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Phase determination of the UDP-N-acetylmuramic acid:L-alanine ligase (MurC) crystal from *Mycobacterium bovis*

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Bacterial peptidoglycan is necessary for bacterial survival against environmental osmotic pressure and is a relevant target for development of anti-bacterial drugs. Formation of a covalent bond between a carbohydrate and an amino acid is a key chemical process for peptidoglycan bio-synthesis. UDP-N-acetylmuramic acid (UDP-MurNAc):L-alanine ligase (MurC) is an ATP-dependent amide bond ligase to form an UDP-MurNAc-L-alanine required in bacterial peptidoglycan. To provide a structural background for development of tuberculosis-specific antibiotics, *Mycobacterium bovis* MurC (MbMurC), which has sequence identity of 100% to *M. tuberculosis*, was cloned and expressed. The purified protein was crystallized from the precipitant of 0.1 M HEPES (pH 7.0), 0.2 M NaCl, 24% (w/v) polyethylene glycol 1.5K, and 10% (v/v) 2-methyl-2,4-pentanediol. Diffraction data were collected to 2.3 Å resolution. The crystal belonged to the primitive monoclinic space $P2_1$ with unit-cell parameters a = 65.30 Å, b = 76.70 Å, c =103.96 Å, $\alpha = \gamma = 90°$, and $\beta = 106.0°$. The spatial positions of the two protein molecules in the asymmetric unit were determined by molecular replacement using the sequentially related *Yersinia pestis* MurC structure.

INTRODUCTION

Peptidoglycan is a major cell wall component, which confers prokaryote for overcoming the osmotic pressure and maintaining its distinctive cell shape. In this regard, study on the bacterial peptidoglycan structure may provide a molecular basis for developing anti-bacterial agents and information on cell division and immune responses. Peptidoglycan is known to be bio-synthesized through three main steps. First, UDP-N-acetylmuramic acid (UDP-MurNAc) are formed from pyruvate and N-acetylglucosamine (GlcNAc) aided by two enzymes of MurA and MurB. Second, ATP-dependent amide bond ligases of MurC, MurD, MurE, and MurF help the addition reactions of several amino acids, for example, L-Ala, D-Glu, m-DAP and D-Ala-D-Ala, to UDP-MurNAc. At the third step, a second GlcNAc is attached to the formed UDP-MurNAc-pentapeptide at the previous step (Smith, 2006; Hrast et al., 2014).

Four Mur ligases in the second step share structural homology, since they commonly have MurNAc-binding sites and the ATP-binding sites. They are also mechanically related because all enzymes catalyze addition reaction of amino acids to the common saccharide derivative, through the activation of the free carboxylate using ATP, formation of an acyl phosphate intermediate, and formation of an amide bond with the next amino acid (Smith, 2006; Silver, 2013). Nonetheless, sequence identity among these four enzymes is relatively low (between 15% and 22%), indicating a possibility of separate studies on each enzyme as a good strategy for the development of a new antibiotics.

MurC ligase aids the addition reaction of L-alanine to UDP-MurNAc and forms UDP-MurNAc-L-alanine (UMA) in an ATP-dependent manner. Structural studies of MurC enzymes revealed that they are composed of three structural domains: an N-terminal domain for taking up the UDP-MurNAc substrate, a central domain providing for an ATP-binding site and a C-terminal domain for accommodating an amino acid substrate. However, their active sites show many minute differences in many loops that interact with substrate as well as their amino acid composition (Mol et al., 2003; Spraggon et al., 2004; Deva et al., 2006).

Mycobacterium bovis is closely related with outbreak of tuberculosis in animal species and man (O'Reilly and Daborn, 1995). Interestingly, the genome sequence of *M. bovis* is >99.95% identical to that of the human tuberculosis-causing *M. tuberculosis*, although its genome size is smaller than that of *M. tuberculosis* (Cole et al., 1998; Garnier et al., 2003). Therefore, study on the Mur ligases of *M. bovis* will be helpful to develop a wide range of antibiotics for humans as well as animals. However, no structural and biochemical study on *Mycobacterium* MurC enzymes has been reported till now. In order to get a structural clue for controlling tuberculosis, we started a structural study of MurC ligase from *M. bovis* (MbMurC, Mb2176c). Its amino acid composition is exactly same as that of Rv2152c gene product of *M. tuberculosis* and it has a sequence identity to ~38%, compared to known MurC enzymes, such as from

Haemophilus influenzae (PDB ID 1P31, 1GQQ), *Acinetobacter baumannii* (PDB ID 6CAU), *Yersinia pestis* (PDB ID 4HV4), and *Escherichia coli* (PDB ID 2F00).

RESULTS AND DISCUSSION

Mur ligases (MurC, MurD, MurE, and MurF) mediate a key reaction in peptidoglycan synthesis by catalysis of step-wise additions of five amino acids to UDP-MurNAc, and are attractive potential multi-drug targets because of their necessity for bacterial survival and no structural homology with the mammalian enzymes (Hrast et al., 2014). MurC catalyzes an addition reaction of the first amino acid to the UDP-MurNAc. With an expectation to get a structural background for developing a wide range of antibiotics against tuberculosis for humans as well as animals, we performed structural study of MurC ligase from *M. bovis*. The MbMurC crystals suitable for diffraction experiments were obtained within 3 days using the hanging drop vapor-diffusion method at 294 K by mixing 0.5 µl protein solution and 0.5 µl reservoir solution and equilibrating against 200 µl reservoir solution, which consisted of 0.1 M HEPES (pH 7.0), 0.2 M NaCl, 24% (w/v) PEG 1.5K, and 10% (v/v) MPD (Table 2). A single crystal with a dimension of approximately 0.1 x 0.05 x 0.02 mm (Figure 1) was used for X-ray diffraction experiment and it diffracted to ~2.3 Å resolution (Figure 2). The crystal belonged to the primitive monoclinic space $P2_1$ with unit-cell parameters a = 65.30 Å, b = 76.70 Å, c = 103.96 Å, $\alpha = \gamma = 90°$, and $\beta = 106.0°$. Assuming the presence of two molecule in the asymmetric unit gave a Matthews coefficient of 2.27 $Å^3Da^{-1}$, corresponding to a solvent content of 46% (Matthews, 1968). A molecular replacement by AutoMR program of the PHENIX suite using *Y. pestis* MurC (PDB ID 4HV4, sequence identity 38%) as a search model clearly indicated two protein molecules in the asymmetric unit with initial Z-scores of 5.3 and 10.6 after translation functions for the first and second molecules, respectively, which gave an interpretable map for determination of MbMurC structure (Figure 3). Model building and refinement are being performed for the complete structure that will provide a spatial rationale for designing an anti-tuberculosis agent.

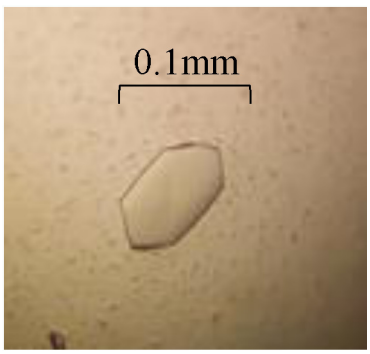

FIGURE 1 | Photograph of a representative MbMurC crystal. The crystal grew at 294 K within 3 days to maximum dimensions of approximately 0.1 x 0.05 x 0.02 mm.

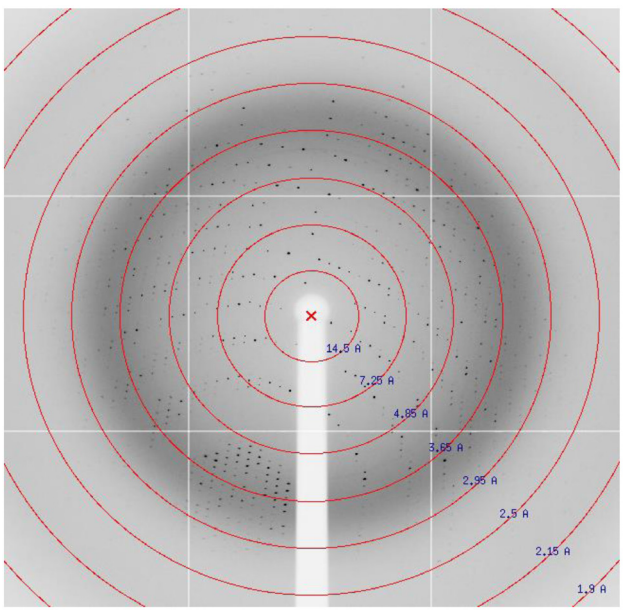

FIGURE 2 | A representative X-ray diffraction image from a MbMurC crystal. The crystal was exposed for 1 s per 1° oscillation range. The edge of the detector corresponds to a resolution of ~2.3 Å.

METHODS

Cloning, expression, and purification of MbMurC

The gene coding for MbMurC (Mb2176c) was amplified from *Mycobacterium bovis* chromosomal DNA by the PCR (polymerase chain reaction) using two designed primers (GATCATATGAGCACCGAGCAGTTGCCGCCC and GATCTCGAGTCATCCCAGCACCCCCGGACGGC) that contains *Nde*I and *Xho*I restriction enzyme sites (underlined), respectively. The PCR product was ligated with a modified pProEX HTa vector (Invitrogen), which is

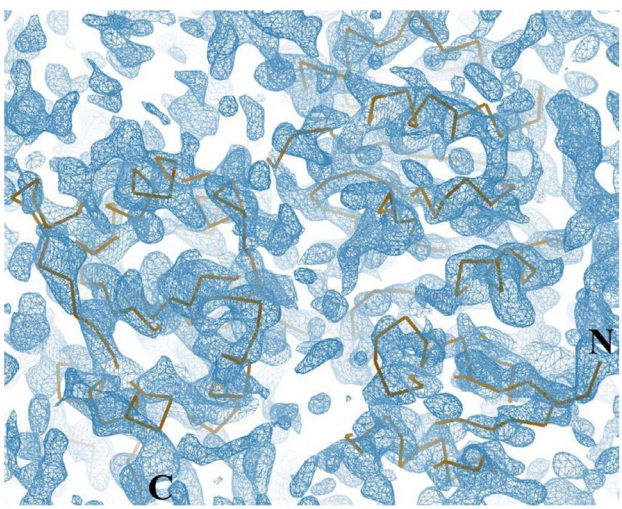

FIGURE 3 | The initial electron density map of MbMurC. The blue mesh represents a calculated electron density for a protein model that is displayed with a Cα-tracing.

TABLE 1 | Macromolecule production information

Source organism	*Mycobacterium bovis*
DNA source	Chromosomal DNA
Forward primer	GATCATATGAGCACCGAGCAG TTGCCGCCC
Reverse primer	GATCTCGAGTCATCCCAGCAC CCCCGGACGGC
Cloning vector	Modified pProEX HTa
Expression vector	Modified pProEX HTa
Expression host	*E. coli* BL21(DE3)Star
Complete amino acid sequence of the construct produced	MSYYHHHHHHDYDIPTTENLYFQGHMSTEQ LPPDLRRVHMVGIGGAGMSGIARILLDRGGL VSGSDAKESRGVHALRARGALIRIGHDASSL DLLPGGATAVVTTHAAIPKTNPELVEARRRG IPVVLRPAVLAKLMAGRTTLMVTGTHGKTT TTSMLIVALQHCGLDPSFAVGGELGEAGTNA HHGSGDCFVAEADESDGSLLQYTPHVAVITN IESDHLDFYGSVEAYVAVFDSFVERIVPGGAL VVCTDDPGGAALAQRATELGIRVLRYGSVPG ETMAATLVSWQQQGVGAVAHIRLASELATA QGPRVMRLSVPGRHMALNALGALLAAVQIG APADEVLDGLAGFEGVRRRFELVGTCGVGK ASVRVFDDYAHHPTEISATLAAARMVLEQG DGGRCMVVFQPHLYSRTKAFAAEFGRALNA ADEVFVLDVYGAREQPLAGVSGASVAEHVT VPMRYVPDFSAVAQQVAAAASPGDVIVTMG AGDVTLLGPEILTALRVRANRSAPGRPGVLG

The underlined characters in the primers indicate restriction-enzyme sites.

designed to express the target protein with a 6xHis-TEV recognition site at the N-terminus (Table 1). For expression of the recombinant protein, the cloned plasmid was transformed into *E. coli* BL21(DE3)Star (Thermo Fisher Scientific). Cells were cultured in LB medium containing 50 μg/ml ampicillin at 37°C. The protein was expressed by adding 0.05 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) (final concentration) for additional 15 h at 18°C when the culture media reached OD 0.5 at 600 nm. The harvested cells were re-suspended and disrupted by ultrasonication in a lysis buffer [20 mM Tris (tris(hydroxymethyl)aminomethane)-HCl (pH 7.5), 0.5 M NaCl, 1 mM β-ME (β-mercaptoethanol), and 1mM PMSF (phenylmethane sulfonyl fluoride)]. The supernatant was loaded onto 5ml His-bind agarose resin column (Elpis Biotech) equilibrated with buffer A [20 mM Tris-HCl (pH 7.5), 0.5 M NaCl and 1 mM β-ME]. The bound protein was eluted by a discontinuous imidazole gradient. The target protein was mixed with a recombinant TEV protease at 4°C for overnight at a ratio of 1:50 to remove the 6xHis-tag, and was dialyzed against buffer B [20 mM Tris-HCl (pH 7.5)]. MbMurC was further isolated by using a HiTrap Q column (GE healthcare) operated with a linear NaCl gradient. The eluted target protein was loaded onto HiLoad 26/600 Superdex 200pg (GE Healthcare) which is equilibrated in 20 mM Tris-HCl (pH 7.5) and 0.2 M NaCl.

Crystallization

For crystallization, the purified MbMurC protein was concentrated to 3.7 mg/mL in a buffer consisting of 20 mM Tris-HCl (pH 7.5) and 0.2 M NaCl. The protein concentration was determined considering extinction coefficient of 0.343 g/l at 280 nm. Searching for initial crystallizing condition were attempted at 294 K by the sitting-drop vapor-diffusion method using commercial screening kits and 96 well crystallization plate (Corning, USA). A drop composed of a mixture of 0.3 μl protein solution and 0.3 μl reagent was placed in each well of sitting-drop plate and equilibrated with a 55 μl reservoir solution. The initial crystals were obtained from a precipitant solution consisting of 0.1 M HEPES (pH 7.5), 0.2 M NaCl, 30% (w/v) PEG (polyethylene glycol) 1.5K, and 5% (v/v) MPD (2-methyl-2,4-pentanediol). This initial condition was further modified by changing the precipitant concentration, the buffer pH, and the vapor-diffusion method to obtain suitable crystals for X-ray diffraction experiments.

TABLE 2 | Crystallization

Method	Hanging-drop vapor diffusion
Plate type	VDX™ Plate without sealant (24-well)
Temperature (K)	294
Protein concentration (mg ml^{-1})	3.7
Buffer composition of protein solution	20 mM Tris-HCl (pH 7.5), 0.2 M NaCl
Composition of reservoir solution	0.1 M HEPES (pH 7.0), 0.2 M NaCl, 24% (w/v) PEG 1.5K, and 10% (v/v) MPD
Volume and ratio of drop	1 μl; 1:1 ratio of protein and reservoir solution
Volume of reservoir (μl)	200

TABLE 3 | Data collection statistics

Data collection	MbMurC
Space group	$P2_1$
Unit cell dimensions	1.2818
a, b, c (Å)	65.30, 76.70, 103.96
α, β, γ (°)	90, 106.0, 90
Wavelength (Å)	0.9796
Resolution (Å)	50-2.33 (2.34-2.33)
R_{sym}	11.1 (46.0)
$I/\sigma(I)$	9.0 (3.0)
Completeness (%)	98.6 (95.1)
Redundancy	2.6 (2.6)

The numbers in parentheses are the statistics from the highest resolution shell.

X-ray diffraction data collection

For X-ray diffraction experiments, crystals were immersed into the precipitant solution containing an additional 10% (v/v) PEG 300 as a cryoprotectant for 5 s and immediately placed in a 100 K nitrogen-gas stream. The diffraction data set of MbMurC was collected at the wavelength of 0.9796 Å on the beamline MX5C of the Pohang Accelerator Laboratory (PAL; Pohang in Korea) using a Quantum 315r CCD detector (USA) (Park, S.Y. et al., 2017) with an oscillation of 1° per frame, an exposure of 1 s per frame and a crystal-to-detector distance of 300 mm. A data set of 270 images was collected and the data collection statistics are summarized in Table 3. The indexing, integration, and scaling of the reflections of the MbMurC were conducted using HKL2000 suite (Otwinowski and Minor, 1997). The phase problem was solved by AutoMR program of the PHENIX suite (Adams et al., 2010) using *Y. pestis* MurC (PDB ID 4HV4) as a search model.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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