Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification, crystallization and preliminary X-ray analysis of the lytic transglycosylase MltA from *Escherichia coli*

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Received 18 December 2003
Accepted 2 February 2004

The lytic transglycosylase MltA from *Escherichia coli* with its membrane anchor and signal sequence deleted has been purified to homogeneity by means of cation-exchange chromatography. The enzyme was crystallized using the hanging-drop vapour-diffusion method. The crystals belong to space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 103.70$, $c = 109.84$ Å and one molecule per asymmetric unit. Crystals diffract to 2.2 Å resolution on a synchrotron-radiation source.

1. Introduction

Lytic transglycosylases are muramidases that participate in the maintenance and processing of the bacterial murein (peptidoglycan) sacculus as required during cell division and septation, DNA conjugation and virulence-related processes (Höltje & Schwarz, 1985; Bayer *et al.*, 1995; Höltje, 1995, 1998; Moak & Molineux, 2000; Rydman & Bamford, 2000; Bayer *et al.*, 2001). Specifically, they catalyze the cleavage of the β-(1,4)-glycosidic bonds between *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) residues in murein, forming non-reducing 1,6-anhydro-muropeptides. In this respect, they differ from lysozyme, which cleaves the same bonds but produces free reducing ends.

In *Escherichia coli*, at least six different lytic transglycosylases occur: one is soluble (Slt70) and five are outer-membrane-bound (MltA–MltD, EmtA). EmtA is the only endolytic transglycosylase, while the other five are exolytic enzymes. The lytic transglycosylases share little sequence identity with each other. On the basis of differences in the consensus signature sequence motifs around their catalytic regions (Blackburn & Clarke, 2001), these enzymes can be classified into three families. Family 1 forms a superfamily containing subfamilies involving sequences with identity to Slt70, MltC, MltD and EmtA. They all have consensus motifs with homology to goose-type lysozyme. The sequences of the other two lytic transglycosylases from *E. coli*, MltA and MltB, form two separate families not related to each other or to family 1. Family 2 contains hypothetical enzymes with homology to MltA. The sequences included in family 3 are from hypothetical enzymes with homology to MltB.

The precise functions of the lytic transglycosylases in murein metabolism are still unclear. Studies of *E. coli* mutants with multiple deletions of murein hydrolases, including the lytic transglycosylases, show that these enzymes are not essential for bacterial growth but are required for the cleavage of the murein septum following cell division (Heidrich *et al.*, 2002). Interestingly, the cells that are affected in murein septum cleavage show an increased outer membrane permeability, thereby making them sensitive to high-molecular-weight antibiotics that normally cannot pass the outer membrane (Heidrich *et al.*, 2002). Moreover, *E. coli* mutants lacking one or more of the three lytic transglycosylases Slt70, MltA and MltB show a decrease in the formation of low-molecular-weight murein-turnover products, which normally function as signals for inducing the expression of β-lactamases (Kraft *et al.*, 1999). Consequently, these mutants display a dramatic reduction in β-lactamase induction, thus suggesting that inhibition of lytic transglycosylases may increase the efficiency of penicillins. Indeed, the glycopeptide bulgecin A, which is a specific inhibitor of the lytic transglycosylases Slt70, MltC and MltD (Templin *et al.*, 1992; Dijkstra, 1997), induces potent growth inhibition and enhances bacteriolysis when used in combination with β-lactam antibiotics (Imada *et al.*, 1982; Kitano *et al.*, 1986; Nakao *et al.*, 1986; Kraft *et al.*, 1999). For this reason, as well as the fact that peptidoglycan is unique and essential for bacteria and because of the unique intramolecular transglycosylation reaction that these enzymes catalyze, they seem interesting targets for novel antibiotic/antibacterial drug design.

Structural research on the *E. coli* lytic transglycosylases has so far resulted in the X-ray structures of the family 1 lytic transglycosylases Slt70 (Thunnissen *et al.*, 1994; van Asselt, Thunnissen & Dijkstra, 1999) and EmtA (Gliubich & Thunnissen, in preparation) as well as of a soluble fragment of MltB (van Asselt *et al.*, 1998; van Asselt, Dijkstra *et al.*, 1999) from lytic transglycosylase family 3.

Despite differences in overall structure, these three enzymes share a catalytic domain that resembles the fold of goose-type lysozyme (Thunnissen *et al.*, 1995; van Asselt, Thunnissen & Dijkstra, 1999). Binding studies with several murein-derived compounds (Thunnissen *et al.*, 1995; van Asselt, Dijkstra *et al.*, 1999; van Asselt, Thunnissen & Dijkstra, 1999; van Asselt *et al.*, 2000) gave insight into the interactions of these three enzymes with oligosaccharides and defined the location and number of the saccharide-binding sites. Furthermore, these X-ray structures and site-directed mutagenesis experiments have indicated the important role of Glu478 (Slt70 numbering) in the catalytic mechanism and suggested that this residue functions as a single catalytic acid/base similar to Glu73 in goose-type lysozyme. So far, no structure of a lytic transglycosylase from family 2 has been solved.

To increase our knowledge of the molecular details of the mechanism of cleavage and the typical 1,6-anhydromuropeptide production by these enzymes, we have started to study the three-dimensional structure of the family 2 lytic transglycosylase MltA from *E. coli* by X-ray crystallography. MltA has a molecular weight of 38 kDa and is composed of 345 amino-acid residues after removal of the signal sequence and membrane anchor. MltA lacks the family 1 lytic transglycosylase motifs that define the catalytic lysozyme-like domain. Like Slt70 and Slt35, MltA is an exomuramidase. However, it differs from Slt70 and MltB in that it is capable of degrading both insoluble murein sacculi and isolated poly-(MurNAc-GlcNAc) glycan strands (Ursinus & Höltje, 1994). Sequence comparison of *E. coli* MltA with MltA-like enzymes from other Gram-negative bacteria shows that there is no conserved glutamate present but that there are three invariant aspartic acid residues (Blackburn & Clarke, 2001). Since the optimal activity of MltA is between pH 4.0 and 4.5, this suggests that one of these three conserved aspartic acids might serve as the catalytic acid/base.

In this paper, we describe the purification, crystallization and preliminary X-ray analysis of a 345-residue soluble form of the lytic transglycosylase MltA which lacks the signal sequence and lipoprotein anchor.

2. Experimental procedures and results

2.1. Expression and purification

Controlled overexpression in *E. coli* of a soluble form of MltA lacking the 20-amino-acid leader sequence and with a methionine residue at the N-terminus replacing the lipoyl-carrying cysteine was achieved from pMSS, an expression vector based on the pBR322 derivative pJFK118EH (Lommatzsch *et al.*, 1997). Cells were cultured at 310 K by shaking at 200 rev min^{-1} in Luria–Bertani (LB) medium containing 50 µg ml^{-1} kanamycin. Expression was induced by the addition of 1 m*M* isopropyl-1-thio-β-D-galactopyranoside (IPTG) at an OD_{600} of 0.3. After IPTG induction, cells were grown for an additional 4 h until an OD_{600} of 1.8 was reached. Cells were harvested by centrifugation at 8000*g* for 10 min at 277 K and resuspended in 200 m*M* NaCl, 10 m*M* $MgCl_2$ and 10 m*M* Tris–malonate pH 5.2 (buffer *A*). After addition of 2 m*M* PMSF and catalytic amounts of DNAse, the cells were ruptured by sonication and cell debris was removed by centrifugation at 12 000*g* for 20 min at 277 K. The MltA-containing supernatant was further clarified by ultracentrifugation at 145 000*g* for 60 min at 277 K. The clear supernatant was applied onto an 85 ml SP-Sepharose fast-flow cation-exchange column (Pharmacia) pre-equilibrated with buffer *A* at 280 K. The column was washed with buffer *A* until the baseline remained constant. MltA was eluted with 425 ml of a linear salt gradient from 200 m*M* to 1 *M* NaCl at a flow rate of 6 ml min^{-1}. MltA eluted at ∼550 m*M* NaCl. Fractions were analyzed by SDS–PAGE. The MltA-containing fractions (molecular weight 38 kDa) were collected and diluted to 200 m*M* NaCl and loaded onto a 1 ml MonoS 5/5 HR cation-exchange column (Pharmacia) pre-equilibrated with buffer *A*. The column was washed with 5 ml of buffer *A* and eluted with 20 ml of a linear salt gradient from 200 m*M* to 1 *M* NaCl at a flow rate of 1 ml min^{-1}. MltA eluted at ∼420 m*M* NaCl as a single peak. Fractions containing pure MltA were collected. MltA was subsequently diluted and concentrated on a 10K Microsep microconcentrator to a concentration of 25 mg ml^{-1} in 30 m*M* NaCl, 10 m*M* $MgCl_2$ and 10 m*M* Tris–malonate buffer pH 5.2. The amount of protein was determined using UV-absorption spectroscopy at 280 nm, with a theoretical molar extinction coefficient of 1.348 g l^{-1}. About 45 mg of pure protein could be obtained from a 3 l culture.

2.2. Crystallization

Preliminary crystallization experiments were carried out at both 280 and 293 K using the microbatch technique (D'Arcy *et al.*, 1996). Crystallization conditions were first investigated using Hampton Research Crystal Screens I and II (Hampton Research, Maumee, USA) and Decode Genetics Wizard Screens I and II (Decode Genetics, Reykjavik, Iceland). Drops were prepared by mixing 0.5 µl protein solution (25 mg ml^{-1}) with 0.5 µl standard crystallization screen solution and covered with Al's oil (D'Arcy *et al.*, 1996) to allow vapour diffusion. Crystalline material and clusters of needle-like crystals were obtained from several conditions with PEG as precipitant at both 280 and 293 K. Many small three-dimensional crystals grew within 3 d from the precipitate using 20%(*w*/*v*) PEG 8000, 200 m*M* NaCl and 100 m*M* phosphate–citrate buffer pH 4.2 at 280 K. Intergrown crystals with a few loose three-dimensional crystals were obtained from 10%(*w*/*v*) PEG 3000, 200 m*M* NaCl and 100 m*M* phosphate–citrate buffer pH 4.2 at 280 K. The crystals could be reproduced using the hanging-drop vapour-diffusion method (McPherson, 1982). Drops were prepared by mixing 3 µl of reservoir solution with an equal volume of protein solution (25 mg ml^{-1}) and were allowed to equilibrate against 0.5 ml of the crystallization solution. Optimization to improve the crystal quality was achieved by testing different PEGs and by lowering the PEG concentration. Single hexagonal rod-like crystals were obtained with 5%(*w*/*v*) PEG 8000, 200 m*M* NaCl and 100 m*M* phosphate–citrate buffer pH 4.2 at 280 K. The best crystals were finally obtained by replacing the phosphate–citrate buffer with an acetate buffer, slightly increasing the NaCl concentration and adding 10 m*M* $MgCl_2$. These conditions resulted in single rod-like crystals with sharp hexagonal faces, which grew from 0–7.5%(*w*/*v*) PEG 8000, 250–400 m*M* NaCl, 10 m*M* $MgCl_2$ and 100 m*M* acetate buffer pH 4.2 at 280 K as the final crystallization condition (Fig. 1).

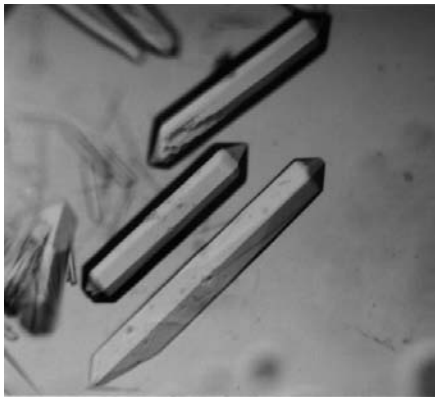

Figure 1
Crystals of lytic transglycosylase MltA. The crystals have approximate dimensions of 0.15 × 0.15 × 0.6 mm.

Table 1
Summary of the diffraction data.

Values in parentheses refer to the outer resolution shell.

Beamline	ID 14-4
Wavelength (Å)	0.9330
Resolution (Å)	30–2.19 (2.27–2.19)
Space group	$P3_121$ or $P3_221$
Unit-cell parameters (Å)	$a = b = 103.7$, $c = 109.8$
No. of observed reflections	199474
No. of unique reflections	35889
Completeness (%)	99.9 (100.0)
$R_{\rm merge}$ (%)	5.5 (51.2)
Average $I/\sigma(I)$	10.0 (3.6)
Redundancy	5.5 (5.5)

2.3. Data collection and preliminary X-ray analysis

For data collection, a crystal was transferred for a few seconds into a cryoprotectant solution containing 10%(*w*/*v*) PEG 8000, 22% ethylene glycol, 360 m*M* NaCl, 10 m*M* $MgCl_2$ and 100 m*M* acetate buffer pH 4.2. The crystal was mounted in a cryoloop and subsequently flash-frozen in liquid nitrogen. X-ray data were collected at 100 K on beamline ID 14-4 at the ESRF, Grenoble. Diffraction data to 2.2 Å were suitable for processing and scaling using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The crystal belongs to the trigonal crystal system, with unit-cell parameters $a = b = 103.7$, $c = 109.8$ Å. The space group was determined to be either $P3_121$ or $P3_221$ on the basis of systematic absences and scaling statistics. Table 1 lists the data-collection statistics. On the basis of calculation of the Matthews (1968) coefficient $V_{\rm M}$, the MltA crystal could contain one or two molecules per asymmetric unit, with a solvent content of 72 or 45%. Examination of the self-rotation function did not reveal the presence of any obvious non-crystallographic symmetry. Calculation of a Patterson map gave only one large peak in the origin but did not show any other peaks, suggesting that there is one molecule in the asymmetric unit.

Since MltA shows no sequence homology to other lytic transglycosylases, we intend to obtain experimental phases by the multi-wavelength anomalous diffraction (MAD) approach using selenomethionine-substituted MltA (Doublié, 1997; Walsh *et al.*, 1999). Recently, a selenomethionine derivative of the enzyme has been purified and crystallized and structure determination using MAD is currently in progress.

The authors thank Professor J.-V. Höltje for providing the *E. coli* cells harbouring the expression vector pMSS for producing soluble MltA. This work was supported by The Netherlands Foundation for Chemical Research (CW), with financial aid from The Netherlands Foundation for Scientific Research (NWO).

References

Asselt, E. J. van, Dijkstra, A. J., Kalk, K. H., Takacs, B., Keck, W. & Dijkstra, B. W. (1999). *Structure Fold. Des.* **7**, 1167–1180.
Asselt, E. J. van, Kalk, K. H. & Dijkstra, B. W. (2000). *Biochemistry*, **39**, 1924–1934.
Asselt, E. J. van, Perrakis, A., Kalk, K. H., Lamzin, V. S. & Dijkstra, B. W. (1998). *Acta Cryst.* D**54**, 58–73.
Asselt, E. J. van, Thunnissen, A. M. W. H. & Dijkstra, B. W. (1999). *J. Mol. Biol.* **291**, 877–898.
Bayer, M., Eferl, R., Zellnig, G., Teferle, K., Dijkstra, A., Koraimann, G. & Hogenauer, G. (1995). *J. Bacteriol.* **177**, 4279–4288.
Bayer, M., Iberer, R., Bischof, K., Rassi, E., Stabentheiner, E., Zellnig, G. & Koraimann, G. (2001). *J. Bacteriol.* **183**, 3176–3183.
Blackburn, N. T. & Clarke, A. J. (2001). *J. Mol. Evol.* **52**, 78–84.
D'Arcy, A., Elmore, C., Stihle, M. & Johnston, J. E. (1996). *J. Cryst. Growth*, **168**, 175–180.
Dijkstra, A. J. (1997). PhD thesis, University of Groningen, The Netherlands.
Doublié, S. (1997). *Methods Enzymol.* **276**, 523–530.
Heidrich, C., Ursinus, A., Berger, J., Schwarz, H. & Höltje, J.-V. (2002). *J. Bacteriol.* **184**, 6093–6099.
Höltje, J.-V. (1995). *Arch. Microbiol.* **164**, 243–254.
Höltje, J.-V. (1998). *Microbiol. Mol. Biol. Rev.* **62**, 181–203.
Höltje, J.-V. & Schwarz, U. (1985). *Molecular Cytology of Escherichia coli*, edited by N. Nanninga, pp. 77–119. London: Academic Press.
Imada, A., Kintaka, K., Nakao, M. & Shinagawa, S. (1982). *J. Antibiot.* **35**, 1400–1403.
Kitano, K., Tuomanen, E. & Tomasz, A. (1986). *J. Bacteriol.* **167**, 759–765.
Kraft, A. R., Prabhu, J., Ursinus, A. & Höltje, J.-V. (1999). *J. Bacteriol.* **181**, 7192–7198.
Lommatzsch, J., Templin, M. F., Kraft, A. R., Vollmer, W. & Höltje, J.-V. (1997). *J. Bacteriol.* **179**, 5465–5470.
McPherson, A. (1982). *Preparation and Analysis of Protein Crystals.* New York: Wiley.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Moak, M. & Molineux, I. J. (2000). *Mol. Microbiol.* **37**, 345–355.
Nakao, M., Yukishige, K., Kondo, M. & Imada, A. (1986). *Antimicrob. Agents Chemother.* **30**, 414–417.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Rydman, P. S. & Bamford, D. H. (2000). *Mol. Microbiol.* **37**, 356–363.
Templin, M. F., Edwards, D. H. & Höltje, J.-V. (1992). *J. Biol. Chem.* **267**, 20039–20043.
Thunnissen, A. M. W. H., Dijkstra, A. J., Kalk, K. H., Rozeboom, H. J., Engel, H., Keck, W. & Dijkstra, B. W. (1994). *Nature* (*London*), **367**, 750–753.
Thunnissen, A. M. W. H., Rozeboom, H. J., Kalk, K. H. & Dijkstra, B. W. (1995). *Biochemistry*, **34**, 12729–12737.
Ursinus, A. & Höltje, J.-V. (1994). *J. Bacteriol.* **176**, 338–343.
Walsh, M. A., Evans, G., Sanishvili, R., Dementieva, I. & Joachimiak, A. (1999). *Acta Cryst.* D**55**, 1726–1732.