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N-terminal Domain of *tly*A from *Mycobacterium tuberculosis* Displayed Concentration Dependent Ordered Structure

V.B. Shivaleela[1], Srihari Prathapaneni[2], P. Sharada[1*] and K. Giri Gowda[2]

[1]Department of Biotechnology, Basaveshwara Engineering College, Bagalkot-587102, Karnataka, India
[2]Sampoorna International Institute of Agri. Science & Horticulture Technology, Mandya-571433, Karnataka, India

**Corresponding author*

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A B S T R A C T

Mycobacterium tuberculosis (Mtb), the causative agent of the disease tuberculosis, is an ancient pathogen and a major cause of death worldwide. Although various virulence factors of *M. tb* have been identified, its pathogenesis remains incompletely understood. TlyA is a virulence factor that is evolutionarily conserved in many gram-positive bacteria, but its full length structure and function in the pathogenesis of infection with *Mtb* has not been elucidated. In the present study, we cloned, expressed and purified N-terminal domain of *tly*A, which play a crucial role in the binding of the co-substrate *S*-adenosyl-L-methionine. We characterized the protein by SDS-PAGE and Circular Dichroism. *Tly*A model generated using *tly*A crystal structure, clearly indicates E59 separates between N-terminal domain (NTD) and C-terminal domain (CTD).

Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), most successful gram-positive bacterial pathogen, primarily infects human lungsand is a major global public health problem, with approximately 9 million new cases and nearly 2 million deaths each year (WHO, 2018).

Efforts to search for virulence factors of *M. tuberculosis* (*Mtb*) is unrelenting, many researchers have identified genes that may serve as potential targets for vaccine development. Among the unexplored gene products of *Mtb*, *tly*A (Rv1694) was recently identified as a possible virulence factor. *Tly*A protein have a haemolysin activity and *tly*A is a 268 amino acid polypeptide (Martino MC *et al.*, 2001). The *tly*A gene is also present in several pathogenic mycobacterial species, including *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Although, *M. tuberculosis* and *M. leprae* evolved from a

common ancestor, *M. leprae* possesses fewer genes. Genes conserved between the two species are hence considered important for pathogenicity and virulence.Almost all *tly*A homologues have K-D-K-E domain for 2′-hydroxy-ribose methylation in ribosomal RNA (Wren *et al.,* 1998).

When *Tly*A is introduced into non-haemolytic *M. smegmatis* strains, and cloned into *E.coli*, it showed contact dependent haemolytic activity (Wren *et al.,* 1998). It has been previously shown that in H37Rv, the *tly*A gene may be a part of an operon containing at least three other genes: *tly*A (Rv1694), ppnk (Rv1695) and RecN (Rv1696), homologous to *E.coli* RecN (Wren *et al.,* 1998). *Tly*A is also known to function as a ribosomal RNA methyltransferase. It is known to methylate 50S and 30S ribosomal RNA and makes *Mtb* susceptible to the peptide antibiotic capreomycin (Monshupee *et al.,* 2012).

Despite intense research on *Mtb* pathogenesis, detailed molecular mechanisms of the role of distinct mycobacterial virulence factors remain in completely understood. To understand its mechanism of pathogenesis, the functions of numerous *M. tuberculosis* gene products are being characterized in animal models. Recently, Rahman *et al.,* (2010) reported that *tly*A (Rv1694) of *M. tuberculosis* possesses haemolytic activity by binding with and oligomerizing into host cell membranes.

Resistance to antibiotics in *Mtb* can aquire via mutation of *tly*A, protein belongs to a unique group of methyltransferases for which the loss of function confers bacterial antibiotic resistance. Many bacterial genera lack *tly*A, the potent antibiotic activity of capreomycin is specific against *Mtb*. (Kumar *et al.,* 2011). In this study, our aim was to understand the structure and possible role of *tly*A N-terminus in the interaction of SAM binding in*Mtb*.

Materials and Methods

Strains and plasmid

pCDF vector system was obtained from Invitrogen (California, USA) and was used according to the manufacturer's instructions. *E.coli* DH5α competent cells were obtained from Invitrogen (California, USA).

Isolation of genomic DNA

Bacterial culture (50ml) was harvested at optical density of A_{600}0.5-0.6 at 37°C by centrifugation at 4150 rpm for 7 mins. The pellet was resuspended by adding 6ml of freshly prepared chloroform-methanol (3:1) solution and vortexed until the bacteria were lysed as evident by a clear bottom layer. 6ml of Tris-buffered phenol (pH 8) was added and vortexed. 9ml of guanidinium thiocyanate buffer (GTC) solution was added and vortexed. The sample was centrifuged at 10000x g for 10-15 mins and a clear supernatant was collected. DNA was precipitated out by adding equal volumes of isopropanol, mixed gently and centrifuged at 13-14,000 rpm for 10-15 mins. The pellet was suspended in 4 ml TE buffer and transferred to an eppendorf tube. The DNA was used for PCR with primers for *tly*A gene.

PCR amplification of *tly*A N-terminal domain (NTD)

Oligonucleotide primers used for amplification of *Mtb*-*tly*A NTD were designed based on the *tly*A sequence from *mycobacterium tuberculosis* strain H37Rv deposited in genome database (NCBI accession no.AQO55200.1). Primers were designed based on its sequence for generating a truncation of *tly*A NTD. The sequence of the forward primer was 5′-GCGGAATTCA TGGCACGACGTGCCCGCGTT-3′ and the reverse primer was 5′- TATGGTACCTTC

ACTGTCGGTCACCAC-3′. The PCR reaction mixture consisted of 5 µl of 5X Phusion buffer supplied with the enzyme, 200 µM of each dNTPs, 0.5 µM of each primer, 500ng of DNA template, and of 0.02 U/µl Phusion DNA polymerase (New England Biolabs, Massachusetts, USA) and water to a final volume of 50 µl. The gene was successfully amplified using the following PCR conditions: 98 °C for 30 sec followed by 30 cycles of denaturation at 98 °C for 10 sec, annealing at 60 °C for 30sec, extension at 72 °C for 30sec and the final extension was carried out at 72 °C for 10 min on a PTC-100 Thermocycler (M.J. Research, Watertown, MA). The PCR product was analyzed on a 1 % agarose gel electrophoresis and DNA band corresponding to the expected size was purified using a gel extraction kit (ThermoFisher Scientific, Waltham, Massachusetts, USA).

Cloning and DNA sequencing

The PCR product was subcloned into plasmid DNA using the modified MBP-His-pCDF vector system (Novagen, Wisconsin, USA)and TEV recognition site upstream of the MCSI (multiple cloning site I). The PCR product and MBP-His-pCDF vector was digested with restriction enzymes EcoRI and KpnI for 2h at 37^{o} C, product was purified. 2 µl of purified PCR product was mixed with 0.5 µl linearized MBP-His-pCDF cloning vector in presence of 0.5 µl T4 DNA ligase (ThermoFisher Scientific, Waltham, Massachusetts, USA) and incubated overnight at 16 °C. Then the ligation mixture was directly used for the transformation of $CaCl_2$ competent DH5α cells by heat shock method (Inoue *et al.,* 1990). Colony PCR was performed to screen positive colonies. Positive colonies were picked, grown overnight in 5 ml of LB broth at 37 °C and plasmids were isolated using commercial mini-prep kit (GCC Biotech, West Bengal, India). Restriction digestion screening of the isolated plasmids was done to select the construct containing the correct size insert and selected constructs were sequenced. Sequencing was performed in both the directions using vector specific T7 promoter primer.

Expression and purification

The MBP-His-pCDF vector containing *Mtb-tly*ANTD was transformed into *E. coli* BL21 (Star) competent cells. For protein expression, transformed BL21 (Star) cells were grown at 37ºC to an optical density of 0.6 at 600 nm (OD600) and induced with 200µM isopropyl-ß-thiogalactopyranoside (IPTG). Induced cultures were transferred to 16° C and cells were grown for 12-14 h. Cells were harvested by centrifugation at 18,000 rpm at 4ºC and cell pellets were stored at -20ºC until further use. For protein purification, cell pellets from 1 litre culture were resuspended in 20 ml of ice cold binding buffer containing 50 mM TrisHCl (pH 7.5), 300 mM sodium chloride, 10% glycerol (v/v) and 5 mMβ-mercaptoethanol. PMSF was added immediately after the lysis(0.2 mM). Cells were disrupted by sonication on ice with 50% amplitude and a pulse of 20 sec on and 60 sec off for 15 min. The lysate was centrifuged at 18,000 rpm for 1h at 4ºC to separate supernatant from cell debris. The supernatant was loaded onto 5 ml Ni-NTA affinity column pre-equilibrated with the binding buffer. Protein was eluted by running a linear gradient of 0–1000 mM imidazole in 60 ml of buffer A (50 mM TrisHCl (pH 7.5), 1 M imidazole, 300 mM sodium chloride and 10% glycerol (v/v)] at a flow rate of 1 ml/min. Eluted fractions were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fractions containing *tly*A NTD were pooled and dialyzed against the buffer A (50 mM TrisHCl (pH 7.5), 300 mM sodium chloride and 10% glycerol(v/v).

TEVprotease cleavage of MBP-His tag

Dialyzed *tly*A protein was transferred to 50 ml falcon tube and subjected to TEV proteolysis (Yarden *et al.,* 2003), TEV to protein ration used was (1:100) and incubated at 18ºC for overnight and the sample was loaded onto 5 ml Ni-NTA affinity column pre-equilibrated with the binding buffer. TEV cleaved protein was eluted by 30 ml of buffer A [50 mM TrisHCl (pH 7.5), 300 mM sodium chloride and 10% glycerol (v/v)] at a flow rate of 1 ml/min. MBP-His tag bound to Ni-NTA column, whereas unbound protein without tag was eluted out.

Gel filtration chromatography

Size exclusion chromatography was performed using Hi-Load 16/60 prep grade Superdex75 column pre-equilibrated with buffer containing 20mMTris-HCl (pH 7.5), 1M NaCl, 10% (v/v) glycerol and 5mM β-mercaptoethanol using AKTA purification system (GE Healthcare). Protein was concentrated up to 5 ml and injected using 5 ml injector, flow rate of the column was fixed at 0.8 ml/min. Fractions collected were analyzed on 15% SDS-PAGE and fractions containing *tly*A NTD were pooled and concentrated. Protein concentration and yield were determined using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970). The expressed soluble fractions were diluted with the sample buffer 1:5 ratio and boiled for 3min before loading. Standard protein marker was used as a broad range protein standard to estimate the molecular weight of the proteins (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The protein sample was isolated at room temperature with a current of 20mA. The proteins were stained with Coomassie brilliant blue G-250 (Bio-Rad, Hercules, California, United States)

Circular Dichroism studies

Measurements were performed using a Chirascan CD spectrometer (Applied Photophysics) according to the method of Whitmore *et al.,* (2008). Cuvette path length used was 1 mm, and sample concentration was 0.30 mg/ml. Protein was dialyzed with the buffer contained 10 mM sodium phosphate, pH 8.0, 200 mMNaCl. The purity of samples was checked by SDS-PAGE and size-exclusion chromatography. Each spectrum was averaged from four repeated scans ranging between 180 and300 nm at a scan rate of 1.25 nm/s. Raw data were corrected by subtracting the contribution of the buffer to the signal.

Results and Discussion

Cloning *tly*A NTD in pCDF vector

The *tly*A NTD (residues 1 to 59) was subcloned into pCDF vector (Invitrogen, California, USA) containing a TEV recognition site upstream of the MCS. The expression vectors encode a Maltose binding protein and hexahistidine tag on the N-terminus, followed by a TEV protease site and ensuing desired coding sequence. It was than expressed as a MBP-His fusion protein in *E. coli* (star) strain as described.

Expression and purification

Modified MBP-His-TEV-pCDF vector system was used as the expression vector which harbors a strong promoter, T7. Maltose binding protein (MBP) tag helps in protein folding and pCDF-*Mtb-tly*A was transformed to *E.coli* strains BL21 (star). The recombinant protein expression level was high when over produced. Soluble form of the protein was

detected with the BL21 (star) strain, MBP and His-tagged tlyA was confirmed by analyzing the protein on 15% SDS-PAGE (Figure 1A). Temperature and IPTG concentration for protein production were optimized and optimum temperature for obtaining maximum protein production was 16°C, whereas optimum concentration of IPTG was found to be 200 μM. The MBP fusion protein was purified using standard affinity chromatography with Ni-NTA beads. Both maltose binding protein and histidine tag were removed by cleavage with TEV protease. The TEV protease is highly specific and does not cleave other sites on the protein. Gel filtration profile showed single predominant peak indicating the *Mtb-tly*A NTD, eluted protein is homogenous and protein eluted after 85 ml on Superdex 75 column, which was further confirmed by SDS-PAGE (Data not shown). Mass of the protein was further confirmed by MALDI-TOF (Figure 1B). Fractions corresponding to protein on SDS-PAGE were pooled, concentrated and stored in -80°C.

Figure.1(A) Purification of *Mtb-tly* ACTD **(B)** MALDI-TOF studies of purified *Mtb-tly* ACTD

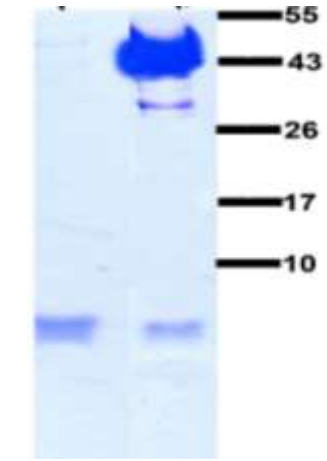


Figure.1B

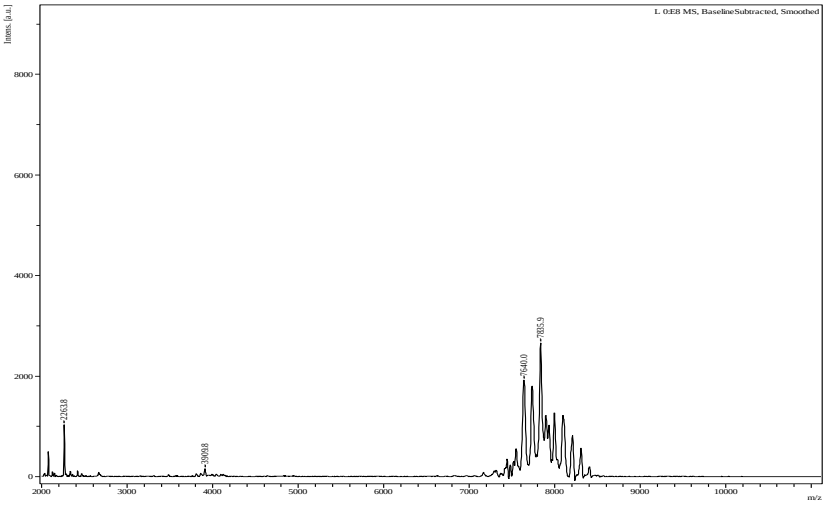


Figure.2 (A)CD spectroscopic analysis *Mtb*-tlyACTD at 0.4 mg/ml concentration. (B) CD spectra of *Mtb-tly*ACTD at concentration of 2 mg/ml.

Figure.2A

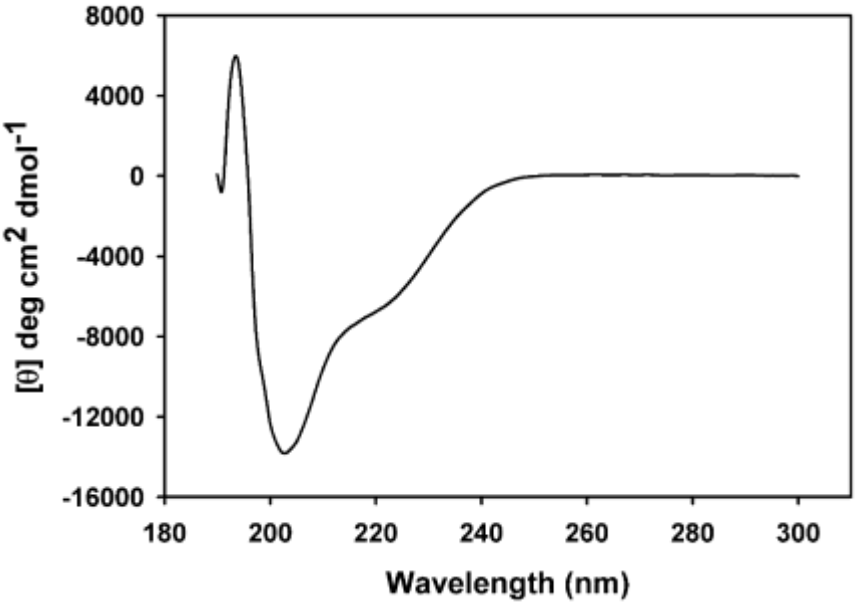


Figure.2B

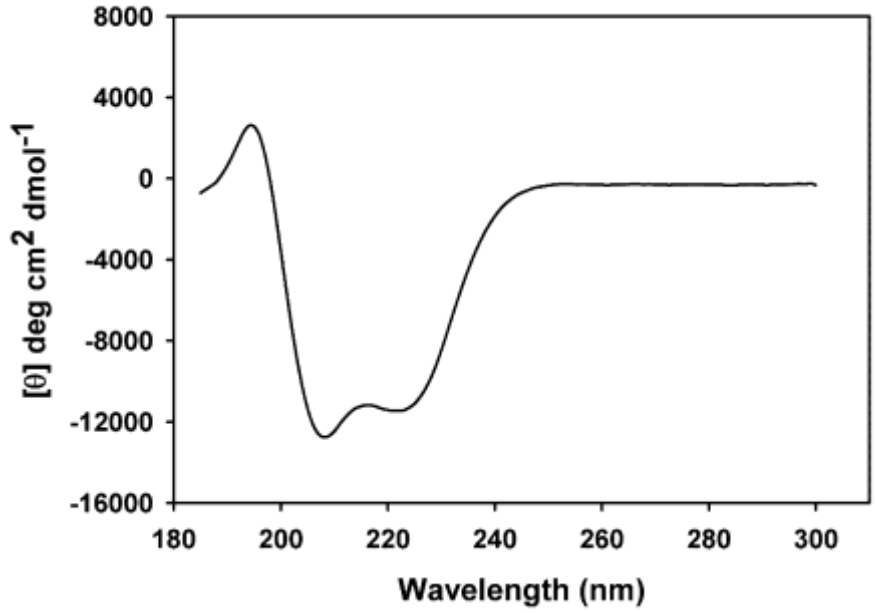
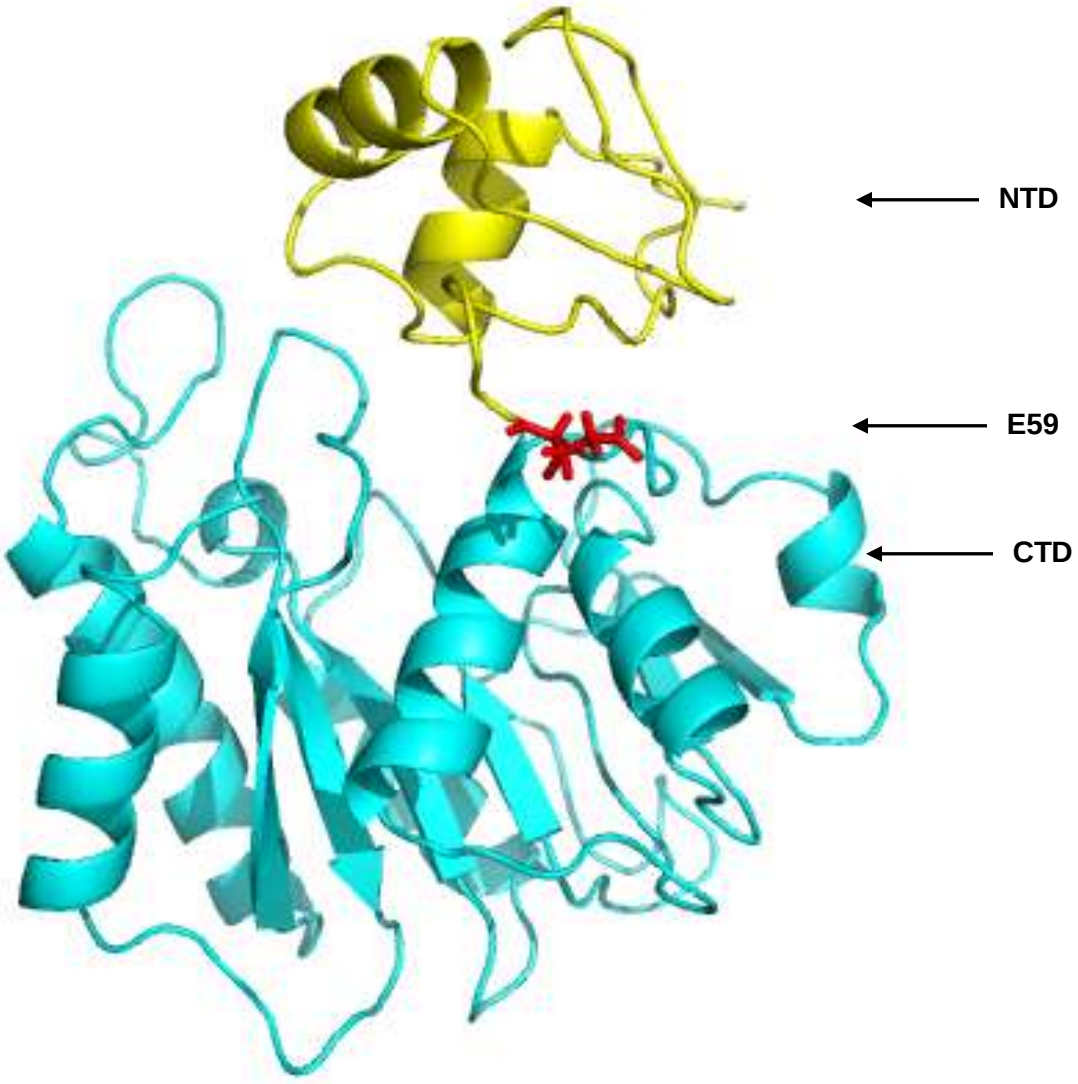


Figure.3 Mtb-*Tly*A model generated using I-Tasser server using CTD crystal structure as a template showing NTD and CTD separates at E59.

Figure.3



Secondary structure studies using circular Dichroism

To explain more precisely about the SAM binding site, we purified the tlyA NTD, residues 1–59, and measured the CD spectrum in the concentration of 0.4 to 2.0 mg/ml. Disordered structure of the truncated N-terminus *tly*A was observed at 0.4 mg/ml (Figure 2A), whereas increasing concentrations indicated increasing fractions of helical secondary structure (Figure 2B). Such behaviour is consistent with intrinsically disordered protein that upon association with protein undergoes a structured transition, facilitating binding with its target, *tly*A CTD containing methyltransferase domain (Witek *et al.*, 2017). Amino acid identity shared among bacterial *tly*A NTD is not so high. The full-length *tly*A protein comprises a

methyltransferase domain, extending from residue64 to 268, and NTD, from residues 1 to 59. What might be the structure of the N-terminal domain? The CD spectrum strongly suggests that the *tly*A NTD is predominantly a disordered structure, although it may have small fractions of helical and extended conformation (Figure 3). Bioinformatics analysis of the *tly*ANTD is equivocal, with one algorithm predicting some secondary structure and others predicting disorder. The structural attributes of the *tly*A NTD suggest that while alone it is largely disordered, it nevertheless may provide a target for protein interactions that would perforce induce structure upon binding.

Based on the previous studies by Witek *et al.*, (2017), homology model prediction studies indicated, Glu^{59}surface exposed amino acid separates between NTD and CTD of *Mtb*tlyA. Studies also indicated that NTD and CTD were intact even after cleavage under the solution conditions.

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Conflicts of interest

The authors declare no conflict of interest.

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