 Structural and functional characterisation of a lytic transglycosylase and two ω-transaminases
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 2

Biochemical and crystallographic characterization of the lytic transglycosylase MltF from *Pseudomonas aeruginosa*

Eswar Reddem, Bauke W. Dijkstra and Andy Mark W. H. Thunnissen

A C-terminally truncated, but active form of MltF from *P. aeruginosa* was expressed in *E. coli*, purified and crystallized. A native 1.85 Å resolution dataset was collected as well as a 2.45 Å resolution SAD dataset from a crystal soaked in a NaI solution to be used for phasing.
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Abstract

Lytic transglycosylases (LTs) cleave the β-1,4-glycosidic linkages between MurNAc and GlcNAc residues in peptidoglycan with concomitant formation of an α-1,6-anhydro-bond in the MurNAc residue. MltF is an unusual LT, which possesses, besides a C-terminal LT-like domain, an N-terminal domain of unknown function that shares sequence similarity to ABC-type periplasmic binding proteins. As a first step to elucidate the precise functions of both domains and their interaction, we cloned the mltF gene from *P. aeruginosa*, and expressed, purified and crystallized a truncated form of *paMltF*, lacking the last 28 C-terminal amino acid residues. Crystals were obtained that belong to the orthorhombic space group *P2_1*2_1, with unit cell parameters *a* = 58.3 Å, *b* = 82.5 Å and *c* = 96.9 Å. They contain one molecule per asymmetric unit, and diffract to 1.85 Å resolution, suitable for a detailed structure-function analysis.
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Biochemical and crystallographic characterization of poMltF

Introduction

Most eubacteria have a cell wall of peptidoglycan (PG; murein) that encloses their cytoplasmic membrane. PG is built up of linear glycan chains consisting of alternating β,1,4-linked N-acetylglycosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) residues, which can be cross-linked by short peptides (Höltje, 1998; Beveridge, 1999; Vollmer & Bertsche, 2008). These glycan chains and peptides form a mesh-like structure, the sacculus, which completely surrounds the bacterial cell. The main function of PG is to preserve cell integrity by conferring resistance to the internal turgor pressure of the cell (Höltje, 1998; Beveridge, 1999; Vollmer & Bertsche, 2008). In addition, PG serves as a scaffold to anchor other cell envelope components including proteins (Dräsi et al., 2008) and teichoic acids (Neuhaus & Baddiley, 2003).

The PG sacculus is not a static structure, but it undergoes continuous remodeling and turnover by the action of various PG synthesizing and cleaving enzymes (Park & Uehara, 2008). The activity of these enzymes must be strictly controlled and coordinated, not only to maintain the integrity of the sacculus, but in particular to preserve cell viability. Because of their importance for cell viability and cell morphogenesis PG-metabolizing enzymes are extensively studied, not the least because several of them are important targets for antibiotics against bacterial infections (van Heijenoort, 2011).

A key class of peptidoglycan degrading enzymes are the lytic transglycosylases (LTs; EC 4.2.2.n), which cleave the same β,1,4-glycosidic linkages between MurNAc and GlcNAc residues as lysozymes (Figure 1). However, unlike lysozymes, which are hydrolytic enzymes, LTs are transglycosylases that catalyze the cleavage of glycosidic linkages with concomitant formation of an α,1,6-anhydro-bond in the newly formed terminal MurNAc residue (anhydroMurNAc) (Höltje et al., 1975; Höltje, 1998; Blackburn & Clarke, 2001; Vollmer & Bertsche, 2008). Genomic sequence analyses have revealed that LTs are ubiquitous in bacteria and occur also in several bacteriophages. Based on sequence similarity and consensus motifs, the LTs have been classified into four families (Blackburn & Clarke, 2001; Scheuwerwter et al., 2008).
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Figure 1: Typical reaction catalysed by LTs, targeting the cleavage of the β-1,4-glycosidic bonds between MurNAc and GlcNAc residues in peptidoglycan, with concomitant formation of a 1,6-anhydro-MurNAc residue.

*Escherichia coli* is known to produce one soluble LT (Slt70; Höljt et al., 1975) and six LTs that are bound to the outer membrane through a lipoyl anchor at their N-terminus (MltA, MltB, MltC, MltD, MltE, and MltF (Scheurwater & Clarke, 2008; van Heijenoort, 2011)). Crystal structures of *E. coli* LTs belonging to families I (Slt70, MltC, MltD, MltE) and III (MltB) revealed that all these enzymes possess a catalytic domain that resembles goose-type lysozyme (Thunnissen et al., 1995a; Thunnissen et al., 1995b; van Asselt et al., 2000; Artola-Recolons et al., 2011; Fibriansah et al., 2012; Artola-Recolons et al., 2014). In contrast, MltA, which belongs to family II, has a β-barrel structure resembling the fold of endoglucanase V (van Straaten et al., 2005; Powell et al., 2006).

A few years ago Scheurwater and Clarke (2008) showed that the hypothetical *E. coli* protein YfhD was localized to the outer membrane and exhibited lytic transglycosylase activity. YfhD was therefore renamed membrane-bound lytic transglycosylase F, MltF. Its gene sequence indicated that MltF possesses two domains, an N-terminal domain with sequence similarity to periplasmic substrate-binding proteins of ABC transporters, and a C-terminal domain that bears sequence similarity to family I LTs. Indeed, the ability of MltF to bind peptidoglycan and its lytic activity were only associated with the isolated C-terminal domain. The function of the N-terminal domain is unknown.
To obtain more insights into the role of the N- and C-terminal domains of MltF, we re-initiated our crystallographic studies with *E. coli* MltF (ecMltF). Previously, diffraction data to 3.5 Å resolution were collected for a soluble form of ecMltF, missing the N-terminal signal peptide (Madoori & Thunnissen, 2010). Unfortunately, all attempts to obtain phase information by molecular replacement, using search models based on available structures of LTs and periplasmic binding proteins, failed. Moreover, we were unable to reproduce the ecMltF crystals, prohibiting us to obtain phases by isomorphous replacement or anomalous dispersion methods. Therefore, we decided to focus on the MltF homologue from *P. aeruginosa* (paMltF), which has 33% sequence identity to ecMltF. In this chapter we describe the cloning, expression and functional analysis of full-length paMltF and of paMltFΔC, a mutant lacking residues 462-490 (the last 29 residues). Crystals were obtained of the truncated protein that diffracted to 1.85 Å resolution.

**Experimental procedures**

**Cloning** — The gene encoding MltF from *P. aeruginosa* (UniProt entry Q9HXN1) was amplified by PCR from genomic DNA and cloned into the plasmid pBADnLIC by following published protocols (Geertsma & Poolman, 2007). DNA primers used for PCR are shown in Table 1. The resulting plasmid, pBADnLIC-paF, allowed expression of a recombinant form of paMltF, containing residues 28-490 of the gene-derived amino acid sequence, preceded by a cleavable N-terminal histidine-tag (M(H)_{10}GENLYFQG) and a serine residue, replacing Cys27 (the N-terminal lipoyl-modified residue of mature paMltF). A plasmid for expressing a C-terminally truncated form of paMltF (paMltFΔC, deletion of residues 462-490) was constructed by introducing a stop codon at the appropriate position in the pBADnLIC-paF plasmid, following the Quick Change site-directed mutagenesis protocol (Agilent Technologies) with suitable DNA primers (data not shown).
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**Expression and purification** — To express His-tagged *pa*MltF, plasmid pBADnLIC-paF was transformed into *E. coli* BL21 (DE3) cells. The transformed *E. coli* BL21 (DE3) cells were grown in 2 ml of LB medium, supplemented with ampicillin (100 μg/ml). After overnight growth at 310 K the culture was transferred into 2 L of fresh medium, further grown at 310 K until the OD<sub>600nm</sub> reached 0.8, and induced with 0.2% arabinose and incubated for an additional 3.5 h at 310 K. Cultured cells were harvested by centrifugation at 9,000 *g* for 10 min at 277 K, and the resulting bacterial pellet was resuspended in 50 ml ice-cold lysis buffer containing 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.2% nonidet P-40, 5 mM β-mercaptoethanol, and appropriate amounts of DNase (Roche) and protease inhibitors (Roche).

Cells were lysed using a French press and the soluble proteins were collected by centrifugation at 36,000 *g* for 45 min at 277 K. The supernatant was loaded onto a 5-ml HisTrap HP column (GE Healthcare), equilibrated with 20 mM Tris-HCl, pH 8.0, 20 mM imidazole, 300 mM NaCl, 5 mM β-mercaptoethanol (buffer A), using an ÄKTA express FPLC system (GE Healthcare). The column was washed with about 4-5 column volumes of 50 mM imidazole in buffer A to remove unbound proteins, and *pa*MltF was eluted with a gradient of 50-500 mM imidazole in buffer A. Column elution fractions containing *pa*MltF were diluted 6-fold with 20 mM Tris-HCl, pH 8.0, 1 mM EDTA and then loaded onto a 5-ml Resource Q column (GE Healthcare). Elution was carried out with a gradient of increasing NaCl concentration from 50 to 500 mM in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA. Peak fractions containing *pa*MltF were pooled and TEV protease (Invitrogen) was added in a mass ratio of 1:1000 relative to *pa*MltF, followed by overnight incubation at 293 K, to cleave off the N-terminal histidine-tag. The protein was passed through a 1-ml His Trap HP column, to remove the His-tag and any traces of uncleaved protein. The protein was concentrated to 50 mg/ml and injected onto a Superdex 200 10/300 GL column (GE Healthcare), equilibrated with 20 mM Bis-Tris-HCl, pH 7.5, and 200 mM NaCl.

The *pa*MltF containing fractions were pooled, concentrated to 30 mg/ml, and either used immediately for crystallization, or frozen in liquid nitrogen for storage at -80 °C. Expression and purification of *pa*MltFΔC followed identical procedures as described above. The proteins from these preparations were at least 98% pure as judged by SDS-PAGE followed by silver staining.
Pre-crystallization analysis — Optimal pH and solution conditions for sample stability and monodispersity were identified using the ThermoFluor thermal shift assay (Ericsson et al., 2006) and by dynamic light scattering (DLS) measurements (Wyatt). ThermoFluor assays were performed in 96-well PCR plates using a Bio-Rad MyiQ Thermal Cycler (Bio-Rad). Screening for the optimal pH was performed from pH 4.0 to 9.0 using the 50 mM MMT buffer system (a mixture of DL-malic acid, 2-(N-morpholino)-ethanesulfonic acid and Tris in a 1:2:2 molar ratio; (Newman, 2004)). Other molecules tested for their effect on paMltF stability were NaCl, glycerol and MgCl₂.

Peptidoglycan cleavage assays — Peptidoglycan lytic activities of the paMltF and paMltFΔC proteins were analyzed by zymography and turbidimetry, following published protocols (Scheurwater & Clarke, 2008; Fibriansah et al., 2012). The SDS-PAGE gels used for zymography contained 0.1% insoluble Micrococcus luteus peptidoglycan and 12.5% acrylamide. Protein renaturation was carried out in 0.1 M Tris-HCl, pH 7.0, 0.5% Triton X-100; cleavage of peptidoglycan was visualized by staining with methylene blue/KOH. The turbidimetric assay was carried out with 0.35 mg/ml suspensions of insoluble M. luteus or E. coli peptidoglycan in protein storage buffer (20 mM Bis-Tris-HCl, pH 7.5, 200 mM NaCl). Cuvettes were filled with 900 µl peptidoglycan suspension and 100 µl protein solution, and the rate of peptidoglycan solubilisation was followed by monitoring the decrease in A₆₀₀. All reactions were performed at different protein concentrations and carried out in triplicate.

Crystallization — Crystallization experiments were carried out in a 96-well plate using the sitting-drop vapour-diffusion technique, by means of a Mosquito (TTP LabTech) crystallization robot. Various commercially available crystallization screens, e.g. JSCG⁺ (Qiagen), Pact premier, Structure, Cryo (Molecular Dimensions), Wizard Screen (Emerald Biosystems) were used for the identification of initial crystallization conditions. Droplets of 0.1 µl in volume (with a 1:1 protein:precipitant ratio, 30 mg/ml protein stock) were equilibrated against 50-µl reservoir solution at 277 K and 293 K for both full-length paMltF and paMltFΔC. X-ray quality crystals of paMltFΔC were reproduced manually using the hanging-drop vapour-diffusion method and optimized by following a streak seeding protocol (Bergfors, 2003).
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Data collection and analysis — For X-ray diffraction data collection, crystals were briefly rinsed in cryoprotectant solution (40% (v/v) PEG 400 in reservoir solution) and flash-cooled in liquid nitrogen. Native diffraction data to 1.85 Å resolution were collected at ESRF beamline ID23-1, Grenoble, France. In addition, iodine-SAD data to 2.45 Å resolution were collected in-house, on a Bruker Microstar X-ray generator equipped with a Mar345 dtb detector. To obtain the iodine-SAD data, a crystal was soaked for 30 min in cryo-solution supplemented with 0.5 M NaI, before mounting it in the X-ray beam. Data processing (indexing, integration, scaling and merging) was performed with the programs XDS (Kabsch, 2010) and SCALA (Evans, 2006).

Results and Discussion

The gene encoding full-length paMltF (amino acid residues 28-490, with the N-terminal signal sequence replaced by a TEV-cleavable histidine-tag) was successfully cloned into the pBADnLIC expression vector and overexpressed in E. coli BL21 (DE3) pLysS cells. Purification of paMltF to homogeneity was achieved through three chromatographic steps, Ni-affinity, anion-exchange, and size-exclusion chromatography. Unfortunately, the purified full-length protein did not yield any crystals. Since a secondary structure analysis (Jones, 1999) suggested that the 29 C-terminal residues were likely disordered (data not shown), a DNA construct expressing a truncated variant of paMltF was prepared (paMltFΔC, encoding residues 28-461 together with an N-terminal histidine tag). Expression and purification of paMltFΔC followed the same protocols as for full-length paMltF. The N-terminal His$_{16}$-tag was successfully removed by TEV protease treatment, leaving only a glycine and serine residue as extra, non-gene derived amino acids at the N-terminus. Thus purified paMltFΔC (with a calculated molecular weight of 49.0 kDa) was judged as highly pure (>98%) on the basis of silver-stained SDS gels (Figure 2).
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![Silver-stained SDS gels](image)

**Figure 2:** Silver-stained SDS gels, showing the final purity of full-length *poMltF* and *poMltFΔC*, with apparent molecular weights of ~52 kDa and 50 kDa, respectively.

Dynamic light scattering analysis showed that the protein sample was monodisperse (Figure 3).

![Dynamic light scattering](image)

**Figure 3:** Dynamic light scattering analysis of purified *poMltFΔC* in 20 mM Bis-Tris buffer, pH 7.5, 200 mM NaCl. The hydrodynamic radius, apparent molecular weight, and polydispersity of the protein sample are 3.2 nm, 50 kDa, and 11%, respectively.
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Using a thermal shift assay (Ericsson et al., 2006), the protein was found to be optimally stable in Bis-Tris propane buffer, pH 7.5, 200 mM NaCl (Figure 4).

**Figure 4:** Thermal shift assay of poMltFΔC in 50 mM MMT buffer at varying pH values, revealing an optimal stability at a pH ranging from 6.5 to 7.5. b) Thermal shift assay of poMltFΔC in 50 mM MMT buffer at pH 6.5, 7.5 and 8.5, and with varying NaCl concentrations, revealing an optimal stability at pH 7.5 and a NaCl concentration ranging from 0-500 mM.

This condition was henceforth used for storage of the protein. The truncated protein poMltFΔC was shown to have the same lytic activity on peptidoglycan as the full-length protein (Figures 5a, b and c).

**Figure 5:** Turbidometric assay using Micrococcus luteus cell walls as substrate for a) full-length poMltF and b) poMltFΔC.
Figure 5c: Zymography assay using Micrococcus luteus cell wall as substrate for full-length paMltF, paMltFΔC and lysozyme.

The paMltFΔC protein yielded single crystals after three months at 277 K from a Wizard screen condition containing 0.2 M MgCl₂, 0.1 M Tris-HCl, pH 8.5, 20% (w/v) polyethylene glycol (PEG) 8000, with approximate average dimensions of 200×75×40 μm. The crystals diffracted to 1.85 Å at beamline ID23-1 at the European Synchrotron Radiation Facility, Grenoble. A summary of the crystallographic data collection statistics is show in Table 2. The crystals belong to the orthorhombic crystal system with unit cell parameters a = 82.0 Å, b = 58.3 Å and c = 96.9 Å. The space group was determined as P2₁2₁2₁ on the basis of systematic absences and scaling statistics. Analysis of the collected data further indicated that the crystals contained one ~49 kDa protein molecule per asymmetric unit with a Matthews’ coefficient Vᵣ of 2.37 Å³Da⁻¹ (Matthews, 1968), and a solvent content of 48%.

Following the protocol published by Dauter et al. (2000), crystals were derivatized by soaking them in 0.5 M sodium iodide, and a single-wavelength anomalous diffraction data set was collected (Table 2). From the anomalous differences, 10 iodide sites could be clearly identified by
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Autosharp (Vonrhein et al., 2007), allowing phase determination and calculation of an electron density map. The overall figures of merit before and after solvent flipping, using SOLOMON (Abrahams & Leslie, 1996), were 0.32 and 0.80, respectively, for reflections in the resolution range 48.4–2.45 Å. The resulting experimental electron density map showed distinct solvent–protein boundaries, and features of secondary structure elements were clearly visible in the protein-associated densities. Using automated model building with ARP/wARP (Langer et al., 2008), it was possible to fit a partial model of nearly 380 amino acid residues (~87% of the polypeptide). The final model of paMltFΔC was completed after several cycles of restrained positional and B-factor refinement with the program Refmac5 (Murshudov et al., 1997), alternated by manual model building using Coot (Emsley et al., 2010). The analysis of the structure of paMltFΔC, complemented with crystallographic ligand-binding studies, is discussed in chapter 3.
**Table 1**: X-ray data collection statistics.

<table>
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<th>Protein</th>
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<th>Iodine soak</th>
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<td>In house</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
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<td>No. of recorded images</td>
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<td>Space group</td>
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<td>P2_1_2_1</td>
</tr>
<tr>
<td>Unit cell parameters (Å)</td>
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<tr>
<td>Resolution range (Å)</td>
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<td>Total no. of observations</td>
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<tr>
<td>No. of unique reflections</td>
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<tr>
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<td>99.7 (97.8)</td>
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<tr>
<td>(R_{merge}) (%)</td>
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<tr>
<td>Mean (I/\sigma)</td>
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<td>18.0 (9.0)</td>
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<tr>
<td>Redundancy</td>
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<td>8.2 (8.0)</td>
</tr>
</tbody>
</table>

Values in parentheses refer to the outer resolution shell.

\(* R_{merge} = \frac{\sum_i \sum_j |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_i \sum_j I_i(hkl)}, \) where \(I_i(hkl)\) is the \(i\)th observation of reflection hkl and \(\langle I(hkl) \rangle\) is the weighted average intensity for all observations \(i\) of reflection hkl.
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References


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