Controlled Expression and Functional Analysis of the Iron-Sulfur Cluster (Isc) Biosynthetic Machinery in *Azotobacter vinelandii*

Deborah Cumaraswamy Johnson

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

APPROVED

_____
D. R. Dean, chairman

_____
T. J Larson

_____
B.S.J. Winkel

_____
R. H. White

May 5th, 2006
Blacksburg, Virginia

Keywords: [Fe-S] cluster biosynthesis, Isc, Nif, *Azotobacter vinelandii*

Controlled Expression and Functional Analysis of the Iron-Sulfur Cluster (Isc) Biosynthetic Machinery in *Azotobacter vinelandii*

Deborah Cumaraswamy Johnson

(ABSTRACT)

A system was developed for the controlled expression of genes in *Azotobacter vinelandii* by using genomic fusions to the sucrose catabolic regulon. This system was used for the functional analysis of the *A. vinelandii isc* genes, whose products are involved in the maturation of [Fe-S] proteins. For this analysis the *scrX* gene, contained within the sucrose catabolic regulon, was replaced by the *A. vinelandii iscS, iscU, iscA, hscB, hscA, fdx, iscX* gene cluster, resulting in duplicate genomic copies of these genes, one whose expression is directed by the normal *isc* regulatory elements (P*isc*) and the other whose expression is directed by the *scrX* promoter (P*scrX*). Functional analysis of [Fe-S] protein maturation components was achieved by placing a mutation within a particular P*isc*-controlled gene with subsequent repression of the corresponding P*scrX*-controlled component by growth on glucose as the carbon source.

This experimental strategy was used to show that IscS, IscU, HscBA and Fdx are essential in *A. vinelandii* and that their depletion results in a deficiency in the maturation of aconitase, an enzyme that requires a [4Fe-4S] cluster for its catalytic activity. Depletion of IscA results in null growth only when cells are cultured under conditions of elevated oxygen, marking the first null phenotype associated with the loss of a bacterial IscA-type protein. Furthermore, the null growth phenotype of cells depleted for HscBA could be partially reversed by culturing cells under conditions of low oxygen. These results are interpreted to indicate that HscBA and IscA could have functions related to the protection or repair of the primary IscS/IscU machinery when grown under aerobic conditions. Conserved amino acid residues within IscS, IscU, and IscA that are essential for their respective functions and/or display a partial or complete dominant-negative growth phenotype were also identified using this system. Inactivation of the IscR repressor protein resulted in a slow growth phenotype that could be specifically attributed to the elevated expression of an intact [Fe-S] cluster biosynthetic system.

This system was also used to investigate the extent to which the two [Fe-S] biosynthetic systems in *A. vinelandii*, Nif and Isc, can perform overlapping functions. Under normal laboratory growth conditions, no cross-talk between the two systems could be detected. However, elevated expression of Isc components as a consequence of inactivation of the IscR repressor protein results in a modest ability of the Isc [Fe-S] protein maturation components to replace the function of Nif-specific [Fe-S] protein maturation components. Similarly, when expressed at very high levels the Nif-specific [Fe-S] protein maturation components could functionally replace the Isc components. Oxygen levels were also found to affect the ability of the Nif and Isc systems to perform common functions. Nevertheless, the lack of significant reciprocal cross-talk between the Nif and Isc systems when they are produced only at levels necessary to satisfy their respective physiological functions, indicates a high level of target specificity with respect to [Fe-S] protein maturation.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dennis Dean for providing me with an excellent education in scientific research and the opportunities to gain the professional skills necessary for a successful academic career. His high expectations, enthusiasm and scientific wisdom have allowed me to mature professionally and personally, to appreciate the possibilities and limitations of research in the life sciences and to gain the confidence and motivation to pursue an academic career path. I am also very grateful for all the positive guidance and support I received from my current and former committee members, Tim Larson, Brenda Winkel, Robert White and Sunyoung Kim.

I have had the privilege of working with and learning from an extremely competent group of women scientists: I owe a special thanks to my lab colleague and friend Patricia Dos Santos who has helped me in so many ways. Her scientific sharpness and enthusiasm have both challenged and inspired me continuously for four years. I thank Valerie Cash for improving my molecular biology skills and for her saintly patience. I thank my former and current lab colleagues and friends: Suzy Mayer and Mihaela Unciuleac, for teaching me biochemistry and for always reminding me to take one step at a time; Callie Raulfs, Ina Puleri, Brook Ragle, Lauren Stone and Melissa dela Cuesta for their ideas, enthusiasm, help and patience; Milagros Perez for being such a reliable and helpful undergraduate research assistant.

I would like to acknowledge the personal contribution of many family members and close friends who have been my greatest cheerleaders. In particular, I want to thank my sister Shanthi and my close friends, Samantha Kirwan, Ujwala Warek and Jennifer/Steve Brauner for always being willing to listen and offer valuable advice during the more challenging times. Thanks to the most special woman in my life, my mother Rosy, for her unwavering support / encouragement and for helping me balance my time between completing my PhD and caring for my wonderful son, Liam. Finally, I want to acknowledge my husband, Marty Johnson, without whom none of this would have been possible or worth it. Thanks for being my pillar of strength and for believing in me.

TABLE OF CONTENTS

ABSTRACT……..ii
ACKNOWLEDGEMENTS……...……..iv
TABLE OF CONTENTS……..v
LIST OF FIGURES……...…….….vii
LIST OF TABLES……....x

CHAPTER 1: Introduction to [Fe-S] Cluster Biosynthesis:
the requirement of a controlled expression system in *A. vinelandii*.....1

CHAPTER 2: Literature Review: Structure, Function, and Formation of
Biological Iron-Sulfur Clusters……...5
2.1 Introduction and Perspectives…….....6
2.2 Functions of Biological [Fe-S] Clusters……..6
2.3 Structures and Properties of Biological [Fe-S] Clusters……....11
2.4 Formation of Biological [Fe-S] Clusters……...12
2.5 Nitrogenase Maturation – The First Paradigm for Biological [Fe-S]
Cluster Assembly……...12
2.6 Systems Involved in Generalized [Fe-S] Protein Maturation……....21
2.7 Biochemical Features of Proteins Involved in [Fe-S] Protein Maturation……28
2.8 IscS has a General Role in Intracellular Sulfur Trafficking……..39
2.9 [Fe-S] Protein Maturation in Eukaryotes……...41
2.10 Summary and Outlook……...……43

CHAPTER 3: Controlled Expression and Functional Analysis of Iron-Sulfur
Cluster Biosynthetic Components within *Azotobacter vinelandii*.....45
3.1 Introduction…….46
3.2 Materials and Methods……49
3.3 Results…….59
3.4 Discussion……...78

CHAPTER 4: Cross-Talk Studies: Analyzing Possible Overlapping Functions between the Isc and Nif Systems…….…….84

Part 1: NifU and NifS are Required for the Maturation of Nitrogenase and Cannot Replace the Function of *isc*-Gene Products……...85

4.1 Introduction…….86

4.2 The *isc* and *suf* Systems also have [Fe-S] Cluster Biosynthetic Functions…….....87

4.3 Controlled Expression of *isc* and *nif* Genes……...89

4.4 NifU and NifS Cannot Functionally Replace IscU and IscS……...90

Part 2: 'Cross-talk' revisited: Conditions under which the Nif and Isc [Fe-S] Protein Maturation Systems Exhibit Full or Partial Functional Equivalence……94

4.5 Introduction……..95

4.6 Materials and Methods…….96

4.7 Results and Discussion……..……100

CHAPTER 5: Summary and Future Directions……111

REFERENCES.....118

APPENDIX I: Evidence of the accumulation of apo-forms of the [2Fe-2S] enzyme, Benzoate Dioxygenase, in cells depleted for IscU……....152

APPENDIX II: Plasmids constructed during this project……..160

APPENDIX III: Strains constructed during this project……169

VITA……...205

LIST OF FIGURES

CHAPTER 2

Figure 1 Structures, core oxidation states and spin states of crystallographically defined Fe-S clusters…….9

Figure 2 Salient mechanistic feature of the NifS/IscS class of cysteine desulfurases…….....15

Figure 3 Organization of genes from various organisms whose products are known or suspected to be involved in [Fe-S] protein maturation……...18

Figure 4 Comparison of primary sequences of representative members of theNifU/IscU/SufU family of proteins…….....19

Figure 5 Comparison of primary sequences of members of the IscA/SufA family of proteins from *Azotobacter vinelandii* and *Escherichia coli*……23

CHAPTER 3

Figure 1 Schematic representation of the relevant genetic organization of key strains used in this work…….....61

Figure 2 Effect of depletion of Isc components in *A. vinelandii*……...63

Figure 3 Depletion of Isc components in *A. vinelandii* has a detrimental effect on aconitase activity…….....66

Figure 4 Primary sequence comparisons for IscU from *A. vinelandii* (Av) and *E. coli* (Ec), and SufU from *Clostridium acetobutylicum* (Ca) and *Thermotoga maritima* (Tm)…….....68

Figure 5 Growth phenotypes exhibited by strains having selected residues of the IscR-regulated copy of IscU substituted by alanine…….69

Figure 6 Comparison of the primary amino acid sequences of three IscA homolgs encoded within *A. vinelandii* ……...71

Figure 7 IscA is essential under elevated oxygen conditions and cannot be replaced by IscA2…….....72
Figure 8 Growth phenotypes exhibited by strains having mutations within the IscR-regulated copy of IscA…….....74
Figure 9 Effect of low oxygen on growth in cells depleted for Isc components…….....75
Figure 10 Deletion of *iscR* (DJ1601) results in a slow growth phenotype that can be reversed by deletion of *iscU* (DJ1603) or *hscBA* (DJ1609)…….....78

CHAPTER 4

Figure 1 Organization of the *isc* gene cluster and *nifUS* genes in *A. vinelandii* strains used in this work…….88
Figure 2 Growth of *A. vinelandii* strains cultured under different conditions……..91
Figure 3 Key features of plasmids, pDB1551 and pDB1562, which respectively contain the *A. vinelandii nifUS* and *iscSUAhscBAfdxiscX* genes under control of the strong *ara* transcriptional and translational elements from *E.coli*…….98
Figure 4 Expression of the Nif system under low oxygen concentrations rescues the null growth phenotype of a strain with an in-frame deletion in *iscU*……...102
Figure 5 Arabinose-dependent and abundant expression of NifU and NifS in *A. vinelandii* strain DJ1626……104
Figure 6 Arabinose-induced, elevated expression of NifU and NifS provides permissive growth conditions for a strain with an in-frame deletion in *iscU*……105

Figure 7 Elevated expression of NifU and NifS does not provide permissive growth conditions for a strain with an in-frame deletion in *iscU* under oxidative stress conditions…….107

Figure 8 Elevated expression of the Isc system, resulting from inactivation of *iscR*, improves the growth rate of a strain deleted for *nifU*…….....108

Figure 9 Arabinose-induced, elevated expression of the *isc* operon provides permissive growth conditions for a strain with an in-frame deletion in *nifU*…….110

APPENDIX I

Figure 1 The complete degradation of benzoate by aerobic bacteria is initiated by the conversion of benzoate to benzoate diol by a multicomponent benzoate 1,2-dioxygenase (XylXYZ)…….....154

Figure 2 Transcriptional organization of the *xyl* genes in *Azotobacter vinelandii* whose products catalyze the initial reactions of the benzoate catabolic pathway……...155

Figure 3 Q-sepharose elution profiles of the crude extracts of WT cells grown in the presence and absence of 25 mM sodium benzoate……...156

Figure 4 Q-sepharose elution profiles of the crude extracts of WT cells compared to cells depleted for IscU in glucose media supplemented with 25 mM sodium benzoate.....158

LIST OF TABLES

CHAPTER 2

Table 1 Functions of some biological [Fe-S] clusters…….....7

CHAPTER 3

Table 1 Key parent plasmids and relevant derivatives of these plasmids used for the construction of *A. vinelandii* mutant strains……..51

Table 2 Mutant strains constructed and/or used in this study……56

Table 3 β-galactosidase activity of *A. vinelandii* strains with mutations in *iscR*……..76

CHAPTER 4

Table 1 Mutant strains used in this study……97