Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Expression, purification, crystallization and preliminary characterization of uridine 5′-diphospho-*N*-acetylmuramoyl L-alanyl-D-glutamate:lysine ligase (MurE) from *Streptococcus pneumoniae* 110K/70

Anne M. Blewett,[a] Adrian J. Lloyd,[a,b] Aude Echalier,[a] Vilmos Fülöp,[a] Christopher G. Dowson,[a] Timothy D. H. Bugg[b] and David I. Roper[a]*

[a]Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, England, and [b]Department of Chemistry, University of Warwick, Coventry CV4 7AL, England

Correspondence e-mail: droper@bio.warwick.ac.uk

Received 4 November 2003
Accepted 2 December 2003

An ORF designated sp1530 (*murE*) in the *Streptococcus pneumoniae* TIGR4 genome sequence, identified as uridine 5′-diphospho-*N*-acetylmuramoyl-L-alanyl-D-glutamate:L-lysine ligase (MurE; EC 6.3.2.7), was cloned into the high-expression plasmid pET21b and overexpressed in *Escherichia coli* BL21 (DE3) Star. The enzyme was purified in three steps to 99% purity. Crystals were obtained by the hanging-drop vapour-diffusion method at 291 K from solutions containing 25%(*w*/*v*) polyethylene glycol 2000 monomethylether, 0.2 *M* potassium thiocyanate, 0.1 *M* MES pH 6.5 in the presence of uridine 5′-diphospho-*N*-acetylmuramoyl alanyl glutamate (UDP-MurNAc-L-Ala-D-Glu) with and without 5′-adenylyl imidophosphate (AMP-PNP), a non-hydrolysable analogue of ATP. Diffraction data to 1.5 and 2.7 Å, respectively, were collected at the European Synchrotron Radiation Facility (ESRF). Crystals grown in the presence of two ligands belong to space group *P*1, with unit-cell parameters $a = 68.4$, $b = 71.4$, $c = 74.8$ Å, $\alpha = 73.4$, $\beta = 80.5$, $\gamma = 72.3^\circ$. Crystals grown in the presence of UDP-MurNAc-L-Ala-D-Glu alone belong to space group $P2_1$, with unit-cell parameters $a = 71.1$, $b = 129.4$, $c = 74.6$ Å, $\beta = 106.3^\circ$.

1. Introduction

Peptidoglycan (murein) is the major structural component of the bacterial cell wall, which resists internal osmotic pressure to prevent lysis. It is a continuous covalent macromolecule of polymerized linear glycan chains of *N*-acetylglucosamine residues alternating with *N*-acetylmuramic acid residues, to which is appended a pentapeptide chain. In the pneumococci and other Gram-positive pathogens, the pentapeptide consists of residues with alternating stereochemistry in the form L-Ala-D-*iso*-Gln-L-Lys-D-Ala-D-Ala and is cross-linked laterally by transpeptidation between the third (L-lysine) residue of a donor and fourth (D-alanine) residue of an acceptor pentapeptide chain. The pentapeptide is constructed by sequential addition of L-Ala, D-Glu, L-Lys and D-Ala-D-Ala dipeptide to UDP-MurNAc by the MurC, MurD, MurE and MurF enzymes, respectively, which are members of the ATP-dependent ligase family and act during the cytoplasmic stages of peptidoglycan biosynthesis. The conversion of D-Glu to D-*iso*-Gln occurs subsequently in the lipid-linked stage of peptidoglycan biosynthesis. MurE is the third ligase in the pathway and catalyses the addition of L-lysine to the nucleotide precursor UDP-MurNAc-L-Ala-D-Glu (Fig. 1), according to the reaction UDP-MurNAc-L-Ala-D-Glu + L-lysine + ATP ↔ UDP-MurNAc-L-Ala-D-Glu-L-Lys + ADP + P_i. The reaction is likely to occur by a similar mechanism to the other Mur ligases. This involves (i) phosphorylation of the C-terminal carboxylate of UDP-MurNAc-L-Ala-D-Glu by the γ-phosphate of ATP, with concomitant release of ADP to form an acyl phosphate intermediate, and (ii) nucleophilic attack of the latter by the α-amino group of L-lysine to form the tripeptide, with the release of inorganic phosphate (Gordon *et al.*, 2001; Falk *et al.*, 1996; Bouhss *et al.*, 1999).

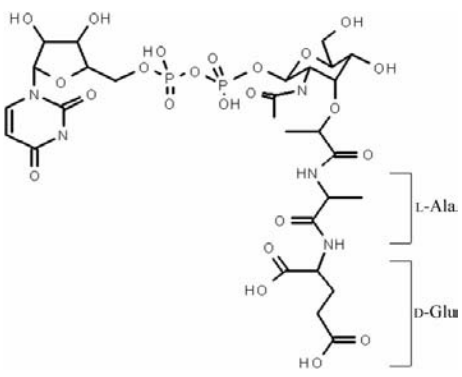

Figure 1
UDP-MurNAc-L-Ala-D-Glu, the substrate used in the crystallization of *S. pneumoniae* MurE 110K/70.

The incorporation of L-lysine in the third position of the pentapeptide is specific to *Streptococcus pneumoniae* and most other Gram-positive organisms, whereas in Gram-negative bacteria such as *Escherichia coli*, *meso*-diaminopimelic acid (m-DAP) is generally incorporated. MurE is highly specific in its choice of amino acid and only permits incorporation of either L-Lys or m-DAP into the pentapeptide, despite the coexistence of pools of both amino acids (Mengin-Lecreulx *et al.*, 1999). This ensures the presence of the specific amino acid (either m-DAP or L-Lys) at the third position of the pentapeptide, required for the lateral cross-linking that is vital for peptidoglycan integrity. Consequently, MurE has been shown to be essential for growth in the Gram-positive pathogen *Staphylococcus aureus* (Jana *et al.*, 2000) and has 50% amino-acid similarity to *S. pneumoniae* MurE. (Alignment carried out using *ClustalW*; Thompson *et al.*, 1994.)

The subtle difference in substrate specificities between MurE from *E. coli* (m-DAP specific) and *S. pneumoniae* MurE (L-Lys specific) is not reflected in the protein sequence, which shows only 28% identity between these two enzymes (Gordon *et al.*, 2001). Recently, Gordon *et al.* (2001) published the structure of m-DAP-specific *E. coli* MurE in complex with its substrates UDP-MurNAc-L-Ala-D-Glu and m-DAP. To determine the molecular basis of substrate binding, we undertook crystallization studies of lysine-specific MurE from *S. pneumoniae* in complexes with its substrates UDP-MurNAc-L-Ala-D-Glu and AMP-PNP in an attempt to compare the mechanism of substrate discrimination in these two enzymes.

Furthermore, *S. pneumoniae* MurE represents an important potential target for antibacterial chemotherapy, as penicillin resistance in this Gram-positive pathogen is a major public health concern (Filipe & Tomasz, 2000). Elucidation of the structure of a lysine-specific MurE may permit the design of novel specific inhibitors effective against antibiotic resistant Gram-positive pathogens.

2. Materials and methods

2.1. Cloning, overexpression and purification

The gene encoding *S. pneumoniae* MurE (481 amino acids; 53 828 Da) was amplified by PCR from *S. pneumoniae* 110K/70 chromosomal DNA with primers incorporating an *Nde*I site at the 5′ end of the gene and an *Xho*I site at the 3′ end. The PCR fragment was digested with these enzymes and ligated into a pET21b vector (Novagen) also digested with *Nde*I and *Xho*I. After selection of recombinants containing the cloned gene by single-colony PCR screening, *E. coli* BL21 (DE3) Star (Invitrogen) harbouring pRARE (Novagen) was transformed with MurE-pET21b. Cells were grown at 310 K in 650 ml LB medium containing 25 μg ml^{-1} carbenicillin and 3 μg ml^{-1} chloramphenicol. Protein expression was induced by addition of IPTG to a final concentration of 0.5 m*M* and growth was continued at 298 K for 4 h. Cells were harvested by centrifugation at 6400*g* and resuspended in 50 m*M* 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) pH 7.5, 1 m*M* $MgCl_2$, 2 m*M* β-mercaptoethanol, 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF), 2 μ*M* leupeptin, 2 μ*M* pepstatin A and 2.5 mg ml^{-1} chicken egg-white lysozyme prior to lysis by sonication.

The sonicated extract was clarified by centrifugation at 50 000*g* prior to overnight dialysis at 277 K into 50 m*M* sodium phosphate pH 7.0, 0.2 m*M* PMSF, 2 μ*M* leupeptin, 2 μ*M* pepstatin A (phosphate buffer) containing 300 m*M* NaCl. Dialysed protein was clarified by centrifugation at 50 000*g* and applied at 277 K to a 25 ml Talon (Clontech) affinity column charged with three column volumes of 50 m*M* cobalt chloride and equilibrated in phosphate buffer containing 300 m*M* NaCl. The column was raised to room temperature and washed with isocratic steps of phosphate buffer containing 1 *M* NaCl, 1 *M* NaCl and 5 m*M* imidazole, followed by 1 *M* NaCl and 10 m*M* imidazole. Protein eluted in phosphate buffer containing 1 *M* NaCl and 50 m*M* imidazole. Fractions containing MurE were pooled, concentrated in centrifugal concentrators and applied to a Superdex 75 HR 26/60 gel-filtration column (Amersham Biosciences) equilibrated in 20 m*M* 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris–HCl) pH 8.2, 100 m*M* NaCl, 1 m*M* $MgCl_2$, 2 m*M* β-mercaptoethanol, 0.2 m*M* PMSF, 2 μ*M* leupeptin, 2 μ*M* pepstatin A (Tris buffer). A single peak was resolved and fractions containing MurE were pooled and applied to a MonoQ HR5/5 anion-exchange column (Amersham Biosciences) equilibrated in Tris buffer. The column was developed by washing with a sodium chloride gradient in Tris buffer from 0 to 300 m*M* NaCl over two column volumes (CV), then from 300 to 600 m*M* NaCl over 30 CV and finally from 600 m*M* to 1 *M* NaCl over two CV. MurE eluted at 350 m*M* NaCl and the pooled peak fractions were judged by SDS–PAGE to be greater than 99% pure (Fig. 2). Protein was dialysed into 20 m*M* HEPES pH 7.5 and 50 m*M* NaCl and concentrated to 12 mg ml^{-1}, as estimated by protein absorbance at 280 nm. An eightfold molar excess (relative to MurE) of each substrate was added to the concentrated protein prior to crystallization. MurE was shown to be active *in vitro* using an assay to monitor the production of inorganic phosphate (data not shown).

UDP-MurNAc-L-Ala-D-Glu was synthesized *in vitro* (A. J. Lloyd, unpublished data) using recombinant *Pseudomonas aeruginosa* MurA, B, C and D enzymes (El Zoeiby *et al.*, 2001).

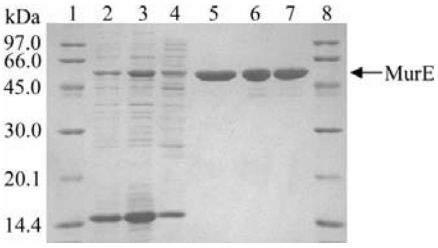

Figure 2
12% SDS–PAGE gel showing purification of *S. pneumoniae* MurE 110K/70. Lane 1, low-molecular-weight markers; lane 2, total protein lysate; lane 3, insoluble fraction; lane 4, soluble fraction; lane 5, peak fraction after immobilized cobalt-affinity chromatography; lane 6, peak fraction after size exclusion on Superdex 200; lane 7, peak fraction after anion exchange on MonoQ; lane 8, low-molecular-weight markers. 10 μg protein was loaded in lanes 2–7. The prominent band at ∼15 kDa visible in lanes 2–4 corresponds to the lysozyme used in preparation of the soluble extract.

2.2. Crystallization

All crystallization experiments were performed using the hanging-drop vapour-

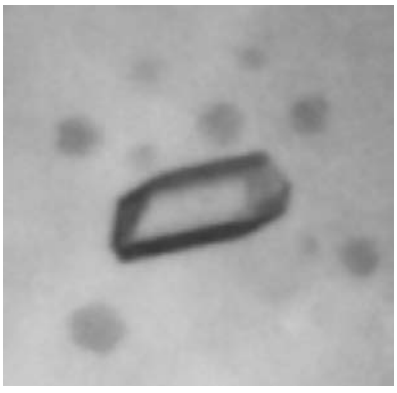

Figure 3
Photograph of single MurE crystal grown in the presence of UDP-MurNAc-L-Ala-D-Glu and AMP-PNP obtained in 25%(*w*/*v*) polyethylene glycol 2000 monomethylether, 0.2 *M* potassium thiocyanate, 0.1 *M* MES pH 6.5. The maximum dimension of the crystal is 0.2 mm.

Table 1
Data-collection and processing statistics.

Values in parentheses correspond to the outer resolution shell.

	UDP-MurNAc-L-Ala-D-Glu	UDP-MurNAc-L-Ala-D-Glu + AMP-PNP
Wavelength (Å)	0.931	0.931
Space group	$P2_1$	$P1$
Unit-cell parameters		
a (Å)	71.1	68.4
b (Å)	129.4	71.4
c (Å)	74.6	74.8
α (°)	90.0	73.4
β (°)	106.3	80.5
γ (°)	90.0	72.3
Matthews coefficient (Å^3 Da^{-1})	3.1	3.1
Molecules per AU	2	2
Solvent content (%)	60	60
Resolution range (Å)	59–2.70 (2.80–2.70)	47–1.50 (1.55–1.50)
Total observations	168513	881055
Unique reflections	34668	199950
Average $I/\sigma(I)$	9.1 (3.1)	30.6 (2.8)
$R_{\rm merge}$	0.156 (0.449)	0.048 (0.393)
Completeness (%)	97.6 (99.7)	96.8 (95.1)

diffusion method in a 24-well tissue-culture Linbro plate at 291 K. Initial crystallization trials were carried out using 0.5 ml reservoir solutions taken from the Hampton Research Crystal Screen (Jancarik & Kim, 1991) or using 0.5 ml solutions taken from the Clear Strategy Screen buffered with 0.1 *M* MES pH 6.5 (Brzozowski & Walton, 2001). Drops consisting of 1 µl of protein and 1 µl of reservoir solution were used throughout. Crystals were obtained after several days in condition 5 of Clear Strategy Screen I [25%(*w*/*v*) polyethylene glycol 2000 monomethyl ether, 0.2 *M* potassium thiocyanate, 0.1 *M* MES pH 6.5] in the presence of UDP-MurNAc-L-Ala-D-Glu, with and without AMP-PNP, a non-hydrolysable analogue of ATP in which the O atom bridging the β to the γ P atoms is replaced by an N atom. These crystals were of a quality suitable for X-ray data collection, with dimensions in excess of 0.05 × 0.07 × 0.2 mm (Fig. 3).

2.3. X-ray crystallographic studies

Preliminary diffraction data were collected at 100 K in-house using a Enraf–Nonius Cu $K\alpha$ X-ray generator operating at 45 kV and 115 mA equipped with Osmic focusing mirrors and a DIP 2030b image-plate collector and at SRS Daresbury beamline 14.1. Complete data sets were collected at ESRF ID14.3. The crystals were vitrified in liquid nitrogen using 15%(*v*/*v*) glycerol in the mother liquor as cryoprotectant and maintained at 100 K throughout data collection using an Oxford Cryosystems Cryostream. Diffraction data to 1.5 Å were collected at ESRF ID14.3 using crystals of space group $P1$ which were grown in the presence of both UDP-MurNAc-L-Ala-D-Glu and AMP-PNP.

Crystals of space group $P2_1$ grown in the presence of UDP-MurNAc-L-Ala-D-Glu alone diffracted to 2.7 Å resolution at ESRF ID14.3. This data set shows relatively poor merging statistics owing to increased mosaicity and smaller size. However, there is a strong indication that the correct space group is $P2_1$, because there are clear systematic absences along b^* and processing in $P1$ gives similarly poor merging statistics, 0.123 (0.397). All data-collection statistics are shown in Table 1. Intensity data for both crystals were indexed, integrated and scaled using the *HKL* programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

We are currently in the process of attempting a molecular-replacement solution using the coordinates of *E. coli* MurE (Gordon *et al.*, 2001; PDB code 1e8c), which shares 28% sequence identity with *S. pneumoniae* MurE. In the event that this is unsuccessful, we will obtain the phases by collecting sulfur SAD data or by MAD phasing using incorporated selenium.

We are grateful for access to and user support at the synchrotron facilities of the ESRF, Grenoble and SRS, Daresbury. We would like to thank Emilie Ugochukwu and Scott White (University of Birmingham) for assistance with data collection. We would also like to thank Roger Lévesque and Ahmed El Zoeiby (Université Laval, Ste-Foy, Québec, Canada) for their kind gift of recombinant enzymes used for *in vitro* synthesis of substrate. AMB is funded by a BBSRC studentship, AJL is funded by The Wellcome Trust and VF is a Royal Society University Research Fellow.

References

Bouhss, A., Dementin, S., van Heijenoort, J., Parquet, C. & Blanot, D. (1999). *FEBS Lett.* **453**, 15–19.
Brzozowski, A. M. & Walton, J. (2001). *J. Appl. Cryst.* **34**, 97–101.
El Zoeiby, A., Sanschagrin, F., Havugimana, P. C., Garnier, A. & Levesque, R. C. (2001). *FEMS Microbiol. Lett.* **201**, 229–235.
Falk, P. J., Ervin, K. M., Volk, K. S. & Ho, H. T. (1996). *Biochemistry*, **35**, 1417–1422.
Filipe, S. R. & Tomasz, A. (2000). *Proc. Natl Acad. Sci. USA*, **97**, 4891–4896.
Gordon, E., Flouret, B., Chantalat, L., van Heijenoort, J., Mengin-Lecreulx, D. & Dideberg, O. (2001). *J. Biol. Chem.* **276**, 10999–11006.
Jana, M., Luong, T. T., Komatsuzawa, H., Shigeta, M. & Lee, C. Y. (2000). *Plasmid*, **44**, 100–104.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Mengin-Lecreulx, D., Falla, T., Blanot, D., van Heijenoort, J., Adams, D. J. & Chopra, I. (1999). *J. Bacteriol.* **181**, 5909–5914.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). *Nucleic Acids Res.* **22**, 4673–4680.