Structure-based Epitope Mapping of *Mycobacterium tuberculosis* Secretary Antigen MTC28**1**

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Secretory proteins of *Mycobacterium tuberculosis* are key players of the mycobacterial infection pathway. MTC28 is a 28-kDa proline-rich secretory antigen of *Mycobacterium tuberculosis* and is only conserved in pathogenic strains of mycobacteria. Here we report the crystal structure of MTC28 at 2.8- and 2.15-Å resolutions for the structure-based epitope design. MTC28 shares a “mog1p”-fold consisting of seven antiparallel β strands stacked between α helices. Five probable epitopes have been located on a solvent-accessible flexible region by computational analysis of the structure of MTC28. Simultaneously, the protein is digested with trypsin and the resulting fragments are purified by HPLC. Such 10 purified peptide fragments are screened against sera from patients infected with pulmonary tuberculosis (PTB). Two of these 10 fragments, namely 127ALDITLPMPPR137 and 138WTQVDPPNPDAFVV1ADR156, are found to be major immunogenic epitopes that are localized on the outer surface of the protein molecule and are part of a single continuous epitope that have been predicted in silico. Mutagenesis and antibody inhibition studies are in accordance with the results obtained from epitope mapping.

The dreaded pathogen of tuberculosis, *Mycobacterium tuberculosis* (*Mtb*), continues to wreak havoc worldwide and is still one of the most pressing concerns for a major global health burden. Despite several curative regimens, the rapid emergence of the bacillus strains (multidrug resistant and extremely drug resistant) resistant to frontline antimicrobials with HIV co-infection has further exacerbated the scenario. According to a report from the World Health Organization global tuberculosis report published in 2015, tuberculosis (TB) is found to be a leading cause of worldwide death alongside HIV. Tuberculosis claimed 1.5 million lives worldwide in 2014 with 1.1 million HIV-negative and 0.4 million HIV-positive (1). The detection, diagnosis, and treatment of TB are still a major bottleneck in some parts of the developing countries. Although the disease has plagued mankind for millennia, there are still no markers available to detect the response to the administered drugs and symptomatic remission in patients (2). Thus, there is not only a continuous growing demand of designing new vaccines of higher efficacy than the currently used *Bacillus* Calmette-Guerin but also to develop new, sensitive immunodiagnostic agents specific for *Mtb* (3).

Extensive research in the last two decades is focused upon the development of (a) the antigens of *Mtb*, which elicit strong cellular and humoral immune responses against TB, and (b) antigens that allow measurement of the induced responses specific for *Mtb*. Among other various proteins, culture filtrate of *Mtb* contains many secreted antigens, which are biologically important diagnostic markers for the detection of tuberculosis in humans. Many of these culture filtrate proteins are identified and annotated (4, 5). Detailed analysis shows that these secreted antigens, primarily responsible for the survival of the pathogen within the host, interact with the host cells, regulate different signaling pathways, and elicit immunological responses during tubercular infection. These targets are thus not only important tools for the diagnostic skin test and serological diagnosis of TB but are lucrative potential drug or vaccine candidates against TB as well. Thus, probing the atomic details from crystal structures of any of the member of this important class of proteins may hold the key to designing inhibitors or vaccines.

MTC28 (Rv0040c) is one of the major secreted antigens in *Mtb* (6). This 28-kDa *Mtb* complex-specific antigen is rich in proline and alanine amino acid residues. The animals immu-
nized with *Mycobacterium avium* do not show antibody responses and delayed type hypersensitivity with respect to the animals immunized with bacillus Calmette-Guerin when MTC28 is introduced (3). The specificity of the immune response can be attributed to the absence of any B- and T-cell epitopes cross-reactive with a common environmental mycobacterial species like *M. avium*. Previous studies show that when MTC28 is administrated individually, it has sensitivity and specificity of 72.4 and 71.4%, but when it is used in an antigenic mixture with other antigens, it shows better immunogenic response with increased specificity and sensitivity of Tb detection (7, 8). This strong yet specific immunogenic response lists MTC28 as a prime candidate for vaccine design and immunodiagnosis of *Mtbc*.

Here we report the crystal structure of MTC28 at 2.8-Å resolution, solved by the multiple isomorphous replacement and anomalous scattering method. A high-resolution dataset of 2.15 Å later collected was used to improve the quality of the model initially obtained from *de novo* phasing. The epitope region of MTC28 is predicted from *in silico* analyzes. Molecular dynamics and simulation studies reveal that the predicted epitopes reside in the flexible region of the protein and is solvent accessible. For further validation, experimental epitope mapping is performed by separating the trypsin-digested fragments of MTC28 by high performance liquid chromatography (HPLC) followed by enzyme-linked immunosorbent assay (ELISA) studies of the separated peptide fragments against human serum samples from TB-infected patients. Two peptides of 1240.861 Da (127ALDITLPMPPR137) and 2141.102 Da (138WTQVPDPNVPDAFVVIADR156) were found to be reactive against pulmonary tuberculosis patient sera. These peptides are experimentally mapped and matched with the computationally predicted probable epitopes. Antibody inhibition and deletion mutagenesis studies further confirm that 127-Ala-Arg156 is the single major epitope of MTC28.

**Experimental Procedures**

**Cloning, Overexpression, and Purification of MTC28**—The open reading frame of *rv0040c* was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA as template using primers 5′-ATATATCGAGCTCGCGATCCCCTGCTGCGCC-3′ (forward primer with SacI restriction site) and 5′-ATATCCCCAGCTTCTAGCCGGCGGCGGACTG-3′ (reverse primer containing HindIII restriction site). The primers were designed to exclude the N-terminal 31-residue long signal peptide as predicted by SignalP 4.1 (9). The amplified PCR product was digested with SacI and HindIII, purified, and subsequently ligated into the SacI and HindIII sites of the expression vector pQE30 (Qiagen), which adds a His tag to the N terminus of the protein. The chemically competent cells of *Escherichia coli* M15 (pREP4) were transformed with the recombinant DNA and consequently selected on ampicillin/kanamycin plates. Clones harboring the desired construct in the correct ORF were confirmed by DNA sequencing using apQE30 specific primer.

One of the positive clones of MTC28 was grown at 37 °C in Luria-Bertani broth containing 100 µg ml⁻¹ of ampicillin and 25 µg ml⁻¹ of kanamycin until *A₆₀₀* reached 0.6, induced with 100 µM isopropyl 1-thio-β-D-galactopyranoside and further grown for 4 h at 37 °C. The harvested cells from a 1-liter culture were then resuspended in buffer A (10 mM Tris-Cl, pH 8.0, 300 mM NaCl, and 10 mM imidazole) containing 0.1 mM each of pepstatin, leupeptin, aprotinin, and 0.02 mM phenylmethylsulfonyl fluoride (PMSF), and lysed by sonication. The lysate was then centrifuged for 40 min at 14,000 rpm (22,830 × g). The supernatant was subjected to affinity chromatography by loading it onto a nickel-Sepharose high performance affinity matrix (GE Healthcare Biosciences) that was pre-equilibrated with buffer A. Buffer A was used for extensive washing of the column followed by buffer B (10 mM Tris-Cl, pH 8.0, 300 mM NaCl, and 50 mM imidazole). Finally, the protein was eluted with buffer C (10 mM Tris-Cl, pH 8.0, 300 mM NaCl, and 300 mM imidazole). The nickel-nitrilotriacetic acid eluate was subjected to size exclusion chromatography using Superdex 75 prep-grade matrix in a 16/70 C column (GE Healthcare Biosciences) on a AKTA prime Plus system (GE Healthcare Biosciences) pre-equilibrated with buffer D (10 mM Tris-Cl, pH 8.0, 100 mM NaCl) at 4 °C. The concentration of the protein was estimated using the Bradford assay (10) and the purity was verified by 12% SDS-PAGE. The buffer composition used during the protein purification was identified using the Thermofluor method (11). MALDI-TOF analysis further confirmed the molecular weight and purity of the sample (supplemental Fig. S1A). A dynamic light scattering experiment was performed to confirm the homogeneity of the protein prior to crystallization trial (supplemental Fig. S1B). The protein solution was filtered through a 0.22-µm membrane (millex, Millipore) and subjected to Zetasizer Nano ZS at 25 °C (Malvern Instruments Ltd., Worcestershire, United Kingdom).

**Crystallization, Data Collection, and Processing**—The purified homogeneous protein was concentrated to 19.6 mg ml⁻¹. Screening of initial crystallization conditions for MTC28 was done by the sitting-drop vapor-diffusion method at 20–25 °C by mixing an equal volume of concentrated protein and reservoir solution. Initial crystallization trials were performed at the Indian Institute of Technology Kharagpur, India, and at the High-throughput Crystallization facility at European Molecular Biology Laboratory (EMBL), Hamburg (12). Of 735 tested conditions, 21 crystallization hits were identified. Most of the crystallization conditions contained high concentrations of sodium chloride (around 2.5 M) in different buffer systems, optimally between pH 7.0 and 8.5. All crystals had a needle-shaped or rod-shaped appearance. Additionally, a crystallization hit was obtained from the condition 0.2 mM trimethylamine N-oxide dihydrate, 0.1 M Tris, pH 8.5, 20% (w/v) PEG MME 2000. The optimized crystallization conditions were 2.25 M NaCl, 100 mM BisTris propane, pH 7.0 (type 1), and 0.2 mM trimethylamine N-oxide dihydrate, 0.1 M BisTris propane, pH 9.0, and 20% (w/v) PEG MME 2000 (type 2). For derivatization, crystals of type 2 were soaked in 10 mM potassium tetrachloroplatinate (K₂PtCl₄) and 2 mM trimethyl lead acetate (Me₃PbOAc) for 24 and 1 h, respectively. A long-wavelength x-ray diffraction data set was collected from crystal type 1 at beamline ID29 at ESRF, France (13), using a wavelength of 1.771 Å and an ADSC Quantum Q315r detector. Helical data collection was performed using an oscillation range on 1.5°, resulting in a high resolution data set expanding to a resolution...
of 2.15 Å. X-ray diffraction data of crystal type 2 (native and heavy-atom derivatives) were collected on an in-house Rigaku R-AXIS IV+ image-plate detector using CuKα x-rays generated by a Rigaku Micromax HF007 rotating anode generator supplying a wavelength of 1.54 Å. The crystal-to-detector distance was kept at 170 mm and the crystal was rotated through a total of 360° in 0.5° increments with 3 min exposure time. The crystal diffracted to a resolution of 2.8 Å and a total of 720 images was collected. The diffraction data were indexed and integrated with XDS (14, 15) and scaled with XSCALE (16) or SCALA (17). POINTLESS from the CCP4 interface (17) was used for analyzing the reflection conditions to determine the screw axes. Data collection statistics are summarized in Table 1.

Structure Determination and Refinement—The structure of MTC28 was solved using multiple isomorphous replacement with anomalous scattering. Two platinum sites and one lead site were located using SHELX (18). SHARP (19) was used to calculate and improve the phases. The sequence of MTC28 (residues 32–310) was then used in Buccaneer software (20) for automated model building. The model obtained from the 2.8-Å resolution data were used as a search model in Phaser (21) to determine the high-resolution structure (crystal type 1). This was followed by iterative cycles of restrained refinement in Refmac (22) alternating with manual model building in Coot (23) to improve the quality of the models and the electron density maps. The stereochemical quality of the model was validated using PROCHECK (24). The phasing and the refinement statistics are tabulated in Table 1. The final structures were deposited in the PDB under the accession codes 4PWS (crystal type 1) and 4OL4 (crystal type 2). PDBePISA (25) was used to analyze the biological assembly of the protein in the crystal structure.

CD Measurements—The secondary structural variations of MTC28 and MTC28 A127_156Rdel were monitored at a wavelength range of 195–250 nm (supplemental Fig. S1C). The molar ellipticity at 220 nm was used for secondary structural analyzes. A spectral bandwidth of 2 nm with step resolution of 0.2 nm, the time constant of 2 s, sensitivity of 10 mdeg, and scan speed of 50 nm/min were used for all measurements. Each spectrum was recorded with an average of two scans with the subtraction of appropriate buffer blank (10 mM Tris-HCl, pH 8.0, 100 mM NaCl).

Molecular Dynamics Simulation—Molecular dynamics simulations and analysis of MTC28 trajectories were performed using the GROMACS (gromingen machine for chemical simulations) 4.5.5 software package with OPLS/AA force field (26, 27). The procedure employed here is fully described under supplemental “Experimental Procedures,” B.

In-gel Protein Digestion and Peptide Separation—MTC28 was subjected to in-gel digestion following the protocol of (28) with minor modification. In brief, the protein bands in SDS-PAGE were excised with a sharp scalpel, rinsed with distilled water, and collected in a 1.5-ml Eppendorf tube. A solution of ammonium bicarbonate and acetonitrile (100 mM of 100 mM NH₄HCO₃, pH 9.0, 50% ACN) was added to each gel piece and incubated for 30 min at room temperature with occasional agitation to denaturation gel. This was followed by addition of 500 μl of 100% ACN and incubation at room temperature until the gel pieces turned white and were reduced in volume. Finally, ACN was removed completely prior to digestion. Trypsin (Promega Trypsin Gold, 1-1-tosylamido-2-phenylethyl chloromethyl ketone treated) solution in 40 μl of 40 mM NH₄HCO₃, 9% ACN was added to the gel pieces (200 ng/spot) in an Eppendorf tube for digestion and incubated at 4 °C for 1 h. Finally, the digestion was carried out at 37 °C for 16 h. The digested peptide mixture was pooled and passed through 10-kDa concentrator (Vivaspin 500, GE Healthcare, United Kingdom) to separate the trypsin. The peptide mixture was then fractioned by reversed-phase HPLC on a Beckman C18 column (300 Å, 4.6 × 150 mm) using a linear gradient of 5–60% acetonitrile in 0.08% trifluoroacetic acid with a flow rate of 1 ml/min. The peptide elution was monitored at 215 nm and all the fractions were collected. The separated peptide fractions were lyophilized at −50 °C for 6 h and further analyzed for peptide mass and purity using mass spectrometer (Bruker, ultrafleXtreme).

MALDI-TOF MS and MS/MS Analysis—MALDI-TOF MS analysis was done to determine the molecular weight of the intact protein and the identity of the protein through peptide mass fingerprinting followed by search in the MASCOT Peptide Mass Fingerprint database search software. MS/MS analysis was done to obtain the sequence of the desired peptide. The details of the experiment are described under supplemental “Experimental Procedures,” C and D.

Human Blood Sera from Healthy and TB-infected Individuals—An informed consent form was obtained in writing from the patients and healthy individuals as per the Institutes ethical norms. This study was approved by human ethical committee of Nil Ratan Sirkar (NRS) Medical College and Hospital, Kolkata, India (No/NMC/378/2016), and the Indian Institute of Technology Kharagpur, India (IIT/SRIC/SAO/2016). For collecting the serum samples, a total of 30 pulmonary TB cases were chosen. All of them were sputum positive and acid-fast bacilli smear positive. A total of 10 healthy controls with the same age and sex groups were selected as tuberculin skin test negative. Individuals suffering from an immunosuppressive disease or transplant cases were excluded from this study.

Reactivity of Serum Antibodies against MTC28—The sequences predicted by computational methods were then cross-validated by experimental epitope mapping using recombinant MTC28, sera was from pulmonary tuberculosis (PTB) patients and healthy individuals. The presence of specific antibodies in a PTB patient serum against MTC28 was determined by ELISA using sera from 30 patients and 10 healthy controls. 0.5 μg/ml of MTC28 in bicarbonate buffer (pH 9.0) was coated in microtiter plates (MaxiSorp®, Nunc-Immuno™, USA) and incubated overnight at 4 °C. 2% BSA was used for blocking at 4 °C overnight. After washing the wells adequately with TBST buffer, the serum samples from each patient were added to the plates and incubated at 4 °C for overnight. The bound antibodies were detected by incubating with horseradish peroxidase-conjugated anti-human IgG antibodies (Sigma) for 1 h at room temperature. The plates were allowed to stand in the dark for 30 min after adding 3,3′,5,5′-tetramethylbenzidine liquid substrate to each well to develop the color. Finally, the stop solution was added to each well and absorbance of the supernatant was measured at 450 nm using an ELISA plate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific).
**Structure-based Epitope Mapping of MTC28**

**Experimental Epitope Mapping by Peptide Screening**—A total of 10 peptides covering the broad area of the MTC28 sequence obtained from the structure was used in ELISA for experimental epitope mapping. Approximately 40 pmol of each peptide from MTC28 were coated in each well of the ELISA plate overnight in bicarbonate buffer, pH 9.0, at 4 °C. After completion of the peptide coating, the wells were washed with 200 µl of 1× TBST for 5 times with an interval of 5 min. 150 µl of serum samples from 5 preselected TB patients and 5 healthy individuals were then distributed in each well and incubated overnight at 4 °C to allow the binding of the antibodies to the target peptide. The sera samples were then discarded and the bound antibodies from serum were detected following the same steps as described above.

**Antibody-binding Inhibition Assay**—An antibody-binding inhibition assay was performed as previously reported (29) with minor modifications. In brief, five PTB patient sera that were previously found to be highly reactive against MTC28 were chosen for this study. The E1, E2, and E1 + E2 epitopes were coated overnight at 4 °C. The unbound peptides were washed with 1× TBST and incubated with preselected patient serum samples overnight at 4 °C to allow the binding of antibodies to the peptides. These serum samples were collected from each well and reincubated overnight at 4 °C in MTC28-coated wells. The rest of the method was as described above. To calculate the inhibition capacity, albumin and MTC28 are used as positive and negative controls, respectively.

**ELISA Displacement Assay**—A competitive ELISA was performed in two sets with peptides E1 and E2. In each set two different concentrations (20 and 40 pmol) of the peptides were taken. Five preselected patient serum samples were added in each well of the microtiter plate (MaxiSorp®, Nunc-Immuno™, USA) precoated with 0.5 µg/ml of MTC28. After an extensive wash with 1× TBST to remove any unbound antibodies, E1 (20 and 40 pmol), E2 (20 and 40 pmol), E1 + E2 (20 and 40 pmol), MTC28 (5 µg/ml), and BSA were added in each well followed by overnight incubation at 4 °C. After extensive washing with 1× TBST the remaining antibodies were detected as described above. The amount of antibodies displaced by the peptides was determined by calculating the amount of antibodies that remain bound to the native protein. MTC28 and BSA were used as positive and negative controls, respectively. The experiments were done in triplicate.

**Cloning, Expression, and Purification of MTC28 A127_156Rdel**—Plasmid containing rv0040c was purified from *E. coli* DH5α cells with a Qiagen plasmid purification kit. The purified plasmid was used for PCR amplification of MTC28 A127_156Rdel using the following primers: MTC28A127_156RdelFp, 5’-GGATTCTTGGGCA-ACAGGCTTACAGCTCG-3’ and MTC28A127_156RdelRp, 5’-GCCCAAGAACCCGTGGCCTCGTGTCG-3’. The amplified product was digested with Ndel and Xhol and cloned in PET28a in *E. coli* BL21 cells using kanamycin as a selectable marker. Clones harboring the desired construct were confirmed by DNA sequencing using a gene-specific primer. The positive clones were selected. For purification, 10 ml of the overnight culture containing MTC28 A127_156Rdel was transferred to 1 liter of LB medium supplemented with 30 µg of kanamycin and grown at 37 °C until the OD value reaches 0.6. The culture was induced with 500 µM isopropyl 1-thio-β-D-galactopyranoside and further grown at 37 °C for 4 h. The protein was purified by the same protocol as described above. Recombinant MTC28 A127_156Rdel was successfully cloned and purified (see supplemental Fig. S4, B1 and B2).

**ELISA Studies with MTC28 A127_156Rdel**—MTC28 A127_156Rdel was coated overnight in bicarbonate buffer (pH 9.0) at 4 °C in 96-well microtiter plates (Nunc). The plates were blocked with 5% BSA (SRL) dissolved in TBST overnight at 4 °C. Each well then received 150 µl of TB patient serum and incubated in 37 °C for 2 h. Extensive washing with TBST was performed to remove any unbound primary antibody for the serum sample. Rest of the steps were followed as described above.

**Results**

**The Overall Structure of MTC28**—MTC28 crystallized in space group *P*3₁2₁ with one molecule in the asymmetric unit. The biological assembly of the protein is monomeric. Initial attempts to solve the 2.8-Å structure using molecular replacement were unsuccessful due to lack of homologous structures. The sequence identity with the closest homolog was only 28% with query coverage of 27%. Phase was determined experimentally by multiple isomorphous replacement and anomalous scattering using potassium tetrachloroplatinate(II) and triethyl lead acetate derivatives of the crystals led to successful experimental phase determination whereby a model of interpretable electron density was finally obtained. The amino acid residues from 97 to 274 are traced in a continuous electron density map resulting in a final model of the structure having *R*ₜₚₑₑₑₑ and *R*ₚₑₑₑₑ value of 18.2/22.3% (Table 1). Three heavy atom binding sites are found in the structure with two Pb positioning around Gln202 and Thr203, and one Pt positioning around Pro101. However, a dataset was collected at 2.15 Å (PDB code 4PWS) and the structure was solved with MR using 2.8 Å (PDB code 4OL4) structure as template. The high resolution structure was refined with *R*ₜₚₑₑₑₑ and *R*ₚₑₑₑₑ value of 19.1/21.5%. Structures determined at both the resolutions satisfy the stereochemical criteria.

The structure of MTC28 contains 10 β strands, three α helices and one 3_10_helix (η). The structure has a β sandwich-like fold where seven antiparallel β strands are stacked centrally between α₁, α₂ on one side, and between the 3_10_helix and α3 on the other side (Fig. 1, A1 and A2). The topology diagram of MTC28 shows the orientation of α helices and β strands arranged as αβββββαββββαβ (Fig. 1B). However, the main polypeptide chain could not be traced for the 65 N-terminal amino acid residues (32–96) and 36 C-terminal residues (275–310) due to lack of interpretable electron density. A secondary structure prediction by DALI (30) shows that the N- and C-terminal ends of the protein constitute an unstructured region. This unstructured region of MTC28 consists of several Pro residues. In general, proteins with high Pro content are often involved in binding to extracellular matrix proteins, cell signaling, and protein-protein interactions (31). It is initially presumed that the high content of Pro in these unstructured parts forms poly-Pro II helices (32, 33), but circular dichroism (CD)
spectra do not show any characteristic minima at 205 nm (supplemental Fig. S1C), which suggests the absence of a poly-ProII helical structure in MTC28. The minima at 205 nm depict the poly-ProII helical conformation (34).

The structure of MTC28 has a V-shaped cleft on its surface with a calculated area of 345.6 Å² and a volume of 428.3 Å³. The side of the cleft is generated by H9251 and H9252, whereas the base of the cleft constitutes H9252, H9258, and H927. The cleft is surrounded by Arg107, Arg111, Arg229, and His231 resulting in a distribution of positive charge around the cleft (Fig. 1C). Although positioned on the same side of the molecule, the N and C termini of the protein are not in close proximity.

**Structural Similarity to PsbP and mog1p-like Proteins**—A DALI (30) search identified several structural homologs of MTC28. It shares the same fold as photosystem-binding proteins (PsbP) and the Ran-binding protein (Fig. 1D). The PsbP of cyanobacteria (PDB entry 2XB3 (35); Z score 13.8), spinach (PDB entry 4RTI (36); Z score 13.1), and Zea maize (PDB entry 4RTH (36); Z score 12.6) are the extrinsic subunits of water oxidizing complex of photosystem II and are required for retention of calcium and chloride ions (37), maintenance of thylakoid membrane (38), and assembly of photosystem II complex (39), respectively. MTC28 also shares a significant structural homology with a Ran-binding protein Mog1p (PDB entry 1EQ6 (40); Z score 9.0), which is a regulator involved in nuclear import and export of RanGTPase in yeast (41). Despite sharing the same structural fold, there is no detectable sequence homology between MTC28 with PsbP and mog1p (supplemental Fig.

### TABLE 1
Diffraction data collection and processing statistics

| Data Set | Crystal type 1 | Crystal type 2 | 2-Pt derivative | Crystal type 2-Pt derivative |
|----------|----------------|---------------|-----------------|----------------------------|
| Source   | ID29 (ESRF)   | n/a           | n/a             | n/a                        |
| Wavelength (Å) | 1.77 | 1.54           |                 |                            |
| Space group | P 3 2 1     |               |                 |                            |
| Unit cell parameters (Å) | a = b = 101.41, c = 67.69 | a = b = 102.22, c = 68.72 | a = b = 101.92, c = 68.69 | a = b = 101.50, c = 68.68 |
| Resolution range (Å) | 43.91 - 2.15 (2.21 - 2.15) | 19.48 - 2.80 (2.96 - 2.80) | 19.42 - 3.0 (3.16 - 3.00) | 19.35 (3.17 - 3.0) |
| Total number of reflections | 240343 (17342) | 70942 (10239) | 56540 (8131) | 76345 (10966) |
| Unique number of reflections | 42287 (3117) | 10415 (1500) | 8479 (1214) | 8394 (1210) |
| Completeness (%) | 99.8 (99.6) | 99.7 (100) | 99.6 (100) | 99.6 (99.8) |
| Rmerge(%) | 10.4 (117.1) | 8.1 (46.1) | 13 (79.5) | 10.9 (42.1) |
| I/σ(I) | 14.2 (1.98) | 24.7 (5.0) | 13.8 (3.1) | 23.8 (6.3) |
| Redundancy | 5.7 (5.6) | 6.8 (6.8) | 6.7 (6.7) | 9.1 (9.1) |
| No. of molecules in Asymmetric unit (Z) | 1 | | | |
| No. of heavy atom binding sites | 1 | 2 |
| Rmerge (%) | 84/76.9 | 77.3/83.2 |
| Phasing power isoacentric/centric | 0.906/1.006 | 0.270/0.280 |
| Phasing power ano | 0.354 | 0.058 |
| Rwor (% ) | 19.1 | 18.2 |
| Wilson B-factor (Å²) | 36.42 | 48.36 |
| Rfree (%) | 21.5 | 22.3 |
| No. of protein atoms | 1389 | 1369 |
| No. of water molecules | 47 | 23 |
| R.m.s.d. bond lengths (Å) | 0.026 | 0.018 |
| R.m.s.d. bond angles (°) | 2.5 | 2.1 |
| Most favored region (%) | 98 | 93 |
| Allowed region (%) | 1 | 5 |
| Outlier region (%) | 1 | 2 |
| PDB-Id | 4PWS | 40L4 |

* Values within parentheses represent corresponding to highest resolution shell.
Therefore, for more detailed structural analysis, we superimpose all the four structures (PDB ID 4PWS, 2XB3, 4RTI, and 1EQ6) and found that α1 and β1 of MTC28 are unique and do not superpose with the other structures (Fig. 1D). Interestingly, α1 is heavily involved in forming the positively charged V-shaped cleft suggesting a functional importance of this cleft for MTC28.

**Prediction and Evaluation of MTC28 Epitopes**—The predicted linear epitopes (by BepiPred, SVMTrip, and ABC pred) are overlapped with the predicted conformational epitopes (by Ellipro, Discotope V2.0, EPDES, EPSVR, and PEPITOPE). The predicted residues that are common to both of these methods are considered as probable epitopes (see supplemental “Experimental Procedures,” A, and Fig. S2B). Therefore, Gln89-Asp98, Pro1102-Asp117, Asp124-Ser133, Pro179-Leu194, and Leu220-Asp232 are considered the predicted epitopes of MTC28 (Fig. 2A). The location of the predicted epitope sites of MTC28 is further evaluated by molecular dynamics and simulation studies. Structural or functional epitopes in protein often reside in surface-exposed, flexible regions. Therefore local conformational flexibility is another major criterion for the prediction of the probable epitope site. Fig. 2, B and C, depict the root mean square fluctuation and root mean square deviation of the protein structure. The molecular dynamics (MD) simulation of MTC28 in an explicit solvent is performed to analyze the local conformational flexibility of the predicted epitope regions. The MD study shows significant equilibration and stability of the MTC28 structure as evident from the analysis of root mean square deviation values. The root mean square deviation values lie between 0.1 and 0.15 nm throughout the time course of the simulation. The local conformational flexibility of the Ca atoms is analyzed through essential dynamics. In essential dynamics, analysis of the covariant matrix of the Ca atomic positions from the MD trajectory allows isolating structural movements that are important for a physiological function of irrelevant local fluctuations. The first five Eigenvectors were analyzed because of their high significant values. They represent the most dominant correlated atomic displacements in a molecule. The Eigenvectors 1 and 2 indicate local structural
motion in surface-exposed loops. The other three Eigenvectors (3, 4, and 5) indicate breathing motion in the MTC28 structure.

Among the predicted epitope regions, region 155–164 shows significant local conformational flexibility along with all five principal Eigenvectors. Region 120–129 has higher fluctuation in Eigenvectors 2, 3, 4, and 5; whereas, region 133–138 shows structural fluctuation in Eigenvector 2, 4, and 5. The C-terminal loop part (residue 220–224) of the region 210–225 also has significant structural flexibility evident from the peak in Eigenvectors 1, 3 and 5. Therefore, MD simulation studies ultimately conclude higher local conformation flexibility of all the five predicted epitope sites. Thus, the epitope sites predicted by web-based servers and molecular dynamics simulation studies are shown in the structure (Fig. 2D).

Maximum Antibody Reactivity against MTC28—ELISA studies are performed with purified MTC28 to select the highly reactive serum samples from a pool of 30 patient sera. The reactive serum samples are selected based on their absorbance (Fig. 3A). Seventy percent (21 individuals among 30 individuals) of the total individuals show reactivity above the cut-off value, which are twice the mean absorbance values of the healthy controls. Sera samples from PTB patient numbers 9, 16, 22, 26, and 28 give a maximum reactivity against MTC28 and are selected for the further epitope mapping studies.

The mass spectra of the trypsin-digested peptide fragments of MTC28 were obtained from MALDI experiments. The digested peptide fragments were then purified by reverse phase HPLC (see supplemental Fig. S4A). A total of 10 peptide fragments ranging from 1181.700 to 3337.925 Da were successfully purified covering the maximum sequence (see supplemental Fig. S3) of the protein that was obtained from the structure. The purity and mass of the purified peptide fragments were further analyzed by MALDI (Fig. 3B). The purified peptide fragments were then subjected to ELISA for epitope selection. Peptide 6 (1240.861 Da) and peptide 10 (2141.102 Da) were found to give

FIGURE 2. Computational epitope prediction of MTC28. A, the epitopes of MTC28 are theoretically determined and 5 color (violet, red, green, blue, and yellow)-coded sequences are the most probable epitope sites of MTC28, predicted by the epitope prediction servers. B, root mean square fluctuation as a function of residue number along five principal Eigenvectors is calculated from Cα coordinates covariance matrix. Violet color depicts for the region 120–129, brown color for the region 133–148, green color for the region 155–164, cyan color for the region 210–225, and yellow color for the region 251–263. C, time evolution of the backbone room mean square deviation of MTC28 with respect to the starting structure. Root mean square deviation is calculated over 10 ns during MD simulation. D, the five epitope sites determined through both epitope prediction server and simulation studies are shown in the structure (also see supplemental Fig. S2B).
a significantly high absorbance than the other peptides upon incubation with preselected TB patient serum (Fig. 3C). Peptides incubated with healthy control serum samples, especially peptides 6 and 10, were found to be less reactive. Lower absorbance values were recorded in the ELISA studies for both peptides with respect to the native protein (Fig. 3A), which further signifies its specificity toward antibodies derived from pulmonary TB patients (Fig. 3D). To determine the sequences of the selected peptides, MS/MS spectra obtained followed by Mascot search and the corresponding sequences were found to be $^{\text{127}}$ALDITLPMPPR$^{\text{137}}$ for Epitope 1 (E1) and $^{\text{138}}$WTQVPDPNPVF$^{\text{156}}$ for Epitope 2 (E2) (see supplemental Fig. S4, C and D).

**Confirmation of E1 and E2 as Major Epitope of MTC28**—To verify the epitope sequences determined in the ELISA studies, an antibody inhibition ELISA was performed. Five preselected patient sera samples that are preincubated with peptides 2141.102 Da and 1240.861 of MTC28 were analyzed for antibody binding to the MTC28. Results show that the peptides inhibit antibody binding up to about 44 (E1) and 35% (E2). The extent of inhibition was much higher (66%) when E1 and E2 were used together (Fig. 4A). Results obtained from ELISA displacement assay further suggests that with increasing concentration of the peptides, the amount of displacement of the bound antibodies also significantly increased (Fig. 4B). The outcome of this study shows that the amount of antibodies dis-
placed by E1 + E2 is 41.20 (20 pmol) and 55.80% (40 pmol). The antibodies displaced by E1 were 22.40 (20 pmol) and 31.20% (40 pmol). E2 displaces 15.60 (20 pmol) and 26.80% (40 pmol) of antibodies. When MTC28 itself is used as a competitor, 83.54% of the antibodies are found to be displaced, whereas for BSA it is only about 5.22%. This result further suggests that the combined displacement capacity of E1 and E2 for serum antibodies is much higher in comparison to each separate entity and this further supports the result obtained from the antibody inhibition assay. Although the overall antibody displacement capacity of E1 and E2 is found to be lower than their antibody binding capacity. Therefore from both the studies it is shown that E1 + E2 have the maximum antibody binding (~66%) and displacement (55.8%) capacity toward the patient serum antibodies. Thus E1 and E2 were found to be the major epitope of MTC28.

Thus E1 and E2 were found to be the major epitope of MTC28. From the structure it was observed that both of these epitopes are located on the same region and present successively thereby constructing a single major epitope (Fig. 4C). Therefore a deletion mutagenesis was performed for validation of this single major epitope site. A significant reduction in the reactivity of MTC28 A127_156Rdel in comparison to the full-length protein against patient sera samples was found (Fig. 4D). Although there is no significant change in the secondary structure and overall protein folding with respect to the full-length protein as indicated in CD spectra (see supplemental Fig. S1D). Therefore the deletion of amino acid position A127_R156 in MTC28
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leads to loss of its immunogenicity. It further signifies that, this region is the single major immunodominant epitope of MTC28 comprising of two individual epitopes.

Discussion

Structure-based epitope discovery and computer-aided epitope mapping increasingly play a central role in the development of novel vaccines and optimization of the biochemical and/or immunological properties of vaccine components (42, 43). Computational analysis of the epitope, structure determination, and validation through experimental footprinting is an established process of designing vaccines based on the identification of the highest immunogenic part. Previously epitopes are mapped for two secreted antigens: Ag85b and TB 10.4 of M. tuberculosis (44, 45). Very few crystal structures of the secreted antigens of Mtb are available in the database. This necessitates the determination of crystal structures of MTC28 and eventually will help to understand the topology of the antigenic part on its surface. From the structural point of view the epitope region of a protein may form a folded or a linear sequence of amino acids. Linear epitopes sometimes have an elevated level of antibody reactivity than the parental antigen. As for example, two acute phase antigens (BPSL 2765 and OppA) of Burkholderia pseudomallei (46, 47). An antigen contains several epitopes, some of which are specific to the parental organism. Other epitopes may be similar to the epitopes of proteins expressed by other bacteria. This may lead to the increased sensitivity of the assays, but a lack of specificity may occur (48). The peptide-based diagnostic markers can be an alternative to whole protein assays as it will increase the specificity. The feasibility of peptide-based assays is shown in the case of VlsE protein from Borrelia burgdorferi where a 25-amino acid peptide (C6 peptide) is used in ELISA for the detection of Lyme disease (49, 50). Two other linear epitopes of OspC and BBK07 are also identified and are shown to have a diagnostic potential in Lyme disease detection (51, 52). The strategy of vaccination proved to be a successful approach in preventing many infectious diseases (53) and was highly successful for eradication of smallpox and polio. The vaccine development includes the exploitation of attenuated strains of pathogenic organisms, recombinant proteins, and a mixture of several antigens or peptides. The attenuated strains are sometimes difficult to culture in in vitro conditions. There is a high risk of conversion into its pathogenic form and subsequently, some of its surface components may trigger unknown immune responses in the host. The major advantage of peptides in designing vaccines is that only the immunogenic region of the antigen is used, thus the immune reaction in the host is followed in a precise direction. Lipids, carbohydrate, or phosphates could be attached in a controlled manner with the peptide to achieve the desired immunogenicity and stability. Despite such novelty and promise, the peptide vaccines are not a successful venture until now due to three reasons: (i) the administration of peptides in the human is a challenging task as it requires suitable carrier protein or adjuvants in complex with the desired peptide for mounting an immune response, (ii) the three-dimensional conformation of the peptides do not remain the same as in the native protein therefore antibodies that are generated against those peptides often do not recognize the native protein (54), (iii) peptides that are shorter in length are often a poor elicitor of immune response (55). However, the present scenario of peptide vaccine is now changing, as a large number of peptide vaccines are under clinical trials (56) and a multiepitope vaccine strategy is found to be successful in the treatment of hepatitis C infection (57). Several pharmacies are trying to develop a suitable peptide-based vaccine for TB detection as no such vaccine for humans is available (58).

Therefore despite so many glitches peptide-based vaccines remain a good target to explore. Whereas peptide-based diagnostic markers may be used as a diagnostic tool for increasing the specificity of the assays for detection of diseases in near future. In this context we have focused our research on MTC28 and determined its immunogenic part for development of a novel detection marker or an epitope-based peptide vaccine for tuberculosis. MTC28, which owns the potential for being a vaccine candidate as well as a diagnostic marker for TB, is found to evoke a strong antibody response in TB patients (59) and is conserved in tubercular strains (M. tuberculosis, Mycobacterium bovis, and Mycobacterium africanum) of mycobacteria. The crystal structure of MTC28 is solved at 2.15-Å resolution. The high content of Pro residues imparts extreme flexibility to the N- and C-terminal part of the protein, which is subsequently not visible in the structure. The surface of the structure contains a deep “V” shaped cleft having a positively charged electrostatic potential. The overall structure of MTC28 shares a mog1P-like fold and bears a significant homology to PsbP and Ran-binding protein although there is no sequence homology between these proteins.

To identify the immunogenic sites of MTC28 we first identified the epitopes of MTC28 computationally by MD and simulations. Experimental epitope mapping was done to purify and identify the highest immunogenic peptide fragment of MTC28. Epitopes that are selected computationally are based on the following criteria such as, (i) the length of an epitope should be a 9–22 amino acid residue (60), (ii) epitope should be in the solvent accessible surface of a protein to get recognized and interact with the respective antibody (61, 62), and finally (iii) the residues should be present on unstructured regions of the protein such as loops with high conformational flexibility (63). The conformational flexibility of the predicted regions is firmly supported by MD simulation study. Thus based on these criteria, Gln89, Asp96, Pro102, Asp117, Asp124, Ser133, Pro179, Leu194, and Leu220 are considered the predicted epitopes of MTC28. To validate this prediction, experimental mapping of the epitopes of MTC28 was performed with sera from pulmonary tuberculosis patients. An experimental mapping shows peptides 1240.861 Da (127ALDITLPMPPR137) E1 and 2141.102 Da (138WTQVDPNPVDAFVIA DR156) E2 are the most reactive with among other peptides. E1 is present in the β2 region accompanied by a loop region. This is followed by E2, which is present in the β3 region adjacent to a loop part and eventually imparts flexibility to this region (Fig. 4B). Both E1 and E2 are exposed to the surface of MTC28 and are solvent accessible (Fig. 4, C1 and C2). These two epitopes belong to the five epitopes that are predicted by the computational methods. Interestingly E1 and E2 are parts of a single continuous epitope.
sequence, which is the major epitope of MTC28. Moreover, due to the highly disordered and flexible nature of the N-terminal and C-terminal proline-rich parts it may be possible to have epitope parts in this region although no peptide fragments were found to be reactive in peptide ELISA (see “Experimental Procedures”) confirming the absence of epitope sites in the terminal region. E1 and E2 together constitute a single major epitope of MTC28 as evidenced by structural and mutational studies. These individual peptides of 11 and 19 amino acids could serve as a peptide-based vaccine as well as a diagnostic marker against tuberculosis in human.

In conclusion, the crystal structure of MTC28 was determined at 2.15-Å resolution. From a computational approach using the structure, five epitope sites are predicted. To validate the predictions, experimental mapping was performed. It was found that two successively placed epitopes of this antigen constitute a single major epitope of MTC28. The epitopes were found to be located on the surface of the protein molecule. These epitopes may serve as a novel peptide-based vaccine candidate or a diagnostic marker in complex with other antigenic determinants against tuberculosis in human.

Author Contributions—S. M. did the cloning. P. K., L. R., and S. M. performed the purification and crystallization of the protein. L. R., J. M. D., and M. W. performed data acquisition and processing of the synchrotron data sets. P. K. and R. B. performed in-house data collection and processing, structure solution, and interpretation of the model. P. K. designed and performed the in silico as well as experimental epitope mapping studies. A. D. performed the MD and simulation studies. N. K. P. supervised the epitope mapping study using human sera. P. K., S. M., L. R., and R. B. drafted and revised the manuscript. All the authors reviewed the manuscript. A. K. D. supervised the research.

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