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ORIGINAL PAPER

# Probing ligand binding modes of *Mycobacterium tuberculosis* MurC ligase by molecular modeling, dynamics simulation and docking

**C. M. Anuradha · Chaitanya Mulakayala · Banaganapalli Babajan · M. Naveen · Chikati Rajasekhar · Chitta Suresh Kumar**

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**Abstract** Multi drug resistance capacity for *Mycobacterium tuberculosis* (MDR-Mtb) demands the profound need for developing new anti-tuberculosis drugs. The present work is on Mtb-MurC ligase, which is an enzyme involved in biosynthesis of peptidoglycan, a component of Mtb cell wall. In this paper the 3-D structure of Mtb-MurC has been constructed using the templates 1GQQ and 1P31. Structural refinement and energy minimization of the predicted Mtb-MurC ligase model has been carried out by molecular dynamics. The streochemical check failures in the energy minimized model have been evaluated through Procheck, Whatif ProSA, and Verify 3D. Further torsion angles for the side chains of amino acid residues of the developed model were determined using Predictor. Docking analysis of Mtb-MurC model with ligands and natural substrates enabled us to identify specific residues viz. Gly125, Lys126, Arg331, and Arg332, within the Mtb-MurC binding pocket to play an important role in ligand and substrate binding affinity and selectivity. The availability of Mtb-MurC ligase built model, together with insights gained from docking analysis will promote the rational design of potent and selective Mtb-MurC ligase inhibitors as antituberculosis therapeutics.

**Keywords** Docking · Gromacs · Modellar9v.3 · Mtb · MurC ligase · NAM · RMSD · Tuberculosis

C. M. Anuradha
Department of Biotechnology,
University College of Engineering and technology,
Anantapur, India

C. Mulakayala · B. Babajan · C. Rajasekhar · C. S. Kumar (✉)
Department of Biochemistry, Sri Krishnadevaraya University,
Anantapur 515003 A.P, India
e-mail: chitt34c@gmail.com

M. Naveen
Institute of Life Sciences, University of Hyderabad Campus,
Gachibowli,
Hyderabad, India

## Introduction

Tuberculosis (TB) continues to be the leading cause of death, worldwide which is mediated with an infectious agent Mtb. Approximately 8 million people have been found to develop new infections of TB every year and almost 2 million of these ultimately die from the disease [1]. A recent survey in 72 countries suggests that multidrug resistant TB is more widespread than previously thought and is likely to be worsening [2]. So far, 50 million people have been infected with drug-resistant Mtb strains, and very few drugs have been developed in the past 40 years [3]. Thus there is a profound need for the identification and development of novel chemotherapeutic compounds active against TB [4]. In this context here we have chosen cell wall biosynthesis of Mtb for designing novel anti tuberculosis therapeutics [5]. The cell wall plays a vital role in bacterial growth and survival in hostile environments. Its functions include support of the delicate cytoplasmic membrane against the high internal osmotic pressure; control of cell shape; mediation of adhesion to surfaces and other cells; involvement in the export of cellular products.

The mycobacterial cell wall has a more complex structure than other Gram + ve bacterial cell walls [6, 7], and is rich in high molecular weight lipids that form a

protective barrier [8]. Mycobacterial cell envelop is made up of 3 major constituents, plasma membrane, covalently linked mycolic acids and arabinogalactan and peptidoglycan complex (MAPs) and a polysaccharide rich capsule. Among these the MAPs have a unique nature, which lead to the conclusion that the enzymes synthesizing MAPs yield a number of potentially unique drug targets [9]. Thus we have considered the machinery of peptidoglycan biosynthesis for designing of novel antituberculosis therapeutics. Peptidoglycan is composed of a β-1, 4- linked glycans of alternating N-acetyl glycosamine (NAG) and N-acetyl muramic acid (NAM) sugars [10]. To the third position of NAM is attached a penta peptide side chain of L-Ala-D-Glu-X-D-Ala-D-Ala, where X is either L-Lys or meso diamino pimelic acid (DAP). During this biosynthesis a series of ATP-dependent amino acid ligases (Mur ligases) add amino acids sequentially on to the lactyl side chain of UDP-NAM. Of the enzymes involving in peptidoglycan biosynthesis MurA, MurB, MurC, MurD, MurE, MurF, MurX, MurG, and ddlA do not have the human homologues [5]. In this scenario we have chosen one enzyme involved in peptidoglycan biosynthetic pathway of Mtb for the designing of novel antituberculosis therapeutics, *i.e.*, UDP-N-acetyl muramate-L-Alanine ligase (MurC ligase). Mtb-MurC ligase catalyses the first step of addition of peptide molecule, *i.e.*, addition of L-Alanine to NAM.

In this paper, a 3-D structural model of Mtb-MurC ligase was constructed using homology modeling techniques. Structural refinement and energy minimization of built Mtb-MurC model was done using Gromacs. The structural quality of the predicted Mtb-MurC model was verified using Procheck, Whatif, Prosa, and Verify 3D. The protein torsion angle restraints for the side chains of each amino acid were predicted using Predictor. Validity of the structural model was assessed by docking with a series of designed isoniazid (INH) inhibitors and natural substrates. Detailed analyses of inhibitor Mtb-MurC ligase interactions were done and the residues in binding domain responsible for binding to the inhibitors and natural substrates with high binding affinity were identified. This information is highly useful for guiding the rational design of safe and effective inhibitors of MurC ligase, as potential anti-tuberculosis therapeutics.

## Materials and methods

All the calculations were conducted by AMD 64 bits dual processing hi end server machines. The sequence of Mtb-MurC ligase (gi: 15609289) was obtained from the National Centre for Biotechnology Information (NCBI) [11]. Local alignments were predicted using Blastp (Basic Local Alignment Search Tool) [12] at the NCBI and the homologous entries were obtained from the protein data bank [13]. The Blastp alignment was further refined using sequence alignments in the ClustalW 1.83 with default parameters [14]. This alignment was used for comparative modeling built in the Modeller9v3 [15] which generated structures by applying spatial restraints. A bundle of 100 models from random generation of the starting structure was calculated and subsequently the best model (with the low RMS value of superposition using Swiss-pdb viewer [16]) was subjected for further analysis. To gain a better relaxation and more correct arrangement of the atoms, refinement was done on the built Mtb-MurC model by energy minimization (EM) and molecular dynamic (MD) stimulations using 43A1 force field of Gromacs96 implemented in the Gromacs 3.2.1 package [17]. A truncated octahedron box with the SPC water model [18] and ions ($Na^+$ and $Cl^-$) was built. The box model, first with explicit water and then with ions, was submitted to 50 rounds of energy minimization using the steepest descent algorithm till an energy gradient was reached and it was found to be the most appropriate energy gradient to relax the models and afford well Ramachandran plots. Mtb-MurC ligase was subjected to a full MD simulation of 5000 ps at 300 k with no restrictions using two fs of integration time. Lincs constraints were used on all protein covalent bonds to maintain constant bond length [19] and the Settle algorithm was used to constrain the intra molecular water bonds to their equilibrium length [20]. Co-ordinates and energy terms (total, kinetic and potential for the whole system and electrostatic, distance-dependent, distance-independent reaction force field) were saved for each ps. With the aim of evaluating the system stabilization throughout the molecular dynamics time, the total, kinetic and potential energy was plotted versus time. The stabilization was assessed by graphics visualization. The stereochemical parameters of the energy minimized Mtb-MurC model were assessed by Procheck [21], Whatif [22] ProSA [23] and Verify 3D [24, 25]. Verify 3D was used to assess whether a primary sequence is compatible with the current 3D structural model. The compatibility between the amino acid side chains in the model is a validation criterion. Torsion angle restraints for side chains of each amino acid in the predicted Mtb-MurC model were determined using a web server Predictor [26]. Predictor assigns an error to the predicted chi (χ) torsion angle including *trans*, *gauche* + and *gauche*- by combining its confidence scores with predicted or identified secondary structures and local sequence identity. Secondary structural conformations for the developed Mtb-MurC model were predicted by pdbsum [27].

Keeping the aim of constructing a novel ligand for Mtb-MurC ligase, a library of 100 molecules were constructed based on the seed structure of INH and implementing structural manipulations and optimizations on to it by Chemdraw (Cambridgesoft Inc.) [28]. The generated library of INH modified ligands were tested for Lipinski's Rule of

Five using Molinspiration server [29] for their ability to follow ADME rules. The Auto Dock 3.0/ADT [30] program was used to investigate ligand binding to structurally refined MurC ligase model using a grid spacing of 0.375 Å and the grid points in X, Y and Z axis were set to 60×60×60. The search was based on the Lamarckian genetic algorithm [31] and the results were analyzed using binding energy. For each ligand, a docking experiment consisting of 100 stimulations was performed and the analysis was based on binding free energies and root mean square deviation (RMSD) values, and the ligand molecules were then ranked in the order of increasing docking energies. Substrate docking with natural substrates, *i.e.*, NAM and L-Alanine was also performed on to Mtb-MurC ligase model with same parameters and PMV 1.4.5 viewer was then used to observe the interactions of the docked compounds to the Mtb-MurC ligase model. The developed 3D model of Mtb-MurC ligase was submitted to Protein Model Data Base (PMDB) [32], which collects 3D models obtained by structure prediction methods.

## Results

### Sequence analysis and homology modeling of Mtb-MurC ligase

The crystal structures of A-chain of the UDP- N -acetyl muramate-L-Alanine ligase (MurC) from *H. influenza* (PDB ID: 1P31) [33] and A-chain of Apo enzyme of UDP- N -acetyl muramate-L-Alanine ligase from *H.influenza* (PDB ID: 1GQQ) [34] were the two structurally homologous proteins that were found by Blastp analysis and hence chosen as templates for developing the Mtb-MurC ligase model. The multiple sequence alignments were followed by pair wise sequence alignment among Mtb-MurC sequence and the templates 1GQQ, 1P31 (Fig. 1a). A total of 100 models of Mtb-MurC ligase were generated using Modellar 9.v3 and were superposed on template crystal structures 1GQQ and 1P31 to calculate the RMS-superposition values and to check their structural compatibility with templates.

### MD simulation studies

MD simulations were performed to determine the stability of the predicted 3D structure of Mtb-MurC. The trajectories were stable during the whole production part of the 5000 ps MD simulation run. The trajectory stability was monitored and was confirmed by the analysis of backbone RMSD (Fig. 2a) and the total energy (Fig. 2b) as a function of time for the Mtb-MurC ligase. Figure 2a revealed that the RMSD values for the Mtb-MurC showed a rise in the first 1000 ps and then remained stable in the following simulation time. The average RMSD for the Mtb-MurC model when measured from 5000 ps was found to be 0.51586198 nm and the total energy (KJ $mol^{-1}$) (Fig. 2b) was found to be stable throughout the simulation time. The total RMSF (peptidic backbone plus side chains), (Fig. 2c

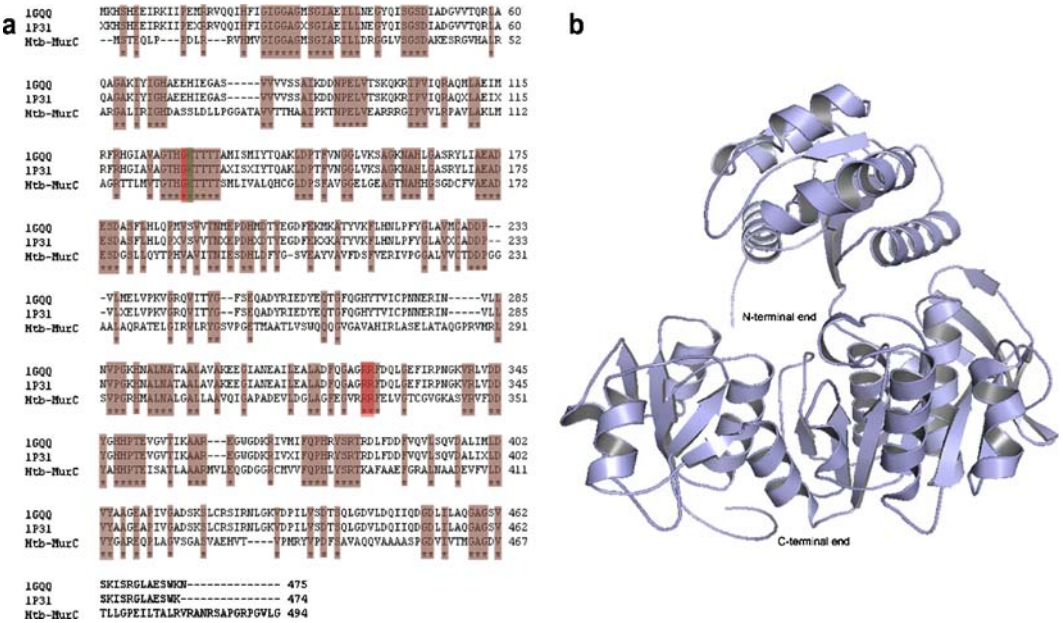


**Fig. 1** **a**) Multiple sequence alignment of Mtb-MurC ligase and the templates 1GQQ and 1P31. Dashes represent insertions and deletions: conserved residues which are not involved in active site, ligand binding and metal binding are shaded in brown. Conserved residues which are involved in active site are shaded in green, conserved residues involved in ligand binding are shaded in red. **b**) A cartoon representation of the 3-D structure of predicted Mtb-MurC ligase model

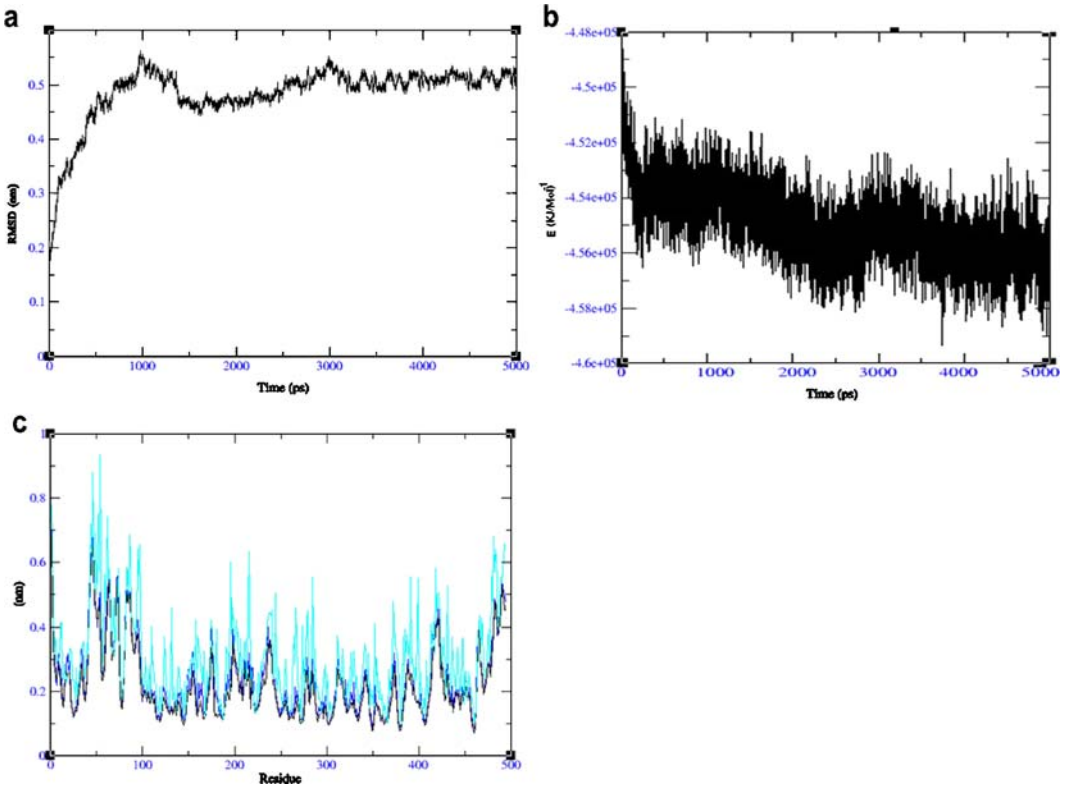


**Fig. 2** **a**) Graphical representation of RMSD of back bone carbons from starting structure of Mtb-MurC model as a function of time. **b**) Plot of energy *vs*. simulation time for Mtb-MurC ligase. **c**) RMS fluctuations for the total protein (blue lines), back bone (black dashed line) and side chains (cyan) of Mtb-MurCligase

in blue) the RMSF of the peptidic back bone (N, Cα, C), (Fig. 2c in black) and the RMSF of the side chains (Fig. 2c in cyan) were represented for the developed model in Fig. 2c. The graph showed that the residues at N-terminal regions have larger RMSF values where as the active site amino acid has lower RMSF value. It was found that throughout the dynamic simulations very few fluctuations exceeded 0.5 nm and even less fluctuations over passed 0.6 nm for total protein. The residues (51–60) with fluctuations close to 0.6 nm observed in the dynamic plots were located at the N-terminal regions.

## Structural validation

The Procheck analysis of the energy minimized model of Mtb-MurC ligase showed that 100% of the residues were found in allowed regions of the Ramachandran plot [20]. Moreover among the total amino acids 94.2% of residues were positioned in the most favored regions of the Ramachandran plot and was found to compare favorably with data for the crystal structures: 1GQQ 89.4%, and 1P31 94.6% (Table 1). The total quality G-factor was −0.2, which indicates a good quality model (acceptable values of the G-factor in procheck

**Table 1** Procheck values for the predicted Mtb-MurC ligase and the template structures

| Ramachandran plot statistics | 1P31 (A chain) | 1GQQ(A chain) | Mtb-MurC |
|---|---|---|---|
| % Amino acids in most favored regions | 94.6% | 89.4% | 94.2% |
| % Amino acids in additional allowed regions | 5.2% | 9.9% | 5.1% |
| % Amino acids in generously allowed regions | 0.0% | 0.3% | 0.7% |
| % Amino acids in disallowed regions | 0.2% | 0.4% | 0.0% |
| ProSA Z-score | −11.37 | −9.69 | −10.78 |
| RMS Z-Score | | | |
| Bond angles | 0.355 | 1.281 | 0.746 |
| Bond lengths | 0.629 | 1.312 | 0.986 |

are between 0 and −0.5, with the best models displaying values close to zero). Analysis of the energy minimized Mtb-MurC model with Whatif web interface [22] reveals that RMS Z-Scores for bond angles and bond lengths are all close to 1 and also within the limits of templates (Table 1). Evaluation of the energy minimized model of Mtb-MurC ligase with ProSA-web reveals that the Z-score value is −10.78 (Fig. 3a) in the range of native conformations of the crystal structures (Table 1). ProSA-web analysis (Fig. 3b) had showed that overall the residue energies of the Mtb-MurC model were largely negative except for some peaks in the middle region. The final structure has been further evaluated for overall quality by Verify 3D. The compatibility scores are obtained using Verify 3D and results for the final structure are shown in Fig. 4a. The compatibility scores for all the residues in the developed model are above zero. Prediction of torsion angle restraints for the side chains of the developed Mtb-MurC model using Predictor had shown that the confidence score and the similarity score for almost all the amino acids is ≥ 0.7. The confidence scores for catalytic amino acid and the amino acids involved in substrate and ligand binding are represented in Fig. 4b. Detailed secondary structural investigation of the predicted Mtb-MurC model with Pdbsum, a secondary structure prediction server reveals 166 (33.6%) residues were in α-helices and 109 (22.1%) residues were in β-sheets, 10 (2.0%) residues were in 3–10 helix and 209 (42.3%) residues were in other conformations (Fig. 3c). The tertiary structure of Mtb-MurC shows close resemblance to crystallized 1GQQ and 1P31, with a back bone RMS values between Mtb-MurC-1GQQ and Mtb-MurC-1P31 are 0.58 Å and 0.72 Å, respectively. The low overall RMS values for backbone superposition reflect the high structural conservation of this complex through evolution, making it a good system for homology modeling.

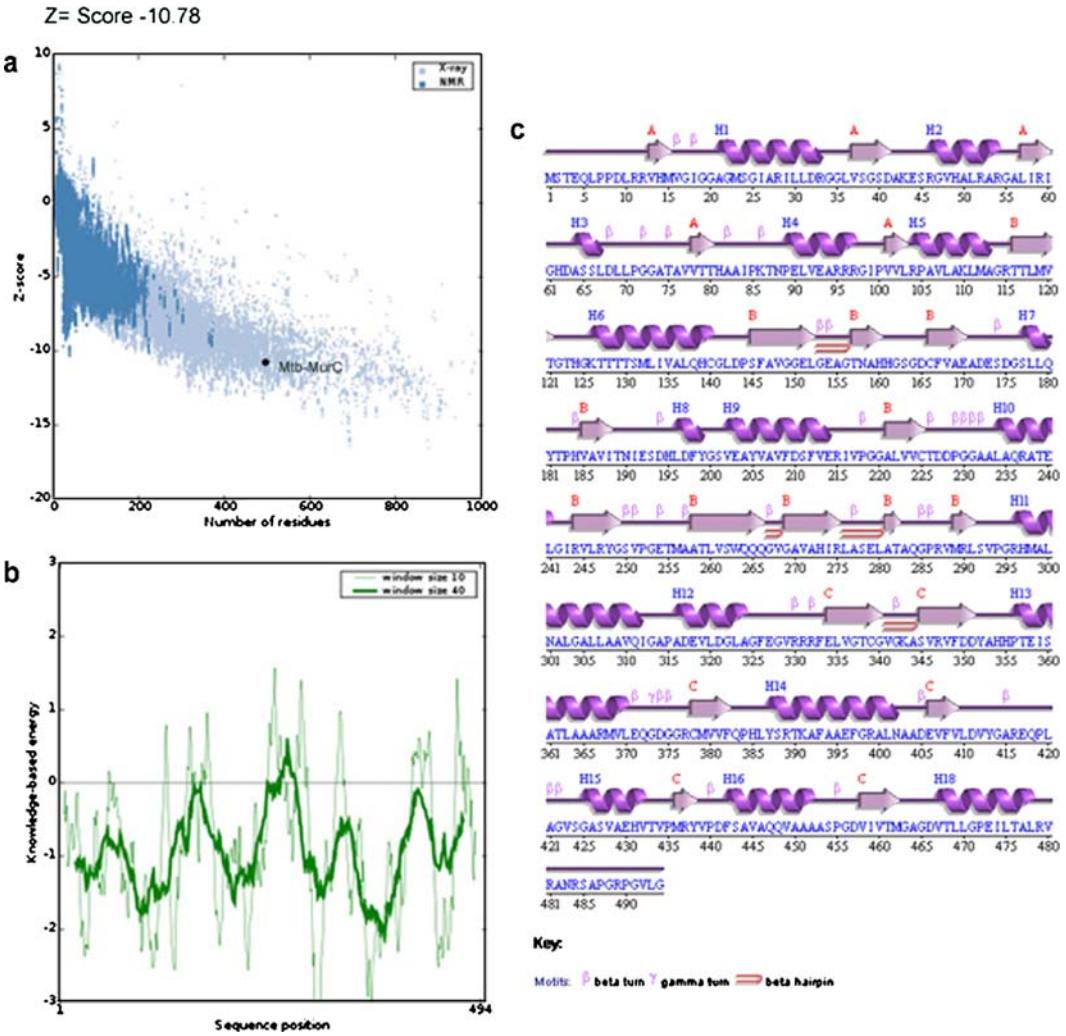


**Fig. 3** **a**) ProSA-web Z-scores of all protein chains in PDB determined by X-ray crystallography (light blue) and NMR spectroscopy (dark blue) with respect to their length. The Z-score of Mtb-MurC was present in that range represented in large black dot. **b**) Energy plot for the predicted Mtb-MurC ligase. **c**) A secondary structure wiring diagram for the Mtb-MurC ligase showing the location of secondary structure elements

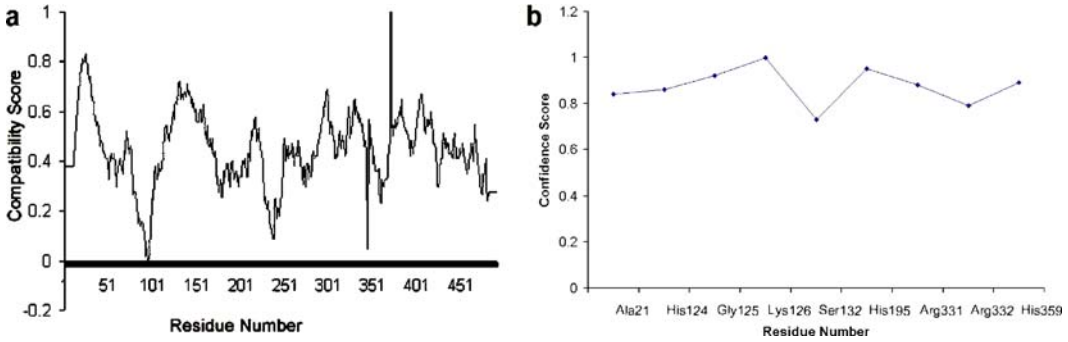


**Fig. 4** **a**) Predicted confidence scores for amino acid residues involved in active site, ligand and substrate binding. **b**) 3D profile verified results of Mtb-MurC model

Design, validation and docking studies of Mtb-MurC inhibitors

Docking studies were performed to gain insight into the binding conformation of pharmacophore models derived from structural manipulations on isoniazid (INH). A library of 100 lead molecules were constructed and screened for satisfying the minimal criteria of ADME for further analysis, using Molinspiration [29]. Among the 100 lead molecules, 10 molecules were selected based on the criteria of satisfying Lipinski's rule-of-five with zero violations, for docking on Mtb-MurC model. All docking calculations were carried out using AutoDock 3.0/ADT and the dlg files generated were analyzed for their binding conformations. Analysis was based on free energy of binding, lowest docked energy, and calculated RMSD values (Table 2). The total clusters of docking conformations, with the 10 docked lead molecules showed negative binding energies. Among all docking conformations INH-1 (isonicotinic acid N-(4-acetyl benzyl) - hydrazide) gave the best predicted binding free energy of −10.59 kcal $mol^{-1}$ to the Mtb-MurC ligase (Fig. 5b). To confirm the mode of binding of designed lead molecules, natural substrate docking with NAM and L-alanine was performed on the Mtb-MurC model with the same parameters. Natural substrate docking revealed that the amino acids Gly125, Lys126, Arg331, and Arg332 along with Ala21, His124, Ser132, His195 and His359 (Fig. 5a) played vital role to bind the natural substrates. To test the validity of our hypothesis, we made a mutant of Mtb-MurC ligase in which Lys126 was mutated to His126 by employing the mutation prediction at WHAT IF Web interface [22], and docking was performed with INH-1. The binding free energy of this complex was −9.89 kcal $mol^{-1}$.

## Discussion

Sequence alignment of Mtb-MurC ligase revealed that, the templates showed 40% identity to the Mtb-MurC ligase and is adequate for developing homology model. A total of 32

**Table 2** Summary of docking results high ranked lead molecules with Mtb-MurC model

| Compound | Lys 126 (Å) | Gly 125 (Å) | Arg 331 (Å) | Arg 332 (Å) | Ala 21 (Å) | His 124 (Å) | Ser 132 (Å) | His 195 (Å) | His 359 (Å) | Free Energy Of binding (-KJ/mol) | Docked Energy (-KJ/mol) | RMSD (Å) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| INH-1 | 2.244 | 2.162 | ___ | 1.972 | ___ | ___ | ___ | ___ | ___ | 8.59 | 10.59 | 0.721 |
| INH-2 | 1.930 | ___ | 1.631 | 1.693 | ___ | ___ | ___ | ___ | ___ | 7.26 | 9.04 | 0.672 |
| INH-3 | 2.240 | 1.185 | ___ | 1.940 | ___ | ___ | ___ | ___ | ___ | 8.12 | 10.03 | 0.565 |
| INH-4 | 1.672 | 1.784 | 2.182 | 2.145 | ___ | ___ | ___ | ___ | ___ | 7.08 | 8.53 | 0.484 |
| INH-5 | 1.912 | 1.631 | ___ | 1.930 | ___ | ___ | ___ | ___ | ___ | 7.29 | 8.68 | 0.524 |
| INH-6 | 2.244 | ___ | ___ | 1.660 | ___ | ___ | ___ | ___ | ___ | 7.59 | 9.63 | 0.515 |
| INH-7 | 2.036 | ___ | 1.631 | 1.680 | ___ | ___ | ___ | ___ | ___ | 6.96 | 8.21 | 0.325 |
| INH-8 | 1.921 | ___ | 1.680 | 1.623 | ___ | ___ | ___ | ___ | ___ | 3.40 | 4.89 | 0.684 |
| INH-9 | 2.32 | ____ | 2.102 | ____ | ____ | ____ | ____ | ____ | ____ | 5.48 | 6.32 | 0.579 |
| INH-10 | 1.732 | 1.80 | 2.345 | ____ | ____ | ____ | ____ | ____ | ____ | 7.42 | 9.32 | 0.825 |
| Natural substrates (NAM + L-Ala) | 1.762 | 1.968 | 1.852 | 2.115 | 2.25 | 2.194 | 1.925 | 1.946 | 1.933 | 8.54 | 12.48 | 0.360 |

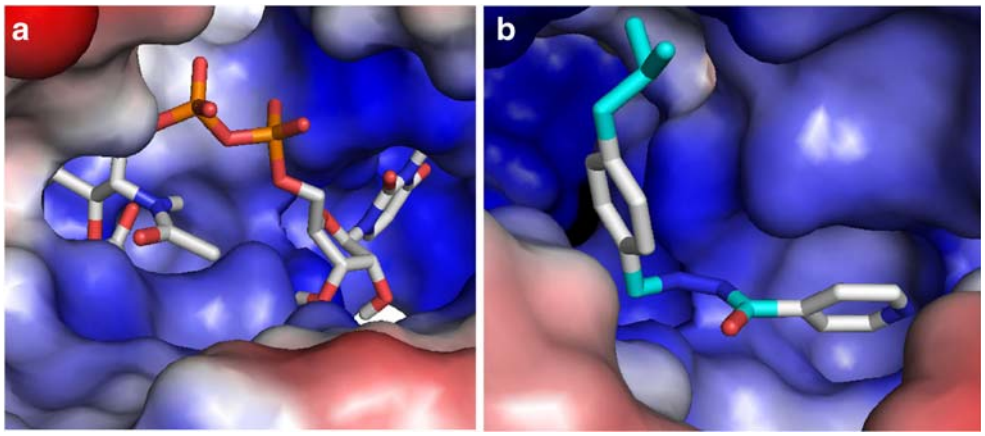


**Fig. 5** The electrostatic surface representation of the binding site of (**a**) Mtb-MurC model and natural substrates (NAM and L-Alanine) and the (**b**) Mtb-MurC model and INH-1 ligand. The surface residues are color coded based on their nature: electronegative (red) and electropositive (blue)

residues were inserted into the Mtb-MurC ligase at various regions in the sequence and 13 residues were found to be deleted from Mtb-MurC ligase; also the active site amino acid in the templates was conserved in the Mtb-MurC ligase (Lys126) (Fig. 1a). Among the 100 developed models the one having lowest RMS-superposition of carbon alpha and carbon back bone (0.58 Å & 0.78 Å) was chosen for further analysis, confirming that the model was satisfactory regarding the utilization of chosen templates for the homology modeling process. The overall stability of the model structure was finally investigated using an unconstrained MD simulation. Result shows that total, potential, and kinetic energies always remained constant during the simulation and the protein size also remained constant. It can be seen that the system remains in equilibrium during the entire simulation. Then, we conclude that the MD simulation has produced an improved and more relaxed structure which can be analyzed for further analysis. As shown in Table 1, the quality of the Ramachandran plot as well as the G-factor was found to be better for the predicted Mtb-MurC. RMS Z-score values for bonds and angle parameters (Table 1) for the developed model of Mtb-MurC are within values typical of highly refined structures [22]. The fact that the RMS Z-score values of bonding distances and angles for the crystal structures are small might indicate that too-strong of constraints have been used in the original refinement of 1GQQ and 1P31 and there is no significant difference observed between the calculated values of the bond lengths and bond angles with that of known proteins for total residues. The compatibility scores obtained by Verify 3D had shown that predicted Mtb-MurC model had acceptable side chain environment [24, 25]. The interaction energy of each residue was checked by Prosa. Prosa analysis of Mtb-MurC model revealed that the residue energies including pair energy, combined energy and surface energy are all negative and has similar energy tendency with templates (Fig. 3a and b). So we can see that Mtb-MurC model reaches the energy criteria of Prosa. Confidence score and the similarity scores predicted for each amino acid side chains in the developed Mtb-MurC model reveals that almost all residues attained the confidence rating of $\geq 0.7$. It indicates that the predicted model is good [24] and there is no error in the predicted secondary structures. Through this assessment and analysis process, we can conclude that the 3D structure of Mtb-MurC we constructed is reliable. The secondary structures assigned to the theoretical and experimental models resulted very similar and very well overlapped (Fig. 3c). By applying structural superposition and RMS evaluations, our model appears very similar to the experimental one.

In summary, the quality of the backbone conformation, the residue interaction, the residue contact, and the dynamic stability of the structure are well within the limits established for reliable structures. Passing all tests by predicted model suggests that an adequate model for Mtb-MurC is obtained to characterize protein-substrate and protein-ligand interactions and to investigate the relation between the structure and function. With all these evaluations the predicted Mtb-MurC model was submitted to PMBD and it has accepted the model with less than 3% stereochemical check failures. PMDB ID for the developed Mtb-MurC model was PM0075016.

Docking results of the designed INH ligands and natural substrates on to Mtb-MurC ligase model showed that a hydrogen bonding network was found to exist between docked molecules and Mtb-MurC model. Almost all the docked molecules were observed to form hydrogen bonding with the Lys126 side chain and this is the active site amino acid even in the templates [33, 34]. Docked molecules exhibited hydrogen bonding interactions with Gly125, Arg331, and Arg332. These included Lys126 amino group formed hydrogen bonds with the carbonyl group of ligands, Gly125 carbonyl formed a hydrogen bonding with the imino group of ligands and the Arg331, Arg332 guandino group showed interactions with the ligand molecules. The docking results revealed that the domain constituting

Lys126, Gly125, Arg331, and Arg332 acts as the catalytic domain in Mtb-MurC ligase. Indeed, the mutated Mtb-MurC model was found to accommodate INH-1 in a pose similar to that in Mtb-MurC model although the two rings of INH-1 were slightly distorted. It should be noted, however, that minute changes in the shape of the catalytic cleft can be influenced by subtle changes of backbone residues that do not reside in the interface. It is highly conceivable that these hydrogen bonding interactions hold the key for the design of potent and selective Mtb-MurC ligase inhibitors.

The purpose of this study is to generate a structural model for the Mtb-MurC ligase, one of the enzymes of peptidoglycan biosynthesis in Mtb and also to generate novel inhibitor compounds for Mtb-MurC ligase. It should be kept in mind that MurC ligase is absent in humans, thus, the compounds identified here-in may also be relevant in the rational development of novel drugs specific against bacterial human pathogens of public health importance. In the present work several novel compounds that exhibit *in vitro* inhibitory activity against Mtb-MurC ligase have been identified. Thus, understanding the structural and energetic basis for inhibitor specificity is of crucial importance. The present work forms the basis for further molecular studies on Mtb-MurC inhibitor design. Furthermore, with the high-quality Mtb-MurC model, computational docking study on Mtb-MurC substrates and designed lead molecules become feasible which may provide new insights into mechanisms of specificity in target.

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