CHAPTER FOUR

Biochemical characterization of Isd11 protein and its interaction with IscS of Leishmania donovani
4.1 Abstract

Iron-Sulfur (Fe-S) clusters are essential and labile cofactors of proteins that facilitate central metabolic processes in all organisms. The Iron-Sulfur Cluster (ISC) system performs the general house-keeping function of Fe-S cluster biogenesis in bacteria and higher eukaryotes. Leishmania spp. has retained the ISC system but lacks other systems, and for this reason we decided to study the ISC system and its components. However, no information regarding the biochemical aspect of Isd11, its interaction partners and regulation was reported in Leishmania parasite an evolving pathogen model with rapidly developing drug resistance. In this study, we have characterized Isd11 protein of Leishmania donovani (Ld-Isd11), investigated a potential interaction with other components of ISC machinery and further tried to identify its effect on the activity of cysteine desulfurase (IscS) protein. We cloned, expressed and purified recombinant L. donovani (rLd) Isd11 protein by affinity column chromatography. Subcellular localization was performed using digitonin fractionation and immunofluorescence. We also checked the protein-protein interaction and carried the physical interaction between Ld-Isd11 and Ld-IscS proteins through co-purification and pull down assay. Analysis of Ld-IscS/Isd11 complex revealed that Ld-IscS/Isd11 retained their activity towards L-cysteine and the presence of Isd11 aid in the stabilization of Ld-IscS. Moreover, intrinsic tryptophan fluorescence measurements showed that Isd11 binding induces conformational changes in Ld-IscS. As expected, Isd11 was found to be co-localized with Ld-IscS within the mitochondria further supporting our interaction and activity studies. Thus, this is the first report in Leishmania parasite where we characterized Isd11 protein and showed its biochemical properties and interaction with IscS protein.


4.2 Introduction

The biogenesis of Fe-S clusters is orchestrated by ISC system; the sulfur donor IscS, scaffold protein IscU, iron carrier Frataxin and Lsd11 novel eukaryotic desulfurase interacting protein being its core components. Biosynthesis of mitochondrial Iron-Sulfur Cluster (ISC) in yeast requires more than a dozen protein components [1] and among them, the cysteine desulfurase, IscS (yeast Nfs1), was first discovered when the entire DNA sequence of S. cerevisiae chromosome III was determined [2] and named Nfs1 on bearing certain sequence identity to NifS of nitrogen-fixing bacteria. IscS homolog is conserved and indispensable sulfur donor that requires cofactor pyridoxal phosphate (PLP) for binding of the substrate L-cysteine as well as to catalyze the formation of persulfide residue on active site cysteine [3]. Each IscS monomer contains two domains, the larger bearing the PLP cofactor binding site and the smaller bearing the active site cysteine residue in the middle of a highly flexible loop involved in catalysis [4, 5, 6]. The persulfide formation on IscS has recently been proposed to involve a significant conformational change in the enzyme, induced by interaction with Lsd11, to bring the bound substrate and active site cysteine on loop in close proximity [3]. Earlier, there was a report that transfer of sulphur from Nfs1 to the Isu1 scaffold protein occurs spontaneously. However, two independent groups reported that S. cerevisiae Nfs1 required binding of a small (11 kDa) protein called Lsd11 to fulfil its regular function for the delivery of sulphur in vivo [7, 8]. They found that Lsd11 was an essential protein of the mitochondrial matrix, loosely associated with the mitochondrial inner membrane. Although, isolated Nfs1 exhibited enzymatic activity as a cysteine desulphurase and released sulphide from cysteine in vitro, but the Nfs1/Lsd11 complex is the active sulphur donor in vivo. Lsd11, therefore, was suggested to act as a stabiliser of Nfs1. Further studies revealed that Lsd11 transiently bound to the proposed iron donor Yfh1 (Frataxin) both in vitro and in vivo, suggesting that the interaction of Frataxin with the Nfs1/Isu1 scaffold complex was mediated by Lsd11 [9, 10]. It has been suggested that Nfs1 protein is prone to aggregate and/or degrade in the absence of Lsd11 [7]. Though, Lsd11 is not required for desulfurase activity of Nfs1 in vitro, but the Nfs1-Lsd11 complex represents the functional sulfur donor, in vivo [8]. In humans, Lsd11 ortholog ISD11 is believed to play a similar conserved role in Fe-S cluster biogenesis [5] and loss of ISD11 function
resulted in a mitochondrial disorder termed as Combined Oxidative Phosphorylation Deficiency 19 (COXPD19) [11]. COXPD19 in the neonates is characterized by respiratory stress, hepatomegaly, etc.

Isd11 belongs to the LYR family of proteins, which is named for the presence of a conserved leucine-tyrosine-arginine tripeptide motif near the N-terminus. Isd11 is unique to eukaryotes, absent in prokaryotes, and required for the biogenesis of Fe-S proteins in both the mitochondria and the cytosol. Isd11 functions closely with the ß-proteobacterium-derived IscS [12]. So the absence of Isd11 from prokaryotic genome implies that its derived from endosymbiotic ancestry to eukaryotic ancestor and which installed IscS as a functional partner of Isd11 [8]. Also the functionality of Nfs1/Isd11 complex and their localization in mitosome was reported in microsporidians Encephalitozoon cuniculi and Trachipleistophora hominis and suggested essential role for the mitosome in the biosynthesis of Fe-S proteins [13]. Isd11 protein only identified in the mitochondria in yeast cell but could not detected in nucleus and cytosol. However, in human cells both the mitochondrial and nuclear localization of endogenous ISD11 have been reported [5].

In eukaryotes i.e. yeast and human models, a homologous ISC system with conserved components exists in the mitochondria except the presence of an additional Nfs1, and Isd11 has been proposed to activate and/or stabilize Nfs1 [7, 8]. Further, apart from the role in Fe-S cluster assembly in yeast, human Isd11 has been reported to regulate the normal cellular iron levels [5, 7]. The mitochondrial ISC system is solely responsible for biosynthesis of Fe-S clusters and maturation of Fe-S proteins localized in the mitochondria as well as cytosol. The role of Isd11 homologs did not explore in protozoan parasites except single report. The Isd11 is essential for tRNA thiolation has been reported in Trypanosoma brucei showing Isd11 indispensable for the thiolation of cytosolic and mitochondrial tRNAs [14]. So, we searched for the Isd11 sequence in Leishmania genome which was found to be present and thus we characterize its biochemical role in Fe-S cluster machinery. Also, we check the localization of Leishmania Isd11 protein and its influence on the conformation of its interacting partner proteins. For this, purification, biochemical and biophysical approach were used to provide insight into the binding partner of Isd11 and its possible role.
4.3 Materials and Methods

4.3.1 Ethical statement

Balb/c female mice 8-10 weeks old were used for raising antibodies after prior approval of Animal Ethical Committee, Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Indian Council of Medical Research (ICMR). The RMRIMS, ICMR follows “The Guide for the Care and Use of Laboratory Animals,” 8th edition by the Institute for Laboratory Animal Research.

4.3.2 Clinical isolates and parasites culture

L. donovani strains, Ag83 (MHOM/IN/1983/Ag83), Dd8 (MHOM/80/IN/Dd8) promastigotes used in experiments (designated, S1 & S2) were cultured at 24±1 °C in RPMI-1640 media (pH 7.2, containing 25 mM HEPES buffer) supplemented with 10% heat inactivated fetal bovine serum (FBS) (HiClone, USA) described previously [15]. Culture were initiated at 1×10^5 parasites per millilitre and grown in BOD incubator for 5-6 days.

4.3.3 Isolation of DNA and RNA

Total RNA was extracted from 2 x 10^6 L. donovani promastigotes using Trizol method (Invitrogen), according to the manufacturer’s instructions. Total DNA was isolated from promastigotes using phenol/chloroform as described previously [16]. The quality and quantity of DNA and RNA were assessed using agarose/formamide gel electrophoresis and spectrophotometer. cDNA was synthesized from 2 µg of total RNA isolated for L. donovani promastigotes using anchored oligo(dT) primer (GenHunter; H-dT11M) following the manufacturer’s instructions.

4.3.4 Multiple sequence alignment

Leishmania genome databases were extensively searched for Isd11 homologs using BLAST algorithm and LdIsd11 sequence was obtained from the Sanger sequencing project of L. donovani. Clustal W version 1.81 was used for multiple sequence alignment of LdIsd11 protein sequence with the sequences of their homologs in various organisms obtained from NCBI/DDBJ/EBI databases.
4.3.5 PCR amplification and cloning of L. donovani lsd11 gene

The polymerase chain reaction (PCR) relies on the general principle of primer-directed DNA synthesis by DNA polymerases. In the present study, L. donovani genomic DNA was used as a template for the PCR amplification of the target gene lsd11 with gene-specific primers and the Pfu DNA Polymerase.

Nfs1 and lsd11 homologs localize to the mitochondrion, and expected to carry an N-terminal mitochondrial signal sequence, which directs the proteins to the organelle. Primer synthesis was achieved externally by Primer 3 input software. Based on the nucleotide sequences of the protein-encoding region of the putative Ld-lsd11 gene, which correspond Gene ID-XM_003863922 at chromosome 33 in the genome database, two primers (shown below) were designed. The Ld-lsd11 open reading frame (ORF) was PCR amplified from genomic DNA with forward (5`ATA GGATCC ATG GTTTTCCTAG3`) and reverse (5` TTA CTCGAG CTCTATGACTGGC-3`) primers, where BamHI and XhoI-sites are underlined and the translation initiation codon is italicized.

a) Reaction mixture

The PCR reagents were kept on ice throughout the sample preparation and mixed in appropriate amounts in thin-walled PCR tubes.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq Buffer (10x)</td>
<td>4µl</td>
</tr>
<tr>
<td>dNTP (10mM each)</td>
<td>4µl</td>
</tr>
<tr>
<td>L. donovani Genomic DNA (100 ng µl⁻¹)</td>
<td>1µl</td>
</tr>
<tr>
<td>Forward Primer (10µl)</td>
<td>1µl</td>
</tr>
<tr>
<td>Reverse Primer (10µl)</td>
<td>1µl</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>38.5 µl</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>0.5µl</td>
</tr>
<tr>
<td><strong>Total reaction volume</strong></td>
<td><strong>50 µl</strong></td>
</tr>
</tbody>
</table>

b) Thermal cycling conditions

Thermal cycler was programmed using the recommended cycling parameters for the Taq DNA Polymerase. Hot lid option was chosen in order to prevent evaporation.
c) Agarose gel electrophoresis and gel-purification of PCR products:
Amplified product of Isd11 gene was run on 1% (w/v) horizontal agarose gel, as shown in Fig 4.1 A. The bands corresponding to the PCR product was further amplified in 50 µl reaction mixture and run on 1% agarose gel prepared which were excised on a UV illuminator using a clean scalpel. The resulting pieces of agarose containing the PCR product were treated with the QIA quick Gel Extraction Kit according to the manufacture’s instructions. The clean PCR product was eluted in 40 µl of Qiagen’s elution buffer. The purified PCR product was either stored at -20 °C or directly used in subsequent cloning reactions.

d) Directional cloning and Double digestion
The insert was prepared by double digesting the PCR product with appropriate BamHI and XhoI restriction endonucleases. Corresponding pET-28a vector was also double digested with the same set of restriction endonucleases. New England Biolab Double Digest Calculator programme (http://www.neb.com/nebecomm/DoubleDigestCalculator.asp) was used to determine the double-digest conditions.

Each reaction was incubated for 2 h at 37 °C. After the incubation period, both the insert and the vector were purified using the specified reagents from the QIA quick Gel Extraction Kit. 30 µl of Qiagen’s elution buffer was used to elute digested DNA. The purified digestion product was either stored at -20 °C or directly used in subsequent ligation reactions.
e) Sticky-end ligation

Ligation of the insert into the vector was performed using Takara’s DNA Ligation Kit, which involves an optimised buffer system and T4 DNA Ligase. The standard protocol was employed for the sticky-end ligation.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Vector DNA</td>
<td>3µl</td>
</tr>
<tr>
<td>Insert DNA</td>
<td>9µl</td>
</tr>
<tr>
<td>Ligation Mix</td>
<td>3µl</td>
</tr>
<tr>
<td><strong>Total reaction volume</strong></td>
<td><strong>15µl</strong></td>
</tr>
</tbody>
</table>

Each reaction was incubated for 30 min at 16 °C. Following ligation, the reaction mixture was either stored at 4 °C or directly used in subsequent transformation reactions.

A single ~ 470 bp PCR product was observed on 1.0% agarose gel electrophoresis that was double digested with BamHI and XhoI, electrophoresed, purified with gel extraction kit (Qiagen), and cloned into BamHI and XhoI-double digested pET-28a vector (Novagen, Darmstadt, Germany) in the same orientation as the T7 promoter. The ligated mixture was transformed in DH5± competent Escherichia coli cells to yield plasmid pET-28a-Ld-Isd11. The colonies obtained were screened by various methods as described below:–

i. Rusconi

ii. Colony PCR.

iii. Restriction digestion.

i). Rusconi method:

12 µl rusconi mixture was taken in 1.5 ml centrifuge tube and a single colony picked from LB- Kanamycin plate with the help of sterile tip was resuspended in the rusconi mixture. Bacterial cell suspension was mixed well and incubated for 15 minutes at room temperature for complete lysis. Removed the tip from the tube and used to make streak on the LB-Kanamycin plate for backup of the colony & incubated at 37 °C overnight. After this 2µl of phenol: Chloroform (1:1) mixture was added into it, vortexed and centrifuged for 2 minutes at 5000 rpm. The supernatant (~10 µl) was applied onto 1%
agarose gel (containing ethidium bromide) and runned at constant voltage as described above.

**ii). Colony PCR:**

The colony PCR was done to determine the presence of positive clones in the colonies by using different combinations of primers. This technique is also helpful in the determination of correct orientation of the insert in the construct. The 50 µl PCR reaction mixture consist 2X master mix PCR buffer-24 µl, 0.5 µl forward and reverse primer each. A single colony was added to the 25 µl PCR reaction mixture without Taq polymerase contained in the PCR tube and PCR was performed using the conditions; hot start at 95 °C for 5 min., denaturation at 95 °C for 30 sec., annealing at 55 °C for 40 sec., elongation at 72 °C for 1 min., and final elongation at 72 °C for 10 min.

**iii). Restriction digestion:**

Restriction enzymes, which were used in the ligation of the insert into the vector, were taken to digest the construct and to determine whether there the restriction site was intact or not in the construct. To determine this following procedure was done:

Plasmid was isolated from the pellet using QIAGEN miniprep kit as per the manufacturer’s protocol. The isolated plasmid was subjected to double digestion using BamHI and XhoI restriction enzymes. The digestion reaction mixture 20 µl consist of 3 µl of 10X fast digest buffer, 10 µl of construct of Ldlsd11 in pET-28a, 1.5µl of each of BamHI and XhoI & 4 µl water. The digested product was visualized by separating on 1% agarose gel (containing 0.5µg/ml of ethidium bromide) in TBE buffer. After this screening of the positive clone the plasmid from positive clones was isolated, & was used for transforming BL-21(DE3-plain) competent cells.

### 4.3.6 Expression and purification of recombinant Ldlsd11 protein

The Ldlsd11 gene was cloned in vectors pET-28a (+) (Novagen) and pGEX-4T-1 (Amersham Biosciences) and recombinant Ldlsd11 (rLdlsd11) fusion proteins with a N-C-terminal histidine tag (rLdlsd11-his) or a N-terminal glutathione-S-transferase tag (GST-rLdlsd11), respectively, was expressed and purified as described previously [15].
IPTG titration of 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM was performed to the individual tubes with incubation at three different conditions viz 37 °C for 4 hours, 30 °C for 6-8 hours, 25 °C and 18 °C overnight. The rLdIsd11 proteins were expressed in Escherichia coli BL21 (DE3) cells and purified using affinity chromatography as described previously [15]. Briefly, the overexpression of rLdIsd11-his protein was induced with 1 mM IPTG at 18 ºC/200 rpm for 18 hours and overexpressed proteins purified from soluble fraction using Ni²⁺-NTA matrix. Solutions containing 10-50 mM imidazole in E. coli lysis buffer (50 mM Tris-Cl, pH 8.0, 300 mM NaCl, 0.1 % Triton X-100) were used for washing column, whereas 100-400 mM imidazole solutions in E. coli lysis buffer were used for elution of Ni²⁺-NTA matrix bound. rLdIsd11-his tag protein was used for raising polyclonal antibodies in mice, described previously [15].

4.3.7 Raising polyclonal antibodies and immunoblot analysis

Polyclonal antibody against purified rLdIsd11-his protein was raised in mice as described previously [17]. Briefly, four doses of 100 µg purified rLdIsd11-his protein (supplemented with appropriate Freud’s adjuvant) was injected subcutaneously at 2 weeks interval and anti-LdIsd11 titre checked after 2 weeks of final immunization dose. Finally, the mice was sacrificed for serum collection and antibodies stored at -30 ºC in small aliquots. The procedures adopted were strictly as per the guidelines of Animal ethical committee, RMRIMS, Patna.

4.3.8 Substrate-binding assay by spectroscopic measurement

Substrate binding spectra can be used to investigate the degree of interaction between an enzyme and its substrate. Many cofactors of enzymes absorb light in the UV-visible region of the electromagnetic spectrum. For instance, the PLP cofactor of cysteine desulphurases absorbs at 415 nm when it is covalently bound to the enzyme through a Schiff base. The binding of free cysteine to the enzyme perturbs the spectrum by displacing PLP. Therefore, the activities of cysteine desulphurases can be measured spectrophotometrically by following the decrease in A₄₁₅ of time. The activities of rLdIscS and rLdIscS-rLdIsd11 complex were assayed in vitro by adding free L-cysteine at a final concentration of 10 mM (incubation allowed at 4 ºC) and the reaction was monitored by UV-visible absorbance spectrophotometry using (50 mM Tris HCL, 200
mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, pH 7.0). Cysteine desulphurase activity was measured in terms of the decrease in A$_{415}$ as a function of time.

a) Cysteine desulphurase assay: Cysteine desulphurase (CDES) activity was assayed by monitoring sulphide production at 670 nm as described previously [17, 18]. Appropriate amount of purified proteins were used (1 equivalent of rLdIscS: 1 equivalent of rLdIsd11).

b) Intrinsic Fluorescence Measurement of rLdIscS upon rLdIsd11 binding

Fluorescence spectra were recorded in 0.5 µl quartz cuvette at room temperature with a Cary eclipse fluorescence Spectrophotometer (Agilent), as described previously [19], with some modification. The excitation 291 nm and emission wavelengths range were and 330-370 nm, respectively. Spectra were obtained on a 2.5 µM solution of rLdIscS protein alone and in complex with rLdIsd11 protein. For a control titration, intensity data consisting of a similar solution containing only the buffer solution (50 mM Tris-HCl, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, pH 7.0) in the sample cell, was subtracted from the experimental data obtained.

4.3.9 Co-purification of rLdIsd11 and rLdIscS by affinity chromatography

In vitro interaction between LdIsd11 and LdIscS was checked by co-purification from E. coli lysates overexpressing the individual proteins as described previously [17]. The purified proteins were extensively dialyzed in E. coli lysis buffer and rLdIsd11-his tag was allowed to bind with Ni$^{2+}$NTA matrix followed by washing with 10-50 mM imidazole solutions. Purified rGST-LdIscS was now added to the column and incubated for 30 minutes at RT. Then, the column was again washed with 10-50 mM imidazole solutions and bound complex eluted with 500 mM imidazole. After washing with 10-50 mM imidazole solutions, the complex was eluted with 500 mM imidazole twice and recombinant proteins identified by probing western blot with monoclonal anti-his (Santa Cruz, 1:4000) and polyclonal anti-GST (Santa Cruz, 1:2500) antibodies.

4.3.10 Pull down assay using Leishmania lysate

Pull down of LdIscS protein (prey) from Leishmania lysate by rIsd11-his (bait) was performed as described previously [17] with minor modifications. Ni$^{2+}$NTA bound
rlsd11-his was obtained as described in the previous section except that 5 mM β mercaptoethanol was used in the E. coli lysis buffer and imidazole wash buffers. For preparing L. donovani lysate, late log phase promastigotes were lysed in buffer containing PBS pH 7.4, 0.5 mg/ml digitonin, 1 X protease inhibitor cocktail, following with 3-4 freeze thaw cycles and brief sonication (4-5 pulses, 2-3 times). The cleared lysate obtained by centrifugation at 13000 rpm for 10 minutes was incubated with rlsd11-his tag bound Ni²⁺-NTA matrix for 60 minute at 4 °C. After washing with 10-50 mM imidazole solutions, the bound complex was eluted with 500 mM imidazole buffer. All the fractions were resolved on a 10% SDS-PAGE and proteins identified by probing the western blot with monoclonal anti-His and polyclonal anti-LdIscS antibodies.

4.3.11 Localization of LdIscS

(a) Digitonin fractionation - The differential membrane permeabilization was performed with 4×10⁹ cells (~5×10⁸ cells contain 1.0 mg total protein) [20] of stationary phase promastigotes. Protocol were adapted same as described previously [21]. The supernatant and pellet fractions were run on 10% SDS-PAGE and analysed by western blot using anti-IscS (1:800) and LdTryS (L. donovani trypanothione synthetase) (1:2000) antibodies respectively.

(b) Indirect immunofluorescence microscopy: Protocol followed as described previously [21], with minor modification. The fixed parasites were incubated with anti-LdIscS antibody diluted in TB buffer (1:500) for 1 hr at RT and secondary antibody FITC-conjugated goat anti-mouse IgG at 1:2000 dilution (Santa Cruz) for 1 hr at RT and immunofluorescence was observed under a microscope (Model BX 41 Olympus fluorescence microscope). Image analysis was done using IPelite software (Olympus). Immunofluorescence microscopy for subcellular localization of LdIscS protein was performed as described previously [21]. Briefly, promastigotes in the late log phase were labelled with 500 nM of Mitotracker deep red (Invitrogen) in serum free M199 media for 30 minutes, washed twice with PBS and fixed-permeabilised with Cytoperm/Cytofix solution (BD) as per manufacturer’s instructions. The permeabilised parasites were stained with anti-LdIscS serum at 1:500 dilution in cytoperm wash buffer at 4 °C, overnight and secondary antibody goat anti-mice IgG-FITC (Santa Cruz) at 1:2000.
dilution in cytoperm wash buffer, 1 hour, RT. DAPI (1:1000, Sigma) was used for labelling DNA. The mounted parasites were observed under BX 41 Olympus fluorescence microscope and image processing was done using ImageJ software.
4.4 RESULTS

4.4.1 Isd11 homolog of Leishmania

The sequence of the target protein (LdIsd11) was annotated as a hypothetical retrieved from NCBI database. We annotated this protein as Isd11 protein of L. donovani with accession number AB678407 based on conserved sequence and domain analysis. Later on, the L. donovani genome sequence was published and a similar protein sequence was reported (XM_003863922, available in NCBI database). Identification of homologous proteins was performed with the help of BLASTP server. In addition to the amino acid sequence, the nucleotide sequence of L. donovani Isd11 retrieved from SGD was also analysed to determine the occurrence and distribution of rare E. coli codons. Rare codons are those codons that are rarely found in highly expressed genes of a given host and include AGG (which encodes Arg), AGA (Arg), AUA (Ile), CUA (Leu), CGA (Arg), CGG (Arg), and CCC (Pro) in E. coli [22]. The presence of rare codon clusters or a large number of single rare codons in the cloned Isd11 gene could introduce translational problems in E. coli. A search of the nucleotide sequence for rare codons by Gene Script codon analysis revealed that the inferred mature Isd11 protein has a Codon Adaptation Index (CAI) of 0.56 which is considered low from the normal value of 1.0 and therefore the gene has the higher chance of poor expression. Based on the codon analysis it was determine that the gene has low frequency codon of 17% (< 30%) based on the target expression in E. coli host. This un-optimized gene employs tandem rare codons that can reduce the efficiency of translation or even disengage the translational machinery. Also, the large number of hydrophobic and positive charged amino acids may interfere protein expression in soluble form as we mentioned in later section 4.1B. Further, the prediction of secondary structure from the LdIsd11 protein sequence was performed on analysis from psipred software which shows the protein is mostly formed of helix-coil fold (Fig. 4.1 A) The multiple sequence alignment of target protein with other putative closely related proteins sequences including yeast was performed using ClustalW program. LdIsd11 showed highest nucleotides homology of 98% with L. infantum, 94% with L. major, 93% with L. mexicana, 56% with T. cruzi, 64% with T. brucei and 62% homology with S. cerevisiae. Further, L. donovani Isd11 amino acid sequence was aligned with the amino acid sequence of four homologues from Leishmania spp., two from Trypanosoma
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and S. cerevisiae (an alignment of only representative members is shown in Fig. 4.1B). L. donovani Isd11 showed 100% a.a. identity with L. infantum; 95% a.a. identity with L. major; 93% a.a. identity with L. mexicana; 40% a.a. identity T. cruzi; 47% a.a. identity with T. brucei and 23% a.a. identity with S. cerevisiae (an alignment of only representative members is shown in Fig. 4.1B). Alignment among different organism shows that L. donovani, L. infantum and L. major represent an overhang N-terminal residue. Overall, the sequence alignment shows most of conserved amino acids between the Isd11 homolog that manifest the probability of these amino acids to be part of the main structural features, including structurally important domain. Indeed, the amino acids involved in Isd11 binding to IscS are strictly conserved across species which has been represented in the alignment (Fig. 4.1B).

The Isd11 gene was amplified via PCR from 100 ng genomic L. donovani DNA as template resulting 492-bp fragment was obtained (Fig. 4.2A). The amplified Isd11 gene was cloned into pET-28a(+) giving the plasmids pET-28a-LdIsd11. The Isd11 gene cloned in pET-28a vector was further confirmed by rusconi mix and colony PCR followed by the restriction digestion analysis and thus confirmed the positive clone of Isd11 construct and with proper 5´-3´ integration (Fig. 4.2 B-D). The LdIsd11 ORF 492 nucleotides encodes 163 amino acids with predicted molecular weight 18.75 kDa and the protein showed theoretical calculated isoelectric point (pl, 10.42) as this protein has many basic amino acids and higher positive charge. Mitochondrial targeting probability was found to be high as 0.9584 calculated by Mito Prot II – v1.101.

A

Target Isd11 protein sequence:

\[
\begin{align*}
10 & MVFLRITLHC \\
20 & LCFTALLPLR \\
30 & RSLAHISLTQ \\
40 & AYPSALASPS \\
50 & TLFHQRQRAK \\
60 & NGEKGSMSAA \\
70 & AKTVQKSVDR \\
80 & LRGKMIRTR \\
90 & RFRDNMFQY \\
100 & FVQHKDDFA \\
110 & ALAKLSEEQ \\
120 & RKFLATEGRD \\
130 & KLRQLQRMAL \\
140 & VNOQMYAKRPV \\
150 & YFDTAAKPH \\
160 & RRQDDGTGKP \\
\end{align*}
\]
Figure 4.1. A) Target sequence Isd11 and secondary structure prediction by psipred software B) Multiple sequence alignments of deduced amino acid sequences of Isd11 from L. donovani and other organisms. The Isd11 homologues of indicated organisms were aligned using the ClustalW program and gaps were introduced to optimize the alignment. Among the residues implicated in E. coli IscS-Isd11 interaction, ‘#’ denotes interacting residues conserved in all eukaryotes including kinetoplastids, whereas, closed boxes denote the semi-conserved amino acid, red shade represents conserved residue. Signal peptide sequence in Leishmania spp. is shown by a black bold line above the alignment. Black Accession numbers of sequences used in the alignment are L. donovani (XP_003863970), L. infantum (XP_001468226), L. major (XP_001685896), L. mexicana (XP_003878389), T. cruzi (XP_807608), T. brucei (XP_823406), S. cerevisiae (NP_010968)
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4.4.2 Expression and Purification of Recombinant LdIsd11

We have standardized protein expression experiments using various IPTG concentrations (0.4 mM & 1 mM) and temperatures (37 °C, 30 °C or 18 °C) for different incubation times (4 h, 8 h or 24 h) to get Isd11 fusion protein in soluble form (Fig. 4.3). We have tried several colonies at 37 °C and 30 °C with 0.5 and 1.0 mM IPTG concentrations but failed to get protein in soluble form in E. coli lysate as shown in Fig. 4.3 B. Hence, it was concluded that recombinant Isd11 has a tendency to form insoluble aggregates. Finally, we checked expression at 18 °C with 0.4 mM IPTG for 24 h (Fig. 4.3 A) showed some
fusion protein is likely present in soluble fractions. These conditions were used for large scale culture to get protein in soluble form. The rLdIsd11-his tag protein expressed in E. coli BL21 (DE3) cells was purified from soluble fraction by Ni^{2+}-NTA affinity matrix as described in details in previous chapter and homogeneous bands of approx ~25 kDa was observed in 200 mM and 300 mM elute fractions on a coomassie stained 12% SDS-PAGE (Fig. 4.3 C). Polyclonal antisera against purified recombinant Ld-Isd11 (rLd-Isd11) were raised in balb/c mice and titre measured at final bleed was 1: 800 dilution.

**Figure 4.3. SDS PAGE analysis of expressed Isd11 transformed E. coli:** A) Induction at 18 °C; B) Induction at 37 °C. Lanes: M, protein markers; Lane: US (Uninduced Supernatant), UP (Uninduced Pellet); Lane: S1, S2, S3, soluble fraction; Lane: P1, P2, P3, insoluble fraction. Arrows indicate the bands of his tagged Isd11 recombinant protein.
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Figure 4.3. Purification of recombinant Ld-Isd11 protein: C) rLd-Isd11-His purification under native conditions using Ni²⁺NTA agarose: The total cell lysate and samples at each purification step were electrophoresed on 12% SDS-PAGE gel and stained with coomassie brilliant blue. Lane 1 - protein marker, Lane 2 - Total lysate, Lane 3 - Soluble supernatant fraction, Lane 4 - Flow through, Lane 5 - fractions of wash with 40 mM imidazole; Lane 6 & 7 - eluted fractions with 200 mM and 300 mM imidazole elution buffer.

4.4.3 rLdIscS and the rLdIscS-Isd11 complex are active towards L-cysteine

The spectra were recorded at room temperature for rLd-IscS (10 µg) and rLd-IscS/Isd11 complex (1:1 molar equivalent). The activity of recombinant IscS and IscS/Isd11 complex were assayed by incubating the protein with high molar excess of free L-cysteine and following the reaction periodically using a spectrophotometer. IscS was assayed in vitro in the absence of a specific sulphur-acceptor protein but in the presence of DTT using L-cysteine as substrate. DTT in the buffer reacts with the enzyme-bound persulphide, regenerating the active-site cysteine residue and liberating sulphur in the S redox state [23]. This is the reason why IscS was assayed in vitro in the absence of a specific sulphur-acceptor protein but in the presence of DTT, using L-cysteine as substrate. Because the substrate was used in high molar excess, the system displayed absorption bands corresponding to all long-living species. Upon addition of L-cysteine, the intensity of the absorption band at 415 nm, assigned to the protonated internal aldime, decreased with a concomitant increase of absorbance at 330 nm or 334 nm. The newly formed absorbing species represents the ketimine intermediate [24].

These results suggest that rLdIscS and rLdIscS/Isd11 complex follows the same reaction scheme as its bacterial and yeast homologues [4, 25]. The change in absorbance at 415 nm (or 330/334 nm) was considerable for the first ~2-minute interval, but then in subsequent 2-minute intervals the activity got reduced. Clearly, the rLdIscS-PLP adduct is not being regenerated, or else the peak at 415 nm would persist. In either case it is clear that the rLdIscS-bound PLP is reacting with the free cysteine and forming the ketimine, revealing that the active centre of the recombinant enzyme is correctly formed (Fig. 4.4). In addition, the complex follows the same reaction scheme as IscS (and its bacterial homologues) raising the question of what is the specific function of bound Isd11. Further,
rLdIscS was found to be precipitated after 16 hrs incubation with cysteine at 4 °C. This may be because the resting enzyme (IscS-SH) is more soluble than the persulphide compound (IscS-S-SH). Unlike rLdIscS, rLd-IscS/Isd11 complex showed no signs of precipitation following overnight incubation with cysteine at 4 °C. rLd-Isd11, therefore, seems to stabilize rLdIscS during and/or after the L-cysteine desulphuration reaction. Similarly, report has been published where the ortholog of ISD11 significantly play a role in stabilizing NFS1 [26].

![Graph A](image1)

![Graph B](image2)
Figure 4.4. A) Time-dependent spectral analysis of L-cysteine binding to IscS. Addition of free cysteine leads to a loss of absorption at 415 nm. B) Time-dependent spectral analysis of L-cysteine binding to IscS/Isd11. Addition of free cysteine leads to a loss of absorption at 415 nm.

4.4. Isd11 affect the cysteine desulphurase (CDES) activity of Ld-IscS

We have measured the CDES activity of IscS in the presence of Isd11 and observed that comparing to Ld-IscS alone, addition of rLd-Isd11 protein to the rLd-IscS stimulates cysteine desulphurase activity by 1.7 fold (Fig. 4.5), which is accompanied by an increase in sulphide production. This might be happen by inducing conformational changes in LdIscS which is similar to yeast but distinct from prokaryotes which do not possess any Isd11 homolog. However, previous studies have demonstrated stimulation of cysteine desulphurase activity by the binding partners. In E. coli, IscS activity is stimulated 6-fold by IscU binding [27] and SufS activity is increased 50-fold by SufE interactions [28]. SufE is a sulphur transfer protein with some structural similarities to IscU. Cysteine desulphurases are also stimulated by the molecular chaperones [29] involved in Fe-S cluster assembly and by proteins involved in tRNA thiolation and molybdopterin biosynthesis where IscS is stimulated up to 1.6 fold [30].

Figure 4.5. Cysteine desulphurase activity in presence of rLdIsd11: Sulfide production by IscS was determined in the presence of Isd11. Reaction mixture was read at 670 nm by double beam spectrophotometer (U-3900, Hitachi, Japan). The experiment was repeated twice in duplicate.
4.4.5 Fluorescence measurements of intrinsic tryptophan indicate a conformational change in IscS upon Isd11 binding

Tryptophan and a few cofactors exhibit this emission phenomenon, and their presence allows intrinsic fluorescence to be measured in proteins. The binding and release of ligands, at sites close to the fluorophores, give rise to alterations in the related fluorescence spectra. Thus, useful information about conformational changes and unfolding can be gained. So, rLdIsd11 protein lacks tryptophan in contrast to rLdIscS which contains four tryptophan residues at positions 82, 300, 425, 439. Therefore, the emission spectra of LdIscS alone and in complex with LdIsd11 were recorded to obtain information regarding the accessibility and microenvironment of these intrinsic fluorophores (Fig. 4.6). Purified protein amount (2.5 µM) was used for the fluorescence measurements on a Cary eclipse fluorescence Spectrophotometer (Agilent) at room temperature. The excitation wavelength was set at 291 nm, and the emission scans were acquired from 315 nm to 400 nm, using excitation and emission slit widths of 5 nm.

Inspection of the spectra of rLdIscS and rLdIscS/Isd11 complex showed that in presence of Isd11, IscS adopts a different conformation as compared to its free form. Upon Isd11 binding, the emission maximum for IscS shifted from 347 nm to 344 nm, suggesting that one or more tryptophans become less exposed to the buffer. With bound Isd11, intrinsic tryptophan fluorescence is also quenched by 22%, which is possibly due to the varying interaction of tryptophans with peptide bonds and amino acid side-chains within the complex structure. These findings explain that either the buried tryptophan is normally situated in the rLdIscS-Isd11 binding site or the binding of rLdIsd11 induces a conformational change in IscS so that the tryptophan becomes more buried.
Chapter Four: Biochemical Characterization and Interaction of LdIsd11

4.4.6 Copurification of rLdIscS-GST with his-rLdIsd11

The interaction between IscS and IscU (or their homologs) proteins is one of the key and conserved early step in the biosynthesis of Fe-S clusters. To identify a similar mechanism for Fe-S cluster biogenesis in L. donovani, in vitro interaction between LdIsd11 and LdIscS was checked by copurification of rLdIsd11-his with rGST-LdIscS, using E. coli lysate overexpressing the individual protein in E. coli. In the copurification experiment using E. coli lysate of rGST-LdIscS was added to the Ni$^{2+}$-NTA bound rLdIsd11-his (described in materials and methods), both proteins were observed in the elute fraction on a coomassie stained SDS-PAGE and their identity was confirmed by western blot of relevant fractions with anti-his or anti-GST antibody. Thus, the results show that rGST-LdIscS remained bound to the column during washing steps and coeluted with rLdIsd11-his in the elute fraction suggesting a physical interaction between these two proteins. As shown in Fig. 4.7 A, bands corresponding to rLdIsd11-his as well as rLdIscS-GST were observed in the elute fraction on a coomassie stained SDS-PAGE which was confirmed by western blot with anti-his (1:4000) or anti-IscS (1:2000) antibodies (Fig. 4.7B). Thus, the results showed that LdIsd11 selectively pulls down
LdIsd11 and LdIscS interact with each other, as indicated by the co-purification of rGST-LdIscS with rLdIsd11-his, suggesting a specific physical interaction. This interaction is confirmed by in vitro co-purification experiments using E. coli lysate overexpressing rLdIsd11 or rLdIscS proteins. A) Upper panel: 12% coomassie stained SDS-PAGE, B) Lower panel: Western blot of relevant fractions using indicated antibodies. Lane 1- protein marker, Lane 2- E. coli cell lysate supernatant expressing His-Isd11 protein, Lane 3- flow through, Lane 4- wash, Lane 5- final elute, Lane 6- E. coli cell lysate supernatant expressing GST-IscS, Lane 7- flow through, Lane 8- final wash.

**4.4.7 Pull down of LdIscS from Leishmania lysate by rLdIsd11-his**

The in vivo evidence for interaction between LdIscS and LdIsd11 was gained by pull down of LdIscS (prey) from L. donovani lysate by rLdIsd11-his (bait). As shown in (Fig. 4.8 A) LdIscS were observed along with rLdIsd11-his protein in the elute fraction on the coomassie stained SDS-PAGE (upper panel) and among them, LdIsd11 and LdIscS was identified by western blot (lower panel) (Fig. 4.8 B) of relevant fractions with anti-
LdIscS sera [15] whereas anti-his monoclonal antibody confirmed the presence of rLdIsd11-his protein. The identification of other bands in the elute fraction remains to be identified.

Figure 4.8. Pull down of LdIscS by rLdIsd11-his proteins from *Leishmania* lysate. A) Upper panel: 12% coomassie stained SDS-PAGE; B) Lower panel: Western blot of relevant fractions from lysate using indicated antibodies. Lane 1- protein marker, Lane 2- *E. coli* cell lysate supernatant expressing His-Isd11, Lane 3- flow through, Lane 4- wash, Lane 5- final elute, Lane 6- *L. donovani* cell lysate supernatant, Lane 7- flow through, Lane 8- final wash.

**4.4.8 Localization of Ld-Isd11**

Digitonin permeabilization of the cell depends on the cholesterol content of membrane. Intact promastigotes were therefore exposed to different concentration of digitonin and the resulting supernatant and pellet fractions were analysed by western blot. As shown in (Fig. 4.9 A) Ld-Isd11s is released from the cells at relatively high digitonin concentration (above 0.5 mg digitonin per mg total protein), compared to protein LdTtryS starts to release at 0.1 mg digitonin per mg total protein. Also, analysis of the pellet fractions indicates that higher concentrations of digitonin (over 0.5 mg per mg total
protein) is required to completely release Ld-Isd11 whereas cytosolic proteins are completely released up to 0.2 mg digitonin. Since Ld-Isd11 release pattern is different to the cytosolic marker LdTryS, so we conclude that Ld-Isd11 is localized in the organelle. Also, immunofluorescence staining of L. donovani promastigotes with anti-Isd11 antibody showed staining in the organelle of the cells (Fig. 4.9B). The mitochondrion was labelled by mitotracker and the composite image overlapped with anti-Isd11 labelled image suggesting Ld-Isd11 is localized in the mitochondria. Thus, our results based on subcellular digitonin fractionation and immunofluorescence microscopy revealed that Isd11 is predominantly found in the mitochondria of L. donovani similar to yeast [8], human [5]. MitoProt II [31], and Target-P analysis a dedicated software for calculating the N-terminal protein region supporting a mitochondrial targeting sequence and the associated cleavage site, inferred that the first 10 amino acids form a basic amphipathic helix containing the mitochondrial targeting information which supports its mitochondrial localization.

Figure 4.9. A) Localization of Ld-Isd11 protein in L. donovani promastigotes: (a) Supernatant and pellet fractions were run on 12 % SDS-PAGE, Supernatant and pellet fractions obtained after L. donovani promastigote permeabilisation with increasing digitonin concentrations were analyzed by immunoblot. (B) Immunofluorescence microscopy of promastigotes with anti-Ld-Isd11 sera.
5 DISCUSSION:

Maintenance of proper Fe-S cluster homeostasis must be one of the key metabolic demands for survival of the parasites. The above peculiarities make it worth investigating the Fe-S cluster biogenesis pathway of Leishmania with an aim to decipher key adaptations of novel molecules within protozoan parasite. Previously, we identified the presence of a classical eukaryote prototype ISC system for Fe-S cluster biogenesis in Leishmania [32] and later, demonstrated the ROS regulated expression of LdIscS [15]. In the present study, we characterized the Isd11 homolog in L. donovani (LdIsd11) which is the small accessory protein involved in assisting the sulfur transfer during Fe-S cluster biogenesis. Our results indicate that Isd11 interacts with cysteine desulfurase, LdIscS, which is the only sulfur donor for Fe-S cluster assembly in Leishmania and belongs to the aminotransferase subgroup of the pyridoxal 5 phosphate (PLP) dependent enzymes. Moreover, as shown previously for LdIscS by our group [15], LdIsd11 was also found to be localized in the mitochondrion of L. donovani promastigotes, supporting the rationale of their observed interaction. Further, rLdIsd11 was found to stabilize rLdIscS and stimulate its desulfurase activity highlighting the conserved role of Isd11 in Leishmania spp.

Our genome wide analysis on trypanosomatids and previous reports by other groups revealed that Isd11 was selective to eukaryotes including mitosomal and hydrogenosomal lineages [12, 13, 32] and therefore may perform a unique function in mitochondria which is not needed in prokaryotes. Homologues of yeast Isd11 were detected in fungi, plants, and animals, but no prokaryotic homologues were found, supporting the previous reports on the eukaryotic addition of Isd11 to the prokaryote-derived ISC-assembly machinery [7, 8, 12]. Although, it cannot be excluded that prokaryotes may possess a distant homolog or analog of Isd11, the current results suggest that Isd11 is a eukaryotic addition to the prokaryote-derived ISC-assembly machinery of the mitochondrial matrix. Previous report demonstrated that Isd11 is essential for the Fe-S cluster assembly of mitochondria and cytosolic proteins in yeast [7], human [5, 8], procyclic trypomastigotes of T. brucei, and other eukaryotes [13]. In addition, Isd11 was reported to be localized in the mitochondrion of T. brucei, in line with its strong association with Nfs1 [14]. Similarly,
our analysis by sub-cellular fractionation and immunofluorescence analysis suggested that Ld-Isd11 is localized in the mitochondrion of L. donovani.

Iron-sulfur cluster assembly in mitochondria is a complex process requiring multiple proteins [1]. Here, we have purified rLdIsd11 and rLdIscS protein to determine the role of LdIsd11 in LdIscS activity. Earlier, it was reported that Nfs1 catalyze sulfur transfer by itself [33], but it was recently shown to additionally require Isd11 for the formation of Fe-S clusters on the IscU scaffold [7]. Recently, we have also reported that in the presence of LdFrataxin and iron, the LdIscS desulfurase activity is stimulated [17]. In the present work, we also observed a 1.7 fold increase in IscS activity in presence of Isd11. However, as compared to previous reports in other organisms, the observed stimulation of desulfurase activity is less. The most plausible explanation may be because LdIscS/Isd11 complex exhibits only basal cysteine desulfurase activity, and that LdFrataxin is needed to act as a positive stimulator, thereby inducing the optimal activated level of cysteine desulfurase. Further, LdFrataxin might enhance substrate binding to IscS rather than persulfide formation by the enzyme, which depends on the initial step of the IscS/Isd11 complex. This view is supported by the previous observation in yeast that in the absence of Isd11 homolig, Nfs1 can bind to cysteine but cannot form the persulfide at the active site residue and thus Isd11 is required for the activation for Nfs1 [3].

Result of UV-visible spectroscopy analysis shows that PLP binds to LdIscS and its active centre closely resembles that of bacterial IscS and yeast Nfs1 suggesting the conservation of the catalytic mechanism [5, 8] and play essential role in mitochondrial Fe-S cluster synthesis. Similarly, our in vitro studies showed that LdIsd11 forms a stable complex with LdIscS and stabilizes the complex. The mutual co-dependence of these two proteins might be essential for the initiation of Fe-S cluster biogenesis on the scaffold protein LdIscU and Ldfrataxin would get involved in the later stages to stimulate the rate of Fe-S cluster biogenesis, thus acting as an iron-dependent regulator of the biogenesis process in concert with other vital Fe-dependent metabolic processes. The interaction between LdIsd11 and LdIscS was readily observed by co-purification and pull down assay in agreement with the previous report in yeast [7] and human [5] where Isd11 has
been shown to interact with both Nfs1 and IscU of the mitochondrial iron-sulfur cluster biogenesis machinery. In the phylogenetically related pathogenic trypanosomatid T. brucei also, pull-down by TAP-tagged Isd11 showed the presence of IscS in the pulled down complex, as analyzed by mass spectrometry [14]. Similarly, in the microsporidian Trachipleistophora hominis, by co-expression of Nfs1 and Isd11 in E. coli, it was revealed that both proteins indeed form a tightly bound complex with enhanced stability and cysteine desulfurase activity representing the functional cysteine desulfurase complex [13].

In conclusion, for the first time in Leishmania parasites, our study on L. donovani Isd11 homolog has shed light on the properties of LdIsd11 as an adaptor or stabilizer protein for LdIscS. The existence of LdIscS-LdIsd11 and Ld-Frataxin-LdIscU interaction in conjunction with their stimulatory effect on cysteine desulfurase activity may exert regulatory role during Fe-S cluster biogenesis delicately synchronized with fluctuating iron availability and metabolic demands during the digenetic life cycle of the unicellular protozoan parasite L. donovani. This mechanism would be of profound significance during infection stages in the macrophage where the iron acquisition is a major challenge for the persistence/survival of the intracellular parasite and desperate but vital metabolic reshuffling occurs to overcome the hostile conditions and cause pathogenesis.
4.6 References:


27. Kato, S, H Mihara, T Kurihara, Y Takahashi, U Tokumoto, T Yoshimura, and N Esaki. Cys-328 of IscS and Cys-63 of IscU are the sites of disulfide bridge formation in a covalently bound IscS/IscU complex: implications for the


