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Characterization Of The Yeast Cysteine Desulfurase Complex Within The Mitochondrial Fe-S Cluster Biogenesis

Dulmini Pabasara Barupala
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CHARACTERIZATION OF THE YEAST CYSTEINE DESULFURASE COMPLEX WITHIN THE MITOCHONDRIAL FE-S CLUSTER BIOGENESIS

by

DULMINI P. BARUPALA

DISSERTATION

Submitted to the Graduate School of Wayne State University
Detroit, Michigan
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

2016

MAJOR: BIOCHEMISTRY AND MOLECULAR BIOLOGY

Approved By:

______________________________
Advisor

______________________________
Date
DEDICATION

To my parents for being the pillars of success I stand on today.
ACKNOWLEDGEMENTS

Looking back from where I stand today, I cannot be happier for what I have accomplished in life. As with many of my accomplishments, this dissertation would have not been possible without the help of so many wonderful people whom I have been very fortunate to be around with. So this opportunity is invaluable for me to thank them.

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# TABLE OF CONTENTS

DEDICATION ........................................................................................................................................ ii

ACKNOWLEDGEMENTS ................................................................................................................... iii

LIST OF TABLES .................................................................................................................................. xii

LIST OF FIGURES .............................................................................................................................. xiii

CHAPTER 1-SYNTHESIS, DELIVERY AND REGULATION OF EUKARYOTIC HEME AND FE-S CLUSTER COFACTORS ................................................................................................. 1

1.0 Prelude .......................................................................................................................................... 1

1.1 Abstract ......................................................................................................................................... 3

1.2 The role of iron in biology ............................................................................................................ 3

1.3 Heme cofactors ............................................................................................................................ 7

1.3.1 Introduction ............................................................................................................................. 7

1.3.2 Heme structure and types of heme in nature .......................................................................... 7

1.3.3 Heme biosynthesis pathway .................................................................................................. 8

1.3.3.1 Important steps in heme biosynthesis .............................................................................. 13

1.3.3.2 Regulation of heme biosynthesis .................................................................................... 16

1.3.4 Incorporation of heme into apoprotein recipients ................................................................... 19

1.3.5 Heme function ......................................................................................................................... 20

1.3.6 Diseases of heme synthesis .................................................................................................... 21

1.3.6.1 Porphyrias ....................................................................................................................... 21

1.3.6.2 Additional diseases ........................................................................................................ 25

1.4 Fe-S clusters ................................................................................................................................ 26

1.4.1 Introduction ............................................................................................................................. 26

1.4.2 Fe-S cluster structure ............................................................................................................. 26
1.4.3 General Fe-S cluster biogenesis pathway .......................................................... 29
1.4.3.1 Iron sulfur cluster (ISC) pathway ................................................................. 29
1.4.3.2 Cytosolic iron sulfur assembly (CIA) pathway ................................................. 34
1.4.3.3 Sulfur assimilation (SUF) pathway ............................................................... 35
1.4.4 Fe-S cluster biogenesis regulation .................................................................... 39
1.4.5 Fe-S cluster function .......................................................................................... 40
1.4.6 Fe-S clusters in human disease .......................................................................... 42
1.4.6.1 Friedreich’s ataxia ......................................................................................... 42
1.4.6.2 ISCU myopathy ............................................................................................. 43
1.4.6.3 GLRX5 sideroblastic anemia ......................................................................... 44
1.4.6.4 Additional diseases ....................................................................................... 44
1.5 Summary ............................................................................................................... 45
1.6 Acknowledgements ............................................................................................... 46

CHAPTER 2-COOPERATIVITY BETWEEN CYSTEINE DESULFURASE “NFS1” AND ITS ACCESSORY PROTEIN “ISD11” PROMOTES STABILITY AND ACTIVITY OF THE YEAST PROTEIN COMPLEX ................................................................. 47

2.0 Prelude .................................................................................................................. 47
2.1 Abstract .................................................................................................................. 48
2.2 Introduction ............................................................................................................ 48
2.3 Materials and methods ......................................................................................... 52
2.3.1 Expression of yeast Fe-S cluster synthesis proteins ........................................... 52
2.3.2 Purification of yeast Fe-S cluster synthesis proteins .......................................... 54
2.3.3 Protein size and stoichiometry characterization ............................................... 56
2.3.4 Biophysical properties of proteins .................................................................... 57
2.3.5 Functional properties of proteins ..................................................................... 59
2.3.6 Structural studies of Nfs1 ........................................................................................................61
2.4 Results .......................................................................................................................................61
2.4.1 Co-expression of Nfs1 with Isd11 leads to stable complex formation ........61
2.4.2 Molecular weight and protein stoichiometry of Nfs1-Isd11 complex..........64
2.4.3 Stabilization energetics of the Nfs1-Isd11 complex ........................................68
2.4.4 Nfs1-Isd11 complex is more folded than Nfs1 itself ......................................71
2.4.5 Yeast Nfs1-Isd11 binds the substrate L-cysteine with millimolar affinity ....73
2.4.6 Nfs1-Isd11 is a moderately efficient enzyme ..................................................74
2.4.7 Nfs1-Isd11 complex undergoes a conformational change during L-cysteine binding ................................................................................................................................77
2.5 Discussion ............................................................................................................................82
2.6 Supplementary material .......................................................................................................88
2.7 Acknowledgements ..............................................................................................................92

CHAPTER 3-EFFECT OF YEAST FRATAXIN “YFH1” ON THE STRUCTURE AND FUNCTION OF THE NFS1-ISD11 COMPLEX........................................................................................................93

3.0 Prelude .................................................................................................................................93
3.1 Introduction ..........................................................................................................................94
3.2 Frataxin’s function in Fe-S cluster assembly .................................................................95
3.2.1 Iron binding ability and iron chaperone function of frataxin .........................95
3.2.2 Frataxin interacts with other key proteins in Fe-S cluster assembly pathway ..........................................................................................................................97
3.2.3 Frataxin as a regulator of Fe-S cluster synthesis through controlling cysteine desulfurase activity ...........................................................................................................98
3.3 Experimental methods ......................................................................................................101
3.3.1 Bacterial expression and isolation of proteins .................................................101
3.3.1.1 Expression of yeast Fe-S cluster synthesis proteins .........................101
3.3.1.2 Purification of yeast Fe-S cluster synthesis proteins ......................... 102
3.3.2 Isothermal Titration Calorimetry ............................................................. 105
3.3.3 In vitro pull-down assay ........................................................................ 106
3.3.4 Differential Scanning Calorimetry .......................................................... 106
3.3.5 UV-visible spectroscopy ........................................................................ 107
3.3.6 Cysteine desulfurase activity assay ....................................................... 108
3.4 Results ......................................................................................................... 109
3.4.1 Purified proteins are > 95% pure ............................................................. 109
3.4.2 ITC experiments suggest a possible interaction between Nfs1-Isd11 complex and Yfh1 .......................................................... 111
3.4.3 In vitro pull-down assay indicates no interaction between Nfs1-Isd11 and Yfh1 ........................................................................ 112
3.4.4 Association of Yfh1 with Nfs1/Nfs1-Isd11 does not affect the stability of the proteins .......................................................... 115
3.4.5 Yfh1 does not alter substrate binding affinity of Nfs1-Isd11 ............... 118
3.4.6 Effect of Yfh1 on cysteine desulfurase activity of Nfs1-Isd11 is minimal .. 118
3.5 Discussion .................................................................................................... 121
3.6 Acknowledgements ..................................................................................... 126

CHAPTER 4 - SOLUBILITY BARRIER PREVENTS STRUCTURAL AND FUNCTIONAL STUDIES ON ISD11 ........................................................................ 127

4.0 Prelude ....................................................................................................... 127
4.1 Introduction .................................................................................................. 127
4.1.1 Structure and oligomeric state of Isd11 .................................................. 129
4.1.2 Functional interactions of Isd11 in Fe-S cluster assembly ................... 130
4.1.3 Role of Isd11 in Fe-S cluster assembly ................................................. 131
4.1.3.1 Isd11 prevents aggregation of Nfs1 ............................................... 132
4.3.3.4 Liquid chromatography desalting column ...................................................... 148
4.3.4 Circular dichroism (CD) of refolded Isd11 ...................................................... 153
4.4 Discussion ........................................................................................................... 156
4.5 Acknowledgements ............................................................................................ 160

CHAPTER 5-CONCLUSIONS AND FUTURE DIRECTIONS ........................................ 161

5.0 Prelude .................................................................................................................. 161
5.1 Summarization of dissertation and conclusions ................................................... 162
  5.1.1 Molecular characterization of yeast Nfs1-Isd11 complex ............................... 162
  5.1.2 The transient effect of frataxin on Nfs1-Isd11 activity ............................... 164
  5.1.3 Challenges in expression and purification of Isd11 ................................... 164
5.2 Future directions .................................................................................................... 165
  5.2.1 Elucidation of x-ray crystal structure of Nfs1-Isd11 .................................... 165
  5.2.2 Role of Acyl Carrier Protein in cysteine desulfuration and Fe-S cluster assembly .................................................................................................................. 166
  5.2.3 Elucidation of cysteine desulfurase mechanism of Nfs1-Isd11 ............... 168
  5.2.4 Effect of frataxin on the cysteine desulfurase activity of Nfs1/Isd11/Isu1/Yfh1 complex ........................................................................................................ 174
5.3 Acknowledgements ............................................................................................... 179

REFERENCES .............................................................................................................. 180

ABSTRACT ...................................................................................................................... 206

AUTOBIOGRAPHICAL STATEMENT ......................................................................... 208
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>Comparison of different heme types in nature</td>
<td>11</td>
</tr>
<tr>
<td>Table 2.1</td>
<td>Melting temperatures (°C) for each domain/unfolding event obtained from Differential Scanning Calorimetry data for Nfs1 and Nfs1-Isd11</td>
<td>70</td>
</tr>
<tr>
<td>Table S2.1</td>
<td>Secondary structural elements by percentage for Nfs1-Isd11 and Nfs1(C421A)-Isd11 protein complexes from circular dichroism</td>
<td>89</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>Melting temperatures (°C) for each domain/unfolding event obtained from Differential Scanning Calorimetry data for Nfs1 and Nfs1-Isd11 in the absence/presence of Yfh1</td>
<td>117</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Protein concentrations measured in each dialysis buffer in the sequential dialysis method to refold urea denatured Isd11</td>
<td>147</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Protein concentrations measured in each dialysis buffer in the spin chromatography desalting method to refold urea denatured Isd11</td>
<td>149</td>
</tr>
<tr>
<td>Table 4.3</td>
<td>Isd11 concentrations measured before and after passing through HiTrap desalting column</td>
<td>152</td>
</tr>
<tr>
<td>Table 4.4</td>
<td>Secondary structure of refolded Isd11 determined by far-UV CD</td>
<td>155</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1.1: Major cellular Fe utilization pathways in humans ........................................... 6
Figure 1.2: Structure of heme ............................................................................................. 9
Figure 1.3: Important types of heme found in nature .......................................................... 10
Figure 1.4: Heme biosynthesis pathway ............................................................................. 12
Figure 1.5: Structure for the 3 most common forms of Fe-S Cluster: 2Fe-2S, 3Fe-4S, and 4Fe-4S ................................................................................................................. 28
Figure 1.6: Diagram depicting de novo Fe-S cluster formation and the main Fe-S cluster transfer steps in the ISC, SUF, and CIA systems ............................................. 33
Figure 1.7: Illustration of suf and isc operons, with corresponding promoters (Pisc and Psuf), which play a central role in regulation of SUF and ISC at the genetic level in bacteria ................................................................. 38
Figure 2.1: SDS-PAGE gel of purified proteins ................................................................. 63
Figure 2.2: Size exclusion chromatograms of purified Nfs1 and Nfs1-Isd11 complex compared to the gel filtration standard proteins ...................................................... 66
Figure 2.3: Gel densitometric analysis of the Nfs1-Isd11 complex ................................. 67
Figure 2.4: Differential Scanning Calorimetric thermal profiles for purified Nfs1 and the Nfs1-Isd11 complex ......................................................................................... 69
Figure 2.5: ANS fluorescence spectra for purified Nfs1 and for the Nfs1-Isd11 complex .......................................................................................................................... 72
Figure 2.6: Titration of L-cysteine to Nfs1(C421A)-Isd11 monitored by UV-visible spectroscopy .............................................................................................. 75
Figure 2.7: Rate of persulfide formation by Nfs1-Isd11 complex shown as a function of L-cysteine concentration .............................................................. 76
Figure 2.8: Homology modeling of Nfs1 ............................................................................ 80
Figure 2.9: Change in ANS and tryptophan fluorescence intensities of Nfs1(C421A)-Isd11 upon addition of L-cysteine ............................................................... 81
Figure S2.1: Circular dichroism spectra of purified Nfs1-Isd11 and Nfs1(C421A)-Isd11 protein complexes ......................................................................................... 88
Figure S2.2: Differential Scanning Calorimetric thermal profiles of purified Nfs1-Isd11 and Nfs1(C421A)-Isd11 protein complexes ............................................. 90
Figure S2.3: Persulfide forming activity of Nfs1(C421A)-Isd11 ..................................................91
Figure 3.1: SDS-PAGE gel of purified proteins .................................................................110
Figure 3.2: Isothermal titration calorimetry data for Yfh1 titration into Nfs1-Isd11 complex ..................................................................................................................113
Figure 3.3: Poly-histidine tag pull-down assay for Nfs1-Isd11 interaction with Yfh1. 114
Figure 3.4: Differential Scanning Calorimetric thermal profiles for purified Nfs1 and Nfs1-Isd11 complex in the presence or absence of Yfh1 ...............................116
Figure 3.5: Titration of L-cysteine to Nfs1(C421A)-Isd11 in the presence/absence of Yfh1 monitored by UV-visible spectroscopy .........................................................119
Figure 3.6: Persulfide formation by Nfs1-Isd11 in the presence/absence of Yfh1 .... 120
Figure 4.1: SDS-PAGE for purification of Isd11 from soluble fraction of cell lysate... 142
Figure 4.2: SDS-PAGE for purification of Isd11 from insoluble fraction of cell lysate144
Figure 4.3: Western blot for Isd11 purified from soluble fraction .................................146
Figure 4.4: Desalting chromatogram for urea denatured Isd11 .....................................150
Figure 4.5: SDS-PAGE of Isd11 containing fractions from desalting column ............151
Figure 4.6: Circular dichroism spectra of refolded Isd11 ..............................................154
Figure 5.1: Proposed chemical mechanism for the cysteine desulfurase reaction ...171
Figure 5.2: Absorbance measurements for three variants of yeast Nfs1-Isd11 ...........172
Figure 5.3: Titration of L-cysteine to Nfs1(K299A)-Isd11 monitored by UV-visible spectroscopy .................................................................................................................173
Figure 5.4: Absorbance change measured at 340 nm for Nfs1(C421A)/Isd11, and Nfs1(C421A)/Isd11/Yfh1/Isu1 reconstituted complex upon addition of substrate L-cysteine .............................................................................................................177
Figure 5.5: Persulfide forming activity of Nfs1-Isd11 and reconstituted Nfs1/Isd11/Yfh1/Isu1 complex measured by acid labile sulfide detection assay .................................................................178
CHAPTER 1
SYNTHESIS, DELIVERY AND REGULATION OF EUKARYOTIC HEME AND FE-S CLUSTER COFACTORs

1.0 Prelude

Iron plays an important role in all biological systems. Its utility in biology comes from its unique electrochemical characteristics that impart the ability of the biomolecule for which it binds to mediate complex chemical reactions required to sustain life. Iron participates in a myriad of indispensable reactions including oxidation-reduction, electron transfer, and substrate activation used in a variety of cellular processes including cellular respiration, metabolism, and photosynthesis. In humans, the majority of iron is incorporated into heme, but a minority gets stored bound either within the iron storage protein ferritin or incorporated into proteins either as metal alone or as a metal containing cofactor\(^1\). Among the several iron containing cofactors, Fe-S clusters are the second prominent class after heme and they can be found ubiquitously in all organisms. These inorganic cofactors exist in a number of different configurations but their main function is the transfer of electrons for redox reactions. When bound to proteins, the range of reduction potentials of Fe-S clusters is expanded and fine-tuned by solvent accessibility and by the amino acids in the vicinity of the cluster\(^2,3\). Hence Fe-S clusters associated with proteins can function as excellent electron carriers in almost all cellular processes. As a few examples, they are present in primary and secondary metabolic enzymes, some DNA repair enzymes, and iron regulatory proteins (IRPs)\(^4\). The list of Fe-S proteins is numerous and their function is a thesis by itself, so I will limit my description of Fe-S clusters in this chapter to their assembly and steps related to their utilization.
The remarkable and diverse electronic potentials of Fe-S clusters allow them to be exploited in many systems, and this makes their assembly indispensable for cell viability. This is best highlighted by the number of human diseases associated with defects in Fe-S cluster assembly proteins, disease states of which are outlined in detail below. Studying the characteristics of proteins involved in Fe-S cluster assembly has been a main focus of research within Stemmler lab. My research was centered on uncovering the structural and functional details of cysteine desulfurase, a protein that provides sulfur for the Fe-S cluster assembly within the mitochondria. My original project in this context proposed to individually characterize Isd11, the accessory protein associated with cysteine desulfurase enzyme Nfs1 in yeast mitochondria. But due to inherent insolubility of Isd11, an issue described in detail in chapter 4, continuation of that project was not feasible. Since then, my research focus deviated to study the characteristics of Nfs1-Isd11 as a complex and the effect of frataxin (a proposed iron delivery protein) on its function. Chapter 2 and 3 describes what I accomplished in those regards.

This first chapter serves to deliver the required background information on the field and it highlights the main characters within the Fe-S cluster bioassembly pathway; essential for one to follow the details within the subsequent chapters. It discusses two major iron cofactor assembly pathways: heme and Fe-S cluster synthesis. This report has already been published as a review article which I was the primary author: "Barupala, D. P., Dzul, S. P., Riggs-Gelasco, P. J., & Stemmler, T. L. (2016). Synthesis, delivery and regulation of eukaryotic heme and Fe–S cluster cofactors. Archives of biochemistry and biophysics, 592, 60-75". My role in writing this review included
organizing the entire content and submitting the article for review in addition to writing the text within the introduction and heme assembly sections. Although the discussion about heme cofactor assembly is far from my research interest, it is included here to emphasize the significant amount of time and effort spent to draft that section solely by myself.

1.1 Abstract

In humans, the bulk of iron in the body (over 75%) is directed towards heme- or Fe-S cluster cofactor synthesis, and the complex, highly regulated pathways in place to accomplish biosynthesis have evolved to safely assemble and load these cofactors into apoprotein partners. In eukaryotes, heme biosynthesis is both initiated and finalized within the mitochondria, while cellular Fe-S cluster assembly is controlled by correlated pathways both within the mitochondria and within the cytosol. Iron plays a vital role in a wide array of metabolic processes and defects in iron cofactor assembly leads to human diseases. This review describes progress towards our molecular-level understanding of cellular heme and Fe-S cluster biosynthesis, focusing on the regulation and mechanistic details that are essential for understanding human disorders related to the breakdown in these essential pathways.

1.2 The role of iron in biology

Iron’s abundance and unique chemical characteristics are often exploited by nature to drive the complex chemistry required by cells to maintain life. Iron is the fourth most abundant element on the earth’s crust, so its high prevalence during early evolution is certainly a factor for its current ubiquitous presence in nature. The human body contains 3 to 4 grams of the metal and absorbs 1 to 2 mg of it each day. The
reactivity and ability of the metal to cycle between the Fe(II), (III) and (IV) oxidation states makes it extremely useful for driving intricate reactions in biology that include substrate activation, electron transfer, and oxidation-reduction reactions. Because of this chemical versatility, iron plays a role in nearly every biological pathway. While its utility within biology is apparent, the Achilles heel of this essential metal is its tendency to precipitate in aqueous solutions. Through coordination to biomolecules, however, the solubility of the metal can be controlled and its reactivity attenuated. At the elemental level, first coordination sphere ligands stabilize the solubility of the metal while at the same time tune its chemical properties for selective participation in only desired reactions. Ligand variability in this first coordination sphere helps control how the metal behaves, however at the cellular level a complex network of proteins, controlled at the genetic level, helps ensure metal availability to specific protein partners in the cell.

Pathways related to eukaryotic iron homeostasis are shown in Figure 1.1. Iron is brought into cells either via the transferrin Fe uptake pathway or through direct membrane transporter (ex. the DMT1 pathway). Once in the cell, imported iron can bind to iron regulatory proteins where it serves to control concentration, it can be stored within the ferritin complex, it can be exported, or it can be incorporated as cofactors into apoprotein partners. In humans, the bulk of iron in the body (over 75%) is directed towards heme or Fe-S cluster cofactor biosynthesis. Assembly of the Fe-cofactors follows highly regulated pathways that have evolved to safely build and load these inorganic Fe-containing cofactors into the apoprotein partners while controlling metal reactivity. In eukaryotes, heme biosynthesis is initiated and completed within the
mitochondria, while Fe-S cluster assembly is controlled by separate but correlated pathways within both the mitochondria and the cytosol. Iron plays a central role in a wide array of metabolic processes, so it is not surprising that defects in cofactor assembly leads to human diseases. This review details progress towards elucidating the molecular details of cellular heme and Fe-S cluster biosynthesis. A focus on both regulation and mechanism will be critical for understanding human disorders related to the breakdown in these essential pathways.
Figure 1.1: Major cellular Fe utilization pathways in humans. Under physiological conditions, most Fe is internalized as the Tf-bound form, which undergoes receptor-mediated endocytosis via binding to Tfr1. Decrease in endosomal pH releases Fe(III) from Tf, which is then reduced by the endosomal reductase STEAP3 to Fe(II). Fe(II) is then transported to the cytosol via DMT1 to join the LIP. Fe in LIP can be utilized for storage in ferritin, import to mitochondria for storage and heme and Fe-S cluster synthesis, export to the cytosol via ferroportin, cellular Fe regulation via IRPs and incorporation into other Fe proteins in the cytosol. Under non-physiological conditions Fe(III) can enter the cell after being reduced to Fe(II) and imported by DMT1. Tf: Transferrin, Tfr1: Transferrin receptor 1, DCYTB: duodenal cytochrome b, DMT1: divalent metal transporter 1, STEAP3: six transmembrane epithelial antigen of the phosphate 3, LIP: labile iron pool, PCBP: poly (rC)-binding proteins, OMM: outer membrane of mitochondria, IMM: inner membrane of mitochondria, MFRN: Mitoferrins, FTMT: mitochondrial specific ferritin, ABCB7: transporter for unknown source of sulfur X-S from mitochondria to cytoplasm, IRP: iron regulatory proteins, IRE: iron-responsive elements of mRNA.
1.3 Heme cofactors

1.3.1 Introduction

Among the many metalloporphyrins found in nature, heme is one of the most abundant. It is utilized in many vital biological processes including photosynthesis, oxygen transport, biological oxidation and reduction, and many more. Most of the total iron content in human body is incorporated into heme-containing proteins such as hemoglobin, myoglobin, catalases, peroxidases, nitric oxide synthases, and cytochromes. The unique structure of heme, which consists of the iron cation coordinated to four nitrogen atoms from a tetrapyrrole ring, allows for fine tuning of the metal's reactivity to carry out the function of the biomolecule to which it is attached. Here we discuss the different heme types, their biosynthesis and regulation, their function in biology, and finally the diseases linked to a loss of heme function.

1.3.2 Heme structure and types of heme in nature

Modifications to the basic heme molecule allow for the diverse array of heme functions found in nature. The parent heme molecule, also known as heme b, protoheme IX or protoporphyrin IX, serves as the platform in each case. The tetrapyrrole unit of heme consists of four pyrrole units linked by four methine bridges. A nitrogen atom from each pyrrole coordinates the iron atom in the center of the planar tetrapyrrole ring. Distortions from planarity can be critical to heme function and reactivity (examples include hemoglobin and myeloperoxidase, MPO). Fifth and sixth ligands to the Fe can be provided by amino acid side chains from the apoprotein or by small inorganic molecules coordinating above and below the plane of the ring. Eight positions in the tetrapyrrole carry side chain modifications, particularly methyl groups on carbons...
2, 7, 12, and 18, vinyl groups on carbons 3 and 8, and propionyl groups on carbons 13 and 17 (Figure 1.2). Substitutions to the same carbons with various other side chains create several biologically important heme types found in nature (Figure 1.3). Heme A, B, and C are found in a wide spectrum of organisms and take part in vital biochemical processes such as respiration, photosynthesis and oxygen transport. The additional cofactor types (Heme D, D1, I, M, and O) are species-specific and carry out highly specialized functions. Table 1 summarizes the structural features, occurrences, and known functions of each heme type.

1.3.3 Heme biosynthesis pathway

Since heme serves as an essential cofactor to several proteins involved in central metabolic processes, all organisms have established a conserved biosynthetic pathway to synthesize the cofactor. Atomic detail is available for many of the enzymes involved in bioassembly of heme, and these enzyme structures have provided key insights into reaction mechanisms. The general assembly process consists of four stages: the synthesis of a single pyrrole, the assembly of four pyrroles to make the tetrapyrrole ring, modification of the side chains, and the insertion of iron into the ring (Figure 1.4)\textsuperscript{15}. Specific details for this conserved pathway are outlined below.
Figure 1.2: Structure of heme. Standard 1-24 IUPAC numbering system is used to number the carbon atoms of the tetrapyrrole. 

\begin{figure}
\centering
\includegraphics[width=\textwidth]{heme_structure.png}
\caption{Structure of heme. Standard 1-24 IUPAC numbering system is used to number the carbon atoms of the tetrapyrrole.}
\end{figure}
Figure 1.3: Important types of heme found in nature. Side chain differences with respect to the parent heme molecule (heme B) are shown in blue.
### Table 1.1: Comparison of different heme types in nature.

<table>
<thead>
<tr>
<th>Type</th>
<th>Structure</th>
<th>Remarks</th>
<th>Organisms</th>
<th>Proteins</th>
<th>Function</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heme A</td>
<td>C3= hydroxyethylfarnesyl group</td>
<td>C18= formyl group</td>
<td>Bacteria, archaea, plants and animals.</td>
<td>Cytochrome a containing heme-Cu oxides. E.g. Cytochrome c oxidase/Complex IV in mammalian mitochondria.</td>
<td>Terminal reaction of aerobic respiration. Reduction of oxygen to water.</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>C18= formyl group</td>
<td>Conversion of heme to heme A is catalyzed by Heme A synthase.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Heme B</td>
<td>Parent heme molecule also known as protoheme IX, Fe-protoporphyrin IX or heme. Binds to apoproteins non-covalently.</td>
<td>In a variety of organisms.</td>
<td></td>
<td>Cytochromes b and hemoglobin.</td>
<td>Diverse array of functions associated with heme b such as electron transport and oxygen carrier.</td>
<td>18</td>
</tr>
<tr>
<td>Heme C</td>
<td>C3 and C8= thioether bonds with cysteine residues. Binds to apoproteins covalently.</td>
<td>In a variety of organisms.</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Heme D</td>
<td>C12= hydroxylated, in a trans conformation to the γ-spirolactone. C13= hydroxylated, propionyl group forms a γ-spirolactone with the hydroxyl group.</td>
<td>In diverse bacteria including Azotobacter, Proteus, Acetobacter, Salmonella, Bacillus, Pseudomonas, Haemophilus and E. coli.</td>
<td>Terminal respiratory oxidases. E.g. Cytochrome d oxidase.</td>
<td></td>
<td>Terminal reaction of aerobic respiration. Reduction of oxygen to water at low oxygen levels.</td>
<td>19-21</td>
</tr>
<tr>
<td>Heme D1</td>
<td>C3 and C8= an acetyl group and a methyl group on each carbon. C2 and C7= keto groups on each carbon. C13 propionyl side chain has reduced to incorporate a vinyl group between second and third carbons.</td>
<td>In Pseudomonas perfectomarinus, Alcaligenes faecalis, Paracoccus denitrificans, Thiobacillus denitrificans, and Thiosphaera pantotropa.</td>
<td>Nitrite reductase that functions in dissimilatory nitrogen metabolism. E.g. Cytochrome cd1 nitrite reductase.</td>
<td></td>
<td>Catalyzes the one-electron reduction of nitrite to nitric oxide and four-electron reduction of oxygen to water.</td>
<td>22,23</td>
</tr>
<tr>
<td>Heme I</td>
<td>C2 and C12= hydroxymethyl groups, they form ester bonds with carboxyl side chains of amino acids. Binds to apoproteins covalently.</td>
<td>In mammalian secretory fluids.</td>
<td>Peroxidases such as bovine milk enzyme lactoperoxidase, eosinophil peroxidase and thyroid peroxidase</td>
<td></td>
<td>Peroxide-driven oxidation of halide and pseudohalide ions as a nonspecific antimicrobial defense mechanism for the protection of mucosal surfaces.</td>
<td>26</td>
</tr>
<tr>
<td>Heme M</td>
<td>C2 and C12= hydroxymethyl groups, they form ester bonds with carboxyl side chains of amino acids. C3= vinyl group on C3 forms a sulfonium ion linkage with the sulfur of a methionyl residue.</td>
<td>In mammalian neutrophils</td>
<td>Myeloperoxidase</td>
<td></td>
<td>Peroxide-driven oxidation of chloride and bromide ions as an antimicrobial defense mechanism.</td>
<td>14,25</td>
</tr>
<tr>
<td>Heme O</td>
<td>C3= hydroxyethylfarnesyl group.</td>
<td></td>
<td>Bacteria such as E. coli.</td>
<td>Cytochrome o containing quinol oxidases. E.g. Cytochrome bo3</td>
<td>Terminal reaction of aerobic respiration. Reduction of oxygen to water.</td>
<td>24</td>
</tr>
</tbody>
</table>
1.3.3.1 Important steps in heme biosynthesis

The first step in eukaryotic heme biosynthesis is the mitochondrial formation of 5-aminolevulinic acid (ALA), which is the precursor that serves as the only source of carbon and nitrogen atoms required to build the basic heme unit. Depending on species, one of two major pathways directs formation of this precursor leading to production of tetrapyrroles. In the Shemin pathway, the enzyme ALA synthase (ALAS) that resides on the matrix side of the mitochondrial inner membrane catalyzes the condensation of glycine and succinyl-CoA\(^{27-30}\). ALAS is found in animals, fungi, non-photosynthetic eukaryotes, and \(\alpha\)-proteobacteria (organisms that resemble bacterial ancestors of mitochondria). Mammals have two ALAS isoforms; the first isoform (ALAS1) provides housekeeping functions and a second erythroid specific isoform (ALAS2) provides for the more robust heme requirements in erythrocytes\(^{31}\). Both forms of ALAS are homodimers and require pyridoxal 5\'-phosphate (PLP) bound as a Schiff base covalent adduct to a catalytic lysine. During the joining of glycine and succinyl CoA on the PLP scaffold, both CO\(_2\) and Coenzyme A are released as byproducts\(^{32}\). Upon formation, ALA exits the mitochondria to serve as the substrate for the subsequent four enzymatic conversions that occur in the cytosol. Little is known about the specifics of export, but it has been suggested that SLC25A38, a member of the SLC25 family of transporters of the mitochondrial inner membrane, facilitates import of glycine into the mitochondria in exchange for ALA transfer across the mitochondrial inner membrane\(^{33}\).

In the second major pathway for ALA synthesis, also known as the C\(_5\) pathway, glutamyl-tRNA reductase (GluTR) converts glutamyl-tRNA to glutamate-1-semialdehyde (GSA) in the first of the two-step ALA production pathway\(^{34}\). The second step involves
the conversion of GSA to ALA by the enzyme glutamate-1-semialdehyde-2, 1-amino mutase (GSAM)\textsuperscript{35-37}. This second major pathway is found in plants, archaea and most bacteria.

Once exported to the cytosol, ALA serves as the building block to synthesize uroporphyrinogen III following three consecutive steps. The first step involves condensation of two ALA molecules to form porphobilinogen (PBG) by porphobilinogen synthase (PBGS), also known as ALA dehydratase (ALAD)\textsuperscript{38,39}. PBGS is a homooolymer in which each dimer contains one catalytic site\textsuperscript{40}. Each active site binds an ALA molecule at two distinct sites and each subunit binds one zinc atom\textsuperscript{41}. Of the eight Zn atoms on PBGS, four zinc atoms stabilize the enzyme structure while the other four engage in catalytic activity\textsuperscript{42,43}. Four molecules of porphobilinogen undergo polymerization to form 1-hydroxymethylbilane in a reaction catalyzed by porphobilinogen deaminase (PBGD). PBGD uses a covalently attached dipyrrromethane cofactor (made of two linked PBG molecules) to prime the polymerization of four PBG molecules. Six PBG molecules form a linear hexapyrrole covalently bound to PBGD, which is then cleaved to yield 1-hydroxymethylbilane and the protein bound cofactor\textsuperscript{44}. This unstable tetrapyrrole serves as the substrate for uroporphyrinogen synthase (UROS), the enzyme that synthesizes uroporphyrinogen III. UROS functions as a monomer and completes ring inversion and the subsequent closure of the tetrapyrrole, yielding uroporphyrinogen III\textsuperscript{45,46}. Spontaneous ring closure is also possible, but the resulting product uroporphyrinogen I cannot be converted to heme. Uroporphyrinogen III also serves as the branching point for the synthesis of chlorophylls and corrolins. In heme biosynthesis, this cyclic intermediate is converted to coproporphyrinogen III, a product
that lacks four acetic side chains. Subsequent decarboxylation steps are carried out by uroporphyrinogen decarboxylase (UROD) within the cytosol to yield four methyl groups in place of the acetic side chains\textsuperscript{47}. UROD is a homodimer with each subunit carrying an active site cleft that faces the other in the dimer interface\textsuperscript{48}.

Three enzymes associated with the mitochondrial inner membrane complete the terminal steps in the eukaryotic heme biosynthesis. Their arrangement suggests they act as a multiprotein complex that facilitates substrate channeling\textsuperscript{49}. Coproporphyrinogen III entry into the mitochondria is likely mediated by the peripheral-type benzodiazepine receptor (PBR), located on the mitochondrial outer membrane\textsuperscript{50}. In the first of the three terminal steps, coproporphyrinogen III undergoes oxidative decarboxylation of its propionyl side chains on two pyrrole rings to form protoporphyrinogen IX. This reaction is completed in higher eukaryotes and in a few bacterial species by the oxygen-dependent coproporphyrinogen III oxidase (CPO), an enzyme located in mitochondrial intermembrane space\textsuperscript{51}. CPO requires molecular oxygen as the terminal electron acceptor\textsuperscript{52}.

In the next step, protoporphyrinogen IX is oxidized to protoporphyrin IX, following the removal of six hydrogen atoms from the porphyrinogen ring, to provide an alternating double bond structure to the macrocycle; this reaction is catalyzed by protoporphyrinogen IX oxidase (PPO)\textsuperscript{53}. In eukaryotes, the oxygen-dependent PPO is a homodimer that utilizes molecular oxygen as the terminal electron acceptor and functions as an integral membrane protein located in the mitochondrial inner membrane\textsuperscript{54}. The active site of the protein faces the intermembrane space and uses a
FAD cofactor for electron transfer. The complete reaction uses three $O_2$ molecules that are reduced to three $H_2O_2$ molecules.

Ferrochelatase (FC) is responsible for the insertion of ferrous iron into protoporphyrin IX to form Fe-protoporphyrin IX or heme, in the next step of heme biosynthesis. FC is a mitochondrial inner membrane protein with its catalytic site located on the mitochondrial matrix side. Channeling of protoporphyrin IX from PPO to FC may occur via direct interaction between the two proteins that allows efficient substrate transfer\textsuperscript{54}. FC functions as a homodimer and each monomer contains a 2Fe-2S cluster whose function is unknown\textsuperscript{55,56}. The reaction mechanism involves distortion of the planar porphyrin molecule into a saddle conformation to facilitate the insertion of ferrous iron\textsuperscript{57}. Once synthesized, heme can undergo side chain modifications (e.g. heme A and heme O synthesis) or covalent attachments (e.g. heme C in cytochrome c biogenesis) to produce additional heme types depending on cellular needs.

1.3.3.2 Regulation of heme biosynthesis

In humans, heme is synthesized at two locations, in erythroid progenitors within bone marrow to provide for developing red cells, and in the liver to provide for numerous heme-containing enzymes. Liver responds to various metabolic states in the body, hence heme synthesis in liver is regulated accordingly. Erythroid progenitors on the other hand maintain heme production at a steady pace to meet the demand of red blood cells. Mechanisms for regulation of heme synthesis in these two origins therefore differ.

The first step, catalyzed by two different isoforms of ALAS in liver (ALAS1) and bone marrow (ALAS2), is the rate-limiting step of heme synthesis. Both isoforms are post-transcriptionally regulated by two different mechanisms. Heme has a negative
feedback effect on ALAS1 transcripts and two alternate splice forms of ALAS1 facilitate this regulatory mechanism. One splice form is subjected to heme-mediated decay but the other is resistant to this effect and requires translation in order to be regulated by heme-mediated decay. Heme itself blocks translocation of a precursor form of ALAS1 from cytoplasm to mitochondria contributing to its downregulation. Transcription of ALAS1 is upregulated by the peroxisome proliferator activated coactivator 1α (PGC-1α) in correlation to cellular glucose levels. In contrast, erythroid-specific transcriptional factors such as GATA1 regulate ALAS2 transcription by binding to the promoter region. In translational regulation of ALAS2, iron responsive elements (IRE) in ALAS2 transcripts can bind iron regulatory proteins (IRP). This prevents ALAS2 mRNA translation, allowing heme synthesis in differentiating red cells to be regulated in relation to cellular iron availability and mitochondrial function.

In addition, three enzymes within the heme biosynthesis pathway (PBGS, PBGD, and UROS) are transcriptionally regulated, yet they utilize a dual promoter system that allows erythroid specific or non-erythroid specific regulation of a single gene. Alternative splicing of exons creates two different PBGS transcripts, either with a housekeeping promoter or with an erythroid-specific promoter that binds erythroid-specific transcription factors including GATA1. Both PBGS transcripts encode identical proteins because they share the same translational start site despite their difference in lengths. On the contrary, alternative splicing of transcripts of the PBGD gene produces proteins of different lengths. Erythroid-specific promoters of PBGD contain several specific cis-acting sequences (including GATA1, NF-E2 and CACCC motifs) that are not seen in the housekeeping promoter. Similarly, alternative splicing of UROS gene
creates two transcripts. Erythroid-specific promoters of UROS contain eight GATA binding sites while the non-erythroid promoter contains NF1, AP1, Oct1, Sp1 and NRF2 binding sites. Both transcripts produce identical proteins\(^72\). The remaining enzymes within the pathway have single promoters; regardless, they exhibit erythroid and non-erythroid expression differences. The UROD gene carries a promoter of non-erythroid origin but UROD levels in erythroid tissue are significantly elevated compared to ubiquitous tissue and the specific mechanism resulting in this upregulation has yet to be determined\(^73\). The human CPO gene contains a single active promoter with six Sp1 sites, four GATA binding sites and a novel regulatory element named CPRE that aids in the elevated expression in erythroid tissue\(^74\). The PPO gene contains a GATA1 binding site in its single promoter, suggesting potential erythroid-specific regulation\(^50\). Cis-elements like NF-E2, GATA1 and the Sp1 binding sites are present in the human FC gene promoter and they have been found to induce FC expression during erythroid differentiation while the GC box maintains housekeeping expression of the gene\(^75\). Expression of ferrochelatase is also regulated by iron availability of the cells, related to the Fe-S cluster of FC\(^76\).

It is evident that the heme biogenesis enzymes in the erythroid pathway are transcriptionally induced by erythroid-specific transcription factors in coordination with iron uptake. Liver on the other hand maintains sufficient heme levels by combining synthesis and degradation in response to changes in cellular heme pools. Both systems are important however for maintaining cellular iron homeostasis and they are therefore areas of interest for understanding heme related diseases.
1.3.4 Incorporation of heme into apoprotein recipients

Although diverse in their function, heme-containing proteins share a structurally conserved element, the heme cofactor. Primary factors contributing to the functional diversity of heme proteins are the protein ligands coordinating Fe in the proximal/distal positions and the covalent linkage of the heme to the biomolecule. Insertion and stabilization of the heme unit within a heme containing protein has been studied extensively within cytochrome c. Heme C forms two thioether bonds between C3 and C8 vinyl groups of heme and the cysteine residues of CXXCH motifs in the apocytochrome c. Three systems driving this association have been studied extensively (reviewed in[77,78]). System I/CCM (Cytochrome C Maturation) is most prevalent in α and γ proteobacteria, all plant mitochondria, some protozoal mitochondria and red algae, and typically involves nine assembly proteins including CcmA through CcmH[79]. Although system II/CCS (Cytochrome C Synthesis) was originally studied in green algae Chlamydomonas reinhardtii, it can also be found in chloroplasts, most Gram-positive bacteria, cyanobacteria and some β, δ, and ε proteobacteria. Several of the components in system II vary in different organisms but the major components include CcsA, CcsB and CcsX. System III/CCHL (Cytochrome C Heme Lyase) is mostly restricted to fungal, vertebrate and invertebrate mitochondria and some protozoal mitochondria. This system employs a cytochrome c heme lyase enzyme, which is now known as holocytochrome c synthase (HCCS)[80] to convert apocytochrome c to holocytochrome c. These three systems carry out a similar function: to keep both Fe in heme and sulfur in cysteine residues of the apoproteins in the reduced state to facilitate correct covalent attachment. Mechanisms of incorporation of additional heme types into
different heme binding proteins can be quite diverse and species specific: hence their discussion was eliminated from this review but is discussed in additional sources. 

1.3.5 Heme function

Heme proteins are ubiquitous in nature and perform a wide variety of functions. One abundant class of heme proteins is the photosynthetic and respiratory cytochromes. Other classes include globins, catalases, peroxidases, cytochrome P450s, oxygenases and others. Here we discuss the versatile chemistry of the heme unit in several categories of heme proteins.

Peroxidases use $\text{H}_2\text{O}_2$ to oxidize substrates without oxygen transfer. Their catalytic cycle includes three steps: 1) $\text{H}_2\text{O}_2$ oxidizes $\text{Fe}^{3+}$ and porphyrin to generate a porphyrin $\pi$ cation radical with water as a product; 2) oxidation of substrate reduces the porphyrin $\pi$ cation radical; and 3) a second substrate reduces $\text{Fe}^{4+}$ to $\text{Fe}^{3+}$. Examples from mammalian peroxidases include myeloperoxidase (MPO), eosinophil peroxidase (EPO), and lactoperoxidase (LPO), and these enzymes can oxidize a wide variety of substrates due to their high reduction potentials. The covalent vinyl sulfonium heme linkage in heme M of MPO enables heme distortion and the resulting reduction in electron density in the heme best explains the unusually high reduction potential of MPO. These peroxidases are capable of generating oxidants such as hypohalous acids, hypothiocyanous acid, reactive nitrogen species, singlet oxygen, phenoxy and hydroxy radicals, all which are key components in antimicrobial properties exerted by the immune system.

Cytochromes P450 represent another subgroup of heme proteins in high abundance in nature; humans alone carry more than fifty P450 enzymes. These
enzymes are mostly known for xenobiotics detoxification in liver, where drugs and other xenobiotics are hydroxylated and made more soluble, facilitating their conversion to easily eliminated products. In addition, P450s also participate in the biosynthesis of steroids, highlighting their importance in metabolism.

Additional noteworthy heme enzymes include nitric oxide synthase (NOS) and heme oxygenase (HO). NOS catalyzes the oxidation of L-arginine to L-citrulline and nitric oxide (NO), a signaling molecule important for regulation of the cardiovascular and nervous systems as well as participating in immune response. Heme oxygenase is responsible for the degradation of free heme, resulting in its efficient elimination and the recycling of iron\textsuperscript{85}. The complete list of heme proteins is extensive and exceeds the limits of this review. Instead, we will focus on human disorders that result from loss of heme protein function, many of which are severe, and merit detailed discussion.

1.3.6 Diseases of heme synthesis

Several human diseases are associated with disruption of the heme biosynthetic pathway. Many of these diseases are associated with inherited mutations in heme biosynthesis genes, however some are caused by environmental factors affecting their enzyme products. Along with nine major porphyrias, we will focus on diseases associated with disruption of the heme biosynthesis pathway.

1.3.6.1 Porphyrias

The clinical presentation of porphyria includes skin lesions and acute neurovisceral attacks which are related to the accumulation of specific intermediates in the heme biosynthetic pathway. Nine such diseases have been identified and they are categorized as hepatic or erythropoietic, pertaining to the organ in which the
intermediates accumulate. However, a more clinical classification of porphyria divides these into three groups: acute, cutaneous and rare recessive.

Even though no disease causing mutations for ALAS1 has ever been found in humans, many mutations affecting the function of the ALAS2 isoform have been identified. Although not commonly seen as loss of function mutations in the heme biosynthesis pathway, gain of function deletions in ALAS2 are found to be causative of a cutaneous porphyria called *X-linked dominant erythropoietic protoporphyria (XLDPP)*. This disease is characterized by increased ALAS2 activity and excessive protoporphyrin production. ALA production is increased such that the final step catalyzed by ferrochelatase is rate-limiting, resulting in the accumulation of protoporphyrin. Liver damage and photosensitivity are the clinical manifestations of this disease\(^8^8\).

Deficiency of ALAD causes a rare recessive porphyria named *ALAD porphyria*, for which only less than ten cases have been reported. In this autosomal disorder, loss of ALAD activity in liver and erythroid precursors leads to excretion of ALA and coproporphyrinogen III into the urine. Patients suffer from intermittent acute neurovisceral attacks and/or chronic neuropathy, and onset of this disorder ranges from childhood to adulthood\(^8^9\).

An additional disease related to the first steps in heme biosynthesis is *acute intermittent porphyria (AIP)*. As in all acute porphyrias, acute life-threatening complications occur mostly in the adulthood, however in rare cases severe attacks can occur in the childhood. Urinary excretion of ALA and porphobilinogen is increased due to decreased PBGD activity. Activity loss is caused by mutations in the PBGD gene and
over 200 mutations have been identified. This disorder is seen in homozygous dominants for the trait and a majority of heterozygotes remain disease-free\textsuperscript{90}.

Congenital erythropoietic porphyria (CEP) is another rare recessive type of porphyria caused by the deficiency of UROS. This autosomal recessive disease that is a result of loss of UROS function leads to the spontaneous formation of uroporphyrinogen I, the isomer of uroporphyrinogen III that cannot be converted to heme, so it is accumulated and excreted\textsuperscript{91}. Clinical features of this disease include chronic hemolysis and cutaneous photosensitivity caused by diffusion of uroporphyrinogen I to plasma, and these conditions begin to manifest in early infancy. There have been over twenty UROS mutations that cause this disease identified to date\textsuperscript{92}.

Porphyria cutanea tarda (PCT) is the most common porphyria and it falls under the category of cutaneous porphyrias. This disease only shows symptoms in the skin, including lesions in areas exposed to sun, skin fragility leading to secondary infections, and hypertrichosis. Ocular pain and photophobia have also been reported rarely\textsuperscript{93}. The cause of these symptoms is accumulation of excessive porphyrin in the skin. Tetrapyrroles are highly photoreactive and hence they absorb energy from the visible region of electromagnetic spectrum. Excited ring structures reach ground state by transferring energy, which drives peroxidation and oxidation of biological macromolecules such as membrane lipids, nucleic acids and proteins. Familial PCT, one of the two forms of PCT, is an autosomal dominant trait similar to AIP in that only a minority of heterozygotes is affected. In familial PCT, uroporphyrinogen III is accumulated and excreted in urine due to UROD mutations. Patients with sporadic
PCT, although not associated with UROD mutations, show decreased UROD activity and hepatic Fe overload, similar to that seen in familial PCT\textsuperscript{94}. Hepatoerythropoietic porphyria (HEP) is a rare porphyria associated with UROD deficiency. Symptoms are similar to that seen in PCT (skin lesions, red urine, hypertrichosis and scarring) however they can be much more severe. This rare recessive porphyria often onsets in infancy or childhood and shows high porphyrin concentrations in erythrocytes and less than 10% UROD activity\textsuperscript{95}.

Hereditary coproporphyria (HCP), another acute porphyria, is caused by mutations that destabilize the enzyme CPO. Clinical characteristics are very similar to other acute porphyrias (i.e., AIP) and show a high coproporphyrinogen III content in urine and feces, as well as increased photosensitivity. Also similar to other acute porphyrias, HCP is mostly seen in dominant homozygotes for the trait, with only some heterozygotes developing the disease\textsuperscript{96}.

An additional porphyria caused by mutations in a heme biosynthesis enzyme includes variegate porphyria (VP), characterized by dysfunctional PPO. Symptoms are again very similar to any acute porphyria, showing acute neurovisceral attacks and cutaneous photosensitivity. Onset of the symptoms is in adulthood and over 100 PPO gene mutations (including nonsense, missense, deletion, insertion and splice mutations) have been identified\textsuperscript{97}. As in AIP, urinary excretion of ALA and porphobilinogen and fecal content of protoporphyrinogen IX and coproporphyrinogen III is increased during acute attacks. In addition, PPO activity is dramatically reduced (50%) in tissues of VP patients\textsuperscript{96}. 
The last of the nine porphyrias associated with heme synthesis is another cutaneous porphyria named *erythropoietic protoporphyria* (EPP). Mutations in the ferrochelatase gene cause a deficiency in mitochondria leading to accumulation of free protoporphyrin IX, primarily in erythrocytes. Excess protoporphyrin IX is behind the predominant clinical feature of photosensitivity, which begins in childhood\(^{99,100}\).

### 1.3.6.2 Additional diseases

Loss of function mutations of ALAS2 is the cause of the disorder *X-linked sideroblastic anemia* (XLSA). A group of point mutations alter the protein's ability to bind the PLP cofactor, however other mutations affect protein domains outside the cofactor-binding region. The decrease in heme synthesis efficiency prevents normal erythroblast development in the bone marrow. The resulting overload of iron results in characteristic iron granules surrounding the nucleus (a sideroblast). This disease is characterized by microcytic hyperchromic anemia, the presence of mature but pale and smaller than normal erythrocytes and Fe overloaded mitochondria in erythroblasts in the bone marrow\(^{101}\).

ALAD requires Zn for function, and Zn deficiency makes ALAD susceptible to Pb inhibition. Pb replaces Zn in ALAD and the inhibited protein leads to high levels of ALA in blood, which is responsible for neurological manifestations due to its toxicity at high concentrations\(^{102}\). A similar disease, named *hepatorenal tyrosinemia*, shows neurotoxicity due to high ALA levels, with abnormal liver and kidney function. This disease is caused by mutations in fumarylacetoacetate hydrolase gene, which hydrolyzes fumarylacetoacetate to fumarate and acetoacetate during the tyrosine catabolism pathway. In the event of enzyme deficiency, fumarylacetoacetate is
metabolized to succinylacetone, which is structurally similar to ALA, hence it acts as a potent inhibitor for ALAD\textsuperscript{103}. Excess ALA and succinylacetone can be seen in patients’ urine and blood samples.

1.4 Fe-S clusters

1.4.1 Introduction

Iron-sulfur (Fe-S) clusters are the second major form of complex iron cofactors found in biology. Due to the high abundance of iron and sulfur on the earth’s surface, and the easy association of these atoms under anaerobic conditions, Fe-S clusters likely developed early in evolution before the earth’s transition to an aerobic atmosphere. Consequently, these cofactors are ubiquitous in all organisms and play a role in almost every biological pathway. Here we provide an overview of the structure, formation, and function of Fe-S cluster cofactors.

1.4.2 Fe-S cluster structure

In many ways, Fe-S clusters are simpler than heme. While heme is a mixture of organic (protoporphyrin) and inorganic (iron) components, Fe-S clusters are strictly inorganic. Iron atoms in biological Fe-S clusters interact directly with protein residues and sulfur atoms bridge neighboring iron atoms. Fe-S clusters exist in a variety of configurations depending on their respective number of iron and sulfur atoms. The three most common forms (2Fe-2S, 3Fe-4S and 4Fe-4S) are illustrated in Figure 1.5. More complex Fe-S clusters have also been observed, including the 7Fe-8S and 8Fe-8S clusters identified in ferredoxins from \textit{Desulfovibrio africanus}\textsuperscript{104}.

The Fe atoms within the Fe-S cluster can exist in either ferric or ferrous forms and cycling between these redox states allows the transfer of electrons for redox
reactions. The tendency of the oxidized Fe-S cluster to gain an electron is termed the "reduction potential". By convention, this potential is expressed in comparison to a reference standard hydrogen electrode, which is assigned a potential of 0V. Depending on Fe-S cluster type, interactions with neighboring amino acids, and solvent accessibility, a single Fe-S cluster can take up to two electrons with a reduction potential spanning ~1000 mV$^{105}$. This remarkable range of accessible reduction potentials can largely explain the biological utility of the Fe-S cluster.

Fe-S clusters do not exist freely but are intimately connected to their apoprotein partner. Free iron will form an insoluble complex when bound to sulfide, so the protein plays a critical role in solubilizing the Fe-S unit. Fe-S proteins usually bind their corresponding Fe-S cofactor via ionic interactions between cysteine thiols and iron in the Fe-S cofactor. In some cases, Fe-S clusters are alternatively ligated via histidine residues. Subsets of 2Fe2S clusters, such as those found in Rieske proteins (see section 1.4.5), are coordinated by two cysteine and two histidines (Cys$_2$His$_2$)$^{106}$ and a common coordination theme of proteins involved in Fe-S cluster biogenesis is Cys$_3$His$_1$ coordination$^{107}$. 


Figure 1.5: Structure for the 3 most common forms of Fe-S Cluster: 2Fe-2S, 3Fe-4S, and 4Fe-4S.
1.4.3 General Fe-S cluster biogenesis pathway

Production of Fe-S clusters must be highly regulated to prevent unwanted reactions of both free iron and sulfur. Similar to heme-proteins, Fe-S proteins are synthesized in their apo-state and obtain their Fe-S cluster cofactor from a dedicated Fe-S cluster formation pathway. At present, there are three known general pathways for Fe-S cluster formation: the iron sulfur cluster (ISC), cytosolic iron sulfur assembly (CIA), and sulfur assimilation (SUF) pathways. These three pathways provide Fe-S clusters for the majority of Fe-S proteins in almost all organisms. While dedicated Fe-S cluster forming pathways can exist for individual Fe-S proteins, such as the nitrogen fixation (Nif) pathway that provides an Fe-S cluster for the nitrogenase enzyme in nitrogen-fixing bacteria\textsuperscript{108}, this review will focus only on the general Fe-S cluster production pathways.

1.4.3.1 Iron sulfur cluster (ISC) pathway

The most robust and best-characterized pathway for Fe-S cluster biosynthesis is the iron sulfur cluster (ISC) pathway. A simplified description of this pathway is provided in Figure 1.6. The ISC pathway, present in bacteria and in the mitochondria of eukaryotes, provides general housekeeping Fe-S clusters to a large number of Fe-S proteins. In eukaryotes, this pathway provides Fe-S clusters for several key mitochondrial Fe-S proteins. ISC was initially identified in the Azotobacter vinelandii and Escherichia coli bacterial species, where ISC genes are arranged in the isc operon. Additional early work in eukaryotes (in Saccharomyces cerevisiae and human proteins) revealed a highly homologous system localized to the mitochondria. In addition to providing for mitochondrial Fe-S proteins, the ISC system provides a component to the
cytosolic and nuclear Fe-S cluster formation pathways, thus ISC is essential for the maturation of all cellular Fe-S proteins in eukaryotes\textsuperscript{109,110}.

ISC serves as a template for understanding general Fe-S cluster production. In human ISC, \textit{de novo} 2Fe-2S synthesis occurs on the dedicated scaffold protein ISCU\textsuperscript{111}. Sulfur for this reaction is provided by ISC’s dedicated cysteine desulfurase enzyme ISCS via a persulfide intermediate that gets transferred to ISCU\textsuperscript{112} upon formation of an ISCU-ISCS complex. In eukaryotes, ISCS has a dedicated protein cofactor ISD11 that is essential for ISCS function\textsuperscript{113}. Electrons for ISCS persulfide release are provided by the 2Fe-2S cluster containing ferredoxin FDX, which in turn gets reduced by the ferredoxin reductase FDXR that uses NADPH as its final electron source\textsuperscript{114,115}. FDX also interacts with ISCU, providing 2 reducing equivalents for assimilation of two 2Fe-2S clusters on an ISCU dimer to form a single 4Fe-4S cluster\textsuperscript{111}. An additional Fe-binding protein Frataxin interacts with the ISCS-ISCU complex and regulates ISCS activity\textsuperscript{116}. Additionally, there are alternate scaffold proteins (ISCA\textsuperscript{117} and NFU1\textsuperscript{118}) that interact with ISC proteins and these are believed to be required for the maturation of a specific subset of Fe-S proteins.

Despite intense study, the physiologic source of iron for ISC remains a subject of debate. Several potential iron donors have been investigated and iron delivery to ISCU has been demonstrated from several potential sources \textit{in vitro} within a variety of systems. The protein frataxin\textsuperscript{119,120} interacts with the ISCU-ISCS complex and could be the source of iron for the pathway\textsuperscript{120}. In the bacterial system, two additional members of the \textit{isc} operon have been investigated as potential iron donors, IscX and IscA. The small acidic protein IscX binds iron and regulates cysteine desulfurase activity in a
manner very similar to Frataxin\textsuperscript{121}. The alternative scaffold IscA is also an interesting candidate because of its tight (\(K_D \approx 10^{19}\)) binding affinity for mononuclear iron\textsuperscript{122} and its capability of delivering iron to IscU\textsuperscript{123,124}. An additional interesting hypothesis is that iron may come from a glutathione-glutaredoxin complex\textsuperscript{125}. The lack of conclusive evidence for a specific iron source suggests that \textit{in vivo}, there could be multiple iron sources or that the mode of iron delivery may be atypical.

A detailed mechanism for Fe-S cluster delivery from ISCU to downstream targets is currently under investigation. The Fe-S cluster bound to ISCU is transferred to the glutaredoxin GLRX5\textsuperscript{126,127}. Efficient transfer from ISCU to GLRX5 requires involvement of the ATPase SSQ1, which binds to both ISCU and GLRX5\textsuperscript{128}. Binding of SSQ1 to ISCU, along with the interaction of a DnaJ-like co-chaperone JAC\textsuperscript{129}, destabilizes the Fe-S cluster on ISCU facilitating its transfer from ISCU to GLRX5\textsuperscript{130}. GLRX5 is considered the end of the ISC pathway because it is the last common Fe-S cluster carrier for all mitochondrial Fe-S proteins. GLRX5 continues the cluster transfer process, however, and it is able to interact with a variety of downstream Fe-S proteins\textsuperscript{131-133}. The specific recipient depends on the ultimate destination of the Fe-S cluster. For example, 4Fe-4S cluster conversion and delivery is facilitated by GLRX5’s interaction with two other Fe-S proteins, ISCA and IBA57\textsuperscript{134}.

Despite being mostly localized to mitochondria\textsuperscript{135}, ISC is required for maturation of all cellular Fe-S proteins\textsuperscript{110}. The mechanism by which cytosolic Fe-S proteins depend on mitochondrial ISC is actively being investigated. Because Fe-S clusters are not able to cross the mitochondrial inner membrane\textsuperscript{136}, this mechanism likely involves the transport of an Fe-S cluster precursor out of the mitochondria and into the cytosol.
Recent work has identified an unknown compound produced by mitochondrial ISCS (named ‘X-S’) that may provide reduced sulfur for cytosolic Fe-S cluster formation\textsuperscript{137}. 
Figure 1.6: Diagram depicting *de novo* Fe-S cluster formation and the main Fe-S cluster transfer steps in the ISC, SUF, and CIA systems. Protein names used are from the human system for ISC and CIA, and from the bacterial system for SUF.
1.4.3.2 Cytosolic iron sulfur assembly (CIA) pathway

Recent studies have revealed the involvement of another essential and highly conserved Fe-S biosynthetic pathway that is present within the cytosol and nucleus of eukaryotes. This pathway is called cytosolic iron-sulfur assembly (CIA). This pathway has been identified in many eukaryotic systems and is essential in almost all cases.

CIA is unique among Fe-S maturation pathways in that it does not obtain reduced sulfur via a dedicated cysteine desulfurase. A simplified description of the CIA pathway is provided in Figure 1.6. Instead of an ISCS analog, CIA relies on mitochondrial export of a sulfur-containing compound, 'X-S', via the mitochondrial export protein ABCB7 and the intermembrane space protein ALR. The identity of X-S is currently unknown, but may be glutathione-complexed to an Fe-S cluster. In human CIA, the primary scaffold for de novo Fe-S assembly is a tetrameric complex formed between CFD1 and NBP35, which binds a bridging 4Fe-4S cluster between the CFD1 and NBP35 subunits.

Reducing equivalents for this reaction are provided by an Fe-S containing protein CIAPIN1 (similar to FDX in ISC), which in turn gets reduced by the diflavin reductase NDOR1 (similar to FDXR in ISC), utilizing reducing equivalents from NADPH.

The 4Fe-4S clusters from the NBP35-CFD1 complex get transferred to another Fe-S protein IOP1, which binds two 4Fe-4S clusters per monomer. IOP1, in turn, delivers its Fe-S clusters to a multi-component complex called the CIA targeting complex, which consists of at least three proteins CIA1, CIA2B, and MMS19. The CIA targeting complex is able to interact with a variety of recipient Fe-S proteins, likely an indication of its function in downstream Fe-S cluster delivery.
1.4.3.3 Sulfur assimilation (SUF) pathway

Of the three Fe-S general cluster formation pathways, the sulfur assimilation (SUF) pathway is probably the most ancient. SUF predominantly exists in prokaryotes, however it is found in specific locations in eukarya, including the chloroplasts in some plants\textsuperscript{148} and the apicoplasts in some plasmodium species\textsuperscript{149}, and recently proteins homologous to bacterial SUF were discovered in the cytosol of a blastocystis species\textsuperscript{150}. At present, SUF has been best characterized in the Gram-negative bacteria \textit{Escherichia coli} and \textit{Erwyna chrysanthemi} where its genes are organized into the \textit{suf} operon (Figure 1.7).

The SUF pathway is similar to ISC in many ways. Like in ISC, SUF provides general Fe-S cluster formation to accommodate a variety of Fe-S proteins. In fact, SUF and ISC seem to be redundant in Gram-negative bacteria, as the removal of the entire \textit{isc} or \textit{suf} operon results in no deleterious effects. Simultaneous \textit{suf}/\textit{isc} operon deletion, however, is lethal\textsuperscript{151}. While ISC and SUF follow the same general mechanism for Fe-S cluster formation (Figure 1.6), SUF seems to be favored under conditions of oxidative stress and iron limitation\textsuperscript{152} and the SUF proteins are correspondingly more stable under adverse conditions \textit{in vitro}\textsuperscript{153}. In \textit{E. coli}, the SUF pathway centers around two heteromeric complexes called SufBC and SufSE.

The primary scaffold SufB requires a binding partner SufC for activity, forming the SufBC complex in a SufB\textsubscript{2}C\textsubscript{2} arrangement. The SufBC complex can form a 4Fe-4S cluster on SufB that can be transferred to recipient proteins\textsuperscript{154}. The exact role of SufC in this process is unknown, but it has ATPase activity that is essential for Fe-S cluster formation on SufB\textsuperscript{155}. SufB, on its own is relatively unstable and prone to spontaneous
oligomerization. There is also a paralogue of SufB, named SufD that is able to replace a SufB in the SufBC complex, resulting in the SufBCD complex. However, SufBC is likely the most active form. The SUF cysteine desulfurase SufS functions in a similar manner to ISCS, accepting sulfur from cysteine via a persulfide intermediate. SufS has an essential binding partner SufE, which is required for activity, forming the SufSE complex. While it may seem SufE is similar to ISD11 from eukaryotic ISC, SufE functions differently from ISD11 in that it accepts the persulfide from SufS and allows the SufS enzyme to complete its turnover.

Details of SUF’s downstream cluster delivery are not as well established as in the ISC pathway. The 4Fe-4S cluster formed by SufBC can be transferred to the A-type carrier protein SufA in vitro, but SufBC also may be able to transfer directly to recipient apo-proteins. In vivo, SufA is functionally redundant with the ISCA bacterial homologue and possibly acts as an intermediate carrier of the 4Fe-4S cluster from SufB, passing it off to downstream apoproteins. Another protein involved in this process (ErpA) has redundant function with SufA but is necessary for the development of active Fe-S proteins.

Several important details of the SUF pathway remain to be identified. As in ISC, the in vivo source of iron is unknown. Also of interest is SufD’s incorporation in the SufBCD complex, which allows SufB to accept iron in vivo and facilitates binding of a FADH$_2$ cofactor. This cofactor may be able to reduce ferric iron, facilitating potential Fe$^{3+}$ sources such as ferritins or ferric citrate. While SufA can deliver mononuclear iron to SufBC in vitro, it is currently believed to function downstream of de novo Fe-S
formation (Figure 1.6). The source of electrons for SufS turnover and for Fe-S formation remains in question\textsuperscript{3}.
Figure 1.7: Illustration of \textit{suf} and \textit{isc} operons, with corresponding promoters ($P_{isc}$ and $P_{suf}$), which play a central role in regulation of SUF and ISC at the genetic level in bacteria. Operons depicted are from the \textit{E. coli} model system. Steps are colored based on the encoded protein’s function as follows: red (regulatory), yellow (sulfur delivery), green (primary scaffold), blue (downstream Fe-S cluster delivery), cyan (electron transfer), and gray (unknown).
1.4.4 Fe-S cluster biogenesis regulation

The best-developed model for Fe-S biogenesis pathway regulation comes from work done in Gram-negative bacterial systems, where both the ISC and SUF pathways are present. This work reveals a fascinating interplay between ISC and SUF, where the necessary ISC and SUF genes are organized into their respective *isc* and *suf* operons (Figure 1.7). While this review has focused on eukaryotic systems, regulatory mechanisms in bacteria may provide insight into how this regulation occurs in eukaryotes. At the center of *E. coli* Fe-S cluster biogenesis regulation is a DNA-binding protein IscR, the first member of the *isc* operon, which directly regulates both the ISC and SUF systems.

Under non-stressed conditions, ISC is favored over SUF for general housekeeping of Fe-S cluster biosynthesis\(^{165}\). The transcriptional regulator IscR can bind a 2Fe-2S cluster (forming *holo*-IscR), obtaining its Fe-S cluster from the same ISC machinery utilized by other Fe-S proteins\(^{166}\). In the *holo* configuration, IscR binds to the *isc* promoter and prevents binding of RNA polymerase\(^{167}\). Thus, *holo*-IscR acts as a feedback regulator, inhibiting transcription of the entire *isc* operon when ISC activity is sufficient\(^{168}\). IscR is a relatively poor substrate for ISC-mediated Fe-S cluster loading\(^{169}\) and *holo*-IscR can only form after the ISC proteins have exhausted their interactions with other apo-Fe-S proteins. In addition to being a weak ISC substrate, IscR does not bind its Fe-S cluster tightly and effectively acts as a sensor of cellular iron and oxygen conditions\(^{170}\). Under high-oxygen or low-iron conditions, *holo*-IscR quickly reverts to apo-IscR. Therefore under typical aerobic conditions, high oxygen levels cause *holo*
IscR to revert to apo-IscR. Apo-IscR dissociates from the isc promoter and isc is uninhibited.

In the apo configuration, IscR does not bind to the isc promoter but instead favors binding to the suf promoter, activating transcription of SUF genes\textsuperscript{171}. Appropriate interaction of apo-IscR with the suf promoter involves two additional transcription factors: the ferric uptake regulator (Fur) and the peroxide responsive regulator (OxyR). Suf expression is constitutively repressed by Fur, which binds Fe\textsuperscript{2+} under non-stressed conditions when iron levels are sufficient and oxidative stress is low. With its Fe\textsuperscript{2+} cofactor, Fur binds to the suf promoter at the same site as apo-IscR, inhibiting suf expression\textsuperscript{172}. When the cell faces iron deficiency, Fur loses its iron cofactor, dissociates from the suf promoter, thus triggering transcription of suf. The cell’s preference for SUF over ISC in the presence of oxidative stress also reveals the involvement of another transcription factor (OxyR), as oxidized OxyR recruits RNA polymerase to the suf promoter.

There are additional regulatory mechanisms for cluster bioassembly beyond the level of gene expression. The small non-coding RNA RyhB, for example, is encoded just upstream of the SUF promoter and can bind to the iscRSUA mRNA to prevents its translation\textsuperscript{173}. RyhB expression, however, is constitutively repressed by Fur-Fe\textsuperscript{2+}, so RyhB effectively inhibits ISC when conditions favor SUF\textsuperscript{174}.

1.4.5 Fe-S cluster function

Fe-S clusters are versatile biological cofactors found in the most fundamental biochemical pathways, including aconitase and succinate dehydrogenase of the citric acid cycle and respiratory complexes I-III of the electron transport chain\textsuperscript{135}. Nuclear Fe-
S proteins also have a unique role in DNA damage recognition and repair. Several forms of DNA polymerase, helicase, glycosylase, and primase all contain Fe-S clusters\(^\text{139}\). Considering their remarkable range of functions, a thorough summary of various Fe-S proteins is well beyond the limited scope of this review. New Fe-S proteins continue to be discovered but in many cases, the role of the Fe-S cluster within the Fe-S protein remains unknown, even if the cluster’s presence is essential for proper protein function.

Fe-S clusters can be found in the active site of many essential enzymes and usually are involved directly in catalysis. Being stable in a variety of redox states, Fe-S clusters are best known for their role as electron carriers. Fe-S clusters can carry usually one, but sometimes two electrons and are, subsequently, stable in various reduced states. 2Fe-2S clusters, for example, can exist in oxidized (Fe\(^{3+}/Fe\(^{3+}\)) or reduced (Fe\(^{3+}/Fe\(^{2+}\)) forms while 4Fe-4S clusters are stable in oxidized (Fe\(^{3+}/Fe\(^{3+}/Fe\(^{3+}/Fe\(^{2+}\))), intermediate (Fe\(^{3+}/Fe\(^{3+}/Fe\(^{2+}/Fe\(^{2+}\))), and reduced (Fe\(^{3+}/Fe\(^{2+}/Fe\(^{2+}/Fe\(^{2+}\))) forms\(^\text{175}\). The reduction potential of an Fe-S cluster is often modulated by interactions with nearest neighbor protein residues and by access to solvent, allowing for a large range of biological functions. Ferredoxins are considered the archetypical Fe-S cluster electron carriers and were the earliest Fe-S proteins to be functionally characterized\(^\text{176}\). Ferredoxins are involved in many essential biochemical pathways, transferring electrons for cellular respiration, photosynthesis, and nitrogen fixation\(^\text{177}\). A ferredoxin is even involved in the ISC iron sulfur cluster biogenesis pathway, as discussed previously (see section 3.3.1)\(^\text{178}\).
But Fe-S cluster-mediated electron transfer is not limited to ferredoxins. In fact, one of the most the fundamental electron transfer processes, the electron transport chain (ETC), utilizes numerous Fe-S clusters. Respiratory complexes I, II, and III of the ETC all contain Fe-S clusters. Respiratory complex I uses a network of 8 Fe-S clusters for step-wise electron transfer\textsuperscript{179}. Similarly, complex II contains an Fe-S protein component called SDHB with a 2Fe-2S, 3Fe-4S, and 4Fe-4S cluster\textsuperscript{180}. Lastly, complex III utilizes a unique Fe-S cluster called a “Rieske center”\textsuperscript{181}. The Rieske center is a 2Fe-2S cluster where one of the iron atoms is coordinated by histidines instead of cysteines, resulting in a Cys\textsubscript{2}His\textsubscript{2} coordination\textsuperscript{182}.

Fe-S clusters can also be involved in non-redox reactions. The 4Fe-4S cluster in aconitase, for example, catalyzes a hydration-dehydration reaction, ligating directly to the citrate substrate\textsuperscript{183}. In some cases, Fe-S clusters appear to only serve a structural function and not participate in chemistry directly, as is the case in endonuclease III\textsuperscript{184}.

1.4.6 Fe-S clusters in human disease

Unlike in Gram-negative bacteria, where ISC/SUF redundancy allows for removal of an entire pathway, in humans the absence or mutation of a single component is often incompatible with life. In select cases there are human diseases that have been linked to defective Fe-S cluster biogenesis pathways. Below we describe several diseases directly linked to dysfunctional Fe-S cluster formation.

1.4.6.1 Friedreich’s ataxia

With an incidence of 1 in 50,000\textsuperscript{185,186}, and a carrier prevalence of 1 in 100\textsuperscript{187}, Friedreich’s ataxia (FRDA) is by far the most prevalent disease linked to defective Fe-S cluster formation. FRDA is an autosomal recessive genetic disease caused by a GAA-
trinucleotide repeat expansion in an intron of the frataxin gene, a protein involved in the ISC pathway. This trinucleotide repeat expansion leads to under-expression of the frataxin gene and subsequently, low levels of frataxin. These insufficient frataxin levels are responsible for the pathophysiology of FRDA, but the precise role of frataxin is still unknown. Frataxin may deliver iron to the ISC pathway, be an allosteric activator of the ISCU-ISCS complex, or may have a combination of roles. FRDA tissues demonstrate increased mitochondrial iron deposits which leads to increased oxidative stress and cell death in metabolically active tissues such as cardiomyocytes and neurons of the dorsal root ganglia. FRDA presents early in adolescence with progressive ataxia, or difficulty coordinating movement, sensory loss, weakness, and dysarthria. FRDA patients are usually wheelchair bound in their teens with a significantly reduced quality of life and life expectancy. Median age of survival is 35 years with cardiac dysfunction usually being the cause of death.

1.4.6.2 ISCU myopathy

ISCU myopathy (IM) is an additional condition related to a defect in Fe-S cluster biogenesis. It is the 2nd most common disorder linked to defective Fe-S cluster synthesis but is much less common than FRDA with only 25 known cases. To date, all patients identified with IM have come from families of Swedish ancestry. Similar to FRDA, the IM phenotype is inherited in an autosomal recessive pattern. IM is caused by a splicing defect during ISCU post-transcriptional processing that leads to defective ISCU protein. Loss of ISCU leads to lower ISC activity and a resulting deficiency of essential Fe-S proteins, including succinate dehydrogenase and aconitase of the citric acid cycle. Symptoms are exacerbated in cells that are metabolically active, such as
the myocytes of skeletal muscle during exercise, and patients with IM experience exercise intolerance\(^{198}\). Prolonged activity can lead to tachycardia, tachypnea, and muscle pain\(^{199}\). Unlike FRDA, IM is not progressive and most cases have a normal life expectancy.

1.4.6.3 GLRX5 sideroblastic anemia

GLRX5 Sideroblastic Anemia (GSA), a disease caused by mutated GLRX5, has only been identified in a single patient to date. While GSA is exceedingly rare, this particular case study has revealed a unique mechanism linking Fe-S cluster production to general iron homeostasis\(^{66,200}\). GLRX5, involved in the last step of the ISC pathway, directs Fe-S cluster delivery from ISCU to downstream targets. One target is the iron-responsive protein IRP1, an Fe-S protein activated when its Fe-S cluster is absent\(^{200}\). Defective GLRX5, therefore, leads to constitutively active IRP1. IRP1 regulates several proteins involved in iron homeostasis\(^{201}\), including those involved in heme production. In particular, apo-IRP1 inhibits expression of the final enzyme in heme synthesis, aminolevulinate synthase (ALAS2). Defective GLRX5, therefore, leads to insufficient heme and impaired erythropoiesis, resulting in anemia. Iron that would be directed towards heme production accumulates in the cytosol of erythroblasts, creating the characteristic ringed-sideroblasts\(^3\).

1.4.6.4 Additional diseases

Succinate Dehydrogenase (SDH) subunit B, the Fe-S cluster containing protein of the succinate dehydrogenase complex, is a known tumor suppressor. Succinate, the substrate for SDH, stabilizes hypoxia-inducible factor (HIF), which regulates key processes in cell division and blood vessel growth under hypoxic conditions. Mutations
in SDHB or in fact any of the other main subunits of SDH (SDHA, SDHC, and SDHD) cause susceptibility to tumor formations known as paragangliomas or phaeochromocytomas in a disorder called *Hereditary Paraganglioma-Pheochromocytoma*\(^2\), stemming from the accumulation of succinate and stabilization of HIF. SDHAF2, an assembly protein that flavinates SDH, is also implicated in paragangliomas. Recently, two additional Fe-S assembly proteins, SDHAF1 and SDHAF3, were found to stabilize Fe-S cluster assembly in SDH\(^2\). The later two proteins have LYR-motifs (Leu-Tyr-Arg) common to proteins involved in Fe-S cluster assembly. SDHAF1 deficiency is known to cause leukoencephalopathy\(^2\).

Lastly, mutations in either NFU1 or BOLA3, two different genes involved in Fe-S cluster biogenesis, leads to *multiple mitochondrial dysfunction syndrome*, a condition characterized by defects in Complexes I, II, and III and pyruvate/a-ketoglutarate dehydrogenases. NFU1 is thought to be an alternative to ISCU as a scaffold for Fe-S assembly. Both BOLA3 and NFU1 appear to be involved in lipoate synthesis, possibly related to a role in assembling Fe-S clusters in lipoic acid synthase (LIAS), thus providing an explanation for the reduced PDH and a-KGDH activities characteristic of this syndrome. The impaired energy production results in lactic acidosis, encephalopathy\(^2\) and early death.

### 1.5 Summary

The utilization of complex Fe cofactors in biology requires a tightly controlled process of cofactor assembly and of delivery to the correct apoprotein partner. Numerous ailments, some of which outlined within this review, result when there is a breakdown in the assembly process or in delivery of the cofactor. The development of
treatment strategies for these disorders will require a more advanced molecular understanding of each protein malfunction outlined above. Therefore cooperation of bioinorganic chemists with the cell biologists alike will be essential to provide the broader understanding of how these complex pathways harness the power of iron in complex cofactors within the biological milieu.

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CHAPTER 2
COOPERATIVITY BETWEEN CYSTEINE DESULFURASE “NFS1” AND ITS ACCESSORY PROTEIN “ISD11” PROMOTES STABILITY AND ACTIVITY OF THE YEAST PROTEIN COMPLEX

2.0 Prelude

During eukaryotic de novo Fe-S cluster biogenesis within mitochondria, the Nfs1-Isd11 protein pair serves as the functional cysteine desulfurase complex. Isd11 does not have any prokaryotic orthologs, and was reported to have an activation function on the eukaryotic Nfs1. Lack of association between them leads to deficient sulfur mobilization in vivo, reduced Fe-S protein levels and retarded cell growth. However, molecular and atomic level understanding of how complexation occurs, and how Isd11 exerts its effect is still lacking. This chapter describes the work I have done to provide comprehensive molecular level information on the Nfs1-Isd11 structure and function. The subunit composition and molecular weight of the complex was elucidated as a part of biophysical characterization. Extensive thermal stability imparted by Isd11 on the Nfs1 was discovered as a significant factor in activating Nfs1 within Fe-S cluster assembly while substrate binding affinity was only minimally affected by Isd11. Taken together these results provide comprehensive knowledge on the level of cooperativity between yeast Nfs1 and Isd11. This work is being prepared for publication at the moment and will be referred as: Barupala, D. P., Murugesan, Y., Dancis, A., Pain, D., & Stemmler, T. L. (2016). “Cooperativity between cysteine desulfurase Nfs1 and its accessory protein Isd11 promotes stability and activity of the yeast protein complex”. Biochemistry.”
2.1 Abstract

The versatility of oxidation states in Fe-S clusters is a key reason for their extensive prevalence in biology, serving as electron reaction centers in several functionally indispensable proteins. Due to their essential nature, it is not surprising that Fe-S cluster synthesis is so carefully controlled by a dedicated set of proteins that work in an orchestrated manner. Cluster synthesis and proteins that utilize these cofactors have been studied extensively. During Fe-S cluster biosynthesis, cysteine desulfurases function as the sulfur source and they operate by enzymatically obtaining sulfane sulfur from L-cysteine and subsequently delivering it to the assembly scaffold protein via transfer of a persulfide intermediate. In yeast, the function of the cysteine desulfurase “Nfs1” is supported by an accessory protein “Lsd11”. Although several functional studies support the physiological activity of Nfs1 and Lsd11 working as a complex, less attention has been given on studying the molecular properties of this complex and also on elucidating the biophysical basis of their activity. Here we report that the isolated Nfs1-Lsd11 complex possesses higher thermal stability as a protein complex, and that an Lsd11 induced conformational change on Nfs1 is the key to this profound stabilization. We find that the Nfs1-Lsd11 complex has a millimolar affinity towards its substrate L-cysteine, and that the substrate binding is facilitated by a conformational change on Nfs1. Combined with previously published data, these molecular details assist in helping us understand the role of the Nfs1-Lsd11 complex and its role in Fe-S cluster synthesis.

2.2 Introduction

Fe-S clusters are found ubiquitously in nature and their function is indispensable within almost every cell. As the second most prominent iron cofactor (after heme), Fe-S
clusters play a role in nearly every cellular pathway\(^2\). Their utility is centered on their capability to direct redox chemistry by transferring electrons in a modulated manner. Key factors that allow for their varied activity is their unique protein bound architecture and their evolutionarily adapted coordination environment, which are optimized for their individual activity. Fe-S clusters can fluctuate between oxidized (Fe\(^{3+}\) based) and reduced (Fe\(^{2+}\) based) states and contain a wide distribution of iron and sulfur stoichiometries (different types of clusters include 2Fe-2S, 3Fe-4S, 4Fe-4S, etc.). Their redox versatility, coupled with their structural diversity, allows these clusters to drive several distinctive chemical processes, including mitochondrial respiration (complexes I, II, and III), gene regulation (SoxR) and product catalysis (radical SAM enzymes)\(^3\). Given the importance of these clusters in all of biology, it is not surprising that their bioassembly is tightly regulated, a process that is highly conserved between prokaryotes and eukaryotes.

In all cells, Fe-S cluster assembly is driven by a dedicated set of proteins that function in a coordinated manner to produce the iron containing cofactor. In eukaryotes, cluster assembly is performed by two major pathways: 1) the iron sulfur cluster (ISC) pathway, which operates within the mitochondria, and 2) the cytosolic iron sulfur assembly (CIA) pathway, which operates within the cytosol but is dependent on sulfur from the ISC\(^3,109,110\). The eukaryotic ISC pathway provides Fe-S clusters to meet most of the cellular demand, and proteins within this pathway are generally conserved between eukaryotes and prokaryotes. In *Saccharomyces cerevisiae*, 2Fe-2S clusters are assembled within the mitochondria *de novo* on Isu1, the assembly scaffold protein, using sulfur provided by the cysteine desulfurase Nfs1 working together with its
accessory protein Isd11\textsuperscript{3}. Electrons are provided by the ferredoxin Yah1 which is recharged in an NADPH driven manner by the ferredoxin reductase Arh1\textsuperscript{126}. While the iron source for the process is unclear, yeast frataxin homolog Yfh1 has been shown to serve as an iron chaperone \textit{in vitro} as well as serving as a regulator of Nfs1 activity\textsuperscript{3,206-208}. Assembly of Fe-S clusters is accomplished through the coordinated assembly activity of these proteins, and downstream cluster delivery and utilization proteins drive the targeted transfer of these clusters to recipient proteins\textsuperscript{3,209}. During cluster assembly however, the protein complex constructed in yeast of Isu1, Nfs1, Isd11, Yah1 and Yfh1 accomplish Fe cofactor biosynthesis with assistance from additional proteins including the acyl carrier protein (ACP)\textsuperscript{210}; exact details of how these proteins work together to accomplish product formation are however lacking. Deficiency of activity in even a single protein within this complex has a disastrous effect on cell (and in the case of humans, patient) viability.

Several diseases states related to insufficient Fe-S cluster synthesis have been identified, however the neurodegenerative disorder Friedreich’s ataxia (FRDA) is the archetypal disease. GAA- triplet repeat expansions of variable length found in the first intron of the human frataxin (FXN) gene lead to a stark reduction in protein levels, and this is at the heart of the disorder. Frataxin deficiency gives rise to mitochondrial iron deposits, a reduction in Fe-S cluster bioassembly, and reduced activity for Fe-S containing proteins. FRDA patients suffer from progressive spinocerebellar and sensory ataxia with hypertrophic cardiomyopathy as well as an increased risk of developing diabetes\textsuperscript{211}. While diseases associated with deficits in the other ISC proteins, including the human cysteine desulfurase (NFS1) and its binding partner (ISD11) are rare, NFS1
deficiency and a compromised NFS1-ISD11 complex are suspected to cause infantile mitochondrial II/III deficiency, which is an autosomal recessive disorder characterized by lactic acidemia, hypotonia, and a deficiency in respiratory complex II and III\textsuperscript{212}. A mutation in the \textit{LYRM4} gene encoding for ISD11 in humans causes combined oxidative phosphorylation deficiency (COXPD), a disease that impairs activity of respiratory complexes I, II, and III\textsuperscript{213}. As a result, ISD11 is unable to form a stable complex with NFS1\textsuperscript{214}. These severe human deficiencies related to reduced activity of the ISC proteins underscore the point that understanding the molecular activity of these proteins is fundamental for understanding the pathophysiology and biochemistry underlying crucial events in these disease states. This molecular and atomic level characterization of two of the yeast ISC proteins, Nfs1 and Isd11, is the goal of this study.

In cells, cysteine desulfurase provides sulfur for Fe-S cluster assembly by liberating sulfide from L-cysteine in a pyridoxal 5'-phosphate (PLP) assisted manner. Cysteine desulfurases generally exist as homodimers\textsuperscript{215,216}. Eukaryotic cysteine desulfurases require the accessory protein Isd11 for activity, however interestingly Isd11 is absent in prokaryotes\textsuperscript{113,217}. The reaction mechanism of cysteine desulfurase was first elucidated using the bacterial NifS ortholog from \textit{Azotobacter vinelandii}\textsuperscript{218,219}. Characterization studies on the yeast Nfs1 protein solidified this enzyme’s role in iron homeostasis\textsuperscript{220} and Fe-S cluster assembly\textsuperscript{221}. Unfortunately, structural details for the eukaryotic enzyme, and an understanding of how it interacts with its Isd11 accessory protein, are lacking. Structural studies on this topic have had a reliance on modeling of the Nfs1 architecture from using the bacterial orthologs\textsuperscript{222,223}. While the exact function of Isd11 is unknown, yeast Isd11 was shown to promote persulfide formation on Nfs1 by
bringing the bound substrate in close proximity to the Nfs1 active site cysteine residue\textsuperscript{116,224}.

In order to provide a molecular and atomic level understanding of how the Nfs1-Isd11 complex is formed, and to provide a basis for understanding the effect of Isd11 on Nfs1 activity, we isolated yeast Nfs1 and Isd11, both individually and as a co-expressed Nfs1-Isd11 complex. The optimal Isd11 to Nfs1 protein stoichiometry related to complex activity was characterized. The thermal stability that Isd11 imparts on Nfs1 during complex formation is shown to be a contributing factor in Nfs1 activity related to Fe-S cluster formation. An Isd11 induced conformational change on Nfs1 produces a profound stabilization effect of the enzyme that leads to activity. Finally, the affinity of the substrate L-cysteine for the Nfs1-Isd11 complex is only moderately altered by Isd11 binding. Combined, these studies paint a picture for how Nfs1 and Isd11 work together to produce and deliver the sulfur required for Fe-S cluster biosynthesis within the eukaryotic ISC pathway.

2.3 Materials and methods

2.3.1 Expression of yeast Fe-S cluster synthesis proteins

\textit{Nfs1\text{(and C421A)}-Isd11 co-expression:} The pST39 co-expression vector containing ∆1-33 Nfs1-6xHis-Isd11 or ∆1-36 Nfs1(C421A)-6xHis-Isd11 was transformed into BL21 (DE3) cells. In both these constructs Nfs1 lacks the mitochondrial targeting sequence but Isd11 remains full length. Starter cultures were made by inoculating a transformed colony in Luria Bertani (LB) broth supplemented with 100 μg/ml ampicillin; These cultures were incubated in a rotating shaker for 16 hours (overnight) at 230 rpm and 37 °C. Starting OD of the growth cultures (1 L) supplemented with 100 μg/ml
ampicillin were set to 0.075 by adding the appropriate amount of starter culture and the cells were grown until cell density reached 0.4 $OD_{600}$. Growth temperature was then switched to 18 °C and cells were induced at 0.7 $OD_{600}$ using 0.8 mM IPTG supplemented with 10 µM PLP. Cells grew for 18 hours before harvesting by centrifugation at 7900 x g for 30 min.

*Nfs1 expression:* The pET21b vector containing Δ1-33 Nfs1-6xHis was transformed into BL21 (DE3) codon plus cells. Starter cultures and growth cultures were treated exactly as mentioned above except they were supplemented with 34 µg/ml chloramphenicol in addition to 100 µg/ml ampicillin. Cells were grown at 37 °C in LB broth (1 L) until cell density reached 0.4-0.5 $OD_{600}$. Growth temperature was then switched to 25 °C and cells were induced at 0.7 $OD_{600}$ with of 1 mM IPTG, supplemented with 10 µM PLP. Cells were continued to grow at 25 °C for 3 hours before harvesting by centrifugation.

*Isd11 Expression:* Expression and purification of Isd11-6xHis followed the protocol that was previously published\(^{225}\). Briefly the pET21b vector containing Isd11-6xHis tag was transformed into BL21 (DE3) codon plus cells. A starter culture was made by inoculating a transformed colony in LB broth, supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and it was incubated in a rotating shaker for 16 hours (overnight) at 200 rpm and 37 °C. Growth cultures (200 ml) were also supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and the starter culture was diluted in LB to a 1:40 ratio. Cells were grown at 37 °C in LB broth until cell density reached 0.7-0.8 $OD_{600}$ and then these samples were induced with 0.5 mM IPTG. Cells were grown at 37 °C for 3 hours before harvesting by centrifugation.
2.3.2 Purification of yeast Fe-S cluster synthesis proteins

**Purification of Nfs1 and Nfs1(and C421A)-Isd11 Complexes:** Frozen cell pellets were resuspended in pH 7.5 lysis buffer containing 50 mM Sodium Phosphate, 150 mM NaCl for Nfs1(and C421A)-Isd11 complexes/300 mM NaCl for Nfs1 alone, 5% Glycerol, 20 mM Imidazole, and 5 mM β–ME (4 ml/g of cells). In addition, Complete™ EDTA-free protease inhibitor cocktail (Roche), lysozyme (10 mg/ml), and DNase (10 μg/ml) supplemented with 5 mM MgCl₂ were also included in the lysis buffer. Protein purification was performed at 4 °C. Cell suspensions were incubated at 4 °C for 30 min while stirring at a slow pace before passing through the Emulsiflex cell homogenizer three times for cell lysing. Cell lysate was then centrifuged at 53300 x g for 1 hour. Crude soluble fraction was filtered (0.20 μm) before passing through His-Trap HP 5 ml Ni column (GE Healthcare) using an AKTA FPLC chromatography system (Amersham Biosciences/GE Healthcare); Nfs1 or Nfs1(C421A)-Isd11 was passed through high density Nickel Agarose Beads (GoldBio) manually packed in a bench-top column. Ni columns were first washed with 20 column volumes of binding buffer to eliminate any residual unbound proteins. Bound proteins were then eluted by flowing an imidazole gradient of 20-500 mM across the columns and fractions containing Nfs1 or Nfs1(and C421A)-Isd11 complexes were identified using SDS-PAGE. Active fractions were pooled and concentrated to ~ 1 ml using 10 kDa molecular weight cutoff Amicon centrifugal filter devices (Millipore). All proteins were incubated with excess PLP to ensure 100 % loading of PLP to all Nfs1 units. Concentrated protein(s) was passed through a Superdex 200 size exclusion column to further remove impurities and to transfer to the final buffer at pH 7.5 comprised of 20 mM HEPES and 5mM β–ME plus..
150 mM NaCl for the Nfs1(and C421A)-Iisd11 complexes and 300 mM NaCl for Nfs1 alone. Fractions containing Nfs1 or Nfs1(and C421A)-Iisd11 complexes were pooled and concentrated to store at -80 °C in aliquots until further use for experiments. These purification protocols yielded >95% pure proteins (Figure 1).

**Purification of Iisd11:** The majority of overexpressed Iisd11 was found in bacterial inclusion bodies. Cell pellets were resuspended in a buffer containing 50 mM Tris HCl (pH 8.0), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed by passing through the Emulsiflex cell homogenizer three times followed by six rounds of sonication (20 s each round and 2 min intervals). The lysate was spun at 53300 x g for 30 min and crude soluble fraction was removed. Pellet containing insoluble Iisd11 was dissolved with a buffer at containing 50 mM Tris HCl (pH 8.0), 8M urea, and 1 mM PMSF, and the resulting suspension was spun at 65000 x g at 20 °C for 30 min. Supernatant was mixed with a His-select Ni affinity gel (Sigma P6611), pre-washed with water and 50 mM Tris HCl (pH 8.0) with 1 mM PMSF buffer, for 1 hour at room temperature on a rocking platform to allow end-to-end mixing. After removing unbound protein, the resin was further washed with a 50 mM Tris HCl (pH 8.0) and 1 mM PMSF binding buffer to eliminate any residual unbound protein. Bound protein was then eluted using an elution buffer containing 50 mM Tris HCl (pH 8.0), 8M urea, 400 mM imidazole, and 1 mM PMSF. Fractions containing denatured Iisd11 identified using SDS-PAGE were pooled and stored at room temperature until further use. This purification protocol yielded approximately completely pure protein (see Figure 1).
2.3.3 Protein size and stoichiometry characterization

*Size exclusion chromatography:* Concentrated and purified proteins were passed again through Superdex 200 (120 ml column bed volume) column using AKTA FPLC system (GE Healthcare) to identify the molecular size and oligomeric states of protein(s). The Superdex 200 column was pre-equilibrated with 20 mM HEPES, 300 mM NaCl, and 5 mM β-ME running buffer at pH 7.5, and concentrated protein at a 1 ml volume was injected onto the column. The separation flow rate at 4 °C was set at 0.5 ml/min. BioRad gel filtration standards containing Thyroglobulin (670 kDa), γ-globulin (158 kDa), Ovalbumin (44 kDa), Myoglobin (17 kDa), and Vitamin B12 (1.35 kDa) were passed across the column in the same manner to calibrate the molecular size of the target proteins.

*Gel densitometry:* Gel densitometric analysis was used to identify the relative oligomeric stoichiometry of protein samples. Purified Nfs1-Isd11 complex was subjected to denaturing SDS-PAGE to separate Nfs1 and Isd11 based on their molecular weight. Several samples of Nfs1-Isd11 complex were run across multiple lanes to reduce uncertainty in the measurement. Each lane carried a total of 2-5 µg of protein, as measured by Direct Detect Infrared Spectrometer for protein quantification. After staining/destaining, gels were imaged using Bio-Rad Gel Doc EZ Imaging System and analyzed using Bio-Rad Image Lab software. Bio-Rad gel imaging system provides a tool to measure the volume of a protein band, and then calculates the sum of all intensities within the band boundaries. The band % is the calculated percentage of a particular band’s volume compared to the total of all band volumes in a lane, and this measurement was used to calculate the ratio between Nfs1 and Isd11. Gel signals
corresponding to Nfs1 and Isd11 was divided by the expected molecular weight of the respective protein to calculate the molar band % and the ratio between molar band % values from Nfs1 and Isd11 (per Nfs1 monomer) provided the stoichiometry of Nfs1 and Isd11 in the protein complex. Stoichiometry between Nfs1 and Isd11 in the Nfs1-Isd11 complex obtained here was then compared to the size of the protein complex obtained from size exclusion chromatography.

2.3.4 Biophysical properties of proteins

Differential scanning calorimetry: The thermal stability of Nfs1 and Nfs1(C421A)-Isd11 complexes was assessed using differential scanning calorimetry (DSC). Purified and concentrated proteins were diluted to 2-3 mg/ml, using 20 mM HEPES, 300 mM NaCl, 5 mM β-ME at pH 7.5 buffer degassed to remove dissolved air. Protein samples were loaded in the sample cell of a TA Instruments Nano-DSC calorimeter; the reference cell was filled with matched buffer. The total volume for the capillary cell is 0.3 ml, however an excess of solution (~0.6 ml) is used for loading to prevent trapping air bubbles. Both capillary cells were manually pressurized to maintain a constant pressure of 3 atm during the scan. Each protein sample was scanned at a rate of 1 °C/min from 10 °C to 90 °C and the melting curves for Nfs1 and Nfs1-Isd11 were compared to assess differences in thermal stability resulting in complex formation. In addition, melting curves for Nfs1-Isd11 and Nfs1(C421A)-Isd11 were compared to assess any difference in thermal stability between wild type and mutant protein complexes. Experiments were done in duplicate on independently isolated proteins to ensure data reproducibility. The thermal profiles were simulated using the TA instruments
NanoAnalyze software package to identify the best-fit values for melting temperatures ($T_m$).

**ANS fluorescence spectroscopy:** Fluorescence spectroscopy was used to identify protein structural changes due to complexation and substrate binding. Binding with 1,8-Anilinonaphthalenesulfonate (ANS), a fluorophore which selectively binds to solvent exposed hydrophobic regions in molten globule proteins\(^{226}\), was used to determine structural differences between Nfs1 and Nfs1-Isd11. ANS binding experiments were carried out using 40 µM samples of either purified Nfs1 or Nfs1-Isd11 diluted in 20 mM HEPES, 300 mM NaCl, and 5 mM β-ME at pH 7.5. All buffers were initially degassed to remove dissolved air. Samples were incubated for 10 min after addition of 50 µM ANS. The ANS probe was excited at 371 nm and the fluorescence emission spectra were measured using a Horiba Scientific Spectrofluorometer at 5 nm intervals with 1 s spectral integration time and 1 mm slit width. A fluorescence cuvette (Starna), with 1 ml capacity and 1 cm path length was used to collect fluorescence emission spectra at 25 °C. Spectra were collected in triplicate using different protein preparations to ensure spectral reproducibility. Similarly ANS fluorescence measurements were used to assess the changes in protein fold upon addition of L-cysteine to Nfs1(C421A)-Isd11. Duplicated 20 µM Nfs1(C421A)-Isd11 samples in 20 mM HEPES and 300 mM NaCl buffer at pH 7.5 were incubated with 20 equivalents of L-cysteine for 10 min and then with 50 µM ANS for another 10 min before acquisition of spectra using same experimental parameters as above.

**Tryptophan fluorescence spectroscopy:** Tryptophan fluorescence spectroscopy was used to measure structural changes in Nfs1(C421A)-Isd11 that occur upon binding
of substrate L-cysteine. Freshly made L-cysteine was titrated consecutively to 20 µM Nfs1(C421A)-Isd11 in 20 mM HEPES and 300 mM NaCl buffer at pH 7.5. Protein samples were carefully mixed and incubated for 10 min after each addition of L-cysteine; emission spectra were measured at 25 °C following excitation of the samples at 300 nm as above. Each addition was timed 1 min after the start of spectra acquisition to maintain consistency between data points. All spectra were corrected for dilution. Experiments were performed in duplicate using independently prepared protein samples to ensure spectral reproducibility. The change in fluorescence intensity at 340 nm was plotted against respective L-cysteine concentrations to calculate the binding affinity of Nfs1(C421A)-Isd11 to L-cysteine. Best-fit simulation to data was obtained using the DynaFit curve fitting software.227

2.3.5 Functional properties of proteins

UV-visible spectroscopy: UV-visible spectroscopy was used to measure the affinity of Nfs1(C421A)-Isd11 to its substrate L-cysteine. In this experiment, 50 µM purified Nfs1(C421A)-Isd11 in 20 mM HEPES and 300 mM NaCl at pH 7.5 was titrated consecutively with freshly made L-cysteine. Protein samples were carefully mixed and incubated for 10 min after each addition of L-cysteine; spectra were measured at a 1.0 nm scan pitch using Shimadzu UV-1800 Spectrophotometer. Each addition was timed 1 min after the start of spectra acquisition to maintain consistency between data points. All spectra were collected at 25 °C under anaerobic conditions and corrected for dilution. Experiments were performed in duplicate using independently prepared protein samples to ensure spectral reproducibility. The change in spectral intensity at 340 nm was plotted against respective L-cysteine concentrations to calculate the binding affinity
of Nfs1(C421A)-Isd11 to L-cysteine. Best-fit simulation to data was obtained using DynaFit curve fitting software\textsuperscript{227}.

**Cysteine desulfurase activity assay:** An acid labile sulfide detection assay was performed to calculate the Michaelis-Menten kinetics parameters for the Nfs1-Isd11 complex. In this assay, Fe\textsuperscript{3+} with sulfide liberated from the enzyme aids the conversion of \(N, N\)-dimethyl-\(p\)-phenylenediamine sulfate (DPD) to methylene blue. Its characteristic visible absorption feature at \(\sim 750\) nm is used to quantify sulfide production with the use of a calibration curve. Nfs1-Isd11 complex was incubated with a series of L-cysteine concentrations ranging from 100 \(\mu\)M to 2 mM. Each reaction was handled separately to maintain accuracy of timing and measurements. Assay mixture contained 100 mM HEPES (pH 7.5), 150 mM MaCl, 50 \(\mu\)M PLP, 12 \(\mu\)M purified Nfs1-Isd11 and distilled water and the total reaction volume was 100 \(\mu\)l. Each reaction was prepared by first mixing the assay components together in an eppendorf tube covered with rubber septa (Sigma-Aldrich-Z565717) to prevent air escaping. Respective L-cysteine amounts were then introduced to each tube using a gas-tight Hamilton syringe. After a 5 min incubation at 30 °C, the reaction was stalled by adding 100 \(\mu\)l of 20 mM DPD in 7.2 N HCl and 30 mM FeCl\(_3\) in 1.2 N HCl also using gas-tight Hamilton syringes and each addition was timed 45 s apart to maintain the consistency between each reaction. Distilled water was added up to 800 \(\mu\)l. Each tube was then vortexed for 10 s before incubating for 20 min at 30 °C for efficient color development. All tubes were spun at 14000 \(\times\) g for 2 min to remove protein precipitation before measuring the absorbance at 750 nm using a 1 ml cuvette with Varian Cary-50 Bio UV-Visible Spectrophotometer. The amount of persulfide liberated at each concentration was determined using a
standard plot constructed using known Na₂S concentrations. Measurements were corrected for dilution and the initial rate of reaction (µM/min) was plotted against L-cysteine concentration (µM) to calculate enzyme kinetics parameters.

2.3.6 Structural studies of Nfs1

*Homology modeling:* Homology modeling was used as a tool to model the three-dimensional structure of Nfs1. Published X-ray crystal structure of bacterial IscS (PDB: 1P3W) was used as the input model for the SWISS-MODEL workspace²²⁸ ([https://swissmodel.expasy.org](https://swissmodel.expasy.org)) provided by the SIB ExPASy Bioinformatics Resource Portal. Modeled Nfs1 structure was viewed using the PyMOL Molecular Graphics System (Version 1.8.2.2 Schrödinger, LLC) to inspect the overall structure and identify the residue configuration of the active site.

2.4 Results

2.4.1 Co-expression of Nfs1 with Isd11 leads to stable complex formation

The solution stability of Nfs1 in complex with Isd11 is enhanced relative to pure homogeneous protein. Purified Nfs1-Isd11 complex exhibited a characteristic neon yellow color indicative of PLP bound to the cysteine desulfurase. This complex was soluble at 4 °C for 2-3 days at salt conditions as low as 150 mM. In contrast, Nfs1 and Isd11 purified separately have poor solubility following isolation, as was previously observed with other eukaryotic proteins²²⁹,²³⁰. When expressed in *E. coli* individually, Isd11 is predominately contained within inclusion bodies. Isd11 could be resolubilized using 8 M urea, and the protein could be purified in its denatured form (Figure 2.1). However, as expected, Isd11 precipitated upon removal of urea below 2 M concentrations (data not shown)²³¹. Similarly, in a previous study, Isd11 purified from a
soluble fraction of bacterial lysate aggregated and formed a polydisperse mixture of oligomers in the absence of Nfs1\textsuperscript{231,232}. When overexpressed alone, Nfs1 exhibited the characteristic neon yellow color highlighted above. The protein was comparatively soluble, in contrast to Isd11 alone, and it could be purified from soluble fractions from the bacterial cell lysate. It was, however, extremely important to maintain high salt concentrations (300 mM NaCl) and low temperatures (< 20 °C) to keep Nfs1 in a soluble unaggregated state. It was previously reported that Nfs1, from yeast mitochondria, is prone to aggregation in the absence of its binding partner Isd11\textsuperscript{113,233,234}. These results are consistent with Isd11 coordination to cysteine desulfurase contributing to the solubility and stability of the enzyme, which helps both proteins maintain solubility.
Figure 2.1: SDS-PAGE gel of purified proteins. Lanes represent 1: molecular weight marker, 2: purified yeast Nfs1-Isd11 complex, 3: Nfs1, and 4: Isd11. Nfs1-Isd11 and Nfs1 are present in non-denaturing media whereas Isd11 is present in 8 M urea.
2.4.2 Molecular weight and protein stoichiometry of the Nfs1-Isd11 complex

Co-expressed Nfs1-Isd11 provided a stable protein complex that could easily be characterized for overall size and protein stoichiometry. Purified Nfs1 and Isd11 migrated individually at ~50 kDa and ~12 kDa, respectively, on an SDS-PAGE gel (Figure 2.1). These results are consistent with the predicted molecular weights of the proteins derived from gene sequences, at 52,196.2 Da and 12331.1 Da for Nfs1-6xHis and Isd11-6xHis, respectively. Nfs1 expressed alone appears to run mostly between 158,000 Da (γ-globulin) and 44,000 Da (Ovalbumin) (Figure 2.2, Line B), as judged by size exclusion chromatography. The predicted molecular weight for a Nfs1 homodimer from the gene sequence is 104,392.4 Da, consistent with the bulk of the protein migrating as a homodimer. This is not unique since a homodimer has been seen for other cysteine desulfurases\textsuperscript{216}. A larger molecular weight species at > 600,000 Da is also seen in our data, suggesting Nfs1 alone is prone to higher order oligomerization. The Nfs1-Isd11 complex peak overlaps at a size of ca. 158,000 Da, as judged by size exclusion chromatography (Figure 2.2, Line A).

Gel densitometric analysis of the Nfs1-Isd11 complex revealed a ratio of Nfs1 to Isd11 of 1:2 (Figure 2.3). Since cysteine desulfurase runs as a stable dimer (as judged by the size exclusion chromatography listed above), the Nfs1-Isd11 complex would be consistent with 2 Nfs1 molecules and 4 Isd11 molecules producing the composite protein complex structure. The predicted total molecular weight for this combination, obtained from gene sequence data, adds up to 149,456.4 Da, which is close within error bars of the technique, to the molecular weight deduced from this complex at 158,000 kDa from size exclusion chromatography. This combination is also in close agreement
with results obtained from the human proteins, which also suggest a NFS1:ISD11 stoichiometry of 1:2\textsuperscript{213,229}. Murine proteins have shown a ternary complex with NFS1-ISD11-ISCU at a 1:2:1 ratio, and a quaternary complex with NFS1-ISD11-ISCU-FXN in 1:2:1:1 ratio\textsuperscript{235}, again in close agreement with results from our yeast Nfs1-Isd11 complex, and from our Nfs1-Isd11-Isu1-Yfh1 multiprotein complex recently reported (Stephen Dzul (2016) “Insights into de novo Fe-S cluster biogenesis via the eukaryotic Fe-S cluster pathway (ISC) in vitro”, PhD dissertation). In combination, these results are consistent with yeast Nfs1 existing as a stable homodimer, and it associates with four Isd11 monomers to form a Nfs1\textsubscript{2}-Isd11\textsubscript{4} protein complex when co-expressed.
Figure 2.2: Size exclusion chromatograms of purified Nfs1 and Nfs1-Isd11 complex compared to the gel filtration standard proteins. Solid, dotted, and dashed lines represent chromatograms for 1) gel filtration standard proteins, 2) Nfs1-Isd11 complex, and 3) Nfs1 expressed alone, respectively. Lines A and B indicate that Nfs1-Isd11 and Nfs1 oligomeric structures correspond to molecular weights of ~150 kDa and ~100 kDa, respectively. Gel filtration standard proteins include 1: Thyroglobulin (670,000 Da), 2: γ-globulin (158,000 Da), 3: Ovalbumin (44,000 Da), 4: Myoglobin (17,000 Da), and 5: Vitamin B12 (1,350 Da). *Nfs1-Isd11 and Nfs1 chromatograms also include additional peaks that correspond to higher order oligomeric forms of Nfs1-Isd11 and Nfs1, which elute at a smaller retention volume. Nfs1-Isd11 and Nfs1 chromatograms are offset for clarity.
Figure 2.3: Gel densitometric analysis of the Nfs1-Isd11 complex. Molar band volume is equivalent to percentage band volume (quantified using the Bio-Rad Image Lab software) divided by expected molecular weight of each protein in the complex. The ratio between molar band volumes corresponds to the ratio between Nfs1 and Isd11 in the complex.
2.4.3 Stabilization energetics of the Nfs1-Isd11 complex

Thermodynamic factors that impact the stability of the Nfs1-Isd11 complex have also been characterized in detail within this report. Comparisons of energetic profiles using Differential Scanning Calorimetry (DSC) revealed the Nfs1-Isd11 complex possesses a thermal stability much greater than Nfs1 alone. An average melting temperature for Nfs1 was measured to be 40.70 ± 0.24 °C (Figure 2.4). The raw melting data for the molecule by itself was best simulated using two individual denaturation events, possibly pertaining to the unfolding of two individual domains. In contrast, the average melting profile from the Nfs1-Isd11 complex was 61.75 ± 0.05 °C, indicating a thermal stability of over ~20 °C higher for the assembled complex compared to Nfs1 alone (Figure 2.4). Interestingly, the raw melting data for the Nfs1-Isd11 complex was best simulated using three individual denaturation events, the additional peak likely pertaining to the unfolding of Isd11 (Table 2.1). Our results are in good agreement with the observation that the two proteins within the complex are more stable when co-expressed. Due to the limited solubility, we were unable to reach concentrations required to measure the thermal stability of Isd11 alone. However, it can be concluded from our data that complexation causes an enhancement in the thermal stability of the Nfs1 enzyme.
Figure 2.4: Differential Scanning Calorimetric thermal profiles for purified Nfs1 and the Nfs1-Isd11 complex. Solid black and dotted gray lines represent sum of best-fit simulations and raw data respectively. Dotted black lines represent simulated peaks corresponding to domains and/or individual unfolding events in each structure. Melting temperatures for simulated peaks are depicted in Table 2.1. The thermal profile for Nfs1-Isd11 is offset for clarity.
Table 2.1: Melting temperatures (°C) for each domain/unfolding event obtained from Differential Scanning Calorimetry data for Nfs1 and Nfs1-Isd11.

<table>
<thead>
<tr>
<th>Domain/unfolding event</th>
<th>Melting Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nfs1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>39.05 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>41.95 ± 0.2</td>
</tr>
<tr>
<td>Nfs1-Isd11</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>56.9 ± 0.8</td>
</tr>
<tr>
<td>2</td>
<td>60.25 ± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>62.05 ± 0.4</td>
</tr>
</tbody>
</table>
2.4.4 Nfs1-Isd11 complex is more folded than Nfs1 itself

Structural changes that occur upon protein complexation may contribute to the higher thermal stability seen for the Nfs1-Isd11 complex. ANS fluorescence spectroscopy was used to test the hypothesis that Isd11 imparts stability on Nfs1 by inducing a conformational change on the protein that allows Nfs1 to adopt a more stable, more compact fold. This experiment, performed with Isd11 bound to Nfs1, showed that the ANS fluorescence signal for the Nfs1-Isd11 complex is quenched by 35.0 ± 7.0 %, as compared to the signal from Nfs1 (Figure 2.5). Signal quenching can be attributed to Isd11 displacing the ANS binding surface, or structural changes in local environment of the ANS binding site(s). A previous study using Circular Dichroism (CD) and Tryptophan fluorescence spectroscopies suggested that the Nfs1-Isd11 complex has substantially rearranged structural elements as opposed to Nfs1\textsuperscript{231}. Therefore ANS fluorescence results would be consistent with Isd11 inducing a conformational change on Nfs1, maybe pushing the protein to rearrange its structure profoundly and adopt a compact fold.
Figure 2.5: ANS fluorescence spectra for purified Nfs1 and for the Nfs1-Isd11 complex. Solid and dashed lines represent Nfs1 and Nfs1-Isd11, respectively.
2.4.5 Yeast Nfs1-Isd11 binds the substrate L-cysteine with millimolar affinity

Due to the presence of bound pyridoxal-5'-phosphate cofactor, UV-visible spectra of purified Nfs1 and Nfs1-Isd11 complex contain a characteristic PLP spectral feature at 420 nm\(^{215}\). This feature is attributed to the protonated internal aldimine formed between PLP and the yeast Nfs1 Lysine residue (K299) at the enzyme's substrate-binding site. Upon addition of the substrate L-cysteine, intensity of this feature diminishes while a new spectral feature appears at \(\sim 340\) nm\(^{218,236}\). This new feature is attributed to the L-cysteine-PLP ketimine adduct that will undergo a nucleophilic attack from a catalytic cysteine residue (C421) to form a persulfide intermediate\(^{231,237}\). Because these spectral features represent reaction intermediates before and after substrate binding, both are useful to determine substrate binding kinetics for the Nfs1-Isd11 complex. However, as observed, these features are also sensitive to persulfide formation on the protein\(^{238,239}\) and therefore they do not correlate directly to substrate binding. To isolate the spectral changes associated only with L-cysteine binding on Nfs1-Isd11, we developed a mutant Nfs1-Isd11 complex carrying C421A mutation in Nfs1. The Nfs1(C421A)-Isd11 complex is incapable of making the nucleophilic attack; hence the reaction is anticipated to stall after substrate binding with no acidic labile sulfide being formed (Figure S2.3). We verified by CD spectroscopy and DSC that the Nfs1(C421A)-Isd11 complex is not significantly different in secondary structure and stability as compared to the wild type complex (Figures S2.1 and S2.2, Table S2.1). A quantitative titration of L-cysteine into the Nfs1(C421A)-Isd11 sample was monitored at 340 nm (Figure 2.6-A) to measure ketimine intermediate formation. These data indicated a substrate binding affinity (or \(K_d\)) of 0.76 ± 0.14 mM (Figure 2.6-B). A similar
affinity, with a $K_d$ in the millimolar range, has been reported for several bacterial cysteine desulfurases$^{219,237}$.

### 2.4.6 Nfs1-Isd11 is a moderately efficient enzyme

Michaelis-Menten kinetics was calculated to determine the catalytic efficiency of the Nfs1-Isd11 complex following an acid labile sulfide detection assay. Under these assay conditions, the Nfs1-Isd11 complex exhibited Michaelis-Menten kinetics with a Michaelis constant ($K_M$) of $0.49 \pm 0.07$ mM for L-cysteine, a $k_{cat}$ of $13.1 \pm 0.4$ min$^{-1}$, and a catalytic efficiency ($k_{cat}/K_M$) of $456 \pm 263$ M$^{-1}$s$^{-1}$ (Figure 2.7). The calculated Michaelis constant ($K_M$) for yeast Nfs1-Isd11 complex was in close agreement with published $K_M$ values of $0.34$ mM and $0.43$ mM for human system$^{207,230}$. However, the reported $K_M$ value for the bacterial cysteine desulfurase IscS (an enzyme that is functional in the absence of Isd11) is approximately a hundred fold lesser ($K_M = 0.0027$ mM) than the values reported for eukaryotic homologs$^{112}$. On the other hand catalytic efficiency ($k_{cat}/K_M$) follows the trend of a gradual decrease from *E. coli* IscS ($\sim 52,000$ M$^{-1}$s$^{-1}$)$^{112}$ to yeast Nfs1-Isd11 complex ($\sim 460$ M$^{-1}$s$^{-1}$; our result) to human NFS1-ISD11 ($\sim 100$ M$^{-1}$s$^{-1}$)$^{207}$. 
Figure 2.6: Titration of L-cysteine to Nfs1(C421A)-Isd11 monitored by UV-visible spectroscopy. A: Consecutive spectra were collected after each addition of L-cysteine. Lines represent the following concentrations: bold solid: 0 mM, bold dashed: 0.010 mM, bold dotted: 0.035 mM, bold dash-dot: 0.084 mM, bold dash-dash-dot-dot: 0.183 mM, thin solid: 0.430 mM, thin dashed: 0.919 mM, thin dotted: 1.901 mM, thin dash-dot: 4.346 mM, thin dash-dash-dot-dot: 9.200 mM. Arrows indicate the direction of change of spectral features upon consecutive addition of L-cysteine. B: Plot shows the change in absorbance at 340 nm as a function of L-cysteine concentration. Solid black line indicates the best-fit simulation obtained using DynaFit curve fitting software.
Figure 2.7: Rate of persulfide formation by Nfs1-lsd11 complex shown as a function of L-cysteine concentration. Solid black line indicates the best-fit curve.
2.4.7 Nfs1-Isd11 complex undergoes a conformational change during L-cysteine binding

We have modeled the structure of Nfs1 to help provide a structural understanding of our results. Since three-dimensional structures of an eukaryotic cysteine desulfurase is not yet available, the published X-ray crystal structure of the bacterial cysteine desulfurase served as primary references for modeling the yeast structure. IscS, the cysteine desulfurase within ISC pathway of E. coli, exhibits the highest sequence identity to Nfs1; the two ortholog sequences share a 57% sequence identity and a 74% sequence similarity. We therefore selected the E. coli IscS structure as the template to model the Nfs1 structure. Sequence alignments between E. coli IscS and yeast Nfs1 shows an overhanging group of 98 amino acids at the N-terminal region of yeast Nfs1 (Figure 2.8-A). The first 33 amino acids of this yeast specific region is the mitochondrial targeting sequence (MTS) that facilitates import of the Nfs1 precursor protein into the mitochondria. This sequence gets cleaved by the mitochondrial processing peptidase (MPP) following import. Three additional amino acids are removed from the nascent yeast N-terminus by the Icp55 peptidase to produce the mature Nfs1 molecule. The first 62 amino acids of mature mitochondrial Nfs1 does not overlap with IscS, and the function of this overhang region is still unknown, and this region could not be modeled. Of the region of sequence overlap, the modeled Nfs1 homodimer was shown to be very similar to the E. coli IscS structure. The yeast structure retained most secondary structural elements of IscS (Figure 2.8-B) during the modeling. Each substrate-binding site (including the PLP cofactor binding site) incorporates residues from both monomers, and PLP could be modeled as being
attached to the K299 residue on each monomer via formation of an internal aldimine (Figure 2.8-C). The location of the substrate-binding site in the modeled structure is found buried beneath the dimer interface, suggesting the requirement of a conformational change on Nfs1 in order to bind L-cysteine in this pocket. The distance between catalytic cysteine residue C421 and PLP is over 15 Å, as calculated from the β-SH group of C421 to the aldehyde group on C4 of PLP. In the current state, this distance would be too far for a successful nucleophilic attack from the thiol group of 421 to the Cys-ketimine PLP adduct to occur. Therefore, a second conformational change on Nfs1 would also be required to bring the catalytic C421 to the substrate-binding site to promote persulfide formation.

ANS was used to attempt to detect any change in Nfs1 fold upon substrate binding. The ANS fluorescence signal for Nfs1(C421A)-Isd11 was monitored after addition of L-cysteine. As previously outlined, Nfs1(C421A)-Isd11 allows us to isolate the substrate-binding step from persulfide formation step, and to monitor structural perturbations caused only by L-cysteine binding on the enzyme, albeit in a perturbed (mutant) state. Following substrate addition, the fluorescence signal of Nfs1(C421A)-Isd11 was only quenched by 8.9 ± 0.8 % when L-cysteine was added, however the emission maxima was shifted from 480 nm to 470 nm (Figure 2.9-A). A blue shift in the fluorescence indicates the polarity of the ANS binding environment on Nfs1(C421A)-Isd11 decreased upon substrate binding. This result would be consistent with L-cysteine binding to Nfs1(C421A)-Isd11 as a result of a protein conformational change.

Tryptophan fluorescence spectroscopy was also used to monitor a conformational change on Nfs1 resulting from substrate binding. Each Nfs1 monomer
contains three tryptophan residues that emit an intrinsic fluorescence signal, while Isd11 does not contain a tryptophan. As a result, changes in tryptophan fluorescence during this experiment reflect changes in the tryptophan local environment within Nfs1. The tryptophan fluorescence signal for Nfs1(C421A)-Isd11 simultaneously reduced during the continuous L-cysteine titration. Consecutive addition of L-cysteine to Nfs1(C421A)-Isd11 resulted in a quench in fluorescence intensity at 340 nm (Figure 2.9-B). A best-fit curve for changes in the fluorescence intensity vs. L-cysteine concentration yielded a $K_d$ of $0.46 \pm 0.08$ mM, in close agreement with the $K_d$ value we determined using UV-visible spectroscopy for substrate binding. This further confirms that the overall change in tryptophan fluorescence was due to a conformational change in Nfs1 caused by substrate binding. With this result it could be concluded that a change in protein fold facilitates L-cysteine binding to Nfs1-Isd11.
Figure 2.8: Homology modeling of Nfs1. A: Clustal Omega alignment between yeast Nfs1 and *E. coli* IscS sequences. Asterisks represent, 1: MPP cleavage site, 2: Icp55 cleavage site, 3: PLP binding Lys299 residue, and 4: catalytic Cys421 residue. B: Nfs1 homodimer model based on IscS x-ray crystal structure (PDB: 1P3W). Small domain and large domain of each monomer are shown in magenta and yellow, respectively. C: Substrate/PLP binding region of Nfs1 homodimer. Residues constituting the substrate/PLP binding site are depicted as surfaces to emphasize the pocket nature of the site. PLP bound to the site is shown in magenta and K299 residue which forms a Schiff base with PLP is shown in yellow. Residues from the two dimers are colored in cyan and green. The catalytic C421 residue is shown in red.
Figure 2.9: Change in ANS and tryptophan fluorescence intensities of Nfs1(C421A)-Isd11 upon addition of L-cysteine. A: Change in ANS fluorescence of Nfs1(C421A)-Isd11 after addition of 20 equivalents of L-cysteine. Solid and dashed lines represent spectra before and after addition of substrate, respectively. B: Titration of L-cysteine to Nfs1(C421A)-Isd11 monitored with tryptophan fluorescence signal. Lines represent the following concentrations: bold solid: 0 mM, bold dashed: 0.010 mM, bold dotted: 0.035 mM, bold dash-dot: 0.084 mM, bold dash-dash-dot-dot: 0.183 mM, thin solid: 0.430 mM, thin dashed: 0.919 mM, thin dotted: 1.901 mM, thin dash-dot: 4.346 mM, thin dash-dash-dot-dot: 9.200 mM. Solid downward arrow indicates the direction of change of spectral feature at 340 nm upon consecutive addition of L-cysteine.
2.5 Discussion

Given the indispensable roles of Fe-S clusters in biology, it is no wonder the activity of proteins driving the assembly process are so highly synchronized and very tightly regulated. While every protein within the ISC apparatus works together in an orchestrated manner to drive Fe-S cluster assembly, it is the biophysical characteristics of each molecule that dictates their unique role in the process. Therefore, real insight in the bioassembly process can be gained by studying each molecule separately, followed by investigating how the proteins interact and work together to accomplish the final goal of Fe-S cofactor production. This report explores, in great detail, two of the four ISC proteins from the yeast system (Nfs1 and Isd11) to understand their role in generating the activated sulfur (via the persulfide) that is utilized during Fe-S cluster formation. Since Isd11 is essential only in eukaryotic Fe-S cluster assembly, having an atomic level understanding of Isd11 and Nfs1 alone provides fundamentals for revealing the coordination details that promote activation of the protein complex from the inert starting materials. Our goal in this report was to elucidate how cooperativity between yeast Nfs1 and Isd11 allows these proteins to work together to provide the sulfur substrate required for Fe-S cluster assembly within the ISC pathway.

Details related to the Nfs1-Isd11 complex obtained from this study provide an enhanced understanding of how the cysteine desulfurase reaction is accomplished when these proteins are coordinated, in contrast to being in their inactive homogeneous states. Here we present a molecular picture of the yeast Nfs1-Isd11 where the individual units alone are thermodynamically unstable, however when combined they form a complex that is highly soluble. Within this Nfs1-Isd11 complex, Nfs1 exists as a
homodimer while coordinating with 2 Isd11 molecules per Nfs1 monomer in a quaternary structure of (Nfs1-Isd11)_{2} when combined. This quaternary structure is similar in stoichiometry to that reported in other eukaryotic systems^{213,229,235}, and in the Nfs1-Isd11 subunit structure of our co-expressed Nfs1/Isd11/Isu1/Yfh1 complex [Dzul (2016) et al. Metallomics (in press)]. Our results do not match the observation by Terali et al.^{231} suggesting a Nfs1:Isd11 ratio of 1:1 and a quaternary structure of a (Nfs1-Isd11)_{3} heterohexamer, possibly due to differences in overexpression characteristics, subtle differences in our protein amino acid constructs, or slight variations in solution conditions between the studies. It is interesting that this stoichiometry is preserved when we co-express yeast Nfs1-Isd11 with Isu1 and Yfh1. This Nfs1/Isd11/Isu1/Yfh1 complex shows relatively high cysteine desulfurase activity (~6 fold higher than Nfs1-Isd11 alone) and high correlated Fe-S cluster assembly activity (~3 fold higher than Nfs1-Isd11 alone). With the observation of Nfs1 and Isd11 aggregating in the absence of complexation, it was apparent that these two proteins have a mutual stabilization effect on each other and thermal stabilities of Nfs1 and Nfs1-Isd11 complex assessed using DSC confirmed this fact. Over 40 % of the residues for both Nfs1 and Isd11 are hydrophobic, which is probably linked to the inherent insoluble nature of these two proteins by themselves. Since complexation relieves any insolubility it is reasonable to hypothesize that Isd11 may bind via hydrophobic interactions to minimize solvent exposure of hydrophobic patches on both Nfs1 and Isd11. This notion is further confirmed by ANS fluorescence spectroscopy, which shows exclusion of ANS from binding to Nfs1 during complexation with Isd11. It is possible that the entire complex becomes tightly folded encapsulating the hydrophobic regions within the inner core,
making ANS inaccessible to the complex. Reported CD and Tryptophan fluorescence data also suggested substantial structural rearrangement during complexation providing additional proof for this hypothesis\textsuperscript{231}. Therefore it is clear that association between Nfs1 and Isd11 provides mutual stabilization and a soluble complex.

UV-visible spectroscopy is the best method to measure substrate-binding affinity of the Nfs1-Isd11 complex. As a consequence of the PLP cofactor being bound to Nfs1, reaction intermediates formed upon substrate binding created distinct spectral features that could be used to determine substrate-binding kinetics as the reaction progresses as previously reported\textsuperscript{218,237,239,241}. Upon L-cysteine addition to Nfs1-Isd11, an absorption feature at 420 nm diminished while a new feature gradually appeared at 340 nm representing a mixture of reaction intermediates (i.e. internal aldimine and persulfide) rather than a single intermediate. The Nfs1(C421A)-Isd11 mutant complex allowed monitoring of spectral changes associated only with substrate binding. In addition, this mutant confirmed the spectral feature at 340 nm only represents L-cysteine-PLP ketimine adduct, the product directly consequential to substrate addition. Our $K_d$ for Nfs1(C421A)-Isd11 complex was consistent with the affinities reported for bacterial cysteine desulphurases\textsuperscript{219,237}. Given the pronounced differences between prokaryotes and eukaryotes (i.e., the presence of Isd11), it can be suggested that Isd11 has minimal effect on substrate binding on eukaryotic Nfs1. A previous study reported that Isd11 only took part in persulfide formation step and had no contribution towards substrate binding in the Nfs1-Isd11\textsuperscript{116}. Therefore Isd11’s role in the eukaryotic cysteine desulfurase complex is not directly linked to substrate binding to Nfs1.
Characterization of the kinetics of the yeast cysteine desulfurase enzyme is essential in order to assess activity parameters affected by the evolution of cysteine desulfurases from bacteria to humans. The most used assay to study cysteine desulfurase activity is a colorimetric detection system that quantifies the acid labile sulfide produced during catalysis\textsuperscript{242,218}. This assay has been applied to study cysteine desulfurase activity in several different systems\textsuperscript{112,207,230}. In this assay, a colorimetric measurement of methylene blue formed in proportion to the amount of persulfide produced allows straightforward quantitation of the same using a standard curve developed with inorganic sulfide. Although previous studies have quantified the amount of methylene blue produced by measuring the spectral intensity at 650 nm, we measured the absorbance at 750 nm, which is the second intense absorption maxima for methylene blue. This feature showed the lowest variation (< 5 % of the total signal at the highest L-cysteine concentration) for the blank reactions performed with varied L-cysteine concentrations similar to the actual experiment, and therefore had minutest effect on the readings. Michaelis-Menten parameters calculated for the yeast Nfs1-Isd11 complex from this report suggest that the yeast system falls between bacterial and human systems in the scale of evolution. The Michaelis constant (K\textsubscript{M}) for yeast Nfs1-Isd11 closely resembles that of the human homolog\textsuperscript{207,230}, but largely deviates from that seen for the \textit{E. coli} IscS\textsuperscript{112}, which is a hundred fold lesser than eukaryotes. An increase in K\textsubscript{M} coupled to increasing complexity of the organism may indicate that cysteine desulfurases have evolved to function near physiological L-cysteine concentration of \(~ 0.1 \text{ mM}\textsuperscript{207}. K\textsubscript{M} values near substrate concentrations allow enzymes to catalyze at a rate of V\textsubscript{max}/2 and thereby regulate turn over based on substrate
availability that is often adjusted to meet cellular demands. On the other hand, catalytic efficiency ($K_{\text{cat}}/K_M$) of the yeast Nfs1-Isd11 complex falls between bacterial and human systems, in a series where catalytic efficiency decreases with increasing complexity of organisms. Catalytic efficiency is a measure of enzymatic function at low substrate concentrations and apparently it has been compromised in eukaryotic cysteine desulfurases as opposed to IscS. Such a compromise may indicate an extra level of regulation summoned to control the cysteine desulfurase reaction in eukaryotes. Interestingly, Isd11 is only present in eukaryotes and complexation with Isd11 has an activating effect on Nfs1. Given that IscS turns over at maximum velocity (when $[S] \gg K_M$, $V=V_{\text{max}}$) and exhibits high catalytic efficiency, it is possible that additional regulation was needed for cysteine desulfurase reaction within the mitochondria to eliminate the formation of toxic levels of sulfides.

Although the function of eukaryotic Nfs1-Isd11 complex in Fe-S cluster biogenesis is established, structural details on the complex are lacking. Therefore a modeled structure for yeast Nfs1 developed from the published structure of the bacterial orthologs\textsuperscript{222,223} will have to suffice for now. The SWISS-MODEL homology modeling workspace\textsuperscript{243} was used with the *E. coli* IscS crystal structure to model Nfs1. However, IscS may not be the ideal template since prokaryotic systems do not utilize Isd11. In addition, the first 62 residues in Nfs1 N-terminus were absent from IscS, so the modeled structure lacked that region. A previous attempt to model Nfs1 using a different homology modeling platform yielded a structure without the flexible loop of 10 residues harboring catalytic cysteine residue (C421) due to lack of corresponding segment on IscS\textsuperscript{231}. However our structure modeled with SWISS MODEL contained this missing
segment. Therefore, we were able to measure the distance between the catalytic
cysteine and PLP, and predict that a conformational change is required for catalysis.
Another conformational change was also predicted for L-cysteine binding, as it was
apparent that the substrate-binding site was occluded in the model. Confirming this
hypothesis, ANS and tryptophan fluorescence experiments showed a conformational
change during substrate binding. Since Isd11 does not seem to be involved in the
substrate binding (as seen with UV-visible spectroscopy experiments) but only alters
catalytic activity it is likely that the second conformational change is facilitated by Isd11.
Supplementary Material

Figure S2.1: Circular dichroism spectra of purified Nfs1-Isd11 and Nfs1(C421A)-Isd11 protein complexes. Solid and dotted lines represent Nfs1-Isd11 and Nfs1(C421A)-Isd11, respectively. Experiment was carried out at 25 °C in 5 mM phosphate buffer at pH 7.5 supplemented with 100 NaF. Spectra were collected in the far-UV region (180-260 nm) with a JASCO J-1500 CD Spectrometer using 0.5 µM protein samples with 1 mm pathlength cuvette. Each displayed spectra represent a smoothed average of spectra from three independent experiments and in each experiment ten individual spectra were collected. Buffer scan was subtracted from the averaged spectra to accomplish baseline correction. Simulation of averaged spectra obtained using CDPro Analysis software, which used CONTIN as the method and SP29, SP37, SP43, SMP50, and SMP56 as reference databases yielded the amount of secondary structural elements present in each individual protein as listed in Table S2.1.
Table S2.1: Secondary structural elements by percentage for Nfs1-Isd11 and Nfs1(C421A)-Isd11 protein complexes from circular dichroism.

<table>
<thead>
<tr>
<th>Secondary structural element</th>
<th>Nfs1-Isd11</th>
<th>Nfs1(C421A)-Isd11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helix</td>
<td>33.2 ± 2.6</td>
<td>38.5 ± 2.7</td>
</tr>
<tr>
<td>Strand</td>
<td>18.8 ± 2.9</td>
<td>13.9 ± 1.4</td>
</tr>
<tr>
<td>Turn</td>
<td>20.4 ± 0.5</td>
<td>20.7 ± 0.7</td>
</tr>
<tr>
<td>Undetermined</td>
<td>27.7 ± 0.3</td>
<td>26.9 ± 0.7</td>
</tr>
<tr>
<td>RMSD</td>
<td>0.14</td>
<td>0.15</td>
</tr>
</tbody>
</table>
Figure S2.2: Differential Scanning Calorimetric thermal profiles of purified Nfs1-Isd11 and Nfs1(C421A)-Isd11 protein complexes. Solid and dotted lines represent Nfs1-Isd11 and Nfs1(C421A)-Isd11, respectively.
Figure S2.3: Persulfide forming activity of Nfs1-lsd11 compared to Nfs1(C421A)-lsd11. Columns A and B represent Nfs1-lsd11 and Nfs1(C421A)-lsd11, respectively. Amount of sulfide formed by 15 µM protein is measured using the acid labile sulfide detection assay as described in the main article in a buffer containing 20 mM HEPES, and 300 mM NaCl at pH 7.5.
2.7 Acknowledgements

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CHAPTER 3
EFFECT OF YEAST FRATAXIN “YFH1” ON THE STRUCTURE AND FUNCTION OF THE NFS1-ISD11 COMPLEX

3.0 Prelude

This chapter describes my work studying the effect of yeast frataxin homolog Yfh1 on the structure and function of yeast cysteine desulfurase “Nfs1-Isd11” complex. The function of frataxin within Fe-S cluster biogenesis has been a longstanding discussion beginning with the discovery that its loss of function causes Friedreich’s ataxia. Presently, frataxin is viewed more as a regulator of cluster assembly than as an essential iron delivery protein. Involvement of frataxin in altering sulfur mobilization and cluster assembly in both prokaryotes and mammalian cells has been studied extensively over the past years, as described later in this chapter. But less attention has been paid towards investigating these events in yeast besides the radiolabeling experiments showing Yfh1 directly stimulating cysteine desulfurase by exposing binding sites to facilitate substrate binding\textsuperscript{116}. The objective of my work was to fill in the gaps in knowledge of the Yfh1/Nfs1-Isd11 interaction and the effect of Yfh1 on cysteine desulfurase activity. Isothermal titration calorimetry and surface plasmon resonance studies were proposed to determine the binding affinity of Yfh1 to Nfs1-Isd11. UV-visible spectroscopy and acid labile sulfide detection assays were used to monitor the effect of Yfh1 on L-cysteine binding affinity and catalytic activity of Nfs1-Isd11 complex respectively. Finally, differential scanning calorimetry detected changes in thermal stability upon Yfh1 binding to the complex. My results presented below suggest that Yfh1 has minimal effect on Nfs1-Isd11 structure and function by itself but might be synergistic with other factors, such as Isu1.
3.1 Introduction

Frataxin was first discovered two decades ago as the protein encoded by the gene X25 located in the critical region of the FRDA locus of Friedreich’s ataxia (FRDA) patients. Most patients were homozygous for an unstable GAA triplet repeat expansion in the first intron of this gene\(^{188}\). Since then, a plethora of studies performed pertaining to characterizing the structure and function of frataxin, not only in humans but also in other organisms, has been tremendously useful in understanding the relation of frataxin to the pathophysiology of FRDA. Initial studies on the yeast frataxin homolog protein (Yfh1) encoded by the \(YFH1\) gene reported involvement of this protein in iron homeostasis and mitochondrial function. Loss of Yfh1 function lead to the accumulation of iron within the mitochondria\(^{244,245}\), deficiency in function of specific mitochondrial proteins (including cytochrome c oxidase), and hypersensitivity to \(H_2O_2\), iron and copper\(^{245}\). The frataxin gene is of nuclear origin, and the protein was found localized to the mitochondria\(^{246-248}\) and predominantly expressed in tissues with high metabolic demand\(^{247}\). In the search for frataxin’s function within the cellular milieu, several hypotheses have been postulated and tested. A few postulates of frataxin’s function include 1) regulation of mitochondrial iron efflux\(^{249}\), 2) employment of early antioxidant defenses to prevent oxidative damage in the cell\(^{250}\), 3) activation of mitochondrial energy conversion and oxidative phosphorylation\(^{251}\), and 4) serve as an iron chaperone for proteins such as ferrochelatase\(^{252-256}\). Frataxin’s function, in relation to Fe-S clusters, was discovered only when Fe-S proteins such as mitochondrial respiratory complexes I, II, and III, and aconitase showed reduced activity with the deletion of frataxin gene\(^{257-259}\). A decrease in Fe-S cluster protein maturation in \(\Delta yfh1\) yeast strains\(^{260,261}\), reduction
in mitochondrial Fe-S protein activities and Fe-S cluster association with the cytosolic iron regulatory protein 1 (IRP1) in frataxin-depleted HeLa cells\textsuperscript{262} indicated that frataxin is crucial for Fe-S protein biogenesis. Phylogenetic studies further confirmed the evolutionary lineage of the frataxin gene to be directly involved in Fe-S cluster protein assembly\textsuperscript{263}. Frataxin’s mechanism of action within Fe-S cluster synthesis has been extensively investigated in various systems in the past decade and multiple studies provided evidence for frataxin’s function as an iron donor for Fe-S cluster assembly, as well as a regulatory protein altering the function of other protein partners of the assembly process. Such evidence is discussed in detail in the following sections.

3.2 Frataxin’s function in Fe-S cluster assembly

3.2.1 Iron binding ability and iron chaperone function of frataxin

In relation to frataxin’s proposed function as an iron donor, its ability to bind iron has been tested in several studies. Affinities of all frataxin orthologs towards iron have shown to be weak, within the micromolar range. Human frataxin’s (FXN) ability to bind 6-7 iron atoms with an affinity of $K_d \sim 10-55 \mu$M was quantified by iron-dependent fluorescence measurements and ITC\textsuperscript{119}. Similarly, bacterial frataxin (CyaY) demonstrated binding of two ferrous ions per monomer with a $K_d$ of 4 \mu M\textsuperscript{264}. A more comprehensive study on monomeric Yfh1 characterized its iron binding properties and provided a detailed description of bound iron and the protein’s metal-binding sites. Monomeric Yfh1 was capable of binding two Fe$^{2+}$ atoms in the high spin state with micromolar affinities ($K_{d1} \sim 3 \mu$M and $K_{d2} \sim 2 \mu$M) at the two independent sites. The coordination geometry of the iron binding sites was determined as six-coordinate with Fe-O/N based ligands attributed to the residues within the acidic patches on the
protein. Weak micromolar affinities towards iron have been considered important for frataxin’s function as an iron chaperone within the cell.

Structural studies provided a framework for mapping the key residues involved in iron binding. Both solution and crystal structures are available for the bacterial, yeast, and human frataxin orthologs and these proteins are extremely similar. All frataxins in common have an α-β sandwich structure motif with two terminal α-helices in one plane and five antiparallel β-strands constructing a second plane, which are intersected by a sixth β-strand. Although frataxin has mostly been characterized as an iron binding protein, it somewhat lacks structural features of a typical iron binding protein, such as hydrophilic iron binding pockets or cavities and typical iron chelating residues (histidines and/or cysteines). Iron binding in all frataxins has been confined to a patch of acidic residues mostly comprising of conserved aspartates and glutamates (negatively charged carboxylate side chains serve as iron binding ligands) of N-terminal region of these proteins. These residues are located in the α1 and β1 secondary structural elements. NMR chemical shift perturbation experiments on CyaY and Yfh1 identified amino acid residues which underwent substantial chemical shift changes upon titration of iron.

Requirement for oligomerization for iron loading to frataxin has also been studied extensively. Yfh1’s ability to self assemble into spherical multimers in the presence of iron and to sequester iron in a bioavailable form have also been reported. Similarly human frataxin is capable of making an iron-binding stable homopolymer while bacterial frataxin CyaY behaves similar to Yfh1 and forms aggregates with iron loading capacity. Hence oligomerization was considered an important property of all frataxins
and suggested an iron storage function. However the physiological relevance of higher order multimers was questioned at that time due to the non-conserved nature of aggregation between species\textsuperscript{274} and in due course iron-induced oligomerization and iron storage in Yfh1 was found dispensable for heme and Fe-S cluster assembly \textit{in vivo}\textsuperscript{275,276}. Therefore, frataxin’s function as an iron chaperone was deemed redundant.

In summary, these studies indicated that although weak binding affinity towards iron and iron-dependent oligomerization predicted frataxin’s function as an iron chaperone, this function is not a critical function in cells.

\subsection*{3.2.2 Frataxin interacts with other key proteins in Fe-S cluster assembly pathway}

\textit{De novo} synthesis of Fe-S clusters is a complex process that involves multiple proteins\textsuperscript{3,175}. Since frataxin was found to be essential for this process, initial studies attempted to identify its individual interaction partners within the cell. Direct interactions between frataxin and the respective scaffold protein within the Fe-S assembly machinery in human, yeast and bacterial systems have been reported many times, postulating frataxin’s function as an iron donor for the process\textsuperscript{119,277-282}. In eukaryotes, this interaction is iron dependent, its $K_d$ is nanomolar\textsuperscript{119,282} and requires frataxin oligomerization\textsuperscript{283}. Isu1 binding residues on yeast Yfh1 have been located to the $\beta$–sheet adjacent to the acidic iron binding ridge\textsuperscript{280-282}. Similarly, direct interactions with ISD11 have also been identified defining ISD11 as a mediator for frataxin to interact with other cluster assembly components\textsuperscript{284}. The interaction between frataxin and cysteine desulfurase has been demonstrated in a different context: frataxin as a regulator of cysteine desulfurase activity and of sulfur delivery. Pull-down assays confirmed bacterial frataxin CyaY interacting with cysteine desulfurase IscS\textsuperscript{208,279} and
NMR experiments mapped CyaY residues involved in the interaction into an iron binding region\textsuperscript{208} comprising of $\alpha_1$, $\beta_1$, and $\alpha_1\beta_1$ loop\textsuperscript{285}. Modeled structures identified CyaY bound at a site near the IscS dimer interface and the stoichiometry of CyaY:IscS was reported as 1:1 (from ITC, SAXS and NMR) with a $K_d$ of 18.5 $\mu$M (from ITC)\textsuperscript{285}. This lower affinity CyaY-IscS complex could not be isolated by gel filtration chromatography\textsuperscript{286}. Similarly in the human system, no SDF (NFS\textsubscript{1}-ISD\textsubscript{11}-FXN) complex was observed from gel filtration chromatography and frataxin had no direct effect on cysteine desulfurase activity as well\textsuperscript{207}. Characterization of these individual interactions added a new level of knowledge towards understanding frataxin function, yet the quest to understand frataxin’s function continues.

Besides the direct interactions with each individual component, frataxin’s involvement in core Fe-S assembly complex (consisting of all four components, cysteine desulfurase (S) and its accessory protein (D), scaffold (U), and frataxin (F): SDUF complex) has been in the limelight in the past few years. Evidence of mammalian frataxin binding to the preformed SDU complex\textsuperscript{207,229,235,283} and mutations/other variants of frataxin binding weakly to the same\textsuperscript{287,288} suggested frataxin’s involvement in the core assembly complex. The role of frataxin in the SDUF complex has been studied extensively, and the most common hypothesis is that frataxin stimulates cluster assembly by enhancing sulfur delivery. The next section reviews this hypothesis.

### 3.2.3 Frataxin as a regulator of Fe-S cluster synthesis through controlling cysteine desulfurase activity

In the search for frataxin’s function within the Fe-S cluster assembly process, much evidence is directed towards defining it as a regulator for cysteine desulfurase
activity (i.e. sulfur delivery). Formation of a multiprotein complex between human NFS1, ISD11, ISU2 and FXN enhanced catalytic efficiency and the \( K_m \) for cysteine desulfurase activity within the quad complex dramatically in an ISU2-dependent manner, as compared to the NFS1-ISD11 (SD) complex alone. Addition of one equivalent of ferrous iron further stimulated cysteine desulfurase activity of this complex\(^{207}\). This notion was further confirmed when frataxin mutants/variants showed decreased binding affinities to the SDU complex, diminished Fe-S cluster synthesis, and lowered cysteine desulfurase activities of the SDUF complex\(^{287,288}\). In dissecting the mechanism of activation by frataxin, it was found that FXN stabilized a confirmation of the SDU complex that accelerated the formation of persulfide on NFS1. Simultaneously to that, FXN also facilitated inter-protein sulfur transfer between NFS1 and residue C104 of ISCU2, leading to acceleration of cluster assembly\(^{289}\). Studies on the yeast system suggested that Yfh1 directly and specifically enhances cysteine binding to Nfs1 by exposing its substrate-binding sites\(^{116}\). Notably, in the murine system, FXN enhanced the cysteine desulfurase activity of the SDU complex but the impact of iron on the stimulatory effect was only minimal and not specific to iron. The same study also reported that iron entry into the SDUF complex is contingent upon activation of cysteine desulfurase by FXN; hence suggesting a concerted mechanism for iron and sulfur entry. Assembly of a 4Fe-4S cluster was observed in both SDU and SDUF complexes but cluster transfer was only efficient in the presence of FXN\(^{235}\). Controversially to all of the above, a novel study showed that FXN is not required for persulfide formation on NFS1, but it specifically enhances sulfur transfer from NFS1 to ISCU, thus increasing the rate of sulfide production in the SDUF complex in a global scale. FXN also stimulated sulfide release
from NFS1 by enhancing the reduction of persulfide on NFS1 by thiols such as DTT, although it is not relevant or efficient for Fe-S cluster synthesis\textsuperscript{290}.

On the other hand, bacterial frataxin CyaY is suggested as an iron-dependent inhibitor of 2Fe-2S cluster formation through binding to cysteine desulfurase IscS\textsuperscript{208} by directly inhibiting cysteine desulfurase activity\textsuperscript{291}. In an experiment in which human and prokaryotic cluster assembly components were interchanged, CyaY could functionally replace FXN and activate human cysteine desulfurase while FXN failed to activate bacterial IscS. Therefore the inhibitory effect was determined by the identity of the cysteine desulfurase, not frataxin\textsuperscript{292}.

Taken together, these studies support the model that frataxin is a regulator of Fe-S cluster synthesis in all organisms. Eukaryotic frataxin most probably facilitates sulfur transfer from the cysteine desulfurase to the scaffold protein. The observed stimulation of cysteine desulfurase activity on the SDUF complex could either be a direct repercussion of frataxin on the cysteine desulfurase enzyme, or a global scale effect observed due to enhanced sulfur transfer. Therefore the explicit function of frataxin in mammalian Fe-S cluster synthesis still remains elusive. In contrast prokaryotic frataxin is an inhibitor of cluster formation via decreasing cysteine desulfurase activity. The functional discrepancy of frataxin between eukaryotic and prokaryotic systems is surprising yet interesting and requires careful rationalization through further investigations.

My goal in this report was to fill in the gaps in knowledge of the Yfh1/Nfs1-Isd11 interaction and the effect of Yfh1 on structure and function of Nfs1-Isd11 in the yeast system. Experiments outlined below were carried out in such context.
3.3 Experimental methods

3.3.1 Bacterial expression and isolation of proteins

3.3.1.1 Expression of yeast Fe-S cluster synthesis proteins

*Expression of Nfs1 (and C421A)-Iisd11:* pST39 co-expression vectors containing Δ1-33 Nfs1-6xHis-Iisd11 or Δ1-36 Nfs1(C421A)-6xHis-Iisd11 were transformed into BL21 (DE3) cells. In both these constructs Nfs1 lacks mitochondrial targeting sequence but Iisd11 remains full length. Starter cultures were made by inoculating a transformed colony in Luria Bertani (LB) broth supplemented with 100 µg/ml ampicillin. These cultures were incubated in a rotating shaker for 16 hours (overnight) at 230 rpm and 37 °C. Starting OD of the growth cultures (1 L) supplemented with 100 µg/ml ampicillin were set to 0.075 by adding appropriate amount of starter culture and the cells were grown until cell density reached 0.4 OD_{600}. Growth temperature was then switched to 18 °C and cells were induced at 0.7 OD_{600} using 0.8 mM IPTG supplemented with 10 µM PLP. Cells grew for 18 hours and then they were harvested by centrifugation at 15900 x g for 30 min. If not lysed immediately, cell pellets were flash frozen and stored at -80 °C for future use.

*Expression of Nfs1:* pET21b vector containing Δ1-33 Nfs1-6xHis was transformed into BL21 (DE3) codon plus cells. Starter cultures and growth cultures (1 L) were treated as mentioned above except they were supplemented with 34 µg/ml chloramphenicol in addition to 100 µg/ml ampicillin. Cells were grown at 37 °C until cell density reached 0.4-0.5 OD_{600}. Growth temperature was then switched to 25 °C and cells were induced at 0.7 OD_{600} with of 1 mM IPTG, supplemented with 10 µM PLP. Cells were continued to grow at 25 °C for 3 hours before harvesting, again by
centrifugation. If not lysed immediately, cell pellets were flash frozen and stored at -80 °C for future use.

Expression of Yfh1 and Yfh1-6xHis: Mature full length Yfh1 was expressed as described previously. Briefly pET11a vector containing Δ1-52 Yfh1 or Δ1-52 Yfh1-6xHis was transformed into BL21 (DE3) Star cells. Starter cultures and growth cultures (1 L) were treated as mentioned above and were supplemented with 100 µg/ml ampicillin. Cells were grown at 37 °C until cell density reached 0.8 OD<sub>600</sub> and were induced with 1 mM IPTG. Cells were continued to grow at 37 °C for 6 hours before harvesting by centrifugation. If not lysed immediately, cell pellets were flash frozen and stored at -80 °C for future use.

3.3.1.2 Purification of yeast Fe-S cluster synthesis proteins

Purification of Nfs1, Yfh1-6xHis, Nfs1-Isd11, and Nfs1(C421A)-Isd11 proteins: Frozen cell pellets were resuspended in pH 7.5 lysis buffer containing 50 mM Sodium Phosphate, 150 mM NaCl for Yfh1-6xHis, Nfs1-Isd11, and Nfs1(C421A)-Isd11 complexes/300 mM NaCl for Nfs1 alone, 5% Glycerol, 20 mM Imidazole, and 5 mM β–ME (4 ml/g of cells). In addition, cOmplete™ EDTA-free protease inhibitor cocktail (Roche), lysozyme (10 mg/ml), and DNAse (10 µg/ml) supplemented with 5 mM MgCl<sub>2</sub> were also included in the lysis buffer. Protein purification was performed at 4 °C. Cell suspensions were incubated at 4 °C for 30 min while stirring at a slow pace before passing through the Emulsiflex cell homogenizer three times for cell lysing. Cell lysates were then centrifuged at 53000 x g for 1 hour. Crude soluble fractions were filtered (0.20 µm) before passing through Ni affinity columns. Nfs1-Isd11 soluble fraction was passed through His-Trap HP 5 ml Ni column (GE Healthcare) using an AKTA FPLC
chromatography system (Amersham Biosciences/GE Healthcare); Yfh1-6xHis, Nfs1 or Nfs1(C421A)-Isd11 soluble fractions were passed separately through pre-equilibrated high density Ni-NTA Agarose Beads (GoldBio) manually packed in bench-top columns. Ni columns were first washed with 20 column volumes of binding buffer to eliminate any residual unbound proteins. Bound proteins were then eluted by flowing an imidazole gradient of 20-500 mM across the columns and fractions containing Yfh1-6xHis, Nfs1, Nfs1-Isd11, and Nfs1(C421A)-Isd11 complexes were identified using SDS-PAGE. Active fractions were pooled and concentrated to ~ 1 ml using 10 kDa molecular weight cutoff Amicon centrifugal filter devices (Millipore). Nfs1 containing protein preparations were incubated with excess PLP to ensure 100 % loading of PLP to all Nfs1 units. Similarly, Yfh1-6xHis protein preparation was incubated with 5 mM EDTA to ensure removal of any bound iron. Concentrated Nfs1, Nfs1-Isd11, and Nfs1(C421A)-Isd11 proteins and Yfh1-6xHis were passed through HiPrep 16/60 Sephacryl S-200 HR and HiLoad 16/600 Superdex-75 size exclusion columns (both GE Healthcare) respectively to further remove impurities and to transfer to pH 7.5 final buffer comprising of 20 mM HEPES, 150 mM NaCl for Yfh1-6xHis, Nfs1-Isd11, and Nfs1(C421A)-Isd11 complexes/300 mM NaCl for Nfs1 alone, and 5mM β-ME. Fractions containing Nfs1, Nfs1-Isd11, and Nfs1(C421A)-Isd11 complexes were pooled and concentrated to store at -80 °C in aliquots until further use in experiments. Yfh1-6xHis fractions were concentrated and stored under oxygen depleted nitrogen atmosphere at 4 °C and was used within two weeks of isolation to ensure protein integrity. All purified proteins were >90% pure (Figure 3.1).
Purification of Yfh1: The mature form of Yfh1 was purified as described previously\textsuperscript{252}. Briefly frozen cell pellets were resuspended in pH 8.0 lysis buffer containing 25 mM Tris HCl, 10 mM EDTA, and 5 mM β–ME (4 ml/g of cells). In addition, cOmplete™ EDTA-free protease inhibitor cocktail (Roche), lysozyme (10 mg/ml), and DNase (10 µg/ml) supplemented with 5 mM MgCl\textsubscript{2} were also added to the cell suspension. Protein purification was performed at 4 °C. Cell suspensions were incubated at 4 °C for 30 min while stirring at a slow pace before passing through the Emulsiflex cell homogenizer three times for cell lysing. Cell lysates were then centrifuged at 53300 x g for 1 hour. Crude soluble fractions were filtered (0.20 µm) and subjected to two ammonium sulfate precipitation steps: (1) contaminating proteins precipitated by incubating with 40% ammonium sulfate for 20 min and centrifugation at 15900 x g for 20 min and (2) Yfh1 precipitation from resulting supernatant by incubating with 60% ammonium sulfate for one hour and centrifugation at 15900 x g for 20 min. Resulting Yfh1 pellet was resuspended in a minimal volume of lysis buffer before dialyzing twice for 3 hours each in 2 L of the same buffer to remove any ammonium sulfate. Then the protein mixture was passed through HiLoad 26/10 Q-Sepharose column (Amersham Biosciences/GE Healthcare) pre-equilibrated with pH 8.0 buffer containing 25 mM Tris HCl, 10 mM EDTA, and 5 mM β–ME and eluted using an increasing 1 M NaCl gradient. Fractions containing Yfh1 were identified using SDS-PAGE, pooled, and dialyzed as before to remove any salt. Further purification of Yfh1 involved passing through pre-equilibrated HiLoad 26/10 Phenyl-Sepharose column (Amersham Biosciences/GE Healthcare) and eluting with a decreasing 1 M ammonium sulfate gradient at pH 8.0. Solid ammonium sulfate was added to the protein mixture
prior loading to the column to make the starting ammonium sulfate concentration 1 M. Fractions containing Yfh1 identified by SDS-PAGE were concentrated to 1 ml, before treating with 5 mM EDTA at pH 8.0 for 30 min. Metal-free protein was then loaded on to pre-equilibrated HiLoad 16/600 Superdex-75 size exclusion column (GE Healthcare) for further purification and buffer exchange to final 20 mM HEPES, 150 mM NaCl, and 5 mM β–ME pH 7.5 buffer. Pure Yfh1 (>90%) (Figure 3.1) was concentrated using a 10 kDa molecular weight cutoff Amicon centrifugal filter device (Millipore) and was stored under oxygen depleted nitrogen atmosphere at 4 °C. Purified frataxin was used within two weeks of isolation to ensure protein integrity.

3.3.2 Isothermal Titration Calorimetry

ITC experiments were executed to determine the Yfh1 binding affinity to the Nfs1-Isd11 complex, as well as the maximum Yfh1/Nfs1-Isd11 stoichiometry. Titrations were carried out at 30 °C on a TA instruments Nano-ITC calorimeter under anaerobic conditions. Concentrated Yfh1 and Nfs1-Isd11 proteins were diluted to 500 µM and 25 µM respectively in degassed pH 7.5 buffer containing 20 m HEPES, 150 mM NaCl, and 5 mM β–ME. Twenty fold excess Yfh1 was loaded on to the syringe while 950 µl of Nfs1-Isd11 remained in the cell stirring at 200 rpm. A total of forty 5 µl injections of Yfh1 were titrated consecutively at 300 s intervals to achieve the thermal titration profile. The TA Instruments NanoAnalyze software package was used to analyze the titration profile and identify the best-fit values for the dissociation constant, stoichiometry, and heat of enthalpy change for the Yfh1/Nfs1-Isd11 interaction.
3.3.3 *In vitro* pull-down assay

A Ni-NTA agarose affinity pull-down assay was performed to observe any complex formation between Yfh1 and Nfs1-Iisd11 proteins. Purified Nfs1-Iisd11 (-6xHis tag on Nfs1) was incubated with 100 µl of high density Nickel-NTA Agarose Beads (GoldBio) pre-washed with Milli-Q water and equilibrated with 20 mM HEPES, 150 mM NaCl, and 5 mM β-ME pH 7.5 buffer, within a BioRad Micro Bio-Spin column. Maximum binding of Nfs1-Iisd11 to the resin was allowed by end-to-end mixing on a rotating platform for 30 min at 25 °C. The sample was spun at 295 x g for 1 min using a tabletop centrifuge to remove the flow through from the column and subsequent washes with the same buffer removed any unbound protein. Beads saturated with bound Nfs1-Iisd11 were then treated with four-times molar excess of Yfh1 for 30 min on the rotating platform at 25 °C to allow maximum binding. Beads were extensively washed with buffer as before until no protein was detected in the collected washes, monitored using the Advanced protein detection solution. After a single 20 mM imidazole wash, bound proteins were eluted with 500 mM imidazole buffer and subsequently detected with SDS-PAGE.

3.3.4 Differential Scanning Calorimetry

Changes in the thermal stability of Nfs1, or the Nfs1-Iisd11 complex, upon addition of Yfh1-6xHis were assessed using differential scanning calorimetry (DSC). Purified and concentrated proteins were mixed and incubated at 25 °C for 10 min to achieve respective stoichiometrically proportioned mixtures (~0.5-3 mg/ml). Samples were prepared in 20 mM HEPES, 300 mM NaCl, 5 mM β-ME at pH 7.5 buffer, degassed to remove dissolved air. Protein samples were loaded in the sample cell of a TA
Instruments Nano-DSC calorimeter; the reference cell was filled with matched buffer. The total volume for the capillary cell is 0.3 ml, however an excess of solution (~0.6 ml) is used for loading to prevent trapping air bubbles. Both capillary cells were manually pressurized to maintain a constant pressure of 3 atm during the scan. Each protein sample was scanned at a rate of 1 °C/min from 10 °C to 90 °C, and the melting curves for Nfs1:Yfh1 (1:2) and Nfs1-lsd11:Yfh1 (1:2) were assessed to identify differences in thermal stability of Nfs1 and Nfs1-lsd11 during complexation with lsd11. Experiments were done in duplicate using independently isolated proteins to ensure data reproducibility. Thermal profiles were simulated using the TA instruments NanoAnalyze software package to identify the best-fit values for melting temperatures ($T_m$).

### 3.3.5 UV-visible spectroscopy

UV-visible spectroscopy was used to identify if the affinity of Nfs1(C421A)-lsd11 to L-cysteine is altered in the presence of Yfh1-6xHis. In this experiment, 50 µM purified Nfs1(C421A)-lsd11 was incubated with 100 µM Yfh1-6xHis (in 1:2 molar ratio) at 25 °C for 10 min. Samples were prepared in 20 mM HEPES and 300 mM NaCl at pH 7.5 and the protein mixture was then titrated consecutively with freshly made L-cysteine. Protein samples were carefully mixed and incubated for 10 min after each addition of L-cysteine; spectra were measured at a 1.0 nm scan pitch using a Shimadzu UV-1800 Spectrophotometer. Each addition was timed 1 min after the start of spectra acquisition to maintain consistency between data points. All spectra were collected at 25 °C under anaerobic conditions and corrected for dilution. Experiments were performed in duplicate using independently prepared protein samples to ensure spectral reproducibility. The change in spectral intensity at 340 nm was plotted against
respective L-cysteine concentrations to calculate the binding affinity of Nfs1(C421A)-Isd11 to L-cysteine in the presence or absence of Yfh1-6xHis. Best-fit simulation to data was obtained using DynaFit curve fitting software\textsuperscript{227}.

### 3.3.6 Cysteine desulfurase activity assay

An acid labile sulfide detection assay was performed to measure the activity of Nfs1-Isd11 complex in the presence or absence of Yfh1-6xHis. In this assay, Fe\textsuperscript{3+} with sulfide liberated from the enzyme aids the conversion of \textit{N}, \textit{N}-dimethyl-\textit{p}-phenylenediamine sulfate (DPD) to methylene blue. The characteristic blue color, i.e. the visible absorption feature at \textasciitilde 750 nm was used to quantify sulfide production along with the use of a calibration curve for signal intensity prepared with a series of known sodium sulfide concentrations. The purified Nfs1-Isd11 complex (12 \textmu M concentration) was incubated with 1 mM L-cysteine in the absence or presence of stoichiometric Yfh1-6xHis (1:2). An assay mixture contained 100 mM HEPES (pH 7.5), 150 mM NaCl, 50 \textmu M PLP, and distilled water, and the total reaction volume was 100 \textmu l. Each reaction was prepared by first mixing the assay components together in an eppendorf tube covered with rubber septa caps (Sigma-Aldrich-Z565717) to prevent air escaping. Respective L-cysteine amounts were then introduced to each tube using a gas-tight Hamilton syringe. After a 5 min incubation at 30 °C, the reaction was stopped by adding 100 \textmu l of 20 mM DPD in 7.2 N HCl and 30 mM FeCl\textsubscript{3} in 1.2 N HCl. This was also performed using a gas-tight Hamilton syringe and each addition was timed 45 s apart to maintain the consistency between each reaction. Distilled water was added up to 800 \textmu l. Each tube was then vortexed for 10 s before incubating for 20 min at 30 °C for efficient color development. All tubes were spun at 14000 x g for 2 min to remove protein
precipitation before measuring the absorbance. Absorbance was measured at 750 nm using a 1 ml cuvette on a Varian Cary-50 Bio UV-Visible Spectrophotometer. The amount of persulfide liberated at each concentration was determined using the standard curve. Measurements were collected in triplicate, averaged, and corrected for dilution.

3.4 Results

3.4.1 Purified proteins are > 95% pure

All proteins purified by following respective protocols listed above were > 95% pure. Nfs1 was present as a homodimer, the typical oligomerization state attributable to cysteine desulfurase enzymes (data shown in chapter 2). Nfs1-Isd11 and Nfs1(C421A)-Isd11 were heteromeric complexes comprising of Nfs1 (wild type or mutant) homodimers and 4 Isd11 units in each complex (data shown in chapter 2). Yfh1 (with/without -6xHis tag) was present in its monomeric form as observed with size exclusion chromatography (data not shown).
Figure 3.1: SDS-PAGE gel of purified proteins. Lanes represent 1: molecular weight marker, 2: purified yeast Nfs1-lsd11 complex, 3: Nfs1(C421A)-lsd11 complex, 4: Yfh1, and 5: Yfh1-6xHis.
3.4.2 ITC experiments suggest a possible interaction between Nfs1-Isd11 complex and Yfh1

To provide evidence that Nfs1-Isd11 and Yfh1 physically interact during Fe-S cluster synthesis, and to quantify the interaction, isothermal titration calorimetry was performed. Titration of Yfh1 into Nfs1-Isd11 yielded the raw binding isotherm (Figure 3.2-A) that could be simulated to obtain the best-fit binding isotherm (Figure 3.2-B) and corresponding binding parameters. The $K_d$, stoichiometry, and enthalpy for the interaction were $7.4 \pm 3.2 \, \mu M$, $1.5 \pm 0.1$, and $-4.9 \pm 0.7 \, kJ/mol$ respectively. Detection of a binding event with ITC suggested a physical interaction of Yfh1 with Nfs1-Isd11 under these experimental conditions. Weak binding affinity in the micromolar range was consistent with a previously published micromolar $K_d$ for the interaction between bacterial orthologs IscS and CyaY$^{285}$. Stoichiometry of $1.5 \pm 0.1$ indicated that approximately 2 Yfh1 units associate with Nfs1-Isd11 complex, in agreement with the 1:1 Nfs1:Yfh1 (per Nfs1 monomer) stoichiometry observed in SDUF quaternary complexes in several systems$^{229,235}$. Although this result suggested a physical interaction between Nfs1-Isd11 and Yfh1, it was also questionable due to few concerns. The Nfs1-Isd11 complex was unstable under these experimental conditions and precipitation of protein was observed during continuous stirring of Nfs1-Isd11 within the cell, regardless of measures taken to prevent precipitation (i.e. decreased stirring speed, addition of stabilizers such as glycerol). Protein precipitation created a shifted but stable baseline with minimal noise. Due to these reasons, reproduction of these results was impossible. Therefore the validity of ITC results was subjected to skepticism and the interaction observed here could be an outcome of aggregation during
experiment and be superficial. Therefore, more evidence to strengthen the validity of these results is required.

3.4.3 *In vitro* pull-down assay indicates no interaction between Nfs1-Isd11 and Yfh1

To ascertain the existence of an interaction between Yfh1 and Nfs1-Isd11, an *in vitro* Ni-affinity pull-down assay was performed. Nfs1-Isd11 bound to the resin could not retain any Yfh1 upon incubation, as Yfh1 readily eluted with the subsequent washes (Lanes 5-7, Figure 3.3). No Yfh1 was found bound to Nfs1-Isd11 when eluted with imidazole (Lanes 12-14, Figure 3.3). Even though a weak interaction with micromolar affinity was observed between Yfh1 and Nfs1-Isd11 with ITC under similar conditions, the same interaction was not observed with pull-down assay. This indicated that an interaction was unlikely under these experimental conditions, and ITC results were indeed superficial. An interaction between human orthologs NFS1-ISD11 and YFH1 was also not detected in a previous study. Hence, results from ITC and pull-down assay may suggest that an interaction between Nfs1-Isd11 and Yfh1 is mostly not a tight interaction, but rather they could be transient/weak and impossible to detect within the sensitivity limits of these techniques.
Figure 3.2: Raw isothermal titration calorimetry data (A) and binding isotherm data (B) for Yfh1 titration into Nfs1-Isd11 complex. Solid black line in panel B indicates best-fit simulation to binding isotherm data obtained using fitting programs.
Figure 3.3: Poly-histidine tag pull-down assay for Nfs1-Isd11 interaction with Yfh1. Flow through and washes were collected after addition of each protein and separated on SDS-PAGE along with elution fractions to identify binding partners. Lanes labels read: 1-molecular weight marker, 2-Nfs1-Isd11 flow-through, 3, 4-two consecutive washes after Nfs1-Isd11 binding to the resin, 5-Yfh1 flow-through, 6-11- consecutive washes after Yfh1 addition, 12- 20 mM imidazole wash, and 13-15 consecutive elutions with 500 mM imidazole.
3.4.4 Association of Yfh1 with Nfs1/Nfs1-Isd11 does not affect the stability of the proteins

It is also likely that the association between Nfs1-Isd11 and Yfh1 is transient, and that this flexibility is essential to achieve certain functional goals during the Fe-S cluster assembly process. It is possible that the binding partners alter each other’s stability via such an interaction (similar to the effect Isd11 has on Nfs1, discussed in chapter 2). Such an effect was monitored by comparing the thermal profiles of Nfs1 and the Nfs1-Isd11 complex in the presence and absence of Yfh1 using Differential Scanning Calorimetry (DSC). Thermal profiles of neither Nfs1 (top panel-Figure 3.4) nor Nfs1-Isd11 (bottom panel-Figure 3.4) were substantially affected by incubation with Yfh1 (Table 3.1). Therefore it is likely that either Nfs1-Isd11 and Yfh1 does not interact in Fe-S cluster biosynthesis machinery, or their transient interaction has minor structural perturbations on both partners.
Figure 3.4: Differential Scanning Calorimetric thermal profiles for purified Nfs1 and Nfs1-Isd11 complex in the presence or absence of Yfh1. Letters represent thermograms of: A- Nfs1 only, B and E- Yfh1 only, C- Nfs1 with Yfh1 (1:2), D- Nfs1-Isd11 only, and F- Nfs1-Isd11 with Yfh1 (1:2). Solid black and gray lines represent raw data and sum of best-fit simulations respectively. Dashed lines represent simulated peaks corresponding to domains and/or individual unfolding events in each structure. Melting temperatures for simulated peaks are depicted in Table 3.1. B, C, E, and F thermal profiles are offset for clarity.
Table 3.1: Melting temperatures (°C) for each domain/unfolding event obtained from Differential Scanning Calorimetry data for Nfs1 and Nfs1-Isd11 in the absence/presence of Yfh1. Melting temperatures related to Yfh1 in each case are shown in italics.

<table>
<thead>
<tr>
<th>Protein(s)</th>
<th>Domain/unfolding event</th>
<th>Melting Temperature (°C)</th>
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<tr>
<td>Nfs1</td>
<td>1</td>
<td>39.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>42.0 ± 0.2</td>
</tr>
<tr>
<td>Nfs1+Yfh1 (1:2)</td>
<td>1</td>
<td>39.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>41.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>53.2 ± 0.2</td>
</tr>
<tr>
<td>Yfh1 only</td>
<td>-</td>
<td>53.1 ± 0.2</td>
</tr>
<tr>
<td>Nfs1-Isd11</td>
<td>1</td>
<td>56.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>60.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>62.1 ± 0.4</td>
</tr>
<tr>
<td>Nfs1-Isd11+Yfh1 (1:2)</td>
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<td>56.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>59.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
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</tr>
<tr>
<td></td>
<td>4</td>
<td>51.9 ± 0.1</td>
</tr>
</tbody>
</table>
3.4.5 Yfh1 does not alter substrate binding affinity of Nfs1-Isd11

In order to determine any functional relationship between Nfs1-Isd11 and Yfh1, UV-visible spectroscopy was utilized to monitor any changes in substrate binding affinity of Nfs1-Isd11 in the presence of Yfh1. As described in the previous chapter, the C421A mutant of Nfs1 was used for this assay to isolate the substrate-binding step from catalytic step of the enzyme. Titration of L-cysteine to the Nfs1(C421A)-Isd11 construct incubated with and without Yfh1 yielded binding curves that are not distinctly different from each other (Figure 3.5). Best-fit simulations for binding data revealed $K_d$ values of $0.76 \pm 0.13$ mM and $0.78 \pm 0.14$ mM in the absence and presence of Yfh1, respectively. The almost identical substrate binding affinities, regardless of the presence of Yfh1, agrees along with the prediction that Nfs1-Isd11 and Yfh1 may not interact or interact transiently to alter a different function other than substrate binding in Fe-S cluster biosynthesis machinery.

3.4.6 Effect of Yfh1 on cysteine desulfurase activity of Nfs1-Isd11 is minimal

In order to determine if a transient interaction with Yfh1 may alter cysteine desulfurase activity of Nfs1-Isd11 complex, an acid labile sulfide assay was performed. This assay was used to compare the rates of sulfide liberation in the absence and presence of Yfh1. The rate of acid labile sulfide formation within first five minutes of the reaction was not significantly altered by the presence of Yfh1 (only a slight enhancement of 16% was observed: Figure 3.6). Similarly, in the human system, frataxin had no direct effect on cysteine desulfurase activity of the human complex\textsuperscript{207}. Therefore it is likely that Yfh1 may not interact with Nfs1-Isd11 in Fe-S cluster biosynthesis machinery in a functional point of view.
Figure 3.5: Titration of L-cysteine to Nfs1(C421A)-lsd11 in the presence/absence of Yfh1 monitored by UV-visible spectroscopy. Black and white markers represent the change in absorbance at 340 nm at each L-cysteine concentration in the absence and presence of Yfh1, respectively. Solid and dashed lines indicate the respective best-fit simulations obtained using DynaFit curve fitting software.
Figure 3.6: Persulfide forming activity of Nfs1-Isd11 in the presence/absence of Yfh1.
3.5 Discussion

The exact function of frataxin within mitochondrial Fe-S cluster biogenesis has been a longstanding question since the protein was discovered to be defective in Friedreich’s ataxia, a neurodegenerative disorder affecting 1/30,000 Caucasians. Although initial in vitro studies provided evidence to ascertain the protein’s function as an iron chaperone delivering Fe for the cluster assembly machinery, later studies proved additional functions were also likely. In recent years, frataxin has been identified as a regulator of Fe-S cluster assembly. Evidence was presented to prove that frataxin achieves this by enhancing cysteine desulfurase activity and sulfur delivery of the core assembly complex. Much of the mechanism of this effect was elucidated in the human and murine systems, but studies on yeast orthologs are lacking. The objective of my research was to identify an interaction between Yfh1 and the yeast Nfs1-Isd11 complex, and then to study the effect of Yfh1 on the structure and function of this system. In such a context, it was proposed that we isolate the necessary proteins and utilize techniques such as ITC, pull-down assays, and DSC to identify any interaction. In addition, we planned to use our UV-visible assay and activity assay to study any possible effect. This chapter describes experimental details and results pertaining to those projected studies.

The expression and purification of all proteins required for these experiments were straightforward, since we have already established protocols and our lab is equipped with required instruments. All isolated proteins were > 90% pure (Figure 3.1). Some of the initial experiments used Yfh1 with no additional tag, but later a 6xHis tagged version of Yfh1 became available for use with an easier, less time-consuming
and resource-consuming isolation protocol. Therefore later experiments used Yfh1-Hisx6. There is no detectable difference in the structure and function of these two Yfh1 variants, and this was confirmed by experiments performed by a fellow graduate student in the lab (Stephen Dzul). Therefore, results obtained with both versions are directly comparable. However, for clarity and integrity, each individual experiment was performed only with one version or the other. The use of the Nfs1(C421A)-Iisd11 mutant complex for UV-visible spectroscopy studies was justified in chapter 2, and our CD, DSC, and activity assay experiments confirmed it is not significantly different from the wild type complex.

ITC experiments were proposed to identify a possible interaction between Yfh1 and Nfs1-Iisd11 and elucidate physical parameters such as the dissociation constant and stoichiometry (Figure 3.2). These experiments were plagued with protein precipitation issues that caused us to question the validity of our ITC results. Continuous stirring during the experiments seemed to affect the stability of the Nfs1-Iisd11 complex, although it was more stable as a complex than its constitutive proteins were individually (chapter 2). Experimental conditions (i.e. stirring speed and buffer composition) were changed to prevent precipitation; nonetheless it could not be avoided. Despite protein precipitation, the shifted baseline was surprisingly stable and not noisy. Although after baseline correction, binding data for one experiment yielded probable binding parameters in the range of published values for the bacterial system, subsequent runs failed to reproduce this. Therefore, our results observed here are more likely to be an outcome of aggregation rather than actual interaction between Nfs1-Iisd11 and Yfh1. However the binding affinity obtained from this single experiment was
similar to the published binding affinity seen between the bacterial orthologs\textsuperscript{285}, and the preliminary results reported by a previous lab member (Swati Rawat) for yeast proteins\textsuperscript{*}. These preliminary results were obtained using a different ITC instrument and no precipitation has been documented for her experiment. Since ITC experiments were subjected to technical limitations, a less rigorous method, surface plasmon resonance (SPR) was proposed. SPR involves immobilizing a 6xHis-tagged protein on a Ni-affinity chip and passing the ligand protein over the bound molecule along with the solvent to allow maximum association. In the next step, dissociation is allowed by passing the ligand free solvent and the changes in SPR signal during association and dissociation steps are used to determine equilibrium binding constants. Yfh1 with no tag was passed with PBST (Phosphate Buffered Saline and Tween-20) buffer over Nfs1-lsd11 (-6xHis) complex immobilized on the sample channel as well as the empty reference channel. Interestingly, this technique was also subjected to technical limitations simply because Yfh1 unexpectedly started binding to the reference channel despite the absence of a 6xHis-tag associated with it. Hence, no reference measurement could be made for accurate data analysis (data not shown). Association of Yfh1 with nickel ions is not novel, as reports show nickel serving as a mediator for Yfh1’s interaction with other cluster assembly components within humans, while other metals act as inhibitors\textsuperscript{284}. Presumably, frataxin’s acidic ridge, proposed to serve as the iron-binding region, may facilitate interaction with nickel in this case. Therefore SPR was not capable of verifying the questionable results from ITC. An \textit{in vitro} pull-down assay was performed using purified proteins as the last resort to observe a possible Yfh1 interaction with Nfs1-
Isd11. Nfs1-Isd11 was immobilized to Ni-NTA Agarose resin and subsequent washes removed any adventitiously bound protein before incubation with Yfh1. Yfh1 was used in excess (4 times) to ensure maximum and efficient association with the immobilized complex. Washes immediately after Yfh1 addition contained a significant amount of unbound Yfh1. Interestingly, the first few washes also contained a minute amount of Nfs1-Isd11, eluted with excess unbound Yfh1 (Figure 3.3). This could potentially be due to a displacement of some of the Nfs1-Isd11 complex from the resin by Yfh1 (given that Yfh1-Ni interaction was observed in SPR experiments too). This is likely however not due to Yfh1 pulling-down Nfs1-Isd11. No Yfh1 was present in subsequent elution fractions, indicating no stable association between Yfh1 and Nfs1-Isd11, at least under the assay conditions we used. Similarly, human NFS1-ISD11 and YFH1 showed no physical interaction in gel filtration chromatography\(^{207}\). In that context, ITC results could be confirmed superficial. Overall, these results suggested that a stable interaction between Nfs1-Isd11 complex and Yfh1 is unlikely. It is therefore likely that this interaction is transient under these experimental conditions.

In the event of a transient binding interaction, it is possible that Yfh1 interacts with Nfs1-Isd11 to reach certain functional goals, even though the physical association is beyond the sensitivity limits of our experimental techniques. To identify if Yfh1 had any effect on stability, substrate binding affinity, and activity of Nfs1-Isd11, DSC, UV-visible, and activity assay measurements were performed, respectively. DSC thermal profiles obtained for Nfs1 and Nfs1-Isd11 in the presence of Yfh1 were not significantly different from their individual thermal profiles (Figure 3.4). Melting temperatures obtained for individual denaturation events within each profile were not substantially
affected by Yfh1, indicating that it does not induce changes in protein fold on Nfs1 or Nfs1-Isd11 globally, or in the domains level of the proteins. Similarly, Yfh1 did not have any effect on the substrate binding affinity of Nfs1-Isd11 complex (Figure 3.5). In a previous study, Yfh1 was reported to enhance cysteine binding to Nfs1 by exposing substrate-binding sites to the exterior\(^{116}\). Therefore it is reasonable to expect a change in the substrate binding affinity in the presence of Yfh1. However, the opposite was observed. Yfh1 also had no significant effect on cysteine desulfurase activity of Nfs1-Isd11 (Figure 3.6), similar to that seen in the human system\(^{207}\).

With all these evidence, it is reasonable to assume that no stable physical interaction between Nfs1-Isd11 and Yfh1 exists in yeast Fe-S cluster biosynthesis machinery. No discrepancy in the structure and function of Nfs1-Isd11 in the presence of Yfh1 was measured by assessing the thermal stability, substrate binding affinity, and catalytic activity comparatively. This further supports a lack of any appreciable stable binding interaction. My results in this chapter add an extra level of comprehension on the role of eukaryotic frataxin on regulating the cysteine desulfurase. Since human orthologs show similar behavior\(^{207}\), it is possible to assume that eukaryotic frataxins have no dramatic effect on their respective cysteine desulfurases when viewed as an individual binding event. It is now logical to hypothesize that the effect of yeast frataxin on cysteine desulfurase activity is contingent on the presence of Isu1\(^{229}\). Altered activity by frataxin in the Nfs1-Isd11-Isu1-Yfh1 (SDUF) cluster synthesis complex was reported for other systems (discussed in section 3.2.3). The direction of research in such a context is therefore discussed in detail in a following chapter.
3.6 Acknowledgements

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4.0 Prelude

This chapter describes my initial work on purifying Isd11, the functional partner of Nfs1 within yeast mitochondrial ISC biogenesis pathway. The objective of this study was to characterize the molecular features of Nfs1-Isd11 complex by isolating each protein separately and characterizing their interaction. Isothermal titration calorimetry was proposed to determine the binding affinity and stoichiometry of Nfs1 and Isd11, and nuclear magnetic resonance (NMR) spectroscopy, along with chemical shift perturbations assignments, was to be performed to elucidate the solution structure of Isd11 and to locate Isd11 residues involved in Nfs1-Isd11 interaction. Work presented below describes the strategies I employed to purify Isd11 to make protein available for the experiments suggested above. Isd11 purified partially from soluble fractions contained predominantly higher order species that were confirmed by western blot analysis to be our desired protein. Denatured Isd11 purified from insoluble fractions by urea denaturation failed to recover from urea efficiently in an attempt to refold protein to its native conformation. Therefore, due to its inherent insolubility and typical “orphan-protein” like nature, sufficient amounts of Isd11 could not be obtained with either methods employed. Details of the methods and some preliminary results obtained during the initial characterization are included in this chapter for general knowledge since the time and effort I spent working on this protein was significant.

4.1 Introduction

The accessory protein Isd11, first discovered a decade ago by two groups, was shown to be an 11 kDa protein essential for mitochondrial Fe-S cluster synthesis in
Gene deletion experiments verified Isd11 (Iron Sulfur cluster biogenesis Desulfurase interacting protein) is present in all eukaryotic species but not in any prokaryote. Isd11 is classified in the LYR family of proteins due to the presence of a LYR/K amino acid residue segment near its N-terminus. Cellular localization experiments and the absence of any predicted transmembrane segments confirmed that Isd11 is a mitochondrial matrix protein loosely associated with the inner membrane. It does not contain a cleavable mitochondrial targeting sequence, and it is imported into mitochondria in a \( \Delta \Psi \)-dependent manner. In studies performed to search for the function of Isd11, cells depleted of Isd11 have shown reduced maturation of Fe-S clusters and decreased activity of Fe-S proteins such as aconitase and succinate dehydrogenase. Isd11 depleted cells further expressed iron accumulation characteristics. It is for both reasons that Isd11 was suggested to have a function linked to Fe-S cluster biogenesis and cellular iron homeostasis. Yeast cysteine desulfurase Nfs1 forms a stable complex with Isd11 in vivo and in the absence of Isd11 Nfs1 aggregates. Therefore Isd11 was also predicted to have a stabilizing effect on Nfs1 when the enzyme operates to provide sulfur component for the cluster synthesis. These initial studies also reported that Isd11 is not required for sulfide production activity by Nfs1 in vitro, however Isd11 and complex formation with Nfs1 are needed for the maturation of Fe-S proteins in vivo, implying that Nfs1-Isd11 is the physiological cysteine desulfurase complex.

A similar study in HeLa cells showed localization of human ISD11 to the mitochondria and nuclei, and formation of a complex with human cysteine desulfurase ISCS. Co-expression of ISCS with ISD11 in bacteria recovered ISCS from being
sequestered to inclusion bodies by facilitating correct folding and enhanced cysteine desulfurase activity. ISD11 deficiency caused a cytosolic iron deficiency phenotype and activated iron regulatory proteins IRP1 and IRP2 in cells, leading to the conclusion that ISD11 is important in maintaining cellular iron homeostasis in humans\textsuperscript{293}.

A number of studies that followed examined Isd11’s binding partners and attempted to understand the exact function of Isd11 within the Fe-S cluster biosynthesis pathway as well as understanding its role in maintaining cellular iron homeostasis. The findings of such studies are discussed below.

### 4.1.1 Structure and oligomeric state of Isd11

Isd11 lacks a characterized crystal or solution structure, likely due to the inherent insolubility of the protein that has been reported many times since its discovery. One study attempted to look at Isd11 three dimensionally using an \textit{ab-initio} structure prediction server Robetta to build the best possible structure of Isd11. The model primarily consisted of $\alpha$-helices, as predicted previously (with secondary structure prediction software)\textsuperscript{217}. Docking experiments predicted the attachment of Isd11 to the Fe-S cluster assembly complex containing Isu1, Yfh1, and Nfs1\textsuperscript{234}. A single far-UV circular dichroism (CD) study performed recently indicated that the protein’s structure was comprised of 57\% $\alpha$-helices and 10\% $\beta$-sheets\textsuperscript{232} further confirming the $\alpha$-helical nature of ISD11.

This same study isolated human ISD11 from \textit{E. coli} in the soluble form (by expressing in conjunction with a cleavable N-terminal GST tag)\textsuperscript{232}. This protein was not found to be monomeric, but instead it existed as a heterogeneous mixture comprising of large and small oligomeric units of dimer-tetramer and monomer-dimer species.
respectively. Ratios between oligomers in each species varied with the concentration and purified lifetime of Isd11, and therefore the oligomers were proposed to be in equilibrium. CD thermal denaturation profile for ISD11 revealed a relatively higher melting temperature of ~67 °C, but the integrity of this result is questionable due to the oligomeric nature of purified ISD11\textsuperscript{232}. Besides these reports, no structural details are available for Isd11, and this hinders us from understanding its function better.

4.1.2 Functional interactions of Isd11 in Fe-S cluster assembly

In the search for the precise function of Isd11 during Fe-S cluster synthesis, numerous studies have discovered individual interacting partners on Isd11 as well as multiprotein complexes containing Isd11.

One such study on human system reported that frataxin (FXN) interacts with ISD11 of the NFS1/ISCU complex and a knockdown of ISD11 creates frataxin deficiency like phenotypes, aconitase deficiency and cellular iron overload. Pull-down assays confirmed that I154F and W155R point mutations of FXN inhibit its interaction with ISD11. Therefore it was suggested that ISD11 serves as an adaptor between FXN and NFS1/ISCU complex\textsuperscript{284}. This fact was further confirmed when the same FXN point mutations depicted above promoted a reduced affinity towards the SDU (NFS1/ISD11/ISCU) complex\textsuperscript{287}. Similarly, the yeast Nfs1-Isd11 complex directly interacts with oligomeric yeast frataxin homolog (Yfh1) and the interaction is mediated at least in part by contact between Isd11 and Yfh1\textsuperscript{283}. A modeled structure for yeast Fe-S cluster assembly protein complex (including Yfh1, Nfs1, Isd11, and Isu) with two Isd11 proteins shows that Isd11 and Yfh1 are in close proximity, providing implications on Isd11’s involvement in mediating the Yfh1-Nfs1 interaction\textsuperscript{234}. 
Evidence for interactions of Isd11 with the scaffold protein Isu have only been reported once. This study utilized $^{15}\text{N}$ TROSY-HSQC NMR experiments to show that the addition of unlabeled human ISD11 to labeled scaffold protein ISCU leads to no changes in NMR spectra, and this indicate that ISD11 does not interact directly with ISCU$^{294}$.

Interactions of Isd11 with its functional binding partner Nfs1 has also been studied, but again due to inherent instability of both proteins in the absence of complex formation (as described in chapter 2), the number of such studies is limited. The only reported binding data between the proteins is available for human ISD11, which binds to human NFS1 with a $K_d$ of 100 nM, as measured by surface plasmon resonance (SPR) spectroscopy$^{230}$. Eight ISD11 residues (F40, L63, R68, Q69, I72, Y76, L81, and E84) are critical for the NFS1-ISD11 interaction, and these were identified recently. Mutating them compromised the NFS1-ISD11 interaction and this led to reduced Fe-S cluster assembly and diminished mitochondrial respiration$^{214}$.

Existence of Isd11 within multiprotein complexes with other partners of the Fe-S cluster assembly machinery has been reported, along with their stoichiometry discussed in several systems, most of which are outlined in chapter two.

4.1.3 Role of Isd11 in Fe-S cluster assembly

Since its discovery, Isd11 was found associated with its functional partner cysteine desulfurase in all eukaryotic systems. Elucidating the purpose and role of such interaction is an area of interest within the Fe-S cluster assembly field. Proposed functions of Isd11 include activating catalytic function of cysteine desulfurase and
stabilizing cysteine desulfurase by preventing aggregation. These hypotheses have been tested in several studies over the past years.

4.1.3.1 Isd11 prevents aggregation of Nfs1

The effect of Isd11 preventing Nfs1 aggregation was reported in the two initial studies within yeast. In one report, isolated mitochondria depleted of Isd11 contained higher order aggregates of Nfs1 within the insoluble fraction, following incubation of the system at 37 °C. Moreover, in lysed mitochondria, Nfs1 was prone to proteolytic degradation by endogenous proteases and also showed a lack of association with mitochondrial membranes in the absence of Isd11. Similarly, the other study reported the presence of Nfs1 within the pellet when mitochondria were isolated from heat-shocked conditional Isd11 mutant strains. During prolonged incubation at non-permissive temperatures, Nfs1 readily degraded. Both studies concluded that Isd11 functions as a stabilizer for Nfs1 in vivo.

An additional study suggested that Isd11 binds to Nfs1 in its oligomerization surface in the three-dimensional modeled structure for yeast Fe-S cluster assembly protein complex, and thereby prevents the aggregation seen in the previous examples. More recently, eight human ISD11 residues were identified as important for the NFS1-ISD11 interaction, and they were also marked as being essential in preventing aggregation of NFS1 in vitro.

Chapter two of this dissertation describes the work performed to elucidate the biophysical basis of the stability imparted by Isd11 onto yeast Nfs1. Instability of Nfs1, when expressed and purified not in complex with Isd11, was again observed in vitro. The hypotheses that the Nfs1-Isd11 co-complex must possess higher thermal stability
than the proteins alone, and that complex formation stabilizes Nfs1 by binding and 
inducing a more compact and stable fold in Nfs1 were tested using differential scanning 
calorimetry and fluorescence spectroscopy respectively. The SD complex was over 20 
°C more stable than Nfs1 alone and a conformational change on Nfs1 induced by lsd11 
binding was suggested to alter the fold of the enzyme. Therefore my work provides 
insight into how lsd11 stabilizes Nfs1.

4.1.3.2 Isd11 activates cysteine desulfurase

Although initial studies proposed that lsd11 complexation with Nfs1 is only 
essential for maturation of Fe-S proteins in vivo but not in vitro, recent studies have 
proved otherwise\textsuperscript{213,224}. It was discovered that the assays previously utilized to measure 
sulfide production did not clearly eliminate other sources of sulfide within the assay 
mixture, hence providing false positive results\textsuperscript{224}. A recent study utilized a novel 
approach to prove that Nfs1 by itself was inactive, but that lsd11 was capable of 
activating the cysteine desulfurase function within isolated mitochondria as well as with 
purified proteins. A carefully designed radiolabeling experiment, which separated the 
substrate-binding step of Nfs1 from its persulfide forming step, revealed that Nfs1 is 
capable of binding its substrate cysteine even in the absence of lsd11, but persulfide 
formation is strictly dependent on the presence of lsd11. It was suggested that lsd11 
binding to Nfs1 induces a conformational change that brings the catalytic cysteine 
residue and bound substrate in close proximity for persulfide formation\textsuperscript{116,224}.

4.1.4 Other functions of lsd11 in cellular milieu

Since it was shown that lsd11 enhances the cysteine desulfurase reaction by 
associating with Nfs1, the role of this protein was also deemed important for other
pathways that Nfs1 serves as the sulfur donor. One study reported that yeast Isd11 is essential for tRNA thiolation in addition to its function in Fe-S cluster assembly within the flagellate *Trypanosome brucei* system. Isd11 forms a stable complex with Nfs1 and aids in sulfur delivery to drive tRNA thiolation in both cytoplasm and mitochondria.\textsuperscript{295}

### 4.1.5 Isd11 and human diseases

So far, the number of diseases associated with defective Isd11 is limited to one. A mutation in the *LYRM4* gene encoding ISD11 in humans causes combined oxidative phosphorylation deficiency (COXPD), a disease in which respiratory complexes I, II, and III and several other Fe-S proteins are deficient in muscle and liver.\textsuperscript{213} Consequentially due to defective oxidative phosphorylation, patients develop neonatal lactic acidosis, respiratory distress and hypotonia.\textsuperscript{296} A recent study showed that the R68L mutation that causes COXPD impairs the ability of ISD11 to form a stable complex with NFS1.\textsuperscript{214} Given the importance of Isd11 in activating cysteine desulfurase during cluster assembly, it is no wonder if more diseases associated with Isd11 are discovered in the future.

### 4.2 Experimental methods

#### 4.2.1 Bacterial expression and isolation of Isd11

##### 4.2.1.1 Purification of Isd11 from soluble fraction of the cell lysate

Isd11-6xHis was expressed and purified from the soluble fraction as described previously, only with slight modifications to the protocol.\textsuperscript{225} Briefly, the pET21b vector containing Isd11 with a C-terminal 6xHis tag was transformed into BL21 (DE3) codon plus cells to express Isd11-6xHis. Starter cultures were made by inoculating a transformed colony in LB broth supplemented with 100 µg/ml ampicillin and 34 µg/ml
chloramphenicol, and this was incubated in a rotating shaker overnight at 200 rpm and 37 °C. Growth cultures (200 ml) were supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol, and the starter culture was diluted in LB in 1:40 ratio. Cells were grown at 37 °C in LB broth until the cell density reached 0.6 OD$_{600}$; then the shaker temperature was switched to 25 °C. After a 10 min incubation at the lower temperature, cells were supplemented with 0.5 M filtered sorbitol and induced with 0.2 mM IPTG after incubating for 15 min. Cells were allowed to grow at 25 °C for 72 hours before harvesting by centrifugation. This protocol yielded a significant amount of overexpressed Isd11 within soluble fraction, as opposed to the regular 3-hour induction at 37 °C protocol. Cell pellets were resuspended in lysis buffer containing 50 mM Tris HCl (pH 8.0), 10 % glycerol, and 1 mM PMSF. Cells were lysed by six rounds of sonication (20 s each round and 2 min intervals while samples are kept on ice). Lysate was spun at 12100 x g for 30 min at 4 °C and the supernatant was mixed with His-select Ni affinity resin (600 µl resin, Sigma P6611, pre-washed with water and lysis buffer) for 3 hours at 4 °C on a rocking platform to allow end-to-end mixing. After removing unbound protein, the resin was further washed with 50 mM Tris HCl (pH 8.0), 10 % glycerol, 10 mM imidazole, and 1 mM PMSF at wash buffer to eliminate any residual unbound protein. Bound protein was then eluted using elution buffer containing 50 mM Tris HCl (pH 8.0), 10 % glycerol, 400 mM imidazole, and 1 mM PMSF. Fractions containing soluble Isd11 were identified using SDS-PAGE. All buffers used were made fresh by mixing suitable stock solutions as needed and fresh Ni affinity resin was used each time.
4.2.1.2 Purification of Isd11 from insoluble fraction of the cell lysate

Expression and purification of Isd11-6xHis from insoluble fractions followed a deviated protocol from the one above\textsuperscript{225}. Briefly, same protocol as above was followed to start growth cultures. Cells were grown at 37 °C in LB broth until the cell density reached 0.7-0.8 OD\textsubscript{600}. Cells were then induced with 0.5 mM IPTG. Cells were allowed to grow at 37 °C for 3 hours before harvesting by centrifugation. When cells were grown under these conditions, the majority of overexpressed Isd11 was found in bacterial inclusion bodies due to lack of solubility. Cell pellets were resuspended in lysis buffer containing 50 mM Tris HCl (pH 8.0), and 1 mM PMSF. Cells were lysed by six rounds of sonication (20 s each round and 2 min intervals while samples are kept on ice). Lysate was spun at 12100 x g for 30 min at 4 °C and crude soluble fraction was removed. Pellet containing insoluble Isd11 was dissolved with a denaturing buffer containing 50 mM Tris HCl (pH 8.0), 8 M urea, and 1 mM PMSF, and the resulting suspension was spun at 65000 x g at 20 °C for 30 min. Supernatant was mixed with His-select Ni affinity resin (600 µl resin, Sigma P6611, pre-washed with water and denaturing buffer) for 3 hours at room temperature on a rocking platform to allow end-to-end mixing. After removing unbound protein, the resin was further washed with denaturing buffer to eliminate any residual unbound protein. Bound protein was then eluted using 50 mM Tris HCl (pH 8.0), 8 M urea, 400 mM imidazole, and 1 mM PMSF buffer. Fractions containing denatured Isd11, identified using SDS-PAGE were pooled and stored at room temperature until further use. All buffers used were made fresh by mixing suitable stock solutions as needed and fresh Ni affinity resin was used each time.
4.2.2 Western blot

In preparation for western blot analysis, samples containing purified Isd11 were subjected to SDS-PAGE. Samples were run on a 4-20 % gel (Bio-Rad Mini-PROTEAN-TGX) at 120 V to allow separation of proteins. Nitrocellulose membrane (0.2 µm) was cut to overlap with the dimensions of the gel and then pre-soaked in transfer buffer containing 25 mM Tris, 192 mM glycine, 20 % methanol, and 0.1 % SDS. This was done along with fiber pads and filter papers required to make the transfer sandwich. The SDS-PAGE gel was also pre-soaked in transfer buffer for 15 min. Transfer cassette was assembled so as to sandwich the gel and nitrocellulose membrane between filter paper and fiber pads on both sides without introducing air bubbles; this was done to allow smooth transfer of proteins to the membrane. Protein transfer was performed overnight at 22 V in the cold room (4 °C) under stirring conditions. Membrane was washed with TBST buffer (20 mM Tris, 150 mM NaCl, 0.1 % Tween-20 at pH 7.5) for 5-10 min on a rocking platform. Efficiency of protein transfer was checked by staining the membrane with Ponceau Red solution (0.2 % Ponceau S dye, 3 % trichloroacetic acid, and 3 % sulfosalicylic acid) for 5 min. Background stain was removed by thoroughly washing with water. Once the bands were visualized for successful transfer, Ponceau Red stain was completely removed by washing with water and TBST buffer. Membrane was blocked using blocking solution containing 5 % (w/v) skim milk powder in TBST buffer for 30 min. The membrane was then treated with primary antibody (anti-Isd11 rabbit antiserum in blocking solution, 1:500 dilution) for 3 hours on a rocking platform. Membrane was further washed three times (10 min each) with TBST buffer to remove unbound primary antibody before incubating with the secondary antibody solution (Bio-Rad anti-rabbit
HRP conjugated antibody, 1:10,000 dilution) for one hour at room temperature. Unbound secondary antibodies were removed by washing with TBST buffer three times, 10 min each. Isd11 was identified by incubating the membrane for 5 min with Bio-Rad Clarity Western ECL Substrate and detecting chemiluminescence with a digital imager. Urea solubilized Isd11 served as the positive control for the experiment.

4.2.3 Protein refolding

This section describes techniques I used in the attempt to refold urea denatured Isd11 isolated from bacterial inclusion bodies. All methods were unsuccessful towards yielding soluble monomeric Isd11. Typical results provided protein aggregates that precipitated out of solution. Only one method, using a liquid chromatography desalting column could recover soluble protein, but this method did not yield sufficient protein to perform any of the proposed experiments.

4.2.3.1 Sequential dialysis

Purified urea-denatured Isd11 was dialyzed into a series of buffers with decreasing urea concentrations and then finally into urea free buffer. Isolated protein was originally placed in 50 mM Tris HCl (pH 8.0), 8 M urea, 400 mM imidazole, and 1 mM PMSF buffer. Protein samples were placed in Slide-A-Lyzer dialysis cassettes (ThermoFisher Scientific) and dialyzed into 500 ml of buffer containing 50 mM Tris HCl (pH 8.0), 6 M urea, 100 mM NaCl, 10 % glycerol, and 1 mM PMSF for 3 hours at room temperature while stirring (buffer: protein ratio was maintained at 250:1). Subsequent buffers had the same composition except the urea concentration was gradually lowered to 4 M, 2 M, 1 M, and 0 M. Cassettes were checked visually for precipitation after each dialysis step and any precipitation was removed by spinning down at 14000 x g for 10
min before proceeding to the next buffer. Fresh cassettes were used in any incidents of visible precipitation. Protein concentrations at each step were measured using the Advance Assay to evaluate the amount of protein recovered.

4.2.3.2 Spin chromatography desalting column

In a second method, purified urea-denatured Isd11 in 50 mM Tris HCl (pH 8.0), 8 M urea, 400 mM imidazole, and 1 mM PMSF buffer was desalted to gradually remove urea. Isolated protein samples (75 µl) were placed in a Micro Bio-Spin 6 chromatography column (Bio-Rad), which consists of a special grade polyacrylamide gel designed to desalt proteins. Desalting was carried out by following the manufacturer instructions. Fresh buffer containing 50 mM Tris HCl (pH 8.0), 6 M urea, 100 mM NaCl, 10 % glycerol, and 1 mM PMSF buffer was applied to the column pre-equilibrated with the same buffer before spinning at 1030 x g for 4 min using a microcentrifuge. Flow through was collected and subsequent buffers were applied, which had 6 M, 4 M, 3 M, 2 M, 1 M, and 0 M urea concentrations. Fresh columns were used for each dialysis step. Protein concentrations at each step were measured using Advance Assay to evaluate the amount of protein recovered in each step.

4.2.3.3 Refolding by immobilizing on a nickel chelating column

Cells containing Isd11 were grown using the protocol at section 4.2.1.2. “Purification of Isd11 from insoluble fraction”. The same protocol was followed to separate the supernatant containing urea solubilized Isd11 by centrifugation at 65000 x g for 30 min at 20 °C. The supernatant was then diluted (1:3) with a buffer comprised of 100 mM Tris HCl (pH 8.0), 0.5 M NaCl, 10 % glycerol, 1 mM PMSF, and 0.5 M arginine for the final urea concentration in the supernatant to reach 2 M. His-select Ni affinity
resin (600 µl resin, Sigma P6611) was pre-washed with the above buffer before applying the supernatant to the resin. Isd11 was allowed to bind to the resin by end-to-end mixing on a rocking platform for 3 hours at 4 °C. The flow through was collected and the column was washed with a buffer containing 100 mM Tris HCl (pH 8.0), 0.5 M NaCl, 10 % glycerol, 1 mM PMSF, 0.2 M sucrose, and 0.5 M arginine. To remove non-specifically bound protein, the column was further washed with the same buffer although with 10 mM imidazole added. Isd11 bound to the column was eluted by using 0.4 M imidazole buffer with all the additional components outlined above.

4.2.3.4 Liquid chromatography desalting column

Purified urea-denatured Isd11 was passed through HiTrap Desalting column (GE Healthcare) attached (5 ml) to an AKTA FPLC system (GE Healthcare) as a means to allow refolding by removing denaturant. The column was pre-equilibrated with 50 mM Tris HCl (pH 8.0), 10 % glycerol, 250 mM NaCl, and 1 mM PMSF buffer before applying the denatured Isd11 (1.5 ml of sample made by diluting 1:1 with the same running buffer). The column was then run at 1 ml/min flow rate. Fractions containing refolded protein were identified from the chromatogram and SDS-PAGE gel, and they were spun down at 14000 x g for 5 min to remove any precipitate present. Pooled fractions were concentrated using 10 kDa Amicon centrifugal filter devices. Isd11 concentrations before and after passing through the column were measured using Advanced Assay® for protein detection to calculate the yield after desalting and protein was stored at -80 °C for further use.
4.2.4 Circular dichroism

Circular dichroism spectra were collected to check the integrity of Isd11 structure following refolding. CD data of a 40 µM Isd11 sample in 1 mM sodium phosphate buffer (pH 8.0) were collected at room temperature using the Applied Photophysics π* 180 CD instrument with a 0.2 mm quartz CD cell. Eight independent scans were collected over a wavelength range of 190-260 nm. Buffer baseline was subtracted from averaged spectra to obtain baseline correction and spectral simulations by CDNN software of Applied Photophysics suggested secondary structural elements for refolded Isd11.

4.3 Results

4.3.1 Bacterial expression and isolation of Isd11

4.3.1.1 Isd11 purified from soluble fraction of the cell lysate

Our purification protocol to isolate Isd11 from the soluble fraction of bacterial cell lysate yielded a small amount of monomeric Isd11 (yield of total purified protein was ~150 µg per 1 liter of bacterial cells), but also contained higher order oligomeric structures as well (Figure 4.1, lanes 9 and 10). This observation is in agreement with the results published recently saying Isd11 purified from soluble fraction is polydisperse and comprised of a heterogeneous mixture of oligomers\textsuperscript{232}. This protocol did not yield sufficient amounts of Isd11 (mg quantities) required to perform my suggested experiments. Even if the yield was substantial, due to the heterogeneous nature of purified Isd11, use of such protein in further experiments was questionable. Scaling up preparation was not an option, as it increased the amount of impurities in isolated protein samples (data not shown).
Figure 4.1: SDS-PAGE for purification of Isd11 from soluble fraction of cell lysate. Lane labels read: 1-molecular weight marker, 2-cells pre-induction, 3-cells post-induction, 4-cell lysate, 5-supernatant, 6-pellet, 7-flow through, 8-unbound wash, 9-elute 1, and 10-elute 2.
4.3.1.2 Isd11 purified from insoluble fraction of the cell lysate

Our purification protocol to isolate Isd11 from the insoluble fraction of bacterial cell lysate yielded ~100 % pure (yield of purified protein was ~5 mg per 1 liter of bacteria cells) monomeric Isd11 (Figure 4.2). This protocol could yield sufficient amounts of Isd11 (mg quantities) required to perform suggested experiments, but since Isd11 was present in its denatured form dissolved in 8 M urea, refolding techniques were attempted to refold Isd11 as described below.
Figure 4.2: SDS-PAGE for purification of lsd11 from *insoluble* fraction of cell lysate. Lane labels read: 1-molecular weight marker, 2-cells post-induction, 3-cell lysate, 4-supernatant, 5-inclusion bodies pellet, 6-solubilized inclusion bodies, 7-flow through, 8-unbound wash, 9-elute 1, 10-elute 2, and 11-elute 3.
4.3.2 Western blot

In order to confirm the oligomers present in the Isd11 preparation from soluble fraction of cell lysate are indeed oligomers of Isd11, a western blot was performed against Isd11. The higher order oligomers and monomers of Isd11 transferred to the membrane, expressed chemiluminescence signals upon detection (Figure 4.3), indicating that Isd11 isolated from soluble fraction most certainly forms oligomeric structures. This observation further confirms the results above from SDS-PAGE analysis, which immediately followed purification and results published recently saying Isd11 purified from soluble fraction comprised of a heterogeneous mixture of oligomers\textsuperscript{232}.

4.3.3 Protein refolding

4.3.3.1 Sequential dialysis

In the attempt to refold urea denatured Isd11 isolated from bacterial inclusion bodies using the sequential dialysis method, Isd11 continuously aggregated and fell out of the solution as the urea concentration was gradually lowered. No precipitation was observed when transferred to 6 M urea, but there onwards protein levels gradually decreased, as measured with Advanced Assay\textsuperscript{®} reagent (Table 4.1). The minimum concentration of urea required to detect a significant amount of Isd11 remaining in solution was 2 M and no protein could be detected thereafter.
Figure 4.3: Western blot for Isd11 purified from soluble fraction. Lane labels read: 1-urea denatured Isd11 (positive control), 2 and 3-Isd11 from soluble fraction.
Table 4.1: Protein concentrations measured in each dialysis buffer in the sequential dialysis method to refold urea denatured Isd11.

<table>
<thead>
<tr>
<th>Urea concentration in dialysis buffer</th>
<th>Isd11 concentration remaining after each step</th>
<th>Precipitation observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 M</td>
<td>309 µM</td>
<td>-</td>
</tr>
<tr>
<td>6 M</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>4 M</td>
<td>134 µM</td>
<td>Yes</td>
</tr>
<tr>
<td>2 M</td>
<td>56 µM</td>
<td>Yes</td>
</tr>
<tr>
<td>1 M</td>
<td>Not detected</td>
<td>No</td>
</tr>
<tr>
<td>0 M</td>
<td>Not detected</td>
<td>No</td>
</tr>
</tbody>
</table>
4.3.3.2 Spin chromatography desalting column

With the use of Micro Bio-Spin 6 chromatography columns, Isd11 levels continuously decreased as measured with Advanced Assay® reagent (Table 4.2) when the protein was transferred to lower urea concentrations. The minimum concentration of urea required to detect a significant amount of Isd11 remaining in solution was 3 M and no protein could be detected thereafter.

4.3.3.3 Refolding by immobilizing on a nickel chelating column

In the attempt to refold urea denatured Isd11 while being immobilized to the nickel affinity column, Isd11 eluted as a suspension with white precipitate from the column. The suspension was spun down to remove any precipitated protein and the amount of protein remaining in the supernatant was measured using Advanced Assay® reagent. No remaining protein was detected.

4.3.3.4 Liquid chromatography desalting column

We used a HiTrap Desalting column to elute Isd11 out with the refolding buffer, and monitored the buffer exchange process using conductivity measurements (Figure 4.4). An SDS-PAGE gel of the peak fractions revealed the presence of ~97% pure monomeric Isd11 (Figure 4.5). Most fractions contained visible white precipitate, indicating some level of oligomerization, but once the precipitate was removed only the monomeric species remained in the soluble fraction. Isd11 concentrations measured before and after passing through the column showed that ~90 % of protein present in urea media is lost due to aggregation during refolding (Table 4.3).
Table 4.2: Protein concentrations measured in each dialysis buffer in the spin chromatography desalting method to refold urea denatured Isd11.

<table>
<thead>
<tr>
<th>Urea concentration in dialysis buffer</th>
<th>Isd11 concentration remaining after each step</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 M</td>
<td>309 µM</td>
</tr>
<tr>
<td>6 M</td>
<td>314 µM</td>
</tr>
<tr>
<td>4 M</td>
<td>310 µM</td>
</tr>
<tr>
<td>3 M</td>
<td>186 µM</td>
</tr>
<tr>
<td>2 M</td>
<td>Not detected</td>
</tr>
<tr>
<td>1 M</td>
<td>Not detected</td>
</tr>
<tr>
<td>0 M</td>
<td>Not detected</td>
</tr>
</tbody>
</table>
Figure 4.4: Desalting chromatogram for urea denatured Lsd11. UV intensity and conductivity are depicted as a solid line and a dashed line respectively. Refolded Lsd11 elutes out with the new buffer in the void volume of the column. Buffer containing urea elutes later.
Figure 4.5: SDS-PAGE of Isd11 containing fractions from desalting column. Lanes 1 to 6-fractions containing Isd11, 7-molecular weight marker.
Table 4.3: Isd11 concentrations measured before and after passing through HiTrap desalting column.

<table>
<thead>
<tr>
<th>Attempt</th>
<th>Concentration before column</th>
<th>Concentration after column</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>222 µM</td>
<td>22 µM</td>
<td>10.0</td>
</tr>
<tr>
<td>2</td>
<td>208 µM</td>
<td>21 µM</td>
<td>10.1</td>
</tr>
</tbody>
</table>
4.3.4 Circular dichroism (CD) of refolded Isd11

CD spectra of refolded Isd11 suggested the secondary structure was primarily helical (Figure 4.6). Spectral simulations for eight individual scans estimated the percentage of each secondary structural element in Isd11 as depicted in Table 4.4. Having a greater percentage of ordered structural elements, as opposed to random coils, suggested that Isd11 may have refolded to its native structure. But, due to heterogeneity of Isd11 purified from the soluble fraction, a direct comparison of refolded Isd11 versus soluble Isd11 was not feasible. To make a comparison, no CD structure analysis results have been published previously for the yeast Isd11 either.
Figure 4.6: Circular dichroism spectra of refolded Isd11. CD data of a 40 μM Isd11 sample in 1 mM sodium phosphate buffer (pH 8.0) were collected at room temperature. This spectrum represents the average of eight spectra.
Table 4.4: Secondary structure elements of refolded Isd11 determined by far-UV CD.

<table>
<thead>
<tr>
<th>Secondary structural elements</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helices</td>
<td>26.20 (± 0.12)</td>
</tr>
<tr>
<td>Antiparallel β-sheets</td>
<td>17.90 (±0.50)</td>
</tr>
<tr>
<td>Parallel β-sheets</td>
<td>8.52 (±0.13)</td>
</tr>
<tr>
<td>β-turns</td>
<td>17.90 (± 0.08)</td>
</tr>
<tr>
<td>Random coils</td>
<td>29.51 (±0.59)</td>
</tr>
</tbody>
</table>
4.4 Discussion

Since its discovery, Isd11 has been suggested to function within the eukaryotic mitochondrial Fe-S cluster biogenesis pathway by primarily associating with the cysteine desulfurase Nfs1 and promoting Nfs1 activation towards persulfide formation. Functional association between Nfs1 and Isd11 within a complex has been reported, but no attempts were made to elucidate the characteristics of the interaction. Such information can help explain how Isd11 exerts its modulating effect on Nfs1 stability/activity. Moreover it would also provide insight into the need to have Isd11 exclusively in eukaryotic species, as it is required to aid cysteine desulfurase activity in eukaryotes but prokaryotic cysteine is perfectly self-sufficient. In such context, it was proposed to isolate the two partner proteins (Nfs1 and Isd11) separately and characterize the interaction between them by NMR and ITC. This chapter described the efforts made to isolate Isd11 individually. Despite the number of methods employed, sufficient amounts of protein could not be yielded to perform experiments due to inherent insolubility and/or instability of Isd11 in the absence of Nfs1. Hence the project is currently stalled until a better method of isolating pure homogeneous Isd11 can be determined.

The observed oligomeric nature of purified Isd11 lead to the conclusion that Isd11 could be present as a heterogeneous mixture of oligomers under non-denaturing conditions. Higher order oligomers were present when Isd11 was isolated from the soluble fraction of the cell lysate, in close agreement with the polydisperse nature of Isd11 under reported recently non-denaturing conditions. Oligomerization is inevitable, even when denatured Isd11 is refolded by transferring to non-denaturing
conditions, which further confirms the tendency of Isd11 to cluster when forced to exist in the absence of its binding partner, Nfs1. This nature could very well be attributed to the “complex-orphan protein” like behavior of Isd11 that was proposed recently by another group\textsuperscript{232}. Complex-orphan proteins are difficult to produce in the absence of its native binding partner and/or cofactor, as they tend to aggregate although they may not necessarily be unstable/defective in a thermodynamic standpoint\textsuperscript{286}. Therefore it is possible that the aggregated state of Isd11 may also be functionally significant, although it may not represent its true physiological oligomeric state. This nature is also seen for other proteins involved in Fe-S cluster biogenesis such as IscU\textsuperscript{286} and Isu1 (by experience from ongoing projects our lab). As mentioned in Chapter 2 and reported in previous publications\textsuperscript{113,214,233,234}, the fact that Isd11 prevents the aggregation of Nfs1 by binding to its oligomerization surface can be postulated in reverse in this context: Nfs1 prevents aggregation of Isd11 as well. Therefore, the Nfs1-Isd11 interaction could primarily be functional in activating cysteine desulfurase activity as a complex, and secondarily as a mutual relationship in maintaining protein stability for efficient catalysis.

The fact that Isd11 oligomers could be observed in the denaturing SDS-PAGE is surprising, knowing that SDS denatures proteins and disrupts their interactions. But given the nature of intermolecular interactions, SDS may not necessarily denature all secondary structures and completely disrupt all interactions, especially the stronger ones. The fact that Nfs1-Isd11 is soluble and stable as a complex but the two proteins aggregate in the absence of each other suggests the interaction interface between these two proteins could predominantly be hydrophobic. Covering up hydrophobic regions of both proteins by complexation could very well stabilize them as a single
functional unit. If such notion is correct, oligomerization of Isd11 in the absence of Nfs1 could be an attempt to hide exposed hydrophobic patches in an enclosed environment. Based on the affinity of the interactions between units in the oligomer, it is possible that SDS is not capable of disrupting them completely; hence oligomers can be visualized even in denaturing SDS-PAGE gels. Western blots performed against Isd11 further confirmed these oligomers as comprising of Isd11.

Various methods were employed in the attempt to refold denatured Isd11 isolated from bacterial inclusion bodies. Sequential dialysis allowed the gradual decrease of urea in the protein’s environment, which is essential to drive correct refolding of Isd11 by populating folding intermediates that will most likely aggregate if a direct dilution is performed. Moreover, refolding using any desalting chromatography column with a porous resin provides a favorable pace at which Isd11 could interact with the renaturant, that allows slow folding kinetics and physical separation of refolding intermediates preventing aggregation. Similarly, nickel-chelating columns provide a matrix for unfolded protein to attach during refolding and thereby prevent unwanted intermolecular interactions leading to aggregation. Chemical additives such as L-arginine and sucrose are also commonly used as refolding aiding agents. In sequential dialysis lesser protein was detected even at 4 M urea compared to spin desalting column, which gave higher protein at 3 M urea. Therefore it is apparent that Isd11 prefers the assistance of a porous environment in refolding. But even with the use of a spin desalting column, no Isd11 was remaining in the final non-denaturing buffer probably because applying centrifugal forces to elute Isd11 must not have provided sufficient time for refolding. Immobilization on nickel chelating column yielded no
refolded protein, probably due to incompatibility of Isd11 and chemical additives used. Clearly the best method to refold Isd11 was the use of liquid chromatography desalting column because it allowed recovery of at least 10 % protein in the non-denaturing buffer while other methods failed to recover any. The porous resin environment and controlled flow rate must have provided better conditions to refold Isd11. Overall, none of the techniques were able to recover Isd11 from the denaturing conditions, and therefore failed to provide sufficient amounts to perform experiments.

Structural details of Isd11 are scarce, and a potentially easy technique to obtain a crude estimation of its secondary structure constituents is using circular dichroism. It is also a perfect measurement to observe if Isd11 isolated from insoluble fraction has refolded to its native structure upon removal of urea by comparison with native Isd11 isolated from the soluble fraction. Unfortunately, due to the oligomeric nature of Isd11 in the soluble fraction (as described in section 4.3.1.1.), a direct comparison could not be performed. Having a greater percentage of ordered structural elements, as opposed to random coils, suggested that Isd11 may have refolded to its native structure. But, as mentioned above, the certainty of this fact could not be verified. Although this result agrees with the secondary structure prediction studies and ab-initio protein modeling attempts that suggest yeast Isd11 structure as primarily helical, it is deviated farther from the results obtained for human ISD11 in a recent far-UV circular dichroism (CD) study. That study confirmed a higher α-helical content for ISD11, 57% and 10% β-sheets. Human ISD11 and yeast Isd11 shares ~60 % sequence similarity. This may suggest that the ratios of structural elements we see for refolded yeast Isd11 could be
superficial due to improper folding and yeast Isd11 could be more helical than we observed.

In conclusion, all results point towards the notion that complex formation between Nfs1 and Isd11 is mutual necessity to maintain the structure of both partners as much as it is important functionally. Individual proteins are unstable and the efforts to study them separately may not provide physiologically relevant information as much as for the complex. Therefore the opportunity to understand the reason behind the requirement for the presence of Isd11 for cysteine desulfurase activity in eukaryotes relies on the attempts to obtain the crystal structure of this complex. Significant effort has been made towards that goal in our lab.

4.5 Acknowledgements

This research was supported by T32 HL120822 (DPB) and NIH RO1-DK068139 and NIH RO1-GM107542 (TLS). Special acknowledgement goes towards Dr. Debkumar Pain and Dr. Alok Pandey (Rutgers New Jersey Medical School) for training and guidance provided in protein isolation procedures and western blot and Dr. Andrew Dancis (University of Pennsylvania) for providing Isd11-Hisx6 plasmid and bacterial cell lines.
5.0 Prelude

A number of human disorders show direct correlations to disrupted iron homeostasis within the body. Among them are the most common neurodegenerative disorders (Alzheimer’s, Parkinson’s, Friedreich’s Ataxia and Huntington’s), all of which are complicated due to iron induced oxidative damage of nerve cells that is caused by iron accumulation in the central nervous system. Pertaining to the growing occurrence and devastating burden associated with these diseases, the emphasis towards understanding the pathophysiology related to the role of iron homeostasis within these disorders becomes more and more important every day. As the second most prominent class of iron cofactor within the body, Fe-S clusters play an essential role in helping the body maintain proper iron homeostasis. In the case of Friedreich’s ataxia, caused by a deficiency in the protein frataxin that is indispensable during Fe-S cluster assembly, the impact of iron homeostasis related to the disorder is directly obvious. Friedreich’s ataxia and other disorders associated with defective Fe-S cluster assembly accentuate the need to unravel the structural and functional aspects of this fundamental biochemical pathway. Effective disease treatment has been hampered by the lack of a molecular level understanding for the individual roles key proteins play in Fe-complex formation. The work described in this dissertation attempts to diminish this knowledge gap by providing a comprehensive biophysical characterization of the key proteins involved in sulfur mobilization during the yeast mitochondrial Fe-S cluster synthesis process. The role of the accessory protein “Isd11” within the catalytic activity of the cysteine desulfurase “Nfs1” has been assessed in a detailed manner for the first time as a part of
this work. Our results will lay the groundwork for understanding a possible regulatory function of Isd11 and possibly help identify the evolutionary requirement for its activating function on Nfs1 in eukaryotes only. The effect of Yfh1 on sulfur mobilization by the Nfs1-Isd11 complex has also been evaluated in detail. Our results suggest that frataxin impacts cluster assembly mostly at the level of the assembled complex rather than at the individual protein level. This information adds an extra level of understanding for the regulatory role of Yfh1 within the yeast mitochondrial Fe-S cluster assembly pathway. This work, therefore, provides novel and significant insight into how the cluster assembly proteins function, and sets the groundwork for which additional experiments that need to be designed to further unravel the mechanistic details of sulfur mobilization during Fe-S cluster bioassembly. These molecular level details will assist in future drug design strategies directed at treating the disease states outlined above. In addition, this chapter concludes the dissertation by summarizing the final outcomes of the previous three chapters and also by providing potential routes the project can take. Some preliminary work performed in relation to our future objectives is included in this chapter.

5.1 Summarization of dissertation and conclusions

5.1.1 Molecular characterization of yeast Nfs1-Isd11 complex

Although several studies have provided details regarding how the Nfs1-Isd11 complex functions from a biochemical point of view, many of the biophysical details of how the complex functions are lacking. Chapter 2 provided findings regarding a comprehensive in vitro biophysical characterization of the yeast Nfs1-Isd11 protein pair. Successful purification of these proteins was followed by experiments that were performed to unveil basic properties of the complex. Co-expressing Nfs1 with Isd11 led
to formation of a stable complex and prevented Nfs1 aggregation, indicating Isd11 coordination to Nfs1 contributed to the stability and solubility of the enzyme. Additional studies confirmed the precise molecular weight and stoichiometry of the proteins within the complex, and also provided the exact oligomeric arrangement of the proteins in our overexpressed complex. Subsequent experiments attempted to rationalize the requirement for Isd11 within yeast Nfs1 Fe-S cluster biosynthesis. Biophysical studies revealed that Isd11 imparts a high thermodynamic stability on Nfs1, most likely by altering the structure of Nfs1 in a manner that is not yet clear. Our measurements testing the substrate binding affinity of Nfs1 suggested Isd11 likely does not exert a direct effect on altering Nfs1’s substrate binding affinity. Subsequent activity measurements however proved that Isd11 alters the catalytic activity of eukaryotic Nfs1. Kinetic parameters, such as $K_M$ and catalytic efficiency, were significantly altered during the emergence of Isd11 in eukaryotes in the evolutionary lineage compared to prokaryotes. Homology modeling was used to generate a structure framework for us to predict yeast Nfs1 biophysical properties. The modeled structure suggested that conformational changes would be required for substrate binding and persulfide formation; these results were supported by our Fluorescence experiments. Taken together, the second chapter of this dissertation supports the idea that cooperativity between Nfs1 and Isd11 is essential to bring about the full functional outcome of cysteine desulfurase complex in yeast. Isd11 alters the catalytic activity of Nfs1 in addition to functioning as a stabilizer for the protein. Having this multifaceted level of control within eukaryotes may be a safeguard established by evolution to eliminate unwanted product formation within higher developed organisms.
5.1.2 The transient effect of frataxin on Nfs1-Isd11 activity

Recently, frataxin was identified as a regulator of the cysteine desulfurase within the ISC Fe-S cluster biosynthetic pathway. This function however has not been completely clarified at a molecular level in any eukaryotic system. Experiments in Chapter 3 were designed to test the hypothesis that Yfh1 interacts directly with the Nfs1-Isd11 complex to regulate the complex's activity. In addition, this chapter tested the effect of Yfh1 on the structure and activity of the complex. Isothermal titration calorimetry was used to explore for a stable physical interaction between proteins, however our work was limited in its outcome due to protein aggregation issues. Pull-down assays were used as an alternative method to explore inter-protein interactions, and our results suggested a transient but not stable interaction between Yfh1 and the complex. Additional biophysical experiments tested the ability of Yfh1 to alter the thermal stability, substrate binding affinity and catalytic activity of Nfs1-Isd11 in order to determine if we could detect any evidence of this transient interaction, however no impact of Yfh1 was identified. Therefore, it is plausible that an interaction between Yfh1 and Nfs1-Isd11 does not exist, or that this interaction is not stable enough to be measured under the conditions we explored. In alignment with similar results observed with human orthologs, it is reasonable to assume that eukaryotic frataxin interacts with the cysteine desulfurase only when it is part of the complete cluster assembly complex that also include Isu1 along with Nfs1 and Isd11.

5.1.3 Challenges in expression and purification of Isd11

In an effort to study the functional association between molecules within the Nfs1-Isd11 complex, it was proposed that we isolate the two proteins individually.
Chapter 4 described the attempts we made towards this directive, and how we rationalize our inability to isolate Isd11 separately. All failed attempts pointed towards the notion that complex formation between Nfs1 and Isd11 is a mutual necessity in order to maintain the structure and functionality of both partners. Our differential scanning experiments described in Chapter 2 supported this idea.

5.2 Future directions

Although the Fe-S cluster biogenesis pathway has been under investigation for several decades, many questions regarding how the molecules within the pathway operate together remain unanswered. While genetics and cellular studies on the yeast cluster assembly system are well understood, little is known about the molecular aspects of how the proteins work together during Fe-S cofactor assembly. Given its reduced complexity, the yeast model system serves as a bridge between the prokaryotic and eukaryotic cluster assembly systems. The yeast system therefore provides a unique opportunity to explore the fine details of how proteins within the Fe-S cluster assembly pathway interact. Sulfur mobilization, which is used during de novo Fe-S cluster assembly, contains specific gaps in our knowledge of how the proteins work together to operate, and as a result there are several questions that need to be addressed in this area. I performed several preliminary new experiments in this context, and the results I have obtained during these exploratory but yet to be completed experiments are provided below.

5.2.1 Elucidation of x-ray crystal structure of Nfs1-Isd11

Not having exact structural details for the structure of Nfs1-Isd11 has hindered our progress in understanding how these proteins function. Some of the specific
questions that still remain unanswered include: 1) What are the molecular details regarding the interaction between Nfs1 and Isd11?, 2) What is the location and proximity of the PLP-bound active site relative to the catalytic cysteine residue?, and 3) What are the structural rearrangements that drive chemistry related to the substrate bound cysteine?. In order to address this knowledge gap, we have attempted to crystallize the yeast Nfs1-Isd11 complex. In-house crystal screens were set for purified Nfs1-Isd11 with Qiagen NeXtal Tubes JCSG Core Suites I-IV using the sitting drop method. These crystal trays were set at temperatures of 4 and 25 °C. Screens are currently being monitored for visible crystals and once suitable hits are obtained, more elaborate screens will be developed to find the best crystallization condition(s) to obtain suitable diffracting crystals. Crystal diffraction and structure elucidation procedures are being done in collaboration with Dr. Amy Rosenzweig’s laboratory at Northwestern University.

5.2.2 Role of Acyl Carrier Protein in cysteine desulfuration and Fe-S cluster assembly

Most recently, the Acyl Carrier Protein (ACP) involved in mitochondrial fatty acid synthesis was identified to be an essential component of the yeast Fe-S cluster assembly complex\(^{210}\). This study reported the role of ACP in Fe-S cluster biogenesis as functioning to stabilize the Nfs1-Isd11 complex, because Nfs1 and Isd11 were found as insoluble aggregates in yeast ACP knockdown strains. This work is supported by the fact that Nfs1 and Isd11, expressed and isolated from bacteria, require ACP. As we observed, purified yeast Nfs1-Isd11 always carries a single impurity of ~18 kDa that we could not separate from our constituent proteins (Chapter 2, Figure 2.1). We actually
identified this protein by mass spectrometry several years ago as potentially being the *E. coli* Acyl Carrier Protein (data not shown). In the absence of the yeast ACP ortholog, recombinant Nfs1 and Isd11 do associate with the *E. coli* ACP during our preps, and this indicates the significance of the protein in in helping form the Fe-S cluster assembly complex. Our next step in trying to elucidate the function of ACP will be to assess its effect on the activity of our *in vitro* cysteine desulfurase-Isd11 complex. Interestingly, activity measurements on purified yeast Nfs1-Isd11 performed to date have already been completed in the presence of bacterial ACP (although we have not quantified the stoichiometry), as a result of the protein’s tendency to co-purify with Nfs1-Isd11 at high quantities. If the bacterial ACP is associated with the yeast Nfs1-Isd11 in the correct stoichiometry, or if it fully complements the capabilities of the yeast ACP ortholog are questions that still need to be addressed. To test if increasing the expression of *E. coli* ACP will have any stimulatory effect on yeast cysteine desulfurase activity, auto-induction methods were tested during bacterial cell growth in an attempt to coordinate yeast protein expression with the enhanced expression of bacterial ACP. Unfortunately there was no significant difference in bacterial ACP levels observed by using auto-induction as opposed to growth under IPTG inducible conditions. Therefore, we will try co-expressing yeast ACP with yeast Nfs1-Isd11 directly in *E. coli* using a co-transformed plasmid vector. In this manner, we will try to coordinate yeast ACP expression with the expression of the complex. Additional strategies, such as a ΔACP *E. coli* expression strain, will also be utilized to eliminate the expression of *E. coli* ACP. This should allow us to obtain an ACP free complex that we can use for side-by-side comparison of protein activity.
5.2.3 Elucidation of cysteine desulfurase mechanism of Nfs1-Isd11

At present, the molecular details of the cysteine desulfurase reaction mechanism have been identified in detail in only a small number of systems; the best of which is in the bacterial systems.\textsuperscript{219,237,238,298} Reaction mechanisms followed by different cysteine desulfurase orthologs during their catalytic activity vary only slightly. The resting state of all the PLP containing cysteine desulfurases consist of a functional internal aldimine formed between PLP and an active site lysine residue. The incoming cysteine substrate forms an external Cys aldimine with the PLP cofactor at the active site, which in turn gets deprotonated to form a Cys quinonoid intermediate. The subsequent protonation at the $\alpha$-C4 atomic position converts this intermediate to a Cys ketimine adduct, which then undergoes nucleophilic attack by the deprotonated catalytic cysteine residue. The end result is an enzyme bound persulfide and an Ala enamine. Thereafter, the alanine gets released and the persulfide gets transferred to a sulfur acceptor\textsuperscript{216} (Figure 5.1).

However, eukaryotic cysteine desulfuration requires the accessory protein Isd11 for activity, and therefore Isd11 plays a direct or an indirect role in this enzymatic reaction mechanism. Having a complete characterization of the eukaryotic cysteine desulfurase reaction mechanism will allow for a direct comparison between the prokaryotic and eukaryotic sulfur mobilization mechanisms, something that may be possible since the bacterial reaction mechanism has already been solved. Obtaining this information will therefore provide direct answers regarding why Nfs1 has evolved to catalyze the persulfide product with an aid of a second protein Isd11, in contrast to the bacterial protein, which did not.
In order to begin to elucidate the mechanism of Nfs1-Isd11, we have expressed and purified two mutant forms of Nfs1 and we will use these to monitor the fate of three separate intermediates formed during the reaction mechanism. As mentioned in Chapter 2, monitoring the characteristic 420 nm UV-vis signal from wild type Nfs1-Isd11 revealed that it is a compound signal representing both internal aldimine (between K299 and PLP) and persulfide formation (on C421). As the reaction progresses, the consumption of the aldimine species upon substrate binding causes a diminution in the 420 nm signal, while persulfide formation causes an increase in the signal (Figure 5.2-A). Our Nfs1(C421A)-Isd11 mutant, in which the catalytic cysteine residue essential for persulfide formation is mutated, was incapable of performing the nucleophilic attack leading to persulfide formation. Therefore, the reaction stalled at the Cys ketimine stage (Figure 5.2-B). The decrease in the 420 nm signal observed for this mutant only represents the consumption of internal aldimine leading to Cys ketimine formation, eliminating the signal for the production of a persulfide. In contrast, my Nfs1(K299A)-Isd11 mutant was not expected to make a progression upon substrate addition, as this mutant was incapable of binding PLP in the active site; this Lys-299 residue forms the internal aldimine with PLP at the resting state of the enzyme. However our studies showed that the Nfs1(K299A)-Isd11 complex was still capable of accommodating PLP in the active site, this interaction is likely facilitated by interactions with amino acids in the active site vicinity. Interestingly as a result of the K299A mutation, the signal around 420 nm from the internal aldimine was only slightly viable (only a low intensity peak at 405 nm was present, Figure 5.2-C). Upon substrate addition, this signal steadily increased, indicating persulfide formation, which was masked before by the internal
aldimine signal (Figure 5.3-A, and B). In addition, the increasing L-cysteine concentration caused a red shift in the data that was observed from 405 nm to 424 nm. A previous report attributes the red shift to an increase in the pKₐ of an acidic group on PLP due to persulfide formation²³⁸, supporting the identity of the red shift we observed for the Nfs1(K299A)-Isd11 complex. Therefore, the Nfs1(K299A)-Isd11 complex can be used to observe the fate of the persulfide intermediate in the second half of the reaction. On the other hand, in the wild type and mutant complexes, the signal at 340 nm might represent a single intermediate, which is likely the Cys ketimine (Figure 5.2-A/B, and Figure 5.3-B). Having the capability to monitor three separate reaction intermediates during the reaction process will allow for the explicit design of stopped flow spectrophotometric experiments that could be performed using isotope labeled substrate (deuterated L-cysteine and H₂O) to help elucidate the complete reaction mechanism.
Figure 5.1: Proposed chemical mechanism for the cysteine desulfurase reaction [by Behshad (2009) et al. for bacterial cysteine desulfurases\textsuperscript{237}].
Figure 5.2: Absorbance measurements for three variants of yeast Nfs1-Isd11. Absorbance change measured over time at 340 nm (white circles) and 420 nm (black circles) for (A) Nfs1-Isd11 wild type and (B) Nfs1(C421A)-Isd11 protein complexes upon addition of 20 molar excess L-cysteine. (C) Absorption profiles of Nfs1(C421A)-Isd11 (dashed line) and Nfs1(K299A)-Isd11 (solid line) protein complexes for comparison.
Figure 5.3: Titration of L-cysteine to Nfs1(K299A)-Isd11 monitored by UV-visible spectroscopy. (A) Consecutive spectra were collected after each addition of L-cysteine. Lines represent the following concentrations: bold solid: 0 mM, bold dashed: 0.010 mM, bold dotted: 0.035 mM, bold dash-dot: 0.084 mM, bold dash-dash-dot-dot: 0.183 mM, thin solid: 0.430 mM, thin dashed: 0.919 mM, thin dotted: 1.901 mM, thin dash-dot: 4.346 mM, thin dash-dash-dot-dot: 9.200 mM. (B) Plots show the change in absorbance for Nfs1(K299A)-Isd11 at 340 nm (white circles) and 420 nm (black circles) as a function of L-cysteine concentration.
5.2.4 Effect of frataxin on the cysteine desulfurase activity of Nfs1/Isd11/Isu1/Yfh1 complex

As discussed in Chapter 3, Yfh1 showed no direct interaction with or effect on the stability, substrate binding affinity, and activity of the Nfs1-Isd11 complex alone. These results suggest the effect of frataxin on this complex is transient and/or contingent upon other factors. However, the effect of Yfh1 on the Fe-S cluster assembly activity in the Nfs1/Isd11/Isu1/Yfh1 complex has been established*. To test the hypothesis that Yfh1’s ability to alter cluster assembly within the Nfs1/Isd11/Isu1/Yfh1 complex occurs by altering the cysteine desulfurase function, I completed some initial preliminary experiments. Yfh1’s ability to alter substrate binding affinity of Nfs1-Isd11 within a reconstituted Nfs1/Isd11/Isu1/Yfh1 complex was tested first. The Nfs1/Isd11/Isu1/Yfh1 quad complex was reconstituted by combining individually purified Nfs1-Isd11 (mutant or wild type), Yfh1 and Isu1 proteins together at a 1:2:1:1 stoichiometric ratio. This ratio was shown by others and us to match that identified in vivo\textsuperscript{213,229,235}. Using the protocol outlined in Chapters 2 and 3, UV-visible spectroscopy was used to study the substrate binding affinity of reconstituted Nfs1(C421A)/Isd11/Isu1/Yfh1 complex. The absorbance at 340 nm was monitored upon consecutive titration of L-cysteine to this quad complex. The identical experiment was performed using the as purified Nfs1(C421A)/Isd11 complex alone as a control for comparison for possible alteration in substrate binding affinity within the quad complex. Titration of L-cysteine to reconstituted Nfs1(C421A)/Isd11/Isu1/Yfh1 complex or the as purified Nfs1(C421A)/Isd11 yielded the binding curves that are not distinctly different from each other (Figure 5.4). Best-fit

\textsuperscript{*} Stephen Dzul’s (2016) PhD dissertation “Insights into de novo Fe-S cluster biogenesis via the eukaryotic Fe-S cluster pathway (ISC) in vitro”.
simulations for binding data revealed $K_d$ values of $0.58 \pm 0.13$ mM and $0.55 \pm 0.14$ mM for Nfs1(C421A)/Iisd11/Isu1/Yfh1 and Nfs1(C421A)/Iisd11, respectively. However from these results, no direct conclusion could be made regarding the effect Yfh1 had on substrate binding affinity of Nfs1(C421A)/Iisd11 within the reconstituted Nfs1(C421A)/Iisd11/Isu1/Yfh1 complex, due to two reasons: 1) During reconstitution, as soon as purified Isu1 was added to the Nfs1(C421A)/Iisd11/Yfh1 reaction mixture, protein precipitation was visible and this continued through the entire experiment. No precipitation was observed when the experiment was carried out with just the Nfs1(C421A)/Iisd11/Yfh1 complex (as outlined in Chapter 3), indicating Isu1 is likely causative of aggregation. Wild type yeast Isu1 alone is unstable and prone to aggregation, even at the slightest perturbation of conditions, during and/or after purification (data not shown). Therefore, the similarity of the binding affinity data of Nfs1(C421A)/Iisd11 and Nfs1(C421A)/Iisd11/Isu1/Yfh1 may have caused by the poor solubility of Isu1 during reconstitution; 2) The absorbance values recorded for the reconstituted quad complex were higher than those only for Nfs1(C421A)/Iisd11 complex by a same amount at each point, even after correcting for dilution (Figure 5.4). This too is an indication of the effect of poor solubility of Isu1 on the reading following reconstitution since neither Yfh1 nor Isu1 alone absorb at 340 nm or 420 nm. Therefore this experiment needs to be repeated using an alternative strategy to avoid Isu1 precipitation during the reaction, as reconstitution of separately purified proteins is not the best approach. Nfs1/Iisd11/Isu1/Yfh1 quad complex has been co-expressed and co-purified in our lab for several years with high purity and stability. Therefore, it would be
beneficial to repeat this experiment with a co-expressed and co-purified Nfs1(C421A)/Isd11/Isu1/Yfh1 quad complex.

Similarly, acid labile sulfide production activity within the reconstituted Nfs1/Isd11/Isu1/Yfh1 was tested, as described above. However, no significant enhancement in the persulfide formation activity within the Nfs1-Isd11/Isu1/Yfh1 complex was observed compared to the Nfs1/Isd11 complex (Figure 5.5). As mentioned above, validity of this result is questionable due to aggregation of Isu1 during the assay, and therefore this experiment should be repeated with the co-purified Nfs1/Isd11/Isu1/Yfh1 quad complex, which exists as a more stable.

To summarize, experiments proposed to help elucidate the structure and the reaction mechanism of yeast Nfs1-Isd11 complex will greatly contribute towards closing the knowledge gap regarding the sulfur mobilization step within de novo Fe-S cluster assembly. The experiments that we proposed in order to study the effect of possible regulators of the sulfur mobilization process (such as, ACP and Yfh1) will provide comprehensive information on how cluster assembly if achieved on a global scale within the regulated environment of the mitochondria. Taken together, these experiments will produce a wealth of information that would contribute towards a better understanding of the molecular details of the Fe-S cluster assembly process. This in turn will aid in accentuating the drug design process for those diseases associated with cluster assembly.
Figure 5.4: Absorbance change measured at 340 nm for Nfs1(C421A)/Isd11 (white circles), and Nfs1(C421A)/Isd11/Yfh1/Isu1 reconstituted complex (black circles) upon addition of substrate L-cysteine. Substrate was incubated for 10 min after each addition and spectra were measured at 25 °C.
Figure 5.5: Persulfide forming activity of Nfs1-lsd11 and reconstituted Nfs1/lsd11/Yfh1/lsu1 complex measured by acid labile sulfide detection assay.
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ABSTRACT

CHARACTERIZATION OF THE YEAST CYSTEINE DESULFURASE COMPLEX WITHIN THE MITOCHONDRIAL FE-S CLUSTER BIOGENESIS

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Disrupted iron homeostasis within the human body materializes as various disorders. Pathophysiology of many of them relates to iron induced oxidative damage to key cellular components caused by iron accumulation within the tissues. Pertaining to the growing occurrence, cost of patient care and devastating burden associated with these diseases, the call for understanding the role of iron homeostasis within these disorders becomes inevitable. Being an abundant iron containing cofactor, the role of Fe-S clusters in cellular iron homeostasis is indisputable in the case of Friedreich’s ataxia, a disease caused by a deficiency in the protein frataxin that is indispensable during Fe-S cluster assembly. Friedreich’s ataxia and similar disorders associated with defective Fe-S cluster assembly accentuate the need to unravel the structural and functional aspects of this fundamental biochemical pathway. Effective disease treatment has been hampered by the lack of a molecular level understanding of the individual roles of the key proteins in Fe-S cluster formation.

The work presented within this dissertation diminish this knowledge gap significantly by providing a comprehensive biophysical characterization of the key
proteins involved in the sulfur mobilization step during the yeast mitochondrial Fe-S cluster synthesis process. The role of the accessory protein “Isd11” within the catalytic role of the cysteine desulfurase “Nfs1” in delivering sulfane sulfur for cluster assembly has been studied. The effect of Yfh1 on sulfur mobilization by the Nfs1-Isd11 complex has also been evaluated in detail. Our results suggest a possible regulatory function of Isd11 and possibly rationalize the evolutionary requirement for its role as an additional cofactor introduced in eukaryotes. Frataxin’s effect on cluster assembly was evident to materialize the most at the overall assembled complex level rather than at the individual protein level. This work, therefore, provides novel and significant insight into how the cluster assembly proteins function, and sets the groundwork for which additional experiments that need to be designed to further unravel the mechanistic details of sulfur mobilization during Fe-S cluster bioassembly. These molecular level details will assist in future drug design strategies directed at treating the diseases outlined above.
AUTOBIOGRAPHICAL STATEMENT

I was born and raised in the beautiful pearl of the Indian Ocean, Sri Lanka. I received my Bachelor of Science degree in Biochemistry and Molecular Biology from University of Colombo in 2010. My passion for science persuaded me to move to the USA, the land of opportunities in the fall of 2011 to pursue a doctoral degree in Biochemistry and Molecular Biology at Wayne State University-School of Medicine. Accomplishments I made during my PhD training in Dr. Timothy Stemmler’s lab are listed here.

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