

1-1-2013

# Cysteine Desulfurase And Isd1 1: A Drosophila Model

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**CYSTEINE DESULFURASE AND ISD11: A DROSOPHILA MODEL**

by

**RAHUL RAVINDRAN NAIR**

**THESIS**

Submitted to the Graduate School

Of Wayne State University,

Detroit, Michigan

In partial fulfillment of the requirements

For the degree of

**MASTER OF SCIENCE**

2013

**BIOCHEMISTRY AND MOLECULAR BIOLOGY**

Approved By:

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Advisor

Date

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## DEDICATION

*To,  
My family*

## **ACKNOWLEDGMENTS**

At the end of my Master's Thesis program, I look at myself and think of all the knowledge and skills I have acquired in the last one and a half years. My mind has distinctly recorded every stage of my project as snapshots and in each of them I have vivid memories of people who helped me break barriers that stood in my way. This journey would not have been possible without them. These people are none other than my mentor and advisor, Dr. Timothy L. Stemmler, my wonderful labmates, my friends in the Department, my friends in USA and India, my family in India and my extended family in USA. They complete my learning cycle and deserve to be mentioned before I begin my manuscript.

I have to begin my vote of thanks with my advisor, Dr. Timothy L. Stemmler. Dr. Stemmler has changed my perception of science, reality and research in a positive way. I have to thank him profusely for having faith in me and entrusting an independent research project. Dr. Stemmler insisted that he would never micro-manage my project and encouraged my thoughts wholeheartedly. Through our practice talk sessions, I have managed to get over one of my inherent fears of speaking in front of a crowd. Above all, the monetary support I received for one entire year allowed me to stay focussed on my research. I will always have utmost regard for Dr. Stemmler and cannot thank him enough for his magnanimity and all the positive changes he has brought about in me.

Among my lab members, first of all I wish to thank our Lab Manager, Lindsey Nico for all her valuable suggestions during the course of my project and for training me in using the AKTA instrument and the CD instrument.

When I started my project I had no particular skills in cloning or protein expression or protein purification and feared the possibility of failing terribly. I cannot forget my early days in lab when I felt out of place and inadequate in my research skills. This feeling lasted only for a few days as I made my first friend in lab. She could very well relate to my frustrating experiences and generously accepted me under her tutelage. I am referring to none other than Poorna Subramanian. Despite the fact that my project was not even remotely related to her project, she spent bucket loads of time training me in cloning, protein expression, cell lysis and, protein purification using the AKTA system. I am extremely grateful to Poorna for kickstarting my project and advising me periodically. Third on my list is my bench mate in lab - Andria Rodrigues. I always look up to Andria for her endless patience and diligence. I started having faith in my efforts after constantly seeing Andria invest countless hours of hard work and effort. I cherish all the discussions we have had on the Iron-sulfur cluster biogenesis projects. Fourthly, I have to thank April Kusowski for all the pranks she has played on me and other lab members. These momentary jokes and laughs were an impetus to keep my spirits alive even at the end of a tiresome day. I am not sure if I will ever be able to stay as meticulous as she is; but this is one quality I have to imbibe in me.

Fifthly, my special thanks to Dulmini Pabasara Barupala. In a short span of time she has turned out to be a great friend who I can rely on at any point of time.

Being my partner in the Icd11-Nfs project, she helped me regain my faith in the project when I was literally running out of ideas and struggling with protein purification. I needed Dulmini every time for a second opinion and invariably, her prudence and scientific acumen saved me from jumping into inaccurate conclusions. Sixth on my list is John Rotondo. My fear of using the ITC instrument vanished only because of John and I can today confidently add this skill to my CV. My vote of thanks for my lab members would be incomplete if I do not thank Stephen Dzul. I will remember Steve for enlivening otherwise uninteresting facts, updating my awareness and teaching me to approach research head-on without fearing failure. Finally, I am glad a hardworking student like Nicholas Spellmon has taken up my project and I wish him luck for the project.

Before I move on to the next segment of my essay I need to thank my Committee members, Dr. David R. Evans and Dr. Brian P. Edwards for helping me gauge my progress through their intellectually stimulating ideas and invaluable suggestions. I also have to thank Dr. Sharon Ackerman for allowing me to use her Ultracentrifuge and, Dr. Zhe Yang and Ms. Yuanyuan Jiang for providing me with high quality CCD camera images for my Western Blots. I extend my sincere thanks to Dr. Russell Finley as well for providing me with the starting material for my project. I also want to thank Ms. Yanna Marsh, Ms. Rose Cooper and Mr. Joseph Fiore for their administrative support and the entire BMB Department for making this entire experience memorable.

The second segment of this acknowledgement essay is dedicated to people outside my lab who provided all the emotional support I needed to sustain the rigors

of Grad school. First of all, USA has been a home away from home because of people like Asmita Vaishnav and Ashoka Kandegedara (Shyamalee). I will certainly miss all of Asmita's goodies and her motherly affection. I will miss my close friend, Fatme Hachem who has not only helped me tremendously with my cloning and Western Blot experiments but, has also provided great moral support. I have thoroughly enjoyed all the discussions I shared with Russell D' Souza about our Seminar speakers, Science in general and of course, the Indian and Western cultural disparity.

The final piece of this essay is dedicated to all my extended family members and friends in the US who showered all their love, welcomed me warmly when I needed periodic breaks from work and ensured that I never felt homesick. Lastly, I dedicate this manuscript to my parents, brother and my grandmothers, who spend all their money on prayers and 'pujas' for my well being. I am sure to fail miserably to express my gratitude to them as I do not know where to start from. To keep it short, if at all there is a part of me that believes in good fortune that is only when I think of them. I hope this good fortune lasts forever!

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# Chapter 1 Iron, sulphur and iron-sulphur cluster biogenesis

## 1.1 Introduction

Trace elements constitute only 0.15% of the mass of the human body; yet, more than a dozen of them are indispensable for sustaining life in a cell. Iron is one such vital trace element. In humans, approximately 50-60% of this iron is a constituent of haemoglobin. The remaining iron is incorporated in the cell's ferritin reserves by means of haemoglobin recycling and distributed intracellularly when required [2]. Iron, being a natural electron acceptor or electron donor, is highly reactive and hence, in its free form is extremely toxic to the cell. Any imbalance in human iron metabolism leads to iron deficiency or iron overload and triggers the release of free iron. These possibilities are witnessed through the pathophysiology of diseases like Friedreich's ataxia, ISCU myopathy, a rare form of sideroblastic anemia and Respiratory complex I-associated encephalomyopathy (where iron accumulation is a common feature) and, Anemia (where iron deficiency is the cause) [3].

Another reactive species critical for the survival of a human cell is sulfur and it is of significance because it readily forms bonds with iron and other trace elements. Sulfur reserves in the body are slightly higher than iron reserves as it constitutes the list of elements that make up 0.85% of the human body mass. Of the available non-metals in the body, sulfur is only next to phosphorus in its versatility of reactivity. This makes sulfur an integral part of many vital proteins, carbohydrates, cofactors and metabolites. Consequently, sulfur needs to be regulated through sulphur metabolism. A human cell acquires sulphur in the form of the amino acids -

cysteine and methionine, and any imbalance in sulphur metabolism would lead to sulphur overload or sulphur deficiency. However, the effects of sulphur overload are not prominent in any diseased state because the human body has an efficient mechanism to excrete sulfur in the form of sulphate through urine, unlike iron that does not have a physiological mechanism to eliminate excess iron[4]. In contrast, sulfur deficiency has multiple side effects on human physiology. When methionine is depleted, the transsulfuration pathway for cysteine biosynthesis and transmethylation pathway for homocysteine biosynthesis is affected[5]. Cysteine's bioavailability depends entirely on methionine intake, the depletion of which causes oxidant stress due to glutathione (a tripeptide antioxidant containing cysteine) and taurine (antioxidant) inadequacy[6]. Intestinal villus atrophy and low intestinal crypt depth due to limited cell proliferation and high apoptosis (oxidant stress-associated) are therefore, symptoms of methionine deficiency [6].

Owing to the reactivity of these two chemical species, one would be tempted to wonder what they could form in each other's company and whether this complexation would be beneficial to the human cell in any way. This brings us to the exciting field of iron-sulfur cluster chemistry. Infact, it appears as though nature has evolved an elegant mechanism, by allowing iron sulfur cluster (or Fe-S clusters) formation, to circumvent the problems arising from free iron and free sulfur reactivity in the cell.

## 1.2 Background

Until the mid-60s iron was believed to be a cofactor in all iron-containing proteins. Simple spectral studies which were performed previously had demonstrated that some of these proteins had extinction coefficients lower than heme proteins at around 400nm[7]. With the development of Infrared spectroscopy, Mossbauer spectroscopy, EPR and ENDOR spectroscopy, these intriguing iron-containing proteins were found to be actually iron-sulfur proteins[8], and they constituted such a vital cohort of proteins that they could not be ruled out as 'exceptions'. Structurally, these proteins could accommodate clusters in the form of 2Fe-2S, 3Fe-3S, 3Fe-4S, 4Fe-4S, 8Fe-7S and 8Fe-8S. To add to their diversity, these clusters could exhibit different redox states like  $[2Fe-2S]^{2+}$ ,  $[2Fe-2S]^+$ ,  $[3Fe-4S]^{2+}$ ,  $[3Fe-4S]^+$ , etc.[8] Some of these proteins were found to house more than one type of cluster; others could house one Fe-S cluster and metal ions. This structural diversity constituted by diverse Fe-S protein folds and its link with protein stability is an emerging field in coordination chemistry[9].

Individually, these iron sulfur clusters could be produced *in vitro* and like their precursors were found to be extremely versatile. They were originally thought to be "sensitive" molecules as they degraded in the presence of dioxygen. This notion changed when methods using mild denaturants and carrier thiols were designed to extrude these clusters from different apoproteins[10]. They were also remarkably capable of conversion and interconversion in both the free and protein-bound conditions [10]. This meant that they could swap cysteine ligands in proteins with or without cluster conversion. In the protein-bound form, they could facilitate substrate

activation (Aconitase), electron transfer (Respiratory complexes I, II, III), environmental oxygen sensing (Fumarate-Nitrate Reduction protein), DNA repair (Base excision repair glycosylases), ribosome assembly (ABCE1 protein), DNA-RNA processing (helicases, DNA polymerases, telomere length regulator proteins), tRNA thiolation and even, iron regulation (iron regulatory protein). This exemplifies their usefulness in most of the life sustaining phenomena inspite of constituting only a tiny fraction of the human body mass. Researchers have also identified diseases associated with Fe-S clusters. A list of diseases for Fe-S clusters is provided in Table 1. For its tremendous influence on myriad pathways, it would be surprising if we did not witness these iron-sulfur clusters in the most primitive life forms on earth. Today, they offer excellent cues to predict the molecular basis of the origin of life and reaffirm the concepts of evolution[11].

Despite these major breakthrough discoveries about their nature, their reactivity, their distribution and their usefulness, one prominent question remained, 'Were these clusters made in the cell or were they acquired from the external environment?'. It took the scientific community another thirty years for this question to be answered when Dean and co-workers identified a set of *nif* genes whose proteins were found to participate in cluster formation for the Nitrogenase enzyme in *A.vinelandii*[12]. The puzzle was solved and slowly key players of this process were uncovered one by one!

### **1.3 Cell-specific differentiation of the pathway**

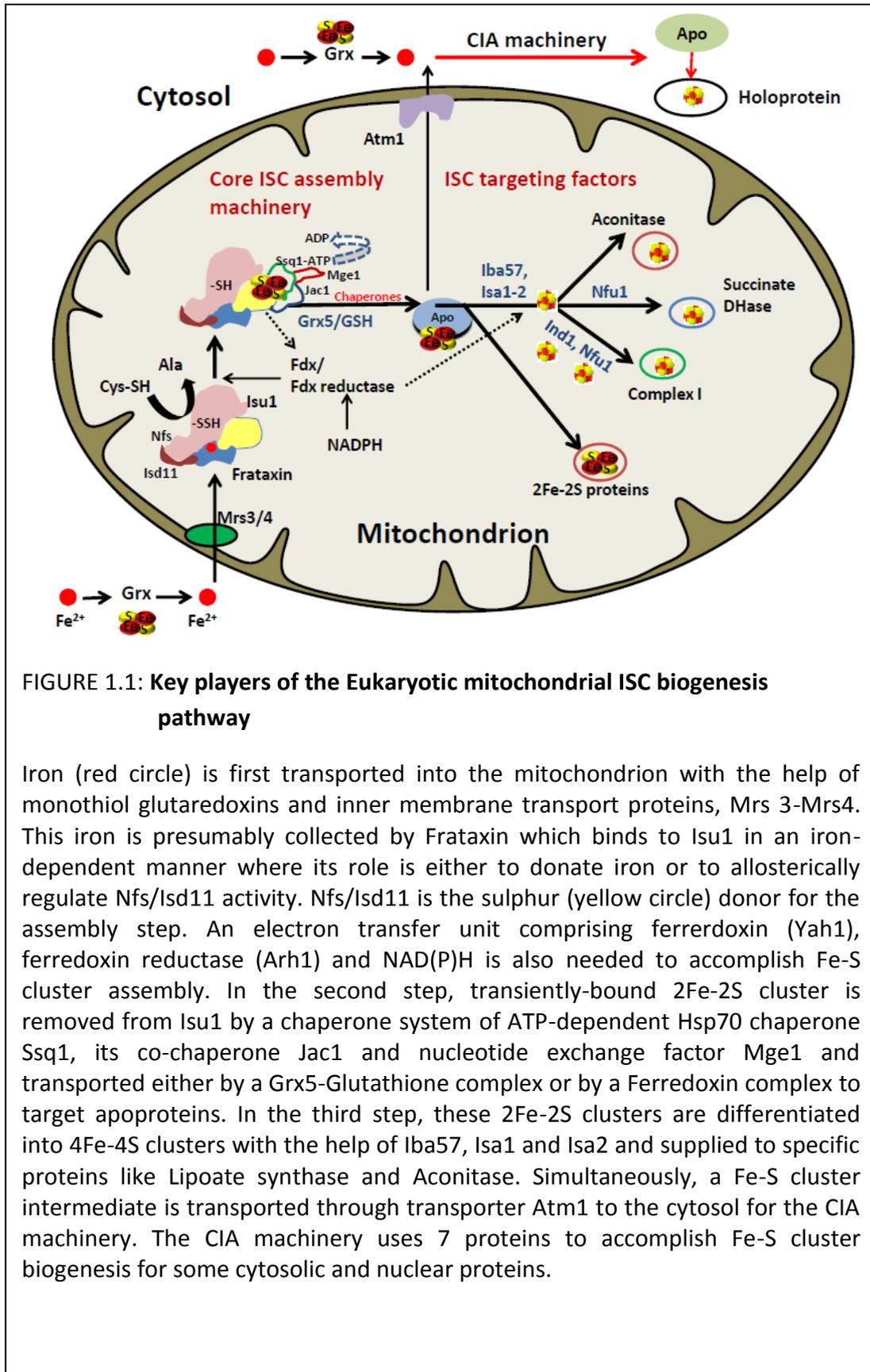
Today, we know that prokaryotes have three different pathways for iron sulfur cluster biogenesis; whereas, eukaryotes have two different iron-sulfur cluster

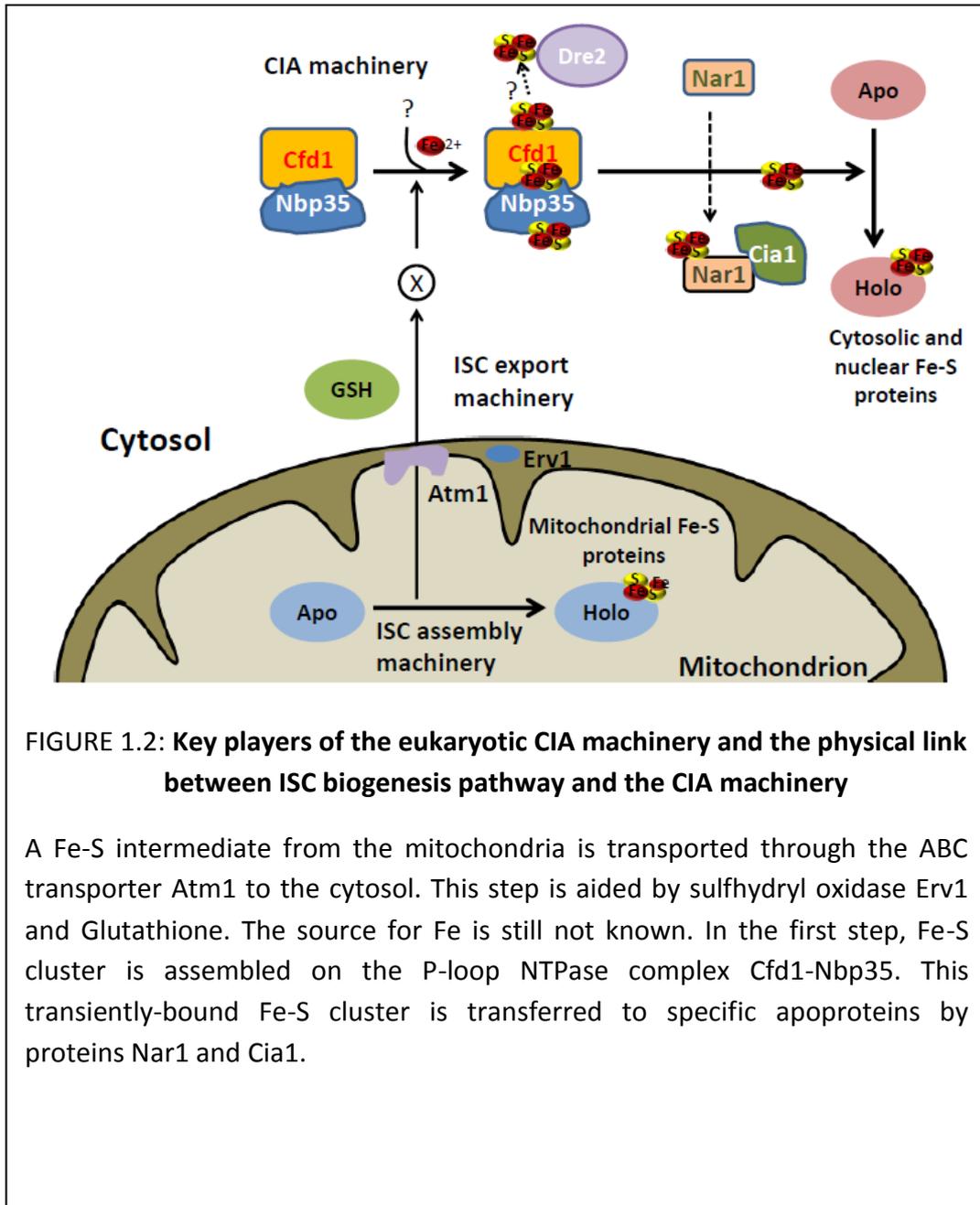
Human protein	Function	Associated disease
<b>Biogenesis components</b>		
Mitoferrin	Putative iron transporter	Erythropoietic protoporphyria
ISCU	Mitochondrial scaffold	Myopathy with exercise intolerance
Frxataxin	Iron donor for ISCU scaffold	Friedreich's ataxia
GLRX5	Transfer of Fe-S cluster from ISCU to apoprotein	Microcytic anaemia
ADR	Electron transfer	Tumor suppressor
ABCB7 (Atm1 homolog)	ISC export machinery component, ABC transporter	X-linked sideroblastic anaemia and cerebellar ataxia
<b>Fe-S proteins</b>		
Complex I	Complex I of respiratory chain	Various mitochondrial diseases such as LHON, MELAS and Leigh syndrome
Complex II	Complex II of respiratory chain	Tumor suppressor
XPD	Fe-S protein involved in nucleotide excision repair	Xeroderma pigmentosum, Cockayne syndrome, trichothiodystrophy
FANCI	Fe-S protein involved in nucleotide excision repair	Fanconi anaemia
MUTYH	Fe-S protein(glycosylase) involved in DNA repair	Colon cancer
<b>Table 1: Diseases associated with Iron-sulfur cluster biogenesis and Fe-S proteins</b>		

biogenesis pathways. In prokaryotes, the pathways are distinguished based on functional significance into the Isc (Iron sulfur cluster) system, Nif (Nitrogen fixation) system and the Suf (Sulfur formation) system. The NIF system is exclusively for iron sulfur cluster maturation for the nitrogenase enzyme in nitrogen fixing bacteria. The SUF system is exhibited under conditions of iron depletion in a cell. The pathway that closely resembles the eukaryotic pathways is the ISC pathway. However, the phylogenetic distribution of these pathways is complicated. For example, organisms such as *Mycobacterium tuberculosis* and few archaea, appear to have the Suf system alone; whereas, in *Escherichia coli* the Isc system is more important than the Suf system[13].

In eukaryotes, however, the pathway is classified into cytosolic and mitochondrial based on subcellular compartmentalization. The cytosolic pathway is known as the CIA machinery. So far, its mitochondrial counterpart – the ISC biogenesis pathway, is regarded as the main pathway for iron sulfur cluster biogenesis in eukaryotes. Interestingly, the human mitochondrion that is believed to have only 13 mitochondrial proteins[14], imports nuclear proteins for the fulfilment of iron sulfur cluster maturation. Figure 1.1 and 1.2 highlight all the key proteins of both the eukaryotic pathways.

Our understanding of all these pathways is very primitive as can be seen from the relaxation in classification strategy for prokaryotes and eukaryotes; while the former is functionally distinguished, the latter is subcellular distribution-based. [No correlation has been postulated between the subcellular occurrence of proteins that are benefited from the pathways and the subcellular predominance of the pathways





**FIGURE 1.2: Key players of the eukaryotic CIA machinery and the physical link between ISC biogenesis pathway and the CIA machinery**

A Fe-S intermediate from the mitochondria is transported through the ABC transporter Atm1 to the cytosol. This step is aided by sulfhydryl oxidase Erv1 and Glutathione. The source for Fe is still not known. In the first step, Fe-S cluster is assembled on the P-loop NTPase complex Cfd1-Nbp35. This transiently-bound Fe-S cluster is transferred to specific apoproteins by proteins Nar1 and Cia1.

Function	Bacterial NIF	Bacterial SUF	Bacterial ISC	Mitochondrial ISC	Eukaryotic CIA
Cysteine desulphurase, sulphur donor	NifS	SufS-SufE	IscS	Nfs1-Isd11	Mitochondrial Nfs1-Isd11
U-type scaffold for assembly	NifU (N-terminal domain)	SufU	IscU	Isu1	-
A-type scaffold for assembly	IscA	SufA	IscA, ErpA	IscA1, Ica2, Iba57?	-
NFU-type scaffold for assembly	NifU(C-terminal domain)	-	NfuA	Nfu1	-
P-loop NTPase scaffold for assembly*	-	-	-	Ind1?	Cfd1-Nbp35
Electron transfer	NifU(middle domain)	-	Fdx	Yah1-Arh1	-
Iron donor	-	-	CyaY	Yfh1	-
Transfer of Fe-S cluster from scaffold to target apoproteins	-	SufC?	HscA, HscB	Ssq1, Jac1, Mge1, Grx5	Nar1, Cia1

\* - Phosphate-binding-loop Nucleoside triphosphatases

**Table 2: Comparison of prokaryotic and eukaryotic proteins involved in the iron sulfur cluster biogenesis pathways**

themselves.] This permits us to look at the similarities in the proteins involved in prokaryotic and eukaryotic ISC biogenesis pathways. Table 2 lists the prokaryotic proteins in the pathways and its eukaryotic counterparts. The mitochondrial ISC biogenesis pathway in eukaryotes will be the focus of this manuscript.

#### **1.4 General mechanism of mitochondrial Iron Sulfur Cluster biogenesis**

The eukaryotic mitochondrial iron sulfur cluster biogenesis pathway is called the ISC biogenesis pathway. The pathway takes place in the mitochondrial matrix and is understood in three major steps – iron-sulfur cluster assembly, iron-sulfur cluster transfer and iron-sulfur cluster delivery. Seventeen proteins have been identified so far in this pathway [15] and a detailed account of the general mechanism of the pathway is required for further discussion. The steps involved are outlined below.

Firstly, Cysteine desulfurase and its accessory protein, Isd11, in a PLP-dependent mechanism, deliver sulfur to a scaffold protein, Isu1. Simultaneously, iron loaded-Frataxin is believed to deliver iron to the same scaffold protein. It is not yet known whether sulfur delivery or iron delivery is the rate-limiting step in iron-sulfur cluster assembly [16]. Once the precursors arrive at Isu1 and are brought into each other's vicinity, through elegant conformational changes of the entire Cysteine desulfurase-Isd11-Isu1-Frataxin complex, the genesis of 2Fe-2S clusters occurs. These events involved in the formation of a 2Fe-2S cluster, also known as 'iron-sulfur cluster assembly' constitute the first step of the pathway.

After assembly of 2Fe-2S clusters on Isu1, these clusters that are transiently bound to Isu1 are first removed, then transported and finally, assembled on target

apoproteins by Cluster Transfer Proteins. Although these three partial steps are difficult to isolate experimentally, iron-sulfur cluster dissociation from Isu1 is believed to occur through the ATP-hydrolysis dependent complexation of an ATPase chaperone, Ssq1 and its co-chaperone Jac1 on the PVK domain of Isu1[1]. The rate-limiting step in the entire pathway is believed to be the cluster transfer step from IscU (bacterial homolog of Isu1) [17]. Apart from these three steps, there is an additional step when some of these 2Fe-2S clusters are differentiated into 4Fe-4S clusters for specific apoproteins. Isa1, Isa2 and Iba57 are few proteins involved in this additional step[1]. This entire event is broadly categorized as the second major step – the ‘iron-sulfur cluster transfer’ step.

In the final step, iron-sulfur cluster-bound proteins are transported outside the mitochondria with the help of ATP transporters and other proteins. Also, some Fe-S cluster intermediate alone is believed to be transported outside the mitochondria probably, to aid the cytosolic pathway.

### **1.5 The organic half of the pathway – Sulfur delivery**

Bacterial NifS was the first protein of the ISC biogenesis pathway that was identified and biochemically characterized[13]. Subsequently, two other cysteine desulfurases IscS and SufS were discovered. All three proteins are similar in sequence and structure and follow a similar persulfide formation strategy to extract sulfur [13]. Biochemical studies suggest that their eukaryotic homologs like Nfs1 (yeast), NFS1 (human-mitochondrial) and ISCS (human-cytosolic) also follow a similar strategy. Figure 1.3 shows the crystal structure of bacterial IscS. Some significant facts derived from the crystal structure of bacterial IscS are [18]:

1. L-Cysteine is its reactive substrate.

2. They are homodimers with a Pyridoxal-5' phosphate (PLP) group attached at the dimer interface.
3. Each monomer has two domains. The larger domain has the conserved active site lysine residue and the smaller C-terminal domain houses a reactive cysteine residue.
4. The reactive cysteine is easily blocked by alkylating agents like N-ethyl maleimide (NEM) and this renders the enzyme inactive.
5. Substitution of this reactive cysteine by alanine also renders this enzyme inactive.

The general steps involved in sulfur delivery by cysteine desulfurase are as follows[19]:

1. First, PLP binds to an active site, lysine rearranges and forms a ketimine adduct with the substrate L-cysteine.
2. Next, the thiolate group of the reactive cysteine residue in the smaller C-terminal flexible loop of the enzyme attacks the PLP-bound cysteine by nucleophile chemistry.
3. A series of bond shifts between the reactive cysteine residue and the PLP-cysteine adduct leads to the extraction of the thiolate group of the substrate by the reactive cysteine to form an enzyme-bound persulfide intermediate.
4. The substrate cysteine devoid of its SH group results in the formation of an alanine-PLP adduct.
5. The mechanism by which alanine is released from PLP is not known yet.
6. Once persulfide is formed, the proximal sulfur/sulfide group is transferred to conserved cysteine residues on Isu1.

7. Latest evidence shows that this transfer of persulfide to Isu1 occurs only in the presence of iron and nucleotides like ATP and GTP[20].
8. The mechanism of this PLP-mediated sulfur extraction reaction of cysteine desulfurase is called beta-elimination.
9. Lastly, the eukaryotic homolog of this enzyme is inactive in the absence of an accessory protein named Isd11.

### **1.6 Other roles of cysteine desulfurase and subcellular distribution**

Apparently, cysteine desulfurase is not an enzyme used exclusively by the cytosol or mitochondria for Iron sulfur cluster biogenesis. The enzyme is also found to play a major role in tRNA thiolation [21]. Moreover, it is involved in the biosynthesis of major cellular cofactors like thiamin, biotin, lipoic acid and molybdenum cofactor[1]. These additional attributes can be considered as the enzyme's moonlighting functions in other cellular processes[1]. Thus, a defect in cysteine desulfurase can have a major impact on multiple pathways within a cell. An alternative viewpoint that was suggested previously was that cysteine desulfurase plays a major role in intracellular sulfur trafficking[13].

With their involvement in multiple cellular processes it would be reasonable to assume that this enzyme is present in all three compartments of the cell – the cytosol, the nucleus and the mitochondrion. Although in humans this is the case, in yeast, the enzyme is found only in the mitochondria and is believed to shuttle between the cytosol and mitochondria for their respective iron sulfur cluster biogenesis pathways[22]. This could possibly mean that in higher eukaryotes the enzyme is more widespread as opposed to the lower eukaryotes like yeast.



**Figure 1.3: Ribbon-shaped diagram showing the Crystal structure of IscS**

The diagram shows the dimeric subunits of cysteine desulfurase with an active site PLP (pyridoxal 5' phosphate) at its dimeric interface. Figure taken from [18]

### **1.7 Cysteine desulfurase's accessory protein - Isd11 and other LYR proteins**

The only major anomaly in the assembly step of eukaryotic Fe-S cluster biogenesis pathways is the presence of an 11kDa protein named Isd11. Isd11 is found exclusively in eukaryotes [23] and is an ancient protein. Homologs are found in the mitosomes of Microsporidia and the hydrogenosomes of Trichomonads, which are organelles that originated before the mitochondrion[11]. Different experimental approaches on cell viability have suggested that yeast Isd11 is a strictly essential protein for the cell [23]. Hence, Isd11 belongs to the small fraction (18%) of cellular proteins that are strictly essential for yeast cell viability [24]. The reason for its indispensable role in the cell can be attributed to its association with cysteine desulfurase and its unique ability to activate the protein. Latest evidence suggests that persulfide formation in the active site of cysteine desulfurase occurs only in the presence of Isd11 [25]. This is one of the main features that distinguishes eukaryotic iron sulfur cluster assembly from its prokaryotic counterpart.

Although Isd11 has no mitochondrial signal sequence, it exists primarily in the mitochondrial matrix and is loosely connected with the inner membrane [23]. Recent evidence suggests that a nuclear version of the protein also exists, consistent with the subcellular distribution of cysteine desulfurase [26]. This finding is consistent with the observation that Isd11 is essential for the biogenesis of mitochondrial and nuclear Fe-S clusters [23], although its involvement in cytosolic Fe-S cluster formation still needs to be addressed.

A notable feature of Isd11 is that it belongs to the poorly conserved LYR family of proteins [27]. Interestingly, a common functional trait for this family of proteins has not been found yet [23]. Other members of this family of proteins

include Mzm1, SDHAF1 (Respiratory Complex II subunit), NDUFA6 (Respiratory Complex I subunit) and NDUF9 (Respiratory Complex I subunit). The functional significance of few of these LYR proteins like Mzm1, SDHAF1, NDUFA6 and NDUF9 suggest that the LYR motif and Fe/S cluster metabolism have some correlation.

### **1.8 Cysteine desulfurase – Isd11 complex**

Some information on the Isd11-Nfs complex is available today. Co-purification of the complex using size-exclusion chromatography has shown that it exists as a 180-200kDa protein complex [27]. In-vivo studies have shown that Isd11 binding on cysteine desulfurase is independent of substrate cysteine binding [17]. In addition, inactivation of cysteine desulfurase by the alkylating agent NEM is not possible at low concentrations. The same group also found that the LYR motif of Isd11 is essential for Isd11 binding and subsequent activation of cysteine desulfurase [17]. Finally, as stated above, recent evidence suggests that Isd11 binding is a prerequisite for eukaryotic cysteine desulfurase activity and persulfide formation [25].

A lot has been known in the past six years about the coordinated functioning of the Isd11-Nfs complex. Still, a lot of questions remain unanswered. Some of the questions are: Why is Isd11 exclusively found in eukaryotes? Why does the eukaryotic system need an extra protein for sulfur extraction? What changes occur in the Iron sulfur cluster biogenesis pathway if methionine or cysteine is depleted? Does Isd11 play a role in other pathways without the involvement of cysteine desulfurase? With steady developments in this field every year, we can remain hopeful that the entire mechanism will be deciphered to a large extent in the near future.

## 1.9 References

1. Lill, R., *Function and biogenesis of iron-sulphur proteins*. Nature, 2009. **460**(7257): p. 831-8.
2. Fleming, R.E. and B.R. Bacon, *Orchestration of iron homeostasis*. N Engl J Med, 2005. **352**(17): p. 1741-4.
3. Rouault, T.A., *Biogenesis of iron-sulfur clusters in mammalian cells: new insights and relevance to human disease*. Dis Model Mech, 2012. **5**(2): p. 155-64.
4. Nimni, M.E., B. Han, and F. Cordoba, *Are we getting enough sulfur in our diet?* Nutr Metab (Lond), 2007. **4**: p. 24.
5. Brosnan, J.T. and M.E. Brosnan, *The sulfur-containing amino acids: an overview*. J Nutr, 2006. **136**(6 Suppl): p. 1636S-1640S.
6. Bauchart-Thevret, C., et al., *Sulfur amino acid deficiency upregulates intestinal methionine cycle activity and suppresses epithelial growth in neonatal pigs*. Am J Physiol Endocrinol Metab, 2009. **296**(6): p. E1239-50.
7. Beinert, H., *Iron-sulfur proteins: ancient structures, still full of surprises*. J Biol Inorg Chem, 2000. **5**(1): p. 2-15.
8. Lamotte, J.-M.M.B., *Iron-sulfur clusters and their electronic and magnetic properties*. Coordination Chemistry Reviews, 1998. **178-180** (Part 2): p. 1573-1614.
9. Meyer, J., *Iron-sulfur protein folds, iron-sulfur chemistry, and evolution*. J Biol Inorg Chem, 2008. **13**(2): p. 157-70.
10. Beinert, H., *Iron-Sulfur Clusters: Nature's Modular, Multipurpose Structures*. Science, 1997. **277**(5326): p. 653-659.

11. Richards, T.A. and M. van der Giezen, *Evolution of the Isd11-IscS complex reveals a single alpha-proteobacterial endosymbiosis for all eukaryotes*. Mol Biol Evol, 2006. **23**(7): p. 1341-4.
12. Zheng, L., et al., *Assembly of iron-sulfur clusters. Identification of an iscSUA-hscBA-fdx gene cluster from Azotobacter vinelandii*. J Biol Chem, 1998. **273**(21): p. 13264-72.
13. Johnson, D.C., et al., *Structure, function, and formation of biological iron-sulfur clusters*. Annu Rev Biochem, 2005. **74**: p. 247-81.
14. Taylor, R.W. and D.M. Turnbull, *Mitochondrial DNA mutations in human disease*. Nat Rev Genet, 2005. **6**(5): p. 389-402.
15. Lill, R., et al., *The role of mitochondria in cellular iron-sulfur protein biogenesis and iron metabolism*. Biochim Biophys Acta, 2012. **1823**(9): p. 1491-508.
16. Tantalean, J.C., et al., *The Geobacillus stearothermophilus V iscS gene, encoding cysteine desulfurase, confers resistance to potassium tellurite in Escherichia coli K-12*. J Bacteriol, 2003. **185**(19): p. 5831-7.
17. Wu, S.P., et al., *Iron-sulfur cluster biosynthesis. Kinetic analysis of [2Fe-2S] cluster transfer from holo ISU to apo Fd: role of redox chemistry and a conserved aspartate*. Biochemistry, 2002. **41**(28): p. 8876-85.
18. Cupp-Vickery, J.R., H. Urbina, and L.E. Vickery, *Crystal structure of IscS, a cysteine desulfurase from Escherichia coli*. J Mol Biol, 2003. **330**(5): p. 1049-59.
19. Zheng, L., et al., *Mechanism for the desulfurization of L-cysteine catalyzed by the nifS gene product*. Biochemistry, 1994. **33**(15): p. 4714-20.

20. Pandey, A., et al., *Identification of a Nfs1p-bound persulfide intermediate in Fe-S cluster synthesis by intact mitochondria*. Mitochondrion, 2012. **12**(5): p. 539-49.
21. Nakai, Y., et al., *Thio modification of yeast cytosolic tRNA is an iron-sulfur protein-dependent pathway*. Mol Cell Biol, 2007. **27**(8): p. 2841-7.
22. Muhlenhoff, U., et al., *Functional characterization of the eukaryotic cysteine desulfurase Nfs1p from Saccharomyces cerevisiae*. J Biol Chem, 2004. **279**(35): p. 36906-15.
23. Wiedemann, N., et al., *Essential role of Isd11 in mitochondrial iron-sulfur cluster synthesis on Isu scaffold proteins*. EMBO J, 2006. **25**(1): p. 184-95.
24. Giaever, G., et al., *Functional profiling of the Saccharomyces cerevisiae genome*. Nature, 2002. **418**(6896): p. 387-91.
25. Pandey, A., et al., *Persulfide formation on mitochondrial cysteine desulfurase: enzyme activation by a eukaryote-specific interacting protein and Fe-S cluster synthesis*. Biochem J, 2012. **448**(2): p. 171-87.
26. Shi, Y., et al., *Human ISD11 is essential for both iron-sulfur cluster assembly and maintenance of normal cellular iron homeostasis*. Hum Mol Genet, 2009. **18**(16): p. 3014-25.
27. Adam, A.C., et al., *The Nfs1 interacting protein Isd11 has an essential role in Fe/S cluster biogenesis in mitochondria*. EMBO J, 2006. **25**(1): p. 174-83.

## Chapter 2 Characterisation of DIsd11

### 2.1 Prelude

Isd11 is a nuclear-encoded, 11kDa mitochondrial protein that localizes in the mitochondrial matrix after its biosynthesis [1]. Surprisingly, the protein has no mitochondrial targeting sequence and yet, enters the matrix of the organelle [1, 2]. Another notable feature of this protein is that it has no cysteines and no tryptophans in its protein sequence. This eliminates the possibility of performing a fluorescence spectroscopy experiment on this protein. Also, every spectroscopic technique that majorly relies on light absorption by the tryptophan residues of proteins to quantitatively estimate its biochemical properties would be inaccurate for Isd11. However, there are certain intrinsic benefits as well. The lack of cysteine residues in the protein eliminates the possibility of a Fe-S cluster binding within it and also, allows us to eliminate the empirical errors that can cause unfolding of the protein by disulfide bond breakage.

Isd11 has been found to activate eukaryotic cysteine desulfurase both *in vivo* and *in vitro*. This was accomplished by A. Dancis et al. and D. Pain et al. [3] through two elegant experiments. Before this study, other research groups claimed that Isd11 has no effect on cysteine desulfurase activity. However, the activity assay used for the earlier claims were highly insensitive as it detected the overall microsulfide concentrations in the mitochondria on Isd11 depletion. The basis for following such a strategy was that Nfs1 is the only known eukaryotic cysteine desulfurase in the mitochondrion [4] and hence, any sulphide produced could be only from cysteine desulfurase. When a radioactive assay that specifically detects persulfide formation

was developed, these assumptions were proven wrong. In the first experiment, a yeast mitochondrion expressing Nfs1p was depleted of Isd11p and tested for persulfide formation and later, purified Isd11 was imported into the same depleted mitochondrion to test if this effected a change. In the first case, no persulfide formed; whereas, in the second case, persulfide appeared and increased in intensity over time. To test the same phenomenon *in vitro*, purified Nfs1p was incubated with  $S^{35}$ -cysteine and tested with and without Isd11. The results of the *in vivo* experiment correlated with the *in vivo* experiment. This confirmed that Isd11p is a prerequisite for yeast Nfs1p activation and persulfide formation and at the same time was not required for binding of substrate cysteine with Nfs1p.

Biochemical characterization of Isd11 is critical for understanding the protein's activity. Like the experiments discussed above, more biochemical experiments are required for further characterization of the protein. Simultaneously, it is also important to perform biophysical characterization of Isd11 to predict the structure of the protein. Prediction of a protein's structure is the ultimate milestone in understanding a protein's functionality. Presently, the crystal structure of Isd11 is not known. Preliminary biophysical characterization before solving Isd11's crystal structure involves studying the folded state of the protein, its oligomeric properties and its thermal stability. To accomplish these tasks, *Drosophila melanogaster* was chosen as a model organism in our lab.

## **2.2 Materials and Methods**

### **2.2.1. Cloning of DIsd11**

DIsd11 cDNA was obtained in a pFLC1 vector, from the Drosophila Genomic Research Center (FlyBase ID: FBcl0223918). DIsd11 cDNA was PCR amplified and sub-cloned into a pET14b vector (Novagen) which has a (His)<sub>6</sub> tag and an Ampicillin resistance 'bla' gene. The cloned plasmid was amplified by transforming into DH5 $\alpha$  cloning strain (Invitrogen). The DIsd11 sequence represents 107-385bp of the complete 705bp open reading frame. Positive clones were verified by DNA sequencing (Genewiz facility). Recombinant plasmid was transformed into BL21 (DE3)-RIL (Stratagene), BL21 (DE3)-Rossetta (Novagen) and BL21 (DE3) E.coli competent cell lines for protein expression.

### **2.2.2. Optimization of soluble protein expression**

The cell line with best soluble protein expression was identified as BL21 (DE3) by growing at 37°C till an OD of 0.8 and then inducing with 0.4mM IPTG for ca. 2 hours. The next step was to optimize protein expression in the selected cell line, BL21 (DE3). DIsd11 soluble protein expression was optimized by varying IPTG concentrations, post-induction time intervals, pre-induction OD and Temperatures. Optimum protein expression was obtained when BL21 (DE3) was grown at 37°C till an OD of 0.8-0.9 and then induced with 0.2mM IPTG for ca. 2.5 hours before harvesting by centrifugation.

### **2.2.3. Soluble DIsd11 protein purification**

Protein isolation steps were all performed at 4° C. Cells were resuspended in 50 mM Na<sub>3</sub>PO<sub>4</sub> (pH 7.4), 300 or 500mM NaCl, 20 mM Imidazole and 5 mM  $\beta$ -Me in the presence of Complete EDTA-free Protease inhibitor cocktail (Roche). Cells were

then lysed by two passes through a French Press cell at high pressure (1100 psi), and centrifuged at high speed (21000 rpm) for 1 hour. Crude soluble fraction was filtered (0.20  $\mu\text{m}$ ) and loaded onto a HisPur Cobalt column (Thermo Scientific) using an Imidazole gradient in the range of 0 – 500 mM (DlSD11 protein elutes between 260 and 380mM). DlSD11 containing fractions were pooled and concentrated to ca. 1mL by centrifugation using 3kDa Molecular Weight Cut Off (MWCO) centricons (Millipore) spun at 5000 rpm. The concentrated retentive solution was run over a HiLoad 16/10 Superdex 75 size exclusion column (General Electronics) equilibrated with 50mM  $\text{Na}_3\text{PO}_4$  buffer (pH 7.4), 500 mM NaCl. Following this protocol, DlSD11 monomer and oligomers were obtained based on SDS-PAGE gel analysis (Bio-Rad). The obtained protein concentration was 0.5 – 0.6 mg/mL and could be stored anaerobically at  $-20^\circ\text{C}$ , for several weeks and slight degradation appeared only after three months.

#### **2.2.4. Western Blot using Anti-His antibody and Anti-DlSD11 antibody**

The western blot experiment was done over two days. On day 1, protein samples were run on SDS-PAGE gels, stained in Coomassie Blue and checked for purity of loaded samples. Simultaneously, another gel containing the same protein samples was left unstained and blotted on a 0.2 $\mu\text{m}$  Nitrocellulose membrane (Bio-Rad). Blotting was performed in three steps: firstly, the Mini Trans-Blot filter paper (Bio-Rad), Mini Trans-Blot filter pads (Bio-Rad), the nitrocellulose membrane and the unstained gel were soaked in Transfer Buffer (60.5g Tris base , 288g Glycine, 10g SDS dissolved in 2L water and further diluted with 600mL methanol and 2.16L of water) for 15 minutes. In the second step, all the above-mentioned blotting components were stacked on a gel plate and placed in a plastic cassette with the gel facing the

black side of the cassette. After assembly, this cassette was placed inside a plastic gel frame with the black side of the cassette facing the black side of the frame. The gel frame was then almost completely immersed in Transfer buffer and allowed to run at 22V overnight in the cold room.

On day 2, the transfer cassette was pulled out of the chamber and a Ponceau red dye staining of the blotted membrane was performed. This was followed by washing the membrane in water and blocking the membrane with blocking buffer [5%(w/v) non-fat dry milk dissolved in 1X TBST buffer – 50mM Tris-HCl(pH8.0), 150mM NaCl and 0.05% Tween20] for 30 minutes to prevent non-specific binding of the primary antibody. The Ponceau staining step was optional and was only used to check the proper transfer of proteins to the membrane. The blotted nitrocellulose membrane was then incubated in a 1:3000 dilution of primary Anti-His antibody or Anti-Isd11 antibody in blocking buffer for 3 hours. After the three-hour incubation period, the membrane was washed 3 times with 1X TBST buffer. After this step, the membrane was incubated in a 1:5000 dilution of secondary antibody in blocking buffer (Non-fat dry milk dissolved in 1X TBST) for 1 hour. Following secondary antibody incubation, the membrane was again washed 3 times with 1X TBST buffer. The membrane was developed using a mixture of Luminol and Peroxidase (1ml each for 1min.) and analysed using a CCD camera.

#### **2.2.5. Circular Dichroism of DIsd11 oligomer-monomer mixture**

Purified DIsd11 protein was completely dialysed into degassed 1mM NaPO<sub>4</sub>. Protein concentration was estimated using Advanced Protein Assay and Lowry Assay. The dialysed protein was then degassed for 15 minutes using a Thermovac degassing system. A Camphor Sulfonic acid control was performed to check the proper

functioning of the UV lamp of the Applied Photophysics CD instrument. After testing the lamp, a 1mM NaPO<sub>4</sub> buffer absorbance spectrum and CD spectrum were taken using  $\pi^*$ 180 software from 340nm to 180nm. Then, six CD spectra of the DIsd11 protein in 1mM NaPO<sub>4</sub> buffer were collected using the same program. Each DIsd11 CD spectrum was buffer-subtracted and the six scans were averaged to obtain an averaged CD profile. The resulting profile was smoothed, saved and then processed using ProData viewer and CDNN softwares.

## **2.3 Results and Discussion**

### **2.3.1. Cloning of DIsd11**

DIsd11 was successfully cloned into pET14b (Figure 2.1), restriction digested after cloning (Figure 2.2) and sequence analysed to confirm the presence of the DIsd11 gene in the vector (Figure 2.3). This confirmed that the selected DH5 $\alpha$  cloning strain transformants and the selected BL21(DE3) expression strain transformants had the right clone.

### **2.3.2. Optimization of soluble protein expression**

The purified plasmid was transformed into three expression strains – BL21(DE3), BL21(DE3)-RIL and BL21(DE3)-Rossetta (Figure 2.4). The protein expression from BL21(DE3) alone was optimal. Hence, BL21(DE3) was used for subsequent expression tests. After a series of expression tests it was found that soluble DIsd11 is obtained in high yields when BL21(DE3) is grown at 37°C at a pre-induction OD of 0.8-0.9 and induced with 0.2mM IPTG for 2 hours (Figure 2.5). Figure 2.6 shows a flowchart for all the expression conditions tried out. The condition for best soluble protein expression was used for large scale protein expression and

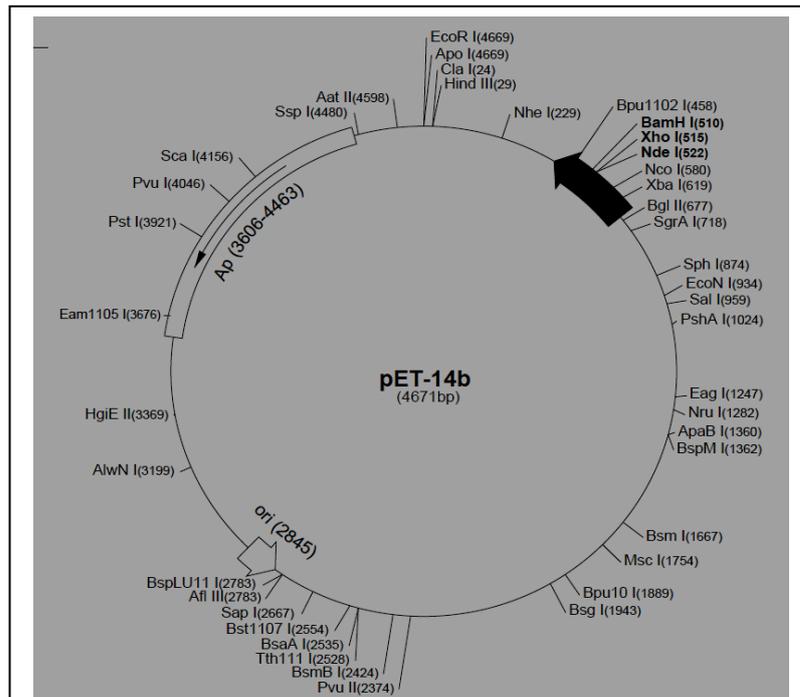
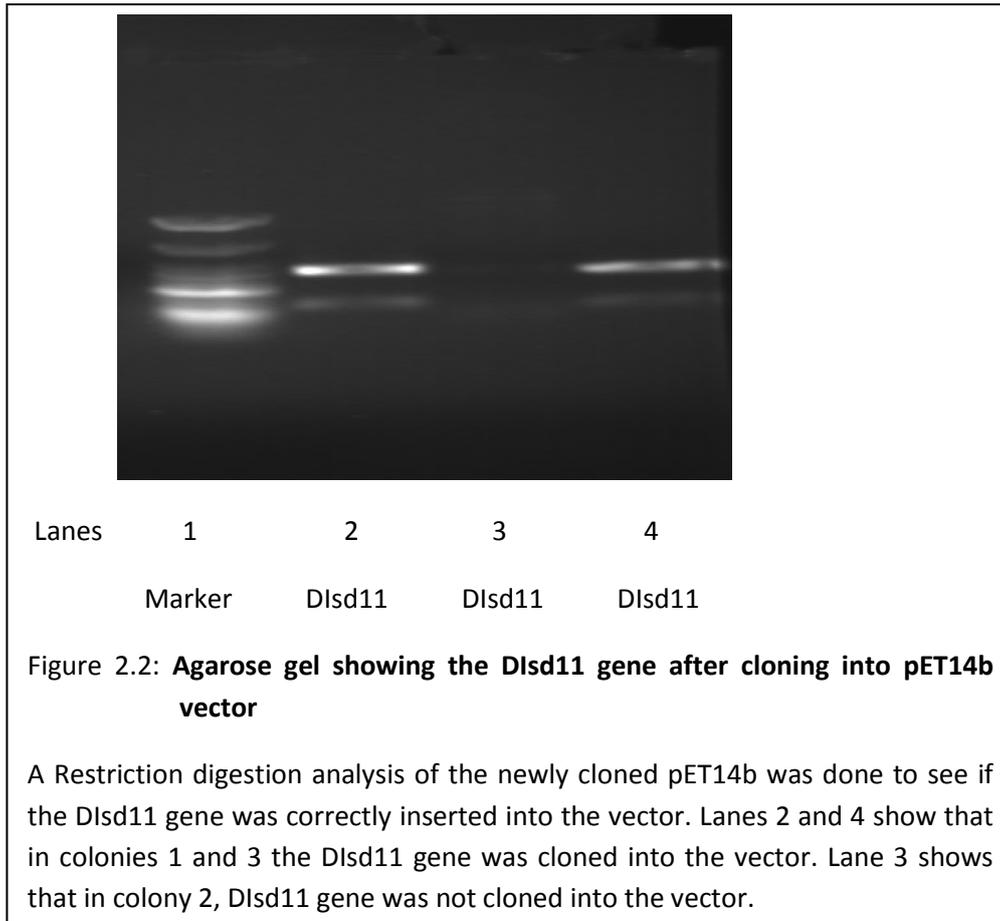


Figure 2.1: Restriction site map of vector pET14b

The vector has an Ampicillin resistance gene and a (His)<sub>6</sub> tag that attaches to the N-terminus of any protein expressed from the gene inserted into its Multiple Cloning Site.



```

RE5789  MSTRRQAITLYRNLLRESEKLPYNFRMYAARKIRDTFRANRSTRDFAEIDRQMAEGQQN 60
gene_Fp MSTRRQAITLYRNLLRESEKLPYNFRMYAARKIRDTFRANRSTRDFAEIDRQMAEGQQN 60
*****

RE5789  LELIRRQVIIGHLYSADKLVENKTKLPSDD 92
gene_Fp LELIRRQVIIGHLYSADKLVENKTKLPSDD 92
*****

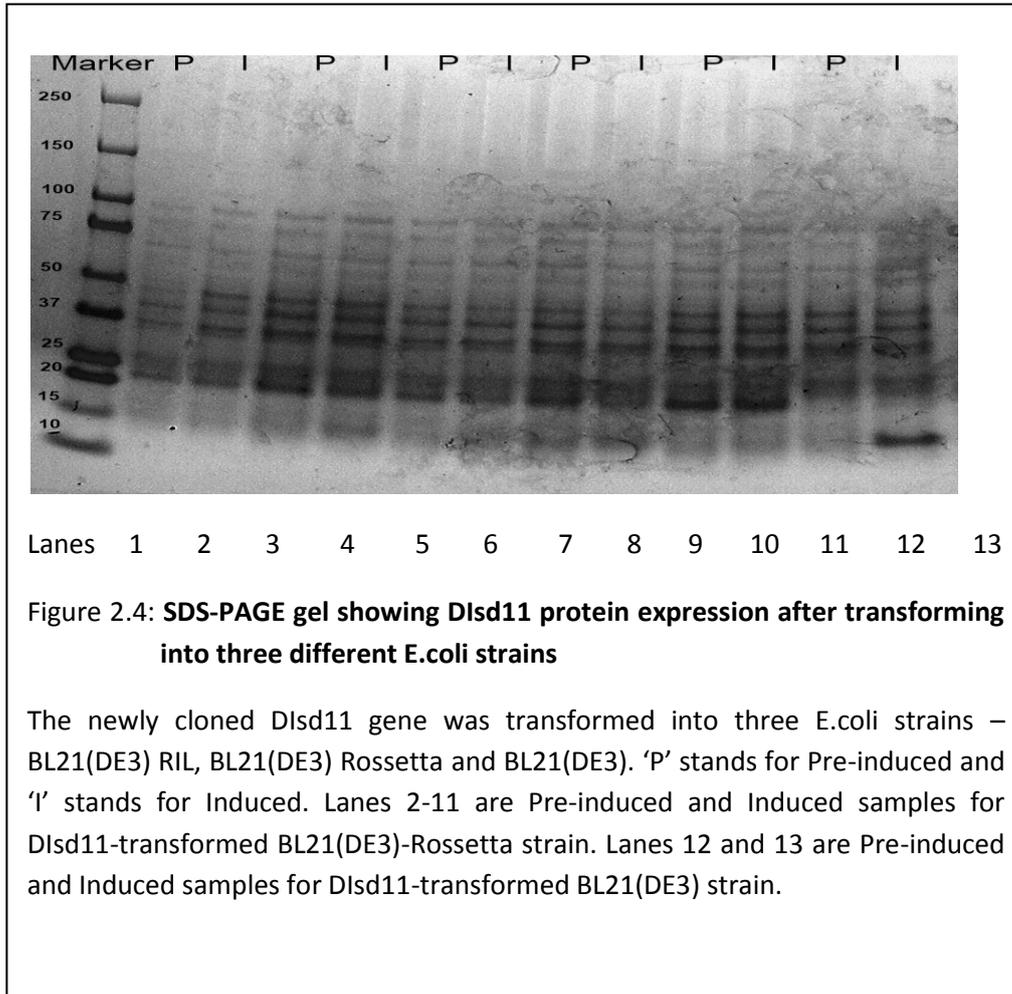
RE5789  MSTRRQAITLYRNLLRESEKLPYNFRMYAARKIRDTFRANRSTRDFAEIDRQMAEGQQN 60
gene_Rp MSTRRQAITLYRNLLRESEKLPYNFRMYAARKIRDTFRANRSTRDFAEIDRQMAEGQQN 60
*****

RE5789  LELIRRQVIIGHLYSADKLVENKTKLPSDD 92
gene_Rp LELIRRQVIIGHLYSADKLVENKTKLPSDD 92
*****

```

**Figure 2.3: Protein sequence alignment of DIsd11 with the protein sequence available on Pubmed**

The DIsd11 gene was sent to the Genewiz facility for sequence verification. The obtained gene sequence was translated into its protein sequence using the Expsy translate program and aligned with the available Pubmed protein sequence for DIsd11 (RE5789) using the ClustalW2 program. The sequence alignment shows 100% identity.



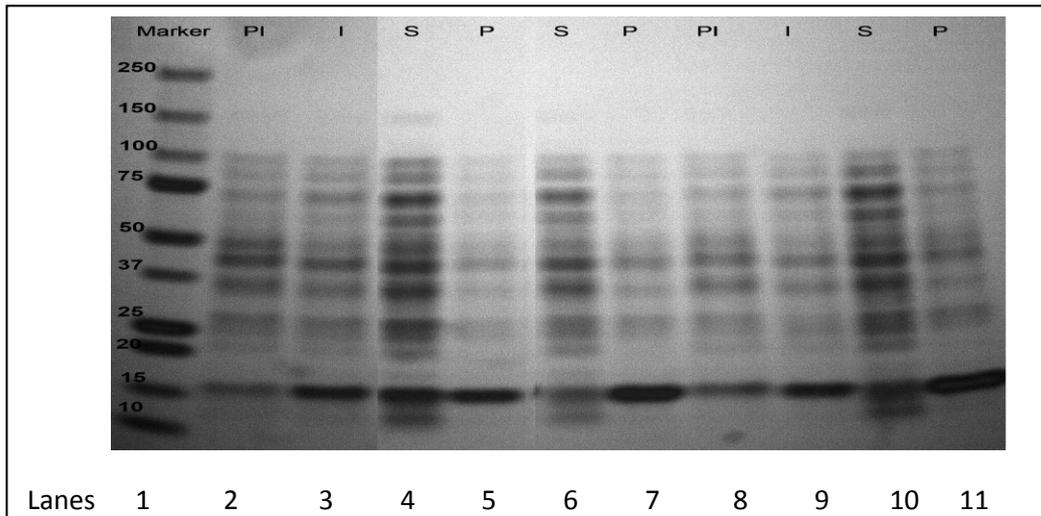
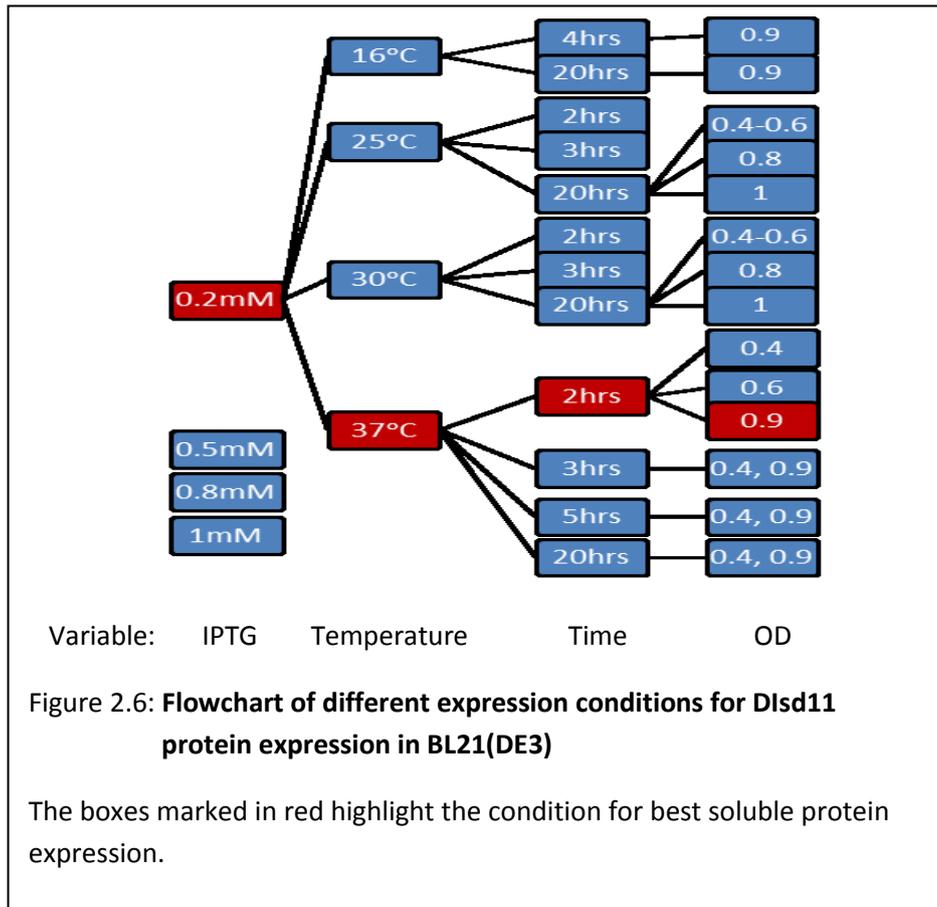


Figure 2.5: **Optimization of soluble DIsd11 protein expression**

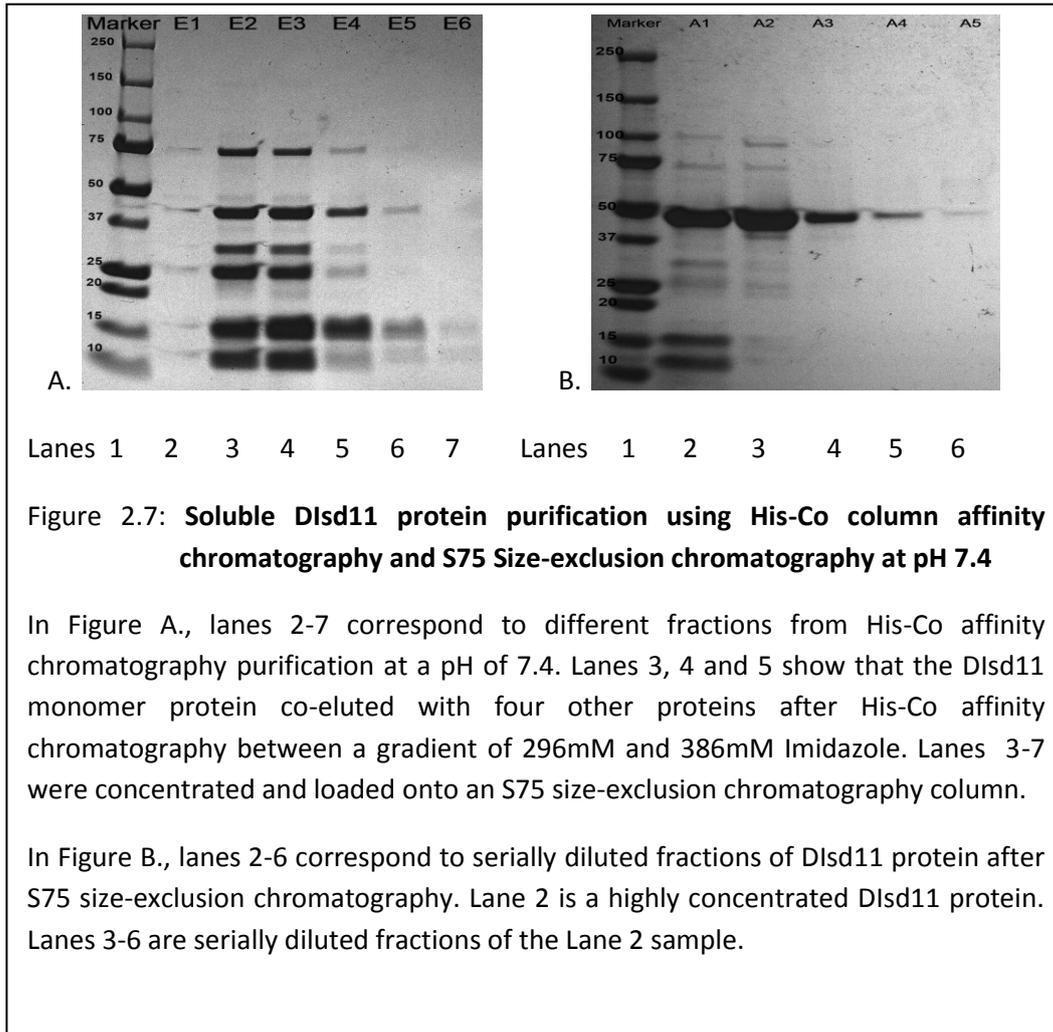
DIsd11 was best expressed at 0.2mM IPTG, 0.9OD, 37°C, 2 hours. Lanes 2-5 correspond to samples from the best expressed condition. Lane 4 shows best soluble protein expression. 'PI' – Pre-induced, 'I' - Induced, 'S' – Supernatant and 'P' – Pellet.

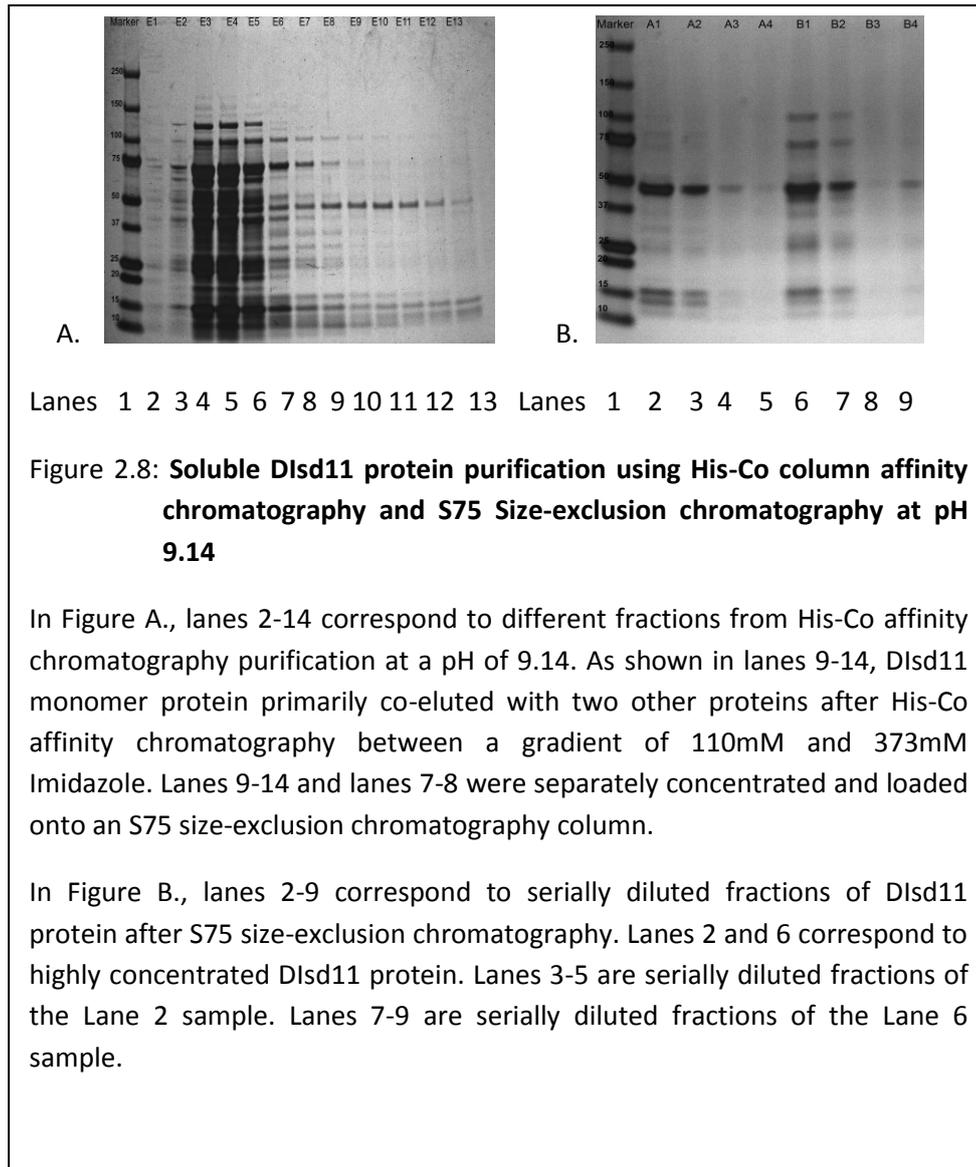


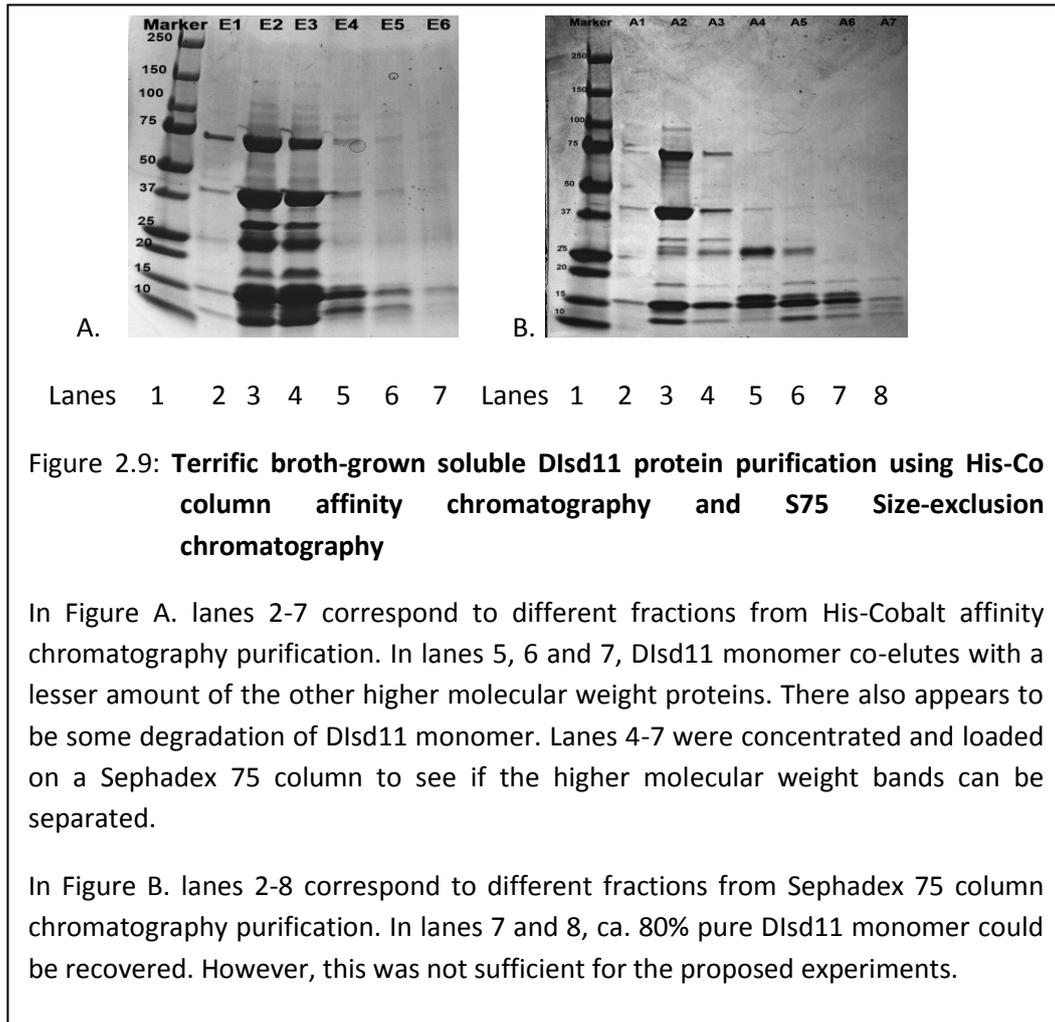
harvesting. A vital point to be noted is that in every SDS-PAGE gel the Isd11 monomer ran at ~15kDa instead of 13.5kDa (with the tag). This was confirmed by comparing the target samples with the induced samples of Isd11.

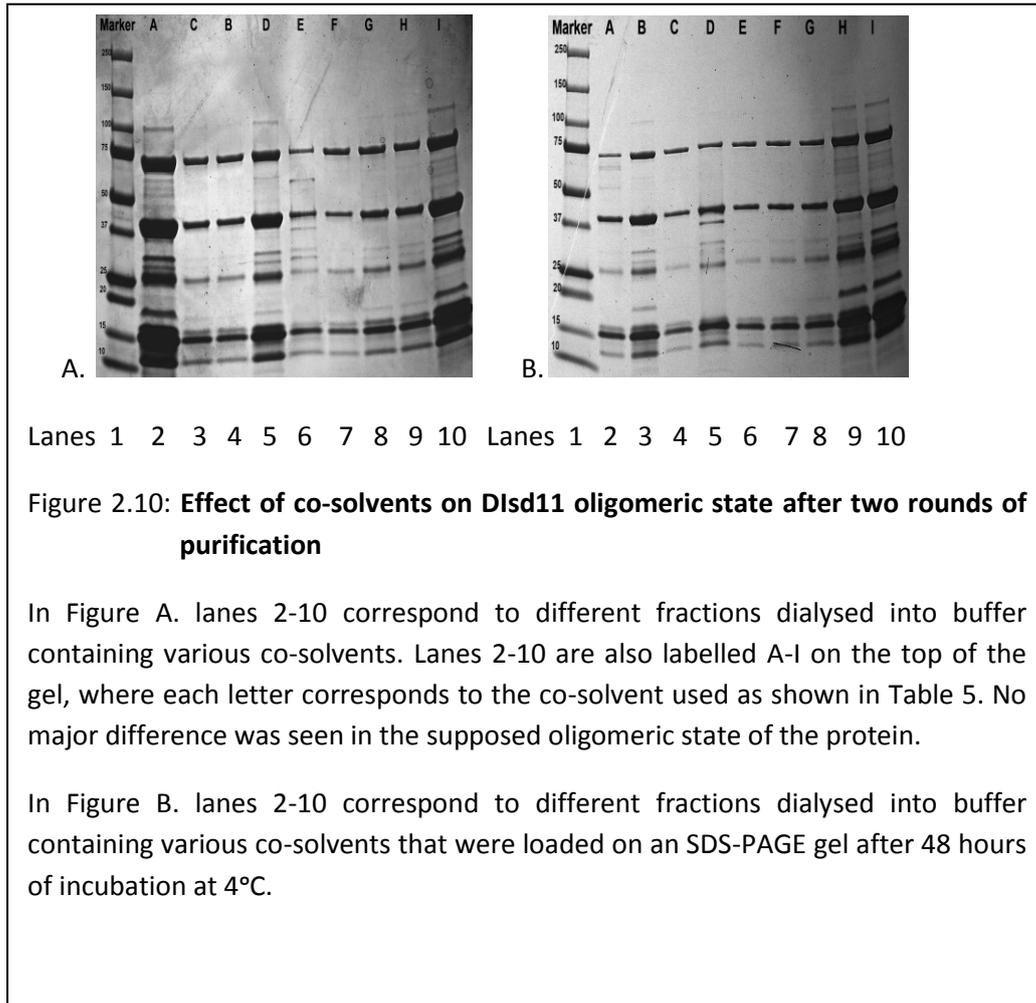
### **2.3.3. Protein purification of soluble DIsd11**

Protein purification was the next step and after two rounds of purification through HisPur Cobalt and HiLoad 16/10 Superdex 75 columns, DIsd11 monomer could not be isolated. The protein consistently eluted out with four other proteins of higher molecular weights (Figures 2.7). On increasing the pH to 9.14 there seemed to be no difference in the purity of the protein (Figures 2.8). Multiple other strategies were adopted for solving the problem of co-elution. In one strategy, a nutrient-rich growth medium, Terrific broth (Mo Bio Laboratories) was used for cell growth. Although the overall soluble protein expression was high, the co-eluted proteins could not be separated effectively (Figure 2.9). There appeared to be some DIsd11 monomer, but this was not sufficient for the proposed experiments. The co-eluted proteins were assumed to be oligomers of DIsd11 monomer because literature suggests that Isd11 has a tendency to exist as oligomers with cysteine desulfurase in the cell. In a second strategy, the purified protein was dialysed using a Micro Bio-spin 6 column into 7 different co-solvents, each of which was dissolved in a 1mM ZnSO<sub>4</sub>, 50mM Tris-HCl (pH 8.0) and 150mM KCl buffer. The co-solvents used were 1M Urea, 10mM DTT, 1%TFA, 1M Cysteine, 10%DMSO, 40%Methanol, 5%  $\alpha$ -D-glucose (Table 3). None of the samples showed a significant difference suggesting that the supposed oligomers were probably not reversible after purification (Figure 2.10). However, a limitation to this conclusion could be that Zinc was a constituent of the









Buffer	Co-solvent	
50mM Tris-HCl, pH 8.0, 1mM ZnSO <sub>4</sub> , 100mM KCl	A	5M Urea (Denaturant)
	B	10mM DTT (Reducing agent)
	C	1% TFA (Stabilizes $\alpha$ helices)
	D	1M Cys (DNfs Reaction substrate)
	E	10% DMSO (Denaturant)
	F	40% Methanol (Salting out)
	G	5% Glucose (Reduces aggregation)
	H	Control

**Table 3: Co-solvent categorization for oligomer stability experiment**

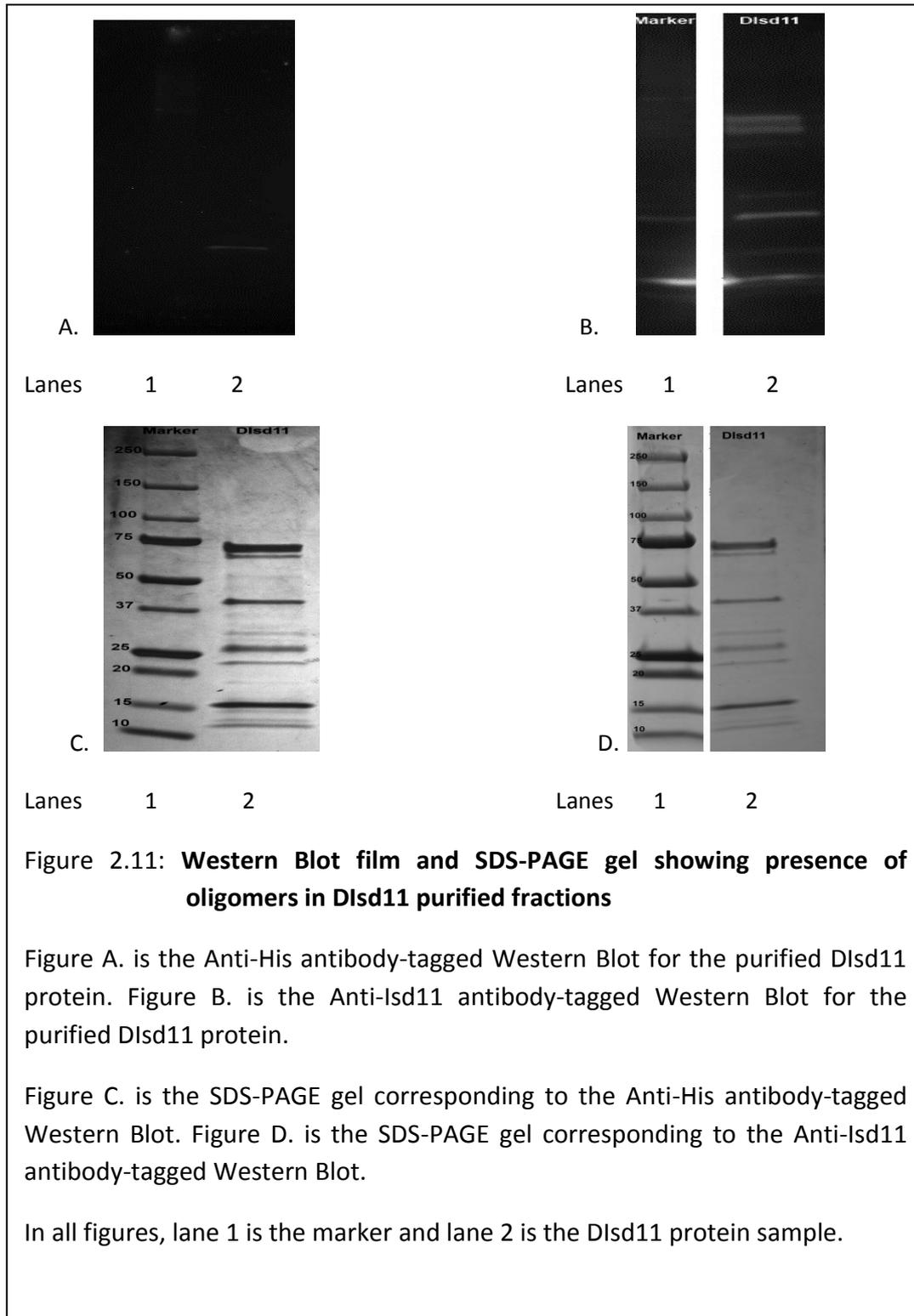
buffer. Many Fe-S cluster assembly proteins are known to bind to Zinc and if this happens with Isd11 as well, the oligomer stability maybe increased by Zinc.

#### **2.3.4. Western Blot using Anti-His antibody and Anti-Isd11 antibody**

The purification results hinted at the immediate need for a Western blot of the purified Isd11 protein fractions. Two blotting experiments were performed. In the first experiment, an anti-His primary antibody was used and in the second experiment, an anti-Isd11 primary antibody was used. The rationale for performing two blots was preceded by our hypothesis that the four proteins that co-eluted could be oligomers of Isd11 and that if oligomerized, the His-tag maybe difficult to detect by the primary anti-His antibody. The anti-His blot revealed only the monomer of Isd11. However, the anti-Isd11 blot revealed all four co-eluted proteins in addition to the monomer of Isd11 (Figure 2.11). This confirmed our hypothesis that the co-eluted proteins are indeed, oligomers of Isd11. However, an alternative explanation could also mean that the four proteins are cross-reactive proteins of the anti-Isd11 polyclonal primary antibody.

#### **2.3.5. Circular Dichroism of DIsd11 oligomer-monomer mixture**

Once the identity of the purified Isd11 fraction was confirmed, our next aim was to characterize the folded state of the protein. The averaged CD spectra from 340nm to 180nm (Figure 2.12) suggested that the protein was primarily alpha-helical in nature (Table 4). However, the saturation of the UV lamp at the end of 180nm slightly distorted the values to favour beta strands over alpha-helices. This could be rectified if the saturation can be avoided by identifying the right protein concentration. Another variable associated with the performed experiment was that

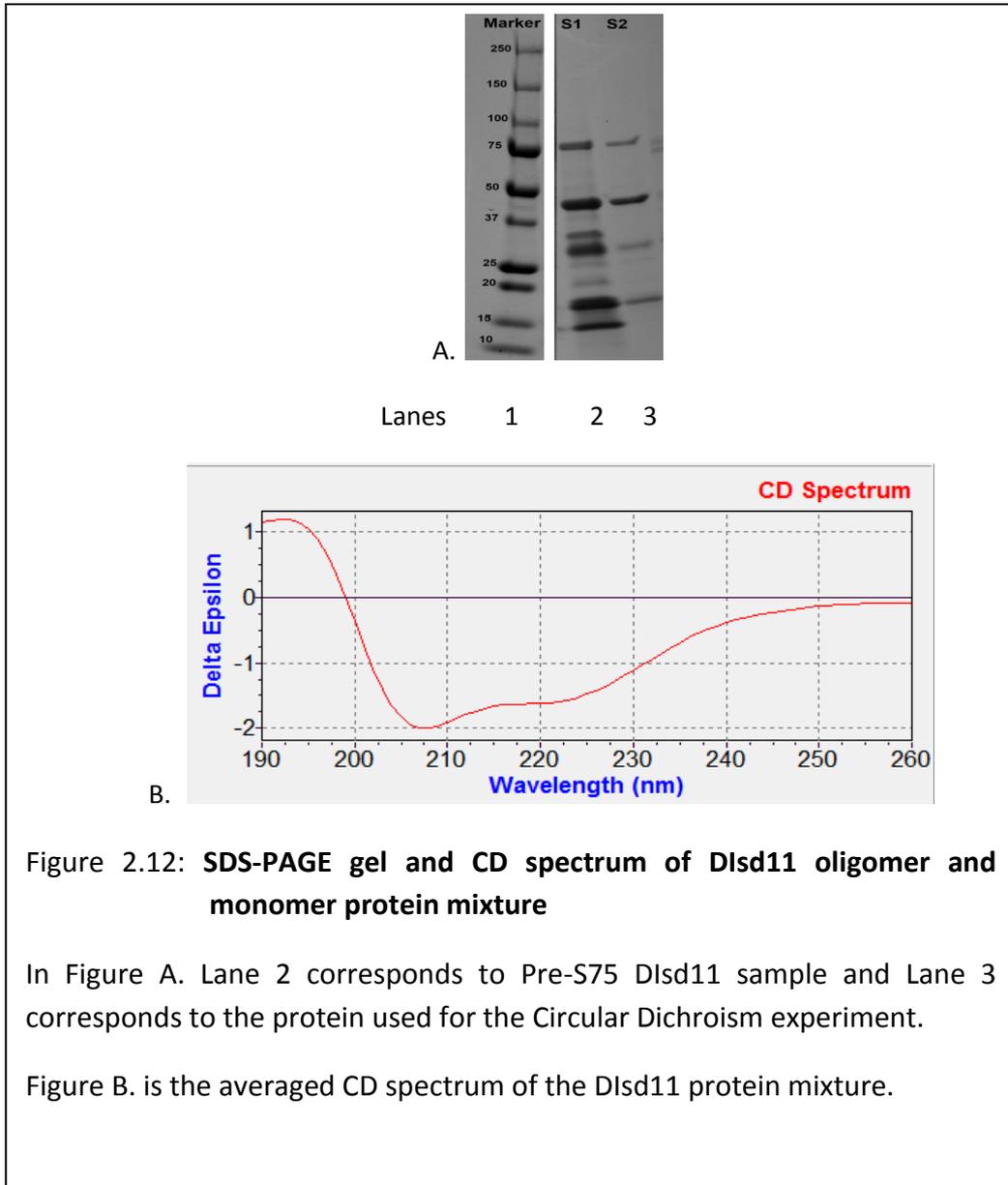


**Figure 2.11: Western Blot film and SDS-PAGE gel showing presence of oligomers in DIsd11 purified fractions**

Figure A. is the Anti-His antibody-tagged Western Blot for the purified DIsd11 protein. Figure B. is the Anti-Isd11 antibody-tagged Western Blot for the purified DIsd11 protein.

Figure C. is the SDS-PAGE gel corresponding to the Anti-His antibody-tagged Western Blot. Figure D. is the SDS-PAGE gel corresponding to the Anti-Isd11 antibody-tagged Western Blot.

In all figures, lane 1 is the marker and lane 2 is the DIsd11 protein sample.



<b>Secondary structure</b>	<b>190-260nm</b>	<b>Standard deviation</b>
$\alpha$ helices	19 %	2 %
Antiparallel $\beta$	24 %	1 %
Parallel $\beta$	9 %	1 %
$\beta$ -turn	17 %	1 %
Random coil	31 %	1 %

**Table 4: Secondary structure content of DIsd11 protein oligomer-monomer mixture**

the profile reflects only the folded state of the protein mixture and not the folded state of the oligomer or monomer individually.

#### **2.4 Future directions**

*Drosophila* Isd11 is an interesting protein that is ca. 40% soluble when grown in BL21 (DE3) strains of *E.coli* cell lines. The soluble protein is however, difficult to purify in any one of its oligomeric states. Another notable feature is that the protein runs at a molecular weight 4kDa higher than its original molecular weight. This is consistent with comparisons to induced samples and also from a western blot experiment. This strange trait could be because of zinc binding to the protein or some other metabolite from the growth media. None of these possibilities have been testified and hence, requires further characterization of the protein.

The limited purity of one state of the protein also makes it difficult to predict the secondary structure of DIsd11 monomer or one oligomer alone using Circular dichroism. Although we have not been able to isolate one form of the protein from the soluble fraction in our lab, we have been able to purify the monomer of Isd11 using 8M Urea (data not shown). This rigorous method usually interferes with the folded state of the protein. Methods to dialyse out the urea using 3kDa MWCO Centricons or 3kDa MWCO Dialysis bags have not been successful. Interestingly, dialysis of Urea-purified Isd11 into Urea-free buffer has been successful in the yeast system when passed through a desalting column and the protein did not oligomerise on Urea removal. A limitation to this method is however, the limited recovery of protein as most of the Isd11p (yeast system) precipitates after dialysis. Yet, enough protein could be recovered for a Circular dichroism experiment. Nevertheless, Urea-purified DIsd11 awaits the same desalting buffer-exchange treatment.

One useful technique for characterising protein-protein interactions is Isothermal Titration Calorimetry (ITC). In one of the several ITC experiments performed in our lab using Urea-purified DIsd11 and Urea-purified DNfs, DIsd11 was found to interact with DNfs (*Drosophila* cysteine desulfurase) with  $\mu\text{M}$  to nM affinity in a two-site binding model (data not shown). It was interesting to see an endothermic profile during the binding event. In addition, DIsd11 showed no binding if left in Urea for 5-7 days and more. Following ITC, both the proteins were tested using a cysteine desulfurase activity assay and this revealed that Urea-purified DNfs was inactive. These results hinted at the limited usefulness of Urea-purified DIsd11 and DNfs for binding characterization and therefore, the ITC experiments were not pursued further. If a definitive solution is found to remove Urea from the two proteins, there could be some success in following this strategy for binding characterization.

In-vivo studies have not yet revealed the exact binding motif of Isd11 with cysteine desulfurase, Isu[5] and Frataxin[6]. In future, experiments should be directed towards creating deletion mutants of Isd11 to understand the binding event in the Fe-S cluster biogenesis complex. The mystery surrounding the functionality of the Isd11 LYR motif could also be resolved.

## 2.5 References

1. Wiedemann, N., et al., *Essential role of Isd11 in mitochondrial iron-sulfur cluster synthesis on Isu scaffold proteins*. EMBO J, 2006. **25**(1): p. 184-95.
2. Adam, A.C., et al., *The Nfs1 interacting protein Isd11 has an essential role in Fe/S cluster biogenesis in mitochondria*. EMBO J, 2006. **25**(1): p. 174-83.

3. Pandey, A., et al., *Persulfide formation on mitochondrial cysteine desulfurase: enzyme activation by a eukaryote-specific interacting protein and Fe-S cluster synthesis*. *Biochem J*, 2012. **448**(2): p. 171-87.
4. Lill, R., et al., *The role of mitochondria in cellular iron-sulfur protein biogenesis and iron metabolism*. *Biochim Biophys Acta*, 2012. **1823**(9): p. 1491-508.
5. Shan, Y., E. Napoli, and G. Cortopassi, *Mitochondrial frataxin interacts with ISD11 of the NFS1/ISCU complex and multiple mitochondrial chaperones*. *Hum Mol Genet*, 2007. **16**(8): p. 929-41.
6. Shi, Y., et al., *Human ISD11 is essential for both iron-sulfur cluster assembly and maintenance of normal cellular iron homeostasis*. *Hum Mol Genet*, 2009. **18**(16): p. 3014-25.

## Chapter 3 Characterisation of DNfs

### 3.1 Prelude

Cysteine desulfurase was the first protein identified and characterised in the Iron sulphur cluster biogenesis pathway. In every pathway dedicated for iron-sulfur cluster biogenesis cysteine desulfurase is a common factor and plays the same vital role of providing sulphur equivalents from cysteine. There is no substitute for cysteine desulfurase in this pathway and therefore, an abrogation of cysteine desulfurase is lethal for cell viability [1]. The enzyme is notated in many ways depending on the pathway or cell-type under consideration. For example, the enzyme is called NifS (in the NIF pathway), SufS (in the SUF pathway), IscS (in the ISC pathway), Nfs1p (in yeast) and NifS (in humans)[2]. For convenience, the enzyme will be called Nfs for the rest of the discussion.

Nfs has been identified in the nucleus, cytosol and mitochondrial matrix. The enzyme's localisation in three distinct parts of a cell is concordant with its functional diversity. This 'moonlighting' property of the enzyme is witnessed in tRNA thiolation and cofactor thiamine biosynthesis[3]. So much has been known about Nfs due to its structural characterisation. A crystal structure of the bacterial isoform of the enzyme, IscS was determined in 2001[4]. This led to a greater understanding of many pathways including the iron-sulfur cluster biogenesis pathway. Structural details show that Nfs is a homodimeric, PLP-dependent enzyme and each subunit can be divided into a small domain and a large domain. This feature is characteristic of  $\alpha$ -family type IV aminotransferases of PLP-dependent enzymes. The relevant features of Nfs with reference to its function are that the large domains of the Nfs dimer

house the PLP cofactor at the dimer interface. The PLP cofactor is held in place by the formation of an internal aldimine Schiff base with a lysine residue in the active site. A reactive cysteine residue present in the small domain of Nfs initiates PLP-mediated catalysis. However, the cysteine residue and the PLP cofactor are  $\sim 17\text{\AA}$  apart and require the enzyme to undergo a major conformational change. This is believed to occur with the help of a flexible loop that connects the large and small domain. The entire reaction is believed to take place in a  $\beta$ -elimination fashion.

All these facets are true for a prokaryotic system. Although IscS shares a 60% sequence identity and an 80% sequence similarity with human NifS[4], it remains to be seen whether the eukaryotic enzyme shares structural similarity with its prokaryotic isoform. This is important because latest evidence suggests that eukaryotic cysteine desulfurase is inactive in the absence of an accessory protein named Isd11, found exclusively in eukaryotes [5]. This intriguing attribute necessitates biochemical and biophysical characterisation of eukaryotic cysteine desulfurase. *Drosophila* cysteine desulfurase was chosen as a model enzyme for eukaryotes in our lab and will be notated as DNfs in the following discussion.

## **3.2 Materials and Methods**

### **3.2.1. Cloning of DNfs**

DNfs cDNA was obtained in a pOT2 vector, from the “*Drosophila* Genomic Research Center” (FlyBase ID: FBgn0032393). The DNfs sequence represents 72bp to 1457bp of the complete 1533bp open reading frame. The first 102bp of the DNfs cDNA were truncated to improve protein expression and solubility. The truncated cDNA with 1284bp was PCR amplified, sub-cloned into a pENTR/SD/D-TOPO vector

(Invitrogen) and then, recombined into pDest15 and pDest17 vectors (Invitrogen) using the Gateway Recombination Cloning technology (Invitrogen). The pDest17 vector has a (His)<sub>6</sub> tag and pDest15 has a GST tag to enhance protein expression and purification. The pDest clones were first transformed into DH5 $\alpha$  cloning strain (Invitrogen) for amplification and later transformed into BL21-AI *E.coli* competent cells. Positive clones were verified by DNA sequencing (Genewiz facility). The recombinant plasmid was transformed into BL21-AI (Invitrogen) and BL21 (DE3) *E.coli* competent cell lines for protein expression.

Alternatively, a DNfs cDNA clone with 1284bp cloned by a previous graduate student, Dr. Swati Rawat, was also available. The truncated cDNA was cloned into pET151/D-TOPO vector (Invitrogen) and transformed into BL21 (DE3) cell line.

### **3.2.2. Optimization of soluble protein expression**

The truncated DNfs protein ( $\Delta$ 1-34 DNfs) was expected to have 429 amino acids and a molecular weight of 47.46kDa (minus the expression tag). None of the clones created by Gateway Recombination cloning technology could be expressed in the transformed BL21 cell lines. The next step was to optimize protein expression using the alternative DNfs clone in pET151/D-TOPO vector (Invitrogen). For convenience, this clone will be referred to as  $\Delta$ 1-34 DNfs2 for the rest of the discussion. Soluble protein expression from  $\Delta$ 1-34 DNfs2 could not be optimized by varying IPTG concentrations, post-induction time intervals, pre-induction OD and temperatures. Based on the overall protein expression (despite the fact that the yield of soluble protein was extremely low), few conditions could be chosen for large scale protein expression and purification. For subsequent  $\Delta$ 1-34 DNfs protein

expression,  $\Delta$ 1-34 DNfs2 in BL21 (DE3) was grown at 37°C till an OD of 0.8-0.9 and then induced with 0.2mM IPTG for ca. 3-4 hours before harvesting by centrifugation.

### **3.2.3. Protein purification of soluble $\Delta$ 1-34 DNfs2**

Protein isolation steps were all performed at 4° C. Cells were resuspended in 50 mM Na<sub>3</sub>PO<sub>4</sub> (pH 7.4), 300 or 500mM NaCl, 20 mM Imidazole and 5 mM  $\beta$ -Me in the presence of Complete EDTA-free Protease inhibitor cocktail (Roche). A 20 $\mu$ g/mL of DNase and 5mM MgCl<sub>2</sub> were added before lysis to reduce viscosity of the medium at the time of filtration. Cells were then lysed by two passes through a French Press cell at high pressure (1100 psi), and centrifuged at high speed (21000 rpm) for 1 hour. Crude soluble fraction was filtered (0.20  $\mu$ m) and loaded onto a HisPur Ni-NTA column (Thermo Scientific) using an Imidazole gradient in the range of 0 – 500 mM. The available soluble  $\Delta$ 1-34 DNfs2 containing fractions were pooled and concentrated to ca. 1mL by centrifugation using 10kDa Molecular Weight Cut Off (MWCO) centricons (Millipore) spun at 5000 rpm. The concentrated retentive solution was run over a HiLoad 16/10 Superdex 75 size exclusion column (General Electronics) equilibrated with 50mM Na<sub>3</sub>PO<sub>4</sub> buffer (pH 7.4), 500 mM NaCl. The fractions obtained were analysed on 10% or 4-20% SDS-PAGE gels (Bio-Rad).

### **3.2.4. Auto-induction protocol for soluble $\Delta$ 1-34 DNfs2 expression**

For the Auto-induction protocol, fresh media was prepared using 5g BactoTryptone, 2.5g Bacto-Yeast extract, 1M MgSO<sub>4</sub>, 50X 5052 (0.5% Glycerol, 0.05% Glucose and 0.2%  $\alpha$ -lactose), 20X NPS (0.5M Ammonium sulphate, 1M Potassium phosphate dibasic and 1M Sodium phosphate monobasic) and 100mg/mL Ampicillin. The medium was inoculated with a starter culture and allowed to grow at 27°C for

24 hours. After 24 hours, the cultures were harvested by centrifugation. Protein purification was then performed by the already described protocol.

### **3.2.5. Double-colony selection for soluble $\Delta$ 1-34 DNfs2 expression**

The  $\Delta$ 1-34 DNfs2 gene in pET151/D-TOPO was first transformed into BL21(DE3) competent cell lines and positive clones were selected. One half of the selected colonies were grown at 37°C till an OD of 0.8-0.9 and then induced with 0.2mM IPTG for ca. 3-4 hours. The other half was then re-plated on a fresh LB agar plate coated with Ampicillin. Fresh positive clones were selected and expressed again at the above-mentioned conditions.

## **3.3 Results**

### **3.3.1. Cloning of DNfs**

N-terminal truncation of proteins is a common strategy used in labs to improve solubility of proteins. Experimental evidence in the yeast model suggests that Nfs is insoluble in its native form. Therefore, the same strategy was used for our Drosophila model. Using 'Mitoprot' software the first 34 amino acids at the N-terminus were found to be the mitochondrial targeting sequence (MTS) and could be safely removed without altering the functionality of the protein. Truncated DNfs ( $\Delta$ 1-34) was successfully cloned into pENTR/SD/D-TOPO vector, recombined into pDest15 and pDest17 vectors, restriction digested after cloning and sequence analysed to confirm the presence of the gene in the vector (Figure 3.1). The sequence analysis of the pDest15 clone required further verification as the T7 forward primer could not detect the first part of the gene due to the large GST tag sequence. On sequence verification of the gene in pDest17, it was confirmed that the selected DH5 $\alpha$  cloning

```

DNfspd      MQKVLGRIQAQVLRSRATNALANVVRHEATSSRTAAKPAATSKEFRERQVRFNIKNEQTE 60
Nfsp17C2    -----MAAKPAATSKEFRERQVRFNIKNEQTE 27
              *****

DNfspd      GRPLYLDAQATTPMDPRVLDAMLPLYLTNFYGNPHSRTHAYGWETE SAVEKAREQVATLIG 120
Nfsp17C2    GRPLYLDAQATTPMDPRVLDAMLPLYLTNFYGNPHSRTHAYGWETE SAVEKAREQVATLIG 87
              *****

DNfspd      ADPKEIIFTSGATESNNIAVKGVARFYGTKKRHVITTTQTEHKCVLDSCRALENEGFKVTY 180
Nfsp17C2    ADPKEIIFTSGATESNNIAVKGVARFYGTKKRHVITTTQTEHKCVLDSCRALENEGFKVTY 147
              *****

DNfspd      LPVLANGLIDLQQLEETITSETSLVSIMTVNMEIGVRQPVDEIGKLCRSRRVFFHTDAAQ 240
Nfsp17C2    LPVLANGLIDLQQLEETITSETSLVSIMTVNMEIGVRQPVDEIGKLCRSRRVFFHTDAAQ 207
              *****

DNfspd      AVGKVPDWNAMNIDLMSISGHKIYGPKGVGALYVRRRPRVRLEPIQSGGGQERGLRSGT 300
Nfsp17C2    AVGKVPDWNAMNIDLMSISGHKIYGPKGVGALYVRRRPRVRLEPIQSGGGQERGLRSGT 267
              *****

DNfspd      VPAPLAVGLGAAAELSLREMDYDKKVVDFL SNRLLDRISSALPHVIRNGDAKATYNGCLN 360
Nfsp17C2    VPAPLAVGLGAAAELSLREMDYDKKVVDFL SNRLLDRISSALPHVIRNGDAKATYNGCLN 327
              *****

DNfspd      LSFAYVEGESLLMALKDVALSSGSACTSASLEPSYVLRAIGTDEDLAHS SIRFGIGRFTT 420
Nfsp17C2    LSFAYVEGESLLMALKDVALSSGSACTSASLEPSYVLRAIGTDEDLAHS SIRFGIGRFTT 387
              *****

DNfspd      VEEVDYTADKCIKHVERLREMSPLWEMVQEGIDLKTIQWSQH 462
Nfsp17C2    VEEVDYTADKCIKHVERLREMSPLWEMVQEGIDLKTIQWSQH 429
              *****

```

Figure 3.1: Sequence verification of pDest17 Δ1-34 DNfs clone

'DNfspd' is the DNfs sequence available in Pubmed and 'Nfsp17C2' is the sequence of the newly cloned DNfs gene.

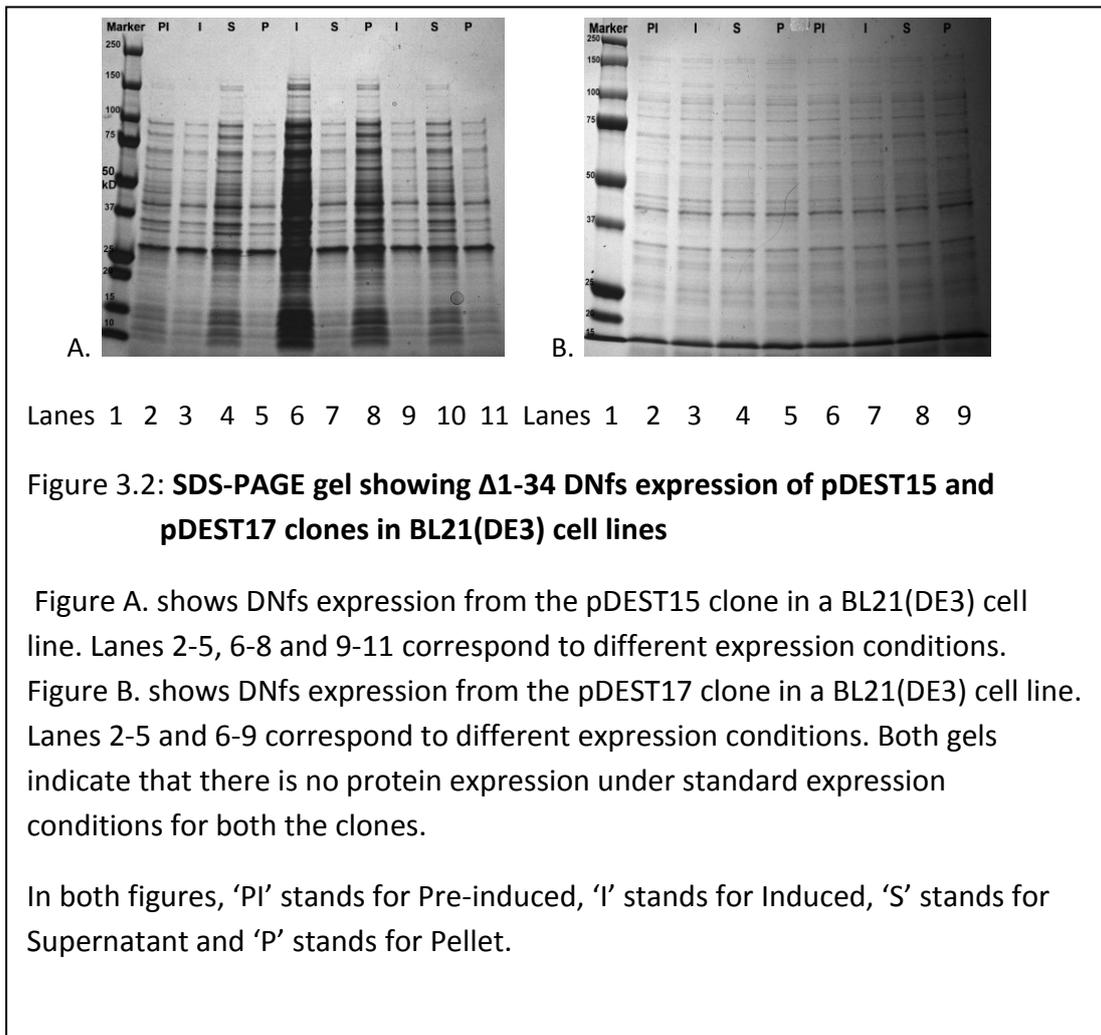
strain transformant and the BL21-AI expression strain transformant had the right clone. The pDest17 positive clones were selected and also transformed into BL21(DE3) expression strain for protein expression. The  $\Delta 1-34$  DNfs2 clone was also transformed into BL21(DE3) for protein expression.

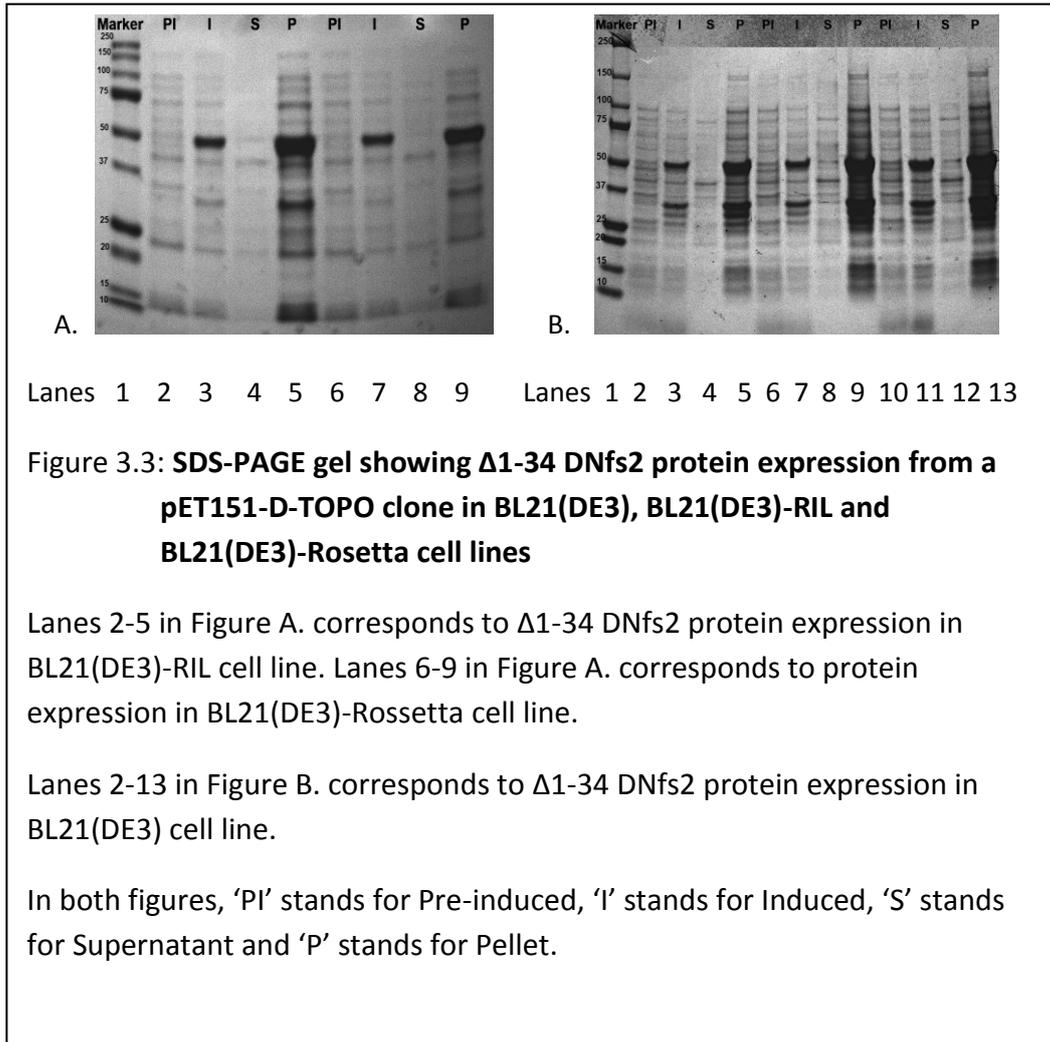
### **3.3.2. Optimization of soluble protein expression**

Different expression conditions were tried and yet, none of the colonies with positive clones expressed  $\Delta 1-34$  DNfs (Figure 3.2). The reason for the lack of protein expression could not be tracked. Contrary to these unexpected results, the  $\Delta 1-34$  DNfs2 clone was able to express protein in the BL21(DE3) expression strain. However, ca. 95% of the protein expressed from BL21(DE3) was insoluble (Figure 3.3B). The clone was also transformed into BL21(DE3)-RIL and BL21(DE3)-Rossetta expression strains; but the results did not vary (Figure 3.3A). Based on overall protein expression the  $\Delta 1-34$  DNfs2 clone in BL21(DE3) was used for subsequent large scale protein expression and purification and was grown at 37°C at a pre-induction OD of 0.8-0.9 and induced with 0.2mM IPTG for 3-4 hours. The protein with its His<sub>6</sub> tag was expressed at a molecular weight corresponding to ca. 48kDa.

### **3.3.3. Protein purification of soluble $\Delta 1-34$ DNfs2**

Protein purification was the next step and after two rounds of purification through HisPur Ni-NTA and HiLoad 16/10 Superdex 75 columns, no pure protein could be isolated. The protein consistently eluted out with ca. 80% of bacterial proteins (Figure 3.4). A possible reason for the inability to purify  $\Delta 1-34$  DNfs2 could be the drop of protein pI from 8.40 in its native form to 6.58 in its  $\Delta 1-34$  truncated





form. With repeated attempts at large scale protein expression and purification we concluded that it was not worth pursuing the large scale expression the truncated form of DNfs using conventional strategies.

#### **3.3.4. Auto-induction for soluble $\Delta$ 1-34 DNfs2 expression**

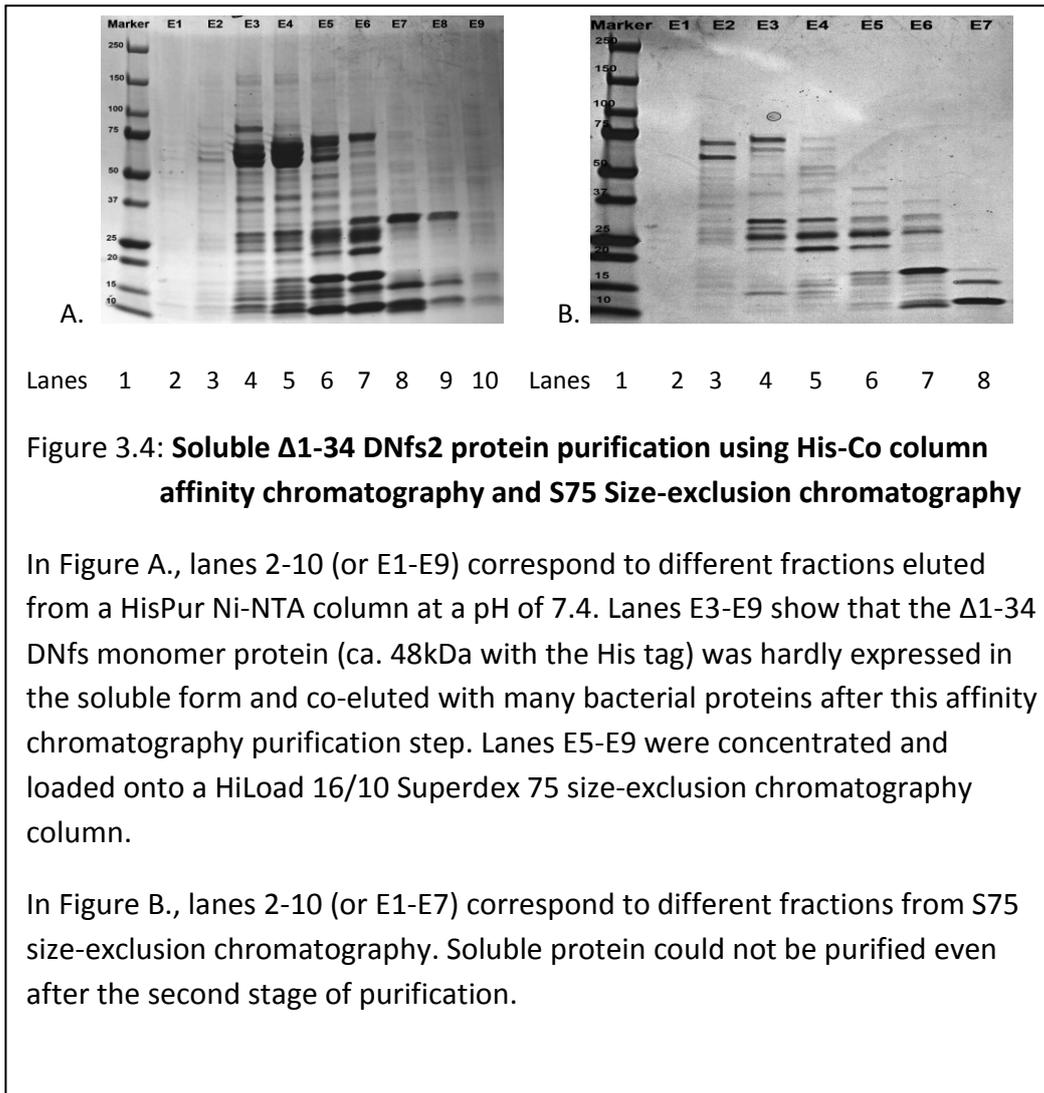
The Auto-induction protocol caused lesser bacterial protein expression than  $\Delta$ 1-34 DNfs2 (Figure 3.5). This was an improvement from the conventional strategy of IPTG-induced expression. However, on lysis and protein purification sufficient soluble protein could not be recovered and purified. This suggests that although the overall protein expression is high, most of the protein is insoluble.

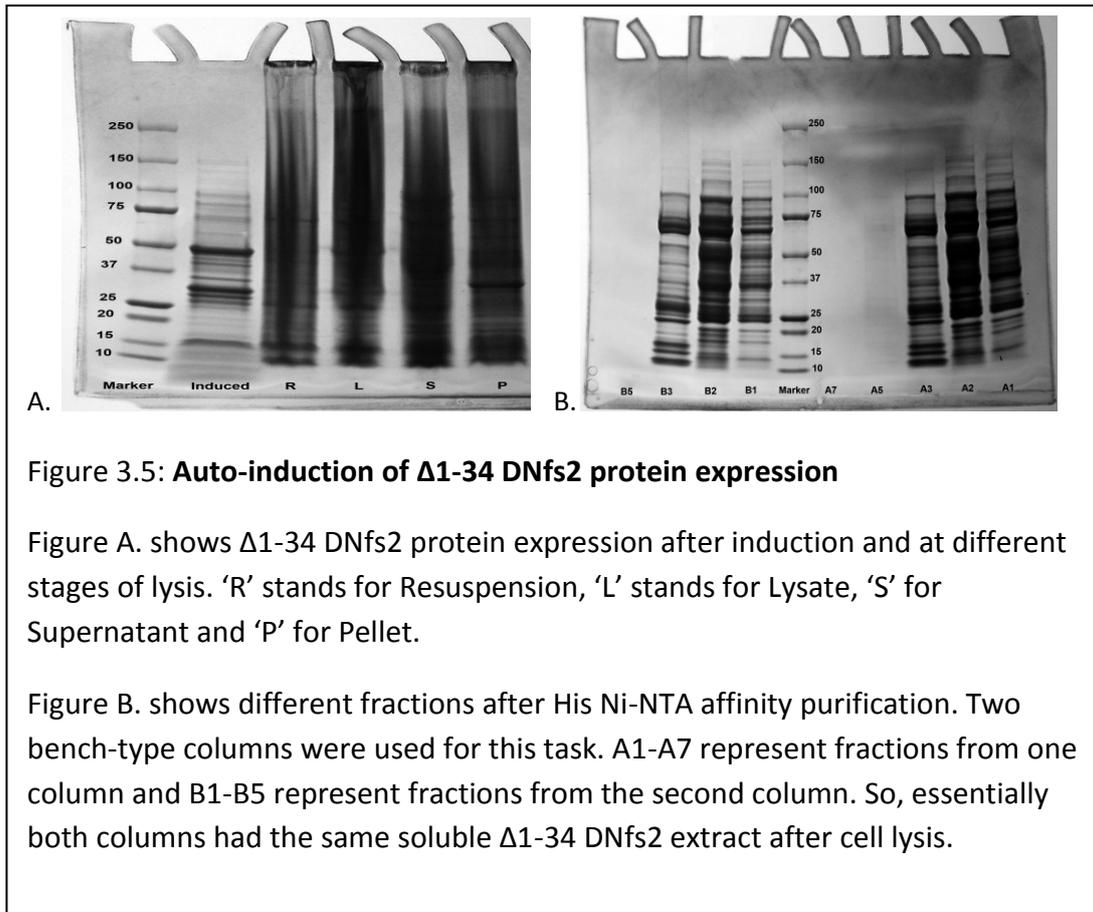
#### **3.3.5. Double colony selection for soluble $\Delta$ 1-34 DNfs2 expression**

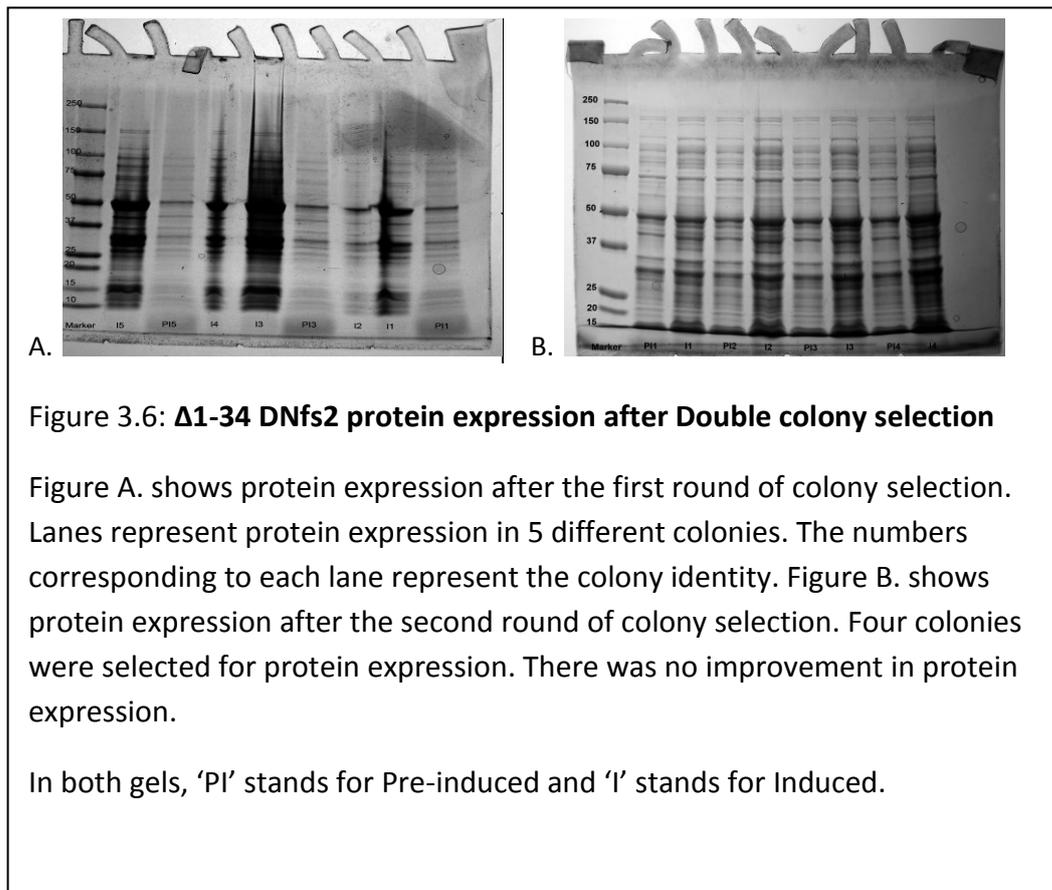
By the end of the second round of colony selection and expression, the intensity of a lower band ca. 30kDa in size was much more than the first round of selection and expression (Figure 3.6). This lower band is speculated to be a degradation product of the truncated protein when expressed in BL21(DE3). However, this hypothesis can be confirmed only if a Western Blot of the expressed protein sample is carried out.

### **3.4 Future Directions**

DNfs is an extremely insoluble protein and could not be purified with a conventional strategy of IPTG induction or even, auto-induction. 26.4% of amino acids in the native state of the protein are positively and negatively charged residues. In the truncated form of the protein as well, 26.57% of amino acids are positively and negatively charged residues (ExPASy ProtParam program). A key point







to be noted is that the theoretical pI of the protein drops from 8.40 in its native state to 6.58 in its truncated form. The impact of this change in pI could influence solubility. After cloning the truncated form of the gene, one feature was noted on close observation. In both the  $\Delta 1-34$  DNfs-pDest17 clone and the  $\Delta 1-34$  DNfs2-pET151/D-TOPO clone, a substitution mutation occurred at exactly one amino acid after cloning. In the first clone, the last amino acid histidine was substituted to glutamine and in the second clone, the 384<sup>th</sup> amino acid, arginine was substituted to cysteine. It is interesting to see if the mutation occurs due to the genetic machinery of the DH5 $\alpha$  cloning strain. Whether this single amino acid mutation causes a major change in expression and solubility is a question that needs to be addressed.

The lack of soluble protein expression in few conventional *E.coli* expression systems renders  $\Delta 1-34$  DNfs2 an unfit clone to work with unless a novel strategy is identified. An alternative strategy could involve creating different truncations of the gene like  $\Delta 1-37$  DNfs,  $\Delta 1-60$ DNfs or any other truncation before the 70<sup>th</sup> amino acid. The reason for restricting truncations to the 70<sup>th</sup> amino acid originates from a comparison with the crystal structure of IscS, *E.coli* cysteine desulfurase. Recent evidence in the yeast model suggests that mitochondrial cysteine desulfurase undergoes two truncations at its N-terminal sequence by two different processing enzymes – MPP (Mitochondrial Processing peptidase) and Icp55[6]. The second truncation apparently occurs after the Mitochondrial Targeting Sequence (MTS) is cleaved off. The Mitoprot program does not account for the second truncation while calculating the MTS. In yeast Nfs1p, Icp55 cleaves off three amino acids after the MTS. The same rationale has been used to suggest a  $\Delta 1-37$  DNfs truncation.

Evidence in the human and yeast systems suggest that different isoforms of cysteine desulfurase arise from a single transcript by alternative utilisation of in-frame AUGs [7]. This means that the mitochondrial and nuclear forms of the protein arise from the same transcript. Therefore, creating a  $\Delta 1-60$ DNfs truncation would be another useful strategy.

### 3.5 References

1. Li, J., et al., *Yeast mitochondrial protein, Nfs1p, coordinately regulates iron-sulfur cluster proteins, cellular iron uptake, and iron distribution*. J Biol Chem, 1999. **274**(46): p. 33025-34.
2. Lill, R., et al., *The role of mitochondria in cellular iron-sulfur protein biogenesis and iron metabolism*. Biochim Biophys Acta, 2012. **1823**(9): p. 1491-508.
3. Mihara, H. and N. Esaki, *Bacterial cysteine desulfurases: their function and mechanisms*. Appl Microbiol Biotechnol, 2002. **60**(1-2): p. 12-23.
4. Cupp-Vickery, J.R., H. Urbina, and L.E. Vickery, *Crystal Structure of IscS, a Cysteine Desulfurase from Escherichia coli*. Journal of Molecular Biology, 2003. **330**(5): p. 1049-1059.
5. Pandey, A., et al., *Persulfide formation on mitochondrial cysteine desulfurase: enzyme activation by a eukaryote-specific interacting protein and Fe-S cluster synthesis*. Biochem J, 2012. **448**(2): p. 171-87.
6. Naamati, A., et al., *Dual targeting of Nfs1 and discovery of its novel processing enzyme, lcp55*. J Biol Chem, 2009. **284**(44): p. 30200-8.

7. Marelja, Z., et al., *A novel role for human Nfs1 in the cytoplasm: Nfs1 acts as a sulfur donor for MOCS3, a protein involved in molybdenum cofactor biosynthesis.* J Biol Chem, 2008. **283**(37): p. 25178-85.

## Chapter 4 Summary

### 4.1 Characterisation of DIsd11

*Drosophila* was chosen as our model organism for two main reasons. Firstly, many geneticists have used *Drosophila* to study neurodegenerative disorders and the organism seems to be an excellent model for these. Secondly, previous studies in our lab with *Drosophila* proteins like Dfh (Fratxin) and DIsu (Isu homolog), both of which are involved in iron-sulphur cluster biogenesis pathway have shown that the proteins are more stable when purified. Such a stable system was required for our characterisation of Isd11 and Nfs, as proteins that form a complex are known to be labile in the absence of its partner. Yeast-based studies have shown signs of degradation or aggregation of Nfs in the absence of Isd11, so there is a question of extended sample integrity for the yeast proteins. Our first protein characterised in this research project was DIsd11.

DIsd11 could be expressed well in an *E.coli* expression strain BL21(DE3). The solubility of the protein improved only at low IPTG concentrations and could be stably expressed at 37°C. The induction time was also lowered to reduce precipitation and the insoluble form of the protein. This observation was consistent even on a large scale. Once soluble DIsd11 was obtained, the next step was to purify the protein, which proved to be a challenging task. Two rounds of purification involving His-Co purification in the first step and Superdex 75 Size exclusion chromatography in the second step were used, and yet pure protein could not be obtained. Several different strategies were adopted and repeated failed attempts

suggested to us that we need to perform a Western Blot to identify the nature of the rest of the co-eluted proteins. Surprisingly, all the major co-eluted proteins were detected on an Anti-Isd11 Western Blot. This gave us increasing confidence to use the protein mixture containing possible oligomers and the monomer of DIsd11 for Circular Dichroism. However, such a CD experiment could only detect the overall secondary structure of DIsd11 and lacked specificity.

Latest developments in our lab using Desalting columns for buffer-exchange have allowed YIsd11 recovery from Urea-solubilised insoluble YIsd11. CD profiles of the so-obtained re-folded protein show a high degree of proper folding. This strategy needs to be followed in future for proper secondary structure characterisation of DIsd11 as well. Interestingly, YIsd11 does not oligomerise on re-folding. Future characterisation of the Isd11 protein should focus on the reasons for Isd11 oligomerisation *in vitro*. In addition, the function of the LYR motif needs to be predicted and also, the exact binding sites of Isd11 on Nfs for cysteine desulfurase activity.

#### **4.2 Characterisation of DNfs**

Simultaneously, DNfs needed to be characterised for any subsequent biophysical characterisation of the Cysteine desulfurase complex with Isd11. DNfs offered a bigger challenge than DIsd11. The protein showed signs of expression but almost 95% of the protein accumulated in the insoluble fraction. Insoluble proteins normally assemble in a cell as inclusion bodies and are difficult to re-fold back to the native state. However, in a series of purification protocols, DNfs was extracted from inclusion bodies using 6 - 8M Urea. All refolding attempts culminated in the

precipitation of the protein and loss of activity. So, expression conditions and purification protocols had to be designed to produce large amounts of soluble protein.

All proteins targeted for the mitochondria show a high degree of floppiness in its N-terminal Mitochondrial Targeting Sequence (MTS) and surprisingly, MTS removal is found to improve protein solubility. So, the first 34 amino acids of DNfs were removed and the newly cloned gene had only 429 amino acids. Expression tests suggested that the protein could be expressed well; but, the issue of solubility persisted even after truncation.

Various expression protocols like the auto-induction protocol and double-colony selection were performed in addition to the conventional method of IPTG induction. None of these protocols showed promising results. With the little protein available, protein purification was also tried. Protein solubility tremendously influenced the outcome of purification and hence, yielded no pure protein. It appears as though the native form of DNfs and its truncated version are poor models for DNfs characterisation using *E.coli*. Site-directed mutagenesis experiments need to be performed to recreate the original DNfs gene from the single amino acid mutated DNfs clones to verify the above conclusion. If mammalian cell lines or other eukaryotic cell lines are not used, DNfs characterisation may continue to pose problems in future. However, this does not mean that alternative truncations of the protein like  $\Delta 1-37$  or  $\Delta 1-60$  DNfs would not improve expression and solubility in *E.coli*. Comparisons with the crystal structure of the bacterial counterpart, IscS allow

us to truncate amino acids till the 60<sup>th</sup> residue without altering the function of the protein. Hence, these strategies are also worth exploring.

### **4.3 Characterisation of both the proteins**

Simple ITC experiments have been performed in our lab using Urea-purified  $\Delta 1-34$  DNfs2 and DIsd11. One of the trials showed a high endothermic heat absorption when Urea-purified DIsd11 was titrated into Urea-purified  $\Delta 1-34$  DNfs2. Unfortunately, these experiments were not pursued further because the enzyme appeared to be thoroughly unfolded. A binding event alone was not enough for our hypothesis. Our aim was to use the native state of both proteins and predict the binding event as well as conformational changes during persulfide formation. The results from this strategy would be more conclusive when coupled with the cysteine desulfurase activity assay as opposed to using Urea-denatured proteins.

A simple remedy to the problem of expressing both the proteins separately and then allowing complexation would be co-expression of both proteins on one vector. This would enable characterisation of the oligomeric state of the Isd11-cysteine desulfurase complex. Other important features of the entire Iron-sulfur cluster assembly complex can also be studied. There are still certain features of the complex that can be estimated only if both the proteins are expressed and purified individually, unless one figures out how to separate both proteins from the complex. One such important study would be an isothermal titration calorimetry experiment which estimates the binding affinity of both proteins for each other. This can also roughly predict the number of binding sites on Nfs for Isd11. This technique is also useful to predict the ability of any of these proteins to bind to metals like zinc or

nickel, which is a quality commonly found among proteins involved in Fe-S cluster biogenesis.

Until a crystal structure is obtained, it would be worthwhile trying to predict the exact binding motifs within Nfs on which Isd11 monomer units bind. For this approach, multiple segments of Nfs will have to be dissected and cloned separately. Following this step, protein pull-down assays can be performed to estimate the exact binding motif. It is interesting to see where exactly Nfs incorporates Isd11 in its dimeric structure because the protein is already known to bind to five other proteins.

**ABSTRACT****CYSTEINE DESULFURASE AND ISD11: A DROSOPHILA MODEL**

by

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Iron-sulfur clusters are cofactors with evolutionary origins that date back to the pre-biotic world. Ever since life originated, these cofactors have intermingled with proteins to play vital roles in sustaining life. My research focuses on one such protein, the cysteine desulfurase (Nfs) that has the PLP cofactor incorporated in its active site and avails of the catalytic property of PLP to provide sulphur for Iron-sulfur cluster biogenesis and assembly in a cell. Interestingly, in a eukaryotic cell, despite the versatility of PLP, cysteine desulfurase's role as a "sulphur-extractor" is incomplete without another protein named Isd11. This interesting piece of evidence led us to perform basic protein characterisation of both the proteins so as to biophysically study the binding event and conformational changes during persulfide formation in future. We chose Drosophila as our model organism. We have been able to successfully create a working model for further characterisation of the binding event during persulfide formation.

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