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RESEARCH ARTICLE

Essential residues for the enzyme activity of ATP-dependent MurE ligase from *Mycobacterium tuberculosis*

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ABSTRACT

The emergence of total drug-resistant tuberculosis (TDR-TB) has made the discovery of new therapies for tuberculosis urgent. The cytoplasmic enzymes of peptidoglycan biosynthesis have generated renewed interest as attractive targets for the development of new anti-mycobacterials. One of the cytoplasmic enzymes, uridine diphosphate (UDP)-MurNAc-tripeptide ligase (MurE), catalyses the addition of meso-diaminopimelic acid (*m*-DAP) into peptidoglycan in *Mycobacterium tuberculosis* coupled to the hydrolysis of ATP. Mutants of *M. tuberculosis* MurE were generated by replacing K157, E220, D392, R451 with alanine and N449 with aspartate, and truncating the first 24 amino acid residues at the N-terminus of the enzyme. Analysis of the specific activity of these proteins suggested that apart from the 24 N-terminal residues, the other mutated residues are essential for catalysis. Variations in K_m values for one or more substrates were observed for all mutants, except the N-terminal truncation mutant, indicating that these residues are involved in binding substrates and form part of the active site structure. These mutant proteins were also tested for their specificity for a wide range of substrates. Interestingly, the mutations K157A, E220A and D392A showed hydrolysis of ATP uncoupled from catalysis. The ATP hydrolysis rate was enhanced by at least partial occupation of the uridine nucleotide dipeptide binding site. This study provides an insight into the residues essential for the catalytic activity and substrate binding of the ATP-dependent MurE ligase. Since ATP-dependent MurE ligase is a novel drug target, the understanding of its function may lead to development of novel inhibitors against resistant forms of *M. tuberculosis*.

KEYWORDS peptidoglycan, MurE ligase, site-directed mutagenesis, *m*-DAP

INTRODUCTION

Tuberculosis is an increasing global health problem. The emergence of totally drug-resistant (TDR) strains of *Mycobacterium tuberculosis* poses a serious challenge, requiring the discovery of new anti-TB drugs against these strains with potential novel mechanisms of action (Velayati et al., 2009). Peptidoglycan, part of the cell envelope of *M. tuberculosis*, plays a major role in preserving the structural integrity of the cell by withstanding the high internal osmotic pressure. It is a giant macromolecule located on the outside of the cytoplasmic membrane in most of eubacteria (Schleifer and Kandler, 1972). In *M. tuberculosis*, the peptidoglycan consists of linear glycan chains with alternating units of *N*-acetyl-α-D-glucosamine (GlcNAc) and *N*-acetyl/*N*-glycolyl muramic acid (MurNAc/MurNGl) substituted with a peptide side-chain that may be cross-linked to peptide of other glycan strands (Mahapatra et al., 2005a). Due to their absence from animal cells, peptidoglycan biosynthesis enzymes are attractive targets for antimicrobials.

Peptidoglycan biosynthesis is a complex process involving

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three major steps. In the first step, MurA and subsequently MurB convert UDP-GlcNAc to UDP-MurNAc. The next step is catalyzed by four ATP-dependent Mur ligases, MurC, MurD, MurE and MurF which catalyze the addition of L-ala, D-glu, *m*-DAP/L-lys and D-ala-D-ala sequentially with the hydrolysis of ATP. These ligases share a common reaction mechanism (Barreteau et al., 2008).

From a multiple sequence alignment of MurC, MurD, MurE and MurF ligases from *E. coli*, conserved residues were identified, which suggested that these genes evolved from a common ancestral gene (Ikeda et al., 1990). Ten invariant amino acids, including 3 residues in the ATP binding consensus sequence GxxGKT/G were recognized by comparing amino acid sequences of 20 Mur synthetases including MurC, D, E, F, Mpl, folylpoly-γ-L-glutamate synthetase (FPGS or FolC) from several bacteria, the C-terminal domain of cyanophycin synthetase (CphA) from *Synechocystis* sp. and the poly-γ-glutamate synthetase (CapB) from *Bacillus anthracis* (Bouhss et al., 1997; Bouhss et al., 1999). To evaluate the roles of these conserved residues in catalysis and binding of the substrates in the Mur superfamily, site-directed mutagenesis of these invariants were studied using MurC and MurD from *E. coli* (Bouhss et al., 1997; Bouhss et al., 1999). This study was facilitated by the availability of 3-D structural information for MurD in the presence of ligands (Bertrand et al., 1997; Bertrand et al., 1999) and these invariants were found to be present in the active site cleft of MurD as observed in its 3-D structure. Similar results showing lack of activity with mutants of some of these invariant residues were reported for *E. coli* MurF (Eveland et al., 1997).

From the alignment of a large number of MurE sequences, a short sequence was identified as having two alternative conserved motifs characterized by the tetrapeptide sequences DNPR (*m*-DAP recognition) and D(D/N)P(N/A) (L-lys recognition) (Dementin et al., 2001). *Thermatoga maritima* MurE, which was shown to incorporate L-lys to UDP-*N*-acetylmuramoyl dipeptide and also to add D-lys and *m*-DAP at 25% and 10% efficiencies respectively relative to L-lysine, has the tetrapeptide sequence DDPR (Boniface et al., 2006). *Staphylococcus aureus* and *Streptococcus pneumoniae* MurE, which incorporate L-lys to UDP-*N*-acetylmuramoyl dipeptide, have DNPA and DDPN as the tetrapeptide sequence respectively.

Construction and characterization of mutants play a major role in understanding the function of specific residues in enzyme activity and substrate binding. This information along with the three dimensional structure of the protein will help in the rational design of inhibitors. In this study, truncation of the first 24 N-terminal amino acids and site directed mutagenesis of some residues that are conserved in MurC, D, E, F, Mpl and FolC and some that are conserved in all MurE orthologs but not in all the other members of the superfamily were carried out in order to investigate their role in *M. tuberculosis* MurE (Mtb-MurE).

RESULTS

Conservation of active site amino acid residues across the bacterial MurE

The sequences of MurC, D, E and F from *E. coli* and MurE from *T. maritima*, *S. aureus* and *S. pneumoniae* were aligned with the MurE of *M. tuberculosis* using the ClustalW program and the final alignment was optimized manually (Fig. 1). The residues in boldface type can be divided into two categories. In the first category are the residues invariant across Mur synthetases, which are similar to those observed in studies on MurC and MurD (Bouhss et al., 1997, 1999). These are D71, K157 belonging to the ATP binding motif GXXGKT/S, E220, N243, H248, N344, N347, R377 and D392, numbered as per Mtb-MurE. Interestingly, N344, which was found to be conserved among the sequences aligned in this study, was not considered an invariant residue when MurD sequences were aligned (Bouhss et al., 1999) but was identified as an invariant residue when MurC sequences were aligned (Bouhss et al., 1997). N243 was identified as a further conserved residue during this alignment. In the second category, the DNPR motif conserved in all *m*-DAP adding enzymes, but not in all the other members of the Mur family, were looked at. The variation in this motif changes the substrate specificity from *m*-DAP to L-lysine in *S. aureus* and *S. pneumoniae* MurE. Intriguingly, *T. maritima* MurE adds D-lys and *m*-DAP in addition to L-lys although less efficiently.

Generation and overexpression of Mtb-MurE mutants

Mutagenesis of Mtb-MurE resulted in the replacement of specific residue N449 by aspartate, R451, K157, E220 and D392 residues by alanine; and the truncation of the first 24 N-terminal amino acid residues. Analysis of the purified proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A) revealed that all the six mutant MurE proteins were overproduced to the same high level as the wild-type MurE and were stable in the host strain without significant proteolysis. Under optimum protein expression conditions around 3–5 mg of protein was purified from one liter of *E. coli* culture.

Enzymatic properties of the MurE mutants

The wild type and mutant proteins were characterized in terms of their substrate binding, catalysis and substrate specificity using an ATPase activity assay. Initial velocity was measured in all the experiments to ensure the inorganic phosphate formed as a result of hydrolysis of ATP was well within the linear response of the reaction.

Specific activity for the mutant proteins using optimized conditions was found to be 0.03, 0.07, 0.11, 0.16 and 0.07 μmoles/min/mg for N449D, R451A, K157A, E220A and

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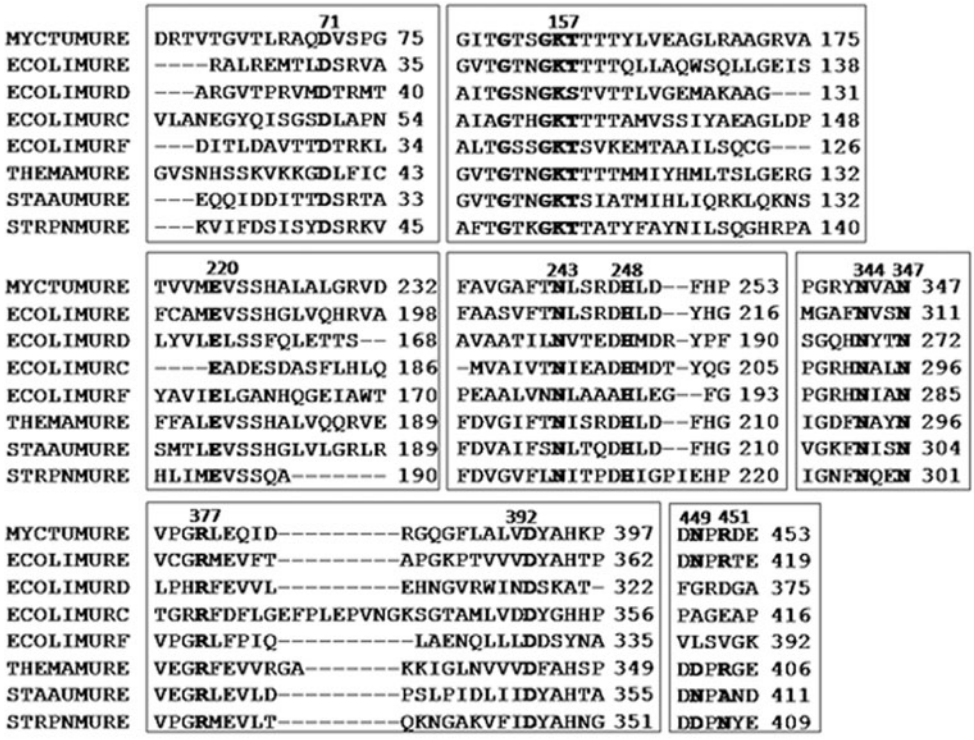

Figure 1. Multiple sequence alignment of MurE protein. MurE from *M. tuberculosis* (MYCTU) was aligned with other homologous proteins from *T. maritima* (THEMA) (28%), *S. aureus* (STAAU) (25%), *S. pneumoniae* (STRPN) (25%), and *E. coli* (ECOLI) (37%) and similarly sequence alignment with other Mur ligases from *E. coli* MurD (16%), MurC (17%) and MurF (23%). Only the clusters containing the conserved amino acid residues and the DNPR motif (in bold) for *m*-DAP adding and L-lys adding MurE of different organisms are shown. Percentage similarity is mentioned in parenthesis. The numbering refers to Mtb-MurE. The alignment function was performed by ClustalW. Hyphens represent a gap in the sequence.

D392A respectively (Fig. 2B) while the wild type showed 1.05 μmoles/min/mg. The specific activity of the mutants was less than 15% of wild type, which nevertheless was still detectable above background. Deletion of the first 24 residues at the N terminus of MurE (MurEtr) did not lead to any significant variation in its activity compared to the wild type. The specific activity obtained for MurEtr was 0.9 μmoles/min/mg protein.

Effect on kinetic parameters

Table 1 shows the kinetic parameters for endogenous substrates. Interestingly all the mutations have led to significant variations in apparent K_m values for more than one substrate. For N449D, 4 fold increases in K_m values were observed for ATP and *m*-DAP whereas a decrease was observed for UDP-MurNAc-L-ala-D-glu. Although there was no significant difference in K_m value of ATP for R451A, the K_m value for *m*-DAP was much larger and that for UDP-MurNAc-L-ala-D-glu slightly lower. For K157A, a large variation in the K_m values for UDP-MurNAc-L-ala-D-glu and *m*-DAP was observed. A major increase was observed for E220A and D392A in their K_m values for ATP with similar increase for UDP-MurNAc-L-ala-D-glu only with the E220A. No significant variations in K_m for any of the three substrates, V_{max} and apparent K_{cat} values were observed in case of MurEtr as compared to the wild type. A decrease in V_{max} was observed for all the other mutants. This is in agreement with the calculated apparent K_{cat} showing more than 10-fold decrease with all single residue replacement mutants except E220A, which showed about a 2 fold decrease.

Substrate specificity of mutant proteins

To better understand the interactions with mutated residues and the influence of the mutation on the substrate specificity, experiments with various analogs of each substrate were carried out (Tables 2–4). Interestingly wild type showed significant activity with DL-lanthionine similar to that of *m*-DAP. N449D and MurEtr showed similar results to the wild type with all the nucleotides, UDP-MurNAc peptides and amino acids, except D-lys, which showed a slightly higher activity for N449D. A significant activity was observed in the presence of L-lys, D-lys, DL-ornithine and D-glu for R451A

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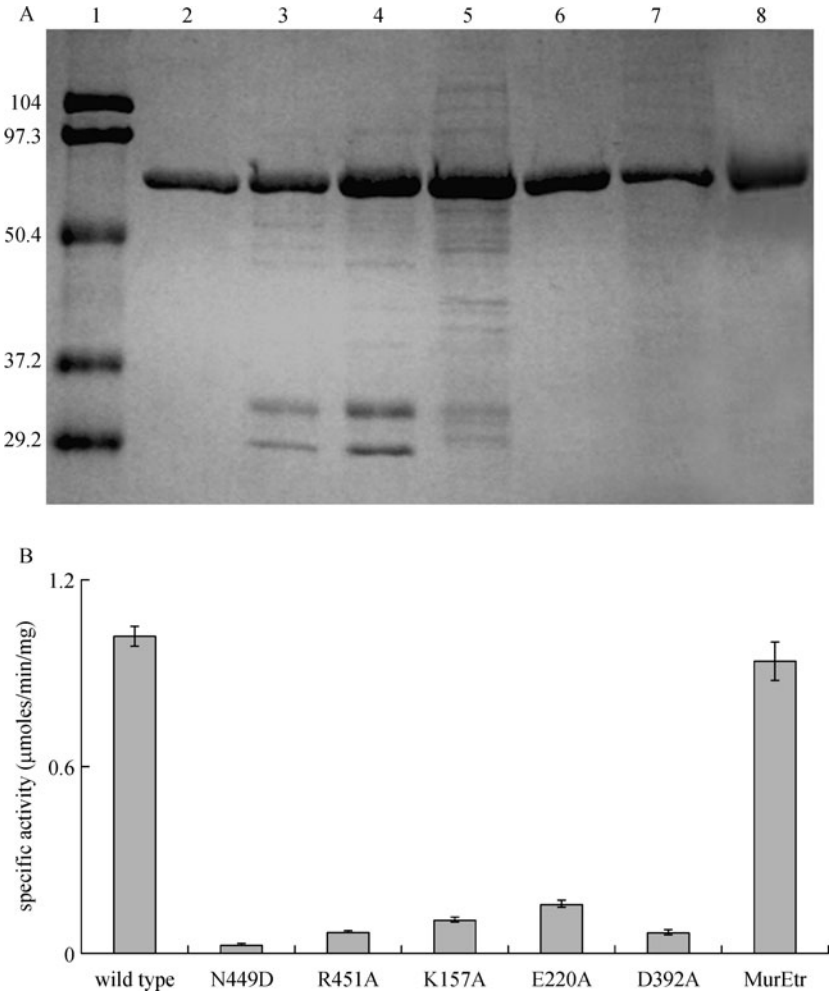

Figure 2. Comparison of wild type and mutant MurE from *M. tuberculosis*. (A) SDS-PAGE of purified fractions showing lane 1: protein molecular mass markers, individual molecular mass in kDa is indicated to the left; lane 2: wild type; lane 3: N449D; lane 4: R451A; lane 5: K157A; lane 6: E220A; lane 7: D392A; lane 8: MurEtr. In all cases 4–6 μg of protein loaded on the gel. (B) Comparison of specific activity of wild type MurE with mutant proteins. The reaction consisted of Bis-tris-propane-HCl (pH 8.5), $MgCl_2$, ATP, UDP-MurNAc dipeptide, *m*-DAP and enzymes at the concentration described in the experimental procedures. In all cases, enzymes were omitted in the control reactions. Y-axis represents the amount of Pi released in μmoles/min/mg of protein. Error bars indicate the standard deviation. SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Table 1 Kinetic parameters of wild type and mutant MurE proteins for endogenous substrates

protein	app K_m^{ATP} (mM)	app K_m^{UMAG} (mM)	app K_m^{m-DAP} (mM)	app V_{max} (nmol/min) [mg of protein]$^{-1}$	app K_{cat} (min^{-1})
wild type	0.017 ± 0.0007	0.024 ± 0.0015	0.078 ± 0.004	790 ± 28	78 ± 2
N449D	0.060 ± 0.005	0.007 ± 0.0003	0.324 ± 0.001	69 ± 3	3 ± 0.2
R451A	0.015 ± 0.001	0.005 ± 0.0001	0.473 ± 0.005	71 ± 4	4 ± 0.1
K157A	0.008 ± 0.0006	< 0.005	0.012 ± 0.001	90 ± 7	5 ± 0.4
E220A	0.123 ± 0.01	0.148 ± 0.005	0.052 ± 0.002	320 ± 20	34 ± 2
D392A	0.100 ± 0.008	0.016 ± 0.0006	0.113 ± 0.008	70 ± 7	6 ± 0.3
MurEtr	0.021 ± 0.004	0.019 ± 0.0022	0.065 ± 0.0016	709 ± 0.5	67 ± 1.2

app: apparent; UMAG: UDP-MurNAc-L-ala-D-glu

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Table 2 Substrate specificity of different amino acids with wild type and mutant proteins

	relative activity (%)[a]						
amino acid	wild type	N449D	R451A	K157A	E220A	D392A	MurEtr
m-DAP	100 ± 7.5	100 ± 6.8	100 ± 8.9	100 ± 9.2	100 ± 9.5	100 ± 5.6	100 ± 1.2
DL-lanthionine	82 ± 5.1	76 ± 4.3	78 ± 3.8	100 ± 8.7	83 ± 6.3	82 ± 4.5	97 ± 2.4
L-cystathionine	12 ± 0.6	0	34 ± 3	100 ± 9.1	100 ± 7.6	81 ± 5.7	3 ± 0.8
L-lys	7 ± 0.6	2 ± 0.12	45 ± 3.6	100 ± 8.6	100 ± 7.9	84 ± 6.8	9 ± 0.43
D-lys	9 ± 0.35	37 ± 3	62 ± 5	100 ± 6.9	100 ± 8.8	78 ± 6.1	7 ± 0.55
L-ala	12 ± 0.82	0	0	100 ± 8.6	100 ± 7.7	99 ± 6.8	3 ± 0.21
D-glu	13 ± 0.42	6 ± 0.4	100 ± 5.9	92 ± 6.8	95 ± 7.2	88 ± 5.8	3 ± 0.36
D-ala-D-ala	5 ± 0.3	1 ± 0.07	18 ± 1.8	100 ± 7.2	98 ± 8.3	88 ± 6.9	1 ± 0.67
Dl-ORNITHINE	8 ± 0.72	8 ± 0.56	8 ± 6.9	85 ± 7.2	90 ± 5.7	86 ± 7	2 ± 0.15

[a] Activity with *m*-DAP was used as 100% for each protein. *m*-DAP: meso-diaminopimelic acid.

Table 3 Substrate specificity of different uridine sugar precursors with wild type and mutant proteins

	relative activity (%)[a]						
nucleotides	wild type	N449D	R451A	K157A	E220A	D392A	MurEtr
ATP	100 ± 6.8	100 ± 8.1	100 ± 7.4	100 ± 6.7	100 ± 5.5	100 ± 4.8	100 ± 5.4
GTP	30 ± 2.5	0	17 ± 1.1	98 ± 3.8	100 ± 6.2	100 ± 7.7	30 ± 1.8
UTP	17 ± 1.5	0	5 ± 0.35	100 ± 6	92 ± 5.8	99 ± 6.8	5 ± 0.55
UTP	14 ± 0.98	0	0	83 ± 7.1	82 ± 5.7	96 ± 4.6	6 ± 0.21

[a] Activity with ATP was used as 100% for each protein.

Table 4 Substrate specificity of different nucleotides with wild type and mutant proteins

	relative activity (%)[a]						
UDP-MurNAc peptides	wild type	N449D	R451A	K157A	E220A	D392A	MurEtr
UDP-MurNAc dipeptide	100 ± 4.7	100 ± 6.9	100 ± 7.5	100 ± 5.8	100 ± 7.7	100 ± 4.7	100 ± 5.4
MurNAc	14 ± 0.87	14 ± 0.94	17 ± 0.77	98 ± 6.6	100 ± 5.8	100 ± 5.8	5 ± 0.68
UDP-MurNAc tripeptide	8 ± 0.73	20 ± 1.1	3 ± 0.2	96 ± 6.8	93 ± 5.7	98 ± 8.8	5 ± 0.37
UDP-MurNAc pentapeptide	15 ± 0.97	16 ± 0.88	18 ± 0.67	97 ± 7.4	99 ± 6.8	83 ± 4.6	4 ± 0.11

[a] Activity with UDP-MurNAc dipeptide was used as 100% for each protein.

with similar activities as wild type for all the nucleotides and UDP-MurNAc peptides tested. Surprisingly, K157A, E220A and D392A showed very high activity with all the amino acids, uridine sugar precursors and nucleotides tested compared to wild type and moreover, reaching up to 100% of the standard assay in many cases.

Effect of ATP, *m*-DAP and UDP-MurNAc-L-ala-D-glu on ATP hydrolysis

The substrate specificity experiments led us to investigate further the effect of ATP alone and in combination with meso-diaminopimelic acid (*m*-DAP) and UDP-MurNAc-L-ala-D-glu (Table 5). Wild type, N449D, R451A and MurEtr showed the lowest absorbance in the presence of only ATP and enzyme, which does not change much in the presence of *m*-DAP, whereas absorbance increased slightly in the presence of uridine sugar precursor similar to all substrates without enzyme. K157A, E220A and D392A showed significant ATP hydrolysis compared to wild type, N449D, R451A and MurEtr in the presence of only ATP and enzyme, in the absence of other substrates. This activity was higher than the control reaction but similar to that in the presence of *m*-DAP. The ATP hydrolysis activity further increased significantly in the presence of uridine sugar precursors to a similar level to that of the enzyme reaction with all the three substrates.

Crystal structure of MurE/ADP/dipeptide ternary complex

The ternary complex structure (PDB: 2xja) shows clear density for ADP with very little other difference from the MurE/dipeptide complex (PDB: 2wtz). The refinement statis-

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Table 5 Activity in the presence of different components of the MurE reaction with the wild type and mutant proteins

	relative absorbance (%)[a]						
different conditions	wild type	N449D	R451A	K157A	E220A	D392A	MurEtr
ATP + enzyme	29 ± 2.4	37 ± 3.6	39 ± 2.7	77 ± 4.1	66 ± 3.8	70 ± 4.2	19 ± 1.3
ATP + enzyme + UDP MurNAc dipeptide	52 ± 3.2	65 ± 4.2	64 ± 3.8	85 ± 4.4	89 ± 6.2	98 ± 7.3	36 ± 2.6
ATP + enzyme + *m*-DAP	28 ± 2.1	34 ± 2.7	38 ± 1.9	73 ± 4.5	66 ± 3.8	67 ± 2.4	21 ± 2.0
ATP + UDP MurNAc dipeptide + *m*-DAP[b]	48 ± 1.4	59 ± 2.5	58 ± 3.1	55 ± 1.9	62 ± 2.3	66 ± 3.2	38 ± 3.5
ATP + enzyme + UDP MurNAc dipeptide + *m*-DAP[c]	100 ± 5.4	100 ± 4.9	100 ± 5.2	100 ± 4.6	100 ± 6.2	100 ± 4.8	100 ± 6.6

[a] Activity with enzyme reaction was used as 100% for each protein.

[b] Control reaction mixture contains ATP, UDP-MurNAc dipeptide and *m*-DAP but no enzyme.

[c] Enzyme reaction mixture contains Mtb- MurE, ATP, UDP-MurNAc dipeptide and *m*-DAP.

tics are in Table 6. The refined structure is depicted in Figs. 3 and 4 showing binding of ADP in an analogous position to that in MurD.

Table 6 Data collection and refinement statistics

ligand combination	UMAG + ADP
space group	P1
chains per unit cell	4
completeness (%)	95.9
multiplicity	3.7
average R_{merge}	0.12
average I/σ	9.1
outer shell resolution (Å)	3.00–3.16
outer shell R_{merge}	0.57
outer shell I/σ	2.6
refined R factor	0.189
final R_{free}	0.258

DISCUSSION

Structural analysis of wild type Mtb-MurE, from our previous study, had revealed that the first 24 N-terminal amino acid residues may not be involved in the active site structure of MurE (Basavannacharya et al., 2010); hence, a truncated version of wild type MurE was generated. The truncated MurE mutant showed activities similar to the wild type MurE, providing further support that this deletion did not affect the activity nor the substrate specificity of the recombinant enzyme.

The *m*-DAP of the peptidoglycan monomer unit plays a pivotal role in the integrity of the peptidoglycan as it is directly involved in peptide cross-linkages (Glauner et al., 1988). *m*-DAP is used in various transpeptidation reactions during the later stages of peptidoglycan biosynthesis, which leads to the formation of D-ala-> *m*-DAP and *m*-DAP-> *m*-DAP cross linkages (Glauner et al., 1988; Nanninga, 1991). Interestingly, peptidoglycan of some bacterial species contain 3-hydroxy *m*-DAP or lanthionine as a natural substitute for *m*-DAP (Inoue et al., 1979; Vasstrand et al., 1980; Kawamoto et al., 1981). It has been shown that in *m*-DAP auxotrophs, L-allo-cystathionine (95%) and *m*-lanthionine (93%), sulfur containing *m*-DAP analogs, were incorporated into the peptidoglycan of *E. coli* in *in vivo* conditions. These are shown to be good substrates in addition to D-allo-cystathionine for the *E. coli* enzyme (Mengin-Lecreulx et al., 1994) concluding that MurE was the cystathionine and lanthionine adding enzyme *in vivo*. *Mycobacterium smegmatis* has been shown to incorporate lanthionine in *m*-DAP auxotrophs. Even though cells grow, they do not produce normal peptidoglycan. The replacement of *m*-DAP with lanthionine but not cystathionine suggested that the peptidoglycan biosynthesis in mycobacteria may have more constraints on peptide composition than in *E. coli* (Consaul et al., 2005). Interestingly, in the case of *M. tuberculosis* wild type MurE showed a similar activity with DL-lanthionine as *m*-DAP, but much lower activity with L-cystathionine or any of the other amino acids. This result is consistent with that observed in the mutant strains of *E. coli* (Mengin-Lecreulx et al., 1994) and *M. smegmatis* (Consaul et al., 2005) demonstrating MurE specificity for the meso isomer.

Asparagine 449, belonging to a well-known consensus DNPR motif (Gordon et al., 2001) conserved in *m*-DAP adding enzymes, was mutated to aspartate in N449D to investigate its role in substrate binding and incorporation of substrates, particularly *m*-DAP. The K_m values for *m*-DAP and ATP were increased by 4 fold whereas for UDP-MurNAc-L-ala-D-glu, there was no significant change. These results suggested that this residue is involved in *m*-DAP and ATP binding. Further evidence comes from the structure of *E. coli* MurE with the product complex (Gordon et al., 2001), where the amino group of amide side chain of Asn414 forms a hydrogen bond with the carboxylic group of *m*-DAP at the

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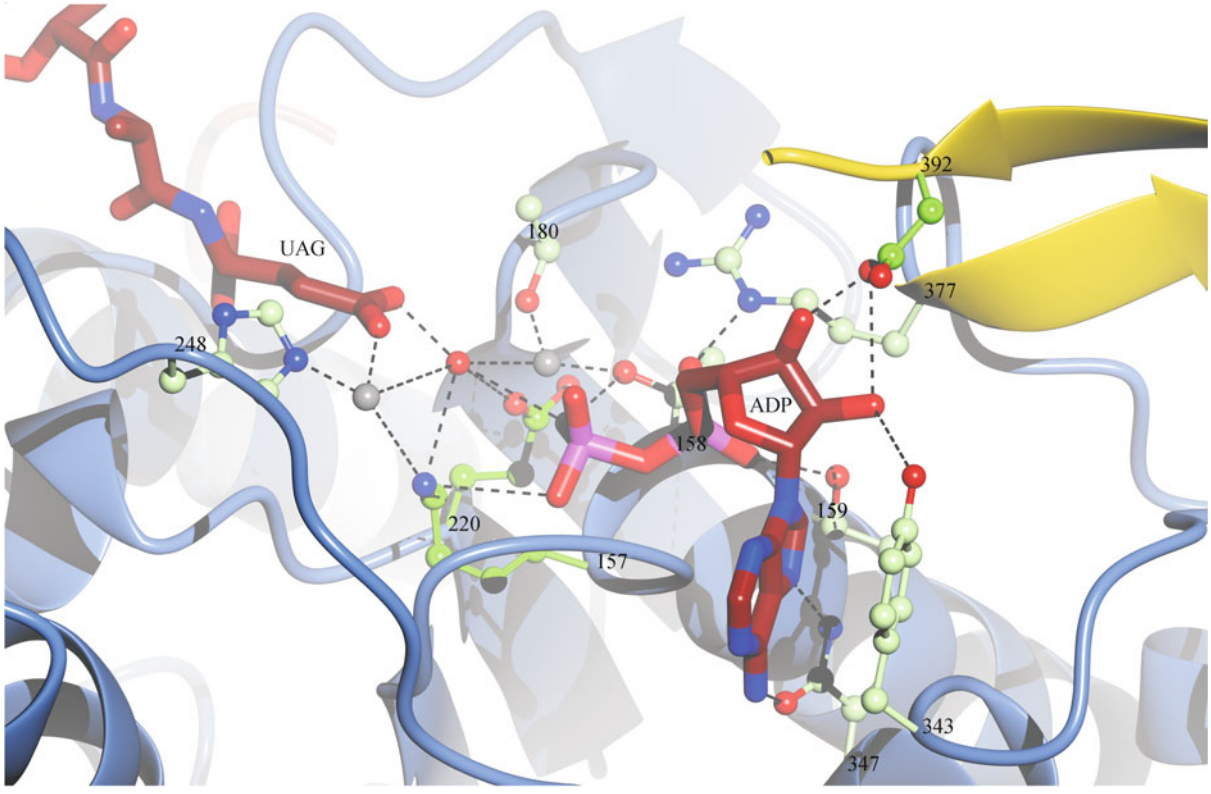

Figure 3. Three dimensional structure of Mtb-MurE/ADP/Mg^{2+} complex. A close up of the active site of *M. tuberculosis* MurE showing the 3 mutated residues close to ADP in dark green and other side chains interacting with ADP in light green labeled with residue numbers at C_{alpha}. ADP and UAG are shown as tan cylinders, magnesium as gray spheres and a highly ordered water as a red sphere. Hydrogen bonds are shown as dotted lines. Domain 1 is in coral, domain 2 is in blue and domain 3 is in yellow and truncated at residue 394.

D-center (Fig. 5). This interaction gets disrupted when the equivalent Asn449 in Mtb-MurE was converted to aspartate and loses the affinity for the substrate. But how this mutation is involved in ATP binding is difficult to explain. A high degree of reduction, by 35 fold in the specific activity, was reflected by 26 fold decrease in the turnover rate. The N449D mutation results in DDPR motif present in *T. maritima*, a thermophilic bacterium which shows 25% and 10% efficiency of incorporation of D-lys and *m*-DAP relative to L-lys (Boniface et al., 2006). However, this mutation neither abolishes the incorporation of *m*-DAP, nor does it show any activity with L-lys. This is not surprising as the D-carbon (distal recognition site) of D-lys is involved in interaction with the residues (Glu468, Gly464, Asp413, Asn414 and Arg416) of *E. coli* MurE (Fig. 5) and the L-carbon gets acylated on its ε-amino group similar to *m*-DAP (Gordon et al., 2001). Similar observations were made in *E. coli in vivo* (Mengin-Lecreulx et al., 1994).

Another residue arginine 451, also part of the DNPR motif, is also conserved in organisms incorporating *m*-DAP at the third position of stem peptide of the UDP-MurNAc pentapeptide. This residue is believed to be responsible for determining the specificity of *m*-DAP incorporation into the peptidoglycan in *E. coli* (Gordon et al., 2001). The R451A mutant was made to investigate further its role in substrate specificity. There was a 6 fold increase observed in K_m value for *m*-DAP whereas a 5 fold decrease was seen for UDP-MurNAc-L-ala-D-glu and no difference for ATP. These results support the involvement of this residue in *m*-DAP incorporation. Evidence from the *E. coli* MurE product structure strongly supports this role for this residue. Two amino groups from guanidinium side chain of Arg416 are involved in hydrogen bonding with carboxylic group of *m*-DAP at the D center as well as at the L center (Fig. 5) (Gordon et al., 2001). The corresponding arginine at position 451 in Mtb-MurE loses these interactions when replaced by alanine, thereby showing a reduced affinity for *m*-DAP, but the negative role of this residue in UDP-MurNAc-L-ala-D-glu binding as seen by decrease in the K_m value is difficult to understand. R451A also showed loss of activity by 15-fold which agrees well with the 20-fold reduction in the turnover rate of this enzyme. The resulting mutation has the DNPA sequence which is present in *S. aureus*. This Gram-positive organism incorporates L-lys at the third position of the stem peptide in the peptidoglycan precursor. This mutation in MurE of *M. tuberculosis* did not abolish the incorporation of

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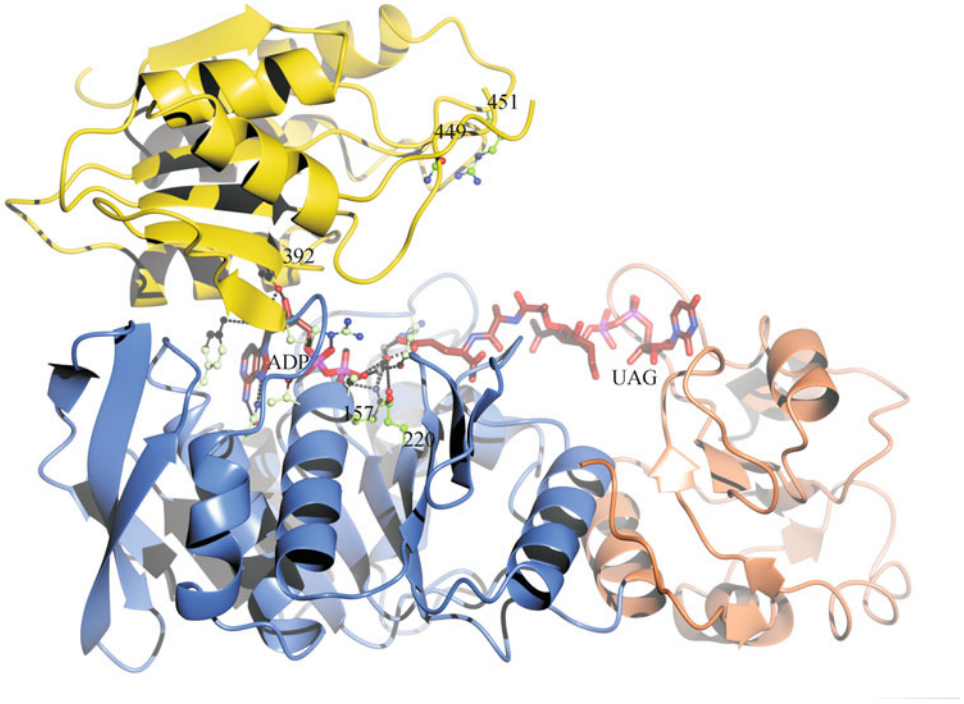

Figure 4. Location of the residues subjected to mutagenesis. Amino acid residues that were mutated are indicted by ball and stick side chains labeled by residue number in green, in the crystal structure of MurE of *M. tuberculosis* colored by domains (1 in coral, 2 in blue, and 3 in yellow). The ligands UAG and ADP are shown by tan cylinder models, and Mg^{2+} is shown as gray spheres.

m-DAP, instead additionally it showed significant activity with L-lys. It also showed substantial activity with DL-lanthionine, D-lys, D-glu and DL-ornithine.

Lysine at position 157 belongs to a well-known consensus ATP binding site (Walker et al., 1982; van der Wolk et al., 1995). Surprisingly, the K_m value of K157A for ATP was reduced 2 fold. In the three dimensional structure of Mtb-MurE/ADP/Mg^{2+} complex (Fig. 3), this lysine forms interacting bonds through its amino group with the β-phosphate of ADP similar to *E. coli* MurD at position 115 (Bertrand et al., 1999). It is clear from the structural data, that the interaction with ε-amino group is lost when lysine is replaced by alanine. The same mutation showed apparent stronger affinity for the other two substrates UDP-MurNAc-L-ala-D-glu and *m*-DAP indicated by their reduced K_m value. This is in contrast to the data observed for mutation of the equivalent residues in *E. coli* MurC and MurD which showed an increase in K_m for ATP and uridine sugar precursor (Bouhss et al., 1997; Bouhss et al., 1999). For K157A, the turnover rate was 16-fold lower than that of wild type with 10-fold reduction in its enzyme activity strongly suggesting its role in catalysis. This is in agreement with the results with MurC and MurD (Bouhss et al., 1997; Bouhss et al., 1999).

The K_m value for E220A was significantly increased by 7 fold for ATP giving a clue that this residue has a crucial role to play in ATP binding. This is predicted from the fact that this residue belongs to the second ATP binding motif (Walker et al., 1982). The residue is conserved across various ATP binding proteins including FolC (Bognar et al., 1987). This residue is generally involved in the chelation of the ATP-Mg^{2+} complexes (Walker et al., 1982; Glaser et al., 1991; Story and Steitz, 1992; Mitchell and Oliver, 1993). The Mtb-MurE/ADP/Mg^{2+} complex reveals that E220 is involved in interactions with Mg^{2+} which in turn binds ADP (Fig. 3) similar to the corresponding E157 in *E. coli* MurD binding to ATP through Mg^{2+} (Bertrand et al., 1999). When this interaction is disrupted by the mutation, the affinity of ATP binding is lowered. The K_m value for UDP-MurNAc-L-ala-D-glu was 6 fold higher whereas *m*-DAP showed an insignificant decrease in K_m value. A similar result showing a drastic increase in K_m for the uridine sugar precursor was observed for the analogous mutation of MurD in *E. coli* (Bouhss et al., 1999). E220A demonstrated a specific activity 6 fold less with 2 fold decrease in turnover rate. This is in contrast to the results obtained with *E. coli* MurC and MurD (Bouhss et al., 1997; Bouhss et al., 1999) and MurF (Eveland et al., 1997) where there was a greater loss of activity with these proteins caused by the analogous mutation.

In D392A where aspartate 392 is replaced by alanine, a 5 fold increase in the K_m value was observed for ATP whereas

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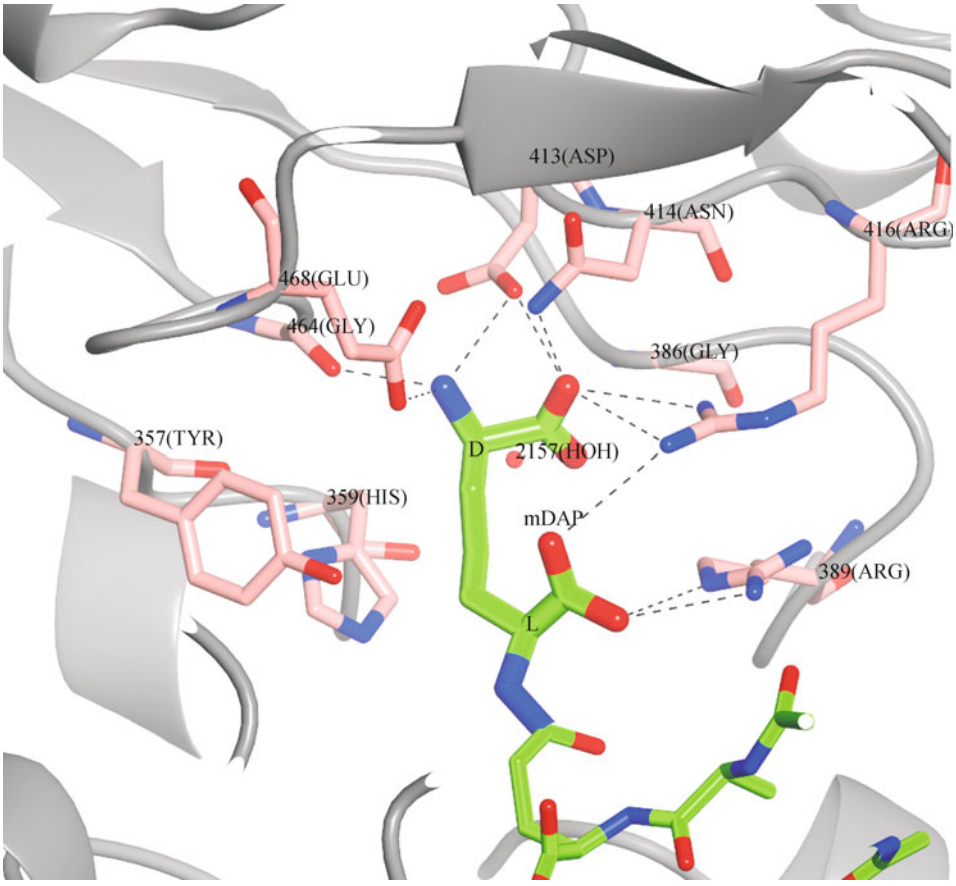

Figure 5. Structure of the *E. coli* MurE product (1e8c). The product UDP-N-acetylmuramoyl-L-alanyl-D-gamma-glutamyl-meso-2,6-diamino-heptanedioate (UMT) is shown in green and contacting side chains in pink. Hydrogen bonds are shown as dotted lines.

for other substrates, it was of the same order of magnitude as that of wild type. In the three dimensional structure of Mtb-MurE/ADP/Mg^{2+} complex (Fig. 3), this residue has been shown to make interactions with the ribose moiety of ADP through its carboxylate oxygens, similar to the equivalent residue Asp317 in *E. coli* MurD (Bertrand et al., 1999). When this residue is mutated to alanine the mutation results in loss of these interactions thereby losing the affinity for ATP. In MurD mutant of *E. coli*, a strong increase in the K_m value for ATP and uridine sugar precursor was detected (Bouhss et al., 1999). The D392 residue appears to play a crucial role in catalysis as there was a reduction in the specific activity and turnover number by 15-fold. This is in agreement with that obtained in analogous mutants of *E. coli* MurC and MurD (Bouhss et al., 1997; Bouhss et al., 1999).

K157A, E220A and D392A showed significant activity with all the amino acids, nucleotides and uridine sugars tested to a level comparable to that of *m*-DAP, ATP and UDP-MurNAc-L-ala-D-glu respectively, indicating that these mutations may have caused ATP hydrolysis to be uncoupled from the enzyme reaction.

Further investigation was done only in the presence of ATP in order to gain more understanding of the above results. The results found from this experiment were intriguing. There seem to be significant ATP hydrolysis taking place with K157A, E220A and D392A even in the absence of other substrates contrary to that of wild type, N449D, R451A and MurEtr. The hydrolysis rate of the mutants remained same after the addition of *m*-DAP, whereas it significantly increased in the presence of uridine sugar precursor. The binding of MurNAc, the common moiety in all the uridine sugars tested, seem to cause more ATP hydrolysis by an allosteric effect. The high rate of phosphate release with different amino acids, nucleotides and uridine sugar precursors with K157A, E220A and D392A is due to the uncoupled hydrolysis of ATP by the enzyme. ATPase contamination in the preparations of these mutants is unlikely as the purification was carried out under identical conditions for these mutants as that for wild type, N449D, R451A and MurEtr. The baseline reactions with only the mutant proteins did not show any phosphate contamination (Table 7). It is well established that the ATPase activity is coupled with ligase activity for the wild type protein

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Table 7 Absorbance of individual assay components at 635 nm

different conditions	$A_{635\,nm}$
ATP alone	0.53
UDP-MurNAc dipeptide alone	0.47
m-DAP alone	0.29
wild type	0.29
N449D	0.28
R451A	0.28
K157A	0.27
E220A	0.29
D392A	0.30
MurEtr	0.26

(Basavannacharya et al., 2010). Hence, the residues involved in these mutations are likely responsible for coupling the ATPase activity with the ligase activity.

The findings in this study contribute significantly to the understanding of the residues essential for catalytic activity, substrate binding and specificity of MurE of *M. tuberculosis*. In particular, it identifies the residues that couple hydrolysis of ATP to the reaction. These results along with the three dimensional structure of Mtb-MurE will aid in the development of inhibitors targeting the drug-resistant strains of *M. tuberculosis*.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

E. coli DH5α (Bioline, UK) and BL21 (DE3) (Promega Corporation) strains were used in this study. Oligonucleotide primers were synthesized by MWG Biotech, Germany. Restriction enzymes were obtained from New England Biolabs, UK. All other media and chemicals were purchased from Sigma unless otherwise mentioned. LB broth and agar media were used for growing cells. Growth was monitored by measuring the culture absorbance at 600 nm. Standard procedures for endonuclease digestion, ligation and agarose electrophoresis were used (Sambrook and Russell, 2001).

Generation of Mtb-MurE mutants

pET28b-*murE* (pSBC1), a plasmid harbouring the wild type *Mtb-murE* gene generated in a previous study (Basavannacharya et al., 2010) was used as the template for the mutagenic PCR. Site-directed mutagenesis was carried out using the QuikChange® Lightning site-directed mutagenesis kit (Strategene) according to the manufacturer's instructions. Truncation of the first 24 amino acid residues at the N-terminal end (72 base pairs) was based on similar principle as site-directed mutagenesis. All primers used in this mutagenesis study are described in Table 8. DNA sequence analysis was done to confirm that mutation has taken place at the desired positions. The plasmids with amino acid residue mutations were designated as pSBC1K157A, pSBC1E220A, pSBC1D392A, pSBC1N449D and pSBC1R451A, and that with the truncated MurE was termed as pSBC1Etr.

Expression and purification of wild type and mutant MurE proteins

The expression and purification of wild type and mutant proteins was carried out as described earlier (Basavannacharya et al., 2010), with slight modifications. On harvesting the cultures, the cell pellets were washed and resuspended in ice-cold buffer A containing 0.02 M Bis-tris propane·HCl (pH 8.5), 0.1 M NaCl and 0.03 M imidazole. Once the bound proteins were eluted from Ni^{2+}-NTA resin with 100 mM imidazole in buffer A, they were diluted in storage buffer [0.02 M Bis-tris propane·HCl (pH 8.5) and 0.1 M NaCl] and reconcentrated to remove imidazole. Protein concentration was estimated by Bradford assay (Bradford, 1976) using bovine serum albumin as a standard and analyzed by SDS-PAGE (Laemmli, 1970). Glycerol was added to a final concentration of 10% (*v/v*) and the proteins were stored in aliquots at −80°C. The resulting mutant proteins were designated as N449D, R451A, K157A, E220A, D392A and MurEtr.

MurE activity assay

MurE enzyme activity was assayed as described previously (Basavannacharya et al., 2010), by measuring the release of inorganic phosphate following ATP hydrolysis using Pi ColorLock Gold kit (Innova Biosciences). The enzyme activity assay was

Table 8 Mutagenic oligonucleotides

plasmid	mutation[a]	oligonucleotide[b]
pSBC1N449D	N449D	5'-GTCACCGACGAC**G**ACCCGCGTGACGAAG-3'
pSBC1R451A	R451A	5'-GACGACAACCCG**GC**TGACGAAGATCCCACG-3'
pSBC1K157A	K157A	5'-AACGTCCGGC**GC**GACCACCACCACCTATC-3'
pSBC1E220A	E220A	5'-ACACCGTGGTCATGG**C**GGTGTCCAGCCACG-3'
pSBC1D392A	D392A	5'-CGCTGGTCG**C**CTACGCGCAC-3'
pSBC1MurEtr	24 residues	5'-CCTGGTGCCGCGCGGCAGCCAggcttgcgccccaacgccgtc-3'[c]

[a] Amino acids are represented by their one-letter abbreviation and the number indicates the location of the mutated residue in the amino acid sequence of MurE from *M. tuberculosis*.

[b] Mutations of the *murE* gene sequence that have been introduced in the oligonucleotides are indicated in bold.

[c] Primers for truncation of N-terminal residues of *murE* with bases in upper and lower case representing sequence flanking the 72-base-pair region to be truncated.

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performed in a final volume of 50 µL in the presence of 25 mM Bis-tris propane·HCl pH 8.5, 5 mM $MgCl_2$, 250 µM ATP, 100 µM UDP-MurNAc dipeptide (UDP-MurNAc-L-ala-D-glu) and 1 mM *m*-DAP at 37°C. The absorbance was measured at 635 nm using FLUOstar Optima plate reader (BMG Labtech). Wild type MurE was used as a positive control in all the experiments for comparison with the mutant proteins. The conditions were kept constant to help compare the results across different mutants and wild type protein. Absorbance values were corrected for background absorbance of reaction mixtures and any non-enzymatic hydrolysis of ATP in the absence of the enzyme. In addition, individual components of the assay were checked with the assay reagents to monitor the background absorbance. A standard phosphate curve was generated using the inorganic phosphate provided along with the kit to calculate the specific activity. All assays were performed in triplicate. The specific activity of the protein is expressed as picomoles of inorganic phosphate released during the reaction per minute per milligram of the recombinant protein.

Protein dependence

Protein dependence was determined for all the mutant proteins in addition to wild type. Protein was diluted in 25 mM Bis-tris propane ·HCl (pH 8.5) according to the quantity to be used. The amount of the protein used for the assay ranged 25–200 ng for wild type, D392A and MurEtr, and 100–1200 ng for rest of the mutants.

Determination of the kinetic constants

For determination of the kinetic constants, the assay was done at various concentrations of one substrate and fixed concentration of the others. For ATP, UMAG and *m*-DAP, varying concentrations of ATP at 10–500 µM, UMAG at 5–250 µM and *m*-DAP at 25–2500 µM with fixed concentrations of 250 µM ATP, 100 µM UMAG and 1000 µM *m*-DAP respectively were used. The protein quantity used for all subsequent assays were 100 ng of wild type, 700 ng of N449D and K157A, 500 ng of R451A, 150 ng of E220A, 600 ng of D392A and 100 ng of MurEtr. Data was fitted to the Hanes' plot equation $[S]/v = 1/V^*_{max}[S] + K_m/V_{max}$ to calculate apparent K_m and V_{max} by linear regression.

Determination of substrate specificity

Different amino acids, nucleotides and MurNAc substitutes were used to determine the substrate specificity of mutants of MurE. 1000 µM *m*-DAP or other amino acids, 250 µM ATP or other nucleotides, 100 µM UDP-MurNAc-L-ala-D-glu or other MurNAc substitutes were added to the assay mix to initiate the reaction at 37°C for 30 min. The rest of the assay set up was as described earlier. In all cases, the experiment was done in triplicate and the standard deviations are indicated by error bars (n = 3).

Effect of ATP, *m*-DAP and UDP-MurNAc-L-ala-D-glu on ATP hydrolysis

Complete and control reactions were set up as described previously. Three reactions were set up, first with ATP and enzyme, second with ATP, UDP-MurNAc-L-ala-D-glu and enzyme, third with ATP, *m*-DAP and enzyme. The rest of the assay set up was as described earlier.

Crystal structure of ternary complex

Crystals of the MurE ternary complex were grown in the presence of 0.25 mM ADP, 0.25 mM UDP-MurNAc-L-ala-D-glu, 0.35 M $MgCl_2$, 0.1 M Tris pH 8.5 and 13% PEG 8000. Data was collected on beamline I04 at the Diamond Light Source. The structure was solved using the MurE/dipeptide binary complex 2 wtz (Basavannacharya et al., 2010) as a starting model for refinement and figures were prepared using CCP4MG.

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ABBREVIATIONS

FolC, folylpoly-γ-L-glutamate synthetase; Mpl, UDP-*N*-acetylmuramate:L-alanyl-γ-D-glutamyl-meso-diaminopimelate ligase; MurC, UDP-*N*-acetylmuramate:L-alanine ligase; MurD, UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase; MurE, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate:*meso*-diaminopimelate ligase; MurF, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamyl-*meso*-diaminopimelate:D-alanyl-D-alanine ligase; UMAG, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate; TDR-TB, totally drug-resistant tuberculosis

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