri plates were placed in BBL GasPak jars and filled with a gas mixture containing 40% O_2 as described previously (Johnson *et al*., 2006).

Aconitase and isocitrate dehydrogenase assays of A. vinelandii crude extracts. *A. vinelandii* cells were grown in liquid cultures containing Burk's medium supplemented with 13 mM ammonium acetate until they reached OD_{600} = 0.5. Cells were, subsequently, harvested by centrifugation at 4225 *g* for 5 min and stored at –20°C, if not

used immediately. To prepare crude extracts, cells were first resuspended in degassed buffer containing 25 mM Tris at pH 7.4 and were lysed using a French Pressure Cell at 12,000 psi. Lysed cells, which were maintained in airtight vials filled with Argon gas, were centrifuged at 235,000 *g* for 45 min. Crude extracts were separated from the cell debris pellet inside a Coy anaerobic glove box containing 4% H_2 gas and 96% N_2 to minimize exposure to oxygen. Aconitase assays were prepared in 1 mL quartz cuvettes – sealed airtight with rubber septa – containing 700 μL of 100 mM Tris-HCl buffer at pH 8.0 and 0.1 mg of crude extract. The reaction was initiated with the addition of 25 mM sodium citrate and activity was monitored at 240 nm (Saas *et al.*, 2000). Isocitrate dehydrogenase assays of crude extracts were performed in quartz cuvettes containing 900 μL of 50 mM MOPS buffer at pH 7.3, 2.5 mM $MgCl_2$, 0.45 mM $NADP^+$, and 0.1 mg of crude extract. The reaction was started with the addition of 80 mM *DL* – isocitric acid (trisodium salt) in the MOPS/$MgCl_2$ buffer described above and monitored at 340 nm (Cribbs & Englesberg, 1964).

Purification of NfuA and its variants. *E. coli* BL21(DE3) cells transformed with plasmids pDB1417, pDB1582, or pDB1583 were used for the heterologous expression of NfuA, $NfuA^{152CA}$, or $NfuA^{155CA}$ respectively. Cells were grown in LB medium containing 0.1 mg/mL ampicillin until they reached OD_{600} = 0.5, at which point, they were induced with 29 mM lactose and allowed to continue to grow for 3 hours at 30°C. Cells were then harvested by centrifugation at 4225 *g* for 5 min and stored at –20°C until being used.

Cells expressing recombinant NfuA or its alanine substituted variants were resuspended in degassed 50 mM Tris-HCl, pH 7.4 buffer (buffer A) and lysed either by sonication (1 min pulse/30 sec pause for a total of 20 min) or by the use of a French Pressure Cell. Crude extracts were prepared as described above. All purification procedures were conducted anaerobically by the use of Schlenk lines filled with argon gas. Crude extracts were loaded at 2.5 mL/min using a peristaltic pump into a 25 mL Q-Sepharose column previously equilibrated with 5 bed volumes of buffer A. The column was subsequently washed with 50 mL of buffer A and 75 mL of buffer A containing 100 mM NaCl. To elute NfuA, a 200 mL of 0.1 - 1.0 M NaCl gradient was applied and the

contents of each fraction were analyzed by 15% SDS-PAGE. The fractions containing NfuA, which eluted between 40 mL and 94 mL of the gradient, were consolidated and $(NH_4)_2SO_4$ was added to a final concentration of 1 M. This sample was then loaded onto a 25 mL Phenyl-Sepharose column that was previously equilibrated with buffer A containing 1 M $(NH_4)_2SO_4$. Following binding of the sample onto the column, the latter was washed with 125 mL buffer A containing 1 M $(NH_4)_2SO_4$ followed by 25 mL of 0.5 M $(NH_4)_2SO_4$ in buffer A. The protein was then eluted using a 100 mL gradient of 0.5 - 0.0 M $(NH_4)_2SO_4$ followed by 50 mL of buffer A. The fractions containing NfuA, collected from the last 28 mL of the gradient, were then concentrated by Amicon ultrafiltration using a YM10 membrane. The concentrated sample was loaded onto a 320 mL Superdex S75 gel filtration column equilibrated with 100 mM Tris-HCl buffer (pH 8.0) with 1 mM DTT (buffer B) and 150 mM NaCl. The purest fractions of NfuA as judged by SDS-PAGE were pooled and concentrated by Amicon ultrafiltration using a YM10 membrane, and frozen as pellets in liquid N2 until used. The resulting samples of NfuA were >95% pure as judged by gel densitometry.

In vitro reconstitution of NfuA. Purified NfuA, $NfuA^{152CA}$, or $NfuA^{155CA}$ (0.24 mM) were incubated with NifS (12 μM), ferrous ammonium sulfate (4.8 mM) and L-cysteine (4.8 mM) in buffer B for 3 hours at room temperature under argon inside a Vacuum Atmospheres glove box (< 2 ppm O_2). Excess reagents were removed from the sample by loading it onto a Hi-trap Q-Sepharose column and eluting with a 0 to 1.0 M NaCl gradient using buffer B. The holo-protein eluted between 0.45-0.55 M NaCl and was concentrated by ultrafiltration using an Amicon YM10 membrane. ^{57}Fe-enriched ferrous sulfate (>95% enrichment) was used in place of natural abundance ferrous ammonium sulfate in the preparation of Mössbauer samples.

Determination of oligomeric state of apo and holo NfuA. The oligomeric states of both apo and holo NfuA were determined by gel-filtration chromatography using a 25 mL Superdex S75 column. The elution buffer was buffer B with 100 mM KCl, which

was applied to the column at a flow rate of 0.4 mL/min. Molecular weight standards used were blue dextran (M_r 2,000,000), β-amylase (M_r 200,000), alcohol dehydrogenase (M_r 150,000), bovine serum albumin (M_r 66,000), carbonic anhydrase (M_r 29,000), and cytochrome *c* (M_r 12,400).

Analytical and spectroscopic analyses. Protein concentrations were determined by the *DC* protein assay (Bio-Rad) using BSA as the standard. Iron concentrations were determined colorimetrically using bathophenanthroline under reducing conditions, after digestion of the protein in 0.8% $KMnO_4$/0.2 M HCl (Fish, 1988) or by using the commercial Quantichrom™ iron assay kit (DIFE-250) from Bioassay Systems. Samples for spectroscopic studies were prepared and handled under Ar in a Vacuum Atmospheres glove box (O_2 < 2 ppm). UV-visible absorption spectra were recorded at room temperature using a Shimadzu UV-3101PC or Cary 50 Bio spectrophotometer. Resonance Raman spectra were recorded as previously described, using an Instruments SA Raman or U1000 spectrometer coupled with a Coherent Sabre argon ion laser, with 20 μL frozen droplets of 2-3 mM sample mounted on the cold finger of an Air Products Displex Model CSA-202E closed cycle refrigerator (Cosper *et al.*, 2004). Signal-to-noise was improved by multiple scans and bands due to the frozen buffer solution were subtracted from all the spectra shown in this work after normalization of lattice modes of ice centered at 230 cm^{-1}. Variable-temperature magnetic circular dichroism (VTMCD) spectra were recorded using samples containing 55% (v/v) ethylene glycol 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 (Johnson, 2000). 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. Mössbauer spectra were recorded by using the previously described instrumentations (Ravi *et al.*, 1994). Analysis of the Mössbauer data was performed with the program WMOSS (Web Research).

Activation of Apo-aconitase by reconstituted NfuA. Apo-aconitase was prepared by incubating recombinantly expressed and purified *A. vinelandii* aconitase A (AcnA) (Unciuleac *et al.*, 2007) with EDTA and potassium ferricyanide as described previously

(Kennedy & Beinert, 1988). Activation mixtures contained 4 μM apo-AcnA and between 0-12 μM of [4Fe-4S] cluster-loaded NfuA in buffer B. The concentration of [4Fe-4S] cluster-loaded NfuA corresponds to the concentration of $[4Fe\text{-}4S]^{2+}$ clusters calculated by using a molar extinction coefficient of $\varepsilon_{400} = 15.0\ mM^{-1}cm^{-1}$ per $[4Fe\text{-}4S]^{2+}$ cluster. Activation mixtures were incubated at room temperature (22 °C) under anoxic conditions, and 10 μL samples were taken at different time points and assayed for AcnA activity. AcnA activity was measured at 240 nm at 22 °C by following the formation of *cis*-aconitate from citrate or isocitrate, using a molar absorption coefficient ε_{240} of 3400 $M^{-1}\ cm^{-1}$ for *cis*-aconitate (Saas *et al.*, 2000). Assays (1 mL) were carried out in sealed anoxic cuvettes containing 900 μL of 100 mM Tris/HCl (pH 8.0) and AcnA and initiated by the addition of 100 μL of 200 mM citrate or isocitrate. Anaerobically reconstituted samples of *A. vinelandii* AcnA containing one [4Fe-4S] cluster per protein monomer exhibited maximal specific activity of 25 units/mg using citrate (~100% activity) and 79 units/mg using isocitrate (~100% activity) *(Unciuleac et al.*, 2007). The time course of holo-AcnA formation at 22 °C was analyzed by fitting to second-order kinetics, based on the initial concentrations of apo-AcnA and $[4Fe\text{-}4S]^{2+}$ clusters on NfuA, using the Chemical Kinetics Simulator software package (IBM).

5.4 Results

***In vitro* assembly of a [4Fe-4S] cluster within NfuA**. When *A. vinelandii* NfuA is heterologously produced in *E. coli* and purified under anoxic conditions it does not contain an [Fe-S] cluster. Gel exclusion chromatography indicated that as-isolated, recombinant NfuA is an approximately equal mixture of dimeric and tetrameric species (data not shown). When as-isolated NfuA is incubated with NifS, L-cysteine and Fe^{2+}, an [Fe-S] cluster is assembled. Gel exclusion chromatography revealed that the cluster-bound form of NfuA is resolved into a single dimeric species. Analytical data, coupled with the UV-visible absorption spectrum and extinction coefficients (Figure 5.2), indicate that the reconstituted sample contains approximately one $[4Fe\text{-}4S]^{2+}$ cluster per NfuA dimer. Repurified samples of reconstituted NfuA contain 2.0 ±0.3 Fe/NfuA monomer and the visible absorption comprises a broad shoulder centered at 400 nm (A_{400}/A_{280} =

0.23 ±0.2) with a molar extinction coefficient (ε_{400} = 7.5 ±0.5 $mM^{-1}cm^{-1}$ based on the concentration of NfuA monomer) that is indicative of approximately 0.5 $[4Fe\text{-}4S]^{2+}$

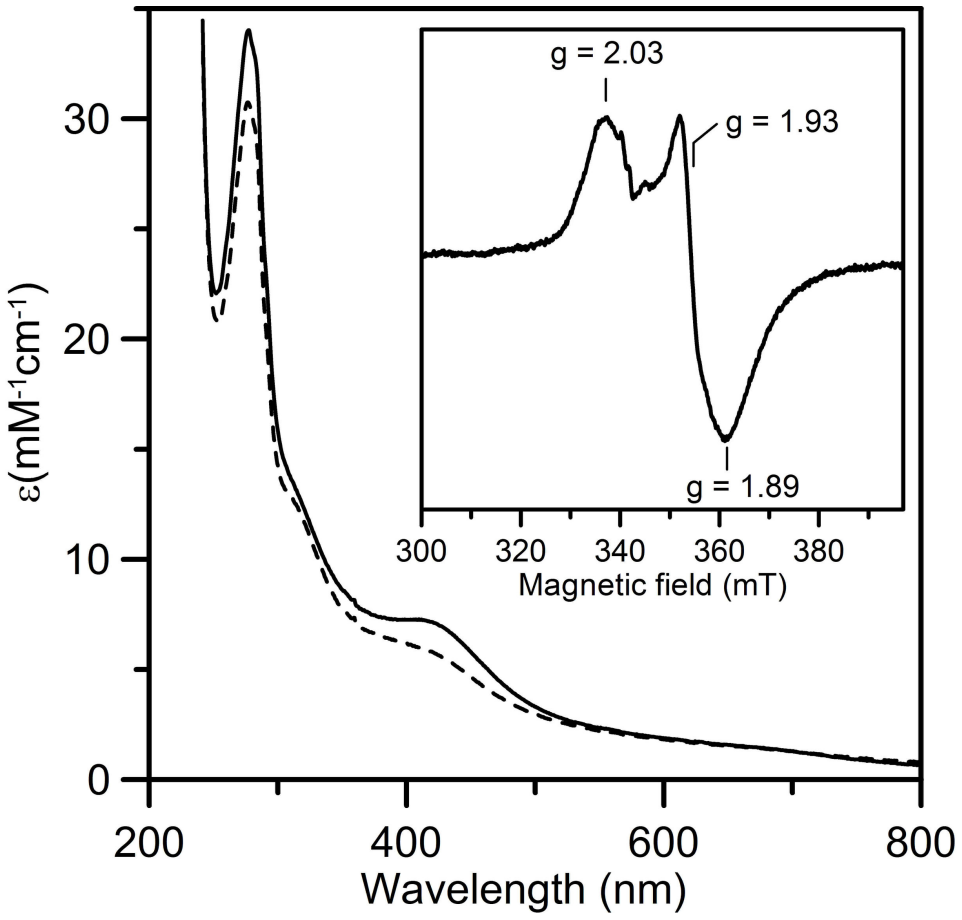

Figure 5.2. UV-visible absorption spectra of reconstituted *A. vinelandii* NfuA as prepared (solid line) and after anaerobic reduction with 1 reducing equivalent of dithionite (dashed line). Molar extinction coefficients are expressed per NfuA monomer. The inset shows the X-band EPR spectrum of the sample reduced with 1 reducing equivalent of dithionite. EPR conditions: microwave frequency, 9.60 GHz; microwave power, 10 mW; modulation amplitude, 0.63 mT; temperature, 10 K.

clusters/NfuA monomer (Agar *et al*., 2000a). More definitive assessments of the [Fe-S] cluster type and content were provided by analysis of the Mössbauer spectrum of a ^{57}Fe-reconstituted NfuA shown in Figure 5.3 (vertical bars), which revealed that 90% of the Fe is associated with a $[4Fe\text{-}4S]^{2+}$ cluster and the remaining 10% of the Fe with an adventitiously bound Fe^{2+} species. The spectrum of the $[4Fe\text{-}4S]^{2+}$ cluster (dashed line) is simulated as a superposition of two equal intensity quadrupole doublets arising from the two valence delocalized pairs with δ= 0.47 mm/s, ΔE_Q =1.25 mm/s for doublet 1 and δ = 0.45/mm/s, ΔE_Q = 0.99 mm/s for doublet 2. The spectrum of the Fe^{2+} species (dotted line) is simulated with an asymmetric quadrupole doublet with δ = 1.38 mm/s and ΔE_Q = 3.25 mm/s. The presence of [2Fe-2S] clusters was not detected.

While the absorption and Mössbauer results provide unambiguous evidence for anaerobic reconstitution of a $[4Fe\text{-}4S]^{2+}$ cluster on NfuA, the vibrational properties, as determined by resonance Raman spectroscopy, are somewhat atypical compared to well-characterized biological $[4Fe\text{-}4S]^{2+}$ centers (Czernuszewicz *et al.*, 1987, Spiro *et al.*, 1988, Brereton *et al.*, 1999) and exhibit characteristics that have been observed previously only for the subunit-bridging $[4Fe\text{-}4S]^{2+}$ cluster in the MgATP-bound nitrogenase Fe protein (Sen *et al.*, 2004). This feature is illustrated in Figure 5.4, which shows a comparison of the resonance Raman spectra of the $[4Fe\text{-}4S]^{2+}$ clusters in reconstituted forms of *A. vinelandii* IscU and NfuA in the Fe-S stretching region using 457-nm excitation. The major difference lies in the anomalously high frequency for the most intense band in the spectrum (353 cm^{-1} for the NfuA $[4Fe\text{-}4S]^{2+}$ center compared to 335-343 cm^{-1} in other biological $[4Fe\text{-}4S]^{2+}$ centers), which is generally assigned to the symmetric breathing mode of the [4Fe-4S] cubane core. Analogous spectra with similar band frequencies and relative intensities were observed for the reconstituted NfuA using 488- and 514-nm excitation, indicating that this behavior cannot be attributed to anomalous excitation profiles for discreet bands. One possible explanation is that the intense bands at 353 and 359 cm^{-1} for the $[4Fe\text{-}4S]^{2+}$ center in NfuA correspond to asymmetric Fe-S(Cys) stretching modes and that the symmetric breathing modes primarily involving the [4Fe-4S] core and the Fe-S(Cys) bonds are only weakly enhanced as a result of distortions of the core and/or the cluster environment. This possibility is tentatively supported by the observation of similar frozen-solution resonance Raman

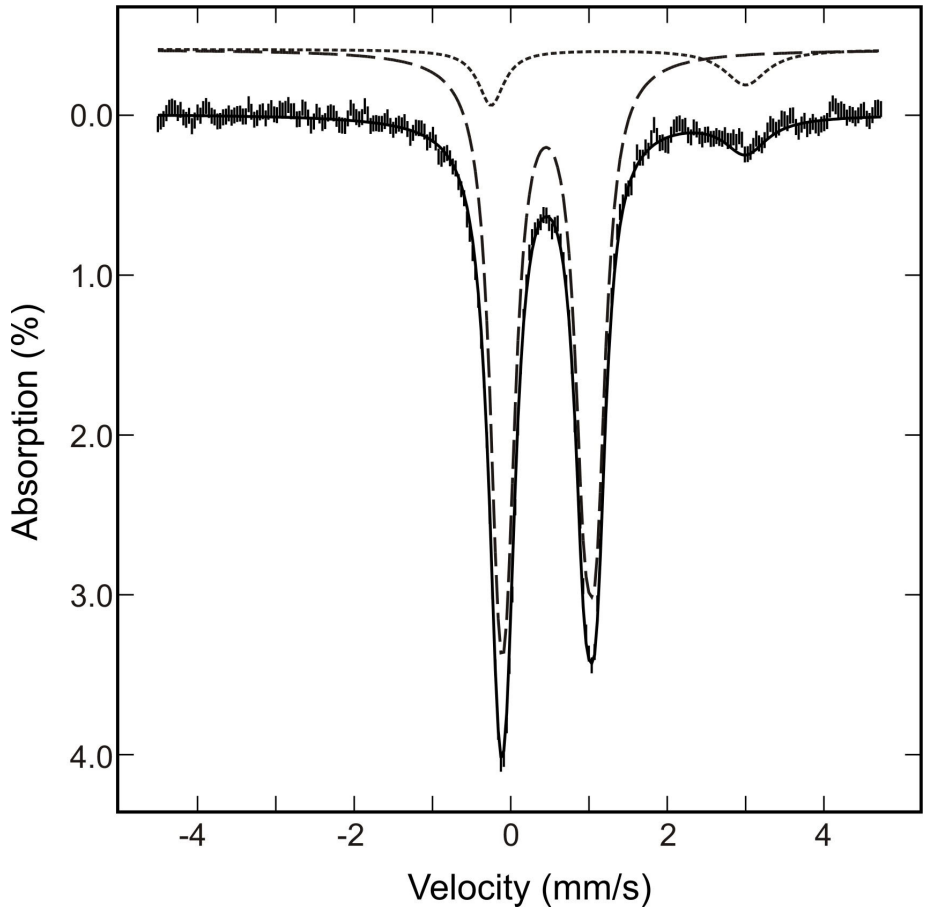

Figure 5.3. Mössbauer spectrum of ^{57}Fe reconstituted *A. vinelandii* NfuA recorded at 4.2 K with a field of 50 mT applied parallel to the γ-radiation. The solid black line is the composite spectrum including the simulated spectrum of a $[4Fe\text{-}4S]^{2+}$ cluster (dashed line), scaled to 90% of the total Fe absorption, and the simulated spectrum of a Fe^{2+} species (dotted line), accounting for 10% of the Fe absorption. The parameters used for the simulations are provided in the text.

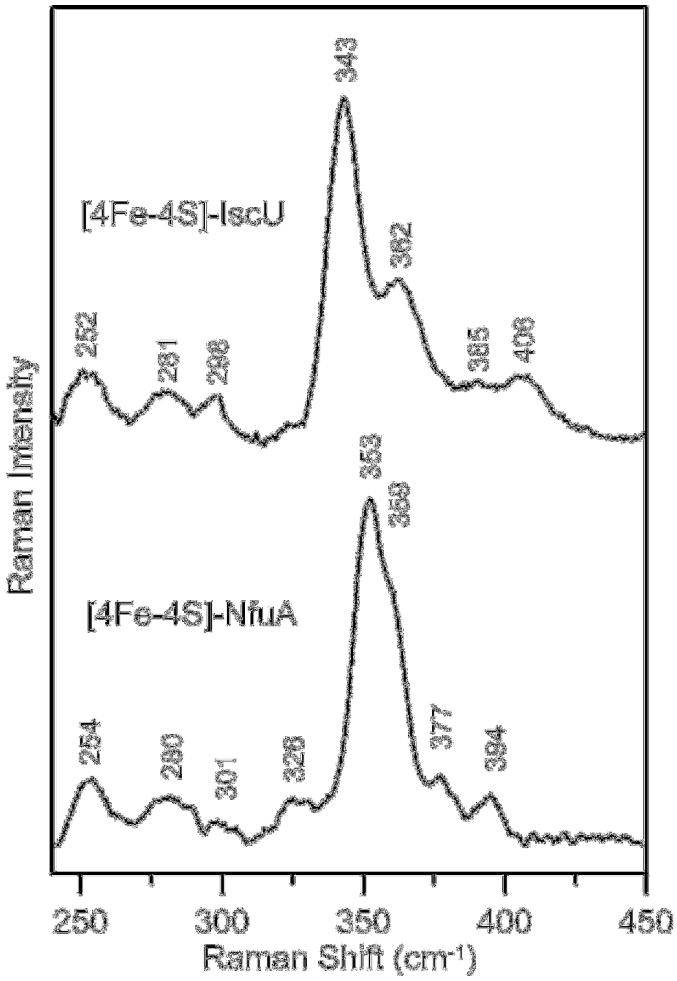

Figure 5.4. Comparison of the resonance Raman spectra for $[4Fe\text{-}4S]^{2+}$ cluster-loaded forms of *A. vinelandii* IscU and NfuA. The samples (~3 mM in [4Fe-4S] clusters) were in the form of frozen droplets at 17 K. The spectra were recorded using 457-nm excitation with 100-mW of laser power at the sample. Each spectrum is the sum of 100 scans, with each scan involving photon counting for 1 s at 1-cm^{-1} increments, with 7 cm^{-1} resolution. Bands due to lattice modes of the frozen buffer solution have been subtracted from both spectra.

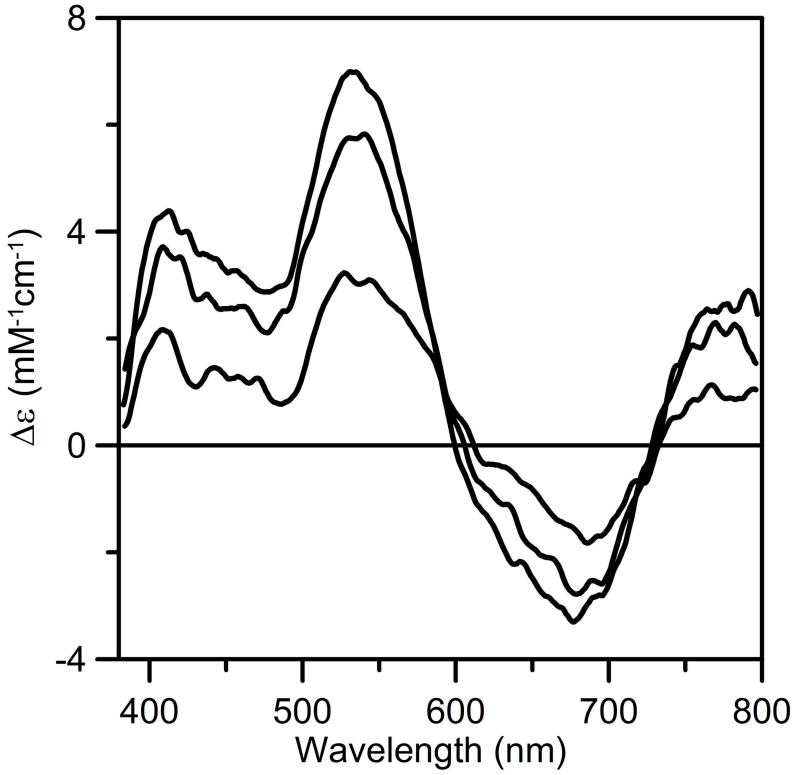

Figure 5.5. VTMCD spectra of dithionite-reduced reconstituted *A. vinelandii* NfuA. Reconstituted NfuA (0.25 mM in [4Fe-4S] clusters) was reduced with 1 reducing equivalent of dithionite after addition of 55% (v/v) ethylene glycol. MCD spectra recorded in a 1 mm cell with a magnetic field of 6 T at 1.7, 4.2 and 15 K. All bands increase in intensity with decreasing temperature and Δε values are expressed per NfuA monomer.

spectra for the $[4Fe\text{-}4S]^{2+}$ cluster in MgATP-bound nitrogenase Fe protein, which was found to be split into two [2Fe-2S] fragments separated by ~5 Å in the crystalline state (Sen *et al.*, 2004). Normal mode calculations coupled with $^{57/54}Fe$ and $^{34/32}S$ isotope shifts and measurement of depolarization ratios are planned to test this hypothesis and to provide vibrational assignments for the $[4Fe\text{-}4S]^{2+}$ center in NfuA.

The nature of the cluster ligation for the [4Fe-4S] center on NfuA was addressed by amino acid substitution of the two conserved cyteine residues located within the Cys-X-X-Cys motif. It was not possible to reconstitute a [Fe-S] cluster on variant forms of NfuA proteins that have either of these two cysteine residues substituted by alanine (data not shown). This information, together with the analytical and spectroscopic data are reasonably interpreted in terms of cluster-loaded NfuA having a $[4Fe\text{-}4S]^{2+}$ cluster that is symmetrically bridged between two identical subunits and coordinated by conserved cysteine residues from opposing subunits.

The redox properties of the $[4Fe\text{-}4S]^{2+}$ cluster assembled on NfuA were assessed using a combination of UV-visible absorption, EPR and VTMCD spectroscopies (Figures 5.2 and 5.5). Anaerobic addition of 1 reducing equivalent of dithionite results in reversible bleaching of the visible absorption (Figure 5.2) due to partial reduction to yield a $S = 1/2$ $[4Fe\text{-}4S]^{+}$ cluster, as evidenced by parallel EPR and VTMCD studies. EPR revealed a near-axial resonance ($g = 2.03, 1.93, 1.89$) with relaxation properties (observable only below 40 K) indicative of a $[4Fe\text{-}4S]^{+}$ cluster, that accounts for 0.4 spins/NfuA dimer (see inset in Figure 5.2). The low spin quantification is in accord with the partial bleaching of the visible absorption and appears to reflect partial reduction. No low-field EPR signals indicative of a $S = 3/2$ $[4Fe\text{-}4S]^{+}$ clusters were observed. In accord with the EPR results, the VTMCD spectrum is consistent with a $S = 1/2$ $[4Fe\text{-}4S]^{+}$ cluster rather than a $S = 3/2$ $[4Fe\text{-}4S]^{+}$ cluster (Onate *et al.*, 1993) and the intensity indicates ~0.4 $[4Fe\text{-}4S]^{+}$ clusters/NfuA dimer. Partial reduction by one reducing equivalent of dithionite implies a redox potential for the $[4Fe\text{-}4S]^{2+,+}$ couple that is close to that of dithionite at pH 8.0 (–450 mV). Such a low potential redox process is unlikely to be physiologically relevant. Moreover, the reduced cluster is unstable and is rapidly degraded within minutes in the presence of excess dithionite even under strictly anoxic conditions. Similar redox behavior has been reported for the subunit-bridging [4Fe-4S]

cluster on IscU (Unciuleac *et al*., 2007). Hence, only the oxidized form of the subunit-bridging [4Fe-4S] cluster on NfuA is likely to be relevant *in vivo.*

Activation of apo-aconitase using [4Fe-4S] cluster-loaded NfuA. Figure 5.6A shows the time-dependent activation of apo-aconitase that occurs when it is mixed with [4Fe-4S] cluster-loaded NfuA. Initial experiments duplicated the conditions established to achieve optimal aconitase activation using [4Fe-4S] cluster-loaded IscU (Unciuleac *et al*., 2007) and involved incubation of cluster-loaded NfuA (12 μM in [4Fe-4S] clusters) with 4 μM of apo-aconitase, i.e. a 3-fold stoichiometric excess of [4Fe-4S] clusters. The activation is remarkably rapid, having a second order rate constant of $6.0 \pm 1.5 \times 10^4\ M^{-1}min^{-1}$, a rate which is almost 10 times faster than that observed for analogous conditions using the [4Fe-4S] cluster-loaded form of IscU (Unciuleac *et al*., 2007). Such rates are consistent only with transfer of intact cluster, as activation with equivalent amounts of Fe^{2+} and S^{2-} ions is at least 15 times slower. Moreover, unlike IscU-directed apo-AcnA activation, which is optimal with a 3:1 stoichiometry, NfuA-directed activation of apo-AcnA occurs with a 1:1 stoichiometry. This feature is illustrated in Figure 5.6B, which shows apo-aconitase activation after 20 min of incubation as a function of the molar ratio of [4Fe-4S] clusters on NfuA to apo-AcnA. The data indicate stoichiometric [4Fe-4S] cluster transfer from NfuA to apo-AcnA. The curvature is a consequence of incomplete cluster transfer after 20 min at near stoichiometric [4Fe-4S] cluster concentrations. Indeed, the observed data are well simulated by theoretical data constructed for [4Fe-4S] cluster transfer occurring with a 1:1 stoichiometry and a second order rate constant of $6.0 \times 10^4\ M^{-1}min^{-1}$ after 20 min of reaction. Hence, cluster transfer and activation of apo-aconitase is much faster and much more efficient with [4Fe-4S]-NfuA than with [4Fe-4S]-IscU.

Phenotypic analysis of mutant strains defective in NfuA function. A strain (designated DJ1707) was constructed that has the *nfuA* gene partially deleted and replaced by a kanamycin gene cartridge insertion. DJ1707 has no obvious phenotypic traits with respect to growth rate when cultured in liquid medium or colony size when cultured on agar plates. However, DJ1707 cell extracts have only ~50% aconitase

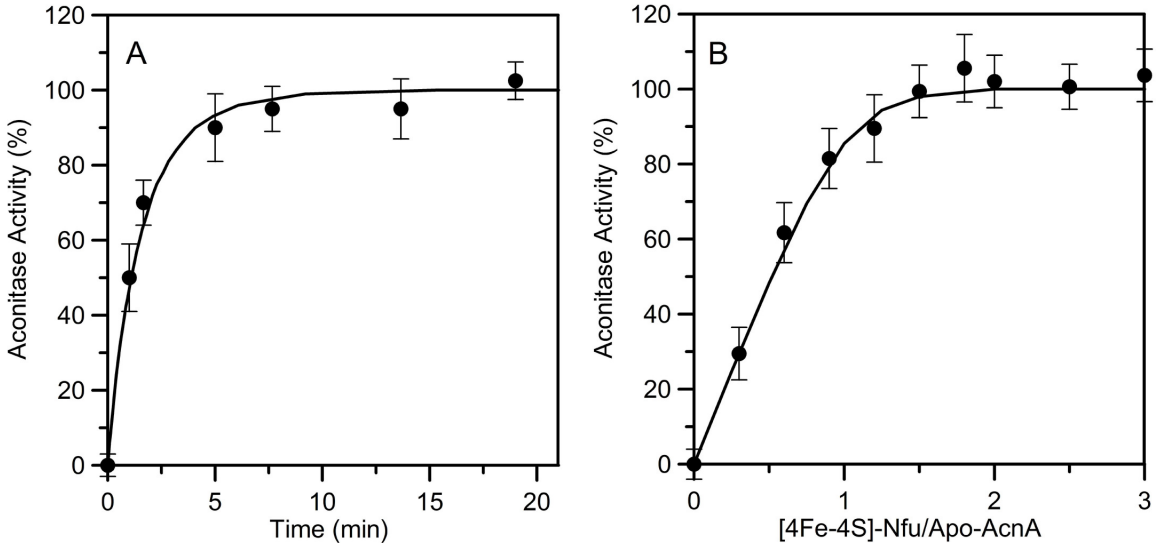

Figure 5.6. Activation of apo-AcnA activity using [4Fe-4S] cluster-loaded NfuA. (A) Apo-AcnA (4 μM) was incubated with [4Fe-4S] cluster-loaded NfuA (12 μM in $[4Fe\text{-}4S]^{2+}$ clusters) at room temperature under anaerobic conditions. Aliquots containing 4 μM AcnA were withdrawn after 0, 100, 150, 300, 460, 820, 1140, and1480 sec, and AcnA activity was immediately measured. The solid line is the best fit to second-order kinetics based on the initial concentrations of $[4Fe\text{-}4S]^{2+}$ clusters on NfuA and apo-AcnA, and corresponds to a rate constant of 6.0×10^4 $M^{-1}min^{-1}$. (B) Apo-AcnA activation as a function of the concentration of NfuA-ligated $[4Fe\text{-}4S]^{2+}$ clusters. The concentration of apo-AcnA was kept constant at 4 μM, and the concentration of NfuA-ligated $[4Fe\text{-}4S]^{2+}$ clusters was varied as indicated on the *x* axis. After 20 min aliquots were withdrawn and assayed for AcnA activity. The solid line is theoretical data computed for [4Fe-4S] cluster transfer from NfuA to apo-AcnA occurring with a 1:1 stoichiometry and with a rate constant of 6.0×10^4 $M^{-1}min^{-1}$ after 20 min of reaction. The data points in (A) and (B) correspond to the average values of three independent measurements and the error bars represent the standard deviation.

activity when compared to the isogenic wild type strain. The diminished aconitase activity of the DJ1707 strain relative to the otherwise isogenic wild-type strain contrasted with the equivalent levels of isocitrate dehydrogenase in the two strains. Isocitrate dehydrogenase, like aconitase, is a TCA cycle enzyme but it does not require an [Fe-S] cluster for its activity. Another phenotype associated with loss of NfuA function is a complete loss of capacity for growth under a 40% oxygen atmosphere (Figure 5.7).

The functional importance of the two cysteine residues located within the Cys-X-X-Cys motif was tested by constructing strains having these residues individually substituted by alanine. These strains, DJ1759 and DJ1769, exhibit the same oxygen-sensitive growth phenotype associated with deletion and insertional inactivation of *nfuA* (Figure 5.7).

NifU replacement of NfuA requires functional NifU N- and C-terminal domains. DJ1707 exhibited the same sensitivity to elevated oxygen concentrations whether or not NifU is also expressed. Thus, NifU is not normally able to supplant the function of NfuA. However, when *nifUS* expression is placed under control of the strong *ara* regulatory elements (Figure 5.8) the oxygen-sensitive phenotype associated with loss of NfuA function is fully corrected. Thus, there is a capacity for functional replacement of NfuA by NifU, but only when NifU is expressed at high levels. In separate experiments it was determined if both the N- and C-terminal domains of NifU are required to rescue the phenotype associated with loss of NfuA function. This possibility was examined by construction of two different strains. One of these (DJ1773) has the NifU-Cys^{35} residue substituted alanine (DJ1773) and the other (DJ1791) has the NifU-Cys^{275} residue substituted by alanine. Previous work has shown that such substitutions respectively eliminate the capacity for [Fe-S] cluster formation within the IscU-like or NfuA-like domains within NifU (Dos Santos *et al*., 2004). Both strains DJ1773 and DJ1791 are also inactivated for *nfuA*. Results shown in Figure 5.8 reveal that a capacity for the assembly of [Fe-S] clusters on both the IscU-like and NfuA-like domains of NifU is required for correction of the oxygen-sensitive phenotype associated with loss of NfuA function.

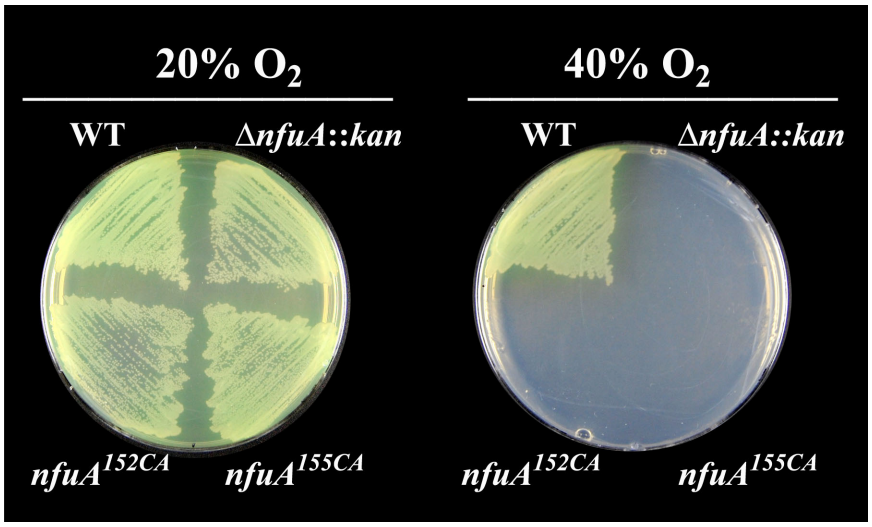

Figure 5.7. Inactivation of *nfuA* and its conserved cysteines results in a null-growth phenotype under elevated oxygen conditions. Strains were cultured in Burk's medium supplemented with 13 mM ammonium acetate under ambient (~20%) O_2 or 40% O_2 for three days. The standard laboratory strain designated as DJ was used as a wild-type control (WT). Strain DJ1707 has a deletion and kanamycin gene cartridge insertion within the *nfuA* gene (Δ*nfuA::kan*). Strains DJ1759 and DJ1769 have respectively NfuA Cys^{152} and NfuA Cys^{155} substituted by alanine.

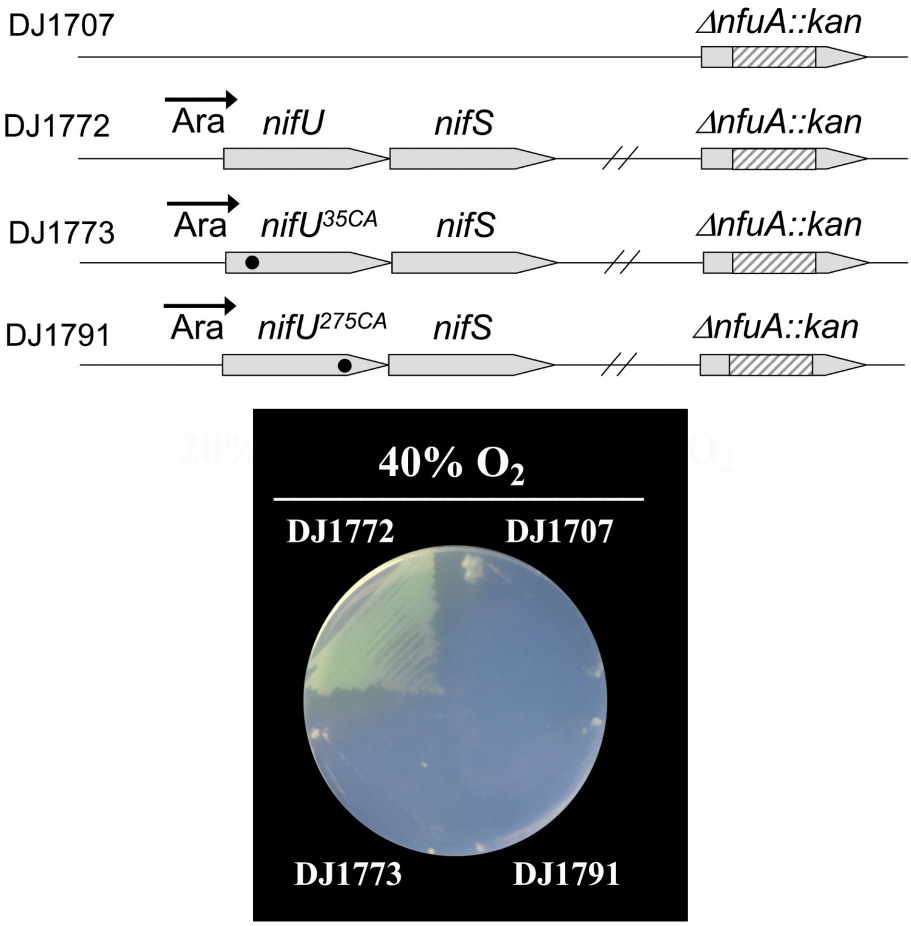

Figure 5.8. Rescue of the null growth phenotype associated with the functional loss of NfuA by elevated *ara*-directed expression of NifUS. (*top*) Schematic representation of the genetic organizations of DJ1707, DJ1772, DJ1773, and DJ1791. (*bottom*) Growth of strains when cultured in Burk's medium supplemented with 13 mM ammonium acetate and 20 mM arabinose and cultured under a 40% O_2 atmosphere. NifU and NifS expression is under control of the *ara*-regulatory elements, resulting in accumulation of high levels when arabinose is added to the growth medium.

5.5 Discussion

NfuA is a modular protein that has an N-terminal domain similar to IscA and a C-terminal domain similar to the C-terminal domain of NifU. Because both IscA and NifU are involved in the maturation of [Fe-S] proteins, such sequence conservation indicates a likely role for NfuA in [Fe-S] protein maturation in *A. vinelandii*, but what is that role? The most pertinent observations from previous work and this study that bear on this question can be summarized as follows: (*i*) IscU is essential for the *in vivo* maturation of [Fe-S] proteins. (*ii*) The [4Fe-4S] cluster-loaded form of IscU is a poor *in vitro* donor for aconitase maturation (Unciuleac *et al.*, 2007), whereas the [4Fe-4S] cluster loaded form of NfuA is very effective for *in vitro* activation of aconitase. (*iii*) NfuA is not essential for *in vivo* [Fe-S] protein maturation but loss of NfuA results in lower activity of at least one key [4Fe-4S] protein, i.e. aconitase. (*iv*) The phenotypic relationship observed between IscU and NfuA is also observed for the IscU-like and NfuA-like domains of NifU. Specifically, functional loss of the IscU-like domain of NifU nearly eliminates the capacity for the *in vivo* maturation of nitrogenase, whereas functional loss of the NfuA-like domain has no obvious effect on nitrogenase maturation (Dos Santos *et al*., 2004). Furthermore, the IscU-like domain of NifU is a relatively poor [4Fe-4S] donor for maturation of the nitrogenase Fe protein (Smith *et al*., 2005b). (*v*) The ability of NifU to correct the oxygen-sensitive phenotype associated with loss of NfuA requires functionality of both the IscU-like and NfuA-like domains within NifU.

These results lead us to propose a model where IscU, and the IscU-like domain within NifU, have essential roles in the *de novo* formation of [Fe-S] clusters, whereas NfuA and the NfuA-like domain of NifU could have auxiliary roles related to the effective transfer of [Fe-S] clusters to specific target proteins. This model suggests that [Fe-S] clusters initially formed on the IscU type of scaffolds in *A. vinelandii* are subsequently transferred to NfuA type proteins. Thus, NfuA type proteins would be considered to serve as intermediate [Fe-S] cluster carriers or, perhaps, as [Fe-S] cluster reservoirs. Given that IscU appears to be required for the maturation of housekeeping [Fe-S] proteins in *A. vinelandii*, the participation of intermediate carriers is a reasonable possibility. Specifically, it is difficult to imagine that IscU would have the capacity to directly and

effectively interact with the large variety of cellular [Fe-S] proteins present in cells. This proposed lack of direct physiological delivery of [Fe-S] clusters from IscU to various client proteins is supported by the relatively poor capacity for cluster-loaded forms of IscU to effect *in vitro* [Fe-S] protein maturation. Nevertheless, the observation that NfuA is dispensable indicates that IscU can serve at some level as a primary [Fe-S] cluster donor to client proteins, or that IscU is able to deliver [Fe-S] clusters to a suite of different intermediate carriers, some of which have overlapping functions. We favor the latter possibility and suggest that a variety of proteins, recently shown to have the capacity to harbor [Fe-S] clusters, for example, Nfu (Tong *et al*., 2003, Nishio & Nakai, 2000, Leon *et al.,* 2003, Yabe *et al.*, 2004), IscA (Krebs *et al*., 2001, Ollagnier-De-Choudens *et al.*, 2004, Morimoto *et al.*, 2006, Abdel-Ghany *et al*., 2005), and glutaredoxins (Rouhier *et al.*, 2007, Lillig *et al.*, 2005, Rodriguez-Manzaneque *et al.*, 2002), could collectively serve as a physiological [Fe-S] cluster reservoir. This possibility is in line with the manifestation of a clear phenotype for NfuA and IscA (Johnson *et al*., 2006) in *A. vinelandii* under conditions of oxygen stress, a situation that could increase the demand for [Fe-S] clusters. Also, the fact that both the N-terminal and C-terminal domains within NifU are required to replace the function of NfuA, indicates the two domains have functional interdependence. Specifically, the observed phenotype is consistent with the possibility that [Fe-S] clusters assembled on the N-terminal (IscU-like) domain within NifU can be transferred to the C-terminal (NfuA-like) domain. The converse is unlikely to occur because maturation of nitrogenase does not require an intact NfuA-like domain within NifU.

Another interesting feature of NfuA is that it shares sequence identity with the IscA family of proteins, but this sequence conservation does not include cysteine residues that are conserved among the IscA family. This feature suggests that amino acid sequence conservation between the IscA family and NfuA could be related to target specificity, rather than [Fe-S] cluster assembly, and supports the possibility that IscA and NfuA could have some overlapping functions. Given the large number of [Fe-S] proteins within the cell, as well as the variety of potential environmental conditions a cell might encounter, a hierarchy of intermediate [Fe-S] cluster carriers that could control the distribution of [Fe-S] clusters would be a reasonable strategy for maximizing metabolic

capacity. There is already precedent for such a strategy in the case of *E. coli* because there are at least two different systems that can function for [Fe-S] protein maturation under different conditions. One of these, the ISC system, operates under standard laboratory conditions whereas the SUF system operates under conditions of Fe limitation or oxygen stress (Outten *et al.*, 2004). It should be noted that *A. vinelandii* does not encode a SUF system for [Fe-S] cluster assembly. Other cases of apparent specialized targeting of preassembled [Fe-S] clusters involve the role of ErpA, an IscA-like protein, necessary for the maturation of IspG (Loiseau *et al.*, 2007), and the roles of NifU and NifS in maturation of nitrogenase (Jacobson *et al.*, 1989b). Future studies will be aimed at testing the hypothesis that NfuA and the NfuA-like domain of NifU function as intermediate carriers of [Fe-S] clusters preformed on the respective IscU and IscU-like scaffolds.

A final important feature to emerge from this and other studies is the apparent organizational and physiological plasticity of [Fe-S] protein maturation systems from different organisms. This aspect is highlighted in Figure 5.1, which compares a variety of proteins having Nfu-like modules. It is particularly noteworthy that the cluster types that can be assembled on Nfu-like modules, as well as phenotypes associated with loss of Nfu-like function, are reported to be different for different organisms. For example, *in vitro* reconstitution of a chloroplastic Nfu-like protein from plants has been shown to result in formation of a [2Fe-2S] cluster and this protein is essential for maturation of chloroplastic [Fe-S] proteins (Yabe & Nakai, 2006, Abdel-Ghany *et al.*, 2005). Thus, it appears that, while Nfu-type modules have auxiliary or specialized functions in certain organisms, such as *A. vinelandii*, they have primary functions in [Fe-S] protein maturation in other organisms. Such variations in the [Fe-S] protein maturation process in different organisms are probably linked to physiological conditions, most significantly, intracellular redox conditions. Results similar to those reported here using *A. vinelandii* have also been found for the NfuA protein from *E. coli* (Angelini *et al.*, 2008a).

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CHAPTER 6. Conclusions

Iron-sulfur clusters are ubiquitous protein cofactors that facilitate vital biological processes. Their synthesis *in vitro* is relatively simple, but their biological assembly is complicated by the inherent toxicity of Fe and S in their free forms. Multiple proteins have been identified to play a role in [Fe-S] cluster biosynthesis although the exact mechanism by which this process is accomplished is unclear. IscU can assemble labile [2Fe-2S] and [4Fe-4S] clusters *in vitro* in the presence of excess Fe^{2+}, L-cysteine, and catalytic amounts of cysteine desulfurase. The observation led to the hypothesis that IscU serves as a scaffold upon which [Fe-S] clusters can be built and subsequently delivered to recipient apo-proteins. However, attempts to purify native IscU as well as recombinantly expressed from *E. coli* have resulted only in cluster-less forms of the protein. To determine whether the accommodation of [Fe-S] clusters by IscU *in vitro* represents a physiological process, it was necessary to test the scaffold hypothesis *in vivo*.

Two methods have been developed in this work that can be used to study the [Fe-S] cluster biosynthetic pathway *in vivo*. The first method exploits the genetic malleability of *A. vinelandii* to construct appropriate strains that allow isolation of IscU from its native host. The second strategy takes advantage of the convenience of recombinant protein technology to isolate IscU from an external vector that allows expression of the entire *isc* operon. Both methods uncouple expression of the *isc* operon from its physiological negative regulator IscR, thus allowing production of the ISC proteins at levels beyond the cellular requirements and increasing the chances of isolating [Fe-S] cluster-bound IscU. Also, all ISC proteins are expressed and their roles in the biosynthesis of [Fe-S] clusters can be examined by introducing various genetic modifications in the *isc* operon.

We have been able to isolate IscU bound to a labile $[2Fe\text{-}2S]^{2+}$ cluster *in vivo* thus validating the scaffold hypothesis. The cluster bound to IscU has similar characteristics to the one assembled *in vitro*. Despite the similarities, several differences between the *in vitro* and *in vivo* methods have emerged. First, recombinantly expressed IscU is isolated bound to a [Fe-S] cluster only when the *isc* operon is expressed at proportionate levels, because hyperexpression of IscU alone or IscU and IscS together results in cluster-less

forms of the protein. Second, $[2Fe\text{-}2S]^{2+}$-IscU isolated *in vivo* is predominantly monomeric, a result that is inconsistent with some studies which showed that recombinantly expressed IscU is a dimeric or trimeric species. This observation demonstrates that a single IscU polypeptide can accommodate a [2Fe-2S] cluster as opposed to two IscU subunits sharing a [2Fe-2S] cluster at their interface. Although [4Fe-4S] clusters were not detected in this work, small amounts of dimeric IscU species were observed supporting previous *in vitro* studies which demonstrated that [4Fe-4S] clusters are formed by the reductive coupling of two [2Fe-2S] clusters on a dimeric IscU molecule (Chandramouli, 2007, Agar et al., 2000a). Third, the $IscU^{39DA}$ variant is produced *in vivo* as a stable complex with IscS in contrast to trimeric forms of the protein that result from recombinant expression of IscU alone. Fourth, the association between IscU and IscS is non-covalent, contradicting previous cross-linking studies which demonstrated that the two molecules interact via disulfide bonds. All these differences affect our interpretation of the mechanism by which the ISC components interact to assemble and deliver clusters.

The studies described in this dissertation also provide several new observations that contribute to the understanding of biological [Fe-S] assembly. A complex between IscU and IscS forms *in vivo* and this association is transient indicating that the two proteins undergo complex formation and dissociation during different stages of [Fe-S] cluster assembly. Interactions between IscU and IscS do not appear to be affected by the chaperones HscA and HscB. IscU was isolated in complex with significant amounts of IscS, the sulfur donor, but no other protein that could serve as the iron donor was present at appreciable amounts. This observation may be a result of several possibilities: *i*) IscS has an additional function beyond its sulfur donation, *ii*) iron atoms are delivered by an agent too small to detect by the methods used in this work (such as glutathione), or *iii*) iron atoms are not donated directly to IscU, but rather Fe may become incorporated into free cysteine, which subsequently undergoes desulfurization and a S-Fe species is donated to IscU (Ding et al., 2005a). The finding that $[2Fe\text{-}2S]^{2+}$-IscU molecules were isolated alone whereas a major portion of apo-IscU species were isolated in complex with IscS leads to the hypothesis that apo-IscU may have a higher affinity for IscS than cluster-loaded IscU. It also suggests that IscU and IscS associate to form a [2Fe-2S]

cluster and dissociate upon its completion. In addition, the presence of unaccompanied $[2Fe\text{-}2S]^{2+}$-IscU supports the hypothesis that [4Fe-4S] clusters can be formed from two [2Fe-2S]-IscU monomers without the requirement of IscS. Formation of [4Fe-4S] clusters, therefore, is likely to occur by combining two [2Fe-2S] clusters rather than by the sequential addition of Fe and S atoms.

Another major observation to emerge from these studies is that both apo and [2Fe-2S] cluster-loaded IscU exist *in vivo* and the two are likely to have distinct conformations. Because the chaperones HscA and HscB have been implicated in cluster release, the effects of depleting the two proteins on the process of [Fe-S] cluster assembly were also examined. Depletion of the chaperones did not affect formation of [Fe-S] clusters – specifically [2Fe-2S] clusters – on IscU. Depletion of the chaperones also did not have an effect on the nature of the clusters bound to IscU indicating that $[2Fe\text{-}2S]^{2+}$-IscU exists in one conformation *in vivo* and any conformational changes are most likely exerted during or after cluster release. Also, conformational changes might be applied only when certain criteria are met such as the presence of a recipient protein. *In silico* experiments demonstrated that a loop of IscU containing the conserved LPPVK region, which is responsible for interactions with HscA, is flexible and could convert from a "closed" position that protects the active site to an "open" position that exposes it. Although the role of the chaperones as mediators of cluster transfer is supported by the results presented here and previous *in vitro* studies, other possibilities cannot be excluded. It is also plausible that the LPPVK loop opens to expose the cluster such that it is able to interact with another cluster to build a more complex structure such as a [4Fe-4S] cluster. In this scenario, energy-consuming structural rearrangements may be required to switch from a cluster that is ligated to four residues of one IscU polypeptide to a cluster that is shared by two polypeptides.

Cluster release from the scaffold protein must be followed by delivery to apo recipient proteins, an aspect that was studied in Chapter 5. It was demonstrated that NfuA, a protein that exhibits sequence similarity with the C-terminal domain of NifU, assembles [4Fe-4S] clusters and transfers them to apo-aconitase at higher rates compared to IscU. Thus, NfuA was proposed to act as a cluster transporter. NfuA is not required for viability during typical laboratory growth conditions but a null-growth phenotype is

associated with the deletion of each gene when cells are cultured at elevated concentrations of oxygen. [Fe-S] clusters are susceptible to oxidative damage at these conditions and the need for their biosynthesis is escalated, thus exacerbating the phenotype associated with impairment of NfuA. The results have led to a model in which IscU acts as a central scaffold and auxiliary proteins, such as NfuA, relay the nascent clusters to their appropriate destinations.

Taken together, the work described in this dissertation provided *in vivo* evidence that IscU serves as a scaffold for [Fe-S] clusters and resolved some of the controversial issues such as the oligomeric state of IscU and the nature of association between IscU and IscS. They have also provided insight into the role of the chaperones and the interplay between IscU and other ISC components during [Fe-S] assembly. Several *in vivo* intermediates in the [Fe-S] cluster biosynthetic pathway were isolated in this work including monomeric apo-IscU, dimeric apo-IscU, monomeric [2Fe-2S]-IscU, dimeric [2Fe-2S]-IscU, dimeric IscS, apo $\alpha_2\beta_2$ IscU-IscS complex, and [2Fe-2S] cluster-loaded $\alpha_2\beta_2$ IscU-IscS complex. *In vitro* work with NfuA demonstrated that NfuA exists as a dimeric and tetrameric species in its apo form but only as a dimer in its [4Fe-4S] cluster-loaded form. On the basis of current literature and findings obtained during this work the following model is proposed (Figure 6.1). Two monomeric apo-IscU molecules associate with homodimeric IscS to form an apo IscS-IscU complex and assemble a [2Fe-2S] cluster on each of the IscU molecules. Upon completion of the [2Fe-2S] cluster, the monomeric [2Fe-2S]-IscU dissociates from IscS and can follow two different paths. One route entails the delivery of the nascent cluster to an apo acceptor target protein, a process that is facilitated by the chaperones. Alternatively, [2Fe-2S]-IscU can be used for the formation of [4Fe-4S] clusters. Two monomeric [2Fe-2S] cluster-loaded IscU molecules can be reductively coupled to form a [4Fe-4S] cluster on a dimeric IscU molecule. This process may be facilitated mechanically and energetically by the chaperones as well as by ferredoxin or another electron source. The possibility of a collaborative role for ferredoxin and the chaperones *in vivo* is favored by the conserved polycistronic relationship of their genes that is often distinct from the *iscSUA* operon. Dimeric IscU containing a [4Fe-4S] cluster can transfer this cluster to a carrier protein such as NfuA which can efficiently relay it to an apo target protein. Cluster-less apo-IscU then dissociates to monomeric apo-IscU and

reforms a complex with IscS to continue the cycle. The methods developed in this work can be used in the future to study different aspects of [Fe-S] cluster assembly by introducing various genetic alterations.

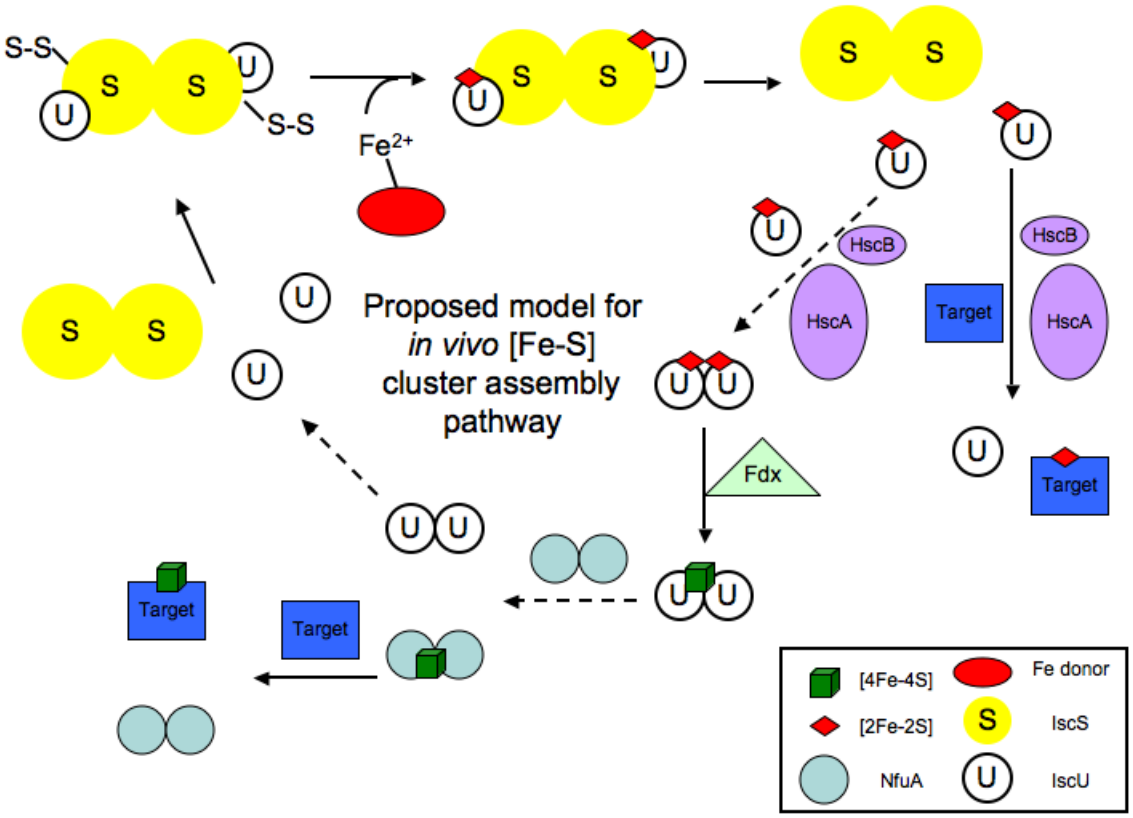

Figure 6.1. A proposed model for *in vivo* iron-sulfur cluster assembly. Starting from top left: an apo $\alpha_2\beta_2$ IscU/S complex detected *in vivo* can form a [2Fe-2S] cluster-containing complex also observed during *in vivo* studies. Upon completion of a $[2Fe\text{-}2S]^{2+}$ cluster on IscU, IscU and IscS dissociate into a dimeric cluster-less IscS species and a $[2Fe\text{-}2S]^{2+}$-IscU species both observed *in vivo*. It is then possible that the cluster formed on IscU is transferred to an apo-target protein with the help of the chaperones (from previous *in vitro* studies) or the chaperones may facilitate formation of a [4Fe-4S] cluster on IscU by exposing the [2Fe-2S]-IscU such that it can interact with another [2Fe-2S] IscU (dashed arrow) and undergo reductive coupling mediated by ferredoxin (shown *in vitro*). [4Fe-4S]-IscU may relay the nascent cluster to a carrier protein such as NfuA, which can efficiently transfer the nascent cluster to an apo target protein (observed *in vitro*). Dashed arrows represent processes for which there is no experimental evidence *in vitro* or *in vivo*.

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APPENDIX I. IscA assembles [Fe-S] clusters *in vivo*

A1.1 Introduction

The role of IscA in [Fe-S] cluster assembly is controversial. Several reports have provided evidence that supports a role for IscA as an iron delivery agent, whereas others demonstrate that IscA is able to accommodate both [2Fe-2S] and [4Fe-4S] clusters *in vitro* and transfer them to apo proteins, suggesting that IscA may serve as an auxiliary scaffold. IscA is strongly conserved as part of the *isc* operon suggesting a significant function for this protein *in vivo* in [Fe-S] cluster assembly. *A. vinelandii* and *E. coli* strains carrying *iscA* deletions do not exhibit any phenotypic differences from the wild type strains during typical growth conditions. However, these deletions have a small effect on the activities of [4Fe-4S] enzymes. A null-growth phenotype associated with inactivation of IscA was shown in *A. vinelandii* during conditions of oxidative stress. Like IscU, IscA is characterized by conserved cysteine residues that may serve as cluster or iron ligands. Substitution of each of conserved cysteine for an alanine results in a similar phenotype as inactivation of the entire protein demonstrating a significant role for these residues in the function of IscA. One of these cysteines is part of a conserved CXXS motif that is typically found in monothiol glutaredoxins. Substitution of the serine residue (Ser104) for a cysteine results in a dominant negative phenotype (Johnson DC, unpublished results). Because cysteine is a stronger cluster ligand than serine, the dominant negative phenotype may be due to the presence of trapped clusters on IscA. Alternatively, it may be due to the formation of inactive complexes between IscA subunits or IscA and other cellular components. In an attempt to understand the role of IscA in [Fe-S] cluster assembly, the protein was isolated *in vivo* using *Azotobacter vinelandii* as the host organism. The findings demonstrate that IscA accommodates [Fe-S] clusters *in vivo* supporting a role for IscA as an auxiliary or specialized [Fe-S] cluster scaffold, an [Fe-S] cluster carrier, or storage protein.

A1.2 Materials and Methods

The parent strain of DJ1737 is DJ1603, a strain in which both *iscR* and *iscU* carry in-frame deletions and does not grow in BN media containing 2% glucose (Johnson *et al.*, 2006). DJ1603 is incapable of growth in glucose but has a second copy of the *isc* operon under control of the sucrose promoter which allows is to grow in sucrose-containing media. DJ1737 was constructed by transforming DJ1603with pDB1647, a plasmid in which a hexahistidine cartridge has been inserted at the N-terminus of IscA and Ser104 has been substituted to an alanine. Transformants were selected for their ability to grow on glucose. DJ1737 does not grow in sucrose at elevated oxygen conditions (40% O_2). DJ1754 was constructed by transforming DJ1737 with pDB1640, a plasmid that is isogenic to pDB1647 but lacks the Ser104Cys substitution. Transformants were selected on the basis of their ability to grow on sucrose-containing media at elevated O_2 concentrations. Genomic DNA from each strain was extracted as described previously (Johnson et al., 2006), and the histidine tag and amino acid substitution were confirmed by DNA sequencing of the relevant PCR-amplified DNA regions.

Purification of IscA or IscA^{104SC} was performed in an anaerobic glove box. Approximately 100 g DJ1737 or DJ1754 cells were resuspended in 150 ml of 50 mM Tris, pH 8.0 degassed buffer containing 0.5 M NaCl (buffer A) and lysed using a French Pressure Cell at 12,000 psi or a NanoDeBee homogenizer. Cell extracts were obtained by centrifuging the lysate in an ultracentrifuge at 235,000 *g* for 1 hour. A 3-ml column packed with iminodiacetic acid resin (Affiland) was charged with 4-5 bed volumes of 50 mM $NiSO_4$ followed by 4-5 bed volumes of buffer A. The cell extracts were loaded onto the equilibrated column and washed with buffer A followed by buffer A containing 50 mM imidazole. IscA or IscA^{104SC} was eluted by reverse flow with buffer A containing 200 mM imidazole. UV-visible absorption spectroscopy, protein concentration, and iron and sulfide were measured as described previously (Raulfs *et al.*, 2008).

A1.3 Results and Discussion

Two strains were constructed that allow isolation of IscA (DJ1754) and IscA^{104SC} (DJ1737) *in vivo*. Both strains carry an in-frame deletion in *iscR*, the gene that encodes

the negative regulator of the *isc* operon. This allows elevated expression of the ISC proteins at levels beyond the physiological requirement, thus increasing the chances of isolating IscA and its variant bound to an iron-sulfur cluster. Both strains carry a poly-histidine tag at the N-terminus of IscA to allow efficient isolation of the protein. Insertion of the tag does not interfere with the function of the protein judging from the ability of DJ1754 to grow at air and elevated oxygen levels. In addition, in both strains, a second copy of the *isc* operon is controlled by the inducible sucrose regulatory elements. The latter allows the identification of any null-growth phenotypes that may be associated with genetic alterations introduced in the endogenous *isc* copy. DJ1737 differs from DJ1754 in that it produces the $IscA^{104SC}$ variant and it exhibits a dominant negative phenotype. DJ1737 also grows slightly slower that DJ1754 at air O_2 levels, indicating that the dominant negative phenotype associated with the Ser104Cys substitution is exacerbated with inactivation of IscR (Figure A1.1).

DJ1754 and DJ1737 cells were lysed using a French Pressure Cell and IscA was purified using immobilized metal affinity chromatography. UV-visible absorption spectroscopy exhibited broad peaks at 320 nm and 415 nm for wild type IscA and 320 nm, 415 nm, and 456 nm for $IscA^{104SC}$ (Figure A1.2). These values are consistent with the presence of [Fe-S] clusters, and although their precise composition could not be established, the spectra resembled those of [2Fe-2S] clusters assembled on IscA *in vitro* (Krebs *et al.*, 2001, Morimoto *et al.*, 2006). Iron analysis revealed 0.5 Fe per $IscA^{104SC}$ dimer, whereas no iron was detected in the wild type IscA fraction. When cells were lysed using a NanoDeBee homogenizer, the IscA-containing fractions had a yellow-green shade suggestive of the presence of [4Fe-4S] clusters (Khoroshilova *et al.*, 1997). The UV-visible absorption spectra of those samples also exhibited peaks at 320 nm and 410 nm. During three separate purifications a protein band similar in size as IscU was present in the $IscA^{104SC}$-containing fractions. A western blot exhibited a band the size of IscU although the overall blot was very noisy. According to the western analysis, lower levels of IscU were also present in wild type IscA from DJ1754. An inactive interaction between $IscA^{104SC}$ and IscU may explain the dominant negative effect exerted by the Ser104Cys substitution. The results demonstrate that both IscA and its Ser104Cys variant assemble iron-sulfur clusters *in vivo*, a finding that is inconsistent with a role for IscA as

an iron donor and supports a role for IscA as a specialized scaffold, cluster carrier or storage protein. They also provide evidence that IscU and IscA interact in vivo, a result that was corroborated in Chapter 4. The purpose of this interaction remains to be established.

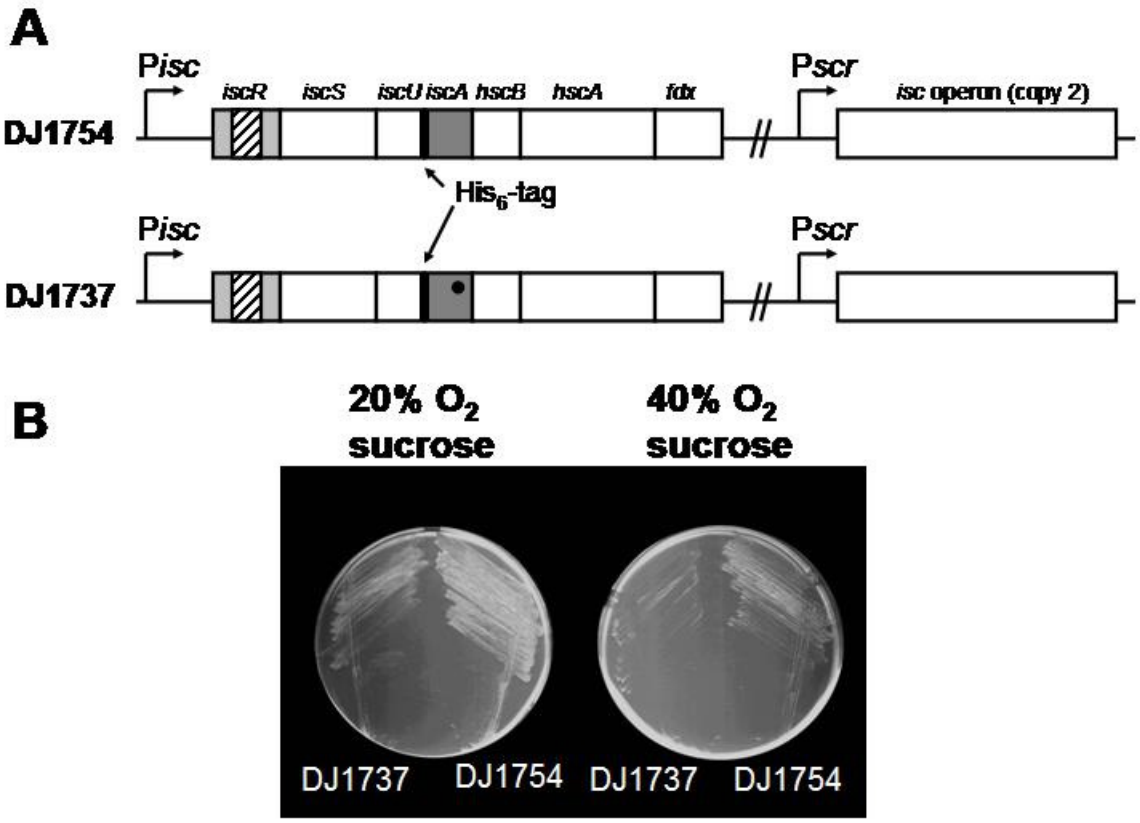

Figure A1.1. Strains constructed for this study. A) Diagrammatic representation of DJ1737 and DJ1754. Rectangle with slanted lines represents in-frame deletion in *iscR*. Black rectangle represents a hexa-histidine tag inserted at the N-terminus of IscA. Black dot represents Ser104Cys substitution in IscA. B) DJ1737 and DJ154 cultured in Burk's medium containing 2% sucrose and 13 mM ammonium acetate at 20% and 40% ambient O_2 concentrations.

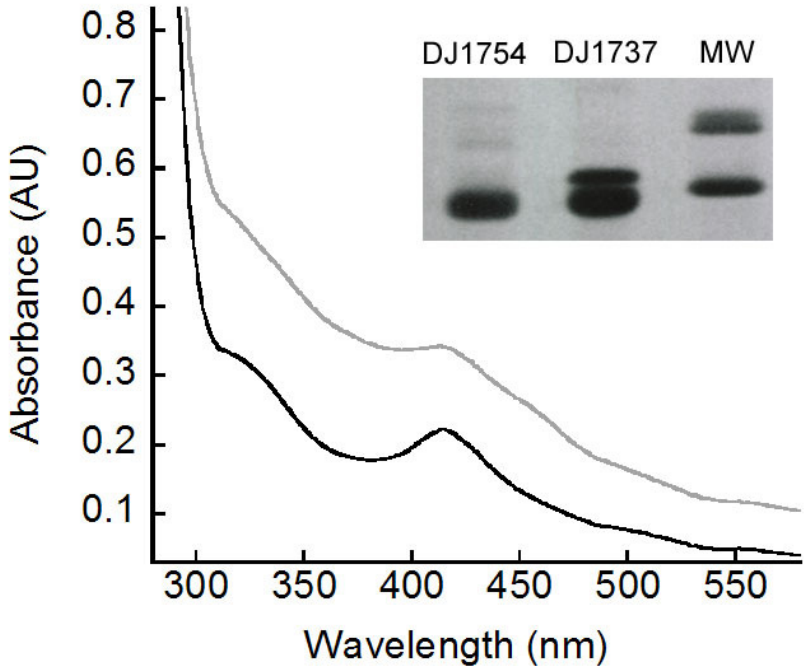

Figure A1.2. UV-visible absorption spectra and SDS-PAGE of purified wild type IscA from DJ1754 (black) and IscA^{104SC} from DJ1737 (gray). Molecular weight lane consists of soybean trypsin inhibitor (21,500), and lysozyme (14,400).

APPENDIX II. Benzoate-1,2-dioxygenase and FeSII proteins as models for monitoring formation of [2Fe-2S] clusters

All cluster transfer experiments from IscU acceptor proteins have utilized [4Fe-4S] proteins such as aconitase and biotin synthase or the [2Fe-2S] ferredoxin of the ISC system. The latter is a convenient choice for monitoring transfer of [2Fe-2S] clusters but transfer kinetics may be different for this particular ferredoxin since it is proposed to be involved in [Fe-S] cluster assembly. This appendix describes the purification and characterization of three proteins that can be used as models for monitoring maturation of [2Fe-2S] proteins *in vitro*. The first two protein systems, XylXY and XylZ, comprise the benzoate dioxygenase complex which catalyzes utilization of benzoate and the third protein, FeSII, is a component involved in protection of *A. vinelandii* nitrogenase.

Benzoate-1,2-dioxygenase catalyzes the first step of the degradation of benzoate by hydroxylating the latter using one molecule of dioxygen and NAD(P)H as an electron source. Two protein components constitute the benzoate dioxygenase system: an oxygenase component (XylXY), which is responsible for the oxygenation of benzoate, and a reductase component (XylZ), which oxidizes NAD(P)H and transfers the resulting electrons to the oxygenase component. The reductase component is a flavoprotein containing FAD as a cofactor as well as a [2Fe-2S] cluster. The oxygenase component has an $\alpha_3\beta_3$ subunit structure and includes a [2Fe-2S] Rieske cluster and a mononuclear iron site.

In order to study the *in vivo* effects of the ISC system on benzoate dioxygenase, a method was developed that allows the isolation of the two protein components from *A. vinelandii* cells. A relatively rapid and effective approach was to place a histidine tag at the N-terminus of the gene of each protein component. In order to provide selection criteria for the his-tagged strains, kanamycin interrupted strains were constructed that were unable to grow on benzoate as a sole carbon source. These strains were transformed with plasmids containing histidine tags at the N-termini of XylX (DJ1613) and XylZ (DJ1638), and plated on benzoate as a sole carbon source. DJ1613 was confirmed by PCR and subsequent sequencing.

DJ1613 and DJ1638 were used to isolate XylXY and XylZ respectively from *A. vinelandii* cells. Passage of the his-tagged XylXY through an IMAC column resulted in a pure form of the protein, although very dilute (< 1mg/ml). Attempts to concentrate the protein by ultrafiltration were unsuccessful because the protein would bind to the filter membrane of the concentrator. Nevertheless, the protein was purified containing an [Fe-S] cluster with UV-visible absorption spectrum similar to the one published for benzoate dioxygenase, and at similar cluster occupancy: $A_{325/280} = 0.11$ compared to the published $A_{325/280} = 0.12$ (Figure A2.1) (Yamaguchi & Fujisawa, 1980).

Purification of the reductase component using the histidine-tagged strain also produced a relatively pure but dilute fraction. No attempts have been made so far to produce the protein at a larger scale or concentrate it. Recombinantly expressed XylZ in *E.coli*, however , resulted in a pure form of the reductase, but of the protein remained in the pellet of the lysed cells. Attempts to increase the solubility of the protein by growing the cells at lower temperatures, including 30°C, 22°C and 4°C were unsuccessful.

FeSII, also known as the Shethna II protein, has been implicated in serving a role in protection of nitrogenase from oxidative damage. FeSII contains a [2Fe-2S] cluster that is stable when exposed to air (Moshiri *et al.*, 1995). The following section describes the purification of FeSII protein and preparations of its apo form. A comparative analysis was performed between the UV-visible absorption and circular dichroism spectra of FeSII and IscU that can be exploited to monitor *in vitro* cluster transfer between the two proteins.

Plasmid pDB1762 was constructed that can be used to drive the T7-based expression of polyhistidine-tagged FeSII. *E. coli* BL21(DE3) cells were transformed with pDB1762 and grown in LB media containing 100 μg/ml ampicillin. Cells were induced with 1% (w/v) lactose at $OD_{600} = 0.5$ for 6 hours and collected by centrifugation. The day of purification, approximately 60 g of cells were resuspended in 150 ml of 50 mM Tris, pH 8.0 buffer containing 0.5 M NaCl (buffer A) and lysed using a NanoDeBee homogenizer at 27,000 psi (passed twice). PMSF (0.5 mM), pestatin A (0.14 mg/L), and DNase were added to limit protein degradation. Dark red cell extracts were loaded onto a 40-ml IMAC column previously equilibrated with 4-5 bed volumes of 50 mM $NiSO_4$ and buffer

A. After washing the loaded column with 60 ml of buffer A and 75 ml of buffer A containing 20 mM imidazole, the protein was eluted with a 300-ml 20-500 mM imidazole gradient. Approximately 230 mg of pure protein were obtained using this one-step procedure. His-FeSII was isolated at full occupancy as judged by its UV-visible absorption spectrum.

To prepare apo-FeSII protein, holo-FeSII was incubated with EDTA and $K_3Fe(CN)_6$ at a molar ratio of 1:75:30 (FeSII:EDTA: $K_3Fe(CN)_6$) for 4 hours after which it was dialyzed overnight in 50 mM Tris, pH 8.0 buffer. Dithiothreitol (1 mM) was added to the dialyzed protein, which was repurified using a DEAE-sepharose column, previously equilibrated with 50 mM Tris, pH 8.0, 1 mM DTT, to remove excess chelating agents. Apo-FeSII was eluted from the anion-exchange column with 500 mM NaCl. The protein-containing fractions were subsequently concentrated by ultrafiltration using a YM10 membrane and 50 mM Tris, pH 8.0, 1 mM DTT to reduce the level of NaCl in the sample. A portion of the protein is lost during this step.

Apo-FeSII has no distict UV-visible absorption or circular dichroism spectroscopic features (data not shown). [2Fe-2S]-FeSII on the other hand has distinct spectroscopic features that differ from [2Fe-2S]-IscU (Figure A2.2). These differences can be used to monitor cluster transfer from [2Fe-2S]-IscU to apo-FeSII spectroscopically. Also, apo-FeSII and holo-FeSII have distinct electrophoretic mobilities in a native gel, which can be exploited to monitor maturation of the protein as well (data not shown).

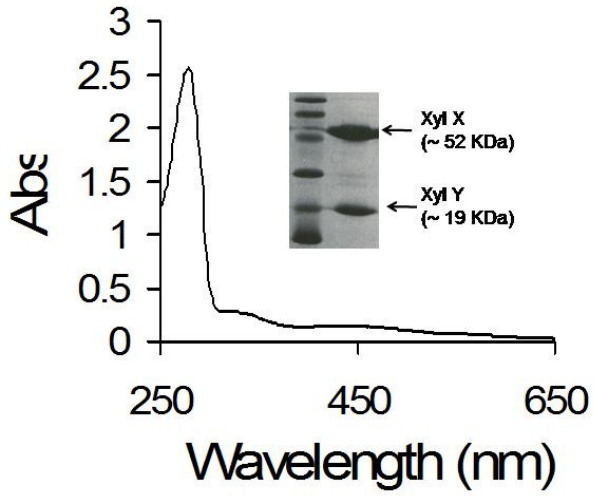

Figure A2.1. UV-visible absorption spectrum and SDS-PAGE of polyhistidine-tagged XylXY DEAE fraction.

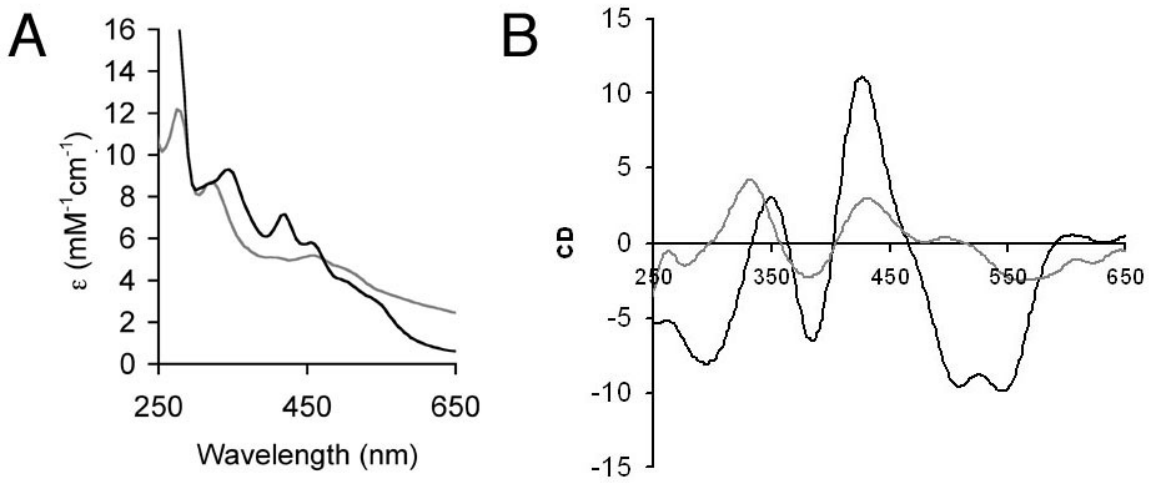

Figure A2.2. UV-visible absorption (A) and circular dichroism (B) spectra of [2Fe-2S]-IscU (gray) and [2Fe-2S]-FeSII (black). Circular dichroism spectra were measured at 30 μM as determined by BioRad assay.

APPENDIX III. Depletion of the ISC ferredoxin hampers proper assembly of [Fe-S] clusters on IscU

The role of ferredoxin (Fdx) in [Fe-S] cluster assembly is unknown. Although it has been purified containing a [2Fe-2S] cluster, it has also been proposed to be required for the assembly of [Fe-S] clusters on IscU. Specifically, Fdx has been proposed to provide electrons for the reduction of sulfane (S^{o}) to sulfide (S^{2-}) or the reduction of ferric (Fe^{3+}) ions to ferrous (Fe^{2+}). It has also been demonstrated *in vitro* to provide electrons for the reductive coupling of two [2Fe-2S] clusters to form a [4Fe-4S] cluster on IscU (Chandramouli, 2007). Johnson *et al*. showed that Fdx is required for cellular viability and maturation of the [4Fe-4S] enzyme aconitase (Johnson et al., 2006).

To understand the role of ferredoxin in [Fe-S] cluster assembly, an *A. vinelandii* strain was constructed (DJ1740) that allows isolation of IscU from a genetic background where the *fdx* gene is deleted. DJ1740 carries an in-frame deletion in *iscR* and endogenous *fdx* copy. It also carries a polyhistidine tag at the C-terminus of IscU and a second copy of the *isc* operon under control of the sucrose promoter. Cells were depleted as described in Chapter 4 for DJ1788 and IscU was purified as described in Chapter 3 for IscU from DJ1697. Depleted DJ1740 cells had a pale brown/pink color compared to DJ1697 cells which typically have a dark brown color.

When IscU was purified from depleted DJ1740 cells, a black precipitate formed on the IMAC column. Passage of 200 mM imidazole through the column, a concentration that is sufficient to elute IscU from the column, resulted in a pale brown/pink fraction that exhibited UV-visible absorption peaks at 325 nm, 405 nm, 455 nm, and 515 nm. This fraction contained IscU and IscS indicating that the absence of Fdx does not affect complex formation between the two proteins. When the IMAC fraction was loaded onto the anion-exchange column and a salt gradient was applied, it resolved into apo-IscU, $[2Fe\text{-}2S]^{2+}$-IscU, and IscS-containing fractions as was observed for DJ1697. The yield of the cluster-loaded IscU anion-exchange fraction was 3 times smaller compared to the yield obtained during the purifications from wild type DJ1697 cells. A negligible IscU yield was also obtained when IscU or IscU variants were purified from strains that were

unable to form clusters on IscU (including DJ1752, DJ1764, and DJ1807 which produce $IscU^{63CA}$, $IscU^{106CA}$, and $IscS^{328CA}$ respectively). The findings suggest that Fdx is either required for the formation of [Fe-S] clusters on IscU or the cluster-loaded IscU species formed in the absence of Fdx is unstable and is degraded. Formation of the black precipitate is unique because it was not observed during purifications of the strains DJ1752, DJ1764, and DJ1807. Also, small but appreciable amounts of cluster-loaded IscU were isolated from DJ1740 (8 μg/g cell paste) compared to the negligible levels observed from DJ1752, DJ1764, and DJ1807 cells. This does not favor the hypothesis that Fdx is involved in cluster formation. Contradictory to this hypothesis is also the fact that Fdx is a [2Fe-2S] protein itself. Precipitation into a black solid is typical for the formation of some metal sulfides. If the black precipitate observed on the IMAC column are due to FeS formation, then it could be hypothesized that [Fe-S] clusters are formed but are unstable and become degraded. Based on the results obtained so far, a new function for Fdx is proposed. It has been demonstrated that reduced $[2Fe\text{-}2S]^{1+}$ clusters assembled on IscU are extremely unstable and become degraded. It is, therefore, possible that Fdx is involved in converting a newly synthesized $[2Fe\text{-}2S]^{1+}$ cluster to the oxidized $[2Fe\text{-}2S]^{2+}$ form that is more stable and has been isolated in vivo. When Fdx is absent, the reduced $[2Fe\text{-}2S]^{1+}$ cluster is degraded into FeS precipitate observed on the column.

Since the IMAC column is charged with Ni^{2+}, it is also possible that the black precipitants observed on the column are due to NiS formation. If the latter is true, it may favor the hypothesis that Fdx is involved in reducing Fe^{3+} to Fe^{2+} and in its absence the sulfides produced by IscS tend to bind to the Ni^{2+} present on the column. To differentiate between NiS and FeS, an elemental analysis of the column contents should be performed or the purification should be conducted on a Zn^{2+}-charged column because ZnS forms a white precipitate.

IscU in the absence of Fdx has not been isolated recombinantly from *E. coli* using the method described in Chapter 4, but a plasmid has been constructed (pDB1749) that is isogenic to pDB1720 with the exception that an in-frame deletion in *fdx* has been introduced. For most relevant results, pDB1749 should be transformed with *E. coli* cells that lack their endogenous *fdx* copy, or the entire *isc* operon.

APPENDIX IV. *A. vinelandii* produces two NADPH-dependent ferredoxins reductase paralogs, one of which is required for viability

This appendix describes studies related to two *A. vinelandii* paralogs of NADPH-dependent ferredoxins reductases Fpr1 and Fpr2. Most of these experiments were performed with the help of two undergraduate students: Deise Galan and Kyle Zingaro, whom I trained and mentored throughout their research experience in the laboratory.

A4.1 Introduction

NADPH:ferredoxin reductase, designated here Fpr1, is a 29 kDa homodimer originally observed in a FdI$^-$ *A. vinelandii* strain where Fpr1 was upregulated under nitrogen fixing conditions (Isas *et al.*, 1995). Although *fpr1* is not preceded by *nif* expression elements it may have a role in providing electrons for NifF (Bittel *et al.*, 2003), a flavodoxin known to provide electrons to nitrogenase (Deistung & Thorneley, 1986). The crystal structure of Fpr has been solved and it contains an NADPH binding domain and an FAD binding domain (Sridhar Prasad *et al.*, 1998). Fpr1 mediates electron flow between $NADP^+$/NADPH and a ferredoxin/flavodoxin, although the direction of electron flow has not been clearly established due to small differences in reduction potentials that may change during physiological conditions (Isas & Burgess, 1994). FdI is believed to be the physiological electron partner of Fpr, based on cross-linking experiments performed by Jung *et al.*, who showed that Fpr1 and FdI form a specific 1:1 complex through a salt bridge between Lys^{258} of Fpr1 and Asp/Glu residues of FdI (Jung *et al.*, 1999b). The $[3Fe\text{-}4S]^+$ cluster of FdI is specifically reduced by NADPH via Fpr1 (Jung *et al.*, 1999b).

Fpr1 has also been implicated to have a role in oxidative stress. In *Pseudomonas putida,* the Fpr1 protein and mRNA levels were shown to be elevated in the presence of paraquat *in vivo* (Lee *et al.*, 2006). A *P. putida* strain deleted for *fpr* exhibits a null-

growth phenotype in the presence of paraquat. *E. coli* Fpr was also demonstrated to be induced in the presence of methyl viologen (Liochev *et al.*, 1994).

In this work, two Fpr paralogs, which have been designated *fpr1* and *fpr2,* have been identified and studied. Both genes were inactivated and Fpr1 was found to be essential for viability whereas inactivation of Fpr2 had no distinct phenotype. Fpr1 was cloned, expressed, purified, and characterized.

A4.2 Methods

Plasmids pDB1709 and pDB1721 were constructed by inserting kanamycin and streptomycin resistance cassettes in *fpr1* and *fpr2* respectively. Plasmid pDB1703, which allows expression of *fpr* by the inducible sucrose regulatory elements of (P*scr*), was constructed. This was used to transform DJ1418 cells (Johnson *et al.*, 2006) to yield DJ1802, which carries two copies of *fpr1*, one under control of its endogenous promoter and the other under control of the inducible sucrose regulatory elements. DJ1802 was transformed with pDB1721 to produce the strain DJ1804, which has the endogenous copy of *fpr1* inactivated by a kanamycin resistance cassette (confirmed by PCR). Amino acid sequence identities were determined by aligning the sequences using the SDSC Biology Workbench program.

To purify Fpr1 a protocol adapted from (Isas & Burgess, 1994) was followed. BL21(DE3) cells were transformed with pDB1653, a plasmid that contains *fpr1* flanked by NdeI and BamHI sites under control of the T7 promoter. Transformants were grown in LB media containing containing 100 μg/ml ampicillin. Cells were induced with 1% (w/v) lactose at OD_{600} = 0.5 for 4 hours and collected by centrifugation. The day of purification, approximately 40 g of cells were resuspended in 80 ml of 25 mM Tris, pH 7.4 buffer (buffer A) and lysed using a French Pressure cell at 12,000 psi. PMSF (0.5 mM), pestatin A (0.14 mg/L), and DNase were added to limit protein degradation. The cell lysate was separated by centrifugation (235,000 *g* for 1 hour) and the resulting supernatant was treated with 40% (w/v) ammonium sulfate. The sample was centrifuged again (35,000 rpm for 30 min) and this supernatant was treated with 60% (w/v) ammonium sulfate. After one more spin at 235,000 *g* for 30 min, the resulting

supernatant was discarded and the pellet was resuspended in 200 ml buffer A. The solution was loaded onto a 23-ml Q-sepharose anion-exchange column that had been equilibrated with 150 ml buffer A. The loaded column was washed with 100 mM NaCl followed by elution of the protein with 200 mM NaCl.

A4.3 Results and Discussion

Sequencing of the *A. vinelandii* genome revealed the presence of two paralogs of the NADPH-ferredoxin reductase Fpr, which have been designated Fpr1 and Fpr2. Fpr1 and Fpr2 exhibit 41% sequence identity to each other. *E. coli* Fpr exhibits 42.5% identity to *A. vinelandii* Fpr2 and 33% identity to Fpr1. From the phylogenetic tree shown in Figure A4.1, it can be deduced that Fpr1 is more similar to NADPH:ferredoxin reductase from the *Pseudomonas* species and Fpr2 resembles the one found in *E. coli*. The apparent divergence may suggest functional differences or substrate specificity. Strains were constructed to inactivate each gene in *A. vinelandii*. DJ1806, in which *fpr2* is interrupted by insertion of a streptomycin resistance cartridge, does not elicit a phenotype when grown in the presence or absence of a fixed nitrogen source, nor when cells are exposed to elevated O_2 (40%) during diazotrophic or non-diazotrophic conditions. In contrast, attempts to inactivate *fpr1* by insertion of a kanamycin resistance cartridge resulted in loss of antibiotic resistance after a few generations in the absence of antibiotic pressure, leading to the hypothesis that Fpr1 is essential for cell survival (data not shown). To establish whether *fpr1* is required for cell growth, a strain was constructed in which the endogenous copy of *fpr1* is inactivated, whereas a second, intact copy is placed under control of the inducible sucrose regulatory elements (DJ1804). This strain grows only when the second, intact copy of Fpr1 is produced, namely when sucrose is present in the growth media. The null-growth phenotype associated with Fpr1 is rescued when DJ1804 is cultured in an environment where ambient O_2 concentrations are lowered to 2%, indicating that Fpr1 is not needed at low O_2 concentrations (Figure A4.2).

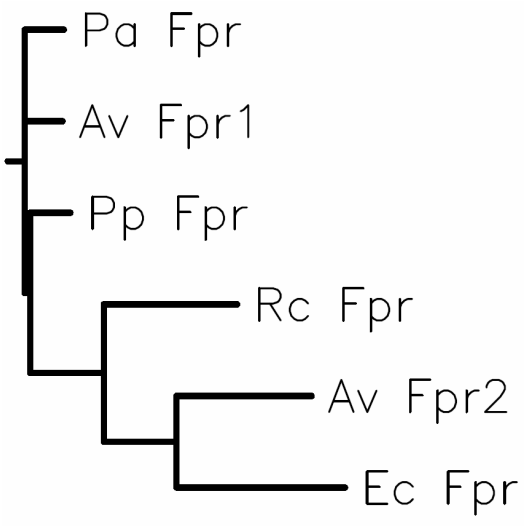

Figure A4.1. Phylogenetic tree showing the relationship between *A. vinelandii* Fpr1 and Fpr2 and the studied Fpr proteins from other organisms. Pa, Av, Pp, Rc, and Ec stand for *Pseudomonas aeruginosa, Azotobacter vinelandii, Pseudomonas putida, Rhodobacter capsulatus,* and *Escherichia coli* respectively.

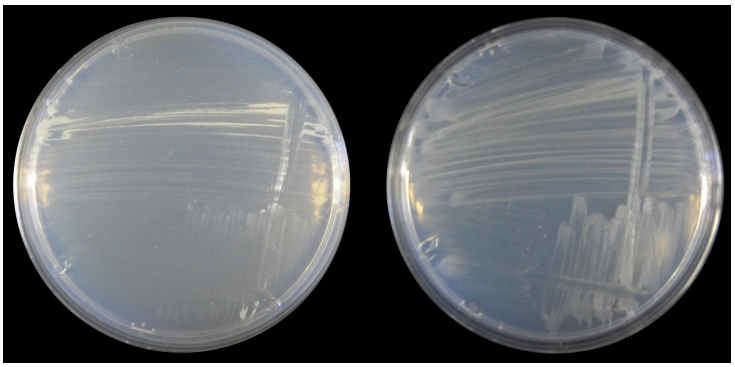

Figure A4.2. Null-growth phenotype associated with inactivation of *fpr1* is rescued at low ambient O_2 concentrations. DJ1804 grown in the absence of sucrose at 20% O_2 (left) and 2% O_2 (right).

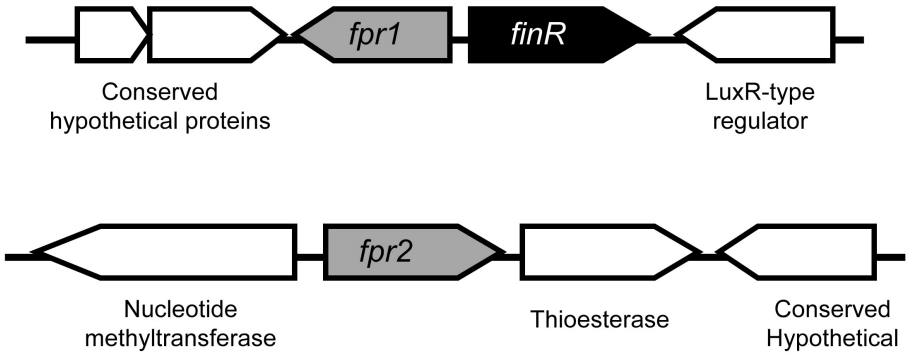

Figure A4.3. *A. vinelandii* genes *fpr1* and *fpr2* have distinct genomic contexts. Arrows represent genes and arrow direction represents transcriptional and translational direction.

The results demonstrate that *fpr1* is required for cellular viability and, thus, has an essential function in *A. vinelandii*. This is the first study to demonstrate that Fpr is required for viability. A yeast NADPH-dependent adrenodoxin reductase has also been shown to be essential, but this protein shares very little sequence identity with *A. vinelandii* Fpr1 (14%), which suggests a low degree of functional conservation. It is interesting that inactivation of *fpr* in *Azotobacter vinelandii* results in a null-growth phenotype but exhibits only sensitivity to paraquat in others (Liochev et al., 1994, Bittel et al., 2003, Lee et al., 2006). It should be noted that, in contrast to the other organisms in which *fpr* has been studied, *A. vinelandii* is an obligate aerobe. This may be an indication that Fpr1 plays a role in respiration or repair during oxidative stress. Supportive of either hypothesis is the finding that the null-growth phenotype associated with DJ1804 is rescued when ambient O_2 levels are lowered to 2% (Figure A4.2) and previous studies demonstrating that Fpr1 is upregulated in the presence of paraquat. Inactivation of the Fpr1 paralog, Fpr2, did not result in a distinct phenotype under the conditions in which it was cultured suggesting either that this copy is not functional, has a redundant role, or functions under specific growth conditions that were not tested in this work.

The regulation of *fpr1* in *A. vinelandii* is unclear. It has been demonstrated that Fpr1 is upregulated in a FdI- strain (Isas & Burgess, 1994), but FdI does not bind to the DNA region upstream of *fpr1,* so regulation by FdI is mediated via other means (Regnstrom *et al.*, 1999). In *E. coli*, *fpr* is controlled by the SoxRS system, which directs expression of genes in response to oxidative stress (Liochev *et al.*, 1994). The region upstream of *fpr* is 50% identical to the SoxS binding site upstream of the *E. coli fpr.* However, there is no SoxS homolog in *A. vinelandii*. In this work, the *fpr1* and *fpr2* genomic contexts were explored revealing that the two genes have distinct genomic context. A LysR-type regulator is located next to the *A. vinelandii fpr1,* which exhibits 82% sequence identity with the *P. putida* FinR. In *P. putida*, FinR is also located next *fpr*, which is 85% identical to *A. vinelandii fpr1*, and has been demonstrated to activate its induction (Figure A4.3). This suggests that *A. vinelandii fpr1* may also be subject to regulation by FinR.

A. vinelandii Fpr1 was cloned, recombinantly expressed in *E. coli*, purified, and characterized spectroscopically. Plasmid pDB1653 was constructed to allow T_7-driven expression of Fpr1. Purified Fpr1 was bright yellow, a color characteristic for oxidized FAD, and its UV-visible absorption spectrum was identical to the one published previously (Isas & Burgess, 1994).

APPENDIX V. Annotation of the *A. vinelandii* genome

This appendix describes my contribution to a project in which the genome of *Azotobacter vinelandii* was sequenced and its genes were automatically and manually annotated. A total of 132 genes were manually "curated", 7 of which were pseudogenes and, therefore, deleted. In addition, I trained three undergraduate students: Kyle Zingaro, Charles Bressan, and Deise Galan and actively helped them annotate at least 51 more genes. Overall, the genes annotated by my group comprise 3.6% of all *A. vinelandii* genes. I initiated and performed a significant portion of the annotation and studies related to Mo and Fe transport, which were continued by Luis Rubio, Jose Hernandez, and Leonardo Curatti from University of California, at Berkeley. Moreover, I was involved in studying the respiratory genes, which were annotated by Lauren Ligon and Dr. Patricia Dos Santos. Specifically, I compared the *A. vinelandii* respiratory genes with other pseudomonads to determine any genomic features that allow *A. vinelandii* to be an obligate aerobe while maintaining its ability to fix nitrogen. Finally, I was also involved in preparations of the manuscript that is planned to be submitted for publication.

Manual annotation of the genome entailed the functional assessment of various proteins. For each gene that was annotated, the translational elements of its protein product were carefully investigated and the start site was modified if necessary. A BLAST search was performed to assess the consistency of the assigned function in the homologous proteins. This was followed by a literature search to determine whether the function of the protein of interest had been experimentally demonstrated in *A. vinelandii*. If no reports were found in *A. vinelandii,* the literature search was expanded to determine whether the function had been experimentally demonstrated in other organisms. If the latter were true, the sequence of the protein from the organism in which the function was assessed was aligned with the sequence of the *A. vinelandii* protein. If the two proteins shared 30% or higher amino acid sequence identity, the *A. vinelandii* protein was annotated to have the same function as the protein, for which a report was available. If the investigated protein did not show significant sequence identity with any proteins for

which the function was experimentally demonstrated, its function was assigned based on the presence of conserved domains and protein motifs. If a function could not be assessed using the latter method, the protein was annotated as a conserved or non-conserved hypothetical protein. If a gene was too small to produce a protein, lacked translational elements such as a ribosome-binding site, and/or had no homologs, it was established to be a pseudogene and removed from the database.

Several biological processes were investigated in this work including nitrogen fixation, molybdenum and iron transport, and respiration. In conjunction with the work performed by Dr. Patricia Dos Santos, we found that the nitrogen fixing genes are separated in two regions, one on each side of the origin of replication. The small distance between each *nif* region and the origin of replication may explain the high levels of nitrogenase proteins produced during nitrogen fixation.

An investigation of the Mo transport elements in *A. vinelandii* revealed genes related to Mo transport are relatively near the nitrogen-fixing genes suggesting co-evolution. The well-characterized *modEABCG* operon lies ~35 kb upstream of the minor *nif* region suggesting a possible co-transfer of the two regions. Interestingly, an additional set of genes was identified ~10 kb upstream of the *major* region. It is possible that the second *mod* region co-evolved with the minor *nif* region, which raises the question whether the *nif* genes joined *A. vinelandii* at different times during its evolution. Moreover, additional putative Mo transporters are scattered near or as part of the *nif* operons (Avin50730 and Avin01580).

Annotation of the Fe transport-related proteins revealed an extraordinarily high number of TonB/ExbB/ExbD systems and TonB-dependent siderophore receptors. TonB proteins work within the periplasmic space to transfer Fe^{+3}-loaded siderophores from the outer membrane TonB-dependent siderophore receptors to the ExbB and ExbD cytoplasmic membrane proteins, which utilize a proton motive force to actively transport siderophores into the cytoplasm (Moeck & Coulton, 1998). *E. coli* possesses one set of TonB/ExbB/ExbD proteins and some Gram-negative bacteria have more than one set but not all are functional (Abbas *et al.*, 2007, Benevides-Matos *et al.*, 2008). In contrast to *E. coli and P. aeruginosa*, which produce one and three TonB systems respectively, six TonB and TonB-like systems were found to be present in *A. vinelandii* (Figure A5.1).

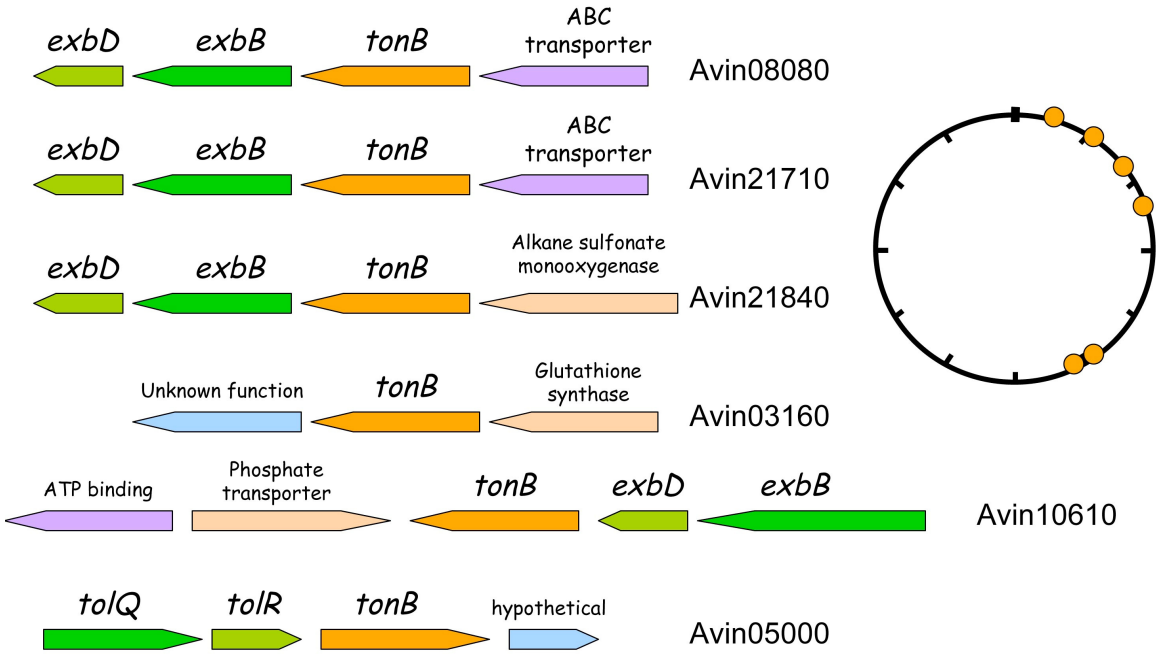

Figure A5.1. *A. vinelandii* encodes 6 putative TonB/ExbB/ExbD complexes. Genes are color-coded based on homology except for light orange and blue genes. Circle indicates location of *tonB* genes within the chromosome in relation to the origin of replication (black rectangle on top of the circle).

This is the highest number of TonB systems found in an organism. Although it is possible that not all TonB copies function in iron transport, it is a very interesting observation. In addition, more than 30 TonB-dependent siderophore receptors were identified. These could be classified into 4 categories based on their phylogenetic relationship and their amino acid sequence similarities to the studied FepA, FecA, FhuA, and FpvA siderophore receptors. It is possible that *A. vinelandii* employs more Fe-uptake systems to supply iron for the biosynthesis of nitrogenase cofactor molecules. Considering that nitrogenase makes up ~10% of total protein produced in *A. vinelandii* during nitrogen-fixing conditions (Dingler *et al.*, 1988), the need for iron is unusually high. This hypothesis would also be consistent with the redundancy observed for the Mo transport elements. It may also be relevant to note here that *A. vinelandii* cell extracts and pellet are darker (brownish red) than their *E. coli* counterparts even when grown under non-diazotrophic conditions. This would suggest that perhaps *A. vinelandii* contains more Fe-containing proteins. It is possible that *A. vinelandii*, an obligate aerobe, exhibits redundancy in Fe-uptake systems in order to produce oxygen-sensitive Fe-containing cofactor molecules in high levels during nitrogen fixation as well as during non-diazotrophic conditions.

Although iron is typically found in the ferric form in the environment, ferrous iron transport genes were also identified in *A. vinelandii*. The protein products of an intact *feoABC* operon (Avin22720-Avin22740) exhibit 29-44% amino acid sequence identity to their *E. coli* counterparts. An additional *feoAB* gene set was also identified (Avin47490 and Avin47500).

The ability of *A. vinelandii* to grow diazotrophically at high oxygen concentrations, led to the investigation of its respiratory genes. A comparison of the respiratory genes between the nitrogen-fixing *A. vinelandii, P. mendocina,* and *P. stutzeri*, and a number of other non-nitrogen fixing Pseudomonas species including *P. aeruginosa*, *P. entomophila*, *P. fluorescens*, *P. putida*, and *P. syringae*. Most of the respiratory genes that were identified were present in all the organisms (Table I). A complex V ATP synthase set was present in all the pseudomonads studied (Avin52150-Avin52230). In addition, *A. vinelandii* produces a second ATP synthase (Avin19670-Avin19750) that is not present in the other pseudomonads. Several proteins were present only in the nitrogen-fixing

bacteria *A. vinelandii, P. mendocina,* and *P. stutzeri*, which suggests their possible implication in nitrogen fixation. The two subunits of cytochrome bd ubiquinol oxidase CydAB (Avin19880-Avin19890) are present in *A. vinelandii* and *P. stutzeri*. Inactivation of the *cydAB* genes results in the inability of *A. vinelandii* to fix nitrogen in air, a phenotype that can be rescued when O_2 is lowered to 1.5% (Kelly *et al.*, 1990). Also, in contrast to the other pseudomonads, which carry one copy of the *rnf* operon, the products of which are involved in nitrogenase electron transfer, *A. vinelandii* and *P. stutzeri* produce an additional Rnf system (Avin50920-Avin50960). Finally, homologs of the genes Avin11160 and Avin11180, which give rise to the two subunits of cytochrome o ubiquinol oxidase, are exclusively present in *A. vinelandii*, *P. stutzeri*, and *P. mendocina*.

In aggregate, the results presented here suggest that *A. vinelandii* exhibits redundancy of Fe and Mo uptake systems in order to fulfill the biosynthesis of Fe and Mo-containing cofactors needed for the maturation of high levels of nitrogenase produced during diazotrophic conditions. *A. vinelandii* may also employ additional respiratory genes to meet its requirement to grow at ambient O_2 concentrations.

Table A5.I. Comparison of respiratory genes present in *A. vinelandii* and other pseudomonads.

Present in only in *A. vinelandii*	Avin19670-19750	COMPLEX V
	Avin21930, Avin21230	Fumarate reductase/succinate dehydrogenase flavoprotein
Present only in A. vinelandii and P. stutzeri	Avin19880-Avin19890	CydAB, Cytochrome bd ubiquinol oxidase, subunits I and II
	Avin50920-50960	*rnfABCDGE* operon
	Avin49080	cytochrome C, class II
Present only in A. vinelandii, P. stutzeri, and P. mendocina	Avin11160	cytochrome o ubiquinol oxidase, subunit II
	Avin11180	cytochrome o ubiquinol oxidase, subunit I
Present in all pseudomonads	Avin20010	cytochrome c oxidase, cbb3-type, subunit I
	Avin20000	cytochrome c oxidase, cbb3-type, subunit II
	Avin19990	cbb3-cyt c oxidase
	Avin19980	cyt c oxidase, cbb3-type, subunit III
	Avin19970	cytochrome c oxidase accessory protein CcoG
	Avin19960	cytochrome c oxidase accessory protein CcoH
	Avin19950	copper-translocating P-type ATPase
	Avin19940	cbbr-cyt oxidase mat. protein
	Avin19930	conserved hypothetical protein
	Avin19920	O_2-independent coproporphyrinogen III oxidase
	Avin19910	Fnr-like negative transcriptional regulator of CydAB
	Avin19530	sodium hydrogen antiporter subunitAB, ShaAB
	Avin19540	sodium hydrogen antiporter subunitC, ShaC
	Avin12000	uncoupled NADH:quinone oxidoreductase
	Avin28440	NADH-ubiquinone oxidoreductase
	Avin29780-Avin29810	sdh operon
	Avin03240	putative cytochrome b561
	Avin47940	cytochrome c5 protein
	Avin01000	cytochrome c oxidase assembly protein CtaG/Cox11
	Avin11040	cytochrome bd ubiquinol oxidase, subunit II
	Avin11060	cytochrome bd ubiquinol oxidase, subunit I
	Avin11090	short-chain dehydrogenase/reductase
	Avin11120	sigma54-dependent regulatory protein; AlgB
	Avin52150-52220	ATP synthase