Isolation of *in vivo* intermediates in iron sulfur cluster biogenesis

Estella Callie Raulfs

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Biochemistry

Dennis R. Dean, Ph.D. Chairperson
Richard Helm, Ph.D.
Timothy Larson, Ph.D.
Robert White, Ph.D.
Brenda Winkel, Ph.D.

April 3rd, 2009
Blacksburg, Virginia

Keywords: Iron-sulfur clusters, *Azotobacter vinelandii*, ISC, scaffold
Isolation of *in vivo* intermediates in iron sulfur cluster biogenesis

Estella Callie Raulfs

**ABSTRACT**

Iron-sulfur clusters are simple inorganic cofactors that are ubiquitous in living systems. The assembly of iron sulfur clusters is an essential process and must be carefully controlled in order to limit the release of toxic free iron or sulfide. Thus far there are three known protein systems for iron sulfur cluster assembly including the *nif*, *suf*, and *isc* systems. The *nif* system makes iron-sulfur clusters for nitrogenase production, while both the *suf* and *isc* systems provide iron-sulfur clusters for general cellular use. In *Azotobacter vinelandii* the *isc* operon contains eight genes which are transcribed together as a single operon: *iscR iscS iscU iscA hscB hscA fdx iscX*. The two central *isc* players include IscS, a cysteine desulfurase, and IscU the proposed site of iron-sulfur cluster assembly.

Using *A. vinelandii* as a model organism, we have sought to better understand the mechanism of *in vivo* *isc* cluster assembly. In order test the scaffold hypothesis, we constructed strains that allowed for quick and rapid isolation of IscU. The purification of IscU with a bound [2Fe-2S] cluster strongly supports the model that IscU serves as the site of cluster synthesis *in vivo*. Additionally, using this same genetic system we isolated an IscU39DA variant with an oxygen stable bound [2Fe-2S] cluster. The IscU39DA scaffold came in tight $\alpha_2\beta_2$ complex with IscS and was not separated by high salt, size exclusion, or reducing conditions. On the other hand, wild-type IscU also associated with IscS in a $\alpha_2\beta_2$ complex, but readily dissociated upon increased salt concentration. The tight association of IscU39DA and IscS was found to occur regardless of the presence of a bound [Fe-S] cluster. We conclude that the IscU Asp-39 residue is essential for mediating the dissociation of IscU and IscS.

In addition to studying IscS and IscU, we were interested to further understand how the *isc* system is regulated in response to external factors. Previous work has
demonstrated that IscR controls expression of the isc operon in *Escherichia coli*. When IscR is holo this protein represses isc expression, while in its apo-form it allows isc expression. In *A. vinelandii* we found that ΔiscR strains exhibit in a 5–7 fold elevation of isc expression. Additionally, ΔiscR strains reveal a small growth phenotype on plates, and a tendency to form spontaneous suppressor mutations allowing reversion to wild-type growth. Loss of apo-IscR function was found to cause a more severe effect on growth than the loss of holo-IscR function, suggesting IscR has cellular roles in addition to the regulation of the isc operon.
ACKNOWLEDGMENTS

I owe my first acknowledgements to my advisor Dr. Dennis Dean who has consistently pushed me to excel and provided me with guidance both professional and personal. The Dean lab truly promotes an environment of scientific team work and much this dissertation would also not have been possible with out the contributions of my laboratory colleagues: Dr. Ina O’Carroll, Valerie Cash, Dr. Patricia Dos Santos, Dr. Deborah Johnson, and Dr. Miheala Unciuleac-Sandu. I would also like to thank my graduate committee: Drs. Timothy Larson, Robert White, Richard Helm, and Brenda Winkel who have been especially helpful and approachable through out my graduate career. Many thanks to Dr. Keith Ray from Dr. Rich Helm’s lab help with protein identification by mass spectroscopy. I have also benefited from the sage advice of many scientists on campus specifically Drs. Robert White, Stephen Melville, Jiann-Shin Chen, Tim Larson, and Birgit Alber. Our collaborators Juan Fontecilla-Champs, Yvian Nicolet, and Frederic Garzoni at the Laboratorie des Cristallographie et Cristallogènese des Proteines in Grenoble, France were instrumental for starting our project with protein crystallography. Dr. Florian Schubot and Nancy Vogelaar have been extremely helpful for the continuance of these crystallization trials on the Virginia Tech campus. Additionally I have mentored several talented undergraduates including Kyle Cromer, Melissa De la Cuesta, Sachi Desai, and Lanessa Byrant, whom have all made contributions to this work.

In conclusion, I’d like to thank my parents Marie and Glenn Raulfs for valuing education above all else and sacrificing to a great extent so that I could have one. Lastly my sister, Mary Disa Raulfs, and many dear friends including, Dongbo Wang, Sher Vogel, Amanda Davis, Krista and Nick Wiggington, Sabine Sibler, Jessica Guiellerm, Neil Norman, Tiffany Adams, Lee Byrant, Thompson Mefford, Kevin Crosby, Stephanie Murray and Maria Bowman, have been like a family for me and a source of constant support and encouragement.
TABLE OF CONTENTS

ABSTRACT iii
ACKNOWLEDGMENTS iv
TABLE OF CONTENTS v
TABLE OF FIGURES viii
TABLE OF TABLES x
ABBREVIATIONS xi

CHAPTER 1
Iron Sulfur clusters were crucial for the evolution of life
1.1 - Iron Sulfur surfaces and the Origins of Life Hypothesis 1
1.2 - Mineral based catabolism of ancient carbon fixation pathways 3
1.3 - The Cellular Revolution and the role of [Fe-S] clusters in Nitrogen assimilation 4
1.4 - Repercussions of Photosystem Evolution 5
1.5 - The need for [Fe-S] Cluster Assembly systems 6
1.6 - Other weapons of defense in the battle with Oxygen 8
1.7 - Mitochondrial Endosymbiosis 8
1.8 - Genetic Diseases and the Evolution of Host /Parasite Defenses 10
1.9 - Conclusions 11
REFERENCES 15

CHAPTER 2
In vivo iron-sulfur cluster formation
2.1 ABSTRACT 19
2.2 INTRODUCTION 19
2.3 MATERIALS AND METHODS 21
2.4 RESULTS 24
   Genetic Constructions and Experimental Rationale 24
   Labile [Fe-S] clusters are assembled on IscU in vivo 24
   Isolation of a stable [Fe-S] cluster-loaded non-covalent αβ2 IscU-IscS complex 26
   The IscU Ala39-IscS complex retains cysteine desulfurase activity 29
2.5 DISCUSSION 30
2.6 ACKNOWLEDGMENTS 32
2.7 FIGURES 33
REFERENCES 38
CHAPTER 3

The Importance of the Aspartate - 39 residue of IscU in dynamic cluster assembly

3.1 ABSTRACT

3.2 INTRODUCTION

3.3 MATERIALS AND METHODS

3.4 RESULTS

- Development of a recombinant expression system for IscU39DA
- Recombinant IscU39DA is similar to native IscU39DA but has lower cluster occupancy
- Recombinant IscU39DAIscS can be reconstituted
- Apo IscU39DAIscS exhibits the same kinetic properties as Holo IscU39DAIscS
- Chaperone proteins, HscB and HscA, are not required for IscU39DA and IscS interactions

3.5 DISCUSSION

3.6 TABLES & FIGURES

3.7 REFERENCES

CHAPTER 4

Characterization of a Deletion IscR phenotype in *Azotobacter vinelandii*

4.1 ABSTRACT

4.2 INTRODUCTION

4.3 MATERIALS AND METHODS

4.4 RESULTS

- IscR regulation of ISC expression is cluster dependent
- Holo-IscR is necessary for native expression of the trmH cysE operon
- IscR dependent expression within the isc operon
- Deletion iscR strains have a ‘small’ phenotype
- Deletion iscR strains over-express aldehyde and alcohol dehydrogenase proteins

4.5 DISCUSSION

4.6 FIGURES AND TABLES

4.7 REFERENCES

CHAPTER 5

Appendices

**Appendix I.** IscU39DAIscS α2β2 complex may interact with other ISC proteins

**Appendix II.** A compromised ISC System causes degradation of IscU
Appendix III. Functional Analysis of IscR

INTRODUCTION

MATERIALS AND METHODS

RESULTS / DISCUSSION

Appendix IV. Growth phenotypes of ΔcysE and ΔiscR strains

INTRODUCTION

MATERIALS AND METHODS

RESULTS / DISCUSSION

Appendix V. Crystallization Trials of the IscU39ΔHisIscS complex
   The C-terminal Histag of IscU is susceptible to degradation
   Project progression using IscU and IscS from Archeaoglobus fulgidus

Appendix VI. Annotation of the Azotobacter vinelandii Genome
   Aconitases and oxidative stress response proteins A. vinelandii genome

REFERENCES

CHAPTER 6

Conclusions and Outlook
TABLE OF FIGURES

CHAPTER 1

Figure 1.1  Schematic representation of iron sulfur clusters  13
Figure 1.2  Model for the origins of life at hydrothermal bioreactor mounds  14

CHAPTER 2

Figure 2.1  Schematic representation of strains used in this work  33
Figure 2.2  Anion-exchange chromatography elution profile of the IscU-containing IMAC fraction prepared from DJ1697  34
Figure 2.3  Comparison of samples that contain IscU or IscU Ala^39-IscS complex prepared by anion-exchange chromatography  35
Figure 2.4  Time-dependent effect of cluster degradation  36
Figure 2.5  Spectroscopic and kinetic features associated with IscS - catalyzed L-cysteine desulfurization  37

CHAPTER 3

Figure 3.1  Sequence alignment of IscU from different organisms  59
Figure 3.2  ISC strains and plasmids used in this study  60
Figure 3.3  UV-visible absorption spectra of apo- and holo- IscU39^DAIscS  61
Figure 3.4  Separation of an equimolar mixture of apo- and holo- IscU39^DAIscS via anion exchange chromatography  62
Figure 3.5  UV-visible spectra of apo-, reconstituted-, and holo-IscU39^DAIscS  63
Figure 3.6  Characteristic blue shift of PLP cofactor upon substrate binding  64
Figure 3.7  Equilibrium and kinetic features associated with IscS catalyzed desulfurization of apo- and holo- IscU39^DAIscS complex  65
Figure 3.8  Separation of IscS and IscU via anion exchange chromatography from crude extracts of arabinose induced plasmids  66
Figure 3.9  Proposal for in vivo [2Fe-2S] cluster assembly  67
Figure 3.10  Structure of H. influenza IscU  68

CHAPTER 4

Figure 4.1  Elevated expression of ISC proteins in ΔiscR strain  92
Figure 4.2  Putative IscR binding sites on the A. vinelandii genome  93
Figure 4.3  IscR dependent expression of lacZ fusion constructs in the A. vinelandii genome  94
Figure 4.4  Relative colony size of A. vinelandii iscR strains  95
Figure 4.5  ΔiscR strain shows improved growth on B plates  96
Figure 4.6  Light micrographs of A. vinelandii cells  97
Figure 4.7  Natural suppressor mutations in ΔiscR strains  98
Figure 4.8  Proteomic level differences between ΔiscR strains grown on
plates: DJ1601 and DJ1877

Figure 4.9 Genomic context of Aldehyde dehydrogenase and Alcohol dehydrogenase proteins

Figure 4.10 Aldehyde and Alcohol dehydrogenase activity of \textit{iscR}+, \textit{iscR}92^{CA} and \textit{\Delta}iscR strains

CHAPTER 5 - Appendices

Figure A.2 Representative SDS-PAGE showing purification fractions for IscU39^{DA}IscS from DJ1766

Figure A.3 \textit{Azotobacter vinelandii} strains used in this study

Figure A.4 IscU, IscS, GroEL western blot from \textit{A. vinelandii} strains

Figure A.6 Growth of \textit{\Delta}iscR and \textit{\Delta}cysE strains on supermedia

Figure A.7 Results related to crystallographic studies of IscU/IscS complex

Figure A.9 Location of aconitase genes in the \textit{Azotobacter vinelandii} genome.
TABLE OF TABLES

CHAPTER 3

Table 3.1  Iron and sulfide analysis of IscU and IscU<sup>39DA</sup> isolated from recombinant and native expression systems 58

CHAPTER 4

Table 4.1  Strains used in this study 91

CHAPTER 5 - Appendices

Table A.1  <i>A. vinelandii</i> proteins isolated from IMAC resin with IscU<sup>39DA</sup>IscS in 20 mM imidazole 110
Table A.5  β-Galactosidase Activity of Φ(<i>hscA-lacZ</i>) strains with IscR substitutions or deletions in <i>A. vinelandii</i> 132
Table A.8  Crystallization Conditions Tested 141
Table A.10  <i>Azotobacter vinelandii</i> Genes Annotated 148
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>β-ME</td>
<td>Beta-mercaptoethanol</td>
</tr>
<tr>
<td>DNASE</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylfluoride</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiotheritol</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>[Fe-S]</td>
<td>Iron sulfur</td>
</tr>
<tr>
<td>I-AEDANS</td>
<td>N-iodoacetaminoethyl-1-naphthylamine-5’-sulfonic acid</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal 5’- phosphate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>psi</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic Acid</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl) aminomethane</td>
</tr>
</tbody>
</table>
CHAPTER 1

Iron Sulfur clusters were crucial for the evolution of life

The pervasive nature of metal-sulfur-clusters in modern cells reflects their ancient origins. In fact, it has often been argued that metal-sulfur clusters are mineral relics from a primordial anaerobic world (Martin and Russell, 2003). Over millennia, iron-sulfur dependent micro-organisms have played a defining role in the intimate co-evolution of Life and Earth, profoundly shaping the geology of our planet and affecting life as we know it. Basic iron sulfur clusters take two forms, the rhombic \([2\text{Fe}-2\text{S}]\) cluster and the cuboidal \([4\text{Fe}-4\text{S}]\) cluster (Figure 1.1). They can also be found in more complex assemblages which include the incorporation of additional metals (such as molybdenum in MoFe protein of nitrogenase), organic ligands (hydrogenase) and inorganic ligands (acetyl-CoA synthetase). Metalloclusters are required for electron transfer in standard cellular processes such as respiration. Three of the eight proteins involved in the Krebs cycle require at least one iron-sulfur cluster for example, aconitase, fumarase, and succinate dehydrogenase. \([\text{Fe-S}]\) Proteins are also key components of essential biosynthetic pathways including the biosynthesis of thiamine, branched chained amino acids, tRNAs, biotin, and membrane bound quinones (Flint et al., 1993; Leonardi et al., 2003; Loiseau et al., 2007; Pierrel et al., 2002; Sanyal et al., 1994). On a global scale, metalloproteins greatly influence the global cycling of nitrogen (nitrogenase), carbon fixation (carbon monoxide dehydrogenase), oxygen production (photosystem II), and the production of H\(_2\) gas (NiFe, FeFe dedydrogenase).

1.1 - Iron Sulfur surfaces and the Origins of Life Hypothesis

Throughout the course of geological history \([\text{Fe-S}]\) clusters have played a crucial role at critical junctures in biological evolution, laying the ground work for their
importance in modern life. About 4.5 billion years ago the primordial earth was a
noxious place containing abundant ammonia, methane, carbon dioxide and hydrogen
sulfide. Experimental evidence provided by Stanley Miller in 1953 revealed that simple
organics could be synthesized from electrically shocking a flask of early earth
components (Miller, 1953). Building on this evidence, Miller and Urey proposed the
‘prebiotic soup’ model for the origins of life, theorizing that simple amino acids
synthesized under early earth conditions would provide the raw material for subsequent
polymerization (Miller and Urey, 1959a, b). While this model still enjoys some
popularity, many have disparaged the theory’s underlying ‘spontaneous generation’
tenets pointing out that equilibrium solutions do not promote productive chemical
reactions. To quote a more recent critic of the Miller-Urey hypothesis, “Once autoclaved,
a bowl of chicken soup left at any temperature will never bring forth life” (Martin and

An alternative origin-of-life theory referred to as the ‘surface-driven metabolist’
hypothesis, was originally put forth in the 1950s (Ycas, 1955). This idea has seen a
resurgence of interest since its re-introduction by the German patent lawyer, Gunter
Wächtershäuser in the early 1990s (Blochl et al., 1992; Wachtershauser, 1990, 1994).
The theory posits that positively charged surfaces found in many iron sulfur minerals
such as pyrite would be ideal for generating life by providing a means to bind and
concentrate negatively charged carbonyls and sulfhydryls. The surface structure would
further limit the rotational degrees of freedom of surface bound molecules (from 6 to 3),
helping to orient molecules for productive condensation reactions and eventually
allowing the generation of higher order compounds (Wachtershauser, 1988). In this way
the mineral surface could act as a catalyst to drive the production of carbon-based two-
dimensional life-forms or ‘surface metabolists’. Building on this idea, Martin & Russell
(2003) proposed that life could have evolved in mineral mounds or ‘biological reactors’
such as those found at hydrothermal vent fields, where small micro-chambers in the Fe-
Ni-S composite would have allowed the accumulation of more complex surface
compounds. In the biological reactor model, hot, reduced, alkaline hydrothermal
solutions from geothermal processes within the earth would come into contact with the
cooler, more acidic and oxidized Hadean ocean. (Figure 1.2) This interaction would have
created a pH/redox/temperature gradient to help drive the abiotic synthesis of organic compounds.

1.2 - Mineral based catabolism of ancient carbon fixation pathways

Supporting the idea that life evolved in biological reactor mounds is the observation that the acetyl-CoA pathway, which is often cited as the most ancient means of fixing carbon, requires multiple Fe-Ni-S clusters (including the C clusters of carbon monoxide dehydrogenase, and the A cluster of acetyl-CoA synthase) (Russell and Martin, 2004). The architecture of these metalloclusters bears striking resemblance to an Fe$_5$Ni$_8$S$_8$ mineral, greigite, which has been found to be present at hydrothermal mounds (Russell and Martin, 2004). The ancient nature of this carbon fixation pathway has been proposed because of its prevalence in deep-branching thermophilic archea. In addition, the only input requirements are the simple inorganic molecules CO$_2$ and H$_2$ and the pathway has a negative delta G value indicating that it is thermodynamically favorable enough to drive the production of cellular ATP. Extant organisms utilizing this mode of carbon fixation produce acetyl-CoA as the final end product. In a primordial world, however, high-energy thioester analogs, such as acetyl-thiols, methyl thioacetate, and methyl-sulphide would have been the most likely end product. As further evidence that porous hydrothermal mounds could have driven the early production of organics, each of these thiol-related end products have each been experimentally produced by various research groups in protein-free Fe-Ni-S reactors (Blochl et al., 1992; Cody et al., 2000; Huber et al., 2003; Huber and Wachtershauser, 1997, 1998). Experimental and evolutionarily derived evidence suggests that aprotic pathways for carbon fixation are possible on Ni-Fe-S surfaces.

Mineral based catabolism provides a powerful model for explaining some of the earth’s earliest biological processes. This roughly 0.5 billion year time frame witnessed the evolution of nucleosides, primitive proteins, tRNA, ribosomes, DNA and RNA polymerase, thus laying the foundations of modern biochemistry prior to the events of cellularization. During the reign of the ‘Fe-S’ world, as it is often referred to, primitive acidic ferredoxins would have accommodated small mineral blocks of iron-sulfide which
could have readily condensed on exposed protein thiol residues in an anaerobic environment. Experimental evidence supporting these ideas comes from a large body of in vitro work showing that in absence of oxygen, \( \text{Fe}^{2+}/\text{Fe}^{3+} \) and \( \text{S}^{2-} \) condense to form basic [2Fe-2S] and [4Fe-4S] cluster subunits (Lane et al., 1977) which can then be used to activate apo-forms of iron sulfur proteins without the addition of auxiliary proteins (Malkin and Rabinowitz, 1966).

Iron sulfur clusters were a mainstay of the prebiotic era. In fact ferredoxins from the Clostridia genera are generally considered to represent the oldest branch of extant proteins, and contain not one, but two [4Fe-4S] clusters (Davis, 2002; Meyer, 2000). The lifting of the mineral world onto a tertiary protein structure would have further limited degrees of freedom of small bound carbon molecules, permitting the first ferredoxins to further control mineral driven reactions. Varying properties of ancient proteins would have modulated the reaction type and efficiency of the small mineral surface, allowing the evolution of specialized protein/mineral functions. It seems likely that if prebiotic life did exist in anaerobic micro-chambers, spontaneous [Fe-S] formation would have allowed cluster insertion on simple protein scaffolds and the evolution of new [Fe-S] clusters functions quite easily.

1.3 - The Cellular Revolution and the role of [Fe-S] clusters in Nitrogen assimilation

The cellular revolution, i.e. the enclosure of primitive biological components into self-replicating membrane-bound systems, divided the world into two distinct lineages: eubacteria and archaea. Carbon isotope signatures indicate proliferic carbon fixation occurred about 4 billion years ago, putting cellularization in a time frame just before this process. At some point following the separation of eubacteria and archaea lineages, available atmospheric ammonium was depleted resulting in a scarce supply of fixed nitrogen (Berman-Frank et al., 2003). As no other means of nitrogen assimilation had yet evolved (nitrification necessarily evolved after the formation of \( \text{NO}_3^- \) and \( \text{NO}_2^- \), an event that followed atmospheric oxidation), available abiotic processes of \( \text{NH}_3 \) production (lightning, meteor impacts, and possibly hydrothermal effluent) would have limited carbon fixation to 0.1% of current levels (Falkowski and Godfrey, 2008). The fate of the
world’s microbial population was therefore dependent on a small group of bacteria containing a multimeric complex, nitrogenase, that was able to catalyze the reduction of the N\textsubscript{2} triple bond (Berman-Frank et al., 2003). The origins the nitrogenase machinery are not clear, although some have postulated a reverse of this system was evolved in the very early NH\textsubscript{3} rich atmosphere as a means to respire off nitrogen, creating N\textsubscript{2} as an electron sink and byproduct (Fani et al., 2000; J. R. Postgate, 1985). Others have cited similarity to hydrogenase systems or cyanide detoxification proteins as nitrogenase progenitors (Berman-Frank et al., 2003). Whatever the origins, nitrogenase is a behemoth of a protein, requiring an enormous amount of energy for function and multiple iron-sulfur clusters including complex metallocenters, P cluster, and FeMo cofactor (Dos Santos et al., 2004a). Not surprisingly, iron sulfur clusters are essential for facilitating the funneling of electrons to the reaction center to properly reduce the high energy N\textsubscript{2} triple bond (Dos Santos et al., 2004a). Despite the high energetic costs required for synthesis and maintenance of this enzyme, nitrogenase provided a distinct ecological niche for diazotrophic bacteria. Except for replacement of the principal metal atom in the FeMo cofactor, an alternative form of nitrogenase protein has never evolved. As a result, the altruistic ammonium ion provided by diazotrophs has continued to be the world’s primary source of fixed nitrogen until the introduction of the Haber-Bosch process during 20\textsuperscript{th} century industrialization.

1.4 - Repercussions of Photosystem Evolution

About 2.5 billion years ago an event took place on earth that has profoundly shaped life on our planet ever since. During this time a rogue group of prokaryotes evolved a novel protein/cofactor system, called photosystem (I & II) for harnessing solar energy. As the basic component parts of the photosynthetic system were already present by this time, the formation of a final photosystem complex required the proper recruitment of chlorophyll centers, Mn atoms for splitting H\textsubscript{2}O, and [4Fe-4S] centers to transfer electrons from the complex center of photosystem I (Nelson and Ben-Shem, 2005). The novel ability to utilize solar energy gave cyanobacterial ancestors a distinct advantage over other prokaryotes, allowing them to proliferate and cover the upper levels
of the sun-drenched earth. The steady accumulation of photosynthesis byproduct, O₂, in the earth’s atmosphere proceeded for over 1 billion years until reaching present day levels of ~20%. The oxygenation of the earth’s atmosphere by cyanobacteria is often referred to as the ‘great oxygenation event’. Due to the fact that O₂ is a limited molecule in the universe, not known to be formed by any known galactic processes, its presence in the atmosphere is thought to represent one of the few clear signs of planetary life (Falkowski and Godfrey, 2008).

The curiously electropositive redox potential (+0.8V) of this new dioxygen molecule greatly expanded the energetic potential of the archeal and bacterial populations able to evolve a means to utilize O₂ as a final electron acceptor. Perhaps surprisingly, this was not a tremendously difficult task as [Fe-S] dependent redox domains were already established for low energy electron transfer (succinate dehydrogenase and ferredoxins), and the upper electropositive domain simply involved evolution of cytochrome c oxidase and recruitment of copper centers to carry high energy electrons (Imlay, 2006).

But dioxygen also brought a dark side to adolescent earth. The high spin valence state of dioxygen is naturally attracted to transition metals such as iron, which have available p-orbitals for interaction, causing a blatant attack of metal enzymes by oxygen. Additionally, the increasing accumulation of O₂ caused the global oxidation of iron from its soluble ferrous (Fe^{2+}) to insoluble ferric (Fe^{3+}) form, leading to wide - spread precipitation and causing this once abundant soluble metal to become a scarce nutrient. Banded iron formations (BIF) found in the geological record around the world are conventionally attributed to the large scale precipitation of ferric iron that occurred following oxygenation of the Earth’s atmosphere.¹

1.5 - The need for [Fe-S] Cluster Assembly systems

Bacteria living in the aftermath of oxygenic photosynthesis dealt with new oxidative stress in a number of ways. Some sought out life in anaerobic environs away

¹ The precise cause of banded iron formations (BIF) has been the subject of much recent debate.
from the destructive force of the high energy O₂ molecule, preferring to eke out a living on the reduction of inorganic substrates. Fortunately for those microbes that could not flee, the slow and steady accumulation of O₂ allowed ample evolutionary time to mount an adaptive response to the changing environment.

As previously mentioned, iron sulfur proteins were particularly impacted by the great oxygenation event. For one, oxidative degradation of protein bound iron-sulfur clusters causes a loss of redox or enzymatic function. Additionally O₂ prevents the spontaneous self assembly of iron and sulfide, and in the same vein, interactions of O₂ with free iron can causes the formation of highly toxic O₂ radicals, rendering formerly benign unchaperoned iron atoms as potentially lethal weapons of cellular terror. Given these parameters, the evolution of machinery designed specifically to protect and replenish assembled [Fe-S] clusters was a necessary response to environmental O₂ accretion. In addition, the evolution of [Fe-S] rich multimeric systems for nitrogen fixation, oxygenic photosynthesis, and cellular respiration likely required iron-sulfur clusters beyond demands that could be meet via spontaneous self-assembly.

Several [Fe-S] cluster producing systems evolved to meet these needs including the *isc*, *suf*, and *nif* machineries. As the relative antiquity of each system is not truly known, the derivation of cluster assembly origins at the onset of oxygenation is a deductive guess. A common feature of cluster assembly systems, however, includes the presence of a highly conserved U-type scaffold protein (Hwang et al., 1996). The U-type scaffold has three conserved cysteines which provide a thiol structure for iron and sulfide insertion. Cysteine desulfurases provide sulfur to the scaffold site via PLP-dependent desulfurization of L-cysteine. The source of iron for cluster assembly is unknown and the subject of much debate (Johnson et al., 2005). In modern organisms the *nif* system specifically produces [Fe-S] modules for nitrogenase protein. Both the *isc* and *suf* systems are generalists, involved in production of [Fe-S] clusters for broad spectrum cellular use. The *suf* system is particularly adapted for cluster production under conditions of cellular stress, as is it the primary cluster assembly machinery in many organisms exposed to heightened O₂ levels such as parasites, plant cytoplasm, or *E. coli* cells under iron depletion and high oxygen tension (Fontecave et al., 2005).
The *isc* system contains ATP hydrolyzing chaperone protein, HscA, and co-chaperone, HscB, which have been implicated in the transfer of clusters to apo-target proteins. The *suf* system also has a convergent ATP utilizing multimer, the SufBCD complex. *Nif* does not have a specific ATP binding domain for the formation of [2Fe-2S] and [4Fe-4S] clusters, but does require ATP-dependent NifY protein for cluster insertion and re-arrangement of NifDK proteins containing the final FeMo cofactor (Dos Santos et al., 2004a).

1.6 - *Other weapons of defense in the battle with Oxygen*

In addition to iron-sulfur cluster machinery, oxygenation of the atmosphere also caused cells to evolve mechanisms to protect themselves from oxidative stress, including superoxide dismutase and H$_2$O$_2$ neutralizing catalase. The limited availability of free iron further prompted the evolution of siderophores, TonB transport receptors, and iron storage proteins like ferritin, to recover and conserve increasingly vanishing amounts of iron (Imlay, 2006).

Over time, iron-sulfur clusters evolved to serve as oxygen sensors for a family of regulatory proteins, including IscR, FNR, CydR, and SoxR. These proteins undergo structural changes upon oxygen-induced degradation of their respective clusters, ultimately effecting gene expression. Other regulatory proteins, such as Fur evolved to maintain cellular iron homeostatis (Imlay, 2006).

1.7 - *Mitochondrial Endosymbiosis*

Around 1 billion years before the Cambrian explosion of multicellular organisms, an autotrophic eubacterium, similar to present day *Rhodobacter* or *Paracoccus*, was phagocytosized by a host cell. For reasons that are still in debate and may never be known, the eubacterium was stabilized in the host cell, forming a mutualistic symbiosis and eventually giving rise to mitochondria. The eubacterial origins of the mitochondrion are now widely accepted. Although numerous evidence exists to support this theory, one
of the most convincing is the observation that eukaryotic glycolytic and citric acid cycle enzymes share a high degree of identity to their eubacterial cousins. The origins of the host cell are more vague - some have proposed that the host cell was an archeabacterium, and indeed the similarity between the genetic and cytoplasmic systems of eukaryotes and archeabacteria would support this hypothesis. The issue is further complicated however by the fact that eukaryotes have a eubacterial (not an archeal) plasma membrane. However, large scale gene transfer from the eubacterial symbiont to host nucleus make plausible the insertion of eubacterial lipid biosynthesis machinery into the host genome, and this rationale is often used to explain this and other discrepancies concerning host cell origins.

An interesting sidenote in the theory of endosymbiosis, involves the presence of iron-sulfur cluster machinery. In present day eukaryotes, the majority of cellular iron sulfur machinery is found in the mitochondrial matrix (Lill and Muhlenhoff, 2008). Early mitochondria might have provided eukaryote host cells with greatly enhanced energy generating capacity, eventually leading to much larger cell size and the evolution of multicellular organisms. Whether or not this original process was aerobic or anaerobic, cluster synthesis would have been required for various redox proteins including aconitase, succinate dehydrogenase, pyruvate ferredoxin oxidoreductase, and ferredoxins. It seems clear then that the original endosymbiont must have had the ability to produce iron-sulfur clusters. Discoveries that eukaryotic organisms containing highly reduced mitochondria such as hydrosomes or mitosomes still retain iron-sulfur cluster synthesizing capacity in these de-evolved organelles (Sutak et al., 2004; Tovar et al., 2003) speaks strongly to the idea that [Fe-S] cluster assembly was of original importance for the stabilization of endosymbiosis. Recent investigations have also revealed that [Fe-S] cluster assembly is not only important for the mitochondria but also for the proteins in the cytoplasm. In a series of studies on the mitochondrial ABC transporter, Atm1p, thought to be responsible for [Fe-S] transport to the cytoplasm, reveal a severe growth defect when this protein is deleted in yeast (Kispal et al., 1999). The defect was eventually pinpointed to Leu1p, a cytoplasmic iron-sulfur requiring protein, indicating the importance of the mitochondrial iron sulfur assembly machinery for synthesis of iron-sulfur proteins (Kispal et al., 1999). Under certain conditions in yeast, mitochondria can
also be forced to respire off substrates other than oxygen, leaving many to propose that the formation of iron-sulfur clusters is the single most essential function of this organelle (Lill et al., 1999).

Taken together these results suggest that mitochondrial [Fe-S] biogenesis is essential for eukaryotic viability and could have provided selective pressure for the stabilization of endosymbiosis. A review by Lill et al. has noted that of the 22 known essential genes in *Saccharomyces cerevisiae*, four are involved in mitochondrial iron-sulfur cluster biogenesis (Lill et al., 1999). These observations underscore the importance of [Fe-S] cluster assembly for both prokaryotic and eukaryotic life.

1.8 - Genetic Diseases and the Evolution of Host/Parasite Defenses

As eukarya have evolved to multicellularity, the fight between iron sulfur proteins and the destructive oxygen molecule has continued. Despite over 1.5 billion years of evolutionary time that has occurred since oxygenation of the atmosphere, all living cells remain highly susceptible to oxidative damage. Host organisms have made use of this weakness by routinely blasting invading pathogens with H$_2$O$_2$ as a means to fight infection, despite a large degree of self-damage inflicted in the process. The ability to produce and repair [Fe-S] clusters is so important that the *isc* and *suf* biosynthetic systems are essential for virulence in a large number of pathogens (Jones-Carson et al., 2008; Rincon-Enriquez et al., 2008; Runyen-Janecky et al., 2008).

In humans, genetic-based malfunctions of the iron sulfur assembly machinery can lead to the accumulation of iron in the mitochondria, which can have disastrous effects. A mutant in the frataxin gene, which serves as the iron donor for cluster assembly, for example leads to a specific type of neurological disorder known as Friedrich’s ataxia (Puccio and Koenig, 2002). X-linked sideroblastic anaemia is caused by a mutation in the Atm1 transporter which functions to deliver [Fe-S] clusters to the cytoplasm (Allikmets et al., 1999; Bekri et al., 2000; Camaschella et al., 2007). Another genetic disorder, known as microcytic anemia, is attributed to a defect in the ISC assembly component human glutaredoxin protein (Camaschella et al., 2007). A common denominator of these disorders is the accumulation of iron in the mitochondria which can
causes a variety of problems including cardiac hypertrophy and neuronal disfunction (Lill and Muhlenhoff, 2008). A more extensive review of human related iron-sulfur disorders has recently been compiled by Lill and Muhlenhoff (2008).

1.9 - Conclusions

The properties of iron sulfur clusters have played a defining role at many critical junctures during the course of biological evolution. During the geological time known as the Archeal eon of early earth, biology evolved in the absence of O₂, laying the foundation for the preeminence of iron-sulfur proteins later in evolution. The fundamental properties of iron-sulfur clusters are implicit with the idea that life originated on a two-dimensional Ni-Fe-S surface. This mineral surface would have promoted the condensation of higher-order organic molecules and primitive carbon fixation pathways. After the evolution of the first proteins, iron-sulfur mineral insertion on the protein scaffold would have prompted further control of biochemical reactions, allowing mineral-protein assemblages to evolve specific functions in an anaerobic world. One of the most important functions of early [Fe-S] proteins was their ability to transfer electrons. The redox property of iron-sulfur proteins provided fine tuned control of reducing power, ultimately allowing cells to respire off inorganic substrates, (as in the case of anaerobic respiration), acquire nutrients (nitrogen fixation), or evolve new means of energy transduction (photosynthesis and respiration).

The oxygenation of the atmosphere severely impacted the anaerobic world from which [Fe-S] proteins evolved. Cells adapted by evolving means to synthesize clusters, prevent oxidative damage, and regulate iron homeostatis. The importance of iron sulfur proteins was not diminished in an oxygen enriched environment however. In fact, the ability to make iron-sulfur clusters likely contributed to the stabilization of endosymbiotic mitochondria and the ultimate evolution of the eukaryotic cell. Ironically, it is precisely because [Fe-S] proteins still play a critical role in modern biology, that their malfunction is the root causes of numerous genetic diseases, making the study of cluster synthesis and iron regulation relevant for medical and disease related fields.
The relatively long anaerobic geological period allowed the perfecting of [Fe-S] redox centers in reductive environments, explaining why cytochrome Cu\(^{+2}\) centers served as better electron conductors for high potential segments of the oxygenic respiration electron transfer pathway. While cells may have adapted to limit the toxic effect of metal/oxygen interactions, it seems that [Fe-S] proteins function did not evolve that much after the great oxygenation event. In a recent review, Jacques Meyer has noted that very few [Fe-S] proteins contain a high redox potential (namely the HiPiPs, and some Rieske proteins) and only four novel [Fe-S] folds have thus far been discovered in eukaryotes (Meyer, 2008).

Instead of evolving new uses for [Fe-S] clusters as likely occurred in the anaerobic world, many living systems have adapted by replacing iron-sulfur proteins with a non-metal dependent isoenzymes capable of the same function. The classic example is the eukaryotic replacement of pyruvate ferredoxin oxidoreductase (PFOR) with clusterless pyruvate dehydrogenase in the decarboxylation of pyruvate (Imlay, 2006). Some bacteria also utilize flavoenzyme ThiO, in place of the oxygen sensitive radical S-adenosyl methionine protein, ThiH, in the biosynthesis of thiamine (Settembre et al., 2003). A large number of aerobes have also evolved an oxygen stable [Fe-S] isomer of aconitase, which is induced under oxidative stress conditions (Gruer and Guest, 1994). Lastly, many eubacteria have substituted ferredoxin electron carriers of the archael Entner-Doudoroff glucose degradation pathway, with the more stable NADPH and NADH molecules (Beinert, 2000). These examples point illustrate that [Fe-S] clusters still play an important role in directing evolutionary recovery to oxygenic photosynthesis and the merging of anaerobically derived life with an aerobic world.
Figure 1.1 Schematic representation of iron sulfur clusters

(A) Cuboidal [4Fe-4S] clusters
(B) Rhombic [2Fe-2S] clusters
Sulfur atoms are denoted in yellow and iron atoms in green.
Figure 1.2 Model for the origins of life at hydrothermal bioreactor mounds

Inset a. 360 million year old hydrothermally formed iron-sulfide chimney showing porous structure. Reprinted with permission from the Royal Society of London (Martin and Russell, 2003).
REFERENCES


CHAPTER 2

*In vivo* iron-sulfur cluster formation


Estella C. Raulfs, Ina P. O’Carroll, Patricia C. Dos Santos, Mihaela-Carmen Unciuleac, and Dennis R. Dean

Dr. Ina P. O’Carroll and I were equal contributors to the work included in this paper. As co-first authors we performed experiments, participated in all stages of manuscript preparation, and prepared figures. My experimental contribution to this work included isolation and characterization of the IscU39\(^{DA}\)IscS complex as well as the comparative spectroscopic and kinetic analysis of IscS and IscU39\(^{DA}\)IscS. Dr. Ina P. O’Carroll’s performed all experiments related to wild-type IscU. Dr. Mihaela-Carmen Unciuleac made the original DJ1601 strain, and Dr. Patricia Dos Santos was involved in experimental design, data analysis, and manuscript preparation.

Department of Biochemistry, Virginia Tech, Blacksburg, VA, 24061
2.1 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.

2.2 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 (Ayala-Castro et al., 2008; Johnson et al., 2005). 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., 2005; Lill and Muhlenhoff, 2008). 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 sulfur 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 (Chandramouli and Johnson, 2006; Muhlenhoff et al., 2003). 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 (Angelini et al., 2008a; Bandyopadhyay et al., 2008a, b).

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.

2.3 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′-GGGCCATCACCATCACCACCATTCAATAT-3′) inserted at the *StuI* 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 GGGHHHHHHHHHLIV. Strain DJ1766 carries the same poly-histidine encoding tag as DJ1697 and also has the Asp<sup>39</sup> codon (GAC) of *iscU* substituted by an Ala<sup>39</sup> 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 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° protein in E. coli was accomplished using a derivative of the plasmid vector pAra13 (Dos Santos et al., 2004b), which has the expression of these genes placed under control of the ara regulatory elements. For construction of this plasmid, pDB1712, a BspHI-HindIII 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., 2004b).

**Growth and purification of IscU and the IscU Ala°-IscS complex produced by strains DJ1697 and DJ1766.** A. vinelandii cells were grown at 30°C in Burk’s medium (Strandberg and 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 35,000 rpm for 1 hr at 4°C in a Beckman Coulter Optima LE-80K ultracentrifuge using a T1 45 rotor. All subsequent IscU purification steps using DJ1697 extracts were performed in a Coy anaerobic chamber containing a gas mixture of 4% H₂ and 96% N₂, whereas purification of the IscU Ala°-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 NiSO₄, 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 HiTrap Q HP column (GE Healthcare) and washed with 10 volumes of the same buffer. IscU was subsequently eluted using a 10 ml 0-200 mM NaCl gradient 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 2.

For further purification of the IMAC fraction that contains the IscU Ala\textsuperscript{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 \textit{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\textsubscript{r} of IscU purified from DJ1697 and the IscU Ala\textsuperscript{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 Bio-Rad protein assay. All analytical analyses were performed in triplicate. Iron was quantified using the commercial Quantichrom\textsuperscript{TM} iron assay kit (DIFE-250) purchased from Bioassay Systems. Sulfide and PLP quantifications were performed as previously described (Chen and Mortenson, 1977; Wada and 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\textsuperscript{39}-IscS complex were separated by SDS-PAGE. Stained bands corresponding to IscU and IscS were excised and delivered to the Keck Biotechnology
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 ΔAbs₄₀₀ elicited by the addition of increasing concentrations of L-cysteine to 50 nmoles of IscS or IscU Ala³⁹-IscS complex. The ΔAbs₄₀₀ values were standardized to the amount of PLP present in each protein sample. The time-dependent relaxation of the ΔAbs₄₀₀ induced by the addition of a 10 fold molar excess of L-cysteine to either IscS or the IscU Ala³⁹-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.

2.4 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 2.1). 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 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 2.1) 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 2.1). 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 three major fractions designated I, II, and III (Figure 2.2). Fraction I is colorless (Figure 2.3A), exhibits no distinctive absorbance in the visible spectrum (Figure 2.3B), and contains only IscU (Figure 2.3C) (Agar et al., 2000b). 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 2.3A), only contains IscU and exhibits a UV-visible absorption spectrum (Figure 2.3B) that has identical features when compared to the [2Fe-2S]-cluster loaded IscU produced by *in vitro* reconstitution methods (Figure 2.3). The maximum extinction coefficients for this sample were $\varepsilon_{456} = 6.0 \text{ mM}^{-1}\text{ cm}^{-1}$ and $\varepsilon_{410} = 6.6 \text{ mM}^{-1}\text{ cm}^{-1}$ 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 2.4), 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 2.3A). SDS-PAGE and quantitative amino acid analysis revealed that IscU-Ala\textsuperscript{39} and IscS are isolated as a nearly exact stoichiometric complex (Figure 2.3C). The identity of IscU Ala\textsuperscript{39} and IscS purified from DJ1766 extracts was further established by mass spectrometry. Size exclusion chromatography indicated that the IscU Ala\textsuperscript{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 2.3B, spectrum 2) from the IscU Ala\textsuperscript{39}-IscS complex (Figure 2.3B, spectrum 3) yields the same characteristic spectrum associated with isolated IscS. Furthermore, subtraction of an equivalent IscS spectrum from the IscU Ala\textsuperscript{39}-IscS complex spectrum (Figure 2.3B, spectrum 3) yields a spectrum that has the same features as the [2Fe-2S] cluster-loaded form of IscU shown in Figure 2.3B. Thus, it can be concluded that the IscU Ala\textsuperscript{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\textsuperscript{39}-IscS complex is not degraded upon prolonged exposure to oxygen (Figure 2.4). Three lines of evidence indicate that the stable \(\alpha_2\beta_2\) IscU Ala\textsuperscript{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 chromatography 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\textsuperscript{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\textsuperscript{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 Ala³⁹ 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 Ala³⁹-IscS complex by the addition of L-cysteine and Fe²⁺. These results indicate that IscU and IscS must be dissociated to permit the initiation of [Fe-S] cluster assembly.

The IscU Ala³⁹-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 2.5A) (Kaiser et al., 2000; Zheng et al., 1998). 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 2.5, Panel C). It should be noted that an accurate Kd value (estimated Kd ~ 0.6 mM) could not be determined by this method because substrate binding is inhibited at high concentrations of L-cysteine (Figure 2.5, Panel C) (Zheng et al., 1998). Addition of L-cysteine to the IscU Ala³⁹-IscS complex also results in the same spectral shift recognized for isolated IscS (compare spectra in Figure 2.5, Panels A & B) and titration with L-cysteine (Figure 2.5, Panel C) reveals that both IscS and the IscU Ala³⁹-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 2.5, 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 2.5, Panel D, also show that regeneration of the resting state spectrum after L-cysteine addition occurs approximately 12 times slower for the IscU Ala<sup>39</sup>-IscS complex \((k = 0.0035 \text{ s}^{-1})\) when compared to IscS \((k = 0.03 \text{ s}^{-1})\). 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 as for the IscU Ala<sup>39</sup>-IscS complex, a subsequent step in the desulfurization reaction is slowed when IscS is captured in a complex with IscU.

2.5 DISCUSSION

Our first experimental strategy was to ask if an [Fe-S] cluster-loaded form of IscU accumulates on the proposed IscU scaffold \textit{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 that is identical when compared to the [2Fe-2S] cluster-loaded form of IscU produced by \textit{in vitro} reconstitution experiments (Agar et al., 2000b). Thus, the present work validates the physiological relevance of previous \textit{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 \textit{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 (Agar et al., 2000a; Chandramouli et al., 2007). 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 (Chandramouli 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 2.2). 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.

2.6 ACKNOWLEDGMENTS

This work was supported by the National Science Foundation grant MCB-071770. We thank Deborah Johnson for contributions to the work and Valerie Cash for expert technical assistance.
Figure 2.1  Schematic representation of strains used in this work

Strains DJ1601, DJ1697 and DJ1766 each carry a 120 bp in-frame deletion within the \textit{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 (filled dot).
Figure 2.2 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).
Figure 2.3 Comparison of samples that contain IscU or IscU Ala\textsuperscript{39}-IscS complex prepared by anion-exchange chromatography

In each panel, (1) indicates apo-IscU prepared from DJ1697 (see fraction I in Figure 2), (2) indicates [Fe-S] cluster-loaded IscU prepared from DJ1697 (see fraction II in Figure 2), and (3), indicates the IscU Ala\textsuperscript{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\textsuperscript{39} substitution. Unlabeled lane: M, standards (ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400).
Figure 2.4  Time-dependent effect of cluster degradation

Time dependent effect at Abs 455nm for [Fe-S] cluster-loaded IscU (filled triangle) and [Fe-S] cluster-containing IscU Ala<sup>39</sup>-IscS complex (filled circle) upon exposure to air. Each sample contained 130 μM of IscU.
Figure 2.5 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-fold excess of L-cysteine (dashed line).

(B) UV-visible spectra of the IscU Ala39-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) ΔmAbs_{400nm} for 50 µM IscS (filled square) and 50 µM of the IscU Ala39-IscS complex (filled circle) 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 ΔmAbs_{400nm} for IscS (filled square) and the IscU Ala39-IscS complex (filled circle) after the addition of a 10-fold excess of L-cysteine.
REFERENCES


CHAPTER 3

The Importance of the Aspartate - 39 residue of IscU in dynamic cluster assembly

I was the major contributor to the experiments described in this section. Valerie Cash constructed some of the plasmids used in this section. Dr. Ina O’Carroll constructed plasmids pDB1719 and pDB1722 and performed cited work with IscU.

Department of Biochemistry, Virginia Tech, Blacksburg, VA, 24061
3.1 ABSTRACT

We recently isolated a cluster-containing $\text{IscU}^{39\text{DA}}\text{IscS} \alpha_2\beta_2$ complex from *Azotobacter vinelandii*. The aspartate-39 to alanine substitution on IscU causes the sequestering of IscS into a tight non-dissociating complex with IscU, a species that might represent a final biosynthetic stage in the process of $[2\text{Fe}-2\text{S}]$ iron-sulfur cluster assembly. The expression of recombinant $\text{IscU}^{39\text{DA}}$ in *E. coli* resulted in the isolation of a cluster-less $\text{IscU}^{39\text{DA}}\text{IscS}$ complex. In this article we compare the properties of holo- and cluster-less forms of the $\text{IscU}^{39\text{DA}}\text{IscS}$ complex. Additionally, the trapping of IscS with $\text{IscU}^{39\text{DA}}$ was used as an indicator to probe questions related to $[2\text{Fe}-2\text{S}]$ assembly, the role of the aspartate residue in complex formation and dissociation, with a goal toward better characterizing the sequential events of *in vivo* cluster formation.

3.2 INTRODUCTION

Iron sulfur proteins are found across all domains of life and participate in a wide range of cellular processes including respiration, central metabolism, redox sensing, and DNA repair. Proteins containing $[2\text{Fe}-2\text{S}]$ or $[4\text{Fe}-4\text{S}]$ clusters have been studied since the 1960s with the discovery of iron and sulfide containing plant ferredoxins (Beinert et al., 1997). Because most iron-sulfur clusters degrade in the presence of oxygen, forming toxic by-products, it has been proposed that [Fe-S] cluster assembly systems evolved with the oxygenation of the atmosphere over 2.5 billion years ago (Imlay, 2006). In the 1970s, Dick Holm and colleagues demonstrated that inorganic iron sulfur assemblages could be synthesized *in vitro* (Lane et al., 1977). Over a decade later the NIF system was discovered to direct iron sulfur clusters assembly of nitrogenase metalloclusters (Jacobson et al., 1989b). Work with the NIF biosynthetic machinery revealed a second iron sulfur producing system that could minimally substitute nitrogenase related cluster production in the absence of essential *nif* genes. This system, referred to as the ISC (iron sulfur cluster) assembly machinery in *Azotobacter vinelandii*, is highly conserved in prokaryotic and eukaryotic genomes and plays a ‘housekeeping’ role in the general
biogenesis of [2Fe-2S] and [4Fe-4S] clusters (Zheng et al., 1998). Subsequently, other cluster production systems have been identified, including a SUF system in *Escherichia coli* involved in the assembly of clusters during periods of cellular stress (Patzer and Hantke, 1999; Takahashi and Tokumoto, 2002).

The NIF, ISC, and SUF machineries are the three primary [Fe-S] cluster producing systems discovered thus far in prokaryotes. They vary in protein composition, the conditions under which they are expressed, and final target destinations. Despite their differences in organization and cellular response, these systems share a common requirement for two central proteins: a cysteine desulfurase to mobilize sulfur and a scaffold protein which serves as the site of cluster biosynthesis. The NIF system is the most simplified of the three systems, requiring only these two proteins to create highly elevated levels of [2Fe-2S] and [4Fe-4S] cluster for the nitrogenase Fe protein. NifS, IscS, and SufS cysteine desulfurase enzymes contain a covalently bound PLP cofactor which binds the substrate, L-cysteine. Activated sulfur on IscS, in the form of an enzyme bound persulfide, is achieved by nucleophilic attack of the L-cysteine::PLP adduct by the conserved thiol group of Cys$^{328}$ (Agar et al., 2000b; Schwartz et al., 2000). The U-type scaffolds, including IscU, SufU, and NifU, contain three conserved cysteines which serve as coordinating sites for cluster assembly (Agar et al., 2000a; Chandramouli et al., 2007; Shimomura et al., 2007). A high degree of sequence similarity from diverse organisms suggests that IscU is one of the most highly conserved proteins in nature (Hwang et al., 1996).

The housekeeping ISC system in *A. vinelandii* contains several other proteins transcribed together as an operon: *iscR iscS iscU iscA hscB hscA fdx iscX*. The source of iron for cluster synthesis is not currently known, but both frataxin homologue, CyaY, and IscA have been implicated as *in vivo* iron donors (Ding et al., 2004; Layer et al., 2006). The chaperone proteins, HscB and HscA, interact with both apo- and holo- forms of IscU (Cupp-Vickery et al., 2004; Hoff et al., 2000) and it is proposed that they facilitate cluster transfer to apo target proteins. Ferredoxin contains a [2Fe-2S] cluster and is thought to serve a redox role during cluster assembly, possibly in the reduction of sulfanes, or the reductive coupling of two [2Fe-2S] clusters to form a [4Fe-4S] cluster (Chandramouli et al., 2007). The role of the small acidic protein IscX has yet to be determined. Holo-IscR
serves as a negative regulator of the \textit{isc} operon, repressing expression of the \textit{isc} genes when the demand for cellular iron-sulfur clusters has been fulfilled (Giel et al., 2006; Schwartz et al., 2001).

Due to the transient nature of iron sulfur clusters intermediates during biosynthesis, our understanding of the assembly process is based upon \textit{in vitro} experiments where cluster assembly is carefully controlled to limit oxidative damage. While these experiments have provided significant insight, they are unable to fully reproduce the physiological process of \textit{in vivo} cluster assembly. Previously we reported a genetic strategy to purify \textit{in vivo} intermediates of the cluster assembly process from host organism, \textit{A. vinelandii} (Raulfs et al., 2008). Using these \textit{in vivo} techniques, our previous work resulted in the isolation of a cluster bound \textit{IscU} protein from \textit{A. vinelandii}, thus confirming the long held scaffold hypothesis of cluster assembly (Raulfs et al., 2008). Using this same genetic system, we also isolated an aspartate-39 to alanine variant of \textit{IscU} that was bound tightly with \textit{IscS}, forming a stable cluster loaded $\alpha_2\beta_2$ tetramer.

The aspartate 39 residue of \textit{IscU} is essential and highly conserved (Figure 3.1) (Johnson et al., 2006). Originally thought to be the fourth ligand for transient cluster assembly, loss of the aspartate residue was found to trap a cluster on the \textit{IscU/NifU} scaffold \textit{in vitro}, indicating that the presence of aspartate-39 may play a cluster destabilizing role \textit{in vivo} (Unciuleac et al., 2007; Yuvaniyama et al., 2000). Additionally, a strain of \textit{A. vinelandii} expressing both \textit{IscU39DA} and \textit{IscU} forms of the scaffold protein reveal a dominant-negative phenotype. This phenotype suggests that the variant form of \textit{IscU39DA} decreases function of either wild-type \textit{IscU}, or some other ISC protein involved in [Fe-S] cluster assembly (Johnson et al., 2006). In answer to this question, the isolation of \textit{IscS} with \textit{IscU39DA} in an \textit{in vivo} $\alpha_2\beta_2$ tetramer suggested that the dominant–negative phenotype associated with this variant may be related to the trapping of \textit{IscS} with \textit{IscU39DA}.

Taking advantage of our ability to isolate an intermediate stop point of \textit{in vivo} cluster assembly we sought to understand:

(I) Is the presence of a cluster essential for \textit{IscU39DA} and \textit{IscS} interaction?

(II) How do other ISC proteins, such as HscB and HscA, affect the formation and dissociation of the $\alpha_2\beta_2$ tetrameric complex?
What is the significance of Asp-39 function on the IscU scaffold protein?

How are intermediate isolates of α2β2 IscU39DAIscS related to in vivo function?


3.3 MATERIALS AND METHODS

Construction of strains and plasmids. DJ1697 and DJ1766 were constructed as previously described (Raulfs et al., 2008). Parent plasmid pDB1720 was constructed in a cut and paste manner to place the entire isc operon: (iscS iscU iscA hscB hscA fdx iscX) under arabinose control. An octahistidine tag (5´-TACCGCATCATCACCATTACCGATTACCG-GATTACCGATCC-3´) was inserted at a StuI site in the C-terminal domain of IscU. Site directed mutagenesis of IscU aspartate 39 was performed using a commercial kit (Gene Editor, Promega) according to the supplier's instructions. The genes for chaperone proteins, HscA and HscB were removed from pDB1720 and pDB1716 by an SbfI in-frame deletion, forming pDB1722 and pDB1719, respectively.

Growth and purification of IscU39DAIscS complex produced from A. vinelandii and E. coli. Native IscU39DAIscS was isolated from A. vinelandii strain DJ1766 as previously described (Raulfs et al., 2008). Recombinant IscU39DAIscS was expressed in E. coli using either pDB1712, a plasmid containing only IscS and IscU39DA or, pDB1716, a plasmid that produces IscU39DA and contains the entire ISC operon under control of the arabinose promoter. TB1 cells transformed with pDB1716 were grown at 30°C in Luria Bertani broth with 100 µg/mL of ampicillin at 300 rpm. Growing cultures were induced with 0.3% w/v arabinose (Spectrum), 0.75 mM ammonium iron (III) citrate, 1 mM pyridoxine-HCl, and 1 mM cysteine, at OD600 = 0.4 - 0.6. Cells were harvested 4 h after induction and resuspended in 1.5 volumes of degassed argon-sparged 50 mM Tris buffer (pH 8) containing pepstatin (0.14 mg/liter) and phenylmethanesulfonyl fluoride (0.5 mM) and DNase. Cells were lysed by passage through French cell press (12,000 psi). Cell lysate was centrifuged for at 95,000 x g for 1 h at 4°C in a Beckman Coulter Optima LE-80K ultracentrifuge using a TI 45 rotor.
Recombinant IscU39\textsuperscript{DA}IscS was purified on the bench top under anoxic conditions maintained by using a Schlenk line and degassed argon-sparged buffers as described previously for IscU39\textsuperscript{DA}IscS purified from \textit{A. vinelandii} (Raulfs et al., 2008).

**Chemical analysis.** Iron, sulfide, and PLP analyses were performed as previously described (Raulfs et al., 2008).

**Separation of apo- and holo- IscU39\textsuperscript{DA}IscS over anion exchange chromatography.** Separately purified apo-IscU39\textsuperscript{DA}IscS and holo-IscU39\textsuperscript{DA}IscS (30 mgs each) were mixed, diluted in 50 mM Tris, 20 mM NaCl, pH 7.4 and passed over an anion exchange column. Loaded protein was washed with the same buffer containing 45 mM NaCl and eluted in a 20 mL gradient from 45 mM to 170 mM NaCl.

**Reconstitution of \textit{E. coli} derived IscU39\textsuperscript{DA}IscS.** \textit{E. coli} cells expressing pDB1716 were grown in LB ampicillin media and induced with arabinose for 4 h at an \textit{OD}_{600} = 0.5 (no iron, cysteine, or pyridoxine was included in the induction cocktail). Harvested cells were resuspended in Buffer A (50 mM Tris, 10\% glycerol, 4 mM MgCl, pH7.4). Crude lysate was processed by French cell press at 12,000 psi, and centrifuged at 95,000 x g for 1 h at 4\^\circC in a Beckman Coulter Optima LE-80K ultracentrifuge using a Ti 45 rotor. Twenty milliliters of crude extract was incubated with 2 mM ferrous ammonium sulfate and 1 mM L-cysteine for 40 min at room temperature. The sample was subsequently loaded onto a Q-sepharose anion exchange column, washed with Buffer A for two column volumes, and eluted with the same buffer containing 200 mM NaCl. UV-visible absorption spectra were taken using anaerobically sealed quartz cuvettes. The molarity of the solution was normalized to the spectrum of native IscU39\textsuperscript{DA}IscS shown for the \textit{A. vinelandii} purified sample.

**Cysteine desulfurase kinetic analyses.** The holo-IscU39\textsuperscript{DA}IscS data set presented in Figure 3.6 has been previously published (Raulfs et al., 2008) but is shown here for comparison purposes. Kinetic assay methods for apo-IscU39\textsuperscript{DA}IscS were performed according to previously published protocols (Raulfs et al., 2008).

**Visualization of IscS and IscU/IscU39\textsuperscript{DA} populations in crude extracts from ISC plasmid expressed in \textit{E. coli}.** \textit{E. coli} cells containing a genomic deletion in \textit{hscA} were transformed with various arabinose plasmids, pDB1720, pDB1716, pDB1722, or pDB1719. The \textit{hscA} minus strain was a generous gift from Dr. Wayne Outten
(University at South Carolina), and was constructed by inserting a chloramphenicol cartridge in hscA of E. coli strain W3110 (unpublished strain). Transformed cells were grown at 30°C in LB broth cultures with 100 µg/mL of Ampicillin at 300 rpm. At an OD$_{600} = 0.4 - 0.6$ cultures were induced with 0.3% w/v arabinose (Spectrum), 0.75 mM ammonium iron (III) citrate, 1 mM pyridoxine-HCl, and 1 mM cysteine. Cells continued to grow and were harvested 4 h after induction. Harvested cells were resuspended in 1.5 volumes of degassed argon-sparged 50 mM Tris buffer (pH 8) containing pepstatin (0.14 mg/liter) and phenylmethanesulfonyl fluoride (0.5 mM). Cells were lysed by french cell press (20,000 lb/in$^2$). Cell lysate was centrifuged for 95,000 x g for 1 h at 4°C in a Beckman Coulter Optima LE-80K ultracentrifuge using a TI 45 rotor. All subsequent purifications were performed in a Coy anaerobic chamber. For each run, 20 mg of cell extract were applied to a 1 mL HiTrap Q HP (GE Healthcare) column using a 2 mL loop. Loaded protein was washed with 10 mL of Buffer A using an ÄKTAprime™ GE Healthcare purification system. Following washing, protein fractions were eluted in a 20mL 0 – 1 M gradient of NaCl at 1.0 mL/min. Elutions were monitored at 280 and 405 nm. Fractions were collected anaerobically, and equal volumes of each fraction were loaded on an SDS-PAGE for visualization.

**Western blotting.** Unstained SDS gels were transferred to nitrocellulose membrane according to previously published methods (Johnson et al., 2006). This protocol was slightly modified to probe for the presence of both IscS and IscU on a single membrane, and thus nitrocellulose was incubated with a 1:600 dilution of primary rabbit anti-sera of both *A. vinelandii* IscU and IscS simultaneously.

3.4 RESULTS

**Development of a recombinant expression system for IscU39DA**

In previous studies we used a system in which the entire ISC operon was expressed under its native promoter in the host organism, *A. vinelandii* (Raulfs et al., 2008). In these strains a second copy of the ISC operon was inserted in the genome under sucrose control, thus allowing manipulation of the endogeneous ISC operon while
maintaining cell viability by growth on sucrose. ISC protein expression was elevated 5-7 fold by the deletion of the negative regulatory protein, IscR. A C-terminal polyhistidine tag was inserted in iscU allowing efficient purification of the scaffold protein.

Although this method was successful for the isolation of cluster loaded scaffold species, it did not allow the accumulation of intermediates at levels amendable to characterization. In order to facilitate protein yield beyond native expression levels, the entire A. vinelandii ISC operon was cloned and expressed recombinantly under an arabinose inducible promoter forming plasmid pDB1720. From this parent plasmid, ISC mutations and deletions were easily constructed, bypassing the additional step of strain construction and selection. Strains and plasmids used in this study are shown in Figure 3.3.

**Recombinant IscU39DA is similar to native IscU39DA but has lower cluster occupancy**

Upon the construction of a recombinant system for IscU purification, we sought to identify if accumulated IscU species were similar to those isolated previously from A. vinelandii. Histidine tagged IscU39DA was purified from E. coli cells over-expressing pDB1716. Like native IscU39DA, recombinant IscU39DA was found to associate with IscS in a 1:1 ratio, forming a non-dissociating $\alpha_2\beta_2$ complex with a size of 117 kDa. The identity of IscS was further confirmed by western blot, and cysteine desulfurase assays. Like the native complex, recombinant IscU39DAIscS tetramer did not dissociate during anion exchange or gel filtration chromatography.

An obvious difference between the two species was the color of the sample upon purification. Native IscU39DAIscS complex is dark brown, whereas recombinant IscU39DAIscS was bright yellow (Figure 3.3, right panel). While ‘brown’ indicates the presence of an [Fe-S] cluster moiety, yellow is the color of the PLP cofactor in IscS implying the isolation of an ‘apo’ form of the $\alpha_2\beta_2$ complex. Not surprisingly these two species also have distinct UV-visible spectra, with native IscU39DAIscS containing an additional absorbance peak at ~ 320 nm and a shoulder at 520 nm (Figure 3.3 left panel). The subtraction of recombinant IscU39DAIscS from native- IscU39DAIscS yields a spectrum with characteristics of [2Fe-2S] cluster loaded IscU (Figure 3.3 inset).
subtraction spectrum is not identical to the [2Fe-2S] IscU spectra, but more closely resembles reconstituted [2Fe-2S] IscU39\textsuperscript{DA} from \textit{A. vinelandii}, which has previously been shown to have a shifted UV-vis absorption spectrum in comparison to wild-type [2Fe-2S] IscU (Unciuleac et al., 2007). Chemical analysis of recombinant IscU39\textsuperscript{DA}IscS revealed variation in iron and sulfide content ranging from 0.1- 0.3 Fe and 0.1 – 0.4 S per αβ subunit. Purification of recombinant IscU39\textsuperscript{DA}IscS using the Nano DeBee homogenizer to produce cell lysates resulted in slightly elevated cluster occupancy (our unpublished results). For reconstitution, kinetic, and conformational studies, however, only IscU39\textsuperscript{DA}IscS samples with occupancy less than 0.10 ± 0.07 Fe and 0.14 ± 0.03 S per αβ subunit were used. The occupancy difference between these samples and native IscU39\textsuperscript{DA}IscS has lead to our loose terminologies of ‘apo’ for plasmid derived IscU39\textsuperscript{DA}IscS and ‘holo’ for natively expressed IscU39\textsuperscript{DA}IscS.

To investigate if apo and holo α₂β₂ are conformationally distinct, equal amounts of apo and holo complex were mixed and passed over a Q-sepharose anion exchange column. The separation of these two species can be clearly seen in Figure 3.4. Since the Q-sepharose resin contains a positively charged nitrogen group, we hypothesized that the more electropositive Holo-IscU39\textsuperscript{DA}IscS would bind less tightly to the column. Despite the added charge of attached clusters, we found the opposite to be true, with holo-IscU39\textsuperscript{DA}IscS eluting after apo-IscU39\textsuperscript{DA}IscS upon the initiation of a shallow salt gradient. This has also been seen with wild-type IscU, in which apo-IscU elutes before holo-IscU via anion exchange chromatography upon initiation of a shallow salt gradient (Raulfs et al., 2008) (See Figure 2.2). These data indicates that the [2Fe-2S]\textsuperscript{2+} cluster of the holo complex may be buried within the α₂β₂ tetramer, perhaps causing exposure of a more electronegative surface for binding to the positive Q-sepharose resin. These data corroborates our previously published results showing that clusters on holo-IscU39\textsuperscript{DA}IscS are resistant to the presence of molecular oxygen, are therefore more likely to be occluded within the scaffold site (Raulfs et al., 2008).

\textbf{Recombinant IscU39\textsuperscript{DA}IscS can be reconstituted}

Recombinant IscU39\textsuperscript{DA} expressed from pDB1716 exhibits low cluster occupancy even when grown in the presence of iron and cysteine. When over-expressed with
elevated iron and cysteine in *E. coli*, IscU39DAIscS has 0.2 ± 0.08 Fe per αβ subunit, compared to 1.2 ± 0.25 Fe per αβ subunit when expressed in *Azotobacter*. On the other hand, wild-type IscU expressed using the arabinose plasmid, pDB1720, was found to contain [Fe-S] clusters at levels similar to those observed with native IscU from *A. vinelandii* (O’Carroll, 2009) (Table 3.1). We hypothesize that low occupancy of recombinant IscU39DAIscS is related to the rapid induction of the P ara promoter, which has been demonstrated to induce significant amounts of protein less than one minute after the addition of arabinose (Guzman et al., 1995). Whereas expression of wild-type IscU allows dynamic association with IscS for cluster assembly, the immediate induction of plasmid expressed IscU39DA may lock the scaffold variant into a complex with IscS, limiting proper insertion of both iron and sulfur. This theory is bolstered by the appearance of *E. coli* cell pellets expressing pDB1720, which are a dark brown color suggesting the presence of iron sulfur clusters, versus pDB1716 cell pellets, which are a light tan color indicating a lack of synthesized [Fe-S].

The apo-IscU39DA scaffold is not permanently inaccessible to small molecules however, because a recombinant IscU39DAIscS sample with low cluster occupancy can be reconstituted to levels near those of native IscU39DAIscS complex upon incubation with excess iron and cysteine (Figure 3.5). Figure 3.5 shows three species of IscU39DAIscS: (1) native complex isolated from *A. vinelandii*, (2) apo complex similarly purified but isolated by over-expressing the isc genes in *E. coli* cells grown with iron, cysteine, and pyridoxine, and (3) reconstituted IscU39DAIscS. The reconstituted sample was obtained by incubating *E. coli* crude extracts expressing pDB1716 with 2 mM ferrous ammonium sulfate and 1 mM L-cysteine for 40 min at room temperature followed by anion exchange purification as described in the materials and methods. The results of this experiment reveal that new [Fe-S] can be constituted on the apo-IscU39DAIscS scaffold. Additionally, the incubation of apo and holo IscU39DAIscS with a fluorescent thiol reagent, I-AEDANS quenched with excess L-cysteine, reveals increased fluorescence for apo vs. holo complex, indicating that the scaffold protein on Apo-IscU39DAIscS has more accessible cysteine residues for cluster assembly (data not shown).
We conclude the $\alpha_2\beta_2$ structure is flexible enough to allow iron sulfur cluster assembly on the scaffold site. Importantly, the isolation of an apo-IscU39$^{DA}$IscS complex indicates that an iron sulfur cluster is not a prerequisite for IscU and IscS association and further suggests that [2Fe-2S] cluster assembly occurs after the association of IscS and IscU. In a separate control experiment an IscU39$^{DA}$IscS328$^{CA}$ complex was isolated, further indicating that the association between IscS and IscU is not dependent on the active site cysteine of IscS, or the ability to mobilize sulfur for cluster synthesis. Our findings imply that the $\alpha_2\beta_2$ tetramer, not IscU alone, serves as the in vivo scaffold for de novo cluster assembly.

**Apo IscU39$^{DA}$IscS exhibits the same kinetic properties as Holo IscU39$^{DA}$IscS**

The activity of the family of group II cysteine desulfurases (SufS) have been shown to be accelerated by the presence of accessory proteins (Outten et al., 2003). Group I cysteine desulfurases (NifS, IscS) are considerably more independent of accessory proteins, although cysteine desulfurase activity has been observed to increase in the presence of target proteins (Zheng and Dean, 1994). Considering the influence of other proteins on desulfurase activity, we sought to understand how interactions with the small 14 kDa IscU protein may affect desulfurase function of IscS. The isolation of an Apo form of the IscU39$^{DA}$IscS complex from *E. coli* provided the opportunity to probe the influence of cluster loaded vs. unloaded scaffold on IscS activity.

The binding of L-cysteine to the PLP active site of IscS causes a characteristic blue shift in the UV/visible spectra. While the exact structural feature that gives rise to the 340 nm absorbance peak is not known, it has been proposed to be related to the formation of a geminal diamine intermediate that occurs as the PLP oscillates between internal and external aldimine states (Counts et al., 2007). The blue shift characteristic of cysteine desulfurases was observed previously when L-cysteine was added to IscS complexed with holo-IscU39$^{DA}$ (Raulfs et al., 2008) and Apo-IscU39$^{DA}$ (Figure 3.6A), indicating that neither IscU nor the cluster affect initial L-cysteine binding to PLP. We have capitalized on this feature using it to measure intermediate kinetic states of IscS, Apo- IscU39$^{DA}$IscS, and Holo- IscU39$^{DA}$IscS. The geminal diamine
intermediate occurs twice during the desulfurization of L-cysteine. The initial geminal diamine occurs upon L-cysteine binding when PLP goes from an internal to external aldimine, and secondly upon alanine release when PLP re-forms an internal aldimine with Lysine 203. Consequently the 340 nm peak represents all the transition steps occurring between substrate binding and product release. The shifted peak at 340 nm, however, was confounded by the absorbance of the [2Fe-2S] cluster for holo IscU39DAIscS, and thus the corollary drop at 400 nm was chosen for monitoring IscS activity.

These data shown in Figure 3.7 has already been published for Holo-IscU39DAIscS, but is shown here for comparison purposes. We previously reported similar L-cysteine affinities (Kd ~ 0.6 mM) for both IscS and holo-IscU39DAIscS. Apo-IscU39DAIscS has the same L-cysteine affinity curve (Kd ~ 0.6mM) as both IscS and holo-IscU39DAIscS (Figure 3.7A). As substrate concentrations increase beyond 3mM, the binding of L-cysteine to the active site of IscS is more likely to be inhibited for holo-rather than apo IscU39DAIscS (Figure 3.7A). The reason for this difference is not clear, although it could be related to structural differences between apo- and holo-IscU39DAIscS. Inhibition of desulfurase activity upon incubation with excess L-cysteine is a common feature of IscS type proteins. (Lacourciere and Stadtman, 1998; Mihara et al., 2000; Zheng et al., 1998). While we are only measuring the apparent K_d and not enzymatic activity, it is worth noting that non-Michaelis Menten behavior of cysteine desulfurases at increasing substrate concentrations has previously been attributed to negative cooperativity effects and the binding of a second ‘blocking’ L-cysteine at the active site (Behshad et al., 2004; Outten et al., 2003).

Our previous results show that following rapid L-cysteine binding and the formation of a characteristic peak at 340 nm, IscS relaxes to the resting state in less than one minute (k_{relaxation} = 0.03 s^{-1}). The holo-IscU39DAIscS complex relaxes much slower than ‘un-complexed’ IscS, taking nearly 10 minutes to fully return to the as-isolated state, at a rate of 0.0035 s^{-1}(Raulfs et al., 2008). With the isolation of an apo-form of the α2β2 complex, we were interested to determine if a clusterless scaffold would allow a relaxation rate similar to un-complexed IscS. The apo-IscU39DAIscS relaxation however was similar to holo-IscU39DAIscS with a rate of 0.0024 s^{-1} (Figure 3.7B). Regardless of
the presence of a cluster, the relaxation is ~10x slower for ‘complexed’ vs. ‘uncomplexed’ IscS. These data reveals that it is the IscU39DA interaction not the presence of cluster, which limits the kinetics of IscS, causing the PLP::substrate adduct to remain in an extended intermediate state.

Since wild-type IscU and IscS form a loosely bound tetramer that readily dissociates it has not been possible to measure the effect of wild-type IscU on the cysteine desulfurase activity of IscS. These results may therefore be relevant only for the IscU39DAIscS scaffold. We conclude that the initial act of L-cysteine binding to PLP is not rate limiting for IscU39DAIscS but that some subsequent step, such as locking of the IscS active site loop, hydrogen abstraction, or alanine release may cause inhibition when IscS is complexed with Apo- or Holo- IscU39DA scaffold.

**Chaperone proteins, HscB and HscA, are not required for IscU39DA and IscS interactions**

The ISC chaperone proteins, HscB and HscA share homology to DnaK and DnaJ, known molecular chaperones that help prevent protein aggregation (Kawula and Lelivelt, 1994; Seaton and Vickery, 1994; Vickery and Cupp-Vickery, 2007). Given that ISC chaperones have recently been shown to be dispensable for [2Fe-2S] cluster assembly (O’Carroll, 2009) and that Hsp70s can be involved in the assembly/disassembly of protein complexes (Vickery and Cupp-Vickery, 2007), we conjectured that HscBA might ‘chaperone’ productive interactions between IscU and IscS. Thus we created arabinose expression plasmids with combinations of ΔHscBA and IscU39DA substitutions. The plasmids include:

- wild-type IscU (pDB1720);
- wild-type IscU and ΔHscBA (pDB1722); IscU39DA (pDB1716);
- and IscU39DA and ΔHscBA (pDB1719) (see Figure 3.3).  *E. coli* W3110 cells containing a deletion in genomic *hscA* were transformed with individual plasmids and grown and induced with arabinose. Twenty milligrams of protein crude extract from each growth was loaded and passed over an anion exchange column. The protein loaded column was subjected to a 20 mL gradient from 0 – 1 M NaCl to separate proteins based on pi. The elution profile was monitored at 405 nm for cluster absorbance and 280 nm
for protein absorbance (Figure 3.8 top panels - 280 nm profiles are not shown). The bottom panel of Figure 3.8 shows eluted fractions analyzed by immunoblot to visualize the ionic strength at which populations of IscS and IscU proteins are found to elute.

Crude extracts from cells transformed with pDB1720 resolved at least three distinct protein populations by immunoblot following anion exchange chromatography: IscU, IscU IscS complex, and IscS (Figure 3.8 middle left panel). Crude extracts lacking HscBA (W3110 cells transformed with pDB1722) also revealed the same pattern of protein elution as cells transformed with pDB1720 (Figure 3.8 middle right panel). The pDB1722 profile reveals little deviation from the pDB1720 profile in both the 405 nm elution profile and immunoblot (bottom panel Figure 3.8, pDB1720 and pDB1722). When *iscU*<sup>39</sup><sup>DA</sup> is expressed however (pDB1716, pDB1719), a dramatic visual difference is apparent both by the anion exchange chromatograph and immunoblot. The presence of the IscU<sup>39</sup><sup>DA</sup> variant, trumps the chaperone deletion, as both pDB1716 and pDB1719 fractions reveal IscU populations that have shifted to be complexed with IscS. Additionally, the line-shapes of IscU<sup>39</sup><sup>DA</sup> containing elution profiles, are more similar to each other than to the wild-type IscU line profiles (top panels Figure 3.8), which is likely a consequence of the tight conformer formed by IscU<sup>39</sup><sup>DA</sup>IscS versus a more loosely bound wild-type IscU IscS complex. While there may be subtle differences in IscU and IscS populations that this experimental method could not resolve, the findings suggest that the chaperone proteins have limited *in vivo* affect on the interactions of IscS and IscU and are not necessary for either the formation or dissociation of the IscS and IscU complex.

Supporting these observations, we subsequently purified IscU<sup>39</sup><sup>DA</sup> from crude extracts of cells transformed with pDB1712, a plasmid containing only IscU<sup>39</sup><sup>DA</sup> and IscS (The C-terminus of IscA is truncated in these plasmids, deleting the monothiol domain, and rendering the protein inactive). Purification of IscU<sup>39</sup><sup>DA</sup> from crude extracts expressing pDB1712 also resulted in the isolation of a tightly bound α<sub>2</sub>β<sub>2</sub> tetramer revealing that only IscS and IscU are necessary for complex association. Regardless of the availability of other ISC proteins, IscS and IscU are able to mediate their own interactions. The aspartate 39 residue of IscU is crucial for the dissociation of
the duo however, as the absence of this residue causes irreversible binding of IscS to the scaffold protein.

3.5 DISCUSSION

The construction of arabinose inducible plasmids expressing the entire ISC operon in *E. coli* has allowed abundant expression of ISC proteins and increased yield of histidine tagged IscU species. The expression and isolation of IscU39DA from this recombinant system has resulted in the purification of an IscU39DAIscS species that has the same oligomeric state, IscS kinetics, molecular size, and tight association as the native α2β2 tetramer. Recombinantly expressed IscU39DAIscS however, has cluster occupancy ranging from 5-15%, and was additionally found to have a different conformation than native IscU39DAIscS. These results suggest that while the presence of a cluster does not affect complex size or association, it could mediate structural rearrangements of the α2β2 tetramer.

Additionally, we have found that other ISC components are not necessary for facilitating IscS and IscU association, but that the highly conserved aspartate 39 residue of IscU is essential for α2β2 dissociation. Several lines of evidence support the idea that the α2β2 scaffold serves as the site of *de novo* cluster synthesis, and that cluster-loaded IscU dissociates from the scaffold upon completed [2Fe-2S] assembly. First, [Fe-S] cluster species can be reconstituted on the IscU39DAIscS scaffold indicating that even the variant platform is flexible enough for dynamic cluster assembly. Secondly, small populations of Apo wild-type IscU IscS complex have been isolated when ISC proteins are abundantly expressed from pDB1720 in *E. coli* (our unpublished results). Third, we have never isolated heterodimers or trimers of IscS and IscU, species that would be expected if the scaffold and desulfurase proteins engaged in a cycle of association, dissociation, and re-association events during cluster assembly. Lastly, it has not been possible to purify holo-IscU IscS α2β2 tetramer, indicating that IscS has a higher affinity for apo- vs. holo-IscU, and bolstering the idea that [2Fe-2S] IscU separates from the wild-type complex upon completed cluster assembly (O'Carroll, 2009).
Due to the labile nature of the IscU IscS assembly scaffold and the transient character of cluster assembly in general, it is extremely difficult to isolate intermediates of the \textit{in vivo} iron sulfur cluster assembly process. However, with the use of an aspartate to alanine substitution on IscU we have been able to isolate stable \textit{in vivo} forms of the $\alpha_2\beta_2$ scaffold providing insight into the process of cluster assembly. We propose that holo-IscU$^{39\text{DA}}$IscS represents an end point of [2Fe-2S] cluster assembly on the $\alpha_2\beta_2$ scaffold that is prevented from final complex dissociation by the absence of an aspartate-39 residue on IscU. Based on these findings, we propose a new model of \textit{in vivo} [2Fe-2S] assembly (Figure 3.9).

A major feature of our model is the independent 1:1 association of IscS and IscU to form an $\alpha_2\beta_2$ complex in the absence of auxiliary proteins. The $\alpha_2\beta_2$ tetramer serves as a functional scaffold \textit{in vivo}, upon which a [2Fe-2S] cluster is assembled. The tetrameric complex likely provides protection from oxygen for the assembly of nascent clusters but also is sufficiently flexible to accommodate new iron and sulfide on the IscU scaffold, which may shuttle back and forth between ‘open’ (exposed) and ‘closed’ (protected) conformers. The dissociation of the $\alpha_2\beta_2$ is partially dependent on the IscU 39 Asp residue upon completed [2Fe-2S] assembly.

While IscU$^{39\text{DA}}$IscS provides a model for the IscU IscS complex, it is clear that the 39$^{\text{DA}}$ intermediates are distinct from isolates of wild-type IscU IscS $\alpha_2\beta_2$ complex. For example, IscS does not stay tightly bound to wild-type IscU when exposed to increasing ionic strength (Raulfs et al., 2008). Additionally, the cluster on the Holo-IscU$^{39\text{DA}}$IscS scaffold is resistant to oxygen for over 24 h suggesting a conformation that protects the iron sulfur cluster from damage. Thus, the IscU$^{39\text{DA}}$IscS complex species should be thought of as intermediate stop points that represents a single snap-shot of a dynamic \textit{in vivo} assembly process.

In the wildtype $\alpha_2\beta_2$ scaffold we imagine an enhanced plasticity of the $\alpha_2\beta_2$ platform for the transport of iron and reducing agents to the IscU scaffold site. Indeed, IscU has been found to be very structurally fluid, a characteristic ascribed to its ‘molten globule’ like state (Bertini et al., 2003). In the NMR structure of \textit{Haemophilus influenza} IscU, the 39 Aspartate residue is situated close to the scaffold site, where three conserved cysteines provide thiol ligands for cluster coordination (Figure 3.10). Structural NMR
analysis of a *T. maritima* IscU40\(^{DA}\) variant found strong hydrogen bonding between Ala40 and Lys34 of the β1 strand of the β-sheet, creating a more structurally rigid protein (Bertini et al., 2003). In the wild-type ISC system we imagine that the conserved Asp 39 could influence the conformation of the IscU protein shifting the entire α\(_2\)β\(_2\) scaffold from a ‘closed’ to ‘open’ conformer to accommodate the addition of iron and sulfide on IscU. The proposed conformational shift of the α\(_2\)β\(_2\) platform may also be externally propagated by chaperone proteins and other interacting factors. Upon completed [2Fe-2S] assembly, the internal positioning of aspartate 39 may help facilitate final release of IscS.

The aspartate 39 residue is highly conserved in U-type scaffold proteins. As previously mentioned, work with Asp 39 variants of IscU have revealed that mutations at this position are transdominant with respect to wild-type IscU in a merodiploid strain of *Azotobacter* (Johnson et al., 2006). Multiple studies have also shown that the IscU39\(^{DA}\) scaffold is more structurally stable and less likely to transfer a cluster to apo-targets than wild-type IscU proteins. (Foster et al., 2000; Unciuleac et al., 2007; Wu et al., 2002a; Wu et al., 2002b; Yuvaniyama et al., 2000). The *in vivo* finding that Asp 39 not only stabilizes a [2Fe-2S] cluster on IscU, but is additionally essential for mediating interactions with IscS is a novel discovery. It expands our understanding of this variant and suggests that *in vivo* the destabilization of cluster loaded IscU is directly translated to the release of IscS from the α\(_2\)β\(_2\) platform.

In conclusion, we have found IscS and IscU to associate forming a stable yet structurally plastic α\(_2\)β\(_2\) scaffold for [2Fe-2S] assembly. It is worth mentioning that we have never isolated a [4Fe-4S] loaded IscU species during our purifications of histidine tagged IscU. The *in vivo* scaffold assembly site for [4Fe-4S] clusters still remains an open question, although it has been proposed that these clusters may be assembled on an IscU dimer or alternative scaffold, such as IscA or NfuA (Angelini et al., 2008a; Bandyopadhyay et al., 2008a; Krebs et al., 2001; Unciuleac et al., 2007). Our model of [2Fe-2S] cluster assembly supports the previously presented idea that the assembly of [2Fe-2S] modules proceeds the synthesis of a more labile [4Fe-4S] cluster (Agar et al., 2000a), which may require additional accessory proteins and enhanced structural protection. Lastly, the presence of the Asp-39 residue of IscU is crucial for facilitating
the dynamic association and dissociation of IscS and IscU and the ultimate release of IscU from the $\alpha_2\beta_2$ scaffold.
### Table 3.1 Iron and sulfide analysis of IscU and IscU39\textsuperscript{DA} isolated from recombinant and native expression systems

<table>
<thead>
<tr>
<th></th>
<th>IscU</th>
<th>IscU39\textsuperscript{DA}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recombinant (pDB1720)</td>
<td>Native (DJ1697)</td>
</tr>
<tr>
<td>Fe/IscU</td>
<td>1.26 ± 0.15</td>
<td>1.29 ± 0.05</td>
</tr>
<tr>
<td>S/IscU</td>
<td>1.14 ± 0.29</td>
<td>1.28 ± 0.19</td>
</tr>
</tbody>
</table>
Figure 3.1 Sequence alignment of IscU from different organisms

Figure 3.2 ISC strains and plasmids used in this study

*A. vinelandii* *isc* strains containing two copies of the *isc* operon, DJ1697 and DJ1766 are shown above. Arabinose controlled *isc* plasmids expressed in *E. coli* are shown below. The black dot in IscU represents an aspartate-39 to alanine substitution. Gray dashed boxes represent gene deletions.
Figure 3.3  UV-visible absorption spectra of apo- and holo- IscU39^{DA}IscS

UV-visible absorption spectra of 67μM apo-IscU39^{DA}IscS (gray dashed line) and 67μM holo-IscU39^{DA}IscS (black line). Inset. Subtraction spectra of holo-IscU39^{DA}IscS minus apo-IscU39^{DA}IscS (dashed line) compared to UV-vis spectra of [2Fe-2S] cluster loaded IscU (black line). Right. Photograph of holo- IscU39^{DA}IscS (top) and apo- IscU39^{DA}IscS (bottom) complex species in sealed cuvette.
Figure 3.4 Separation of an equimolar mixture of apo- and holo- IscU39DAIscS via anion exchange chromatography

(A) Chromatograph showing separation of apo- and holo- IscU39DAIscS from anion exchange column during a shallow NaCl gradient. (B) Appearance of apo- (yellow) and holo- (brown) IscU39DAIscS on protein loaded anion exchange column.
Figure 3.5 UV-visible spectra of apo-, reconstituted-, and holo-IscU39\textsuperscript{DA}IscS

*Inset.* Iron occupancy of included IscU39\textsuperscript{DA}IscS species.

For the reconstituted sample, crude extract of *E. coli* cells expressing proteins from the pDB1716 plasmid were incubated with 2 mM ferrous ammonium sulfate and 1 mM L-cysteine for 40 min at room temperature and purified by anion exchange as described in the materials and methods.
Figure 3.6 Characteristic blue shift of PLP cofactor upon substrate binding

(A) UV-visible absorption spectra of recombinant Apo IscU39DAIscS (34µM) in the as-isolated state (solid line) and immediately after the addition of 30 molar excess L-cysteine (dashed line). (B) Formation of geminal diamine intermediate thought to represent ~340 nm absorbance upon substrate binding to PLP. This figure was taken from Counts et al. 2007 (Counts et al., 2007). (ACS License # 2117130779986)
Figure 3.7  Equilibrium and kinetic features associated with IscS catalyzed desulfurization of apo- and holo- IscU39\textsuperscript{DA}IscS complex

(A) ΔmAbs\textsubscript{400nm} for 50µM Apo IscU39\textsuperscript{DA}IscS (gray circle) and 50µM Holo IscU39\textsuperscript{DA}IscS (black circle) upon increasing concentrations of cysteine. (B) Time dependent relaxation of Abs\textsubscript{400nm} for Apo IscU39\textsuperscript{DA}IscS (gray circle) and Holo IscU39\textsuperscript{DA}IscS (black circle) following the addition of a 10-fold molar excess of L-cysteine.
Figure 3.8 Separation of IscS and IscU via anion exchange chromatography from crude extracts of arabinose induced plasmids

Arabinose plasmids used for expression in this experiment include, pDB1720, pDB1716, pDB1722, and pDB1719. (Top) Anion exchange chromatography profiles monitored at 405nm of pDB1720 (iscSU_{his8}AhscBAfdx) and pDB1716 (iscSU39_{his8}AhscBAfdx) (left panel) and pDB1722 (iscSU_{his8}A\Delta hscBAfdx) and pDB1719 (iscSU39_{his8}A\Delta hscBAfdx) (right panel). Tick marks denote 40 mAbs units (Bottom) IscS and IscU western blot of anion exchange fractions 1-8 of pDB1720 and pDB1716 elutant (left panel) and pDB1722 and pDB1719 (right panel). White arrows point to IscS and gray arrows point to IscU.
Figure 3.9 Proposal for *in vivo* [2Fe-2S] cluster assembly

Model shows that IscS and IscU associate to form a $\alpha_2\beta_2$ complex upon which an iron sulfur cluster may be build. We envision that this complex may shuttle back and forth between ‘protected’ and ‘exposed’ states allowing entry of iron and sulfide. Eventually, a holo-IscU IscS complex is formed which dissociates into holo-IscU and IscS.
Figure 3.10 Structure of *H. influenza* IscU

3.7 REFERENCES


CHAPTER 4

Characterization of a Deletion IscR phenotype in *Azotobacter vinelandii*

I was the major contributor to the experiments described in this chapter, however I am grateful for the help of many colleagues. Dr. Miheala Uniculeac-Sandu constructed the original DJ1601 strain. Dr. Deborah Johnson constructed DJ1421, DJ1525, DJ1524, and DJ1504. Dr. Patricia Dos Santos and Lauren Lignon made the *iscR-lacZYKn-fdx* construction in which *isc* genes: *iscS iscU iscA hscBA* were removed and replaced with *lacZYKn*<sup>R</sup>. This plasmid was necessary for the construction of DJ1731, DJ1739, and DJ1751. Valerie Cash made the Φ(*hscA’-lacZYKn*) plasmid constructions. Dr. Ina O’Carroll gave me the idea to do a 2-dimensional analysis of DJ1601 and DJ1877 from plate grown cultures. Dr. Timothy Larson and Nathan Larson were instrumental in the construction of Δ*cysE3* strains. Dr. Stephen Melville allowed me access to his equipment and instructed me on use of the DIC light microscope. Dr. David Popham gave me the protocol for fixing cells with glutaraldehyde. Dr. Keith Ray performed sequencing of the protein bands from 2-d gel analysis using LC-Mass spectrometry. Visiting scientist, Dr. Birgit Alber and Dr. Jiann-Shin Chen provided very helpful conversations on n-butanol production and aldehyde and alcohol dehydrogenase activity assays.
4.1 ABSTRACT

In this study we investigated the role of IscR regulation in the *A. vinelandii* genome. We determined that this protein plays an important role in regulating the *isc* operon, the neighboring *trmH cysE2* gene region, and an internal promoter upstream of *hscB hscA fdx*. Additionally, we have discovered a ‘small’ colony phenotype associated with the loss of IscR function, which can be partially relieved by diazotrophic growth conditions. We show that deletion of IscR function causes the formation of cyst-like cells and the simultaneous increase of aldehyde and alcohol dehydrogenase proteins. In conclusion, we discuss the role of IscR in [Fe-S] cluster biogenesis and oxidative stress management in *A. vinelandii*.

4.2 INTRODUCTION

Iron-sulfur proteins are crucial for the proper functioning of numerous intracellular processes involved in metabolism, respiration, and DNA repair. As a result, the proper maturation of [Fe-S] clusters is a crucial intracellular task, and is managed by one of three biosynthetic assembly systems: the ISC, SUF, or NIF systems. The ISC system has been termed a ‘housekeeping’ operon and in *E. coli* and *A. vinelandii*, it functions to produce [2Fe-2S] and [4Fe-4S] clusters under standard growth conditions. In *E. coli*, the SUF operon operates as a back-up system, producing [2Fe-2S] and [4Fe-4S] clusters during periods of oxidative stress and iron depletion. In nitrogen fixing organisms, the *nif* system functions in the specialized production of iron sulfur units for the activation of apo-nitrogenase proteins. Not all of these cluster assembly systems are functionally similar in different organisms. For example in gram-positive bacteria, plant plastids, and some archaea the SUF operon has evolved as the primary [Fe-S] producing mechanism. It is likely that continued work in this field of iron-sulfur cluster biogenesis will lead to the discovery of additional novel and adaptive [Fe-S] assembly machineries.

Improper synthesis of iron-sulfur clusters can result in the release of free iron and sulfide in the cytoplasm that results in the formation of toxic oxygen radicals which can bind cytochromes, preventing respiration. As the toxic side effects resulting from the
over- or under-production of [Fe-S] clusters is highly detrimental, the amount of cluster production must be kept in check with cellular demands for [Fe-S]. In many β- and γ-proteobacteria, regulation of cluster production of both the ISC and SUF system is controlled a single protein, IscR, from the Rrf2 family of regulators (Giel et al., 2006; Schwartz et al., 2001; Yeo et al., 2006).

The IscR protein contains an N-terminal helix-turn-helix DNA binding region and a second domain with three highly conserved cysteine residues, suggesting an iron-sulfur cluster binding site. Based on genomic context and sequence homology with members of the AraC/XylS transcriptional family of regulators, IscR was theorized to be involved in the regulation of the iscRSUA operon (Schwartz et al., 2001). The first published work on the protein revealed that IscR can bind a [2Fe-2S] cluster and additionally represses transcription of its own promoter in vitro (Schwartz et al., 2001). This study additionally found IscR mediated ISC expression to be dependent on the presence of essential cluster machinery, IscS and HscA, suggesting the importance of the [2Fe-2S] cluster for IscR function. Based on these findings the authors proposed that [2Fe-2S] cluster loaded IscR represses expression of the ISC system through an auto-regulatory feedback cycle. In this manner, ISC expression is limited when the cellular demand for iron-sulfur clusters is met.

A set of secondary studies revealed that apo-IscR activates expression of the SUF operon in E. coli (Giel et al., 2006). In this case the [2Fe-2S] cluster of IscR also serves a regulatory feature. When the cluster is present, holo-IscR represses expression of the isc operon, when it is absent, apo-IscR activates expression of the suf genes. In this way, IscR dependent regulation allows redundant elevation of both ISC and SUF systems under oxidative stress or iron depletion, conditions in which IscR is more likely to be in found in an apo form.

While no null phenotypes have been reported for IscR, many organisms require this regulatory protein for virulence and pathogenesis. For example, IscR is necessary for maximal suf expression and virulence in Shigella flexneri (Runyen-Janecky et al., 2008) and pathogenicity of Erwinia chrysanthemi (Rincon-Enriquez et al., 2008). The deletion of IscR results in an H₂O₂ sensitive phenotype in Pseudomonas aeruginosa (Choi et al., 2007) and nitric oxide sensitivity in Burkholderia mallei (Jones-Carson et al., 2008).
Because host cells generate superoxide as a mechanism to fight invasion of pathogens, controlled [Fe-S] production is necessary to repair and replenish damaged [Fe-S] proteins essential for cell growth.

In addition to regulation of the *suf* operon, transcriptional profiling analysis revealed that IscR controls the expression of over 40 genes in *E. coli* (Giel et al., 2006), including operons involved in anaerobic respiration such as hydrogenase-1 (*hyaABCDEF*) and hydrogenase-2 (*hybOABCDEFG*) and nitrate reductase (*napFDAGHBC*). IscR was also found to regulate an alternative scaffold protein for [Fe-S] assembly, *nfuA*\(^2\). While these data are informative, it does not indicate if transcriptional control of genes proceeds through a direct or indirect mechanism. Interestingly, one of the operons found to be upregulated in *ΔiscR* strains was *fimAICDFGH*, a gene region involved in the synthesis of type I fimbriae for biofilm attachment. Wu and Outten have recently proposed that regulation of the *fimA* operon is indirect and proceeds through apo-IscR controlled expression of *fimE* recombinase which subsequently turns off expression of the *fimA* promoter through a site specific inversion mechanism (Wu and Outten, 2009). Decreased biofilm formation was also found to take place under nutrient limited conditions specific to the presence of Apo-IscR, such as low iron or oxidative stress.

As more studies are performed with IscR there is a growing understanding of the importance of this protein in iron sulfur cluster regulation and general stress management. The varied and growing reports on IscR reveal that we have much to learn about how this protein dually facilitates [Fe-S] cluster biogenesis and cellular response to environmental stress. Here we discuss IscR regulation of [Fe-S] cluster biosynthesis and oxidative stress management in *A. vinelandii*. This organism makes a particularly interesting model system for addressing these issues because it can fix nitrogen and has been cited as having one of the highest known respiratory rates for any bacteria (Kelly et al., 1990; Sandercock and Page, 2008a). Additionally, the *Azotobacter* genome does not contain a complete *suf* system, and therefore allows study of non-SUF related regulatory effects of IscR. In this study we seek to more fully understand the diverse roles of apo-

\(^2\) Referred to as *yhgf* in Giel et al. (2006)
and holo-IscR in the regulation of [Fe-S]-related biosynthetic genes on the *A. vinelandii* genome, and in serving as a sensor for intracellular oxidative stress.

4.3 MATERIALS AND METHODS

**Growth and media.** *A. vinelandii* strains were grown at 30°C on Burk’s minimal medium containing 2% sucrose or 2% glucose as the sole carbon source as indicated. BN medium refers to Burk’s medium with 13 mM ammonium acetate, B-Urea medium refers to Burk’s medium with 10 mM urea, B medium refers to Burk’s medium without any added nitrogen source. –Mo refers to Burk’s medium with 7 mM ammonium acetate and no added molybdenum. Antibiotics were used at a concentration of: 0.08 µg/mL ampicillin, 0.5 µg/mL kanamycin, 0.05 µg/mL gentamicin, and 0.1 µg/mL streptomycin. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to a final concentration of 60 µM.

**Strain constructions.** Most strains used in this study are derived from DJ1421 (see Table 4.1 for a complete listing strains), which is merodiploid, containing an additional copy of the *isc* operon under the control of an inducible sucrose promoter (Johnson et al., 2006). Parent strain DJ1731 was constructed by the transformation of DJ1421 with pDB1641, a plasmid which was constructed piece-wise by PCR in order to replace the interior *isc* genes (*iscS iscU iscA hscB hscA*) with a 6.2kb SmaI lacZYKn cartridge forming *iscR–lacZYKn–fdx*. DJ1731 was positively selected on BNKn plates and double reciprocal recombination and insertion of the *lacZYKn* cartridge was confirmed by PCR. DJ1504 was constructed by transformation of DJ1421 with pDB1179, a plasmid containing a 5.5kb PstI lacZY cartridge insertion inbetween the first PstI site of *trmH* and PstI site of *cysE2*. DJ1525 and DJ1524 were both constructed by transformation of DJ1421 with pDB1468, a plasmid carrying a 6.1kb SmaI lacZYKn cartridge insertion at the AleI site of *hscA*, forming Φ(*hscA'–lacZYKn*). Transformants of DJ1421 x pDB1468 were selected for on BN-Xgal-Kn plates. DJ1525 transformants, containing Φ(*hscA'–lacZYKn*) in the endogeneous *isc* operon, were glucose sensitive and selected for on BN-Xgal-Kn plates followed by PCR verification of the orientation and location of the insert.
DJ1524 transformants containing Φ(hscA'·lacZYKn) in the merodiploid copy of the isc operon, were selected for on BN-Xgal-Kn plates. These transformants were capable of growth on glucose and exhibited decreased lacZ expression on BG-Xgal-Kn plates. Proper insertion and location of the lacZYKn cartridge was also confirmed by PCR as previously described (Johnson et al., 2006). All ΔiscR and iscR92CA daughter strains in this study were constructed from one of several parent strains: DJ1525, DJ1731, DJ1524, or DJ1504. DJ1532 is a recA::GmR derivative of DJ1525, in which the recombinase A gene has been inactivated by a gentamicin insertion cartridge. All ΔiscR strains were constructed using the plasmid, pDB1490, which contains a 120 bp SphI in-frame deletion in IscR, removing Ala^{21} to Gly^{60}. Genomic substitutions of IscR92CA were constructed by transformation of A. vinelandii strains with pDB1507, a plasmid containing iscR with a sited directed mutation in Cys^{92} (GCA). DJ1819 was constructed from DJ155, a strain containing a deletion in cysE1 with pDB1490 (ΔiscR) and pDER (cysE3::Kn).

Transformants were selected on supplemented media containing BNKn with nutrient broth.

**LacZ activity assays of IscR strains.** β-galactosidase activity of A. vinelandii strains was determined *in vivo* using a whole cell assay adapted from J. H. Miller 1972 (Miller, 1972). Cell cultures were grown in BN media to an OD_{600} of 0.5, harvested and resuspended in Z buffer (40 mM NaH_{2}PO_{4}, 60 mM Na_{2}HPO_{4}, 10 mM KCl, 1 mM MgSO_{4}, and 30 mM β-mercaptoethanol) to give a final resuspended OD_{600} of 1.0. The substrate, ONPG, (o-nitrophenol-β-D-galactopyranoside) was dissolved in 100% DMF and added to resuspended cells to a final concentration of 0.2 mM. LacZ activity was monitored at discrete time points by stopping the reaction with the addition of 1M Na_{2}CO_{3} to a final concentration of 50 mM and measuring absorbance at 414 nm. β-galactosidase activity was expressed as ΔAbs (414 nm) / min / OD_{600} resuspended culture.

**Immunoblot.** Fifty micrograms of crude extract from DJ1525 and DJ1562 strains was loaded per SDS-PAGE lane and transferred to nitrocellulose membrane as previously described (Johnson et al., 2006). Polyclonal primary rabbit antiserum to A. vinelandii IscU was used at a dilution of 1:600. Alkaline phosphate-conjugated anti-rabbit goat immunoglobulin G served as the secondary antibody. A chemiluminscent detection
system was used (LumiPhosWB; Pierce) and the BioRad Gel Doc XRS was used for photographing the immunoblot and quantifying blot density.

**Light Microscopy.** DJ1601 and DJ1421 cells from BN plates were pelleted and resuspended in 840 µL of 0.5 M NaH₂PO₄ solution, pH 7.0. Fifty microliters of 25% EM grade glutaraldehyde was added and the cell solution was allowed to gently vortex at room temperature for 1 h. Five microliters of cell solution was placed on a glass slide with a sealed cover slip and imaged with a Zeiss – Differential Interference Contrast Light microscope.

**Frequency of suppressor mutations.** DJ1601 cells were grown in 500 mL of liquid BN media, at 30°C and 300 rpm. Cell aliquots were taken at an OD₆₀₀ of 0.2, 0.4, 0.6, and > 1. Aliquots were diluted in sterile phosphate buffer (14 mM KH₂PO₄, 46 mM K₂HPO₄) from 10⁻¹ to 10⁻⁶ as appropriate to growth stage, and plated on BN agarose plates. After 5 days of growth incubation at 30°C, large colonies representing spontaneous suppressor mutants were counted per plate and growth condition and plotted for comparison. Four spontaneous suppressor mutant *A. vinelandii* strains were stored, and one, DJ1877 was analyzed further by 2-dimensional gel electrophoresis.

**2-dimensional gel electrophoresis.** DJ1601 and DJ1877 were T-streaked on five BN plates and allowed to grow at 30°C for 5 days. Following this incubation period, plate grown cells were collected and resuspended in 10 mL of cold 10 mM Tris-HCl, 5 mM MgCl₂-H₂O buffer at pH 7.4. Resuspended cells were processed by french cell press at a 12,000 psi and centrifuged for 1 h at 35,000 rpm using a Ti45 rotor and Beckman Coulter Optima LE-80k Ultracentrifuge (95,000 x g). Crude cell lysate (150 µg) was ‘cleaned’ using the 2D gel clean-up kit following the manufacturer’s instructions. The final ‘cleaned’ 150 µg protein sample, was re-suspended in 210 µL of rehydration buffer and loaded onto 11cm GE Health-Care Immobline™ (IPG) DryStrips, pH 3-10. Isoelectric focusing of IPG strips was achieved overnight using a GE Health-Care Ettan IPGphor 3. The next day, IPG strips were equilibrated in SDS equilibration buffer containing 75 mM Tris, 6M Urea, 30% glycerol (w/v), 2% SDS (w/v), bromophenol blue, and 6 mM DTT. Equilibrated strips were loaded onto BioRad 2-dimensional Criterion gel system using Criterion precast Tris-HCl gel cassettes. The upper and lower chambers were filled with
running buffer (66 mM Tris, 5.2 mM glycine, 0.1% (w/v) SDS). The gel was run for 15 minutes at 35 mA and 1.5 h at 70 mA and stained in Coomassie blue dye for 12 - 16 h.

**Alcohol Dehydrogenase protocol.** An activating buffer containing 50 mM Tris, 20 mM NADH, pH 7.5 was made. For each strain, 50 µL of crude extract of plate-grown cells was added to a 1 mL of activating solution. The reaction was initiated by the addition of substrate, 20 µL of 13.8 mM butyraldehyde (dissolved in methanol). Activity was monitored at room temperature using a Cary 50 Bio UV-visible spectrophotometer by measuring a decrease in absorbance at 340 nm. Final activity values are reported relative to isocitrate dehydrogenase activity. Isocitrate activity was measure as described previously (Johnson et al., 2006).

**Aldehyde Dehydrogenase.** An activating buffer containing 50 mM CHES, 2 mM NAD+, 0.5 mM coenzyme A, and 5 mM DTT, pH 8.6 was made. For each strain, 25 µL of crude extract of plate-grown cells was added to a 1 mL of activating solution. The reaction was initiated with the addition of 20 µL of 13.8 mM butyraldehyde (dissolved in methanol) and activity was monitored at 340 nm using a Cary 50 Bio UV-visible spectrophotometer at room temperature. Final activity values are reported relative to isocitrate dehydrogenase activity as previously described (Johnson et al., 2006).

4.4 RESULTS

**IscR regulation of ISC expression is cluster dependent**

Previous work demonstrates that IscR serves as a feed-back regulator of *isc* expression in *E. coli* (Schwartz et al., 2001). In order to determine if the same model of IscR-dependent *isc* regulation holds true in *Azotobacter vinelandii*, we sought to measure *isc* expression in *Azotobacter* strains containing substituted or deleted portions of the IscR protein. To investigate this question, we constructed a set of strains in which indicator genes, *lacZY*, were fused to the chaperone *hscA* (DJ1525, see table 4.1). However, because *hscA* is an essential gene and the *lacZY* fusion causes insertional inactivation of the chaperone protein, a previously described parent strain (DJ1421) was used which contains an additional copy of the entire *isc* gene region under sucrose inducible control
DJ1421 is not viable on glucose, but when grown on growth medium containing 2% sucrose, enough hscA is expressed from the sucrose dependent ISC promoter to allow normal growth. Figure 4.1, right panel, indicates that both IscR92CA and ΔiscR strains have an ~5x over-expression of hscA::lacZY compared to wild-type IscR. In addition, the three conserved cysteines of IscR, and several residues in the DNA binding domain including charged residues: Arg34, Ser38, Lys48, were also found to be crucial for IscR repressor function because they exhibited elevated β-galactosidase activity (for a full functional analysis of IscR see Appendix III).

Densitometric analysis of an immunoblot from wild-type and ΔiscR strains (Figure 4.1 - left panel) also revealed ~ 5x over-expression of IscU in ΔiscR strains. Elevated levels of IscU were also seen in strains containing IscR92CA, 98CA, or 104CA substitutions, revealing that conserved cysteines in IscR are essential for repressor function. In conclusion, our results provide substantial evidence that as in E. coli (Schwartz et al., 2001), holo-IscR serves as a negative regulator of the ISC operon in A. vinelandii.

**Holo-IscR is necessary for native expression of the trmH cysE operon**

Upon scanning the promoter region of the trmH cysE operon, located just upstream of the isc genes, we noticed a DNA sequence with high identity to the putative promoter region of A. vinelandii iscR (Figure 4.2A). Thinking that IscR may regulate this neighboring gene region in the same manner as the isc operon, we constructed strain DJ1504, containing a lacZY cartridge inserted between the PstI sites of trmH and cysE2 genes forming: \( \text{trmH}^{-}::\text{lacZY}::\text{cysE}^{+} \). To match our other constructs, DJ1504 was derived from a strain, DJ1421, which contains a duplicate copy of the iscSUA hscBA fdx iscX operon under control of the sucrose promoter.

To determine if the trmH cysE gene region is similarly up-regulated in ΔiscR strains, DJ1504 was transformed with a plasmid carrying an inframe deletion in iscR (pDB1490). Surprisingly, instead of dark blue transformants representing elevated levels of \( \text{trmH}^{-}::\text{lacZY}::\text{cysE}^{+} \) expression, we discovered small white colonies, which were later confirmed to be ΔiscR transformants. Subsequent construction of IscR92CA and IscR104CA derivatives of the DJ1504 parent also revealed small white transformants,
indicating that apo-IscR regulation follows the same pattern as ΔIscR regulation, and suggests that holo-IscR is the critical species involved in positive control of \textit{trmH cysE} expression. \textit{LacZ} expression assays were attempted for the DJ1504 \textit{iscR} derivatives. However β-galactosidase activity was so low (less than 1 Miller Unit) that it was not possible to derive meaningful expression data from these strains. The expression levels of \textit{trmH::lacZY::cysE2} are best seen in Figure 4.3 by comparing the blue color of wild-type and Δ\textit{iscR} strains streaked on sucrose plates with 60 µM X-gal. Taken together, our results suggest that IscR positively regulates \textit{trmH cysE2} expression and that holo-IscR is necessary for native expression of \textit{trmH cysE2}.

**IscR dependent expression within the isc operon**

In \textit{E. coli} two transcriptional start sites have previously been mapped upstream of the \textit{hscBA fdx} gene region suggesting a transcriptional start separate from the major IscR dependent promoter (Lelivelt and Kawula, 1995). Previous growth phenotypes of Δ\textit{hscBA} strains have also indicated the presence of an internal promoter in the \textit{isc} operon (Johnson et al., 2006). Additionally, as seen with the \textit{trmH cysE2} promoter region, a gene region upstream of \textit{hscBA} in \textit{A. vinelandii} was also found to bear sequence similarity to the putative \textit{iscR} promoter (Figure 4.2). To test the hypothesis that an internal promoter may exist and be regulated by IscR, we constructed a strain in which \textit{hscA´::lacZY} expression was decoupled from IscR control via the P\textit{iscR} promoter, by placing the \textit{hscA´::lacZY} fusion gene construct in the sucrose controlled copy of the \textit{isc} operon, forming strain DJ1524. On glucose plates DJ1524 is white, but turns blue quickly when switched to sucrose indicating tight catabolite repression of the P\textit{sucrose} promoter.

DJ1524 was transformed with a Δ\textit{iscR} plasmid (pDB1490) and a congression plasmid containing streptomycin resistance to form DJ1761 (Table 4.1). Transformants were screened for color change on glucose plates with X-gal. We isolated small blue transformants that were later confirmed to contain a deletion in \textit{iscR}, indicating the presence of an internal promoter negatively controlled by IscR. We theorize that the IscR dependent \textit{isc} internal promoter is weaker than the IscR promoter upstream of \textit{iscR},
because on plates the DJ1761 strain goes blue much more slowly and to a lesser extent than its counterpart, DJ1562. We conclude that there is an additional IscR dependent binding site upstream of hscBA fdx which weakly regulates downstream expression.

In a separate set of experiments, a set of strains was constructed to determine the level of isc expression following the iscR promoter. In these constructs lacZ was inserted directly following iscR. Unlike strains containing an hscA' -lacZY translational fusion, ∆iscR (DJ1739) and iscR92CA (DJ1751) strains resulted only in a minor increase in expression of iscR-lacZY when compared to the iscR+ parent (DJ1731) (see Figure 4.3). We attribute this result to an increased population of apo-IscR in DJ1731 which is lacking a full complement of ISC proteins required for synthesizing clusters. As a result the wild-type expression of iscR-lacZY is elevated, as seen in the left panel of Figure 4.3, thereby decreasing the fold difference of lacZ expression between iscR+ and ∆iscR strains.

**Deletion iscR strains have a ‘small’ phenotype**

Upon constructing iscR+ (DJ1421), iscR92CA (DJ1696), and ∆iscR strains (DJ1601), containing the full complement of isc genes, we noticed a curious phenotype. Cell colonies of strains containing an inframe deletion in iscR were considerably smaller on plates than colonies of wild-type iscR strains. On sucrose plates, colonies of iscR92CA (or ‘apo’ IscR strains) were medium sized between iscR+ (DJ1421) sized colonies and ∆iscR (DJ1601) sized colonies (Figure 4.4). Medium sized colonies were also seen for iscR104CA strains. We had previously noted in our lab that the small ∆iscR phenotype could be relieved by the deletion of another isc gene, for example, ∆iscR/∆iscU, or ∆iscR/∆hscA (Johnson, 2006). The double deletion strains resulted in a wild-type phenotype, suggesting that the overproduction of the ISC proteins causes a toxic level of iron and sulfide production which could only be relieved by disablement of the iron-sulfur cluster machinery. On the other hand, medium-sized colonies of iscR92CA do not change in colony size upon the insertional inactivation of hscA.

Since colony growth phenotypes are only replicable on plates and not in liquid medium - a result we later attributed to the propensity of DJ1601 to form suppressor
mutants allowing reversion to normal growth (discussed below) - we decided on the terminology of ‘small’ rather than ‘slow-growth’ phenotype for DJ1601. Observations of glutaraldehyde fixed cells by light microscopy revealed very different morphologies for individual iscR+ (DJ1421) and ∆iscR cells (DJ1601). DJ1421 cells were rod shaped and many were fixed in the process of active division. On the other hand, DJ1601 cells were large and spherical and no dividing cells were seen (Figure 4.6). The average diameter of fifty individual iscR+ cells from DJ1421 was 2.1 ± 0.4, while the average diameter of fifty ∆iscR cells from DJ1601 was 2.8 ± 0.8 nm.

While ∆iscR strains are ‘small’ on both BN and B-urea plates, they exhibit improved growth under diazotrophic conditions when nitrogen is removed from the growth media (Figure 4.5). Wanting to understand this further, our initial thought was that diazotrophic growth helps rescue the phenotype by elevating levels of cysE1 (cysE<sup>nilf</sup>) to replace low levels of cysE2 in ∆iscR cells. To investigate this question, we constructed a ∆iscR strain in which both remaining serine acetyltransferases, cysE3 and cysE1 were deleted (DJ1819). Both DJ1601 (∆iscR) and DJ1819 (∆iscR, ∆cysE1, ∆cysE3) exhibited increased growth on B plates, indicating that it is not the additional expression of Nif-dependent cysE1 which improves cell growth of ∆iscR strains under nitrogen fixing conditions (see Appendix IV). Additionally, the colony size of DJ1601 did not increase upon the addition of L-cysteine to the media, growth at high (40%), or at low oxygen (5%).

We did note however that DJ1601 cells grown at 40% O<sub>2</sub> have an increased tendency to form suppressor mutants on plates. Additionally, ∆iscR strains are highly prone to form suppressor mutations when grown in liquid culture where the oxygen tension is also high. Figure 4.7A shows DJ1601 cells plated on sucrose after growth in liquid Burk’s medium containing ammonium acetate (14 mM) to an OD<sub>600</sub> = 0.2. At this OD, the percent of suppressor mutants makes up 10% of the total number of cells. Supporting our previous growth results with DJ1601 colonies on B plates, the frequency of suppressor mutants significantly decreases when DJ1601 is grown on Burk’s medium lacking ammonium acetate (Figure 4.7B), indicating that diazotrophic growth helps to alleviate some of the deleterious effects of the ∆iscR genotype. When DJ1601 is grown in liquid -Mo medium, containing no molybdenum and decreased levels of ammonium
acetate (7 mM), the strain reveals a rate of reversion to normal growth in between that of diazotrophic (Burk’s medium, ‘B’) and non-diazotrophic (Burk’s medium + ammonium acetate ‘BN’) growth (Figure 4.7B). On the other hand, the \( iscR92^{CA} \) strain does not exhibit the formation of any suppressor mutants when grown in various liquid media (BN, B, -Mo). In accordance with the medium-sized colony phenotype seen for this strain, our data indicates that the \( IscR92^{CA} \) variant is not as destructive for \( A.\ vinelandii \) cells as \( \Delta IscR \). We conclude that deletion of the IscR protein is highly deleterious and causes an oxygen sensitive phenotype which can be relieved by growth under non-nitrogen fixing conditions and/or the disablement of the ISC machinery.

**Deletion IscR strains over-express aldehyde and alcohol dehydrogenase proteins**

Four DJ1601 large colonies representing suppressor mutants were isolated from BN plates grown from an aliquot of liquid growth medium as shown in Figure 4.7A. In each of these four stains the inframe deletion of \( iscR \) was confirmed to be intact. Since we have previously seen that \( \Delta iscR/\Delta iscU \) and \( \Delta iscR/\Delta hscBA \) strains form large wild-type colonies similar to the suppressor mutants, we were interested to see if some of the suppressor mutants isolated from DJ1601 growth in liquid media were glucose sensitive, indicating the formation of a natural mutations in the \( isc \) operon compromising function. We screened 30 suppressor mutants but did not find any glucose sensitive phenotypes for the suppressor mutants screened. In addition, a western blot revealed equal levels of IscU protein in DJ1601 and suppressor strains (data not shown). From the suppressor strains analyzed, we conclude that a larger colony was not the result of a mutation in the \( isc \) operon.

Wanting to further understanding the difference between the ‘small phenotype’ of DJ1601 and the suppressor mutants, we decided to pursue a proteomics approach, using 2-dimensional gel electrophoresis to screen for varying protein levels between the two crude extracts. Because the high suppressor mutation rate of DJ1601 prevented our ability to obtain a pure culture of this strain from liquid media, we decided to use cells grown on plates (which have a much lower frequency of suppression) for the 2-dimensional gel analysis. DJ1601 and DJ1877 (representing one of the four suppressor
mutant strains) from several BN plates were resuspended in 10 mLs of 50 mM Tris buffer, pH 8.0 and processed by passage through a french cell press and ultracentrifugation at 95,000 x g for 1 h. Cell pellets obtained from the ΔiscR ‘small’ (DJ1601) and ΔiscR ‘suppressor’ (DJ1877) strains were markedly different (Figure 4.8, top panel). The DJ1601 cell pellet was white and hard, while the suppressor pellet appeared more typical of *A. vinelandii* cells showing a tan color and layer of red slime indicative of cytochromes and membranes. Following centrifugation, equal amounts of crude cell extract were loaded and separated by 2-d gel electrophoresis.

The results yielded a surprising discovery. DJ1601 crude extracts revealed two additional proteins (*Figure 4.8 - middle panel*, circled as #1 and #2) that were not visible in the suppressor strain and expressed at a relatively high level. The two proteins were identified by mass spectrometry as Avin 08000 (#1) and Avin 07980 (#2) and mapped to the same operon (The raw data for mass spectroscopy analysis is included after the figure). These genes have been annotated in the *A. vinelandii* genome as aldehyde dehydrogenase (NCBI# ZP_00419081.1) and iron alcohol dehydrogenase (NCBI# ZP_00419082.1), respectively (Figure 4.8). DJ1601 was also found to over-express these proteins when grown on B-urea, but not when grown on B media, lacking a source of fixed nitrogen. Increased levels of aldehyde and alcohol dehydrogenase were not evident for the three other suppressor mutant strains, *iscR*+ (DJ1421), or *iscR92*CA (DJ1696) grown in various media. Thus, we conclude that higher levels of alcohol and aldehyde dehydrogenase only occurs when the ‘small’ ΔiscR strain, DJ1601 is grown nondiazotrophically on plates.

Additionally, we measured the aldehyde and alcohol dehydrogenase activity of crude extracts of DJ1421 (*iscR*+), DJ1601 (ΔiscR), DJ1696 (*iscR92*CA), and DJ1877 (ΔiscR suppressor) grown on various kinds of 2% sucrose plates (BN, B-urea, B) and re-suspended in 50 mM Tris, pH 8.0 buffer. As was consistent with our 2-dimensional gel analysis, only DJ1601 cells from B-urea and BN plates showed elevated levels of alcohol and aldehyde dehydrogenase activity (Figure 4.10). DJ1877 had levels of alcohol and aldehyde dehydrogenase activity similar to the *iscR*+ strain, DJ1421. Our results indicate that the ΔiscR ‘small’ phenotype is correlated with the increased levels of aldehyde and alcohol dehydrogenase, and that restoration of normal growth *via* natural suppressor
mutations are associated with decreased levels of aldehyde and alcohol dehydrogenase proteins.

4.5 DISCUSSION

In this study we have shown that IscR regulates the isc operon in an auto-regulatory negative feed-back loop and that the conserved cysteines of IscR are necessary for repressor function. We have also shown that IscR has a role in regulating the neighboring trmH cysE operon and that holo-IscR is required for proper expression of the trmH cysE genes. Other reports have suggested a connection between IscR regulation and expression of the serine O-acetyltransferase gene, cysE2. In a previous study, an experimentally derived ‘sequence logo’ was used to determine conservation of the IscR promoter in E. coli and other genomes (Giel et al., 2006). As expected, high scoring IscR promoter motifs were found upstream of iscRSUA in multiple organisms. The motif was also found conserved upstream of cysE in E. coli and other enterobacteria suggesting an IscR regulatory site (Giel et al., 2006). It is not clear why cysE2 would be transcribed with an RNA methyltransferase although the genomic organization of trmH cysE iscR iscS is conserved in Pseudomonads. In some gram positive organisms, such as Bacillus subtilis, cysE is co-transcribed with cysS (tRNA synthetase for L-cysteine) (Gagnon et al., 1994). TrmH is a tRNA methyltransferases that methylates guanosine 18 on the D-loop of tRNAs. A recent report has found some specificity for various TrmH proteins, for example preferential methylation of tRNA^{Leu}, tRNA^{Ser}, and tRNA^{Phe} (Hori et al., 2003). The A. vinelandii genome reveals at least two other weak homologues to TrmH (Avin 07630 and Avin 04710). In future studies it would be of interest to address the specificity of TrmH transmethylase and determine if methylation may be related to the function of serine O-acetyltransferase. Our results lead to a model in which IscR differentially regulates isc and trmH cysE2 expression as a mechanism to keep a check on the cellular build-up of toxic metals and sulfides. In other words, when iron sulfur cluster synthesis is repressed, L-cysteine production can be renewed by the holo-IscR activation of the trmH cysE2 operon. During the build up of intracellular L-cysteine, the cluster on
holo-IscR eventually degrades relieving repression of the iron-sulfur cluster biosynthetic machinery, which will now be able to process the excess levels of intracellular cysteine.

IscR also regulates an ‘internal’ promoter upstream of \( hscBA \) \( fdx \), although likely at reduced levels in comparison to the primary promoter upstream of \( iscR \). It is not clear how regulation of this site may fit into an overall scheme of \( isc \) regulation. For example, why does IscR regulate both \( hscBA \) \( fdx \) expression from the internal promoter site and expression from \( P_{iscR} \) promoter? Is there a difference in holo- and apo-IscR regulation at this site? It is likely that multiple regulatory sites (both IscR dependent and independent) exists upstream of the \( isc \) chaperones genes. For example, Lelivelt et al. have demonstrated cold-shock induction of HscBA proteins controlled by a sigma-32 promoter upstream of \( hscBA \) \( fdx \) (Lelivelt and Kawula, 1995). Martens et al. have also shown that independent regulation of \( hscBA \) \( fdx \) in \( Xenorhabdus \) \( nematophila \) is necessary for the mutualistic colonization of juvenile nematode intestines (Martens et al., 2003). We conclude that while primary \( hscBA \) \( fdx \) expression is controlled by the \( P_{iscR} \) promoter, secondary expression of these genes is controlled both by IscR and other regulatory factors to modulate \( hscBA \) \( fdx \) expression in response to cellular growth demands.

Colonies from \( \Delta iscR \) strains exhibit a ‘small’ phenotype on plates, which can be partially relieved by growth under nitrogen fixing conditions. The tendency of DJ1601 to form natural suppressors under conditions of high oxygen tension, as seen on both plates and in liquid cultures, suggests that increased oxygen exacerbates intracellular stress associated with the toxic byproducts of over-produced iron-sulfides. The small phenotype is relieved by conditions which help limit the effect of excess iron and sulfide. This is achieved either by #1- disabling iron-sulfur cluster machinery so as not to produce excess clusters or #2- diazotrophic growth, which may cause an increased demand for iron-sulfur clusters (including nitrogenase, Fe, and respiratory proteins) which would help ‘sop’ up excess iron-sulfides.

While it is clear that the loss of IscR causes intracellular stress, it also evident that the phenotype exhibited by \( \Delta iscR \) strains is not functionally equivalent to the loss of holo-IscR function, via an \( iscR92^{CA} \) substitution. While both \( \Delta IscR \) and \( IscR92^{CA} \) cause a 5 - 7x over-expression of ISC proteins, the \( \Delta iscR \) strain has a small phenotype on plates whereas the \( iscR92^{CA} \) strain has colonies that exhibit a ‘medium’ sized growth phenotype.
Keeping in mind that a deletion of IscR represents the loss of both apo- and holo- IscR functions, while the IscR92$^{CA}$ substitution only represents a loss of holo-function, we conclude that the loss of both IscR functions (DJ1601) is much more severe that the loss of only holo-IscR function (DJ1696). This explains why DJ1696, a strain with only a loss of holo-IscR function does not form suppressor mutations at high oxygen or require the relief of repression by the deletion of the ISC machinery. Our results indicate an as yet uncharacterized intracellular role of apo-IscR, and suggest that loss of apo-IscR function (DJ1601) may be even more detrimental to cell viability and stress management than loss of holo-IscR function (DJ1696).

To further understand the phenotype seen in DJ1601 cells, we performed 2-dimensional gel electrophoresis of crude extracts from DJ1601 cells and a ∆iscR suppressor strain, DJ1877, grown on plates. Interestingly, we found that DJ1601 'small' cells had increased levels of aldehyde and alcohol dehydrogenase protein in the crude extract which mapped to a single operon on the A. vinelandii chromosome. Likewise, both the iscR+ strain DJ1421, and the ∆iscR suppressor mutant DJ1877, did not reveal highly elevated levels of alcohol or aldehyde dehydrogenase (aldA) protein by 2-dimensional gel electrophoresis. Recently, Gama-Castro et al. have shown that the aldA protein is essential for catabolizing n-butanol and inducing cyst formation in A. vinelandii cells (Gama-Castro et al., 2001). The authors found that expression of aldA (Avin 08000) is sigma-54 dependent and elevated when cells are switched from growth on glucose to n-butanol. The divergently transcribed sigma-54 activator protein is located just upstream of aldA. An operon encoding for machinery involved in the import of large Fe/siderophore complexes is also located upstream of aldA. In between the sigma-54 activator protein and the Fe/siderophore import operon is a protein called murein peptide ligase (Mgl) (Figure 4.8). The E. coli homologue of Mgl has been shown to play a role in the recycling of peptidoglycan modules for new cell wall biosynthesis (Herve et al., 2007). Its location near aldA, a gene essential for cyst formation, suggests a common role in cellular differentiation.

In this study, we find that over-expression of aldehyde and alcohol dehydrogenase is correlated with the small phenotype, as these proteins are elevated only in crude extracts from BN or B-urea media. Crude extracts of DJ1601 grown on B plates did not
reveal abundant expression of dehydrogenase proteins. Neither did crude extracts of the apo-IscR strain, DJ1696. The over-expression of these two dehydrogenase proteins indicates that the ‘small’ phenotype of ΔiscR strains may be related to the formation of cysts via the production of n-butanol. Consistent with this hypothesis is the observation that under the microscope, ΔIscR cells form large spherical cyst-like structures. We conclude that the loss of apo-IscR function causes the formation of cysts-like cells and concomitant over-expression of cyst-dependent proteins, aldehyde and alcohol dehydrogenase, in *A. vinelandii*.

We propose that the formation of cyst-like cells is a result of increased intracellular stress in ΔiscR strains, and that the cyst-like structures help decrease metabolism and the potential of O₂ interaction with intracellular iron and sulfide. Consistent with our results, it has previously been noted that diazotrophic growth causes germination of encysted cells in *A. vinelandii* (Sadoff, 1975). Other studies have also shown that the initiation of N₂-dependent growth causes the elevation of RpoS, a sigma factor involved in turning on stress response genes in *Azotobacter* (Sandercock and Page, 2008b). Along with an increased demand for intracellular clusters, these reasons explain why diazotrophic growth alleviates, but does not fully rescue, the ‘small’ growth phenotype of ΔiscR cells.

As IscR has been demonstrated to be important for mediating cellular response to oxidative stress in other organisms, it is not surprising to find it plays a similar role in *A. vinelandii*. We do not know if regulation of the aldA operon is controlled directly or indirectly by IscR, although future studies will address this issue. However, because the ‘small’ phenotype is so strongly correlated to the loss of apo-IscR function, our results point strongly to additional roles of apo-IscR in oxidative stress management in *A. vinelandii*. Additionally it is interesting that aldehyde and alcohol dehydrogenase are induced in the absence of n-butanol or other alcohols present in the media. This indicates that instead of catabolizing the alcohol, *A. vinelandii* may be synthesizing n-butanol as an electron and carbon sink during the process of cyst formation. The bio-production of solvents such a butanol is of great interest to the current biofuel industry and will be pursued in future studies.
### 4.6 FIGURES AND TABLES

#### Table 4.1 Strains used in this study

<table>
<thead>
<tr>
<th>Strain #</th>
<th>Genotype</th>
<th>Genes controlled by P_{scr}</th>
<th>Other mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>DJ1421</td>
<td>iscR+</td>
<td>iscSUAniscBAdfxiscX</td>
<td></td>
</tr>
<tr>
<td>DJ1525</td>
<td>iscR+, Φ(hscA’-lacZYKn)</td>
<td>iscSUAniscBAdfxiscX</td>
<td></td>
</tr>
<tr>
<td>DJ1532</td>
<td>iscR+, Φ(hscA’-lacZYKn)</td>
<td>iscSUAniscBAdfxiscX</td>
<td>recA::GmR</td>
</tr>
<tr>
<td>DJ1731</td>
<td>iscR-lacZYKn-fdx</td>
<td>iscSUAniscBAdfxiscX</td>
<td></td>
</tr>
<tr>
<td>DJ1524</td>
<td>iscR+</td>
<td>iscSUAhscBΦ(hscA’-lacZYKn)</td>
<td></td>
</tr>
<tr>
<td>DJ1504</td>
<td>iscR+, Φ(trmH’-lacZY-cysE2’)</td>
<td>iscSUAniscBAdfxiscX</td>
<td>nif4-11(orf)Δ::SmR</td>
</tr>
<tr>
<td>DJ1601</td>
<td>ΔiscR</td>
<td>iscSUAniscBAdfxiscX</td>
<td>nif4-11(orf)Δ::GmR</td>
</tr>
<tr>
<td>DJ1877</td>
<td>ΔiscR, suppressor mutant(1)</td>
<td>iscSUAniscBAdfxiscX</td>
<td>nif4-11(orf)Δ::GmR</td>
</tr>
<tr>
<td>DJ1562</td>
<td>ΔiscR, Φ(hscA’-lacZYKn)</td>
<td>iscSUAniscBAdfxiscX</td>
<td>nif4-11(orf)Δ::SmR</td>
</tr>
<tr>
<td>DJ1739</td>
<td>ΔiscR-lacZYKn-fdx</td>
<td>iscSUAniscBAdfxiscX</td>
<td>nif4-11(orf)Δ::GmR</td>
</tr>
<tr>
<td>DJ1761</td>
<td>ΔiscR</td>
<td>iscSUAhscBΦ(hscA’-lacZYKn)</td>
<td>nif4-11(orf)Δ::SmR</td>
</tr>
<tr>
<td>DJ1725</td>
<td>ΔiscR, Φ(trmH’-lacZY-cysE2’)</td>
<td>iscSUAniscBAdfxiscX</td>
<td>nif4-11(orf)Δ::GmR</td>
</tr>
<tr>
<td>DJ1823</td>
<td>ΔiscR</td>
<td>iscSUAniscBAdfxiscX</td>
<td>recA::KnR</td>
</tr>
<tr>
<td>DJ1819</td>
<td>ΔiscR, cysE3::Kn, ΔcysE1</td>
<td>iscSUAniscBAdfxiscX</td>
<td>nif4-11(orf)Δ::GmR</td>
</tr>
<tr>
<td>DJ1696</td>
<td>iscR92^{CA}</td>
<td>iscSUAniscBAdfxiscX</td>
<td>nif4-11(orf)Δ::GmR</td>
</tr>
<tr>
<td>DJ1582</td>
<td>iscR92^{CA}, Φ(hscA’-lacZYKn)</td>
<td>iscSUAniscBAdfxiscX</td>
<td>recA::GmR</td>
</tr>
<tr>
<td>DJ1751</td>
<td>iscR92^{CA}-lacZYKn^R-fdx</td>
<td>iscSUAniscBAdfxiscX</td>
<td>nif4-11(orf)Δ::GmR</td>
</tr>
<tr>
<td>DJ1730</td>
<td>iscR92^{CA}, Φ(trmH’-lacZY-cysE2’')</td>
<td>iscSUAniscBAdfxiscX</td>
<td>nif4-11(orf)Δ::GmR</td>
</tr>
</tbody>
</table>

(1) isolated from growth of DJ1601 in liquid media, source of suppression is not known
Figure 4.1 Elevated expression of ISC proteins in ΔiscR strain

(Left panel) Immunoblot of IscU expressed in iscR+ (DJ1532) and ΔiscR strains (DJ1562). (Right panel) β-Galactosidase activity of Φ(hscA′-lacZY) strains relative to wild-type IscR. Φ(hscA′-lacZY) strains assayed in this experiment include wild-type iscR (DJ1532), iscR92CA (DJ1582), and ΔiscR (DJ1562).
Figure 4.2 Putative IscR binding sites on the *A. vinelandii* genome

The putative IscR binding sites on the *A. vinelandii* genome were determined by eye alignment, using a previously identified IscR Type I sequence logo from *E. coli* (Giel et al., 2006). (Panel A) An alignment of the three putative IscR binding sites mentioned in this text (Panel B) Genomic context of each IscR binding site. Each of the diagramed IscR binding sites are contained on the same DNA strand as the ATG codon of the downstream gene and are found in the same 5′ → 3’orientation.
Figure 4.3  IscR dependent expression of lacZ fusion constructs in the A. vinelandii genome

(Left panel) Wild-type iscR strains in this figure include: DJ1731 (iscR–lacZYKn-fdx), DJ1532 Φ(hscA’-lacZYKn), and DJ1504 Φ(trmH’-lacZY-cysE2’). (Right panel) Deletion iscR strains in this figure include: DJ1739 (ΔiscR- lacZYKn-fdx), DJ1562 Φ(hscA’-lacZYKn), and DJ1739 Φ(trmH’-lacZY-cysE2’). Strains are streaked on 2% sucrose plates containing 60 µM X-gal.
Figure 4.4 Relative colony size of *A. vinelandii* *iscR* strains

Colonies of different *iscR* strains were plated the same day from liquid growth media onto BN plates (2% sucrose plates containing 14 mM ammonium acetate) and allowed to grow for 8 days at 30 °C before being photographed. Scale bar of 1 cm is relevant for all three panels.
Figure 4.5 ΔiscR strain shows improved growth on B plates

Colony growth phenotypes of ΔiscR strain, DJ1601, and wild-type iscR strain, DJ1421 when grown on Burk’s medium containing and lacking ammonium acetate (BN and B, respectively).
Figure 4.6  Light micrographs of *A. vinelandii* cells

*(Panel A)* Light micrograph of individual *A. vinelandii* cells from DJ1421 containing wild-type *iscR*. *(Panel B)* Light micrograph of individual *A. vinelandii* cells from DJ1601 cells containing Δ*iscR*. 
Figure 4.7 Natural suppressor mutations in ΔiscR strains

(Panel A) An aliquot from a growth culture of DJ1601 at OD₆₀₀ = 0.2, plated on a sucrose showing ‘small’ colony phenotype typical of the DJ1601 strain as well as larger suppressor colonies from growth in liquid culture. (Panel B) Increase in suppressor mutations of DJ1601 during growth on Burk’s medium with and without 14 mM ammonium acetate (BN, and B respectively) and Burk’s medium with 7 mM ammonium acetate but lacking molybdenum (-Mo).
Figure 4.8 Proteomic level differences between $\Delta iscR$ strains grown on plates: DJ1601 and DJ1877

(Top panel) Centrifuged cell lysate of DJ1601 ($\Delta iscR$) and DJ1877 ($\Delta iscR$, suppressor) showing a difference in cell pellet from these strains. (Bottom panel) 2-dimensional gel electrophoresis of crude cell lysate from DJ1601 (left) and DJ1877 (right) showing elevated levels of two proteins in DJ1601 crude extracts.
Figure 4.9 Genomic context of Aldehyde dehydrogenase and Alcohol dehydrogenase proteins

Elevated levels of Protein #1 (Aldehyde dehydrogenase) and Protein #2 (Alcohol dehydrogenase) as seen by 2-dimensional gel electrophoresis for DJ1601 ‘small’ crude extracts, were identified by LC-MS (Mascot data is included following this figure) and found to reside in the same operon. The broader genomic region gene is shown here. Of special interest is the close proximity of a Sigma-54 transcriptional activator protein, a protein involved in cell wall biosynthesis (Mpl), and an operon involved in the transport of large iron/siderophore complexes. Hypothetical and conserved hypothetical genes are shown in gray.
Mascot Search Results

Protein View

Match to gi|31739799|score: 899
Aldehyde dehydrogenase (EAD+) [Acinetobacter vinelandii A001]
Found in search of C:\Program Files\Applied Biosystems\Keith\Sep 2003\87ppw_A7_122244811.txt

Nominal mass (M): 61946; Calculated pI value: 6.17
NCBI BLAST search of gi|31739799 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Acinetobacter vinelandii
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|31739799 FOR Acinetobacter vinelandii A001

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleave by Trypsin; cuts C-term side of M unless next residue is F
Sequence Coverage: 224

Matched peptides shown in Bold Red

1 MRGFCFYRT GYVFFPPKSC GESQALLQG KTGTSLEAVS KGGKALBRRPE
31 KRRHKHRNN GIAKAFGRG AYTVKIRT YSNQYVPV YKQYTVTTDPR
181 YLDVRQAFR PRTRLHRA ARVAPAAG ADRQPDQV AAVMPIPAS
151 HNLDIHIL VTVWNOFHV RTTMHADVL RHYQRTTAN CRACRKEEG
291 EINSNIVTAE HFVVGVFVQ IIFQFTFQK AQAAKAEA AGRYKLEPA
251 FQYPFTVR VIELGQGDP SYCNYGQPS HGAEKQTPF EILKATPQG
301 STAYVILIK CAEHKISPIF VELGQKRNFI FHDINGAQF AFINMRAKL
351 LSLFYQKVY IQPBQSAFL SLTSDQPM VCRKQKIQAQGRDLTIYQ
401 QGQASQKID PRRLAAEA SGERQAYK RGL seinsqf 5SSTPTDQF
451 KRRHEKVFQ ERISPFPVQV TFFKLSEQA AIKHDTSQG GAGMWTDSIT
501 RAYMNGKIO AGRTYCTRY LYSALAFPG FRSQYTRGL ERMLNMDTQG
551 TKNLVYSDA NPLGTF

Online predicted peptides also

Sort Peptides by: ○ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End Observed Mr(calc) ppm Miss Sequence
63 - 76 1498.5572 1497.5406 1497.7201 -162 0 K. TAMPOGUAGLYLVR (from score 54)
73 - 112 2362.7694 2362.6670 2362.0229 -164 0 K. QYKFMDVYDIEAFPEMEY (from score 81)
112 - 136 1686.4831 1683.5840 1683.8316 -161 0 R. ETLARVPLAHIRFV (from score 417)
412 - 420 1077.6842 1076.4879 1076.6230 -132 0 K. ILSYEAM (from score 85)
415 - 451 1852.7317 1851.7246 1851.9982 -148 0 R. LSHSAHYQTFLKQ (from score 74)
451 - 502 1895.7316 1895.7316 1895.0033 -165 0 R. YQGQEPVPVQTVTPK (from score 95)
475 - 497 2419.8286 2418.8286 2419.1655 -161 0 K. DIAEAALAKIRTELFGMT (from score 82)
543 - 552 1310.4592 1309.3579 1309.5975 -139 0 K. MOLFTRIRK oxidation (M) (from score 31)

LOCUS 27_0041981 ZP_0041981 366 aa LINEAR BCT 01-JUN-2003
DEFINITION Aldehyde dehydrogenase (ND+) [Acinetobacter vinelandii A001],
ACCESSION 27_0041981 ZP_0041981
VERSION 27_0041981 ZP_0041981

http://www.matrixscience.com/cgi/protein_view.pl?pfld=.../data/200010...&pno=1&site=69&threshold=33&a=7&database=0.035&server=nxml&switch=0.001
Mascot Search Results

Protein View

Match to gi|3757932 | Score: 344
Iron-containing alcohol dehydrogenase [Azotobacter vinelandii AvDP]
Found in search of C:\Program Files\Applied Biotechnology\Proteomics\Sep 2003 B1ppp_A1.122232121902.tnn

Nominal mass (Mr): 39965; Calculated pI value: 5.47
NCBI BLAST search of gi|3757932 against nr
Unformatted sequence string for posting into other applications

Taxonomy: Azotobacter vinelandii
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|3757932 from Azotobacter vinelandii 3J

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-terminus of PE unless next residue in PE
Sequence Coverage: 29%
Matched peptides shown in Bold Red

<table>
<thead>
<tr>
<th>Start</th>
<th>End</th>
<th>Observed</th>
<th>Mr(calc)</th>
<th>ppm</th>
<th>Mass</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>165</td>
<td>234</td>
<td>2013.7395</td>
<td>2013.734</td>
<td>-58</td>
<td>0</td>
<td>K.GILAVISHKRENQALPK</td>
</tr>
<tr>
<td>218</td>
<td>227</td>
<td>1088.4989</td>
<td>1084.416</td>
<td>-131</td>
<td>0</td>
<td>K.AVLEHNLNA</td>
</tr>
<tr>
<td>305</td>
<td>327</td>
<td>2286.8177</td>
<td>2285.840</td>
<td>-161</td>
<td>0</td>
<td>K.SNGIDVQLSAEQGQAALTAIR.T</td>
</tr>
</tbody>
</table>

LOCSUS ZP_00190928 382 as linear SCT 09-JUN-2001
DEFINITION Iron-containing alcohol dehydrogenase [Azotobacter vinelandii AvDP].
ACCESSION ZP_00190928
VERSION ZP_00190928.1 GIN.SL17927
REFERENCE ZP_00190928.1.1 GIN.SL17927.1
KEYWORDS
SOURCE Azotobacter vinelandii
ORGANISM Azotobacter vinelandii
DATABASES Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonasales; Pseudomonadaceae; Azotobacter.
REFERENCE 1 (residues 1 to 382)
AUTHORS Copeland,A., Lucas,R., Lapidus,A., Barry,K., Detter,C., Glavina,T.,...
Figure 4.10 Aldehyde and Alcohol dehydrogenase activity of $iscR^+$, $iscR^{92CA}$, and $\Delta iscR$ strains

Aldehyde dehydrogenase activity is denoted by gray bars; Alcohol dehydrogenase activity is denoted by black bars. Error bars denote averages from triplicate analyses. Activity is plotted over isocitrate dehydrogenase activity.
4.7 REFERENCES


CHAPTER 5
Appendices

The results summarized in this section did not easily fit into a research ‘story’ congruous with the previous three chapters. This work however, represents a significant contribution towards my thesis, and will serve as a reference and starting point for future studies.
Appendix I. IscU39$^{DA}$IscS $\alpha_2\beta_2$ complex may interact with other ISC proteins

INTRODUCTION

During purification of the IscU39$^{DA}$IscS complex from Azotobacter strain DJ1766, we noticed multiple proteins being pulled down with IscU39$^{DA}$IscS in the 20 mM imidazole wash, before final elution in 200 mM imidazole. We theorized that background proteins could be weak interactors with the IscU39$^{DA}$IscS complex. To identify these possible proteins, the 20 mM imidazole elutant from five separate purifications was run out by SDS-PAGE and selected bands were identified by mass-spectrometry. The results are summarized in Appendix Table A.1.

MATERIALS AND METHODS

Growth of DJ1766. Native IscU39$^{DA}$IscS was isolated from A. vinelandii strain DJ1766 as previously described (Raulfs et al., 2008). IscU39$^{DA}$IscS complex bound to the Ni$^{2+}$ column was washed with 20 mM imidazole, and fractions from these washes were collected and run out by SDS-PAGE. The results summarized in Appendix Table A.1 represent the combination of 20 mM imidazole washes from 5 separate purifications.

Mass spectrometry. IscU Ala$^{39}$ IscS complex sample was separated by SDS-PAGE. Bands of interest were excised from the stained polyacrylamide gel and washed overnight in solutions of 25 mM NH$_4$HCO$_3$/50% acetonitrile and 10 mM DTT. In-gel tryptic digests were performed overnight and stopped with a small addition of acetic acid at pH 4. Peptides were gently vortexed out of the solution. Following tryptic digestion, peptides were separated via an ultimate capillary HPLC system by LC Packings interfaced with a ThermoFinnigan LCQ DecaXP ion trap mass spectrometer in NSI mode. Peptides were eluted over a linear gradient of 5% to 95% mobile phase B over 30 min at a flow rate of 100 nL/min where mobile phase A was 0.5% AcOH and mobile phase B was MeCN with 0.5% AcOH. Collision induced LC/MS/MS spectra was performed and data was processed by Xcalibur version 1.2 software. MASCOT software
was employed to analyze the tandem mass spectrometry peak lists. Dr. Keith Ray from Dr. Rich Helm’s laboratory performed preparation of protein bands and sequencing using LC-Mass spectrometry.

RESULTS

Poly-histidine tagged IscU39^{DA}IscS from the *A. vinelandii* strain, DJ1766, was purified via immobilized metal affinity chromatography using stepwise imidazole washes at 20 mM and 40 mM imidazole followed by a final elution in 200 mM imidazole. A representative gel showing stepwise purification of IscU39^{DA}IscS, including the 20 mM imidazole wash is shown in Figure A.2. Numerous additional proteins eluted together with weakly bound IscU39^{DA}IscS complex. We were interested in identifying these additional proteins, with the ultimate goal of determining if any of the proteins eluted represented weak physiological interactors with the IscU39^{DA}IscS complex.

The 20 mM imidazole wash of five separate purifications were examined by SDS-PAGE and protein bands of interest were excised and submitted for LC-MS sequencing. The results of the sequencing survey are summarized in Table A.1. The right hand panel of Figure A.2, shows additional protein bands that were identified from a representative 20 mM wash. Many minor SDS-PAGE protein bands in the 20 mM imidazole fraction were not sequenced so the list in Table A.1 is by no means exhaustive. Of the many proteins identified, only those with scores above 50, indicating a robust match, were included in this summary. “Times identified” refers to the number of times that a particular protein band was picked and sequenced and is not necessarily indicative of the likelihood of interaction with IscU39^{DA}IscS complex. In fact, it is probable that many of the proteins identified in Table 1 were bound weakly to the IMAC resin, rather than the IscU39^{DA}IscS complex as several of them (such as pyruvate kinase) are also evident in the flow-through fractions.

The full list of proteins that were identified in the 20 mM imidazole wash is included here for future reference. Several identified proteins are of particular note, including the iron-sensing protein, Fur (*ferric* uptake regulator), which controls global iron homeostatis, and ISC proteins, HscA and Ferredoxin.
DISCUSSION

The *A. vinelandii* genome contains over eight ISC genes which participate in the formation of \([2\text{Fe-2S}]^{\text{+2}}\) and \([4\text{Fe-4S}]^{\text{+2}}\) clusters. Many previous studies have revealed associations between dyads of these proteins including, IscS-IscU, Fdx-IscS, HscB-HscA, and HscA-IscU (Hoff et al., 2000; Tokumoto et al., 2002). Our work with both the wild-type IscU and IscU39\text{DA} variant reveals a strong association between both the scaffold protein and cysteine desulfurase *in vivo* (Raulfs et al., 2008). The identification of additional ISC proteins eluting with IscU39\text{DA}IscS in the 20 mM fraction supports the idea that IscS and IscU complex may form a transient association with other ISC proteins *in vivo* for the assembly of iron-sulfur clusters. Other ISC proteins, HscB and IscA, have also been isolated with wild-type recombinant IscU under similar purification conditions (O’Carroll, 2009).

Thus far, these results are preliminary and need further replication and control experiments to firmly establish that identified proteins such as HscA, ferredoxin, and Fur may form weak protein/protein interactions with the IscU39\text{DA}IscS complex *in vivo*. However, this method is informative in that it provides a means to identify unexpected but physiologically relevant interactors with IscS and IscU39\text{DA} including non-ISC proteins. The identification of both ISC and non-ISC proteins bound to IscU39\text{DA}IscS would confirm their association and supports the idea that rather than dyadic associations, these proteins form a loose multimeric complex for cluster assembly *in vivo*. 
Table A.1 *A. vinelandii* proteins isolated from IMAC resin with IscU39<sup>DA</sup>IscS in 20 mM imidazole

<table>
<thead>
<tr>
<th>NCBI#</th>
<th>Avin #</th>
<th>Protein name</th>
<th>Size kDa</th>
<th>Genomic context</th>
<th>Times identified</th>
<th>Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZP_00415059.1</td>
<td>13920</td>
<td>ATP La protease</td>
<td>89</td>
<td>Next to TonB gene, upstream of operon containing iron alcohol dehydrogenase, and permease</td>
<td>2</td>
<td>229, 440</td>
</tr>
<tr>
<td>ZP_00418128.1</td>
<td>40360</td>
<td>HscA</td>
<td>66</td>
<td>ISC protein</td>
<td>2</td>
<td>252, 136</td>
</tr>
<tr>
<td>ZP_0041928.1</td>
<td>51360</td>
<td>Pyruvate kinase</td>
<td>51</td>
<td>Multiple pyruvate Kinases identified</td>
<td>3</td>
<td>658, 1062, 1123</td>
</tr>
<tr>
<td>ZP_00418947</td>
<td>27240</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZP_00419601.1</td>
<td>42860</td>
<td>Phosphoglucomasine</td>
<td>48</td>
<td>Part of operon <em>ftsJHisPglmMtpiA</em></td>
<td>2</td>
<td>221, 414</td>
</tr>
<tr>
<td>ZP_00420011.1</td>
<td>42500</td>
<td>Acetohydroxy acid isomeroreductase</td>
<td>37</td>
<td>Part of <em>ilvIHC</em> operon</td>
<td>1</td>
<td>381</td>
</tr>
<tr>
<td>ZP_00415096.1</td>
<td>13550</td>
<td>Enoyl-CoA hydratase</td>
<td>33</td>
<td>Near many transposases</td>
<td>3</td>
<td>196, 183, 282</td>
</tr>
<tr>
<td>ZP_00416840.1</td>
<td>34340</td>
<td>KHG/KDPG aldolase</td>
<td>23</td>
<td>In an operon with sigma-54 protein, FleQ</td>
<td>1</td>
<td>76</td>
</tr>
<tr>
<td>ZP_00418410.1</td>
<td>06650</td>
<td>Conserved hypothetical</td>
<td>21</td>
<td>Next to thioredoxin</td>
<td>3</td>
<td>189, 50, 107</td>
</tr>
<tr>
<td>ZP_00416074.1</td>
<td>41110</td>
<td>Petptidylpropyl isomerase, FKBP type</td>
<td>17</td>
<td>Next to glutathione peroxidase, acetate kinase</td>
<td>2</td>
<td>349, 219</td>
</tr>
<tr>
<td>ZP_00419590.1</td>
<td>43000</td>
<td>Ferric uptake regulator</td>
<td>15</td>
<td>Next to RecN, DnaK, DnaJ</td>
<td>2</td>
<td>149, 159</td>
</tr>
<tr>
<td>ZP_00418127.1</td>
<td>40350</td>
<td>Ferredoxin</td>
<td>13</td>
<td>ISC protein</td>
<td>1</td>
<td>173</td>
</tr>
</tbody>
</table>
Figure A.2  Representative SDS-PAGE showing purification fractions for IscU39DAIscS from DJ1766

An enlargement of the SDS-PAGE lane containing the 20 mM imidazole fraction analyzed by mass spectrometry is shown in the panel on the right. Protein bands identified by mass spectrometry from this particular sample are denoted by red arrows.
**Mascot Search Results**

**Protein View**

Match to gi|6153314 Score: 446
Peptidase S16, ATP-dependent protease La [Acinetobacter vinelandii AvP]

### Matched Peptides

<table>
<thead>
<tr>
<th>Residue Number</th>
<th>Increasing Mass</th>
<th>Decreasing Mass</th>
<th>ppm</th>
<th>Mass</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 - 119</td>
<td></td>
<td></td>
<td>-3</td>
<td>1101.6339</td>
<td>VNNQQVAPR VVKTSTNL VLPQCLPGQ YVLYDPPFR FYPPACDPYV</td>
</tr>
<tr>
<td>21</td>
<td></td>
<td></td>
<td>1</td>
<td>1034.8573</td>
<td>VVLYDPPFR FYPPACDPYV</td>
</tr>
<tr>
<td>101</td>
<td></td>
<td></td>
<td>0</td>
<td>1394.7310</td>
<td>1394.7310</td>
</tr>
<tr>
<td>131</td>
<td></td>
<td></td>
<td>0</td>
<td>1526.8997</td>
<td>1526.8997</td>
</tr>
<tr>
<td>151</td>
<td></td>
<td></td>
<td>0</td>
<td>1581.9807</td>
<td>1581.9807</td>
</tr>
<tr>
<td>211</td>
<td></td>
<td></td>
<td>0</td>
<td>1588.7353</td>
<td>1588.7353</td>
</tr>
<tr>
<td>231</td>
<td></td>
<td></td>
<td>0</td>
<td>1634.4935</td>
<td>1634.4935</td>
</tr>
<tr>
<td>453</td>
<td></td>
<td></td>
<td>10</td>
<td>1851.1753</td>
<td>1851.1753</td>
</tr>
<tr>
<td>484</td>
<td></td>
<td></td>
<td>0</td>
<td>1958.2158</td>
<td>1958.2158</td>
</tr>
<tr>
<td>599</td>
<td></td>
<td></td>
<td>0</td>
<td>2169.1875</td>
<td>2169.1875</td>
</tr>
<tr>
<td>678</td>
<td></td>
<td></td>
<td>0</td>
<td>2170.1875</td>
<td>2170.1875</td>
</tr>
<tr>
<td>700</td>
<td></td>
<td></td>
<td>5</td>
<td>2212.1875</td>
<td>2212.1875</td>
</tr>
<tr>
<td>766</td>
<td></td>
<td></td>
<td>5</td>
<td>2308.4305</td>
<td>2308.4305</td>
</tr>
</tbody>
</table>

**Sequence Coverage:** 16%

**Variable Modifications:** Oxidation (M)

**Classify by** Tryptic cuts C-termini side of ER unless next residue is P
Mascot Search Results

Proteina View

Match to: gi|31112297|Score: 136
Chaperone protein bscA homolog

Peptide mass (M.): 66068; Calculated pI value: 5.24
NCBI BLAST search of gi|31112297 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Azotobacter vinelandii

Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|31112297 from Azotobacter vinelandii

Variable modifications: Carbamidomethyl (C); Oxidation (M)

Cleanup by Trypsin: cuts C-term side of P unless next residue is P
Sequence Coverage: 10%

Matched peptides shown in bold red

1 MALIYARVG QESPHRELK AVOJLJITIT IIVHALERGL ASIKETRON
11 DIAYRVEPPD IQGOUOADM AAAYFDVQTVLQKEVNIQ CIANVKIII
101 QKYPYHKH BSHYPSTTT QPKSVYVISL NIWKLLLH AIKAGLAK
111 VIIVYPAFQ EAKQGQKA ALAKLULL LKEHTTAAY MAVQDAGG
291 VRAYLQGQK TDYHILAKK HUEAKDGDQ AIKIANPF
211 QGASAQOKQ GQMVQKHQQ CANNRANL QVYATATQQ QVVLKKEF
391 AIKLUYRE QYNQMDAD QCITEPDQ GYKICSTV PQYIYXAZEL
311 PENGKITAQ PIMQVYQGIQAKLAAQALASVQKRL VKSPRIHCT
401 MERVMEKUI PNTILPDAE QFTTYKDOO TMLYHVLOG REILRVCX
411 LKYNLAEI IPMDAKKIR FQYQIVSOI LSVNSDLS QYEHVQVP
591 SYLQOQHES KMLRDSAYA CGWARIKL AQQYKQNL LQVQLEQAD
511 GAILLCHVE AALRTQQL RYLRQPHOA VIETVHSLT QVTIFAYAAR
621 LQYVRNLLS VQHSHLWES

Show predicted peptides also

Sort Peptides by: 　Residue Number 　Increasing Mass 　Decreasing Mass

Start 　End 　Observed 　Mr (ept) 　Mr (cal) 　ppm 　Miss 　Sequence
15 　37 　1476.3866 　1466.1973 　1466.1320 　-3 　1 　R.LAVVEKTVGEFSLVTRK (Tana score 15)
181 　196 　1174.9251 　1172.8784 　1172.8784 　-3 　1 　R.LAVVEKTVGEFSLVTRK (Tana score 15)
137 　212 　2123.2430 　2123.2430 　2123.2430 　-3 　0 　R.QADQVAYLQGOGISDLR (Tana score 25)
137 　212 　2123.2703 　2123.2620 　2123.2620 　-3 　0 　R.QADQVAYLQGOGISDLR (Tana score 25)
298 　309 　1157.7702 　1156.7709 　1156.7728 　-3 　0 　R.FALIYELVAR (Tana score 10)

RMS error 36 ppm

Loc003 　069221 　614 aa 　linear 　BCT 03-NAR-2009
DEFINITION 　Noclease: Full-Chaperone protein bscA homolog.
ACCESSION 　069221
VERSION 　069221.1 GI:31112297
DEBRECEN 　UniProt: locus bscA, accession 069221,
class: standard.

http://www.matrixscience.com/cgi/protein_view.pl?ice=/ice/data/200711...Flask=1&seq=thrash=52& sg=thrash=0.05& server_module:switch=0.001 　Page 1 of 2

113
**Mascot Search Results**

**Protein View**

Match to: gi:67153463 Access: 1062  
Pyruvate kinase (Acetobacter vinelandii AvOP)

Found in search of C:\Program Files\Applied Biosystems\KeithJolly Methods\WELL\Nov 2007 UT\ppw_H1\1995938620.txt

Nominal mass (Mr): 31721; Calculated pI value: 5.15

WCS C D A S T search of gi:67153463 against nr

Unformatted sequence string for pasting into other applications

**Taxonomy:** Acetobacter vinelandii

**Links to retrieve other entries containing this sequence from NCBI Entrez:**


**Variable modifications:** Carbamoylation (C), Oxidation (M)

**Cleavage by Trypsin:** cuts C-term side of K/R unless text residue is F

Sequence Coverage: 39%

Listed peptides shown in **Bold Red**

<table>
<thead>
<tr>
<th>Start</th>
<th>nL</th>
<th>Observed</th>
<th>Mr(calc)</th>
<th>ppm</th>
<th>Mass</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>61</td>
<td>72</td>
<td>1257.7829</td>
<td>1270.7756</td>
<td>1270.7105</td>
<td>37</td>
<td>RTPSHIFTQ</td>
</tr>
<tr>
<td>68</td>
<td>104</td>
<td>1640.5617</td>
<td>1645.6248</td>
<td>1643.6407</td>
<td>34</td>
<td>1</td>
</tr>
<tr>
<td>107</td>
<td>136</td>
<td>2081.4519</td>
<td>2105.4449</td>
<td>2105.3463</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>224</td>
<td>147</td>
<td>2456.3618</td>
<td>2455.2541</td>
<td>2455.2428</td>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>248</td>
<td>165</td>
<td>2650.3013</td>
<td>2650.4449</td>
<td>2650.3467</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>276</td>
<td>193</td>
<td>2223.1932</td>
<td>2221.1909</td>
<td>2222.1109</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>334</td>
<td>344</td>
<td>1454.9748</td>
<td>1463.4767</td>
<td>1463.2148</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>358</td>
<td>377</td>
<td>1154.1162</td>
<td>1145.1549</td>
<td>1145.2076</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>379</td>
<td>410</td>
<td>1250.4780</td>
<td>1189.6687</td>
<td>1289.6164</td>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>419</td>
<td>471</td>
<td>1227.4767</td>
<td>1236.4721</td>
<td>1236.6351</td>
<td>38</td>
<td>0</td>
</tr>
</tbody>
</table>

**Locke:** ZP_01424296 101 aa linear DEC 09-JUN-2015

**Definition:** Pyruvate kinase [Acetobacter vinelandii AvOP].

**Accession:** ZP_01424296

**Version:** ZP_01424296 1 GI=151315366

**Source:** RIKEN accession RA_AANU1000001

**Organism:** Acetobacter vinelandii

[http://www.matrixscience.com/cgi/protein_view.pl?file=.../dist/200711...&p=1&acc=4箖&thresh=544&sgthresval=0.05&server=rmutpit_switch=0.001](http://www.matrixscience.com/cgi/protein_view.pl?file=.../dist/200711...&p=1&acc=4箖&thresh=544&sgthresval=0.05&server=rmutpit_switch=0.001)
# Mascot Search Results

## Protein View

**Match to:** qj6159413 Score: 181

- **Acetohydroxy acid isomeromutase** (Aerobacteriamelinii AvOP)
- Found in search of "/Users/Documents and settings/Administrative Documents/Proteomics/PSM/AvOP_rep1.fragment.170.txt"

**Nominal mass (m/z):** 3661.1; Calculated P value: 5.16

**NCBI BLAST search of:** qj6159413 against 12

- Unmodified sequence shown for putting into other applications

**Taxonomy:** Aerobacteriamelinii

**Links to retrieve other entries containing this sequence from NCBI Entrez:**
- qj6159413 from Aerobacteriamelinii AvOP

**Variable modifications:** Carbamidomethyl (C), Oxidation (M)

**Coverage by Trypsin: C-terminus and N-terminal side of P, unless next residue in N**

**Sequence Coverage:** 23%

**Matched peptides shown in Bold Red**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Start</th>
<th>End</th>
<th>Observed</th>
<th>Mr(calc)</th>
<th>Pps</th>
<th>Misses</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65</td>
<td>87</td>
<td>2613.325</td>
<td>2613.3150</td>
<td>10</td>
<td>1</td>
<td>Loss score 76</td>
</tr>
<tr>
<td>65-87</td>
<td>2613.325</td>
<td>2613.328</td>
<td>2613.3208</td>
<td>2613.327</td>
<td>17</td>
<td>1</td>
<td>Loss score 58</td>
</tr>
<tr>
<td>66-87</td>
<td>2613.338</td>
<td>2613.358</td>
<td>2613.3477</td>
<td>2613.3471</td>
<td>19</td>
<td>1</td>
<td>Loss score 10</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
<tr>
<td>66-87</td>
<td>2614.160</td>
<td>2614.160</td>
<td>2614.1513</td>
<td>2614.151</td>
<td>29</td>
<td>0</td>
<td>Loss score 54</td>
</tr>
</tbody>
</table>

**DEPOSITION**

- **Acetohydroxy acid isomeromutase** (Aerobacteriamelinii AvOP)

**Accession:** KP_0042601

**Version:** KP_0042601.1

**References:** accession_KP_0042601.1

**Keywords:**

- Aerobacteriamelinii
- Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;

---

Related links:
- [Mascot's website](http://www.matrixscience.com/cgi/protein_view.pl?file=.../data/200711_33&x=x&y=544&sgt=on&calc=on&x=0.058_server=modpsw_switch=0.001)
Mascot Search Results

Protein View

Match 1: gi|97153281| Ref: 183
Enoyl-CoA hydratase/isomerase [Acetobacter vinielandii AvOP]
Found in search of C:\Program Files\Applied Biosystems\Flight\May 2005 Aqppu_K15_19104030904.txt

Nominal mass (M+): 31016; Calculated pI value: 6.09
NCBI BLAST search of gi|97153281 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Acetobacter vinielandii
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|97153284 from Acetobacter vinielandii 53

Variable modifications: Oxidation (%) Cleavage by Trypsin: cuts C-term side of K/R unless next residue is P
Sequence Coverage: 19%

Matched peptides shown in bold red

<table>
<thead>
<tr>
<th>Residue Number</th>
<th>Observed</th>
<th>Mr(calc)</th>
<th>ppm</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>58 - 90</td>
<td>2581.6206</td>
<td>2580.4127</td>
<td>22</td>
<td>28</td>
</tr>
<tr>
<td>115 - 124</td>
<td>1176.6106</td>
<td>1175.5212</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>296 - 297</td>
<td>1511.8121</td>
<td>1511.7821</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>239 - 246</td>
<td>1050.6927</td>
<td>1052.4028</td>
<td>16</td>
<td>0</td>
</tr>
</tbody>
</table>

RMS error 21 ppm

LOCUS  TP_10415096  linear  BCT 09-JUN-2005
DEFINITION Enoyl-CoA hydratase/isomerase [Acetobacter vinielandii AvOP].
ACCESSION TP_10415096
VERSION TP_10415096.1 gi|6711351
SOURCE  RefSeq: accession NW_000592031.1
KEYWORDS
SOURCE  Acetobacter vinielandii
UNIGRAMS  Acetobacter vinielandii
COMMENT  Predicted peptide: This record has not been reviewed and the function is unknown. The reference sequence was derived from

http://www.matrixscience.com/cgi/protein_view.pl?file=.../data/200605...&p=1&acc=thrash=49&_sgte=1&sgte=1&sgte=0.03&svgast=1&svgast=0.001&svgast=0.001
Page 1 of 2
Mascot Search Results

Protein View

Match to: gi|67155212 Score: 76
KEPD and XKG aldolase [Azotobacter vinelandii AvOP]
Found in search of C:\Program Files\Applied Biosystems\Peith\June 2008 C\gpw_M1_111511013003.txt

Nominal mass (M_r): 21065; Calculated pI value: 5.60
NCBI BLAST search of gi|67155212 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Azotobacter vinelandii
Links to retrieve other entries containing this sequence from NCBI Entrez:
|gi|67155212 from Azotobacter vinelandii AvOP|

Variable modifications: Oxidation (M), Oxidation (K)
Clearance by trypsin: cuts C-term side of XR unless next residue is P
Sequence Coverage: 11%

Matched peptides shown in Bold Red

<table>
<thead>
<tr>
<th>Start - End</th>
<th>Observed</th>
<th>Mr(expt)</th>
<th>Mr(calc)</th>
<th>ppm</th>
<th>Miss</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>33 - 47</td>
<td>1200.622</td>
<td>1200.699</td>
<td>1575.8618</td>
<td>1575.8097</td>
<td>-36</td>
<td>R.AHAGS</td>
</tr>
<tr>
<td>194 - 203</td>
<td>1200.621</td>
<td>1200.699</td>
<td>1200.5949</td>
<td>1200.6251</td>
<td>-35</td>
<td>R.AHQ</td>
</tr>
</tbody>
</table>

LOCUS SP_00416840 216 aa linear BCT 09-JUN-2005
DEFINITION KEPD and XKG aldolase [Azotobacter vinelandii AvOP];
ACCESSION SP 00416840
VERSION SP_00416840.1; gi|67155212
SOURCE RefSeq; accession NR_XX0000520152.1
KEYWORDS
ORGANISM Azotobacter vinelandii
AZO细菌：Gammaproteobacteria; Pseudomonadales; Pseudomonadaeae; Azotobacter.
COMMENT PREDICTED RefSeq; This record has not been reviewed and the function is unknown. The reference sequence was derived from

http://www.matrixscience.com/cgi/protein_view.pl?lic=a&data=200607...23&pm=1&z=thmaxv=54&z=value=0.05&server=multiplet_switch=0.001

Page 1 of 2
### Mascot Search Results

**Protein View**

Match 1: gi|67157048 | Score: 107
Conserved hypothetical protein (Azoarcus visn/liadii AVP)

**Nominal mass (M):** 20844; Calculated pI value: 5.24

**NCBI BLAST search of gi|67157048 against nr**

Unformatted sequence string for pasting into other applications

**Taxonomy:** Azoarcus visn/liadii

Links to retrieve other entries containing this sequence from NCBI Entrez:

gi|67157048 from Azoarcus visn/liadii 10

Variable modifications: Carboxymethyl (C), Oxidation (M)
Cleavage by Trypsin cuts C-terminal side of K/R unless next residue is P
Sequence Coverage: 28%

Matched peptides shown in **Bold Red**

1. MELFVAFVL A/LAVFYQAE DORNHEAOX HXEHXAEAL ACHXGHALL
2. NALHDVYL IFIPFAMML LPutherford ERKAXDTK AQLXPEL
3. GOFFVFXGCV DEXLHAFX EZXGDNREX EXEAXTQX GADDLAXQEL
4. IGQVFLQFR G

<table>
<thead>
<tr>
<th>Start</th>
<th>End</th>
<th>Observed</th>
<th>Mz(calc)</th>
<th>ppm</th>
<th>Miss</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>91</td>
<td>111</td>
<td>2262.2625</td>
<td>2262.2667</td>
<td>32</td>
<td>0</td>
<td>R.QLXPELHAXDVFVXGCV.E</td>
</tr>
<tr>
<td>91</td>
<td>111</td>
<td>2253.3028</td>
<td>2252.2467</td>
<td>32</td>
<td>0</td>
<td>R.QLXPELHAXDVFVXGCV.E</td>
</tr>
<tr>
<td>114</td>
<td>136</td>
<td>2577.2107</td>
<td>2577.1592</td>
<td>32</td>
<td>1</td>
<td>R.QLXPELHAXDVFVXGCV.E</td>
</tr>
<tr>
<td>114</td>
<td>136</td>
<td>2577.2149</td>
<td>2577.1592</td>
<td>32</td>
<td>1</td>
<td>R.QLXPELHAXDVFVXGCV.E</td>
</tr>
<tr>
<td>165</td>
<td>173</td>
<td>1022.6179</td>
<td>1022.6237</td>
<td>41</td>
<td>0</td>
<td>R.QLXPELHAXDVFVXGCV.E</td>
</tr>
<tr>
<td>165</td>
<td>173</td>
<td>1022.6474</td>
<td>1022.6237</td>
<td>42</td>
<td>0</td>
<td>R.QLXPELHAXDVFVXGCV.E</td>
</tr>
</tbody>
</table>
Mascot Search Results

Protein View

Match to gi|97596429| Ref: 219
Peptidyl-prolyl isomerase, FERB-type (Acinetobacter viselndii AV01)
Found in search of Citrigen File: Applied Biosystems\Bioph\May 2008 A\spresults\c17_12108030405.txt

Nominal mass (M+) 16673; Calculated P value: 4.7e-11

NCBI BLAST search of gi|81734325| Ref: 20
Automated sequence scoring for posting into other applications

Taxonomy: Acinetobacter viselndii

Links to retrieve other entries containing this sequence from NCBI Entrez:
   gi|97596429| from Acinetobacter viselndii AV01

Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-terminus of K unless residue is P
Sequence Coverage: 56%

Matched peptides shown in bold red

Residues Number   Increasing Mass   Decreasing Mass

Start - End     Observed             Exp(calc)             ppm             Miss          Sequence

40 - 61       2664.3460            2664.3467            2664.3462       23             R.KSIFSAAAQKDVQGWFQKGLKLK.A  (Zone_score 34)
82 - 111      1266.5320            1266.5317            1266.5302       7              R.NPITFLAVGMRHQQGAVQVVRM.G  (Zone_score 18)
111 - 130     1953.9820            1953.9827            1953.9812       30             L.KLDEYGTKAFAKPHQAQKLR.  (Zone_score 18)
141 - 161     3124.7990            3124.7977            3124.7952       16             A.AAKTPRSSARKRRDKD.R  (Zone_score 18)

Locus: 2P_00416074 161 aa
Definition: Peptidyl-prolyl isomerase, FERB-type (Acinetobacter viselndii AV01)
Accession: 2P_00416074
Version: 2P_00416074.1 ID: 67154329
Description: FERB sequence
Source: Acinetobacter viselndii
Organism: Acinetobacter viselndii

Comment: Predicted FERB: This record has not been reviewed and the function is unknown. The reference sequence was derived from KM079146.
Method: Conceptual translation.

Features:
Source: Location/Qualifiers
1..161
/organism="Acinetobacter viselndii"
/stRAIN="FEB"
/db_client="taxon1254"

Domain: 1..161
/product="Peptidyl-prolyl isomerase, FERB-type"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomerase"
/calculated_MW:16673

Regions:
1..161
/organism="Acinetobacter viselndii"
/product="Peptidyl-prolyl cis-trans isomera
Mascot Search Results

Protein View

Match to: gi|67158725 Score: 149
Ferric-uptake regulator (Azotobacter vinelandii AvOP)
Found in search of C:\Program Files\Applied Biosystems\May 2006 A\ppw_C18_121080380704.txt

Nominal mass (Mr): 15143; Calculated pI value: 5.81
NCBI BLAST search of gi|67158725 against sr
Unformatted sequence string for pasting into other applications

Taxonomy: Azotobacter vinelandii
Links to retrieve other entries containing this sequence from NCBI Entrez:
- gi|67158725
- gi|67084602 from Azotobacter vinelandii

Variable modifications: Oxidation (M)
Cleavage by Trypsin cuts C-term side of ER unless next residue is P
Sequence Coverage: 32%

Matched peptides shown in Bold Red

<table>
<thead>
<tr>
<th>Start</th>
<th>End</th>
<th>Observed</th>
<th>Mr(exp)</th>
<th>Mr(calc)</th>
<th>ppm</th>
<th>Miss Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>56</td>
<td>1694.8600</td>
<td>1693.8527</td>
<td>1692.8345</td>
<td>11</td>
<td>K_ALMEAGEDVOLATVR.V</td>
</tr>
<tr>
<td>57</td>
<td>60</td>
<td>1692.8100</td>
<td>1691.8037</td>
<td>1690.7860</td>
<td>3</td>
<td>K_VLYQFFRANLVR.W</td>
</tr>
<tr>
<td>117</td>
<td>121</td>
<td>1810.8400</td>
<td>1809.0379</td>
<td>1808.0206</td>
<td>11</td>
<td>K_GEFELVHNNVTVF.R</td>
</tr>
</tbody>
</table>

PSM error 9 ppm

LOCUS   IP_06419596  124 aa  linear  DEC 09-JUN-2005
DEFINITION Ferric-uptake regulator (Azotobacter vinelandii AvOP),
ACCESSION IP_06419596
VERSION  IP_06419596.1 GI:67158729
SOURCE   KF067; accession NY_AA03310017.1
KEYWORDS 
SOURCE   Azotobacter vinelandii
ORGANISM Azotobacter vinelandii
Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Azotobacter.
REFERENCE
AUTHORS Capeland,A., Irnes,S., Lapidos,A., Barry,K., Beter,H., Clavins,T., Hamon,N., Issani,S., Pitluck,S. and Richardson,P.

http://www.matrixscience.com/cgi/protein_view.pl?id=IP_06419596...
Mascot Search Results

Protein View

Matched to gi:67193324 Accession: 173
Adrenodoxin [Azotobacter vinelandii AvOP]

Found in search of C:\Program Files\Applied Biosystems\Keith\Jody\Methods\WRL\Nov 2007 LT\ppw_m24_1.txt

Nonspecific search of gi:67193324 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Azotobacter vinelandii
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi:256535 from Azotobacter vinelandii
gi:16709293 from Azotobacter vinelandii

Variable modifications: Carbamidomethyl (C), Oxidation (M)
Charge by TrueP: cuts C-term side of R unless C-terminal residue is F
Sequence Coverage: 24%

Matched peptides shown in Bold Red

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Score</th>
<th>Ex</th>
<th>Exp Mass</th>
<th>Calc Mass</th>
<th>Error (ppm)</th>
<th>Ppm Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 QNLQVLYDLV YGEITSGILAAALRALRLAAALRNLV</td>
<td>592</td>
<td>1366.68061</td>
<td>1365.5939</td>
<td>-0.87</td>
<td>2.05E+01</td>
<td>26.5</td>
</tr>
<tr>
<td>2 VOVDELEIWEVSF</td>
<td>310</td>
<td>1326.6593</td>
<td>1325.5934</td>
<td>0.76</td>
<td>2.05E+01</td>
<td>26.5</td>
</tr>
</tbody>
</table>

LOUIS: ZP_00481271

DEFINITIONS: Adrenodoxin [Azotobacter vinelandii AvOP].
ACCESSION: ZP_00481271
VERSION: ZP_00481271.1 G16709293
SOURCE: RefSeq: accession: ZP_00481271.1

COMMENT: Predicted RefSeq: This record has not been reviewed and the function is unknown. The reference sequence was derived from EAM01763.

Method: Conceptual translation.

http://www.matrixscience.com/cgi/protein_view.pl?Species.../200712...4fps=1&amp;thrsh=0.33&amp;siglevel=0.05&amp;server=multiplex_switch=0.001
Appendix II. A compromised ISC System causes degradation of IscU

INTRODUCTION

Using \textit{in vivo} techniques, our previous work resulted in the isolation of a cluster bound $\alpha_2\beta_2$ IscU IscS complex from \textit{Azotobacter vinelandii}, thus proving the scaffold hypothesis of IscU cluster assembly (Raulfs et al., 2008). The bound [2Fe-2S] cluster was labile during further purification, a result attributed to the readily dissociable nature of IscS and IscU in the $\alpha_2\beta_2$ tetramer. Cluster degradation and IscS separation was prevented in a mutant form of the $\alpha_2\beta_2$ complex in which aspartate 39 of IscU was substituted by alanine to form a tight, cluster bound, $\alpha_2\beta_2$ IscS and IscU39$^{DA}$ complex (Raulfs et al., 2008).

Following isolation of both a labile and tight $\alpha_2\beta_2$ IscS and IscU complex, we were interested to determine if, using the same genetic strategy, we could map how other ISC mutations effect interactions with IscU, complex formation, cluster biogenesis, and lability \textit{in vivo}. In order to address some of these questions, we constructed \textit{A. vinelandii} strains shown in Figure A.3. Strains DJ1697 and DJ1766 were constructed previously (Raulfs et al., 2008). DJ1752 (iscU63$^{CA}$) and DJ1764 (iscU106$^{CA}$) were constructed to contain mutations in essential cysteine residues of the IscU scaffold. It should be noted that an alanine substitution of the third cysteine in IscU (Cys37) has previously been shown to be lethal even in the presence of a P$_{sc}$ expressed copy of IscU and thus was not constructed (Johnson et al., 2006). DJ1807 in which the catalytic cysteine of IscS, Cys328 was substituted for alanine, was constructed to determine how the loss of this residue might influence cluster formation and IscS / IscU interactions. DJ1788, a $\Delta$hscBA strain, was also constructed to determine the effect of chaperones on IscS and IscU interactions and cluster formation (O'Carroll, 2009). To prevent possible read through of chaperones from an internal promoter when cultured on glucose, DJ1788 was constructed which deletes the \textit{isc} gene region upstream of \textit{hscBA}.
MATERIALS AND METHODS

**Strain constructions.** DJ1752 (iscU63^{CA}), DJ1764 (iscU106^{CA}), DJ1807 (iscS328^{CA}) were constructed by transformation of DJ1697 with either pDB1658 (iscU63^{CA}), pDB1518 (iscU106^{CA}), or pDB1210 (iscS328^{CA}) and the congression plasmid, pDB528, containing a Kn^{R} insertion in *recA*. As each of these residues has been found to be essential in *A. vinelandii* causing a null growth phenotype in the absence of sucrose (Johnson et al., 2006), transformants were screened for growth on glucose. Final selected strains were sequenced to confirm both the desired IscS or IscU substitution and the integrity of the IscU C-terminal histag.

**Western blot analysis.** *A. vinelandii* strains were grown in sucrose in a 150 L fermentor to an OD_{600} of ~1.5 and collected by centrifugation. Three grams of harvested cells were re-suspended in 10 mLs of 125 mM Tris (pH 8.0) and processed anaerobically using the NanoDebee homogenizer at 25,000 psi (B.E.E International). Lysate was centrifuged at 95,000 x g for 30 minutes. For the immunoblot, 50 µg of crude extract was loaded per SDS-PAGE lane and transferred to nitrocellulose membrane as previously described (Johnson et al., 2006). Crude sera containing Anti – GroEL (*E. coli*) was the generous gift of Dr. Nancy Love (University of Michigan) and used according to previously described methods (Bott et al., 2001).

RESULTS

Unlike our previous work with IscUhis and IscU39^{DA}his, the purification of IscU63^{CA}his, IscU106^{CA}his, and IscUhis with an IscS328^{CA} substitution resulted in negligible amounts of purified IscU variants following IMAC. Indeed, cellular contaminates overwhelmed the Ni^{2+} charged column during chromatography of crude extracts from these strains, and total IscU made up only a small percentage of the final 200 mM imidazole elutant (data not shown). This was not the case for IscUhis (DJ1697), IscU39^{DA}his (DJ1766), or IscUhis with ΔhscBA (DJ1788). In these strains, holo-IscU was isolated at levels of: ~2 (DJ1697), ~10 (DJ1766), or ~10 (DJ1788) nmoles IscU/gram cell paste respectively.
Previous work has confirmed that the deletion of negative regulator, IscR, results in increased expression of IscS, IscU, IscA, HscB, and HscA in \textit{A. vinelandii} (our unpublished results). Thus the isolation of varying amounts of IscU protein from strains compromised in ISC biosynthetic capability was unexpected. As the regulatory protein, IscR, was not present in these strains, we hypothesized that either protein or mRNA levels of IscU were being regulated at a post-transcriptional level.

A western blot of \textit{A. vinelandii} crude extracts from these strains revealed varying amounts of IscU protein (Figure A.4). Relative IscU levels were increased in two strains: DJ1766, containing a 39 aspartate to alanine mutation in IscU, and DJ1788, containing a deletion of HscBA chaperones. Decreased amounts of IscU protein relative to WT IscU were evident in crude extracts in which the essential IscU scaffold sites (Cys63 or Cys106) were mutated. IscU levels were also near negligible in DJ1807 cells, a strain containing a mutation in IscS Cys\textsuperscript{328}, the active site residue essential for cysteine desulfurase activity. Interestingly, anti-IscS immunoblots of the same crude extract did not reveal a significant difference in protein levels of the cysteine desulfurase, indicating that only levels of the iron sulfur scaffold protein, IscU, were affected. Taken together these results suggest that increased levels of IscU protein in the crude extract is correlated with increased levels of purified holo-IscU.

DISCUSSION

Our results indicate variations in intracellular levels of IscU protein when [2Fe-2S] cluster biogenesis is compromised. The present data does not distinguish between mRNA or protein levels of IscU, although it is clear that degradation is specific only for the scaffold, because levels of IscS protein remain constant in ISC mutant strains. Additionally, IscU levels are low in \textit{isc} strains, such as \textit{iscU63\textsuperscript{CA}}, \textit{iscU106\textsuperscript{CA}}, which are compromised in their ability to synthesize iron-sulfur clusters. Mutations not necessary for [2Fe-2S] cluster biosynthesis, such as \textit{iscU39\textsuperscript{DA}} and \textit{ΔhscBA}, but which are in fact associated with cluster stabilization, reveal increased levels of IscU protein.

We propose that varying amounts of IscU protein from ISC strains is the result of preferential degradation of apo- vs. holo-IscU. The absence of HscBA causes build up
of holo-IscU preventing protein degradation and apo-IscU recycling. The HscBA
dependant build-up could be related to a chaperone function which shifts \([2\text{Fe}-2\text{S}]\) IscU
from a ‘protected’ to ‘accessible’ state ready for cluster transfer. The idea that
chaperones may have a role in modulating the conformation of the \(\alpha_2\beta_2\) scaffold was
discussed previously in Chapter 3 of this manuscript.

The proposal that iron sulfur clusters accumulate on the IscU scaffold in HscBA
depleted crude extracts is supported by additional observations detailed in Dr. Ina
O’Carroll’s dissertation (O’Carroll, 2009). For example, when the \(isc\) operon is over-
expressed on pDB1720 by arabinose induction in \(E. coli\), large amounts of iron-sulfide
precipitates are found in the crude cell extract. Conversely, no precipitates are present
when HscB and HscA are absence under the same growth conditions. Since the full \(isc\)
operon is induced in the absence of elevated levels of apo-target proteins, the
precipitation of iron sulfides is attributed to the over-production of iron sulfur clusters on
IscU and their subsequent release in to the cytoplasm. When HscBA are deleted,
stabilized clusters accumulate on IscU, preventing their release into the cytoplasm.

Histidine- tagged IscU species purified from \(E. coli\) cells expressing pDB1720 (\(iscU\)his)
and pDB1722 (\(iscU\)his, \(\Delta hscBA\)) (See Figure 3.1) also reveal different cluster occupancy
following immobilized metal affinity chromatography. IscU isolated from cells deficient
in HscBA, had elevated levels of bound iron-sulfur clusters compared to IscU isolated
from the over-expression of the wild-type \(isc\) operon (O’Carroll, 2009).

A recently published report demonstrates that ISU1 from yeast have a slowed rate
of degradation when HscA homolog, Ssq1, is deleted (Andrew et al., 2008). While
cysteine desulfurase, Nfs1, is required for the ISU1 degradation, the authors find that
only levels of ISU1 are elevated in crude extracts lacking Ssq1. These results suggest the
importance of iron sulfur cluster biosynthesis for ISU1 degradation, but also reveal that
no other ISC assembly proteins, (including Nfs1, Jac1, Mge1, Yah1, Isa1, and Isa2) are
increased protein levels in the absence of eukaryotic chaperone protein. Another study
reveals that iron sulfur proteins in \(Saccharomyces cerevisiae\) missing an intact cluster are
more susceptible to attack by Pim1, a eukaryotic homologue to bacterial Lon1 protease
(Major et al., 2006). Additionally U-type scaffolds have been shown to be stabilized by
the binding of a cluster or metal ion at the scaffold site (Bertini et al., 2003). Together
these results support a growing body of evidence that suggest proteins are structurally stabilized by the presence of [Fe-S] clusters, and that structural modulation could acts as a sensor for detection by proteases. This ‘regulation via degradation’ may be an especially important mechanism to control proper assembly of clusters on IscU during periods of oxidative stress.

Our results have shown a correlation between accumulation of intracellular IscU and the stabilization of a cluster on the IscU scaffold site. Our previous work has also shown that IscU assumes different structural conformations in apo- and holo states (Raulfs et al., 2008). Additionally, we have demonstrated that a 39 Asp to Ala in IscU substitution stabilizes a [2Fe-2S] cluster on an IscSU α2β2 tetrameric complex. Work performed by Dr. Ina O’Carroll suggests that a cluster is also stabilized on the IscU scaffold by the deletion of HscBA proteins. To connect these corollary observations, we propose analyzing the degradation rate of apo- and holo-IscU purified from both pDB1720 (iscUhis), pDB1716 (iscU39DAhis) and pDB1722 (iscUhis, ΔhscBA). If our theory is correct, we would expect to see an increased rate of degradation of apo-IscU vs. holo-IscU when exposed to a protease cocktail.
Figure A.3 *Azotobacter vinelandii* strains used in this study

*Isc* strains used in this study are shown containing two copies of the *isc* operon under endogenous (*isc*) control and inducible sucrose control. The endogenous *isc* operon contains a poly-8-histidine tag at the C-terminus of *iscU*. Gray squares are used to show deleted gene regions and black dots represent site-directed amino acid variants. The specific amino acid residue substituted in the IscU or IscS protein, is denoted by a number above the respective gene.
Figure A.4 IscU, IscS, GroEL western blot from *A. vinelandii* strains

Western blot showing levels of IscU, IscS, and control protein, GroEL from *A. vinelandii* strains containing mutations in the ISC biosynthetic gene region. Extracts from the following strains were analyzed: DJ1697 (wt), DJ1766 (iscU39\textsuperscript{DA}), DJ1752 (iscU63\textsuperscript{CA}), DJ1764 (iscU106\textsuperscript{CA}), DJ1807 (iscS328\textsuperscript{CA}), and DJ1788 (ΔhscA).
Appendix III. Functional Analysis of IscR

INTRODUCTION

IscR is a two domain protein. The N terminus, which shares a high degree of homology to transcriptional regulator MarA, contains a helix-turn-helix binding motif and is therefore thought to be involved in DNA binding (Schwartz et al., 2001). The C terminal domain contains three conserved cysteines that are necessary for cluster coordination. Appendix Table 2 lists strains and plasmids constructed containing mutations in both the DNA and [Fe-S] cluster binding domains. A requirement for the study of IscR in A. vinelandii involved decoupling IscR regulation from isc expression. Because ISC biosynthetic machinery is necessary for cell survival, an isc “merodiploid” parent, DJ1525, was used which contains a second copy of the essential isc genes under the control of an inducible sucrose promoter. Isc expression was determined using an hscA´-lacZYKnR reporter construct in the endogeneous isc operon.

MATERIALS AND METHODS

Cell strains containing mutations in IscR were constructed using a site-directed Gene Editor mutagenesis kit from Promega, according to the manufacturer’s instructions. β-galactosidase activity assays were performed according to the protocols detailed in Chapter 4.

RESULTS / DISCUSSION

In total, 14 new iscR strains containing site-directed substitutions or deletions were constructed and their effect on hscA´-lacZYKnR expression was determined by β-galactosidase assays (Appendix Table A.5). The values plotted in Table 2 represent triplicate lacZ activity measurements of listed strains. Strains with in-frame deletions in iscR in A. vinelandii were found to exhibit a 5-7 fold increase in β-galactosidase activity
in comparison to wild-type iscR strains. Alanine substitutions of three conserved cysteines, \(92^C\), \(98^C\), and \(104^C\) in the [Fe-S] cluster binding domain resulted in \(\beta\)-galactosidase activity similar to \(\Delta\text{iscR}\) strains, suggesting that these cysteines are essential for [Fe-S] cluster co-ordination. These results are significant therefore in reinforcing the idea that [2Fe-2S] cluster coordination of IscR is necessary for isc repression.

Alanine substitutions in the proposed DNA binding domain including residues, \(34^R\), \(38^S\), and \(48^K\), resulted in varying levels of de-repression, suggesting the importance of these amino acids in DNA recognition and binding. Interestingly, an alanine substitution of \(111^C\), a \(4^{th}\) cysteine found in the cluster binding domain of \textit{A. vinelandii}, resulted in decreased \(\beta\)-galactosidase activity in comparison to WT activity levels. This extra cysteine is not highly conserved but is present in IscR from \textit{Azotobacter} and \textit{Pseudomonas} genera. This result suggests that in these taxa \(111^C\) does not play a role in [Fe-S] cluster coordination for IscR, but instead may act as a cluster ‘destabilizing’ factor. An alanine substitution of \(91^R\), in the cluster binding domain also resulted in decreased \(\beta\)-galactosidase activity in comparison to WT activity levels. Both \(111^C\) and \(91^R\) substitutions exhibit 0.5- 0.75 isc expression levels in comparison to WT IscR. The discovery of non-isc inducible forms of IscR suggests these mutants may more tightly bind their clusters or the promoter region than wild-type IscR. These iscR strains have the potential to be useful for studies in which we would like to limit the amount of isc expression. It is also possible that \(111^C\) and \(91^R\) substitutions may provide a source of holo-IscR that remains stable following protein purification.
### Table A.5 β-Galactosidase Activity of Φ(hscA-lacZ) strains with IscR substitutions or deletions in *A. vinelandii*

<table>
<thead>
<tr>
<th>Strain</th>
<th>IscR variant</th>
<th>β-Galactosidase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DJ1532</td>
<td>IscR (WT)</td>
<td>74 (+/- 12)</td>
</tr>
<tr>
<td>DJ1579</td>
<td>IscR34RA</td>
<td>132 (+/- 12)</td>
</tr>
<tr>
<td>DJ1590</td>
<td>IscR38SA</td>
<td>224 (+/- 26)</td>
</tr>
<tr>
<td>DJ1591</td>
<td>IscR48KA</td>
<td>128 (+/- 17)</td>
</tr>
<tr>
<td>DJ1597</td>
<td>IscR91RA</td>
<td>39 (+/- 9)</td>
</tr>
<tr>
<td>DJ1582</td>
<td>IscR92CA</td>
<td>372 (+/- 51)</td>
</tr>
<tr>
<td>DJ1595</td>
<td>IscR93QA</td>
<td>95 (+/- 10)</td>
</tr>
<tr>
<td>DJ1531</td>
<td>IscR98CA</td>
<td>265 (+/- 51)</td>
</tr>
<tr>
<td>DJ1543</td>
<td>IscR101GC</td>
<td>344 (+/- 68)</td>
</tr>
<tr>
<td>DJ1587</td>
<td>IscR104CA</td>
<td>386 (+/- 53)</td>
</tr>
<tr>
<td>DJ1592</td>
<td>IscR111CA</td>
<td>46 (+/- 8)</td>
</tr>
<tr>
<td>DJ1594</td>
<td>IscR120FA</td>
<td>212 (+/- 24)</td>
</tr>
<tr>
<td>DJ1556</td>
<td>ΔIscR (A^{21}-P^{61})</td>
<td>356 (+/- 84)</td>
</tr>
<tr>
<td>DJ1557</td>
<td>ΔIscR (V^{55}-P^{61})</td>
<td>365 (+/- 63)</td>
</tr>
<tr>
<td>DJ1580</td>
<td>ΔIscR (S^{57}-E^{119})</td>
<td>366 (+/- 86)</td>
</tr>
</tbody>
</table>
Appendix IV. Growth phenotypes of ΔcysE and ΔiscR strains

INTRODUCTION

Serine acetyltransferases, which catalyze the conversion of serine to O-acetylserine, perform the rate-limiting step in the cellular production of L-cysteine (Zheng et al., 1998). *Azotobacter vinelandii* contains three serine O-acetyl transferase genes, cysE1, cysE2, and cysE3. As these enzymes are required to produce L-cysteine necessary for [Fe-S] assembly, it is not surprising that two of the three *A. vinelandii* cysE genes are found in the same genomic region as iron-sulfur cluster assembly operons. CysE1 is under NifA promotional control and located down stream of nifUSV. CysE2 is expressed upstream of the isc gene region in a small operon with trmH, a tRNA methyltransferase. Both a 92\textsuperscript{CA} substitution and deletion of the ISC regulatory protein, IscR, reveal decreased levels of trmH cysE2 expression compared to wild-type IscR (Chapter 4), suggesting the importance of holo-IscR for proper trmH cysE2 expression. The final serine acetyltransferase, cysE3, is located between a gene for cysteine desulfurase similar to SufS, and rhdE, encoding a rhodanese protein.

MATERIALS AND METHODS

DJ155 (ΔcysE1), DJ1502 (cysE3::Kn), and DJ1830 (ΔcysE1 cysE3::Kn) were constructed in Dr. Timothy Larson’s laboratory. DJ1818 was constructed by the transformation of DJ1601 with pDER76, a plasmid containing a Kanamycin cartridge replacing most of cysE3. DJ1819 was constructed by the transformation of DJ155 (already containing ΔcysE1) with pDB1490 (a plasmid containing an inframe deletion of iscR), and pDER76 used as a congression plasmid. The recipe for supermedia plates was the same as Burk’s media with the addition of 8 grams of nutrient broth per 1 liter.
RESULTS / DISCUSSION

Triple cysE1, cysE2, and cysE3 deletion strains in A. vinelandii are unviable (Dr. Timothy Larson, communicated results). Figure A.6 reveals that ΔcysE1 (strain #1 DJ155), cysE3´::Kn (strain #2 DJ1502), and ΔcysE1 cysE3´::Kn (strain #4 DJ1830) do not exhibit measurable differences in growth on supermedia plates with or without the addition of ammonium. This result supports the idea that cell viability is maintained as long as one serine acetyltransferase is expressed, and that expression of cysE2 and cysE3 are sufficient to compensate for ΔcysE1 even when there is a high demand for cysteine under nitrogen fixing conditions. Subtle growth differences between strains DJ155, DJ1502, and DJ1830 in Figure A.6 may be more apparent on less rich growth media.

Results from Chapter 4 demonstrate that ΔiscR strains diminish trmH cysE2 expression, and additionally exhibit improved growth under nitrogen fixing conditions. In order to test the hypothesis that improved growth could be due to the elevated expression of cysE1 under diazotrophic conditions, we constructed strains DJ1818 (ΔiscR, cysE3´::Kn (#3)), and DJ1819 (ΔiscR, cysE3´::Kn, ΔcysE1 (#5)). These strains grow significantly worse than ΔiscR strains. In fact, DJ1819 was not viable unless cultured on highly enriched supermedia (data not shown). Since both DJ1818 (#3) and DJ1819 (#5) reveal improved growth under diazotrophic conditions, we conclude that the improved growth of the ΔiscR strains in general is not due to the expression of cysE1 under nitrogen fixing conditions.
Growth of ΔiscR and ΔcysE strains on supermedia

Figure A.6  Growth of ΔiscR and ΔcysE strains on supermedia

Growth of ΔiscR and ΔcysE strains on supermedium under nitrogen fixing (right panel) and non-nitrogen fixing (left panel) growth conditions.
Appendix V. Crystallization Trials of the IscU39DAIscS complex

In Chapter 2, we detailed the isolation of a holo-IscU39DAIscS complex from *A. vinelandii* representing a stable intermediate of the *in vivo* cluster assembly process. Because the α₂β₂ complex is non-dissociating, the isolation of this species provides an opportunity to pursue further structural analysis of the holo-IscU39DAIscS complex. A high resolution crystal structure of the α₂β₂ IscU39DAIscS complex containing natively synthesized cluster would undoubtedly shed significant insight on the process of *in vivo* assembly and provide a better understanding of IscS and IscU interactions in general.

This work was initiated as part of my EIGER internship, at the Laboratoire de Cristallographie et Cristallogénèse des Protéins (LCCP) under the supervision of Dr. Juan Fontecilla-Camps and Dr. Yvain Nicolet in Grenoble, France. Given that our *in vivo* sample was fairly complex, (containing two proteins in the process of transient cluster assembly), this lab was chosen because it is one of the best facilities in the world equipped for large-scale anaerobic crystallization trials. The LCCP team has previously solved the structure of other oxygen sensitive metalloproteins, including [Fe-Fe] hydrogenase, [Ni-Fe] hydrogenase, biotin synthase, and NikA, among many others.

Starting in the spring of 2007, I spent several months in this laboratory learning anaerobic crystallization techniques; preparing, filtering, and degassing solutions for manual set-up of hanging-drop crystallization trays in a large anaerobic chamber. Although I learned a variety of ‘tricks’ for growing crystals (including micro-seeding techniques, oil immersion, and the always trusty ‘dance a crystal jig’), no conditions resulted in the growth of reproducible protein crystals. Solutions tested are listed in Table A.8. During the last month of my stay in France, I also tried a few large-scale aerobic screens using automated crystallography facilities available at the Jean Pierre Ebel Institute.

I continued with our efforts to obtain reproducible protein crystals of the holo-IscU39DAIscS complex in Blacksburg by collaborating with Dr. Florian Schubot and Nancy Vogelaar at the Virginia Tech X-ray Crystallography lab. Deciding to forgo the manual anaerobic strategy, we turned to high-throughput methods available in the Schubot lab for screening different crystal growth solutions simultaneously. We also
followed a protocol to reductively methylate surface lysines of natively expressed IscU39<sup>DA</sup>IscS sample in hopes of limiting charge repulsion and facilitating more stable protein/protein interactions for crystal growth (1997). This technique did not yield any enhancements in crystallization however. Following these lessons, we shifted focus towards obtaining different protein samples for further crystallization trials.

We have seen a limited improvement in crystal growth using recombinantly expressed <i>A. vinelandii</i> IscU39<sup>DA</sup>Isc protein isolated from <i>E. coli</i>. Unlike natively expressed complex which contains cluster, IMAC and anion exchange purification of histagged IscU39<sup>DA</sup> from pDB1712 results in a pure apo-form of the α2β2 IscU39<sup>DA</sup>Isc complex (see Chapter 3 for further details). Following the set-up of several large-scale crystallization screens, we observed very small needle-like crystals in the C1 well (3.5 M Na<sup>+</sup> formate, pH 7) of the INDEX screen. Unfortunately these crystals were sensitive to temperature and dissolved back into the mother solution when analyzed under the microscope! We decided the best approach would be to optimize the Na<sup>+</sup> formate conditions and perform all subsequent crystal analysis at 4°C. As a result, we made a ‘home-made’ sodium formate screen containing over 100 conditions of varying molarity and pH to use with the automated crystallography system. Even when using the exact same Apo-IscU39<sup>DA</sup>Isc protein preparation however, we were only able to reproduce the small needle-like protein crystals one time with our home-made buffer screen. Crystal growth was not enhanced by increased protein concentration.

**The C-terminal Histag of IscU is susceptible to degradation**

In the course of working with the C-terminally histagged IscU39<sup>DA</sup>IscS samples we noticed that the histagged complex, particularly IscU39<sup>DA</sup>his was susceptible to degradation when left for a week or longer at room temperature. This was observed on SDS-PAGE by the formation of two distinct IscU bands (Figure A.7B). Sequencing of the upper and lower bands by LC mass-spectrometry revealed that the cleavage site was located between Lysine125 and Glycine126 of the IscU protein, effectively removing the C-terminal octa-histidine domain. The identical cleavage site was further confirmed in a separate experiment in which both native and recombinantly expressed IscU39<sup>DA</sup>IscS
were incubated with 1 mM of thermolysin protease, revealing the formation of the same upper and lower IscU bands by SDS-PAGE (also confirmed by LC-MS). The result suggests that native IscU39DAIscS may contain residual protease contaminants causing protein degradation and explaining our inability to grow stable crystals over time. We have previously isolated ATP La protease, (Avin 13920) with the holo- IscU39DAIscS complex under low imidazole conditions and theorize that this may be the source of protease contamination in vivo (Appendix I). Crystallographers used thermolysin protease to remove floppy protein extensions in order to probe the most structurally stable ‘core’ of the protein. Our results suggests that the C-terminal octahistidine tag is a floppy extension of IscU available for protease dependent degradation.

**Project progression using IscU and IscS from Archeaoglobus fulgidus**

In the meantime my lab colleagues Dr. Patricia Dos Santos and Dr. Ina O’Carroll lead an undergraduate research class titled, “The Cutting Edge of DNA” in which students cloned IscS and IscU genes from Archeal genomes into an arabinose expression vector. The archeal genomes used by the class for PCR sequencing of IscU and IscS were the generous gift of Dr. Biswarup Mukhopadhyay (Virginia Polytechnic Institute). This project was undertaken to help isolate more compact and thermostable IscU and IscS proteins for future crystallization trials, which could be readily purified using heat-treatment techniques in place of immobilized metal affinity chromatography. One clone from the sulfur-metabolizing hyperthermophile, *Archeaoglobus fulgidus*, exhibited promising expression of both IscS and IscU proteins in *E. coli*. During the summer and fall semester of 2008, I supervised a senior undergraduate student researcher from Animal & Poultry Sciences, Kyle Cromer, on the optimization and purification of these proteins. Like *A. vinelandii*, IscS and IscU from *A. fulgidus* purify together as a loose complex. The *A. fulgidus* proteins can be separated from the *E. coli* crude extract by a single step heat treatment at 85°C for 10 minutes, which removes over 90% of the contaminating proteins (Figure A.7A). *A. fulgidus* IscS and IscU can be further purified via anion exchange chromatography. Kyle has recently constructed an *A. fulgidus* IscU clone containing an alanine substitution of the conserved Aspartate-40 residue.
Purification of this protein sample may provide a more stable non-histidine tagged $\text{IscU}^{\text{DA}}\text{IscS}$ complex for continued crystallization trials.
Figure A.7 Results related to crystallographic studies of IscU/IscS complex

(A) Heat treatment of *E. coli* crude extracts over-expressing *A. fulgidus* IscS and IscU. 65°C treatment for 20, 40, and 60 minutes removes most proteins. Heat treatment at 85°C for 10 minutes is sufficient for removing nearly all *E. coli* proteins. Molecular weight markers shown (from bottom to top): lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5k Da), carbonic anhydrase (31 kDa) and ovalbumin (45 kDa)

(B) SDS-PAGE showing two proteolysis products of IscU39DA scaffold protein after leaving natively expressed IscU39DAIscS complex for one week at room temperature. The top band is the non-degraded IscU39DA protein, containing the C-terminal octahistidine tag. The bottom band shows loss of the C-terminal histag due to a cleavage event between residues K^{125} and G^{126}. The sequence of the top and bottom protein bands were determined by LC- mass spectrometry. Molecular weight markers shown (from bottom to top): lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), and phosphorylase B (97.4 kDa).
<table>
<thead>
<tr>
<th>Date</th>
<th>Protein Sample</th>
<th>Variables tested in Manual Screen</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/15/07</td>
<td>7/27 sample (9 mg/mL, 100 mM Tris, pH 9)</td>
<td>10 - 60% MPD, 0.5-3.0 M Ammonium Sulfate, 5%-30% PEG 6000</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines – Glove Box 3 trays</td>
</tr>
<tr>
<td>4/13/07</td>
<td>7/27 sample (9 mg/mL, 100 mM Tris, pH 9)</td>
<td>PEG 400, 8000, 20000 (varying concentrations) pH 5 - 8 Cadmium (Y/N) PEG 5% - 25%</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines – Glove Box 4 trays, hanging drop</td>
</tr>
<tr>
<td>4/20/07</td>
<td>7/27 sample (9 mg/mL, 100 mM Tris, pH 9)</td>
<td>PEG 6000, 8000, 10,000 PEG 1% - 15% pH 5.5, 5.7, 6.0, 6.3, 6.5, 6.6, 7.0</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines – Glove Box 3 trays – 2 drops each per well (one containing 1/1 ratio of protein to mother liquid, another containing a 2/1 ratio)</td>
</tr>
<tr>
<td>5/3/07</td>
<td>7/27 sample (9 mg/mL, 100 mM Tris, pH 9)</td>
<td>50% PEG: 2000, 6000, 10000 100 mM Tris, pH 9 1 M Na acetate</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines – Glove Box 2 trays 1 tray - 2µL protein / 1µL mother liquid 1 tray – 1µL protein/ 1µL mother liquid precipitate structure in A2, A3, A4 &amp; B6 showed agglomerated protein structures which disintegrated upon ‘fishing’ indicating they are not real crystals, but likely a precipitated structure. A small crystal from A3 was fished and frozen.* (this was the sample I tested on synchrotron*)</td>
</tr>
<tr>
<td>5/3/07</td>
<td>7/27 sample (9 mg/mL, 100 mM Tris, pH 9)</td>
<td>2 – 0.25 M Na malonic acid pH 5 -8 13% PEG 6000 (Y/N)</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines – Glove Box 1 tray – 1µL protein/ 1µL mother liquid</td>
</tr>
<tr>
<td>5/10/07</td>
<td>7/27 sample (9 mg/mL, 100 mM Tris, pH 9)</td>
<td>Trying to optimize A2, A3, A4 cells from 5/3 screen PEG 2000 10%, 20% PEG 10000 14%, 16%, 18% PEG 8000 14%, 16%, 18% 70mM Na citrate (Y/N) 70mM Na malonate (Y/N) 70mM Na acetate (Y/N) 100mM Tris, pH 9</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines – Glove Box 2 trays 2µL protein / 1µL mother liquid</td>
</tr>
<tr>
<td>5/15/07</td>
<td>SuperD prep (6 mg/mL, 50 mM Tris, 400 mMNaCl) 7/27 sample</td>
<td>Same A2, A3, A4, and B6 solutions from 5/3/07 box but with varying concentrations of</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines – Glove Box</td>
</tr>
<tr>
<td>Date</td>
<td>Sample Details</td>
<td>Protein Details</td>
<td>Notes</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>6/5/07</td>
<td>SuperD prep (6 mg/mL, 50 mM Tris, 400 mM NaCl) 7/27 sample (9 mg/mL, 100 mM Tris, pH 9)</td>
<td>Same A2, A3, A4, and B6 solutions from 5/3/07 box</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines – Glove Box Determined that soluble (non-precipitated protein) is only found under these conditions: 14% -22% PEG 10,000 20% -22% PEG 2,000 100 mM Tris, pH 9 (no salt!)</td>
</tr>
<tr>
<td>6/18/07</td>
<td>SuperD prep (6 mg/mL, 50 mM Tris, 400 mM NaCl) 7/27 sample (9 mg/mL, 100 mM Tris, pH 9)</td>
<td>Trying to optimize supersaturation by limiting rate of water because A4 solution precipitates too quickly. Row A- regular protein Row B- regular protein with 500 µL reservoir volume Row C- regular protein with oil Row D- 2/3 concentrated protein</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines – Glove Box 3 trays (one for each protein sample) 2µL protein/1µL additive DDAO/1µL mother liquid</td>
</tr>
<tr>
<td>6/22/07</td>
<td>SuperD prep (6 mg/mL, 50 mM Tris, 400 mM NaCl) 7/27 sample (9 mg/mL, 100 mM Tris, pH 9)</td>
<td>0.1 M Tris pH 8.5 0.2 M Lithium sulfate (Y/N) 25% PEG 8000 20% PEG 3350 DTT (Y/N) DDAO (Y/N)</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines – Glove Box 2µL protein/ 2µL buffer Or 2µL protein/ 1µL mother liquid / 1µL DDAO one tray in glovebox, one outside glovebox</td>
</tr>
<tr>
<td>6/22/07</td>
<td>SuperD prep (6 mg/mL, 50 mM Tris, 400 mM NaCl) 7/27 sample (9 mg/mL, 100 mM Tris, pH 9)</td>
<td>A4 solution DTT (Y/N) DDAO (Y/N) Or 100 mM Tris, pH 8.5, 0.2 M lithium sulfate, 25% PEG 8000 DTT (Y/N) DDAO (Y/N)</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines – Glove Box 2µL protein/ 2µL buffer 2µL protein/ 1µL mother liquid / 1µL DDAO one tray in glovebox, one outside glovebox</td>
</tr>
<tr>
<td>7/24/07</td>
<td>SuperD protein (size exclusion purified)</td>
<td>Replicate D10 and G7 solutions from large scale PEG screen D10 = Calcium acetate, 20% PEG 3350 G7 = 0.2 M Calcium chloride,</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines – Glove Box one tray in glovebox, one outside glovebox All these ‘crystals’ turned out to be</td>
</tr>
<tr>
<td>Date</td>
<td>Description</td>
<td>Solution Details</td>
<td>Notes</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>7/24/07</td>
<td>SuperD protein (size exclusion purified)</td>
<td>20% PEG 3350 Varied 10 – 20% PEG 3350 pH 8 or pH 9, 100 mM Tris</td>
<td>some form of calcium salt crystal according to Yvain.</td>
</tr>
<tr>
<td>2/20/08</td>
<td>11/6 Apo-IscU39\textsuperscript{DA}IscS complex</td>
<td>11/6 Apo-IscU39\textsuperscript{DA}IscS complex</td>
<td>Custom Na\textsuperscript+ formate screens</td>
</tr>
<tr>
<td></td>
<td>5 mg/mL, 25 mM Tris, pH 8, 600 mM NaCl, 1 mM TCEP (pDB1712 transformed and over-expressed in E. coli)</td>
<td>5 mg/mL, 25 mM Tris, pH 8, 600 mM NaCl, 1 mM TCEP (pDB1712 transformed and over-expressed in E. coli)</td>
<td>Schubot Lab, 4°C</td>
</tr>
<tr>
<td></td>
<td>OR Apo-IscSU complex</td>
<td></td>
<td>3 trays</td>
</tr>
<tr>
<td></td>
<td>(pDB1716 transformed and over-expressed in E. coli)</td>
<td></td>
<td>Only one well (B6) had the same needle-like crystals with pDB1712 protein</td>
</tr>
<tr>
<td></td>
<td>B6 solution from Custom Na\textsuperscript+ formate screen + additives</td>
<td></td>
<td>(why is it so difficult to recreate Index C1 condition?)</td>
</tr>
<tr>
<td>3/16/08</td>
<td>11/6 Apo-IscU39\textsuperscript{DA}IscS complex</td>
<td>11/6 Apo-IscU39\textsuperscript{DA}IscS complex</td>
<td>Custom Na\textsuperscript+ formate solution #2 (buffered) + additives</td>
</tr>
<tr>
<td></td>
<td>5 mg/mL, 25 mM Tris, pH 8, 600 mM NaCl, 1 mM TCEP (pDB1712 transformed and over-expressed in E. coli)</td>
<td>5 mg/mL, 25 mM Tris, pH 8, 600 mM NaCl, 1 mM TCEP (pDB1712 transformed and over-expressed in E. coli)</td>
<td>Remade custom screens with 25mM Tris And 0.6M NaCl pH 6.2 – 8.3 Na formate Molarity 2.5 - 4.7</td>
</tr>
<tr>
<td>3/18/08</td>
<td>11/6 Apo-IscU39\textsuperscript{DA}IscS complex</td>
<td>11/6 Apo-IscU39\textsuperscript{DA}IscS complex</td>
<td>Custom Na\textsuperscript+ formate solution #2 (buffered) + additives</td>
</tr>
<tr>
<td></td>
<td>5 mg/mL, 25 mM Tris, pH 8, 600 mM NaCl, 1 mM TCEP (pDB1712 transformed and over-expressed in E. coli)</td>
<td>5 mg/mL, 25 mM Tris, pH 8, 600 mM NaCl, 1 mM TCEP (pDB1712 transformed and over-expressed in E. coli)</td>
<td>Remade custom screens with 25mM Tris And 0.6M NaCl pH 6.2 – 8.3 Na formate Molarity 2.5 - 4.7</td>
</tr>
<tr>
<td>5/16/08</td>
<td>5/15 Apo-IscU39\textsuperscript{DA}IscS complex</td>
<td>5/15 Apo-IscU39\textsuperscript{DA}IscS complex</td>
<td>Custom Na\textsuperscript+ formate solution #2 (buffered) DTT (Y/N)</td>
</tr>
<tr>
<td></td>
<td>16 mg/mL, 50 mM Tris, pH 8, 400 mM NaCl</td>
<td></td>
<td>Schubot Lab, 4°C</td>
</tr>
</tbody>
</table>

**Notes:**
- 20% PEG 3350 Varied 10 – 20% PEG 3350 pH 8 or pH 9, 100 mM Tris
- Some form of calcium salt crystal according to Yvain.
- Laboratoire de Cristallographie et Cristallogenèse des Protéines – Glove Box
- One tray in glovebox, one outside glovebox
- For each drop used: 1µL protein solution / 1µL mother liquid / 0.7µL crystal seed solution
- Schubot Lab, 4°C
- Florian suggests I need higher concentration of protein for bigger crystals
<table>
<thead>
<tr>
<th>Date</th>
<th>Protein Sample</th>
<th>Large scale Automated Screens</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>6/14/07</td>
<td>7/27 sample (9 mg/mL, 100 mM Tris, pH 9)</td>
<td>JCSG</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines</td>
</tr>
<tr>
<td>7/13/07</td>
<td>7/27 sample (9 mg/mL, 100 mM Tris, pH 9)</td>
<td>PEGS PACT JCSG Classic-Qiagen pH Clear Index Hampton</td>
<td>Laboratoire de Cristallographie et Cristallogenèse des Protéines D10 and G7 from PEG screen had crystals</td>
</tr>
<tr>
<td>8/30/07</td>
<td>Super D (size exclusion purified, 6.4 mg/mL) Reductive methylation of lysine residues in this sample</td>
<td>PEG/Ion Index JCSG Wizard I&amp;II MemFrac Classic-Qiagen</td>
<td>Schubot Lab</td>
</tr>
<tr>
<td>1/28/08</td>
<td>11/6 Apo-IscSU complex 5 mg/mL, 25 mM Tris, pH 8 600 mM NaCl, 1 mM TCEP (pDB1712 transformed and co-expressed in E. coli)</td>
<td>Screens set up in duplicate, storing one at RT and one at 4°C Hampton Crystal Screen Complex Screen, MF/PI Index Wizard I &amp; II, JCSG</td>
<td>Schubot Lab C1 condition of Index gave the only thing that Florian says may actually be real crystals Very small needle-like crystals This condition = 3.5 M Sodium formate, pH 7.0, needles disappeared after 2 weeks</td>
</tr>
<tr>
<td>12/9/08</td>
<td><em>A. fulgidus</em> IscSU (wildtype) sample (21 mg/mL, 50 mM Tris pH 8, 500 mM NaCl)</td>
<td>Wizard I &amp; II, duplicate Index JCSG</td>
<td>No crystals, will retry trays with IscU39&lt;sup&gt;Δ30&lt;/sup&gt; clone from <em>A. fulgidus</em></td>
</tr>
</tbody>
</table>
Appendix VI. Annotation of the *Azotobacter vinelandii* Genome

As part of large multi-laboratory effort to thoroughly scrutinize the JGI automated annotations of the recently sequenced *Azotobacter vinelandii* genome, our laboratory became involved in the process of manual gene annotation and analysis. Individual gene annotation can be a very time-consuming process which involves examining the DNA sequence, ribosomal binding site, genomic context, conserved motifs, and primary literature in order to make an educated guess about the protein function and category of the gene in question. The genes that I directly annotated or was directly involved in supervising, via an undergraduate student annotator, Lanessa R. Bryant, are listed in Table 4.

In addition, Drs. Patricia Dos Santos, Ina O’Carroll, and I also completed analysis on genomic regions involved in respiration and oxidative response in the *A.vinelandii* genome. I was assigned to look specifically at aconitase and oxidative stress response proteins and my comments are included below.

**Aconitases and oxidative stress response proteins *A. vinelandii* genome**

*Azotobacter vinelandii* has been cited as having one of the highest respiratory rate of any known bacteria (Jordan et al., 1999; Kelly et al., 1990; Sandercock and Page, 2008a). Feeding the respiratory electron transport chain, are two \([\text{Fe-S}]\) dependent aconitases, the major citric acid cycle enzyme, AcnB (Avin 23470) and iron and oxidative-stress induced enzyme, AcnA (Avin 20040). Studies in *E. coli* have shown that AcnA is the most stable aconitase isoform, and is able to operate over a broad pH and oxygen range whereas the more active and abundant enzyme, AcnB, functions optimally only at physiological pH (Jordan et al., 1999). Although the case is not known for *A. vinelandii*, in *E. coli* both apo-AcnA and apo-AcnB have been shown to bind their own cognate mRNAs (Tang and Guest, 1999). *A. vinelandii* also contains a non-TCA related \([\text{Fe-S}]\) aconitase, AcnD (Avin23230), which has the same domain structure as AcnA, and is found in the *prp* operon required for the utilization of propionate.
The major citric acid cycle enzyme, AcnB, is found in an anomalous region of the chromosome next to the nitrate/nitrite assimilation (nas) system (Figure A.9). Interestingly we find the oxidative response aconitase, AcnA, directly upstream of terminal oxidase complexes, cbb3 oxidase, and the oxygen sensitive regulator, CydR, suggesting mutual induction under elevated oxygen. In *E.coli* AcnA is regulated by both Fur and the SoxRS in response to iron and superoxide stress. The AcnD homolog is not found in *E. coli*, but is contained in most *Psuedomonas* species. AcnD requires PrpF *in vivo*, which is speculated to both protect the [4Fe-4S] cluster on AcnD and to aid in the cis-trans isomerization step of the substrate (Garvey et al., 2007; Grimek and Escalante-Semerena, 2004). The other genes in the prp operon include prpB, which is a methyl-isocitrate lyase, and prpC, which is a 2-methyl citrate synthase. *E. coli* and other enterics replace the function of AcnD and PrpF with AcnC, a non-[Fe-S] dependent dehydratase, which is not found in the *A. vinelandii* genome. A last note is the close genomic proximity of AcnD and AcnB, which are separated by only 30 kilo-basepairs.

*Azotobacter* is predicted to utilize several proteins in response to oxidative stress. The genome contains two superoxide dismutases, an Fe-SOD (Avin37820) and a Cu/Zn-SOD (Avin 30470) (Qurollo et al., 2001). Hydroperoxidases, *ahpC* (Avin 45910) and *ahpF* (Avin 26320), are also found but they are not transcribed in an operon as in *P. aeruginosa*. There are at least three catalase genes, a monofunctional *katE* (Avin 47920), bifunctional (both catalase and peroxidase activity) *katG* (Avin05880), and a novel catalase, ccc (cytochrome c catalase) (Avin 05690). KatG has been shown to be expressed during exponential growth while cytochrome c catalase is RpoS-dependently expressed during nutrient limitation (Sandercock and Page, 2008a). *Azotobacter vinelandii* contains the LysR-type oxidative response regulator, OxyR, (Avin 48440) which is in an operon with RecG, as found previously in *P. aeruginosa* (Ochsner et al., 2000), and the global iron regulatory protein, Fur (Avin 43000). We found evidence of at least one clear Fur binding domain located upstream of the *fluCBD* operon involved in siderophore transport. No SoxRS homologs were found in the *A. vinelandii* genome.
Figure A.9 Location of aconitase genes in the *Azotobacter vinelandii* genome.

The relative locale of *acnA*, *acnD*, and *acnB*, are shown in the color-coded schematic of the *A. vinelandii* genome above. Neighboring gene regions of each of the aconitase genes are also shown.
**Table A.10 Azotobacter vinelandii Genes Annotated**

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene name</th>
<th>Product</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avin03150</td>
<td>Avin05500</td>
<td>Avin12970</td>
<td>Avin19190 Avin23140 Avin23230 Avin28270 Avin29620</td>
</tr>
<tr>
<td>Avin2330</td>
<td>Avin28270</td>
<td>Avin29620</td>
<td>Avin30310 Avin40150 Avin40300 Avin40310 Avin40320</td>
</tr>
<tr>
<td>Avin40380</td>
<td>Avin40400</td>
<td>Avin40400</td>
<td>Avin40410 Avin40420 Avin40420 Avin40420 Avin40420</td>
</tr>
<tr>
<td>Accession</td>
<td>Gene ID</td>
<td>Protein Name</td>
<td>Function/Category</td>
</tr>
<tr>
<td>-----------</td>
<td>---------</td>
<td>--------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Avin40430</td>
<td>trmH</td>
<td>RNA methyltransferase, TrmH group I</td>
<td>Translation/tRNA modification</td>
</tr>
<tr>
<td>Avin40440</td>
<td>suhB</td>
<td>Inositol-1-monophosphatase</td>
<td>Fatty acid and phospholipids metabolism/Other</td>
</tr>
<tr>
<td>Avin40460</td>
<td>secD</td>
<td>SecD membrane export protein</td>
<td>Cellular processes/Sec-dependant protein secretion</td>
</tr>
<tr>
<td>Avin40470</td>
<td>yajC</td>
<td>Preprotein translocase, YajC</td>
<td>Cellular processes/Sec-dependant protein secretion</td>
</tr>
<tr>
<td>Avin40480</td>
<td>tgt</td>
<td>Queuine tRNA-ribosyltransferase</td>
<td>Translation/tRNA modification</td>
</tr>
<tr>
<td>Avin40490</td>
<td>queA</td>
<td>Queuine biosynthesis protein: S-adenosylmethionine tRNA ribosyltransferase</td>
<td>Biosynthesis of cofactors, prosthetic groups, and carriers/ Other</td>
</tr>
<tr>
<td>Avin40500</td>
<td>PagE</td>
<td>PagE intergrase</td>
<td>Other categories/phage related functions and prophages</td>
</tr>
<tr>
<td>Avin40520</td>
<td>Integrase, catalytic domain-containing protein</td>
<td>Other categories/phage related functions and prophages</td>
<td></td>
</tr>
<tr>
<td>Avin40530</td>
<td>Transposase</td>
<td>Other categories/transposon-related functions</td>
<td></td>
</tr>
<tr>
<td>Avin40560</td>
<td>Hypothetical protein</td>
<td>Undefined</td>
<td></td>
</tr>
<tr>
<td>Avin40590</td>
<td>Transcriptional regulator protein</td>
<td>Hypothetical/Conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin40720</td>
<td>Ribosomal protein, S20p</td>
<td>Translation/Ribosomal proteins: synthesis and modification</td>
<td></td>
</tr>
<tr>
<td>Avin40750</td>
<td>CreA family protein</td>
<td>undefined</td>
<td></td>
</tr>
<tr>
<td>Avin41060</td>
<td>Conserved hypothetical protein</td>
<td>Hypothetical/Conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin41070</td>
<td>radA</td>
<td>DNA repair protein, RadA</td>
<td>DNA metabolism/ DNA replication, recombination, and repair</td>
</tr>
<tr>
<td>Avin41080</td>
<td>Conserved hypothetical protein</td>
<td>Hypothetical/ conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin41090</td>
<td>mscL</td>
<td>Large conductance mechanosensitive channel protein</td>
<td>Transport and binding proteins/ porins</td>
</tr>
<tr>
<td>Avin41110</td>
<td>Peptidylprolyl isomerase, FKBP-type</td>
<td>undefined</td>
<td></td>
</tr>
<tr>
<td>Avin41140</td>
<td>OmpA family protein</td>
<td>Cell envelope/Membrane associated receptors</td>
<td></td>
</tr>
<tr>
<td>Avin41150</td>
<td>Metallo beta-lactamase like protein</td>
<td>Energy metabolism/Other</td>
<td></td>
</tr>
<tr>
<td>Avin41170</td>
<td>Hypothetical protein</td>
<td>Hypothetical/Non-conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin41180</td>
<td>Staphylococcus nuclease</td>
<td>DNA metabolism/ Degradation of</td>
<td></td>
</tr>
<tr>
<td>Accession</td>
<td>Description</td>
<td>Functional Category</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>Avin41200</td>
<td>Conserved hypothetical DNA</td>
<td>Hypothetical/Conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin41210</td>
<td>Petidoglycan binding protein</td>
<td>Hypothetical/Conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin41220</td>
<td>Conserved hypothetical protein</td>
<td>Hypothetical/Conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin41230</td>
<td>D-glycerate dehydrogenase</td>
<td>Amino acid biosynthesis/serine family</td>
<td></td>
</tr>
<tr>
<td>Avin41240</td>
<td>rRNA (guanine-N(2))-methyltransferase</td>
<td>Translation/Ribosomal proteins: synthesis and modification</td>
<td></td>
</tr>
<tr>
<td>Avin41250</td>
<td>Conserved hypothetical protein</td>
<td>Hypothetical/Non-conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin41260</td>
<td>Conserved hypothetical protein</td>
<td>Hypothetical/Non-conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin41270</td>
<td>Peptidase M48, Ste24p family</td>
<td>Cellular processes/ proteolysis</td>
<td></td>
</tr>
<tr>
<td>Avin41280</td>
<td>Conserved hypothetical protein</td>
<td>Hypothetical/Conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin41290</td>
<td>Acyltransferase</td>
<td>undefined</td>
<td></td>
</tr>
<tr>
<td>Avin41300</td>
<td>Hypothetical</td>
<td>Hypothetical/Non-conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin41320</td>
<td>Conserved hypothetical protein</td>
<td>Hypothetical/Conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin41340</td>
<td>Conserved hypothetical protein</td>
<td>Hypothetical/Conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin41350</td>
<td><em>hpt</em></td>
<td>Hypoxanthine phosphoribosyltransferase</td>
<td></td>
</tr>
<tr>
<td>Avin41360</td>
<td><em>upp</em></td>
<td>Uracil phosphoribosyltransferase</td>
<td></td>
</tr>
<tr>
<td>Avin41370</td>
<td><em>hemH</em></td>
<td>Ferrochelatase</td>
<td></td>
</tr>
<tr>
<td>Avin41380</td>
<td>Sugar nucleotide epimerase</td>
<td>Hypothetical/Conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin41410</td>
<td>Transcriptional regulatory protein, MerR-family</td>
<td>Regulatory functions/MerR</td>
<td></td>
</tr>
<tr>
<td>Avin41430</td>
<td><em>phr</em></td>
<td>Deoxyribodipyrimidine photolase</td>
<td></td>
</tr>
<tr>
<td>Avin41440</td>
<td><em>pqqE</em></td>
<td>Coenzyme PQQ, biosynthesis E</td>
<td></td>
</tr>
<tr>
<td>Avin41860</td>
<td>Integrase, catalytic domain-containing protein</td>
<td>Other categories/Phage-related functions and prophages</td>
<td></td>
</tr>
<tr>
<td>Avin42030</td>
<td>Antitoxin protein</td>
<td>undefined</td>
<td></td>
</tr>
<tr>
<td>Avin31730*</td>
<td><em>cysT</em></td>
<td>Sulfate ABC transporter, permease protein, CysT</td>
<td></td>
</tr>
<tr>
<td>Avin31740*</td>
<td><em>cysW</em></td>
<td>Sulfate ABC transporter, permease protein, CysW</td>
<td></td>
</tr>
<tr>
<td>Avin39620*</td>
<td>NUDIX hydrolase</td>
<td>Purines, pyrimidines, nucleosides and nucleotides/Nucleotide and nucleoside interconversions</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>------------------</td>
<td>------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Avin39630*</td>
<td>NUDIX hydrolase</td>
<td>Purines, pyrimidines, nucleosides and nucleotides/Nucleotide and nucleoside interconversions</td>
<td></td>
</tr>
<tr>
<td>Avin39640*</td>
<td>ErfK/YbiS/YcfS/YnhG-related protein</td>
<td>undefined</td>
<td></td>
</tr>
<tr>
<td>Avin39650*</td>
<td>Conserved hypothetical protein</td>
<td>Hypothetical/Conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin39670*</td>
<td>Periplasmic binding domain/transglycosylase SLT domain fusion protein</td>
<td>Transport and binding proteins/substrate binding protein</td>
<td></td>
</tr>
<tr>
<td>Avin39720*</td>
<td>guaA</td>
<td>GMP synthase</td>
<td></td>
</tr>
<tr>
<td>Avin39730*</td>
<td>IMP dehydrogenase</td>
<td>Purines, pyrimidines, nucleosides, and nucleotides/Purine ribonucleotide biosynthesis</td>
<td></td>
</tr>
<tr>
<td>Avin39740*</td>
<td>DNA helicase</td>
<td>Hypothetical/Conserved hypothetical</td>
<td></td>
</tr>
<tr>
<td>Avin46920*</td>
<td>Sulfate transporter</td>
<td>Transport and binding proteins/Anions</td>
<td></td>
</tr>
<tr>
<td>Avin41020</td>
<td>Hypothetical protein, 28 amino acids</td>
<td><strong>DELETED</strong></td>
<td></td>
</tr>
<tr>
<td>Avin41050</td>
<td>Hypothetical protein, 31 amino acids</td>
<td><strong>DELETED</strong></td>
<td></td>
</tr>
<tr>
<td>Avin41420</td>
<td>Hypothetical protein, 33 amino acids</td>
<td><strong>DELETED</strong></td>
<td></td>
</tr>
</tbody>
</table>

* annotated by Lanessa R. Byrant
REFERENCES


CHAPTER 6

Conclusions and Outlook

The assembly of iron sulfur clusters is an essential life sustaining process requiring multiple proteins and various levels of regulation. Starting with the original characterization of nitrogenase related iron-cluster assembly proteins, NifU and NifS, the Dean laboratory has been instrumental in the field of iron-sulfur cluster biogenesis for over two decades (Jacobson et al., 1989a). Since these times, the field has seen the discovery of two additional cluster producing systems including ISC, which assembles clusters for general purpose use (Zheng et al., 1998), and SUF which fabricates [Fe-S] clusters during periods of oxidative stress (Takahashi and Tokumoto, 2002). With the discovery of more cysteine desulfurase proteins (CsdA, CsdE) (Loiseau et al., 2005) and alternative scaffold sites (NfuA, ErpA, IscA) (Angelini et al., 2008a, b; Bandyopadhyay et al., 2008a; Krebs et al., 2001; Loiseau et al., 2007) it is likely our understanding of how cells synthesize clusters will only continue to expand.

Despite the evolutionary adaptations of the nif, isc, and suf systems, it is clear that each system shares a large degree of commonality in both form and function. For example, each assembly system involves the use of either a U- or A-type scaffold protein, containing three conserved cysteines, which serve as thiol ligands for cluster assembly in vivo. A PLP-dependent cysteine desulfurase protein required for the mobilization of sulfide is another common requirement of cluster assembly systems. More recently we have learned that some proteobacteria also contain a well-characterized regulatory protein, IscR, which functions to provided negative feed back regulation of isc expression (Schwartz et al., 2001). In bacteria which contain both the isc and suf assembly systems, IscR provides dual control of these two operons (Giel et al., 2006). Though not common to the nif and suf assembly systems, the isc system contains an additional set of assembly genes including potential scaffold protein, IscA, heat shock chaperone-like proteins, HscB and HscA, and a [2Fe-2S] containing ferredoxin. The
exact roles of these proteins in the process of iron-sulfur cluster biogenesis is still a matter of debate.

Upon joining the lab in the summer of 2004, I inherited a rich tradition of iron-sulfur knowledge, anaerobic purification techniques, and genetic know-how. My lab colleague Dr. Deborah Johnson previously constructed and bequeathed to the lab a merodiploid genomic system allowing manipulation of the endogenous copy of the *isc* operon, while still maintaining cell viability by the expression of a second copy of the *isc* operon under sucrose control (Johnson, 2006). Using this genetic system, we found that an IscU39\(^{DA}\) substitution (strain DJ1453) yields a dominant-negative phenotype *in vivo* (Johnson et al., 2006). Previous *in vitro* work with this substitution from our lab had revealed that the IscU39\(^{DA}\) scaffold does not inhibit iron-sulfur cluster assembly, but does limit ready transfer to apo-target proteins (Unciuleac et al., 2007). The discovery of a dominant-negative phenotype reveals that *in vivo* the IscU39\(^{DA}\) variant scaffold restrains the function of another protein. Given these results and *in vitro* evidence, our hypothesis at the time was that IscU39\(^{DA}\) either prevented the function of an apo-target protein or the proper workings of wild-type IscU expressed from the sucrose operon.

The construction of *Azotobacter* strain, DJ1697, containing C-terminally histagged IscU with elevated expression of ISC proteins has allowed us to ‘biochemically’ address some of the phenotypes exhibited by *A. vinelandii* merodiploid strains (for a schematic of this strain, see Figure 2.1). For example, my work has shown that *in vivo* the IscU39\(^{DA}\) mutant is trapped in a tight \(\alpha_2\beta_2\) cluster-containing complex with the cysteine desulfurase protein, IscS (Figure 2.3). Single turn-over kinetic studies reveal that the intermediate PLP::L-cysteine state of IscS is lengthened in the IscU39\(^{DA}\) mutant, suggesting that the variant scaffold inhibits *in vivo* function of IscS (Figure 2.5D). The isolation of this species reveals that it is quite possibly the trapping of IscS with IscU39\(^{DA}\), (not wild-type IscU or an apo-target protein), that causes the detrimental dominant-negative phenotype of this strain *in vivo*. The isolation of a stable wild-type \(\alpha_2\beta_2\) cluster-containing complex has not been possible, suggesting that cluster formation is a dynamic process, and that the IscU39\(^{DA}\)IscS variant complex represents a final ‘snapshot’ of this process *in vivo*.
Studies with the IscU39DAIscS protein expressed from *E. coli* reveal that only IscU and IscS are required for [2Fe-2S] cluster synthesis, $\alpha_2\beta_2$ complex formation and dissociation (see Chapter 3). Reconstitution experiments suggest that the $\alpha_2\beta_2$ complex, not IscU alone, may serve as a scaffold site *in vivo* (Figure 3.4 and Figure 3.8). From this work, it is clear that chaperone proteins HscB, HscA, and ferredoxin, function in cluster assembly following the completed biosynthesis of a [2Fe-2S] cluster.

Our novel genetic/biochemical approach allows a unique understanding of genetic phenotypes and progression in understanding from genetic, to protein, to organismal. But there is still much we do not understand. What happens after the formation of a [2Fe-2S] cluster for example? How is the iron brought to the IscU scaffold? Why is only the sulfur donor (not the iron donor) trapped with the IscU39DA variant? How are [4Fe-4S] clusters assembled? Do chaperone proteins and ferredoxin play a role in this process? How is iron sulfur cluster assembly tied to other forms of cellular regulation, particularly: iron homeostasis, oxidative stress response, respiration, protein degradation, and cellular differentiation?

In regards to this last question, new studies are divulging increased connections between the health of [Fe-S] cluster biosynthesis and the regulation of other key cellular processes. A crucial protein tying [Fe-S] biosynthesis to the rest of the cell is regulatory protein, IscR, which has recently been found to be play a role in: suf expression, biofilm formation, bacterial pathogenicity, oxidative stress management, and anaerobic respiration (Choi et al., 2007; Giel et al., 2006; Rincon-Enriquez et al., 2008; Wu and Outten, 2009; Yeo et al., 2006).

In *A. vinelandii* we have found that holo-IscR is essential for proper regulation of *isc* expression (Chapter 4 of this manuscript). Additionally we have documented IscR dependent effects on the regulation of upstream *trmH cysE2* operon, and downstream *hscBA fdx*. The $\Delta$iscR strain has a small colony phenotype which can be relieved by growth under nitrogen fixing conditions (Figure 4.4). Growth at elevated oxygen tensions increases the probability that the strain will form spontaneous suppressor mutants (Figure 4.6). IscR also plays a role in differentiation because its absence causes *A. vinelandii* to form large cyst-like cells and causes the over expression of a protein, AldA, which has previously been shown to be involved in cyst formation of *Azotobacter*.
cells (Gama-Castro et al., 2001) (Figure 4.5 and Figure 4.7). We hypothesize that the formation of cyst-like structures is a consequence of intracellular stress due to the toxic over-accumulation of iron and sulfide. Supporting a broader understanding of IscR regulation in general, we have found that the loss of apo-IscR function is even more severe than the loss of holo-IscR function. These results suggest that IscR has cellular roles in addition to the regulation of the isc operon, and most likely in the form of apo-IscR. Our findings reveal that [Fe-S] cluster biogenesis and oxidative stress are directly tied to cellular differentiation in *Azotobacter vinelandii*, as has also been seen with biofilm formation in *E. coli* (Wu and Outten, 2009).

In the introduction to this dissertation I discussed how the evolution of the ISC assembly machinery was an adaptive response to oxygenation of the atmosphere. In addition to the fact that [Fe-S] clusters, (and especially transient [Fe-S] clusters) are generally labile, various results from our laboratory over the years have hinted at this evolutionary connection between cluster assembly and oxidative protection. The discovery that both NfuA and IscA are essential at high oxygen (Bandyopadhyay et al., 2008a, b; Johnson et al., 2006) indicates that they either serve as alternative scaffolds under these conditions, or as carriers for target proteins essential during oxidative stress. The finding that chaperone proteins, HscB and HscA, are dispensable at low oxygen gives a clue about their function under ambient conditions (Johnson et al., 2006), suggesting that under these conditions, chaperone proteins may function to protect [Fe-S] cluster assembly and transfer. As our recent work indicates HscB and HscA do not participate in [2Fe-2S] assembly, we assume that the protective mechanism is most likely related to [4Fe-4S] cluster assembly or transfer to apo-target proteins.

How might clusters be protected during [2Fe-2S] assembly? Our work with the IscU39<sup>DA</sup> variant sheds some light on this subject. For example, we have seen that clusters trapped on the IscU39<sup>DA</sup>-IscS scaffold complex are oxygen stable over the course of days, whereas [2Fe-2S] cluster on the IscU wild-type degrades in a matter of minutes upon exposure to air (Figure 2.4). The result suggests that the α<sub>2</sub>β<sub>2</sub> scaffold provides a protected scaffold site for the construction of nascent [2Fe-2S] clusters before dissociation of the IscS/IscU complex. Different structural characteristics of holo-IscU39<sup>DA</sup>-IscS and apo-IscU39<sup>DA</sup>-IscS complex as outlined in Chapter 3, lead to the
hypothesis that in vivo the IscU IscS $\alpha_2\beta_2$ could form ‘protective’ and ‘exposed’
conformations to allow for the assembly of nascent clusters and the transfer of iron and
sulfur components to the IscU scaffold site (Figure 3.8). Additionally, the IscU39$^{DA}$
variant may structurally stabilize the cysteine scaffold, causing stronger bonding of the

While there is much we do not understand in terms of how the ISC machinery has
evolved to assemble oxygen-sensitive clusters in aerobic environments, results from our
laboratory suggest that specialized scaffolds (NfuA, IscA), chaperone proteins, and a
protective $\alpha_2\beta_2$ assembly complex may all help shield nascent iron-sulfur clusters from
oxygen exposure during the cluster assembly and transfer process. This realization
highlights the fact that ISC machinery represents one type of cellular response to a 4
billion year fight between iron, oxygen, and proteins.
6.1 REFERENCE


