Chapter: 6

Cloning, Over-expression, Purification and Crystal Structure studies of N-myristoyltransferase from *Leishmania donovani*
6.1. INTRODUCTION

The leishmaniases, caused by species of the kinetoplastid parasite Leishmania, are a range of diseases associated with immune dysfunction that give rise to more than 2 million new cases each year in 88 countries, with 367 million people at risk (Chappuis et al., 2007; Shaw, 2007; Brannigan et al., 2010). Cutaneous leishmaniasis, the most common form of leishmaniasis includes most prominently disfiguring skin lesions whereas visceral leishmaniasis (VL) is the most serious form, and is potentially fatal if untreated. In VL the parasites leave the inoculation site and proliferate in spleen, liver and bone marrow, resulting in host immunosuppression and ultimately death in the absence of treatment. These symptoms are severe in children and immunocompromised patients, such as those diagnosed as HIV positive. Though there is a limited range of effective drugs in use including Pentostam, amphotericin B and miltefosine, with a few potential ones in clinical trials (Croft et al., 2006), delivery mode can be complex, while miltefosine, the only oral VL drug currently available, is teratogenic in nature. Developing resistance is an increasing problem, therefore a pressing need for new, preferably orally administered drugs, against this globally important but neglected health problem (Branigan et al., 2010).

Previous studies have identified Myristoyl-CoA:protein N-myristoyltransferase (NMT; EC 2.3.1.97) as a suitable candidate for drug development against protozoan parasitic infections, including *Plasmodium falciparum*, *Trypanosoma brucei*, *Leishmania major* and *Leishmania donovani* causative agents of human malaria, African sleeping sickness, cutaneous and visceral leishmaniasis, respectively (Bowyer et al., 2007; Price et al., 2003, 2010; Panethymitaki et al., 2006; Brannigan et al., 2010).

NMT is ubiquitous in eukaryotic cells in which it catalyses the co- and post-translational addition of the C14:0 fatty acid, myristate, via an amide bond formation, to the N-terminal glycine residue of a subset of proteins. N-Myristoylation plays a role in facilitating protein–protein interactions, targeting proteins to membrane locations and stabilizing protein structures (Resh, 1999).
N-Myristoylation by NMT proceeds via an ordered Bi–Bi reaction mechanism; binding of myristoyl-CoA opens up a second pocket for docking of the substrate protein (Rudnick, et al., 1991; Bhatnagar et al., 1998). The myristate group is then transferred to the N-terminal glycine of the substrate in a nucleophilic addition–elimination reaction, followed by stepwise release of first the free CoA and then the N-myristoylated protein (Bhatnagar et al., 1999).

NMT was first characterised in 1987 in *Saccharomyces cerevisiae* (Towler et al., 1987), but subsequently has been isolated and characterised from number of pathogens, parasitic protozoans, plants and mammals. Comparative sequence and biochemical analyses as well as structural studies have demonstrated high conservation of the myristoyl-CoA binding sites in human NMT and parasites but divergent peptide-binding specificities (Johnson, 1994). Therefore, NMT has been the target of a number of antifungal drug development programmes, with the focus on development of selective inhibitors that act at the peptide-binding pocket. Peptide-based and peptidomimetic inhibitors have been developed that show selectivity against the NMTs of fungal species as compared to human NMT (Langner et al., 1992; Lodge et al., 1997, 1998).

NMT from *Leishmania donovani* (*Ld*NMT), the principal causative agent of the most serious form of leishmaniasis, visceral leishmaniasis has been proven to be essential for viability in extracellular parasites (Brannigan et al., 2010). Cloning of the NMT gene from *Leishmania donovani* strain LV9 (*Ld*NMT) into a modified pET28 vector with a cleavable N-terminal hexahistidine tag, its purification and crystallization with non-hydrolysable myristoyl Co-A analogue S-(2-oxo) pentadecyl-CoA (NHM) has been reported in the literature (Brannigan et al., 2010, PDB code: 2WUU). The structure contains one molecule of NHM and 378 well defined water molecules.

With the aim of exploiting *Ld*NMT for drug discovery, this chapter deals with the cloning of a new construct of the *Ld*NMT gene with deletion of a disordered N-terminal region, its overproduction in *E. coli* and the purification of recombinant protein. Since NMT from *Leishmania donovani* strain LV9 is different from Friedlin strain *Lm*NMT in only 11 amino acids, crystals obtained for *Ld*NMT in complex with
non hydrolysable myristoyl CoA analogue were soaked in reported inhibitor bound in 
*Lm*NMT peptide binding site (Frearson *et al.*, 2010). Co-crystallization by addition of a 
non-hydrolysable myristoyl CoA analogue and inhibitor after overnight incubation 
were also tried. Crystal structures in two different crystal forms were determined for 
the purified NMT enzyme bound with its non hydrolysable myristoyl CoA analogue. 
Co-crystallization of NMT with substrate and inhibitor resulted in poorly diffracting 
crystals.
6.2. RESULTS

6.2.1. Cloning of N-terminal mutant of the \textit{LdNMT} gene

A new construct of \textit{LdNMT} gene, an N-terminal deletant with a non-cleavable histidine tag (\(\Delta N \textit{LdNMT}\)), was prepared using the technique of Ligation Independent Cloning (LIC cloning) as described in Section 2.3.2 and as shown in Figure 2.4. Plasmid DNA from \textit{L. donovani} strain LV9 (Brannigan \textit{et al}, 2010) was used to prepare the N-terminal deletion mutant.

The following primer pair was designed to add the LIC specific ends by PCR method using KOD polymerase (Novagen).

\begin{align*}
\text{LdLicF} & \quad 5’-\text{CACCACCACCACATGGCATTCTGGAGCACACAGCCCGT}-3’ \\
\text{LdLicR} & \quad 5’-\text{GAGGAGAAGGCGCGTTACAACATCACCAACAGCCCGT}-3’
\end{align*}

PCR includes one cycle of initial denaturation at 98 °C for 2 min followed by 25 cycles of denaturation at 98 °C for 15 sec, annealing at 55 °C for 2 sec and extension at 72 °C for 20 sec followed by final extension cycle at 72 °C for 3 min. A 1292 bp product was amplified for the \textit{LdNMT} gene with overhangs attached (Figure 6.1).

The amplified and gel purified \textit{LdNMT} gene fragment with LIC overhangs was then treated with 1 unit of T4 DNA polymerase (Novagen/Merck) in the presence of the 2.5 mM dATP to generate the single stranded vector-compatible overhangs. 0.2 pmol of the insert was used in the reaction mixture. The contents were mixed and incubated at 22 °C for 30 min and the reaction was stopped by further incubation at 75 °C for 20min.

The product with compatible single stranded overhangs was then ligated with linearized modified pET28 vector (LICYSBL) prepared using the same protocol as above, without in the use of T4 ligase and the annealed products were transformed into NovaBlue singles competent cells.

Confirmation of the mutant was done by double digestion of the plasmid preparation from random colonies. As N-terminal deletion mutant cloned in LIC vector generated new restriction site of NcoI that was not present in original clone, a restriction digestion with NcoI and NdeI were set up with each plasmid DNA.
preparation in order to ascertain which colonies had the insert mutant DNA. The presence of an insert with size close to 1200bp confirms the presence of N-terminal mutant LdNMT gene which was confirmed by DNA sequencing of the isolated plasmids. Double digestion was done using 1 unit each of NcoI and NdeI.

Figure 6.1: 1% agarose gel showing PCR amplified LdNMT gene with LIC overhangs, lane 1: 1Kb DNA ladder, lane 2: PCR product.

Sequencing of the double digestion confirmed plasmid prep confirms the cloning of N-terminal mutant of LdNMT gene as shown in the Figure 6.2 and 6.3.

Figure 6.2: Translation product of the double digestion confirmed construct isolated from E.coli NovaBlue cells. Residues that were mutated are shown in bold and are underlined.
Figure 6.3: Amino acid alignment of the N-terminal mutant with the original NMT construct (Brannigan et al., 2010). Residues that were mutated are underlined.

6.2.2. Expression Studies for the Cloned Protein

The plasmid harbouring the coding sequence for the truncated NMT was transformed into E.coli pLEMO cells for the expression. 10 ml LB medium supplemented with kanamycin (50 µg/ml) + chloramphenicol (34 µg/ml) was inoculated with 100µl of overnight grown single colony and induced with 1 mM IPTG at OD$_{600}$ of 0.5-0.7. Lysate from induced culture after 3 h at 37 °C of incubation showed the expression of the truncated protein mostly in insoluble fraction (Figure 6.3.4).
6.2.3. Comparison of expression and initial purification of $\Delta N$ LdNMT in LB and in Auto Induction Medium (AIM)

500 ml of LB and Auto Induction Medium each were inoculated with 5ml of overnight primary culture containing LdNMT mutant in pLEMO cells. LB medium was induced with 1mM IPTG after incubating at 37 °C for 2 h. AIM was kept at 37 °C for 7 h. Both medium flasks were shifted to 18°C. LB culture was harvested at 5000 rpm for 15 min at 4 °C after 4 h of incubation while AIM was kept for overnight incubation at 18 °C. Harvested cells for both culture broths were resuspended in minimum volume of lysis on buffer. Unclarified sonicate cells were loaded onto a 1ml HisTrap crude column (GE life Sciences, USA) pre-equilibrated with binding buffer (20 mM Tris-HCl pH 8.5, 10 mM Imidazole and 0.5 M NaCl) and eluted with elution buffer containing 500 mM Imidazole. Eluted fractions along with flow through and lysate were loaded on a 12% SDS-PAGE gel to compare the expression levels and preliminary purification.

The auto-induction method of protein expression in *E. coli* is based on diauxic growth resulting from dynamic function of *lac* operon regulatory elements (*lacO* and *LacI*) in mixtures of glucose, glycerol, and lactose. During the initial growth period,
glucose is preferentially used as a carbon source and protein expression is low as a result of catabolite repression of alternative carbon utilization pathways (Inada et al., 1996; Hogema et al., 1998, 1999) and binding interactions between lac repressors (LacI) and lac operators (lacO). As glucose is depleted, catabolite repression is relieved, which leads to a shift in cellular metabolism toward the import and consumption of lactose and glycerol. Lactose import results in the production of allolactose from lactose by a promiscuous reaction of β-galactosidase. Allolactose then acts as the physiological inducer of the lac operon (Blommel et al., 2007). AIM results in higher protein yields than IPTG induction. Furthermore, if a protein is very toxic or even slightly toxic, AIM has the added advantage of very low to no expression prior to the time of induction. Expression and initial purification of ΔN LdNMT was found to be better in Auto Induction Media (AIM, Studier, 2005) as compared to LB media (Figure 6.5). As seen in the gels, there is more expression of the enzyme in the auto Induction medium and more of the binding to the affinity column. Almost all the protein expressed in LB medium was in the insoluble fraction that could not be available for the binding presumably due to unavailability of histidine tag.

![Figure 6.5: Comparison of expression and initial purification using affinity chromatography of ΔN LdNMT expressed in pLEMO in (A) LB and (B) Auto Induction Medium; Lane 1-low range protein marker, lane 2-lysate 2µl, lane 3- FT 2µl, lane 4- washing 20 µl, lane 5-10-elution fraction 20 µl.]

6.2.4. Purification of ΔN LdNMT Enzyme

The steps ΔN LdNMT enzyme purification were described under Methods in Chapter 2 under section 2.3.2.8. Briefly the enzyme was purified from auto induction
media using a three step purification protocol. Since the enzyme was expressed containing N-terminal histidine tag, the affinity chromatography was employed as a first step of the protocol followed by anion exchange on Q-Sepharose and the polishing step of Size Exclusion chromatography. The elution profiles for the purification protocol are shown in Figure 6.6.
Figure 6.6: Purification protocol followed for the \( \Delta N \) LdNMT Enzyme; (A) Elution profile for the affinity chromatography, (B) Elution profile for Q-Sepharose, (C) Elution profile for Size exclusion Chromatography (HiLoad Superdex 75 16/60pg). OD\(_{280}\) were shown in blue colour, OD\(_{215}\) as red, buffer conductivity as brown and concentration of Elution buffer as green.

The typical yield for the enzyme was 3-4 mg/l of culture. Purified protein showed the presence of more than 90% pure protein of expected size on 12 % SDS-PAGE (Figure 6.7). Pure protein when concentrated tends to precipitate, therefore 4 times molar concentration of non-hydrolysable Myristoyl CoA analogue substrate (NHM) was added during concentration of protein. 50 µl aliquots of concentrated protein were made and stored in 193K after freezing in Liquid N\(_2\).
6.2.5. Crystallization of $\Delta N LdNMT$

$\Delta N LdNMT$ protein was concentrated to 6 mg/ml in the presence of the non-hydrolysable myristoyl CoA analogue (NHM or S-(2-oxo) pentadecyl-CoA). The final concentration of NHM was kept at 4 times the molar concentration of protein and the sample was kept overnight on ice. Crystallization was tried using available commercial sparse matrix screens from Hampton Research Ltd, Qiagen and Molecular Dimensions. Protein samples were centrifuged for 5 min at 10,000 rpm and 278 K immediately prior to the crystallization to ensure that samples were free from any particulate matter. Each experiment consisted of equilibrating a mixture of 150 nl protein solution and 150 nl screen solution over a reservoir of 60 nl screen solution in MRC 96-well plates (Griener bio-one, USA) using a Mosquito nano-litre high throughput robot manufactured by TTP labtech.

Initially, several different precipitants and buffers were used in crystallization attempts. Often, the crystals grew as multiple crystals or their diffraction quality was poor. Further optimization of hits by varying the concentration of precipitant and/or salt and drop size in 24 well hanging-drop vapour-diffusion experiments resulted in multiple crystals and/or single crystals (Figure 6.8). Single crystals used for the soaking experiment and subsequently for the data collection were grown by setting up

![Figure 6.7: 12% SDS-PAGE showing final purified $\Delta N LdNMT$.](image)

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the hanging-drop vapour-diffusion method manually mixing 1.5 µl of protein at 6 mg/ml with 1 µl of well solution. Reproducible single crystals of diffraction quality were obtained primarily in two conditions of 2.0 M ammonium sulphate, 0.2 M sodium chloride in 1-2 weeks and 0.2 M tri-sodium citrate tetra hydrate, 25% PEG 3350, 0.1 M sodium acetate pH 4.6 in 3-4 weeks at 293K (Figure 6.8).

![Figure 6.8](image)

**Figure 6.8**: Crystals obtained in 24 well plate hanging drop-vapour diffusion in two different conditions for \( \Delta N \text{LdNMT} \) with its substrate incubated overnight; (A) Multiple crystals obtained for the mutant (B) Single crystals obtained in ammonium sulphate condition, (C) Single crystal obtained in PEG3350 condition.

### 6.2.6. Soaking of the Crystals in the Reported Inhibitor of \textit{L.major} NMT and \textit{T.brucei} NMT

DDD 85646 is a synthetic molecule containing a pyrazole sulphonamide scaffold known to inhibit the proliferation of the bloodstream form of \textit{T. brucei} in culture (Figure 6.9) (Frearson et al., 2010). Since \textit{LmNMT} has 74% overall sequence identity with \textit{TbNMT} and 94% identity within the peptide-binding site and \( \Delta N \text{LdNMT} \) is 98% identical to NMT from \textit{Leishmania major}, crystals obtained for N-terminal
mutant of LdNMT were soaked in the reported inhibitor of LmNMT. This inhibitor (DDD 85646) is selective against peptide binding pocket for the LmNMT and LdNMT as well (LdNMT IC₅₀ = 3nM, Data not published). Crystals obtained from both the conditions were soaked in mother liquor containing 1-2 mM of DDD85646 for 30 min and flash cooled in liquid nitrogen.

![Figure 6.9: Representation of DDD85646 inhibitor](image)

### 6.2.7. Data Collection for the Soaked Crystals

Diffraction data from crystals soaked with DDD85646 were collected at the Diamond Light Source synchrotron facility at Oxford, UK on beamline I02 (λ = 0.9795 Å) using an ADSC Q315r CCD detector. Data for the crystals obtained from the ammonium sulphate condition were collected as 720 images with 0.25° oscillation and 0.25 sec exposure time per image and the crystals obtained from PEG3350 condition was collected as 400 images with 0.5° oscillation and 0.5 sec exposure per image (Figure 6.10). Data were found to be highly anisotropic from both the crystals and there were indications that the crystals were split. Moreover, diffraction showed overlapping spots at low resolution which mean possibility of twinning effect in the intensity distribution.

Data were automatically processed with xia2/XDS (Winter, 2010; Kabsch, 2010) and scaled using the SCALA programme in CCP4 suite (Winn et al., 2011). The crystal of LdNMT mutant protein from ammonium sulphate condition is found to be triclinic P1 with unit cell a = 70.08 Å, b = 79.79, c = 92.29 Å, α = 75.18°, β = 73.07°, γ = 72.68°, whereas crystals grown from PEG3350 crystallized in the monoclinic space group P2₁ with unit cell dimensions a = 92.58 Å, b = 90.19 Å, c = 121.71 Å, α = γ = 90°, β =
110.45°. The dataset obtained from P1 crystal was found to be twinned with twin fraction of 13%. The data collection and processing statistics are summarized in Table 6.1.

Figure 6.10: Diffraction images for two crystals obtained in two different conditions; (A) Diffraction image of the crystals obtained from the PEG3350 condition indexed in P2_1, (B) Diffraction image of the crystals obtained from the Ammonium sulphate condition indexed in P1.
### Table 6.1: Data collection statistics for crystals obtained for $\Delta N LdNMT$

<table>
<thead>
<tr>
<th></th>
<th>Crystal form 1 with NHM</th>
<th>Crystal form 2 with NHM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beam line</strong></td>
<td>Diamond IO2</td>
<td>Diamond IO2</td>
</tr>
<tr>
<td><strong>Wavelength</strong></td>
<td>0.9795 Å</td>
<td>0.9795 Å</td>
</tr>
<tr>
<td><strong>Space group</strong></td>
<td>P2₁</td>
<td>P1</td>
</tr>
<tr>
<td><strong>Unit cell dimensions (Å)</strong></td>
<td>$a = 92.58$, $b = 90.19$, $c = 121.71$, $\alpha = \gamma = 90^\circ$, $\beta = 110.45^\circ$</td>
<td>$a = 70.08$, $b = 79.79$, $c = 92.29$, $\alpha = 75.18^\circ$, $\beta = 73.07^\circ$, $\gamma = 72.68^\circ$</td>
</tr>
<tr>
<td><strong>Resolution</strong></td>
<td>1.8 Å</td>
<td>2.23 Å</td>
</tr>
<tr>
<td><strong>% Solvent</strong></td>
<td>49.7</td>
<td>49.7</td>
</tr>
<tr>
<td><strong>Matthews Coefficient (Å³ Da⁻¹)</strong></td>
<td>2.44</td>
<td>2.45</td>
</tr>
<tr>
<td><strong>Total Number of observations</strong></td>
<td>695337 (94751)</td>
<td>170076 (24547)</td>
</tr>
<tr>
<td><strong>Total number of unique observations</strong></td>
<td>171280 (24396)</td>
<td>86979 (12571)</td>
</tr>
<tr>
<td><strong>$R_{\text{merge}}$ (%)†</strong></td>
<td>12.3(87.4)</td>
<td>10.0(36.9)</td>
</tr>
<tr>
<td><strong>Completeness (%)</strong></td>
<td>99.0(97)</td>
<td>96.6(95.2)</td>
</tr>
<tr>
<td><strong>Mean I/σ(I)</strong></td>
<td>7.2(1.5)</td>
<td>6.3(2.1)</td>
</tr>
<tr>
<td><strong>Multiplicity</strong></td>
<td>4.1(3.9)</td>
<td>2.0(2.0)</td>
</tr>
<tr>
<td><strong>Molecules in a.s.u</strong></td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

† $R_{\text{merge}} = \frac{\sum_{hkl} \sum_{i} |I_{i}(hkl)| - \{I(hkl)\}}{\sum_{hkl} \sum_{i} I_{i}(hkl)}$, where $I_{i}(hkl)$ is the intensity of an individual measurement of the reflection with Miller indices hkl and $\{I(hkl)\}$ is the mean intensity of redundant measurements of that reflection.

Numbers in the parentheses are for the last resolution shell.
6.2.8. Solvent Content of Crystals

Considering molecular weight of the mutant as 48.7kDa, the calculated Matthews coefficient (Matthews, 1968) suggests four molecules in the asymmetric unit for both P1 and P2₁ datasets (2.45 Å³ Da⁻¹ and 2.44 Å³ Da⁻¹ corresponds to a solvent content of 49.7% respectively).

6.2.9. Structure Solution

Unique solutions were found for each of the two crystal forms taking coordinates of \textit{L.major} NMT bound with substrate and inhibitor (DDD85646) using molecular replacement method in CCP4 suite (PDB code: 2WSA, Frearson \textit{et al.}, 2010). \textit{MOLREP} (Vagin and Teplyakov, 1997) from CCP4 suite using pdb coordinates of \textit{Lm}NMT was used for the P2₁ dataset. \textit{MOLREP} resulted in the detection of non-crystallographic translation vector of 0.000, -0.425, 0.500 (40% of the origin) for monoclinic dataset. \textit{PHASER} (McCoy \textit{et al.}, 2007; Storoni \textit{et al.}, 2004) from CCP4 suite (Collaborative Computational Project, Number 4, 1994) was used as molecular replacement method in the case of triclinic dataset. As suggested by Matthews coefficient, both the resultant structures contained 4 molecules in the crystallographic asymmetric unit.

6.2.10. Refinement of the structures

The initial phases obtained from molecular replacement using the search model were improved by subsequent rigid body refinement keeping the sequence of the input model. Rigid body refinement using Refmac 5.6.0116 (Murshudov \textit{et al.} 2011) gave the R-factor/R_free of ~0.42/ ~0.43 for P2₁ and ~36/35 for P1 structure. This was followed by several cycles of positional refinement using the data in the resolution shell of 48.67-1.8 Å (monoclinic), 86.76-2.3 Å (triclinic) resulting in the improvement of both R_factor and R_free. Since both structures have more than one molecule in their asymmetric units, their initial models were refined taking advantage of the presence of non-crystallographic symmetry. Manual correction was done in \textit{COOT} (Emsley & Cowtan, 2004). Individual B-factors were refined in the final cycles. Electron density maps were calculated at this stage. Side chains were mutated according to the sequence of
∆N LdNMT and their conformations were selected based on the observed density and their new positions were refined.

Water molecules were added using (Fo-Fc) map as reference. Automated water picking programme of the suite was employed for adding the water molecules during refinement. Water molecules were manually checked using following criteria.

(i) The electron density is observed at 1σ level in the (2Fo-Fc) map and correspondingly in the (Fo-Fc) map at 5σ level initially and has been extended up to 3σ level during final cycles of refinement.

(ii) The distance between selected water molecule and interacting protein atoms or water molecules has to be between 1.8 to 3.2 Å. At least one hydrogen bond must be satisfied for selecting a water molecule. The appropriate geometry of hydrogen bonds around water molecule also has been considered.

∆N LdNMT was co-crystallised with NHM, a non hydrolysable analogue of myristoyl-CoA with a methylene group intervening between the sulphur and the carbonyl carbon in what would otherwise be the thioester linkage in myristoyl-CoA (Figure 6.11). A difference map calculated during refinement showed clear density for the NHM in both the structures (Figure 6.12). However conclusive density for the inhibitor was not seen in the difference map in any of the crystal forms (Figure 6.13).

Figure 6.11: (A) Structure of Myristoyl-CoA. The arrow indicates the bond that is hydrolysed in the transfer of myristate to the protein subset. (B) Structure of S-(2-oxo)pentadecyl-CoA (NHM). The arrow shows the extra methylene group intervening between the sulphur and the carbonyl carbon in the Myristoyl-CoA.
Figure 6.12: NHM density in Fo-Fc map at 3σ level showing presence of bound substrate in P2₁. Similar density was seen in all the four chains of monoclinic form and also in the triclinic form (not shown). The substrate was superimposed and added to the difference density obtained after refinement. Figure was prepared in COOT.

Figure 6.13: Inhibitor (DDD85646) superimposed on P2₁ electron density showing its absence in the crystal. The electron density for inhibitor is absent in all the four chains and also in the triclinic form (not shown). Figure was prepared in COOT.
6.2.11. Structure validation

During the final cycles of refinement the stereochemistry and the geometry of the models were checked using PROCHECK (Laskowski et al., 1993). The overall G factors considered to be a measure of stereochemical quality of the model output by PROCHECK were -0.06 and -0.16 for the P2_1 and P1 crystal forms respectively. The values for G factors were within the limits expected for correct structures that are refined at 1.8 Å and 2.23 Å resolution respectively. The refinement statistics and the values of refined parameters are presented in Table 6.2. The Ramachandran (φ, ψ) plots (Ramachandran & Sasisekharan, 1968) showed that most of the residues were placed in the most favoured or partially allowed regions of the map.

Table 6.2: Refinement Statistics for the ∆N LdNMT crystals

<table>
<thead>
<tr>
<th>Crystal System/Space Group</th>
<th>Monoclinic/P2_1</th>
<th>Triclinic/P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution range (Å)</td>
<td>48.72-1.80</td>
<td>86.76-2.23</td>
</tr>
<tr>
<td>Refinement Statistics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R_{cryst}^a</td>
<td>0.254</td>
<td>0.293</td>
</tr>
<tr>
<td>R_{free}^b</td>
<td>0.296</td>
<td>0.327</td>
</tr>
<tr>
<td>Root Mean Square Deviation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond Length (Å)</td>
<td>0.017</td>
<td>0.0133</td>
</tr>
<tr>
<td>Bond Angle (°)</td>
<td>1.8337</td>
<td>1.841</td>
</tr>
<tr>
<td>Chiral Volume</td>
<td>0.1155</td>
<td>0.327</td>
</tr>
<tr>
<td>Ramachandran Plot Analysis (% Residues)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Most favoured region</td>
<td>90.1</td>
<td>86.6</td>
</tr>
<tr>
<td>Additionally allowed region</td>
<td>9.4</td>
<td>12.7</td>
</tr>
<tr>
<td>Generously allowed region</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Disallowed region</td>
<td>0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

^a R_{cryst} = Σ |Fo-Fc| / ΣFo

^b R_{free} = Σ |Fo-Fc| / ΣFo where the F values are test set amplitudes (5 %) not used in refinement.

In addition to the anisotropy in both the datasets, the splitting of the crystals and non-crystallographic translation in monoclinic and possibility of twining in triclinic
crystal form also may be contributing to the comparatively higher values for R-factors, $R_{\text{cryst}}$ and $R_{\text{free}}$.

### 6.2.12. Description of the Structure

In describing the structure, the secondary-structure nomenclature used for *C. albicans* NMT (Weston *et al.*, 1998), *S. cerevisiae* NMT (Bhatnagar *et al.*, 1998) and *L. donovani* wilt type structure (Brannigan *et al.*, 2010) has been adopted in which helices are denoted with uppercase letters and strands with lowercase letters (Figure 6.13). Secondary-structure elements additional to those observed in the *C. albicans* NMT (*CaNMT*) are denoted by one or more primes (').

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**Figure 6.13:** The three-dimensional structure of *LdNMT*. Stereo ribbon representation of *LdNMT* in its complex with NHM, which is shown in cylinder format and coloured by atom type: carbon, cyan; oxygen, red; nitrogen, blue, sulfur, yellow; phosphorus, magenta. The protein chain is colour ramped from residue 11 at the N-terminus (red) to residue 421 at the C-terminus (magenta). The secondary-structure elements are labelled with uppercase letters for $\alpha$-helices and lowercase letters for $\beta$-strands. The ' and " superscripts indicate elements additional to those observed in the *C. albicans* NMT (*CaNMT*, Weston *et al.*, 1998).

Both the crystal forms are very similar to the wild type *LdNMT* structure with rmsd of 0.96Å for 402 superposed $Ca$ atoms (Figure 6.14) with volume occupied by the protein is 227669 Å³. The NHM was also found to be bound with similar
conformation as in wild type LdNMT. Like the wild type, the molecule showed two lobes, each of which has a multistranded β-sheet element at its core flanked by two α-helices on one face and a single α-helix on the other face (Figure 6.13). For the N-terminal lobe, the sheet is made up of strands “acdefg” with helices “A” and “B” packed onto one face and helix “C” onto the other. For the C-terminal lobe, the sheet comprises strands “gmlkjh” against one face of which helices “F” and “G” are packed, with helix “H” packed onto the other face.

Figure 6.14: Comparison of the structures of LdNMT and ∆N LdNMT. The structures of LdNMT–NHM (PDB: 2WUU, Brannigan et al., 2010) and ∆N LdNMT–NHM (P2₁, form) were superposed using the SSM superpose routine in CCP4MG. The structures are displayed as ribbons, LdNMT (light green with turns coloured as coral) and ∆N LdNMT (Light cyan with turns coloured grey), with the ligands represented in cylinder format and coloured by atom type: carbon, cyan; oxygen, red; nitrogen, blue, sulfur, yellow; phosphorus, magenta.

Figure 6.14: Comparison of the structures of LdNMT and ∆N LdNMT. The structures of LdNMT–NHM (PDB: 2WUU, Brannigan et al., 2010) and ∆N LdNMT–NHM (P2₁, form) were superposed using the SSM superpose routine in CCP4MG. The structures are displayed as ribbons, LdNMT (light green with turns coloured as coral) and ∆N LdNMT (Light cyan with turns coloured grey), with the ligands represented in cylinder format and coloured by atom type: carbon, cyan; oxygen, red; nitrogen, blue, sulfur, yellow; phosphorus, magenta.
PROMOTIF analysis using PDBsum (http://www.ebi.ac.uk/pdbsum/) (Hutchinson & Thornton, 1996) showed the monoclinic form of the ΔN LdNMT consist total of 2 β-sheets with 16 strands and 18 α-helices. The secondary structure and topology diagram obtained from PDBSum are shown in Figure 6.14 and 6.15.

![Secondary structure and topology diagram](image-url)

Figure 6.14: Arrangement of secondary structure elements ΔN LdNMT monoclinic form.
Figure 6.15: Topology diagram of ΔN LdNMT with secondary-structure elements labelled and with α-helices represented as cylinders and β-strands as arrows

Most of the residues in the structure have mean atomic temperature factor for main-chain atoms as calculated from a web based suite for Protein Structure Analysis Package (PSAP, http://iris.physics.iisc.ernet.in/psap/) is low at 15-20 Å² with average of 23.1 Å² for entire protein. The regions corresponding to surface loops and loops “Ab” “DE” and “kl” have residues with significantly higher average main-chain temperature factors, suggesting that these regions of the structure have independent mobility.
Structurally deduced mechanism of the N-terminal Myristoylation of subset of proteins by NMT enzymes requires the displacement upon myristoyl-CoA binding of the “Ab” loop, which partially occludes the binding site for the N-terminal peptide of the substrate (Bhatnagar et al., 1998; Farazi et al., 2001; Brannigan et al., 2010). This loop, which serves as a lid that opens and closes over the catalytic site, reported to be having variable conformations among the different structures. It partially occludes the myristoyl-CoA and peptide binding sites in the structure of the uncomplexed CaNMT, while in the binary and ternary complexes of ScNMT the loop has swung upward to different extents, opening up the active site. In this way, the binding of myristoyl-CoA assists peptide binding by ensuring the lid is open and the second substrate can bind unhindered. The different conformation of this “Ab” loop as reported in apo-enzyme and binary and ternary complexes has been depicted in the Figure 6.16 with ΔN LdNMT superimposed. ΔN LdNMT structure showed the loop conformation in between the fully open conformation of ScNMT ternary complex and closed conformation of apo-enzyme structure of CaNMT.
Figure 6.16: Different conformation of Ab loop as shown in apo-enzyme structure (CaNMT PDB code: 1NMT, Weston et al., 1998) and in the ternary complex (ScNMT–NHM–GLYASKLA, PDB code: 1IID, Farazi et al., 2001) superimposed with binary complex of ΔN LdNMT-NHM superposed using the SSM superpose routine in CCP4MG. The structures are displayed as ribbonΔN LdNMT (green), CaNMT (yellow) and ScNMT (blue), with the ligands represented in cylinder format. The Ab loops from the superposed CaNMT apo-enzyme, ΔN LdNMT-binary and ScNMT–NHM ternary complex are shown in grey, green and red respectively.

Out of 4 chains in ΔN LdNMT structure chain A and chain B contains good electron density for the residues forming “Ab” loop but having much higher than average temperature factors, whereas, chain C and D shows no good density for most of the loop residues, suggesting intrinsic mobility of this segment of the structure. Taken together, it seems that the “Ab” loop is closed in the uncomplexed enzyme and
that following fatty acyl-CoA binding the “Ab” loop adopts an ensemble of structures that facilitate the binding of the N-terminal peptide of the substrate protein or inhibitor mimicking the N-terminal peptide.

Figure 6.17: (A) Packing arrangement of the 4 chains in asymmetric unit of $\Delta N \text{LdNMT}$, (B) schematic diagram of interactions between protein chains of $\Delta N \text{LdNMT}$.
Table 6.3: Interface statistics for the four chains of $\Delta N LdNMT$

<table>
<thead>
<tr>
<th>Chains</th>
<th>No. of interface residues</th>
<th>Interface area ($\text{Å}^2$)</th>
<th>No. of salt bridges</th>
<th>No. of disulphide bonds</th>
<th>No. of hydrogen bonds</th>
<th>No. of non-bonded contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td>A : B</td>
<td>17:15</td>
<td>786:822</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>93</td>
</tr>
<tr>
<td>A : C</td>
<td>17:16</td>
<td>913:922</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>69</td>
</tr>
<tr>
<td>B : D</td>
<td>16:16</td>
<td>865:851</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>62</td>
</tr>
</tbody>
</table>

6.2.13. Co-Crystallization of $\Delta N LdNMT$ with substrate and inhibitor

Various commercially available screens were tried for co-crystallization of $\Delta N LdNMT$ with substrate and inhibitor. Condition G5 (20% PEG3350, 0.2 M sodium nitrate, 0.1 M Bis Tris propane pH 7.5) from PACT screen (Qiagen) gave small but single crystals (Figure 6.18). Fine screening of the condition varying either precipitant concentration or pH at a time gave single crystals in many conditions (Figure 6.19). However these crystals were found to be diffracting poorly to $12 \text{ Å}$ even at synchrotron facility.
Figure 6.18: Original hit obtained for co-crystallised ΔN LdNMT with substrate and inhibitor added and incubated overnight.

21% PEG3350, 0.2M Sod. Nitrate, 0.1M Bis Tris Propane pH 7.1
19% PEG3350, 0.2M Sod. Nitrate, 0.1M Bis Tris Propane pH 7.5
19% PEG3350, 0.2M Sod. Nitrate, 0.1M Bis Tris Propane pH 7.3

18% PEG3350, 0.2M Sod. Nitrate, 0.1M Bis Tris Propane pH 7.3
19% PEG3350, 0.2M Sod. Nitrate, 0.1M Bis Tris Propane pH 7.6
19% PEG3350, 0.2M Sod. Nitrate, 0.1M Bis Tris Propane pH 7.3

Figure 6.19: Various crystals obtained in fine screen after optimization of initial hit obtained for ΔN LdNMT with substrate and inhibitor added.
6.3. DISCUSSION

NMT has been shown to be essential for the survival for many parasites making it a potential drug target against many parasitic infections. Targeted gene deletion methods showed NMT is likely to be an essential gene in the *L. donovani* life cycle, thus presenting NMT from *L. donovani* as a suitable target for drug discovery for deadly disease Visceral Leishmaniasis (Brannigan *et al.*, 2010). Industrial antifungal drug discovery programmes, high throughput screening and *in vivo* results have led to the identification of many highly potent selective inhibitors against various fungal and protozoan parasites. Comparative sequence and biochemical analyses as well as structural studies have demonstrated high conservation of the myristoyl-CoA binding sites across species, and the interactions of fatty acyl-CoA with the enzyme are conserved in the structures that have been determined. Attention has therefore been directed toward the development of inhibitors that bind in the peptide binding site. There is conservation of sequences in the peptide binding groove; nevertheless, selective inhibitors of yeast and fungal NMTs have been discovered that bind in this location, exploiting residue differences at a handful of positions and often subtle differences in structure (Bhatnagar *et al.*, 1998; Sogabe *et al.*, 2002). The determination of the crystal structure of the *Ld*NMT–NHM complex (Brannigan *et al.*, 2010) established a foundation both for the structural analysis of inhibitor complexes and for structure-assisted drug discovery. Comparison of *Ld*NMT-NHM bound structure with high-resolution structure of *Hs*NMT-Myristoyl-CoA (PDB code: 3IU1; Structural Genomics Consortium, unpublished data) has shown differences in the loops (e.g., “Ab”, “kl” and “DE”) forming the upper surface of the peptide binding site. Non conservation of predicted residue in *Ld*NMT and *Hs*NMT, binding to peptide substrate or inhibitor and residues further to that may present an opportunity for selective inhibitor action. Also, the residues predicted to form the peptide binding site in *L. donovani* NMT is different in the NMTs of *T. brucei* and *T. cruzi* at two and four positions respectively (Brannigan *et al.*, 2010).

Recent availability of the structures of *Hs*NMT in complex with myristoyl-CoA and three different polycyclic inhibitor molecules (PDB codes: 3IU2, 3JTK and 3IWE, Structural Genomics Consortium, unpublished data), one of which crystallized with same DDD85646 inhibitor, can also present useful information determining the
differences in the conformations and interactions of these inhibitors in different pathogens and in human, hence useful in selective structure based drug discovery efforts.

However, failure to soak in the reported inhibitor (DDD85646) for *T. Brucei* (Frearson *et al.*, 2010) and *LdNMT* (*LdNMT IC_{50} = 3nM, Data not published*) to the new construct (∆N *LdNMT*) and poor diffraction of the co-crystallized crystals shows more considerable efforts to be made before gaining the insight to the binding mode and selective efficacy of this inhibitor with the *LdNMT*. 