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Overexpression, purification, crystallization and preliminary structural study of dTDP-6-deoxy-L-*lyxo*-4-hexulose reductase (RmlD), the fourth enzyme of the dTDP-L-rhamnose synthesis pathway, from *Salmonella enterica* serovar Typhimurium

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L-Rhamnose is an essential component of the cell wall of many pathogenic bacteria. Its precursor, 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. RmlD catalyses the terminal step of this pathway by converting dTDP-6-deoxy-L-*lyxo*-4-hexulose to dTDP-L-rhamnose. RmlD from *Salmonella enterica* serovar Typhimurium has been 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 of native and selenomethionine-enriched RmlD have been obtained using the sitting-drop vapour-diffusion method with polyethylene glycol as precipitant. Diffraction data have been collected from orthorhombic crystals of both native and selenomethionyl-derivatized protein, allowing tracing of the protein structure.

1. Introduction

L-Rhamnose is a key component of the cell wall of pathogenic bacteria (Shibaev, 1986; McNeil *et al.*, 1990). In mycobacteria, this sugar is essential to the integrity of the cell wall, as it connects the inner peptidoglycan layer to the arabinogalactan polysaccharides that are linked to the outer lipid layer (McNeil *et al.*, 1990). In a variety of Gram-negative bacteria, L-rhamnose is often found in the O-antigen part of lipopolysaccharides and these molecules are essential for resistance to complement-mediated serum killing (Joiner, 1988; Burns & Hull, 1998). The L-rhamnose-containing capsule of *Streptoccocus mutans* has also been proposed to play a role in the colonization of tooth surfaces (Michalek *et al.*, 1984) and adherence to kidney, muscles and heart tissues (Stinson *et al.*, 1980).

L-Rhamnose is incorporated into the cell wall from a precursor, dTDP-L-rhamnose (deoxythymidine diphosphate-L-rhamnose; Mikusova *et al.*, 1996). The synthesis of this precursor from α-D-glucose-1-phosphate and dTTP has been described by Kornfeld & Glaser (1961). In bacteria such as *Salmonella enterica* (Jiang *et al.*, 1991), *Shigella flexneri* (Rajakumar *et al.*, 1994), *Xanthomonas campestris* (Köplin *et al.*, 1993) and *Escherichia coli* K-12 (Stevenson *et al.*, 1994), as well as in *Strep. mutans* and mycobacteria (Tsukioka *et al.*, 1997; Ma *et al.*, 1997), four enzymes are required in this biosynthetic pathway (Fig. 1). The first, RmlA (α-D-glucose-1-phosphate thymidylyl transferase), catalyses the transfer of dTMP to α-D-glucose-1-phosphate to generate dTDP-D-glucose. RmlB (dTDP-D-glucose 4,6-dehydratase) then converts dTDP-D-glucose to dTDP-6-deoxy-D-*xylo*-4-hexulose. The third enzyme, RmlC (dTDP-6-deoxy-D-*xylo*-4-hexulose 3,5-epimerase), catalyses the epimerization of dTDP-6-deoxy-D-*xylo*-4-hexulose to dTDP-6-deoxy-L-*lyxo*-4-hexulose. In the final step, RmlD (dTDP-6-deoxy-L-*lyxo*-4-hexulose reductase) converts dTDP-6-deoxy-L-*xylo*-4-hexulose to dTDP-L-rhamnose.

Since dTDP-L-rhamnose synthesis does not occur in humans, any enzyme in its metabolic pathway can be considered a valid target for the design of new therapeutic agents. This is supported by the recent results of Burns & Hull (1998) obtained with mutants of the uropathogenic *E. coli* O75:K5, in which wild-type bacteria were able to grow and multiply in serum, whereas bacteria with *rmlD* mutations were rapidly killed under the same conditions. In order to find new therapeutic drugs, we have initiated the structural study of the four proteins of this pathway. We have recently elucidated the structure of RmlC (Giraud *et al.*, 1999; Giraud *et al.*, in preparation) and here we report the overexpression, purification, crystallization and the preliminary structural study of RmlD. RmlD overexpressed in *E. coli* is active (Graninger *et al.*, 1999) and tightly binds

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NADPH (K_m = 13 ± 2 μ*M*). RmlD shows 15–20% sequence identity with proteins of the reductase/epimerase/dehydrogenase (RED) protein superfamily, which includes the short-chain dehydrogenase family (SDR family; Jörnvall *et al.*, 1999). Like proteins of this superfamily, RmlD contains a typical NAD(P)-binding motif (G*xx*G*xx*G) in its N-terminal part. The crystal structures of two proteins of the RED superfamily, UDP-galactose epimerase (Thoden & Holden, 1998) and GDP-4-keto-6-deoxy-D-mannose epimerase/reductase (Rizzi *et al.*, 1998), have been determined and both contain a catalytic triad consisting of a serine, a tyrosine and a lysine. The catalytic tyrosine and lysine belong to a conserved motif (Y*xxx*K) found in all proteins of this superfamily and the catalyic serine residue is located upstream of this motif. RmlD has also the characteristic (Y*xxx*K) motif and a conserved serine residue.

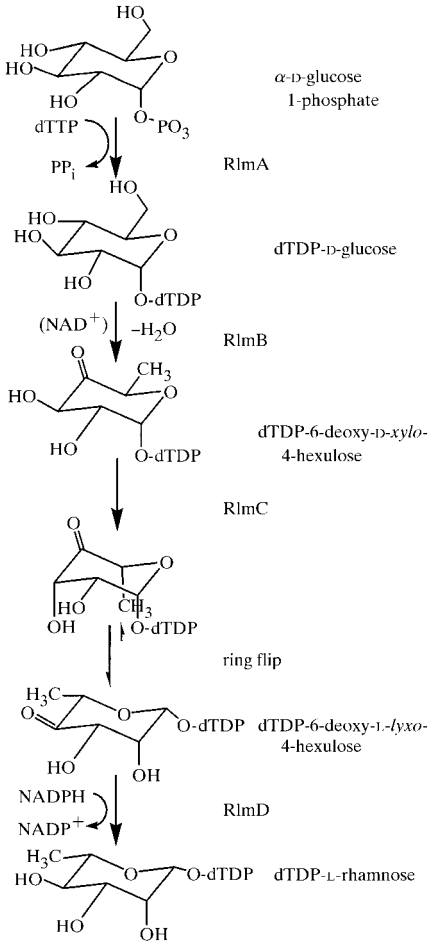

Figure 1
The dTDP-L-rhamnose biosynthetic pathway. RmlA: α-D-glucose-1-phosphate thymidylyl transferase. RmlB: dTDP-D-glucose 4,6-dehydratase. RmlC: dTDP-6-deoxy-D-*xylo*-4-hexulose 3,5-epimerase. RmlD: dTDP-6-deoxy-L-*lyxo*-4-hexulose reductase.

2. RmlD overexpression

The gene encoding the dTDP-6-deoxy-L-*lyxo*-4-hexulose reductase (RmlD, E.C. 1.1.1.133) was amplified by PCR using primers that incorporated a 5′ *Nde*I and a 3′ *Sst*I restriction site to facilitate cloning in the pET30a(+) vector. Expression involves the IPTG (isopropyl-β-D-thiogalactopyranoside) inducible T7 promoter and ribosome-binding sites conferred by the vector, but uses the natural *rmlD* initiation ATG codon. The sequence of the amplified and cloned gene was confirmed to be identical to the chromosomal copy. The expressed protein is identical in amino-acid sequence to the authentic product and carries no extensions or mutations.

For native RmlD overexpression, *E. coli* BL21(λDE3) cells were transformed with this plasmid and grown at 310 K in Terrific Broth (Maniatis *et al.*, 1982) containing 80 mg l^{-1} kanamycin until the OD_{600} reached 0.6–0.8. Overexpression was induced by addition of 1 m*M* IPTG and after 3 h of culture at 310 K the cells were harvested by centrifugation (10 min, 6000*g*, 277 K).

Selenomethionine-enriched RmlD was produced using the protocol described by Budisa *et al.* (1997). Briefly, *met*$^-$ B834(λDE3) cells were transformed with the plasmid and grown on MMN medium: 7.5 m*M* ammonium sulfate, 8.5 m*M* NaCl, 55 m*M* KH_2PO_4, 100 m*M* $KHPO_4$, 1 m*M* $MgSO_4$, 20 m*M* glucose, 1 mg l^{-1} $CaCl_2$, 1 mg l^{-1} $FeSO_4$, 1 μg l^{-1} K_2MoO_4, 1 μg l^{-1} $ZnSO_4$, 1 μg l^{-1} $MnCl_2$, 1 g l^{-1} $CuCl_2$, 1 mg l^{-1} thiamine, 20 mg l^{-1} biotin, 80 mg l^{-1} kanamycin without amino acids. Precultures were grown in this medium containing 0.3 m*M* L-methionine. 2 ml of the preculture were centrifuged and the cell pellet was washed with 1 ml of MMN medium containing 58 mg l^{-1} of L-selenomethionine (Sigma). The cells were then transferred to 1 l of the same medium. 1 m*M* IPTG was added when the OD_{600} reached 0.6 and after 16 h of induction at 310 K the cells were harvested by centrifugation.

3. Purification of RmlD

The cells were lysed in 150 m*M* NaCl, 2 m*M* dithiothreitol (DTT), 5 m*M* phenylmethyl sulfonyl fluoride (PMSF), 20 μ*M* lysozyme, 20 m*M* Tris–HCl pH 9. After 30 min of incubation at 277 K, the viscosity of the mixture was decreased by addition of DNAase (20 μg ml^{-1}) and by sonication (four cycles of 30 s interrupted by 1 min periods on ice).

The mixture was then centrifuged for 30 min at 30000*g* and 277 K. After addition of 1 m*M* ethylenediamine tetraacetic acid (EDTA), 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 two changes of 2 l of 20 m*M* Tris–HCl pH 9. 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 0–500 m*M* NaCl gradient. A protein with a molecular weight corresponding to RmlD (32.5 kDa) was found in a peak eluted at 30 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 7.8. Ammonium sulfate was added gradually to 25% saturation and DTT added to a final concentration of 2 m*M*. The filtered protein sample was loaded on a POROS-HP HPLC column (BiocadSprint system) equilibrated in buffer *A* (25% ammonium sulfate, 20 m*M* sodium phosphate pH 7.8). Elution was performed with an increasing gradient of buffer *B* (20 m*M* sodium phosphate pH 7.8). The 32.5 kDa protein was eluted at 50% of buffer *B*.

Purification protocols for native RmlD and selemothionine-enriched RmlD were the same, except that for the latter all the buffers were degassed and contained 10 m*M* DTT in order to prevent selenomethionine oxidation.

4. 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 a hydrodynamic radius of 3.2 nm and a molecular weight of 49.5 Da. The weight derived from light scattering assumes a spherical molecule. Although the solution species is clearly well defined, it is not possible to confidently assign it as a monomer or a dimer on the basis of light scattering. The solution species is presumably rather anisotropic in shape.

N-terminal sequencing identified the N-terminus MNILL, consistent with that predicted by the nucleotide sequence for RmlD. The molecular weight determined from mass spectroscopy for the native protein was 32539 Da, which is in agreement with the molecular weight deduced from the sequence (32557 Da). The spectrum of selenomethionine-enriched protein

Table 1
Data-collection statistics.

(*a*) Data obtained in-house on a room-temperature crystal. (*b*) Data obtained at the Daresbury Laboratory on a frozen crystal. (*c*) Three-wavelength MAD data set obtained at the ESRF on a frozen selenomethionine-enriched protein crystal. Values in parentheses refer to the highest resolution shell. All data were indexed with *DENZO* and merged with *SCALEPACK* (Otwinowski, 1993).

	(*a*)	(*b*)	(*c*)		
Wavelength (Å)	1.54	0.98	0.9791	0.9790	0.8856
Resolution (Å)	30–3.1	40–2.5	30–2.6	30–2.6	30–2.4
High-resolution shell (Å)	3.21–3.1	2.59–2.5	2.66–2.60	2.66–2.60	2.46–2.40
Space group	$P2_12_12$	$P2_12_12$	$P2_12_12$		
Temperature (K)	293	110	110	110	110
Unit-cell parameters (Å, °)	a = 48.72, b = 72.72, c = 84.93, $\alpha = \beta = \gamma = 90$	a = 47.75, b = 72.40, c = 82.70, $\alpha = \beta = \gamma = 90$	a = 47.49, b = 72.06, c = 82.59, $\alpha = \beta = \gamma = 90$		
V_m† (Å^3 Da^{-1})	2.31	2.20	2.17		
Percentage solvent	46.35	43.54	42.88		
Unique reflections	5746	10411	9237	9247	11614
I/σ	15.3 (2.3)	17.3 (1.9)	20.3 (5.7)	20.3 (5.3)	19.1 (3.8)
Average redundancy	3.6 (3.5)	3.6 (3.4)	2.1 (2.0)	2.1 (2.0)	2.1 (2.1)
Data completeness (%)	93.6 (96.7)	92.1 (94.5)	99.9 (99.9)	99.9 (99.5)	99. (99.1)
$R_{\rm merge}$‡ (%)	8.4 (32.4)	6.2 (40.5)	3.6 (15.9)	3.2 (13.8)	5.2 (21.8)

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

showed only one major peak with an increase of 193 Da. Since RmlD contains four methionines, this is consistent with a full incorporation of selenomethionine.

The final yield of purification was 38 mg l^{-1} of Terrific Broth for native RmlD and 12 mg l^{-1} of MMN for selenomethionine-enriched RmlD, as estimated by the Bradford method (Bradford, 1976).

5. RmlD crystallization

After the final purification step, the protein sample was dialysed against two changes of 2 l of 50 m*M* Tris–HCl pH 7.8 and concentrated to 3 mg ml^{-1}. DTT and NADPH were added to a final concentrations of 4 m*M* and 2.5 m*M*, respectively. Crystals were grown with 10 µl of protein solution and 10 µl of precipitant (0.1 *M* HEPES pH 7.1, 22% PEG 4K, 0.2 *M* $MgCl_2$) using the sitting-drop vapour-diffusion method (Ducruix & Giegé, 1992). Crystals of native or selenomethionine-enriched RmlD (Fig. 2) grew in 6 d.

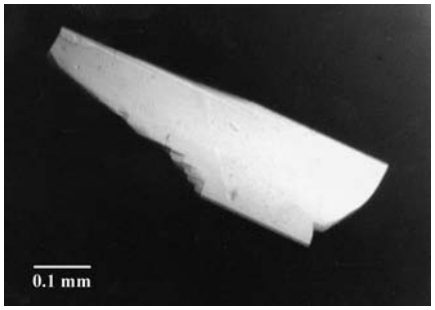

Figure 2
Photograph of a selenomethionine-enriched RmlD crystal (1.8 × 0.6 × 0.1 mm) taken with a polarizer.

6. Data collection

A 3.1 Å resolution data set was collected in-house at 293 K from a single crystal mounted in a glass capillary using a 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 170 mm. Data were recorded as 90 non-overlapping 30 min 1° oscillations. Crystals can be cryoprotected by a 45 s soak in a solution containing 23% PEG 4K, 0.1 *M* HEPES pH 7.1, 0.2 *M* $MgCl_2$ and 15% PEG 600. A 2.50 Å resolution data set from a frozen crystal of the native protein was measured at a wavelength of 0.98 Å at 110 K at Daresbury Laboratory station PX9.5 using a 30 cm MAR image plate as the detector. The data were integrated with the program *DENZO* and merged with *SCALEPACK* (Otwinowski, 1993). RmlD crystals are orthorhombic with space group $P2_12_12$. Unit-cell dimensions were a = 47.75, b = 72.40, c = 82.70 Å for the frozen crystal. Given the above cell, the V_m value (Matthews, 1968) was 2.20 Å^3 Da^{-1}, with a solvent content of 43.54%. The calculated Matthews coefficient for two molecules per asymmetric unit was 1.10 Å^3 Da^{-1}, indicating that the asymmetric unit contains only one molecule. Table 1 summarizes the two sets of data.

We have also collected a three-wavelength MAD data set to a resolution of 2.4 Å on BM14 at the ESRF–Grenoble from a single selenomethionine-enriched RmlD crystal (Table 1) and are currently tracing the structure.

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