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ORIGINAL PAPER

Homology modeling and docking analyses of *M. leprae* Mur ligases reveals the common binding residues for structure based drug designing to eradicate leprosy

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Abstract Multi drug resistance capacity for *Mycobacterium leprae* (MDR-Mle) demands the profound need for developing new anti-leprosy drugs. Since most of the drugs target a single enzyme, mutation in the active site renders the antibiotic ineffective. However, structural and mechanistic information on essential bacterial enzymes in a pathway could lead to the development of antibiotics that targets multiple enzymes. Peptidoglycan is an important component of the cell wall of *M. leprae.* The biosynthesis of bacterial peptidoglycan represents important targets for the development of new antibacterial drugs. Biosynthesis of peptidoglycan is a multi-step process that involves four key Mur ligase enzymes: MurC (EC:6.3.2.8), MurD (EC:6.3.2.9), MurE (EC:6.3.2.13) and MurF (EC:6.3.2.10). Hence in our work, we modeled the three-dimensional structure of the above Mur ligases using homology modeling method and analyzed its common binding features. The residues playing an important role in the catalytic activity of each of the Mur enzymes were predicted by docking these Mur ligases with their substrates and ATP. The conserved sequence motifs significant for ATP binding were predicted as the probable residues for structure based drug designing. Overall, the study was successful in listing significant and common binding residues of Mur enzymes in peptidoglycan pathway for multi targeted therapy.

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Introduction

Leprosy is an infectious disease caused by *Mycobacterium leprae*. The World Health Organization (WHO) estimated that 2 million people worldwide were infected with *Mycobacterium leprae* [1].

Though the combination of drugs Dapsone, Rifampin and Clofazimine proved to be effective [2–7] for the treatment of leprosy, their long term treatment led to resistance. Increased morbidity and mortality are the most dramatic consequences of this resistance [8]. Therefore, there is an urgent need for the development of novel antibacterial agents.

Some of the best known and most validated targets for antibacterial therapy are the enzymes involved in the biosynthesis of bacterial cell wall. The low permeability of the mycobacterial cell wall contributes to the intrinsic drug resistance of mycobacteria [9].

Peptidoglycan is a major component of the cell wall of almost all eubacteria. It provides rigidity, flexibility and strength that are necessary for bacterial cells to grow and divide. It withstands the higher internal osmotic pressure [10]. Peptidoglycan is a complex heteropolymer that is composed of long glycan chains made up of alternating units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). The D-lactoyl group of each MurNAc residue is substituted by a pentapeptide which is composed of L-Alanine, D-Glutamic acid, meso-diaminopimelic acid (meso-A2pm) or L-Lysine and a dipeptide D-alanyl-D-alanine [11]. In *M. leprae* the first amino acid in the pentapeptide is Glycine instead of L-Alanine [12] and the third is meso-diaminopimelic acid [11].

The biosynthesis of peptidoglycan involves a number of ATP-dependant Mur ligases (MurC to MurF), which contribute to the formation of UDP-MurNAc-pentapeptide by successive addition of Glycine (MurC), D-Glutamic acid (MurD), meso-diaminopimelic acid (MurE) and D-Alanyl-D-Alanine (MurF) [11]. The MurC to MurF ligases follows the same reaction mechanism (Fig. 1), which consists of the activation of the carboxyl group of the nucleotide precursor by ATP, generating an acyl phosphate intermediate and ADP. The acyl phosphate is then attacked by an amino group of the incoming amino acid, leading to the formation of a high-energy tetrahedral intermediate. This eventually breaks down into the product and Pi [11].

In our earlier work on comparative genome analysis of metabolic enzymes in *M. leprae* with human reveals that the peptidoglycan biosynthetic enzymes MurC, MurD, MurE and MurF do not have any human homologs and can be used as potential drug targets [13]. As each of these MurC to MurF ligases binds the product of the previous Mur enzyme and follows the same mechanism of action, there is a significant overlap in the sequence and structure in active-site region [11]. Hence, we considered MurC, MurD, MurE and MurF enzymes as ideal multiple targets for developing a multi target inhibitor.

Anuradha et al. considered MurC ligase as a potential drug target and built the model and analyzed its binding site to promote the rational design of potent inhibitor for antituberculosis therapeutics [14]. Sink et al. identified that the N-acylhydrazone derivative has inhibitory activities against MurC and MurD ligases [15]. Perdih et al. predicted the benzene–1,3-dicarboxylic acid has an inhibitory activity against MurD and MurE ligases [16]. Mansour et al. predicted the naphthyl tetronic acids as multi target inhibitors of bacterial peptidoglycan biosynthesis [17]. Tomasic et al. predicted 5-benzylidenethiazolidin–4-one as multi target inhibitor, active against MurD to MurF ligases [18]. Matej sova et al. predicted phosphorylated hydroxyethylamines as novel inhibitors of bacterial cell wall biosynthesis enzymes murC to MurF [19].

Knowledge on the three-dimensional structure of these Mur ligases will provide a clue on its potentially important residues for substrate binding and/or catalytic mechanism. As a case study, we have successfully built the homology model of one of the potential drug targets MurE ligase using homology modeling and analyzed its binding features using docking studies [20]. This work is an extension to find the structural characteristics and binding specificity of all these MurC to MurF ligases in detail. This observation underlines the important residues in these Mur ligases to be considered while designing a better multi target drug for drug resistant strains of *M. leprae*.

Methods

The amino acid sequences of *M. leprae* MurC [EC: 6.3.2.8], MurD [EC: 6.3.2.9], MurE [EC: 6.3.2.13] and MurF [EC: 6.3.2.10] were obtained from UNIPROT database [21] using accession number P57994, P57995, O69557 and O69556 respectively. In our recent publication, we reported in detail about the 3D structure of *M. leprae* MurE developed by homology modeling and its binding features [20]. A similar approach is applied again to find homology model and binding features of the other three Mur ligases, which is briefly outlined below. For complete descriptions and methodology refer to [20].

The amino acid sequence of each of the Mur ligases was subjected to position specific iterated (PSI) BLAST [22] search against protein databank (PDB) [23] to identify the suitable template structures for homology modeling. As the homology modeling relies on the sequence alignment between the target sequence and the template sequence whose structure has been experimentally determined, the target and the template sequences were aligned using ClustalW [24].

After careful examination of the potential alignment errors, the automated comparative protein modeling program MODELLER9v6 was used to build the model [25]. In the first step of model building, distance and dihedral angle restraints on the target sequence were derived from its alignment with the template 3D-structure. The spatial restraints and the energy minimization steps were performed with the CHARMM22 force field for proper stereochemistry of proteins. Then, optimization of the model was carried out by the molecular dynamics simulated annealing method.

The stereo chemical quality of *M. leprae* Mur ligase models generated were evaluated using PROCHECK [26]. The quality of the models is also verified by comparing predicted structures to X-ray solved structures via superposition and measurement of the C-α root mean square deviation assessment (RMSD). The predicted model and the template 3D structure were submitted to the server SUPERPOSE [27] and the RMSD between predicted model and template was identified.

The atomic coordinates of the ligands (substrates and products of mur enzymes) Glycine [DrugBank: DB00145]

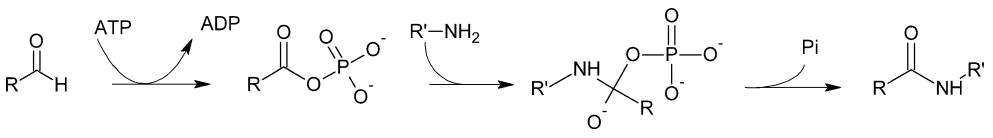

Fig. 1 Mechanism of action of mur ligases

and D-Glutamic acid [DrugBank: DB02517] as well as ATP [DrugBank: DB00171] molecule were obtained from DrugBank [28]. The atomic coordinates of meso A_2pm [PDB: 1E8C] was obtained from Protein Data Bank (PDB). The structures of the remaining ligands D-Alanyl-D-Alanine, UDP-*N*-acetyl muramic acid (UNAM), UDP-*N*-acetylmuramoyl-Glycine (UMG), UDP-*N*-acetylmuramoyl-glycyl-D-glutamate (UMGG), UDP-*N*-acetylmuramoyl tripeptide (UMT) and UDP-*N*-acetylmuramoyl pentapeptide (UMPP) were drawn using ACD/ChemSketch [29] and their SMILES notation was obtained. SMILES notation was translated into PDB file [30] and energy minimized using mmff94 force field and the conjugate gradient optimization algorithm.

To find the binding affinities between each of the *M. leprae* Mur ligases and their substrates, products and ATP molecule, an automated flexible docking of ligands at the active site of an enzyme was carried out using AutoDock 4.0 [31]. The grid maps representing the protein in the actual docking process were calculated with the aid of AutoGrid. The dimension of the grid was 40×40×40 points in each dimension for glycine, D-glutamic acid, meso A_2pm and D-ala-D-ala and 60×60×60 points in each dimension for the remaining ligands with spacing of 0.375 Å between the grid points. Gasteiger charges were computed using ADT (AutoDock tools) on the atoms for each ligand. The AUTOTORS utility, included in the AutoDock software, was used to define all possible torsions of ligand molecules for the docking algorithm. Docking parameters were as follows: ten docking trials, population size of 150, maximum number of energy evaluation ranges of 250,000, maximum number of generations of 27,000, mutation rate of 0.02, cross-over rate of 0.8 and an elitism value of 1. Each job consisted of 100 independent runs. Other docking parameters were set to the software's default values.

1P31 is the crystal structure of *H. influenza* MurC in complex with UMA and ANP. Using the active site information from this structure, the grids were set over Lys126 for docking ATP with MurC and Tyr350 for docking UMG with MurC. As none of the crystal structure of MurC were found to be in complex with Glycine or UNAM, the active site for these ligands were predicted using WHATIF program and the grids were set over Thr363 for docking Glycine and His124 for UNAM with *M. leprae* MurD ligase..

2UAG is the crystal structure of *E. coli* MurD in complex with UMA and ADP. Similarly 2JFF is the crystal structure of *E. coli* MurD in complex with D-Glutamic acid derivative. From the active site information for these ligands for MurD, the grids were set over Thr130 for docking ATP, Asn151 for UMG and Arg389 for D-Glutamic acid. From the active site information obtained from WHATIF program for MurD ligases, the grid was set over Arg332 for docking UMGG with *M. leprae* MurD ligase.

1E8C is the crystal structure of *E. coli* MurE in complex with UAG and A_2pm. 2WTZ is the crystal structure of *M. tuberculosis* MurE in complex with UAG. From the binding interactions observed between the bound ligand and MurE ligase, the grid was set over Arg53 for UMGG, Arg410 for meso A_2pm and Thr181 for UMT. The active site predicted by WHATIF program together with the phosphate binding motif predicted from the sequence alignment, Lys143 was found to be essential for ATP binding and thus the grid was set over Lys143 for docking ATP molecule with *M. leprae* MurE ligase.

In the case of MurF, as the crystal structures of MurF ligases available in the structural databases are not in complex with its natural substrates, the active site was predicted using WHATIF program. From this, the grid was set over Arg193 for docking UMT, Leu213 for D-ala-D-ala, Ser144 for ATP and Asp321 for UMPP molecule with *M. leprae* MurF ligase.

Finally, resulting docking orientations lying within 1.5 Å in the root-mean square deviation (RMSD) tolerance of each other were clustered together and represented by the result with the most favorable free energy of binding (ΔG_b). The docked complex with the highest score was selected to study the binding interactions.

Results and discussion

The amino acid sequence of *M. leprae* MurC [EC: 6.3.2.8], MurD [EC: 6.3.2.9], MurE [EC: 6.3.2.13] and MurF [EC: 6.3.2.10] were retrieved from UNIPROT database using the accession number P57994, P57995, O69557 and O69556 respectively.

The position specific iterated BLAST search of the target sequence *M. leprae* MurC resulted in the crystal structures of an enzyme of UDP-N-acetylmuramate-L-Alanine ligase (MurC) from *Haemophilus influenzae* [PDB: 1GQQ, 1P31] with the highest sequence identity of 38% and 37% respectively. The BLAST score is 266 for 1GQQ and 220 for 1P31. The E-value is 2e–83 and 3e–66 for 1GQQ and for 1P31 respectively. If 1GQQ is used as template the RMSD value of the model from the template is found to be 0.46 Å and if 1P31 is used as template it was found to be 0.6 Å. As the conformations of the *M. leprae* MurC model are almost similar, based on BLAST score the crystal structure of 1GQQ is selected as a potential template structure for modeling *M. leprae* MurC ligase.

M. leprae MurD resulted in the crystal structure of UDP-N-acetylmuramoylalanine-D-glutamate ligase [PDB: 1E0D and 2UAG] from *Escherichia coli* with sequence identity of

30% each. 2UAG is the crystal structure of MurD in presence of its substrate UMG, its product ADP and its co-factor Mg^{2+}, whereas 1E0D is the crystal structure of MurD with no bound substrates. *M. leprae* MurE resulted in the crystal structure of UDP-N-acetylmuramoyl tripeptide synthetase from *E. coli* [PDB:1E8C] with the highest sequence identity of 40%. *M. leprae* MurF resulted in the crystal structure of UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate-D-alanyl-D-alanine ligase [PDB:1GG4] from *E. coli* with sequence identity of 31%. The result of the BLAST search analysis is shown in Table 1.

In all cases, the enzymes predicted with highest sequence identity in PSI-BLAST search belong to Mur ligase family. As both the template structure and the target belong to the same family, the percentage identities predicted between these two are sufficient for developing a good quality model using homology modeling. Hence the 3D structure of MurC ligase from *Haemophilus influenzae* [PDB: 1GQQ], MurD ligase, MurE ligase and MurF ligases from *Escherichia coli* [PDB: 2UAG, 1E8C and 1GG4] were used as the potential template structures for homology modeling of *M. leprae* MurC, MurD, MurE and murF ligases.

Each of the target sequences are aligned with their corresponding template sequence using ClustalW program. Coordinates from the reference protein (PDB: 1GGQ, 2UAG, 1E8C and 1GG4 for MurC, MurD, MurE and MurF ligases respectively) for the structurally conserved regions (SCRs), structurally variable regions (SVRs), N-terminal and C-terminal were assigned to the target sequence based on the satisfaction of spatial restraints. The initial model was generated using MODELLER 9v6. All side chains of the modeled protein were set by rotamers. Structural refinement and energy minimization of built models was done through CHARMM22 force field using steepest descent and conjugate gradient algorithms.

The selected models were subjected to "internal" evaluation of self-consistency checks such as stereo chemical check to find the deviations from normal bond lengths, dihedrals and non-bonded atom-atom distances. The goodness factors (G-factors) from the PROCHECK results confirmed the quality of dihedral, covalent and overall bond/angle distances. The comparable Ramachandran plot characteristics and the goodness factors supported the quality of the modeled structures. The results of Ramachandran plot analysis and the G-factor from the PROCHECK program were shown in Table 2.

The amino acids Thr75, Val77 and Cys485 in MurC, Asp3, Arg117, Trp119, Lys213 and Asp265 in MurD, Ala100, Ser104, Ser293, Ala397 and His398 in MurE and Asp19, Ala25, Ala110, Glu462, Val472 and Arg487 in MurF ligases were in the disallowed region in the Ramachandran plot. As these residues were located too far from the binding pocket, these amino acids will not have any impact in binding of the substrates or products with Mur ligases. Hence these models can be considered for prediction of binding residues.

Further, the overall quality factor predicted by ERRAT and the compatibility of an atomic model (3D) with amino acid sequence (1D) predicted by Verify–3D also confirms that these models were reliable. The results of ERRAT and Verify–3D servers were shown in Table 3.

The root mean square deviation (RMSD) of *M. leprae* MurC model from UDP-N-acetylmuramate-L-alanine ligase (murC) from *Haemophilus influenzae* [PDB: 1GQQ], MurD model from the crystal structure of UDP-N-acetylmuramoylalanine-D-glutamate ligase from *Escherichia coli* [PDB: 2UAG], MurE from the crystal structure of UDP-N-acetylmuramoyl tripeptide synthetase from *E. coli* [PDB:1E8C] and MurF from the crystal structure of UDP-N-acetylmuramoylalanyl-D-glutamyl–2,6–diaminopimelate-D-alanyl-D-alanine ligase from *Escherichia coli* [PDB:1GG4] was found to be 0.45 Å, 0.40 Å, 0.34 Å and 0.55 Å respectively. These RMSD values showed that the 3D structures developed for Mur ligases were similar to their corresponding template structures. Thus these could fit for drug designing and binding affinity tests.

Overall protein structure

All four Mur ligases are topologically similar to one another. Each composed of three evolutionarily conserved

Table 1 Results of BLAST analysis of *M. leprae* MurC to MurF ligases

Mur enzyme	Length of target sequence	Template's PDB ID	Name of the crystal structure	Source	Length of template sequence	Seq identity	BLAST score	E-value
MurC	495	1GQQ	MurC - crystal structure of apo-enzyme	*Haemophilus influenzae*	473	38%	266 bits	2e–83
MurD	490	2UAG	MurD - UDP-N-acetylmuramoyl-L-alanine:D-glutamate ligase	*Escherichia coli*	414	30%	119 bits	2e–29
MurE	530	1E8C	MurE - UDP-N-acetylmuramyl tripeptide synthetase	*Escherichia coli*	493	40%	211 bits	7e–62
MurF	517	1GG4	MurF - UDP-N-acetylmuramyl-tripeptide D-Alanyl-D-alanine-adding enzyme	*Escherichia coli*	450	31%	132 bits	1e–33

Table 2 Results of PROCHECK program for *M. leprae* MurC to MurF ligases

Enzyme	G-factor			Ramachandran plot data			
	Dihedrals	Covalent	Overall	MFR	AAR	GAR	DAR
MurC	−0.06	−0.28	−0.14	94.3%	3.7%	1.9%	0.5%
1GQQ	−0.28	−0.24	−0.26	89.4%	9.9%	0.3%	0.4%
MurD	−0.28	−0.56	−0.38	89.8%	7.1%	1.8%	1.3%
2UAG	−0.12	0.56	0.16	91.1%	8.6%	0.3%	0.0%
MurE	−0.18	−0.36	−0.23	91.7%	4.9%	2.3%	1.1%
1E8C	−0.23	0.54	0.36	90.6%	8.5%	0.9%	0.0%
MurF	−0.15	−0.33	−0.21	90.2%	5.2%	3.0%	1.6%
1GG4	−0.07	0.45	0.23	88.3%	11.5%	0.3%	0.0%

MFA-Most favored region

AAR-Additionally allowed region

GAR-Generously allowed region

DAR-Disallowed region

globular domains: N-terminal Rossmann-fold domain responsible for binding the UDPMurNAc substrate; a central ATP binding domain responsible for binding ATP (similar to ATP-binding domains of several ATPases and GTPases); and a C-terminal domain (similar to dihydrofolate reductase fold) that is associated with binding the incoming amino acid.

N-terminal domain

N-terminal Rossmann-fold domain comprises Residues 1–103 in MurC, 1–98 in MurD, 1–125 in MurE and 1–86 in MurF ligases. In MurC, MurD and MurE ligases this domain consists of five, three and four parallel β- sheets whereas in MurF this consists of five β- sheets where four are found to be parallel to each other and one is anti parallel. In MurC and MurD this β- sheets were surrounded by four α-helices while in MurE and MurF it was found to be six and three α-helices. This domain accounts for the fixation of UDP moiety of UDP substrate. This domain will therefore be referred as *UDP-binding domain*.

Table 3 The overall quality factor from ERRAT and the compatibility of an atomic model (3D) with amino acid sequence (1D) by Verify-3D of *M. leprae* MurC to MurF ligases

Enzyme	ERRAT	VERIFY−3D
MurC	98.31	91.33%
1GQQ	94.61	93.71%
MurD	90.56	90.04%
2UAG	96.42	98.60%
MurE	95.96	94.20%
1E8C	93.71	97.21%
MurF	93.22	97.26%
1GG4	97.53	100%

Central domain

The central domain comprises residues 104–326 in MurC, 99–317 in MurD, 126–358 in MurE and 87–359 in MurF enzyme. In MurC this domain comprises ten β-sheets in which seven were found to be parallel to each other and three were anti parallel. In MurD this ATP binding domain composed of nine β-sheets where seven were parallel to each other and two were anti parallel. In MurE among the 12 β-sheets present in this domain, ten were found to be parallel to each other and two were anti parallel. In MurF this domain comprised of seven β-sheets, in which six were parallel to each other and one was anti parallel. In all these Mur ligases, this domain consists of eight α-helices except in MurE which comprises nine α-helices. The fold of the central β-sheet is similar to the classic "mononucleotide-binding fold" found in many ATP-binding proteins. This domain will therefore be referred to as the *ATP binding domain*.

C-terminal domain

The central domain comprises residues 327–495 in MurC, 318–490 in MurD, 359–530 in MurE and 360–517 in MurF enzyme. In MurC and MurE, this domain consists of six β-sheets where five were parallel to each other and one was anti parallel. While in MurD and MurF, this domain comprises five β-sheets where four were parallel to each other and one was anti parallel. In all these Mur ligases, this domain consists of five α-helices except in MurF which comprises six α-helices. Residues from this ligand-binding domain provide key interactions that orient and position the incoming amino acid ligand with the growing peptidoglycan chain. The N-terminal, central and C-terminal domains arranged in *M. leprae* MurC, MurD, MurE and MurF ligases were shown in Fig. 2

Binding site prediction

Phosphate binding motif

In nucleotide-binding proteins with the classical mononucleotide fold, there is a characteristic fingerprint, GXXGXGKT/S located in the loop between a central β-strand and α-helix. Here G, K, T and S represent the amino acid Glycine, Lysine, Threonine and Serine, whereas X represents any amino acid.

In MurC this loop comprises residues 119–126 with the sequence MVAGTHGKT. In MurD this loop comprises

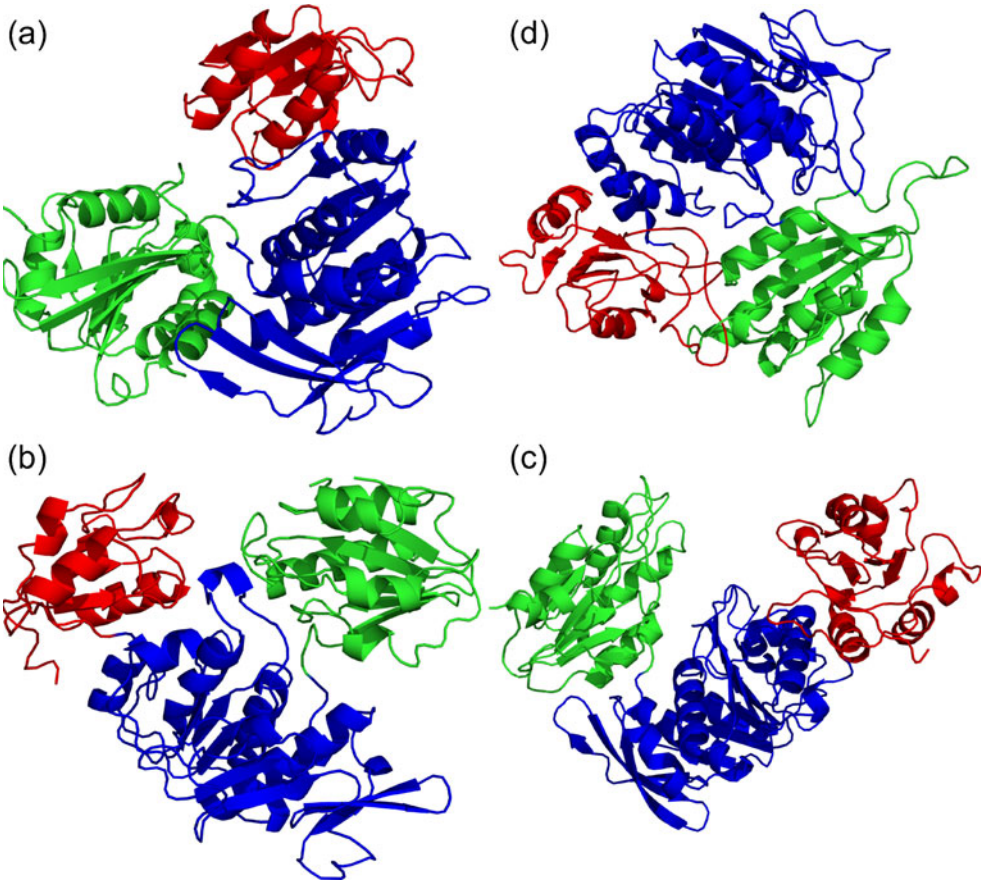

Fig. 2 The domains in the 3D structure of *M. leprae* Mur ligases. The 3d structure of (**a**) MurC ligase (**b**) MurD ligase (**c**) MurE ligase (**d**) MurF ligase is shown in cartoon representation using Pymol. The N-terminal UDP-binding domain, central ATP binding domain and C-terminal ligand binding domain were shown red, blue and green color

residues 121–128 with the sequence VVTGTNGKT. In MurE structure this loop comprises residues 137–144 with the sequence GITGTSGKT. In MurF this corresponds to residues 135–143 with the sequence GITGSSGKT. Apart from the usual nucleotide binding proteins, the length of the phosphate binding motif is increased by one more amino acid in Mur ligases. The additional residue is observed between the second and the third glycine residues of the motif and is located between β6 and α4 of the ATP binding domain. Hence in Mur ligases this has become GXXGXXGKT/S. Instead of the glycine in the first position of the motif GXXGXGKT/S, in MurC and in MurD methionine and valine were located.

As this phosphate binding motif is rich in glycine, this loop is called *Glycine rich loop* which is significant in most of the ADP forming enzymes. Glycine does not have any side chain. This makes the loop flexible and can accommodate the ligands comfortably when they dock with this central domain. Serine and threonine present in the motif contain hydroxyl groups in their side chain. This helps in forming hydrogen bonding interactions with the ligand which binds with them.

Lysine present in this motif is strongly basic in nature and is positively charged at physiological pH. Thus a large anion hole is formed by the loop which accommodates the phosphates of the mono-nucleotide. The ionic and electrostatic interactions between the positively charged lysine and negatively charged phosphates of the ATP molecule stabilize the complex of Mur ligases and ATP. Thus lysine present in this motif plays an important role in binding nucleotides. As this glycine rich G-loop holds the phosphates this may also be called *P-loop*.

Binding site of ATP

The amino acids of MurC, MurD, MurE and MurF interact with ATP through hydrogen bonding whose interactions were given in Table 4. As mentioned above the presence of glycine and proline makes the loop flexible for binding. Lysine, arginine and histidine make the loop positively charged which is essential to hold the negatively charged phosphates of the ATP molecule. These essential basic amino acids were conserved in all ADP forming enzymes.

Residues coordinating and stabilizing ATP include lysine, arginine, histidine, serine and threonine. Lysine, arginine and histidine stabilize the complex through electrostatic and hydrogen bonding interactions. Serine and threonine stabilizes the complex through hydrogen bonding interactions.

Table 4 Residues in *M. leprae* MurC to MurF ligases interacted with ATP

MurC	MurD		MurE	MurF	
THR123	GLY15	THR125	GLY142	ASP147	SER140
LYS126	GLY16	ASN126	LYS143	VAL151	GLY141
THR127	THR19	GLY127	THR144	GLN323	SER144
THR128	ASP38	THR130	THR166	GLY352	LEU213
GLU170	ASP40	ASP194	THR181	PRO353	ASN214
ASP172	ALA42	ASP290	TYR379	ARG358	VAL318
ASP194	SER77	ASP336			TYR319
ASP352	PRO78	ALA339			HIS322
TYR353	GLY79				GLN323
ALA354	ASP100				ASN326
HIS355	GLY150				ARG358
GLU359					MET359
THR363					

In MurD and MurF, ATP molecule binds in a region other than the phosphate binding motifs mentioned above. This indicates that MurD and MurF have two different binding sites for ATP molecule. The MurC, MurD, MurE and MurF ligases bound with ATP molecule were shown in Fig. 3

Binding site of glycine, UNAM and UMG in MurC

The substrate glycine binds with MurC in the cleft between central domain and C-terminal domain. The oxygen atom present in the carboxylic acid of the glycine form electrostatic and hydrogen bonding interactions with His295 present in α11 in the central domain. Both the δ and ε-nitrogen atoms of His 295 were involved in binding the amino acid glycine. In the same manner, glycine also binds with Glu359 and Thr363 present in α13 of the C-terminal domain. In addition to the hydrogen bonding interactions, the substrate also forms electrostatic interac-

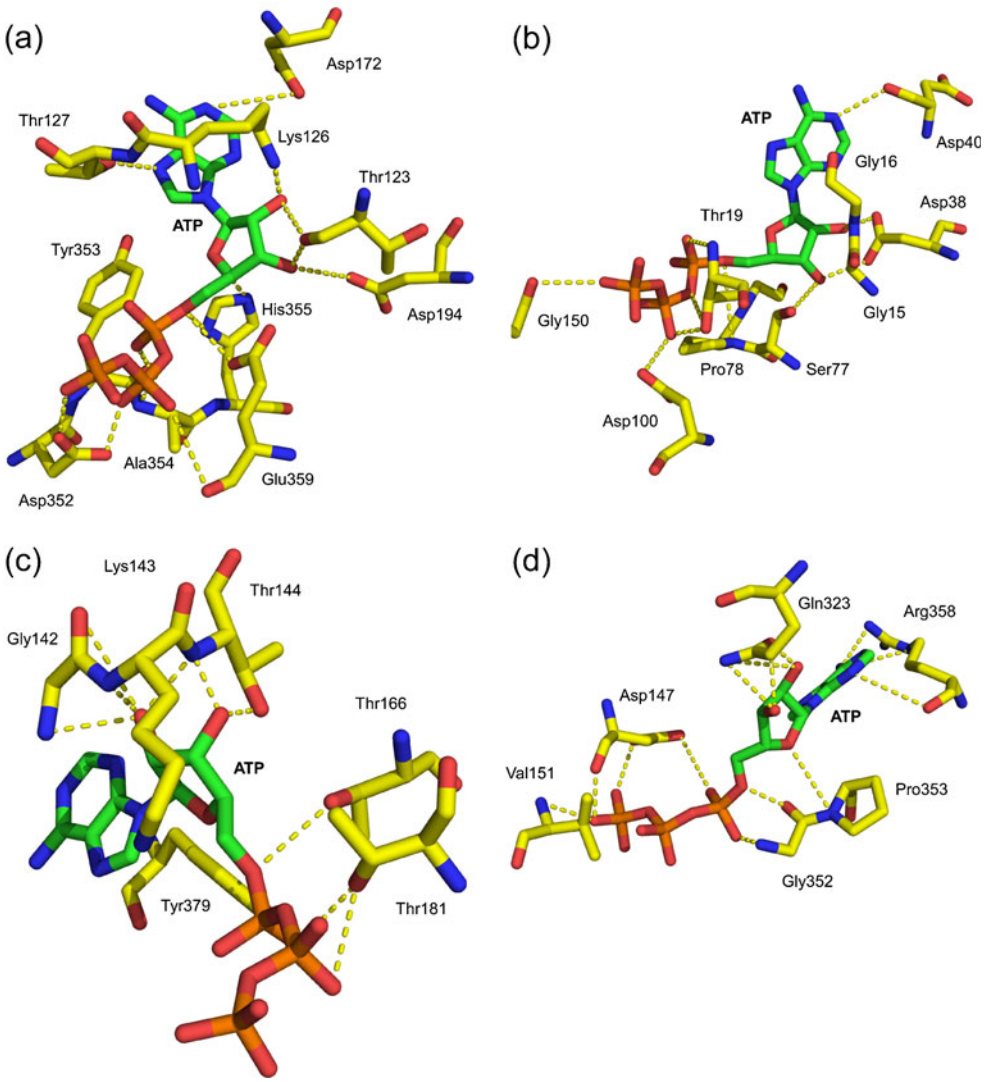

Fig. 3 The docked pose of *M. leprae* Mur ligases with ATP. The docked complex of *M. leprae* (**a**) MurC ligase (**b**) MurD ligase (**c**) MurE ligase (**d**) MurF ligase with ATP mplecule were shown in stick representation using Pymol. For clarity only few of the important residues which bound with the enzymes were shown. The Mur ligases and ATP molecule were in yellow and green color. The nitrogen, oxygen and phosphorous atoms were shown in blue, red and orange color

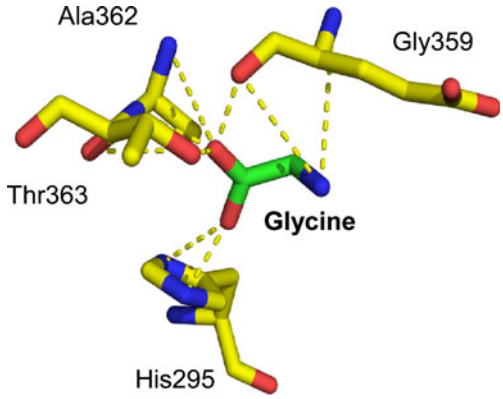

Fig. 4 The binding pose of *M. leprae* MurC ligase with Glycine. The docked complex of *M. leprae* MurC ligase with its substrate glycine was shown in stick representation using Pymol. For clarity only few of the important residues which bound with the enzymes were shown. The MurC ligase and glycine molecule were in yellow and green color. The nitrogen, oxygen and phosphorous atoms were shown in blue, red and orange color

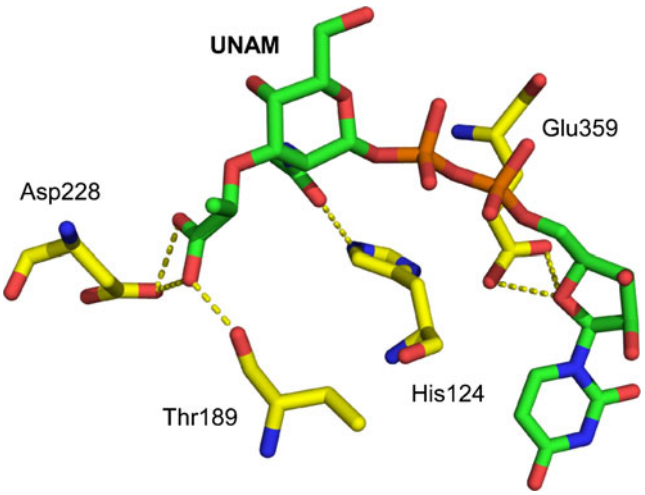

Fig. 5 The binding pose of *M. leprae* MurC ligase with UNAM. The docked complex of *M. leprae* MurC ligase with its substrate UNAM was shown in stick representation using Pymol. For clarity only few of the important residues which bound with the enzymes were shown. The MurC ligase and UNAM molecule were in yellow and green color. The nitrogen, oxygen and phosphorous atoms were shown in blue, red and orange color

tions with Thr363 to stabilize the complex. The docked pose of glycine with MurC ligases was shown in Fig. 4

O(35) in muramic acid moiety of UNAM interacts with the side chain oxygen of Thr189 present in a loop connecting β9 and α9 present in central domain. In addition, O(34) and O (35) in muramic acid moiety of UNAM also interacts with the Oδ of Asp228 present in a loop connecting β10 and α10 present in central domain. The pyrophosphate groups of UNAM interacts with Nζ of His124 via electrostatic and hydrogen bonding interactions. This His124 is located in a loop between β6 and α6 of the central domain. The positive charge of this histidine residue helps in binding the negatively charged pyrophosphates moiety. Oε of Glu359 is also forming hydrogen bonding interaction with the pyrophosphates moiety in UNAM. The docked pose of UNAM with MurC ligases was shown in Fig. 5

The uridine part of the product of MurC ligase UMG is inserted between the α11 in the central domain and α13 in the C-terminal domain. Nε of His295 present in the central domain, Oε of Glu359 and Oγ of Thr363 present in the C-terminal domain fixes this uridine via hydrogen bonding interactions. The negatively charged pyrophosphates of UMG form both hydrogen bonding and electrostatic interactions with the positively charged His355 present in the loop between β17 and α13 of the C-terminal domain. The hydroxyl group of N-acetyl glucosamine part of UMG stabilizes the complex through electrostatic and hydrogen bonding interactions with the Nε of His195 present in the loop between β9 and α9 of the C-terminal domain. The carboxylic acid of glycine were in contact with Gly149.O and Gly22.N. The residues of MurC ligase interacted with the substrate glycine and UNAM and the product UMG is given in Table 5.

Binding site of glutamic acid, UMG and UMGG in MurD

The carboxylic acid of glutamic acid interacts with both the O and Nζ atoms of Lys338 present in the loop between β14 and α14 of the C-terminal domain. The side chain carboxylic acid interacts with Thr340.Oγ present in the loop between β14 and α14 and Lys364.Nζ present in the loop between β15 and α15 of the C-terminal domain. The electrostatic interaction between the basic amino acid lysine and the acidic amino acid glutamic acid plays an important role in binding the substrate D-glutamic acid. The docked pose of glutamic acid with MurD ligases was shown in Fig. 6

The uridine part of UMG is inserted into the loop between β2 and α2 of N-terminal domain and bind with Asp38.O and Pro41.N. The hydroxyl group of the ribose sugar in UMG fixes itself in a loop region in between β2 and α2 of N-terminal domain. Ser77.Oγ plays an important

Table 5 Residues in *M. leprae* MurC ligase interacted with Glycine, UNAM and UMG

Glycine	UNAM	UMG
HIS295	THR189	GLY22
GLU359	HIS124	GLY149
ALA362	ASP228	HIS195
THR363	GLU359	HIS295
		HIS355
		GLU359
		THR363

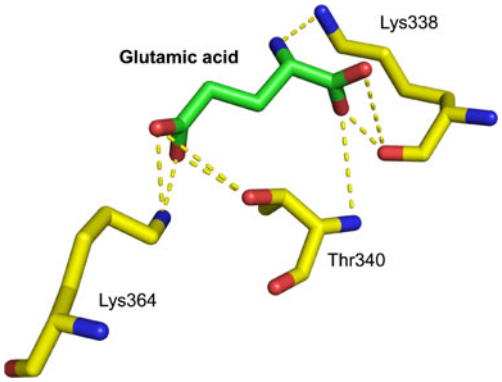

Fig. 6 The binding pose of *M. leprae* MurD ligase with Glutamic acid. The docked complex of *M. leprae* MurD ligase with its substrate glutamic acid was shown in stick representation using Pymol. For clarity only few of the important residues which bound with the enzymes were shown. The MurD ligase and glutamic acid molecule were in yellow and green color. The nitrogen, oxygen and phosphorous atoms were shown in blue, red and orange color

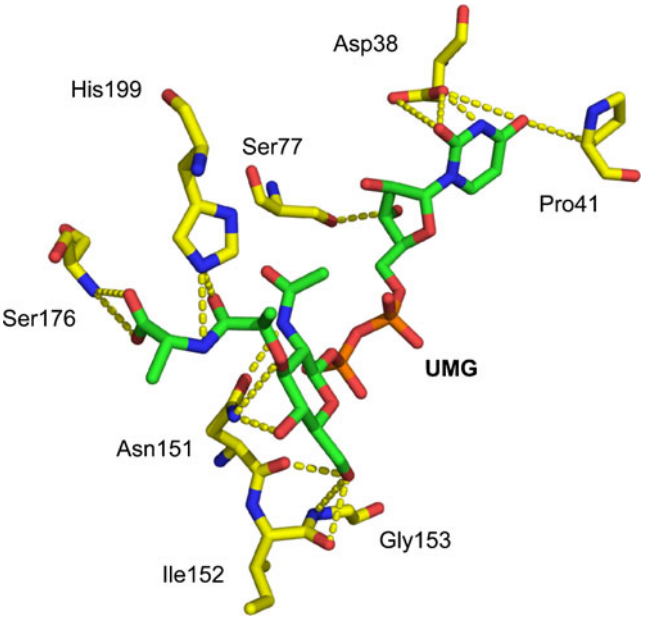

Fig. 7 The binding pose of *M. leprae* MurD ligase with UMG. The docked complex of *M. leprae* MurD ligase with its substrate UMG was shown in stick representation using Pymol. For clarity only few of the important residues which bound with the enzymes were shown. The MurD ligase and UMG molecule were in yellow and green color. The nitrogen, oxygen and phosphorous atoms were shown in blue, red and orange color

role in fixation. The oxygen atoms in the glucose present in UMG is compactly located in a loop region which connects β5 and β6 of the central domain. Oxygen and Nδ of Asn151 in this region forms a greater number of interactions with the many oxygen atoms of glucose units in UMG. These interacting oxygen atoms were also found to form interactions with Ile151 and Gly153. In addition to the above mentioned interactions, glycine present in the growing nucleotide was found to form electrostatic interactions with Ser176.N in α7 and His199.Nε present in a loop connects β7 and α8 in the central domain. The docked pose of UMG with MurD ligases was shown in Fig. 7

The glutamic acid part of UMGG is inserted in between the central and C-terminal domain. The main interactions include the electrostatic interactions with the nitrogen atoms present in Pro285 and Val286 in the central domain and the hydrogen bonding interactions with Ser348 in the C-terminal domain. Ala322 present in the loop connecting α13 and β13 and Val324 present in the β14 in C-terminal domain bind the glucose of UMGG via hydrogen bonding and ionic interactions. The interaction of negatively charged pyrophosphates with positively charged Arg332 is significant in UMGG binding. Glycine in UMGG binds with Ala345 present in β14 via hydrogen bonding and ionic interactions. The residues of MurD ligase interacted with the substrate glutamic acid and UMG and the product UMGG is given in Table 6.

Binding site of meso-A_2pm, UMGG and UMT in MurE

The binding site of the substrates Meso-A_2pm and UMGG and the product UMT in MurE were discussed in detail in our recent publication [14]. The residues that exactly bind with ligands and stabilize the complex through hydrogen bonding interactions were given in Table 7.

Binding site of D-alanyl-D-alanine, UMT and UMPP in MurF

Leu213 and Val215 present in the loop connecting β8 with α8 are significant in binding D-alanyl-D-alanine. The hydrogen bonding interactions together with the electrostatic interactions between Leu213.O with the nitrogen present in the peptide bond and Val215.N with the O(10) present in one of the alanine of the dipeptide stabilizes the docked complex. Asn249.Oδ present in β9 interacts with

Table 6 Residues in *M. leprae* MurD ligase interacted with glutamic acid, UMG and UMGG

D-glutamic acid	UMG	UMGG
LYS338	ASP38	PRO285
THR340	PRO41	VAL286
LYS364	SER77	ALA322
	ASN151	VAL324
	ILE152	ARG332
	GLY153	ALA345
	SER176	SER348
	HIS199	

Table 7 Residues in *M. leprae* MurE ligase interacted with meso-A_2pm, UMGG and UMT [20]

UMGG	MesoA2PM	UMT
ARG53	GLY142	ALA69
ILE167	LYS143	LYS143
PHE179	THR144	THR144
LEU180	ARG363	HIS210
ARG496	ALA385	HIS234
GLU492		ASP378
		ARG410

both O(10) and O(11) of this alanine respectively. Asp252. Oδ present in α9 of the central domain formed a hydrogen bonding interaction with O(10) of this alanine. The nitrogen atom of one more alanine present in the dipeptide interacts with Ser140.O and Gly141.N present in the loop between β6 with α5. The docked pose of D-alanyl-D-alanine with MurF ligases was shown in Fig. 8

The UMT binds in the cavity between the central and the C-terminal domain. The uridine, N-acetyl glucosamine (GlcNAc), ribose, pyrophosphate and glycine part of UMT fixes itself with the amino acids in the central domain. While the diaminopimelic acid (meso-A2pm) in UMT binds with the amino acids in the C-terminal domain. The oxygen and the nitrogen atoms of Asn167 present in the loop connecting α5 with α6 interact with the GlcNAc part of UMT. Arg193.N, Arg193.Nε, Arg.194.N, Ser191. Oγ and Ala192.O present in the loop between β7 and α7 also stabilizes the fixation of the GlcNAc part of UMT. Arg193.O in this loop and Lys233.Nζ present in α8 forms hydrogen bonding interactions with the pyrophosphate moiety of UMT. Together with this the electrostatic interaction between the basic amino acid lysine and the negatively charged pyrophosphate is significant in this

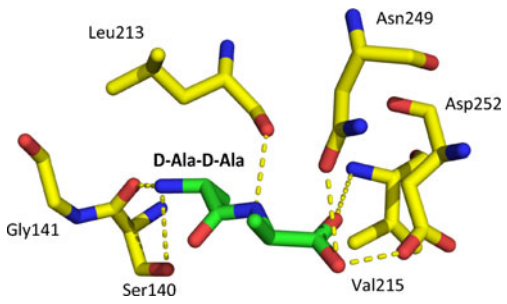

Fig. 8 The binding pose of *M. leprae* MurF ligase with D-Alanyl-D-Alanine. The docked complex of *M. leprae* MurF ligase with its substrate D-Alanyl-D-Alanine was shown in stick representation using Pymol. For clarity only few of the important residues which bound with the enzymes were shown. The MurF ligase and D-Alanyl-D-Alanine molecule were in yellow and green color. The nitrogen, oxygen and phosphorous atoms were shown in blue, red and orange color

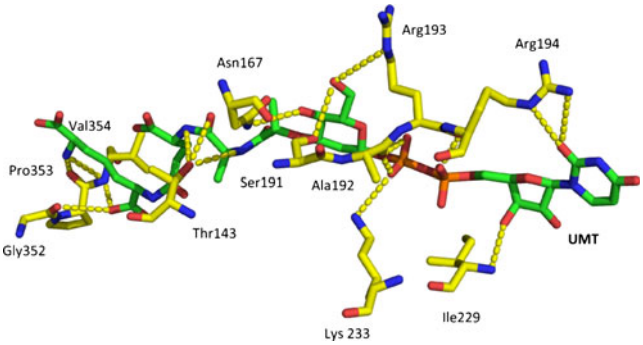

Fig. 9 The binding pose of *M. leprae* MurF ligase with UMT. The docked complex of *M. leprae* MurF ligase with its substrate UMT was shown in stick representation using Pymol. For clarity only few of the important residues which bound with the enzymes were shown. The MurF ligase and UMT molecule were in yellow and green color. The nitrogen, oxygen and phosphorous atoms were shown in blue, red and orange color

UMT binding. The uridine part of UMT is also inserted in the loop region between β7 and α7. The Arg194.Nε present in this loop forms ionic interaction with O(7) of uridine. The glycine in UMT binds with Thr143.Oγ. The O (15) of ribose sugar readily binds with Ile229.N through hydrogen bonding and electrostatic interactions. The O(65) of A_2pm binds with Pro353.N and Val354.N. While N(65) of A_2pm binds with Gly352.O in MurF ligase. The docked pose of UMT with MurF ligases was shown in Fig. 9

The A_2pm of the product UMPP binds with Thr143.Oγ present in α8 in the central domain. The D-alanyl-D-alanine part of UMPP is inserted into the loop region connecting α5 and α6. It forms various interactions with Ser165.Oγ, Asn167.Oδ and Asn168.Oδ. O(8) of uridine part of UMPP form ionic interactions with both the δ and ε

Table 8 Residues in *M. leprae* MurF ligase interacted with D-alanyl-D-alanine, UMT and UMPP

D-Ala-D-Ala	UMT	UMPP
SER140	THR143	THR143
GLY141	ASN167	SER165
LEU213	SER191	ASN167
VAL215	ALA192	ASN168
ASN249	ARG193	ARG297
ASP252	ARG194	TYR319
	ILE229	GLY320
	LYS233	ASP321
	GLY352	HIS322
	PRO353	GLN323
	VAL354	ARG358
		MET359
		GLN387
		ALA388

Fig. 10 The multiple sequence alignment of *M. leprae* MurC, MurD, MurE and MurF ligases. The *M. leprae* MurC, MurD, MurE and MurF sequences were aligned using ClustalW program. The residues were color coded based on ClustalW. The conserved binding residues which should be considered for multi targeted therapy were indicated with red color boxes

nitrogen of Arg297 in the loop between β11 and β12. Thr319.O interacts with both O(23) and O(33) of the pyrophosphate of UMPP. Similarly Gly320.N interacts with O(19) and O(23) of the pyrophosphate of UMPP. These Thr319 and Gly320 were located in the loop connecting β13 and α10 in the central domain. The electrostatic interactions formed together with the hydrogen bonding interactions by Asp321.N and His322.Nε was significant in pyrophosphate binding. Gln323.Nε binds the glucose part of UMPP. Met359.O in loop between α11 and β13 binds the GlcNAc part of UMPP. Gln187.O, Ala388.O and Ala388.N bind the ribose part of UMPP. The residues of MurF ligase interacted with the substrate D-alanyl-D-alanine and UMT and the product UMPP is given in Table 8.

The interactions of Thr127, Lys126, Gly125, Thr128 and Arg330 of *M. leprae* MurC with ATP molecule, Lys126, His195, Tyr350, His352, His385, His386 and Arg390 with

Table 9 Significant common binding residues of Mur enzymes in peptidoglycan pathway for multi targeted therapy

Enzyme	Residue	Functional importance
MurC	Lys126	ATP binding residue
	Thr127	ATP binding residue
MurD	Gly127	ATP binding residue
	Thr130	ATP binding residue
MurE	Gly142	ATP binding residue
		meso-A2pm binding residue
	Lys143	ATP binding residue
		meso-A2pm binding residue
		UMT binding residue
	Thr144	ATP binding residue
		meso-A2pm binding residue
		UMT binding residue
MurF	Gly141	D-Ala-D-Ala binding residue
	Thr143	UMT binding residue
		UMPP binding residue

UMG have equivalent interactions in the crystal structures. Similarly, the interactions of Thr19, Gly79 and Asn151 of *M. leprae* MurD with UMG, Asn126, Gly127, Thr130, Asp336 and Lys338 with ATP, His198, Arg389 and Ser467 with D-Glutamic acid also have equivalent interactions in the crystal structures. In the same manner, the interactions of Tyr75, Thr181, Ser208 and Arg215 of *M. leprae* MurE ligase with UMT, Leu52, Arg53, Ala54, Thr71, Thr181, Glu184, Ser208, His234 and Arg366 with UMGG and His233, His380, Tyr378, Gly407, Arg410, Asp433, Asn434, Arg436, Gly487 and Glu491 with mesoA_2pm have equivalent interactions in the crystal structures. These showed that the conformations of the Mur ligases developed were reliable and the predicted binding features can be useful for rational designing of a novel antibacterial agent.

Significant binding residues for multi targeted therapy

We performed topological analysis and confirmed that the binding sites are in similar locations between different species of Mur family. The 3D structure of *M. leprae* MurC was superimposed upon the crystalline MurC structure 1P31 and MurD with 2UAG, 2JFF and MurE with 1E8C, 2WTZ using Dalilite server. The superimposed structures revealed that all binding residues in each of *M. leprae* MurC, MurD, MurE and MurF ligases were in a topologically similar location among the different species of Mur family except Ala345 and Ser348 from MurD and Arg297 from MurF ligases. This is also verified by superposing the 3D structures in Pymol using the command 'align'.

To find the significant binding resides for multi targeted therapy, the residues conserved among MurC to MurF ligases in *M. leprae* were predicted by sequence alignment method using ClustalW program (Fig. 10) to design a multi target inhibitor. The residues which exactly bind with any of the substrates, products or the co-factors specified in Tables 4, 5, 6, 7, 8 were analyzed for their conservation among the Mur ligases by referring to the sequence alignment file. This results in the residues Lys126, Thr127 and Asp228 in MurC, Gly127 and Thr130 in MurD, Gly142, Lys143 and Thr144 in MurE and Gly141, Thr143 and Gly320 in MurF as the conserved binding residues. Their conservation indicated that they were very important for the catalytic activity of the enzymes. Among these residues the residues Lys126 and Thr127 in MurC, Gly127 and Thr130 in MurD, Gly142, Lys143 and Thr144 in MurE and Gly141 and Thr143 in MurF were the

Table 10 Amino acids in Mur ligases which destabilizes the structure upon site directed mutagenesis

Name of the enzyme	Mutation site	Amino acids destabilizes the structure
MurC	Ly126	Gly, Pro, Ser, Thr, Gln, Asn, Glu, Asp, Arg, His
	Thr127	Gly, Pro, Ser, Gln, Lys, Asn, Arg, His
	Asp228	Gly, Ala, Val, Pro, Trp, Ser, Thr, Phe, Gln, Lys, Tyr, Asn, Cys, Glu, Arg, His
MurD	Gly127	Ala, Val, Leu, Ile, Met, Pro, Trp, Ser, Thr, Phe, Gln, Asn, Cys, Glu, Asp, Arg, His
	Thr130	Met, Trp, Ser, Gln, Lys, Tyr, Asn, Glu, Asp, Arg, His
MurE	GLy142	Ala, Val, Leu, Ile, Met, Pro, Trp, Ser, Thr, Gln, Lys, Tyr, Asn, Glu, Asp, Arg, His
	Lys143	Gly, Pro, Ser, Thr, Gln, Asn, Glu, Asp, Arg, His
	Thr144	Pro, Trp, Ser, Gln, Lys, Tyr, Asn, Glu, Arg, His
MurF	Gly141	Pro, Trp, Asn, Cys, Glu, Asp
	Thr143	Gly, Ala, Pro, Trp, Ser, Phe, Gln, Tyr, Asn, Cys, Glu, Asp, His

conserved sequence motifs significant for ATP binding. The results are summarized in Table 9. The importance of these residues was predicted by insilico site directed mutagenesis studies by CUPSAT server. Upon mutation these residues destabilize the structure. The details of the site directed mutagenesis studies on *M. leprae* Mur ligases was shown in Table 10. This showed that these residues were crucial for the Mur ligases. Hence these are the probable residues for structure based drug designing. If these residues were targeted while designing a novel antibacterial agent, such a drug can easily target MurC, MurE and MurF ligases important for the peptidoglycan biosynthesis of bacterial cell wall. As this novel agent targets more than one enzyme at a time, the bacterium could not be able to develop resistance easily.

Conclusions

MurC, MurD, MurE and MurF ligases essential for the biosynthesis of peptidoglycan biosynthesis are the well validated bacterial targets. As the peptidoglycan is an important component of most of the eubacteria, targeting these enzymes will result in a better anti bacterial agent. Understanding the structural basis and binding specificity of an enzyme is of crucial importance while designing an inhibitor. Hence in this study, we generated the structural model for *M. leprae* MurC, MurD, MurE and MurF ligases using homology modeling and were validated. The binding features of these Mur ligases with their substrates, products and co-factors were also studied using docking. The conserved sequence motifs significant for ATP binding were predicted as the probable residues for structure based drug designing. As these Mur ligases are absent in human, the binding features predicted here would be helpful for the rational development of novel drugs specific against bacterial human pathogens of public health importance. The present work forms the basis for further molecular studies on these Mur ligases while designing a multi target inhibitor.

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