Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification, crystallization and preliminary structural studies of dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase (RmlC), the third enzyme of the dTDP-L-rhamnose synthesis pathway, from *Salmonella enterica* serovar Typhimurium

Marie-France Giraud,[a] Fiona M. Gordon,[a] Chris Whitfield,[b] Paul Messner,[c] Stephen A. McMahon[a] and James H. Naismith[a]*

[a]Centre for Biomolecular Sciences, Purdie Building, University of St. Andrews, Fife KY16 9ST, Scotland, [b]Department of Microbiology, University of Guelph, Ontario N1G 2W1, Canada, and [c]Zentrum für Ultrastrukturforschung, Universität für Bodenkultur, A-1180, Vienna, Austria

Correspondence e-mail: naismith@st-andrews.ac.uk

Received 19 October 1998
Accepted 16 November 1998

L-Rhamnose is an essential component of the cell wall of many pathogenic bacteria. Its precusor, dTDP-L-rhamnose, is synthesized from α-D-glucose-1-phosphate and dTTP *via* a pathway requiring four distinct enzymes: RmlA, RmlB, RmlC and RmlD. RmlC was overexpressed in *Escherichia coli*. The recombinant protein was purified by a two-step protocol involving anion-exchange and hydrophobic chromatography. Dynamic light-scattering experiments indicated that the recombinant protein is monodisperse. Crystals were obtained using the sitting-drop vapour-diffusion method with ammonium sulfate as precipitant. Diffraction data were collected on a frozen crystal to a resolution of 2.17 Å. The crystal belongs to either space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 71.56$, $c = 183.53$ Å and $\alpha = \beta = 90$, $\gamma = 120^\circ$.

1. Introduction

L-Rhamnose is a key component of the cell wall of many pathogenic bacteria (Shibaev, 1986; McNeil *et al.*, 1990). A full listing of the established primary structures is available in the Complex Carbohydrate Structure Databank (http://www.ccrc.uga.edu). In mycobacteria, L-rhamnose is essential for cell-wall integrity as it connects the inner peptidoglycan layer to the arabinogalactan polysaccharides that are linked to the outer lipid layer of mycolic acids (McNeil *et al.*, 1990). In Gram-negative bacteria, L-rhamnose is often found in the O-antigen part of lipopolysaccharides. This portion of the molecule is often responsible for resistance to complement-mediated serum killing (Joiner, 1988). In *Streptococcus mutans*, the rhamnose-containing polysaccharide has been proposed to be responsible for the colonization of tooth surfaces (Michalek *et al.*, 1984) and adherence to heart, kidney and muscle tissues (Stinson *et al.*, 1980).

L-Rhamnose is incorporated in the mycobacterial cell wall from a nucleoside diphosphate precursor, dTDP-L-rhamnose (Mikusova *et al.*, 1996). In Gram-negative bacteria such as *Salmonella enterica* (Jiang *et al.*, 1991), *Shigella flexneri* (Rajakumar *et al.*, 1994), *Xanthomonas campris* (Köplin *et al.*, 1993) and *Escherichia coli* K12 (Stevenson *et al.*, 1994), as in *Streptococcus mutans* and mycobacteria (Tsukioka *et al.*, 1997; Ma *et al.*, 1997), four enzymes, RmlA, RmlB, RmlC and RmlD, are required to synthesize dTDP-L-rhamnose from α-glucose-1-phosphate and dTTP (Fig. 1).

Because of the importance of L-rhamnose in many pathogenic bacteria, all the enzymes involved in its synthesis are potential targets for the design of novel therapeutic inhibitors. To this end, we have initiated the structural study of the four enzymes involved in its synthesis. Here, we describe the purification, crystallization and preliminary structural studies of dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase (RmlC). Bacterial RmlC are not related in sequence to any other known epimerase.

2. RmlC over-expression and purification

The open reading frame of the gene encoding the dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase (RmlC) was amplified by PCR using primers that incorporated a 5′ *Nde*I site and a *Sst*I site to facilitate cloning in the pET30a(+) vector. Expression involves the IPTG-inducible T7 promoter and ribosome-binding sites conferred by the vector, but uses the natural *rmlC* initiation ATG codon. The sequence of the amplified and cloned gene was confirmed to be identical to the chromosomal copy. The expressed protein is therefore strictly identical to the authentic product and carries no extensions or mutations. BL21(DE3) cells transformed with this plasmid were grown at 310 K on Terrific Broth (Maniatis *et al.*, 1982) containing 80 μg ml^{-1} kanamycin until the OD_{600} reached 0.6–0.8. Overexpression was induced by addition of 1 m*M* IPTG. After 3.5 h of culture at 310 K, the cells were harvested by

centrifugation (10 min, 6000*g*, 277 K) and suspended in 100 m*M* NaCl, 2 m*M* DTT, 5 m*M* PMSF, 20 µ*M* lysozyme, 20 m*M* Tris–HCl pH8.

After 30 min of incubation at room temperature, the viscosity of the mixture was decreased by addition of DNAase (20 µg^{-1} ml) and by sonication (four cycles of 30 s interrupted by 1 min periods on ice). After addition of 1 m*M* EDTA, the mixture was centrifuged for 30 min at 20000*g* and 277 K. The supernatant was brought to 20% ammonium sulfate saturation and incubated for 1 h at 277 K. After a second centrifugation (20 min, 20000*g*, 277 K), the supernatant was dialysed against three changes of 2 l of 50 m*M* NaCl, 20 m*M* Tris–HCl pH 8.5. DTT was added to a final concentration of 2 m*M* and the filtered supernatant passed through a POROS-HQ HPLC column (BiocadSprint system). Proteins were eluted with a 50–500 m*M* NaCl gradient. A protein with a molecular weight corresponding to RmlC ($M_r \simeq$ 20.6 kDa) was found in a peak eluted at 250 m*M* NaCl. Fractions corresponding to this peak were pooled, concentrated with an Amicon filter and dialysed against two changes of 1 l of 20 m*M* sodium phosphate pH 7.3. Ammonium sulfate was added gradually to 30% saturation and DTT was added to a final concentration of 2 m*M*. The filtered protein sample was loaded on a POROS high-density phenyl HPLC column (BiocadSprint system) equilibrated in buffer *A* (30% ammonium sulfate, 20 m*M* sodium phosphate pH 7.3). Elution was performed with an increasing gradient of buffer *B* (20 m*M* sodium phosphate pH 7.3); the 20.6 kDa protein was eluted at 75% buffer *B*.

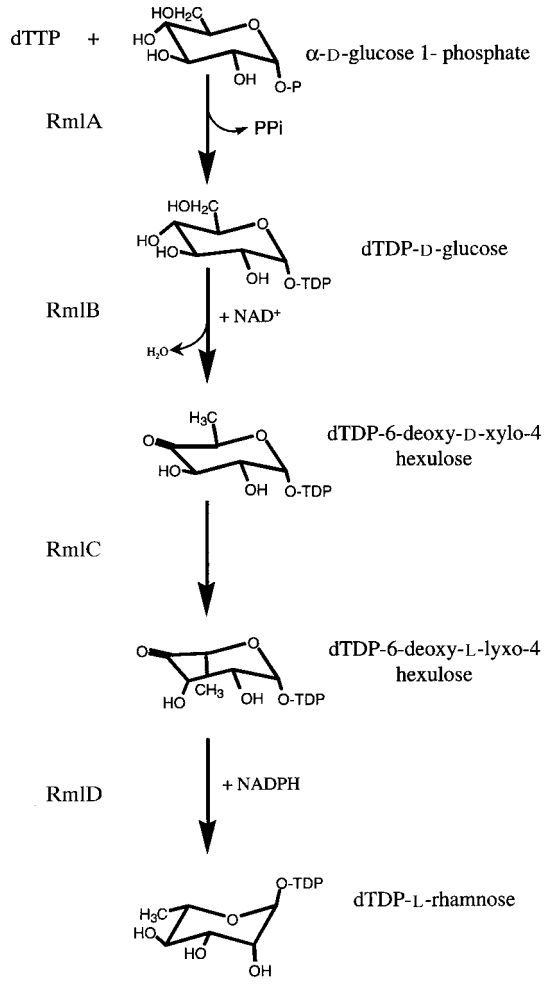

Figure 1
The dTDP-L-rhamnose biosynthetic pathway. RmlA, α-D-glucose-1-phosphate thymidylyltransferase; RmlB, dTDP-glucose 4,6-dehydratase; RmlC, dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase; RmlD, dTDP-6-deoxy-L-xylo-4-hexulose-4-reductase.

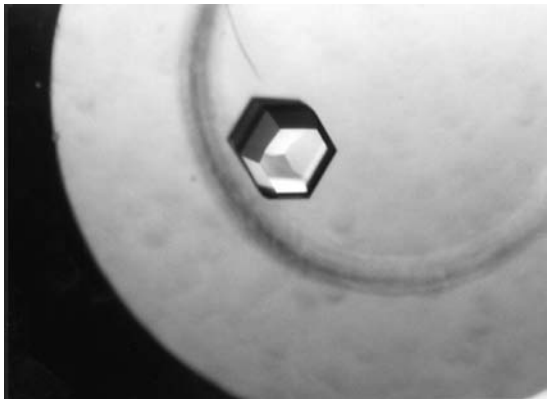

Figure 2
Photograph of an RmlC crystal.

3. Protein analysis

After the two HPLC steps, the protein appeared to be pure as judged on an SDS silver-stained gel. Light-scattering experiments indicated the protein was monodisperse with an apparent molecular weight of 42 kDa, consistent with a dimer. N-terminal sequencing confirmed that the protein was RmlC with an MMIVI N-terminal extremity, with less than 5% of the initiating methionine removed. The final yield of purification was 15 mg l^{-1} of Terrific Broth.

Protein concentration was estimated by the Bradford method (Bradford, 1976).

4. RmlC crystallization

After the final purification step, RmlC was dialysed against three changes of 2 l of 25 m*M* Tris–HCl pH 7.75 and concentrated to 3.75 mg ml^{-1}, and DTT was then added to a final concentration of 5 m*M*. Crystals were grown with 7 µl protein solution and 7 µl precipitant (1.6 *M* ammonium sulfate, 0.1 *M* MES pH 6.1) using the sitting-drop vapour-diffusion method (Ducruix & Giegé, 1992). Hexagonal shaped crystals grew in 6 d (Fig. 2).

5. Data collection

Data on frozen crystals (~0.6 × 0.4 × 0.4 mm) were obtained after a 30 min soak in cryoprotectant (20% glycerol, 2 *M* ammonium sulfate, 0.1 *M* MES pH 6.1). 2.65 Å data were collected at 125 K using the Nonius/Macscience DIP2000 image plate. X-rays were generated at a wavelength of 1.54 Å from a Nonius FR591 rotating-anode generator and focused with MacScience mirrors. The crystal-to-detector distance was 150 mm. Data were recorded as 62 non-overlapping 20 min 1° oscillations.

2.17 Å synchrotron data were recorded on a frozen crystal at 110 K at the ESRF BM14 beamline at a wavelength of 0.979 Å using a 30 cm MAR Research image plate. The crystal-to-detector distance was 300 mm. Data were recorded as 36 non-overlapping 1 min 1° oscillations.

Data on derivatives were recorded at Daresbury Laboratory at 125 K using a 30 cm MAR Research image plate at beamline 9.5.

The reflections were indexed in a hexagonal space group ($a = b = 71.76$, $c = 183.09$ Å; $\alpha = \beta = 90$, $\gamma = 120°$) with *DENZO* and could be scaled in space groups $P3_121$ or $P3_221$ using *SCALEPACK* (Otwinowski, 1993). The V_m value (Matthews, 1968) was 3.3 Å^3 Da^{-1} for two molecules per asymmetric unit and 2.4 Å^3 Da^{-1} for three molecules per asymmetric unit. The self-rotation function (Collaborative Computational Project, Number 4, 1994) has no peak for $\kappa = 120°$ but several peaks for $\kappa = 180°$, strongly suggesting that the asymmetric unit contains a dimer. Table 1 summarizes the two sets of data.

Table 1
Data collection.

Values in parentheses refer to the highest resolution shell.

	Data obtained in-house	Data obtained at the ESRF
Resolution (Å)	25–2.65	40–2.17
Highest resolution shell (Å)	2.74–2.65	2.25–2.17
Space group	$P3_121$ or $P3_221$	$P3_121$ or $P3_221$
Unit-cell parameters (Å, °)	$a = b = 71.56$, $c = 183.5$; $\alpha = \beta = 90$, $\gamma = 120$	$a = b = 71.56$, $c = 183.5$; $\alpha = \beta = 90$, $\gamma = 120$
V_m (two molecules per asymmetric unit) (Å^3 Da^{-1})	3.3	3.3
Percentage solvent	64.4	64.4
Unique reflections	16155	27346
I/σ	15.1 (2.3)	19.5 (1.9)
Average redundancy	3.0 (2.0)	2.2 (2.09)
Data completeness (%)	97.1 (86.2)	92.1 (80.7)
$R_{\rm merge}$† (%)	7.5 (27.3)	4.7 (38.4)

† $R_{\rm merge} = \sum\sum I(h)j - \langle I(h)\rangle / \sum\sum I(h)j$ where $I(h)$ is the measured diffraction intensity and the summation includes all observations.

6. Derivatization

A partial data set (50%) was recorded at Daresbury Laboratory on a derivative obtained with a 1 h soak in 1 m*M* $HgCl_2$. This derivative had four Hg atoms bound per unit cell with a phasing power of 1.6 and Cullis *R* factors of 0.64 for acentric data and 0.5 for centric data. We have produced other mercury derivatives and selenomethionine-enriched protein and will determine the RmlC structure shortly.

The use of the CCLRC Daresbury Laboratory UK and ESRF-Grenoble facilities is gratefully acknowledged. We thank The Wellcome Trust for an international travel grant.

References

Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Ducruix, A. & Giegé, R. (1992). *Crystallization of Nucleic Acids and Proteins, a Practical Approach.* Oxford: IRL Press.
Jiang, X.-M., Neal, B., Santiago, F., Lee, S. J., Romana, L. K. & Reeves, P. R. (1991). *Mol. Microbiol.* **5**, 695–713.
Joiner, K. A. (1988). *Ann. Rev. Microbiol.* **42**, 201–230.
Köplin, R., Wang, G., Hötte, B., Priefer, U. B. & Pühler, A. (1993). *J. Bacteriol.* **175**, 7786–7792.
Ma, Y., Mills, J. A., Belisle, J. T., Vissa, V., Howell, M., Bowlin, K., Sherman, M. S. & McNeil, M. (1997). *Microbiology*, **143**, 937–945.
McNeil, M., Daffé, M. & Brennan, P. J. (1990). *J. Biol. Chem.* **265**, 18200–18206.
Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Michalek, S. M., Morisaki, I., Gregory, R. L., Kimura, S., Harmon, C. C., Hamada, S., Kotani, S. & McGene, J. R. (1984). *Protides Biol. Fluids Proc. Colloq.* **32**, 47–52.
Mikusova, K., Mikus, M., Besra, G., Hancock, I. & Brennan, P. J. (1996). *J. Biol. Chem.* **271**, 7820–7828.
Otwinowski, Z. (1993). *Proceedings of the CCP4 Study Weekend*, edited by L. Sawyer, N. Isaacs & S. W. Bailey, pp. 56–62. Warrington: Daresbury Laboratory.
Rajakumar, K., Jost, B. H., Sasakawa, C., Okada, N., Yoshikawa, M. & Adler, B. (1994). *J. Bacteriol.* **176**, 2362–2373.
Shibaev, V. N. (1986). *Adv. Carbohydr. Chem. Biochem.* **44**, 277–339.
Stevenson, G., Neal, B., Liu, D., Hobbs, M., Packer, N. H., Batley, M. Redmond, J. W., Lindquist, L. & Reeves. P. (1994) *J. Bacteriol.* **176**, 4144–4156.
Stinson, M. W., Nisengard, R. J. & Bergey, E. J. (1980). *Infect. Immun.* **27**, 604–613.
Tsukioka, Y., Yamashita, Y., Nakano, Y., Oho, T. & Koga, T. (1997) *J. Bacteriol.* **179**, 4411–4414.