2.1 Material

The expression vector pET-NH6 used for cloniong of Rv3619c and Rv3620c was a kind gift from Dr. M.Weiner. Oligonucleotides for gene isolation were bought from BIO Serve (Hyderabad, Andhra Pradesh, India). Gene amplification was performed using *Pfu* polymerase from Fermentas (Genetix, New Delhi, India). The restriction endonucleases, T4 DNA ligase and DNA size markers used for cloning were obtained from New England Biolabs (Beverly, MA, USA). The plasmid was isolated using Plasmid Miniprep kit, and the DNA was purified using Gel extraction kit, both of which were obtained from Qiagen (Genetix, New Delhi, India). Luria- Bertani media and agar powder were from Hi-Media (India). For protein purification the Nickel／nitrilotriacetic acid (Ni／NTA) superflow metal-affinity chromatography matrix was obtained from Qiagen (Genetix, New Delhi, India). The protease inhibitors phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (containing AEBSF, pepstatin A, E-64, bestatin, and phosphoramidon) were from Sigma (New Delhi, India). The purified protein was concentrated using Centricon of 3-kDa cut-off from Millipore Corporation (USA). RPMI 1640 and FBS were obtained from Gibco BRL (USA). The interleukins IL-12, IL-4 and IFN-γ were quantified using ELISA kit from BD Biosciences (Mumbai, Maharashtra, India). Rest of the chemical reagents was from Sigma (New Delhi, India).

2.2 Methods

2.2.1 Cloning of genes from Rv3619c and Rv3620c from *M. tuberculosis* H37Rv in *E. coli* expression vector

2.2.1.1 Preparation of genomic DNA of *M. tuberculosis* H37Rv

Genomic DNA of *M. tuberculosis* H37Rv was prepared by the method described by Kremer et al. (2005). Briefly, a volume of 100 µl of packed cells of *M. tuberculosis* H37Rv was taken in a 1.5 ml micro centrifuge tube, washed twice with 500 µl of TE buffer-1 (50 mM Tris.Cl

and 5 mM EDTA, pH 8.0) and suspended in 200 µl of TE buffer. The bacilli were heat killed at 80 °C for 1 h on a dry bath and were centrifuged at 7700 × g for 10 min. The resulting cell pellet was suspended in 200 µl of TE buffer, followed by addition of 26 µl of 10 mg/ml of lysozyme and 2 µl of 1 mg/ml of RNase to the suspension. This was incubated for 2 h at 37 °C. After incubation, 60 µl of 10% SDS and 1 µl of 20 mg/ml proteinase K were added to the cell suspension, mixed properly and further incubated at 60 °C for 1 h. 660 µl of 5 M NaCl and 550 µl of CTAB/NaCl solution (10% CTAB and 0.7 M NaCl) were added to the suspension and it was incubated for 30 min. After incubation, the cell debris were pelleted down by centrifugation at 9500 × g for 20 min at room temperature. The clear supernatant was taken in a fresh eppendorf tube and was extracted first with equal volume of phenol (50% v/v) /chloroform (49% v/v) /iso-amyl alcohol (1% v/v) and then with equal volume of chloroform (98% v/v) /iso-amyl alcohol (2% v/v). Finally, the DNA in the aqueous phase was precipitated with 0.7 volume of isopropanol, washed with 70% ethanol and suspended in 100 µl of TE buffer-2 (10 mM Tris.Cl, 1 mM EDTA, pH 8.0). The concentration of DNA was estimated to be 50 ng/µl.

2.2.1.2 Isolation and amplification of the genes

The primers used for isolation and amplification of the genes by polymerase chain reaction (PCR) from the genomic DNA of *M. tuberculosis* H37Rv are given in the Table 1. The forward primers contained *EcoRI* and the reverse primer contained *HindIII* restriction enzyme sites.

Sl. No.	Oligo Name	Oligo sequence
1	Rv3619cFp	`5 - CCggAATTCATgACCATC AACTATCAATTCgg-3`
2	Rv3619c Rp	`5 - CCCAAgCTTTTAggCCCA gCTggA gCC-3`
3	Rv3620cFp	`5 - CCggAATTCATgACCTCg CgTTTTATgACg-3`
4	Rv3620c Rp	`5 - CCCAAgCTTTCAgCTgCT gAggATCTgCTg-3`

The PCR reaction was performed in 50µL volume. The reaction contained 10ng *M. tuberculosis* H37Rv genomic DNA, 5µM forward and reverse primer and 200 µM dNTP's. The reaction was performed in 1X PCR buffer and nuclease free water (Fermentas, Genetix, New Delhi, India). The PCR reactions were carried out in BIO-RAD minicycler. Each reaction was given an initial denaturation step of 3 min at 95 °C which facilitated melting of the genomic DNA, followed by 25 cycles of denaturation at 94 °C for 1 min, primer annealing at 61 °C for 1 min, and primer extension at 72 °C for 1 min. Each PCR reaction was terminated with a final extension step of 72 °C for 20 min followed by cooling down to 4 °C. The size of the fragments was verified by running the PCR products on a 1.5% agarose gel in 1x TAE (40 mM Tris-acetate, 1 mM EDTA) buffer and comparing their sizes with 100 bp dsDNA NEB ladder. The PCR products were extracted from the gel by using Qiagen Gel extraction kit.

2.2.1.3 Restriction digestion and Ligation of PCR products:

1 µg PCR product of Rv3619c and Rv3620c were digested with 10 U of *EcoRI* and 10 U of *HindIII* in 1x NEB buffer EcoR for 6 h at 37 ºC in a water bath. The digested products were run on 1.5% agarose gel and purified using Gel extraction kit. 1 µg pET-NH6 vector was digested with 10 U of *EcoRI* and 10 U of *HindIII* in 1x NEB buffer EcoR for 2 h at 37 ºC in a water bath. The digested products were run on 1.5% agarose gel and purified using the Gel extraction kit. 300 ng of *EcoRI* ─ *HindIII* digested Rv3619c and pETNH6 were used to set a 15 µl ligation reaction in 1x T4 DNA ligase buffer and 200 U of T4 DNA ligase from NEB on ice. Similar reaction was setup for Rv3620c. The reaction was mixed properly and incubated at 16 ºC for 12-16 h. The ligation mixture was then directly transformed into chemically competent (prepared using $CaCl_2$ method) *E.coli* DH5α cells.

2.2.1.4 Confirmation of the clones

The positive clones were identified by restriction digestion of the clones with suitable restriction enzymes and analyzing the digested products on 1.5% agarose gel. Clones with required insert and vector sizes were selected as positive clones. These clones were subsequently verified by sequencing using T7 promoter primer at the DNA Sequencing Facility at UDSC, Department of Biochemistry, University of Delhi South Campus, New Delhi-110021.

2.2.2 Over-expression and Purification of Rv3619c and Rv3620c proteins

The vectors containing Rv3619c and Rv3620c were transformed into BL21 (DE3) *E. coli* cells and grown in LB medium supplemented with ampicillin (100 µg/ml). BL21 (DE3) cells containing the plasmid pETNH6-Rv3619c and pETNH6-Rv3620c were grown in LB medium to standardize optimum temperature, induction OD_{600}, IPTG concentration and induction time for highest expression of N-terminal hexa-histidine tagged proteins. During the standardization procedure, Rv3619c and Rv3620c were expressed in a temperature range from 20 °C to 37 °C, OD_{600} was varied from 0.6 to 1.2, IPTG was varied from 0.1 mM to 1 mM, and the induction time was varied from 3 h to 16 h. Both Rv3619c and Rv3620c were expressed only in insoluble inclusion bodies. The highest level of Rv3619c was obtained by inducing the protein expression at OD_{600} = 1.0 with a final IPTG concentration of 0.5 mM, post-induction temperature of 27 °C and induction time of 14 h. Rv3620c was optimally induced at higher levels at OD_{600} = 1.0 with a IPTG concentration of 0.5mM at post induction temperature of 37 °C for 6h. The cells were harvested at 4 °C and the cell pellet from 1 L culture was suspended in 20 ml of 50 mM Tris.HCl, 1 mM PMSF, 1 mM EDTA, pH 8.0, and was stored in ice. 20 µl of protease inhibitior cocktail was added to the cell suspension. The cells containing Rv3619c-pETNH6 were lysed by using Constant Cell Disruption System (Labmate (Asia) Pvt. Ltd.) at 22 kpsi at room temperature. Rests of the steps were performed

at 4 ºC. The lysate was centrifuged at 9000 × g for 1h. The pellet thus obtained contained inclusion bodies and cell debris. The inclusion bodies were dissolved in 50 mM NaH_2PO_4, 8 M urea (buffer A), pH 8.0, and centrifuged again at 9000 × g for 1 h. The supernatant obtained was passed through 0.2 μm syringe filter and was loaded on a 7 ml Ni-NTA column pre-equilibrated with buffer A, pH 8.0. Unbound protein was removed by washing the column with 10 CVs of buffer A, pH 6.9 and 2 CVs of buffer A, pH 5.9. The bound Rv3619c was eluted with 4 CVs of buffer A, pH 4.5. Immediately after elution, pH of the buffer containing the eluted protein was adjusted to neutral pH (pH=7.0). Fractions of >95% purity were pooled together and the protein was refolded by dialysis. In a typical refolding procedure, ≈ 30 ml of eluted protein was dialysed against 2 L of dialysis buffer (25 mM NaH_2PO_4, 100 mM NaCl, 1 mM EDTA, and pH 6.5) at 4 ºC. The dialysis buffer was first changed after 24 h, then after 36 h and the dialysis was stopped after 48 hours. The refolded Rv3619c was subsequently dialyzed against NMR buffer (20 mM NaH_2PO_4, 50 mM NaCl, 0.1% NaN3, pH 6.5). Since Rv3620c was also expressed in insoluble inclusion bodies, same procedure was applied for its purification and refolding. Both Rv3619c and Rv3620c were concentrated using the centricon of 3-kDa cut-off from Millipore Corporation (USA).

2.2.3 Determination of protein concentration

The concentration of proteins in aqueous protein samples were determined by Bradford colorimetric method by using Bradford reagent from Sigma (New Delhi, India) and following the user's manual for the 'Micro 2 ml assay protocol'.

2.2.4 Isothermal Titration Calorimetry

Isothermal titration calorimetric (ITC) experiments were performed at 25 °C on a VP-ITC calorimeter from MicroCal™ (Northampton, MA, USA). The calorimeter was calibrated according to the user manual of the instrument. Stock solutions of Rv3619c and Rv3620c were dialyzed extensively against the phosphate buffer (20 mM NaH_2PO_4, 50 mM NaCl, 1

mM EDTA, pH 6.5) and degassed for 20 min prior to each of the ITC experiments. Titrations were performed at least in duplicate using the same set of stock solutions. The ITC experiments were done by adding aliquots of Rv3619c to Rv3620c. The sample cell was filled with 1.43 ml of 0.010 mM of Rv3620c (titrand) and titrated against Rv3619c, which was filled in the syringe at a concentration of 0.1 mM. Thirty injections with an injection volume of 10 µl each were made at an interval of 180 sec. During the titration, the reaction mixture was continuously stirred at 400 rpm. Control experiments were performed by injecting Rv3619c into buffer under conditions exactly similar to the Rv3619c/ Rv3620c titration, to take into account 'heats of dilution' and 'viscous mixing'. The heats of injections of control experiment were subtracted from the raw data of Rv3619c and Rv3620c titration. The ITC data were analyzed using the ORIGIN version 7.0 software provided by Microcal™. The heats of binding were normalized with respect to the titrant concentration and a volume correction was performed to take into account dilution of titrand during each injection. The amount of heat produced per injection was calculated by integration of the area under each peak using a baseline selected by the ORIGIN 7 program.

2.2.5 Recording CD spectra

CD measurements were carried out on a Jasco spectropolarimeter Model J-810 (Jasco International Co., Ltd, Tokyo, Japan) fitted with a thermostatically controlled cell holder having an accuracy of ± 0.1 ºC. Calibration of the spectropolarimeter was performed with (+)-10-camphorsulfonic acid. The protein spectra were recorded with the protein samples in buffer containing 20 mM NaH_2PO_4, 50 mM NaCl, pH 6.5. The concentration of proteins used was in range of 5 -10 µM. Three scans were averaged for each spectrum. Isothermal wavelength scan was recorded in the range of 250-200 nm, pathlength of 2 mm, response time of 1 s, scan speed of 20 nm/min, data pitch of 0.5. The CD results were expressed as mean residue ellipticity (MRE), in degree cm^2/dmol, which is defined as:

$$MRE = (\theta \text{ X } 100 \text{ X } M) / (c \text{ X } d \text{ X } N_A),$$

Where,

θ = observed ellipiticity in degrees,

c = protein conc in $mg.ml^{-1}$,

d = path length in cm.,

M = molecular weight

N_A = No. of amino acid residues.

Percentage secondary structure was calculated using online K2d server (http://www.embl-hidelberg.de/~andrade/k2d/).

Three scans were averaged for each spectrum. All spectra were corrected by subtracting the buffer background. Phosphate buffer (20 mM NaH_2PO_4, 50 mM NaCl, , pH 6.5) was used during the recording of CD spectra.

2.2.5.1 Study of thermal unfolding/refolding using CD

Thermal denaturation studies were performed by recording spectra of protein samples at various temperatures ranging from 25 °C to 80 °C, with a 1°C/ min increment. For refolding, the temperature was reversed at the same speed. Change in mean residue ellipticity values at 222 nm represents change in helical content in proteins. Since two-thirds of Rv3619c-Rv3620c complex is in helical conformation, fractional helicities observed at MRE values at 222 nm corresponds to fraction of protein folded during the thermal heating or cooling process. The fraction of protein folded corresponding to the MRE at 222 nm was calculated from the equation

$$[\theta^{obs} - \theta^{den}] / [\theta^{nat} - \theta^{den}]$$

where θ^{nat} and θ^{den} are mean residual ellipticity at 222 nm when proteins are under native state at 25 °C and under denatured state at 80 °C. θ^{obs} is the observed mean residual ellepticity.

CD unfolding curves were produced by plotting fraction of protein folded vs. temperature. Each thermal denaturation experiment was repeated at least twice with fresh samples. In all cases, after the heating experiment, the samples were tested for their transparency.

2.2.5.2 Stability of complex under variable conditions

To study the effect of pH on Rv3619c, Rv3620c and 1:1 complex of Rv3619c and Rv3620c, we dialyzed the proteins against citrate-phosphate buffer (0.2 M Na_2HPO_4, 0.1 M citric acid) at three variable pH values of 6.5, 5.5 and 4.5. We recorded the CD spectra of individual proteins and of the complex by mixing them at these variable pH values. The curve was produced by plotting the mean residual ellipticity at 222 nm against different pH values. The experiments were performed in triplets.

The effect of denaturant GdmCl was studied by incubating the proteins and 1:1 complex at variable GdmCl concentration ranging from 0M to 3M in 50mM phosphate buffer and recording their CD spectra. The fraction of unfolded and folded protein was shown by plotting mill degree values against GdmCl concentration.

2.2.5.3 Recording the CD spectra in presence of membrane mimetic conditions

To study the effect of membrane mimetic conditions on conformation of proteins, far-UV CD spectra were acquired in the presence of 40% TFE and 20 mM DPC. 200 mM DPC stock was prepared in buffer containing 20 mM NaH_2PO_4, 50 mM NaCl, pH 6.5 and centrifuged at 12000 rpm to remove any suspended particles. DPC was added to 5 μM proteins to a final concentration of 20 mM. For TFE experiments 5 μM of proteins were added to 40% TFE. The spectra were recorded in the wavelength range of 250-200 nm and analyzed on K2d server.

2.2.6 Fluorescence Spectroscopy

Fluorescence spectra were acquired at 25 °C, on a Perkin Elmer Life Sciences LS 5B spectroluminescencemeter (USA) or Varian Cary Eclipse fluorescence spectrophotometer

(USA) fitted to a water bath, using a 5 mm path length quartz cell. Fluorescence spectra were acquired to record the intrinsic tryptophan fluorescence changes of Rv3619c, Rv3620c and the 1 : 1 complex in the presence of 40% TFE and 20 mM DPC. The fluorescence emission spectra were recorded in the range 300–400 nm, with an excitation wavelength of 280 nm.1 μM of Rv3619c and Rv3620c were mixed with 40% TFE and 20 mM DPC and incubated for 30 min before recording the intrinsic fluorescence.

ANS (8-anilino-1-naphthalenesulfonic acid ammonium salt) binding of Rv3619c and Rv3620c were estimated by recording the fluorescence emission of ANS. The stock solution was prepared in phosphate buffer and the concentration of ANS was determined using extinction co-efficient of $\varepsilon = 8000\ M^{-1}cm^{-1}$ at 372 nm (Muro-Pastor et al., 2003). The samples were kept in dark immediately after addition of ANS stock solution to the proteins, and measurements were made within an hour. The ANS binding experiments were carried out with the excitation maximum of ANS (380 nm) and emission spectra were recorded in the range of 400 to 600 nm, with a slit width of 12 nm for excitation and 10 nm for emission. The concentration of the protein samples was 10 μM and the molar ratio of protein and ANS was 1:10 in all experiments.