Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

The purification, crystallization and structural elucidation of dTDP-D-glucose 4,6-dehydratase (RmlB), the second enzyme of the dTDP-L-rhamnose synthesis pathway from *Salmonella enterica* serovar Typhimurium

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Received 27 October 1999
Accepted 9 December 1999

dTDP-D-glucose 4,6-dehydratase (RmlB) is the second of four enzymes involved in the dTDP-L-rhamnose pathway and catalyzes the dehydration of dTDP-D-glucose to dTDP-4-keto-6-deoxy-D-glucose. The ultimate product of the pathway, dTDP-L-rhamnose, is the precursor of L-rhamnose, which is a key component of the cell wall of many pathogenic bacteria. RmlB from *Salmonella enterica* serovar Typhimurium has been overexpressed and purified, and crystals of the enzyme have been grown using the sitting-drop vapour-diffusion technique with lithium sulfate as precipitant. Diffraction data have been obtained to a resolution of 2.8 Å on a single frozen RmlB crystal which belongs to space group $P2_1$, with unit-cell parameters $a = 111.85$, $b = 87.77$, $c = 145.66$ Å, $\beta = 131.53°$. The asymmetric unit contains four monomers in the form of two RmlB dimers with a solvent content of 62%. A molecular-replacement solution has been obtained and the model is currently being refined.

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1. Introduction

L-Rhamnose is a 6-deoxyhexose naturally occurring in several complex carbohydrates (Graninger *et al.*, 1999). It is a vital component of the surface antigens of many microorganisms, specifically being one of the important residues of the 'O' antigen of lipopolysaccharide, an important virulence factor of many Gram-negative bacteria (Klena & Shnaitman, 1994). In Gram-positive bacteria such as the streptococci and mycobacteria, rhamnose is found in the cell-wall polysaccharides (Schleifer & Kilpper-Bälz, 1987; McNeil *et al.*, 1990). For example, *Streptococcus suis* type 2, which was first isolated in 1966, is an important zoonotic pathogen causing a wide spectrum of porcine diseases including arthritis, meningitis and endocarditis (Staats *et al.*, 1997), as well as being one of the major causes of human adult meningitis in Hong Kong (Lutticken *et al.*, 1986). In animals, control of this pathogen by vaccines and conventional antimicrobials has largely been ineffective, partly owing to increased resistance by *Strep. suis* type 2 to antimicrobials and a lack of information regarding its virulence factors (Staats *et al.*, 1997). Recent work by Smith *et al.* (1999) has shown that loss of the *Strep. suis* type 2 capsule correlates with a complete attenuation of the organism's virulence. Although the structure of the *Strep. suis* type 2 capsule is unknown, rhamnose is an important constituent (Elliott & Tai, 1978), making the rhamnose biosynthetic pathway a potential therapeutic target.

Four enzymes, α-D-glucose-1-phosphate thymidylyl transferase (RmlA), dTDP-D-glucose 4,6-dehydratase (RmlB), dTDP-6-deoxy-D-*xylo*-4-hexulose 3,5-epimerase (RmlC) and dTDP-6-deoxy-L-*lxyo*-4-hexulose-4-reductase (RmlD) are required for the synthesis of dTDP-L-rhamnose from α-glucose-1-phosphate and dTTP. Significantly, the enzymes involved in the dTDP-L-rhamnose pathway are highly conserved amongst microorganisms (Marolda & Valvano, 1995; Graninger *et al.*, 1999).

The first step in the dTDP-L-rhamnose pathway is the synthesis of dTDP-D-glucose from α-D-glucose-1-phosphate and dTTP, catalyzed by RmlA. RmlB (E.C. 4.2.1.46) catalyzes the subsequent dehydration of dTDP-D-glucose to dTDP-4-keto-6-deoxy-D-glucose. There is a large body of evidence for the reaction mechanism of RmlB (Fig. 1*a*), which includes the formation of a 4-keto intermediate (Glaser & Zarkowsky, 1971; Snipes *et al.*, 1977; Tonetti *et al.*, 1998). Work on UDP-galactose 4-epimerase has helped to provide further information on the RmlB mechanism (Thoden & Holden, 1998; Thoden

Table 1
Data collection on beamline 7.2, Daresbury SRS.

Values in parentheses refer to the highest resolution shell.

	Data obtained at the SRS
Wavelength (Å)	1.488
Resolution (Å)	30–2.8 (2.9–2.8)
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 111.85, b = 87.77, c = 145.66, β = 131.53
V_m (four molecules per asymmetric unit) (Å Da^{-1})	3.27
Percentage solvent	62
Total measurements	210905
Unique reflections	51729
I/σ	18.65 (3.79)
Average redundancy	4.0 (4.14)
Data completeness (%)	100 (99.9)
R_{merge}† (%)	7.4 (30.93)

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

et al., 1996*a*,*b*, 1997; Yijeng *et al.*, 1997). In common with RmlB, UDP-galactose 4-epimerase is a member of the 'short-chain' dehydrogenase superfamily and contains the catalytic triad of serine, tyrosine and lysine. UDP-galactose 4-epimerase catalyzes the interconversion of UDP-galactose and UDP-glucose (Fig. 1*b*), its mechanism also proceeding through a 4-keto intermediate. The first step for RmlB and UDP-galactose 4-epimerase is an initiation of catalysis, involving the transfer of hydrogen from the C-4 of the sugar moiety to the enzyme-bound NAD^+. The 4-keto intermediate compound produced by RmlB undergoes an elimination of H_2O from C-5 and C-6 to give a 4-ketoglucose-5,6-ene derivative (Snipes *et al.*, 1977); however, with UDP-galactose 4-epimerase there is a putative rotation of the 4-keto intermediate in the active site to facilitate the transfer of the hydride from NADH to the opposite face of the sugar (Kang *et al.*, 1975). RmlB from *S. enterica* exists as a homodimer, comprising two 40.7 kDa subunits each containing 361 amino-acid residues and one bound NAD^+ moiety. Wang & Gabriel (1969) have demonstrated that removal of NAD^+ from *Escherichia coli* RmlB results in the dissociation of the dimer into two monomers and the loss of enzyme activity.

The significance of L-rhamnose in many pathogenic bacteria makes the four enzymes involved in dTDP-L-rhamnose synthesis potential targets for novel therapeutics. Further detailed studies on RmlB and its comparison with UDP-galactose 4-epimerase will hopefully lead to a greater understanding of its catalytic mechanism. Here, we describe the overexpression, purification and crystallization of RmlB from *S. enterica* serovar Typhimurium and its structural elucidation by molecular replacement using RmlB from *E. coli* as a model (PDB code 1bxk).

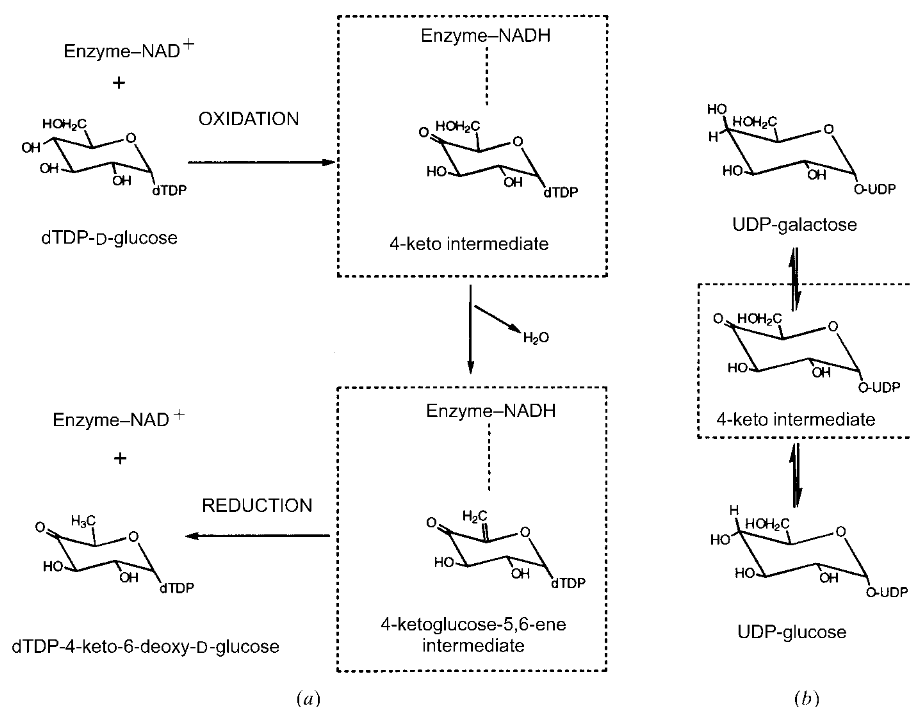

Figure 1
(*a*) The proposed catalytic mechanism of dTDP-D-glucose 4,6-dehydratase (RmlB). The first step is a reduction of the enzyme-bound NAD^+, with the formation of a 4-keto intermediate which remains bound to the protein. This is followed by the elimination of a water molecule from C-6 with the subsequent reoxidation of the dinucleotide and formation of the final product dTDP-4-keto-6-deoxy-D-glucose (Glaser & Zarkowsky, 1971; Snipes *et al.*, 1977; Tonetti *et al.*, 1998). (*b*) The interconversion of UDP-galactose and UDP-glucose *via* a 4-keto intermediate, catalyzed by UDP-galactose 4-epimerase.

2. RmlB overexpression and purification

The open reading frame of the gene encoding RmlB was amplified using PCR with primers that incorporated a 5′ *Nco*I site and a 3′ *Sst*I site to facilitate cloning into the expression vector pET-28a(+). Expression involves the IPTG (isopropyl-β-D-thiogalactoside)-inducible T7 promotor and ribosome-binding sites conferred by the vector, but uses the natural *rmlB* 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 identical in amino-acid sequence to the authentic product and carries no extensions or mutations.

In order to overexpress RmlB, *E. coli* BL21(DE3) cells were transformed with the pET-28a(+) plasmid and grown at 310 K in 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 the addition of 1 m*M* IPTG. After 3 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* dithiothreitol (DTT), 5 m*M* phenylmethyl sulfonyl fluoride, 20 µ*M* lysozyme, 20 m*M* Tris–HCl pH 8.

After 30 min incubation at room temperature, the viscosity of the mixture was decreased by the addition of DNAase I (20 µg ml^{-1}) and sonication (four cycles of 30 s interrupted by 1 min periods on ice). After the addition of 1 m*M* EDTA, the mixture was centrifuged for 30 min at 20 000*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, 20 000*g*, 277 K), the supernatant was dialysed against two changes of 2 l of 50 m*M* NaCl, 20 m*M* Tris–HCl pH 8.3 at 277 K. DTT was added to a final concentration of 2 m*M* and the filtered supernatant was passed through a POROS-1760 HQ/M HPLC column (BioCAD 700E Perfusion Chromatography Workstation). Proteins were eluted with a 50–1000 m*M* NaCl gradient. A protein with a molecular weight corresponding to RmlB

(~41 kDa) was found in a peak eluted at 275 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 7.6 at 277 K. Ammonium sulfate was added gradually to 35% and DTT was added to give a final concentration of 2 m*M*. The filtered protein sample was loaded onto a POROS HP2/M HPLC column (BioCAD 700E Workstation) equilibrated in buffer *A* (35% ammonium sulfate, 20 m*M* sodium phosphate pH 7.6). Elution was achieved with an increasing gradient of buffer *B* (20 m*M* sodium phosphate pH 7.6), RmlB being eluted at 57% buffer *B*.

3. Protein analysis

Following the two HPLC steps, the protein appeared to be pure as judged by a SDS silver nitrate stained gel (single band at an apparent molecular weight of 41 kDa); the calculated molecular weight based on the sequence being 40 722 Da. Dynamic light-scattering results (DynaPro 801) indicated the protein to be monodisperse with a molecular weight indicative of a dimer. N-terminal sequencing was performed and confirmed the protein to be RmlB. The final yield from purification was approximately 18 mg of RmlB per litre of Terrific Broth as estimated at UV_{280}, given that 1 AU corresponds to 0.62 mg ml^{-1} for *S. enterica* RmlB.

4. RmlB crystallization

Following the final purification step, the RmlB protein sample was dialysed against two changes of 2 l of 25 m*M* Tris–HCl pH 7.6 and concentrated to 4 mg ml^{-1}. The protein was incubated overnight at 277 K with 2.5 m*M* DTT and 4 m*M* NAD^{+}. Crystals were grown at 293 K from a solution containing 4 µl protein sample and 4 µl precipitant [0.1 *M* MES (2-morpholino-ethanesulfonic acid monohydrate) pH 6.3, 1.5 *M* lithium sulfate] using the sitting-drop vapour-diffusion technique (Ducruix & Giegé, 1992). Needle-shaped crystals grew within 14 d to maximum dimensions of 1.5 × 0.15 × 0.15 mm (Fig. 2).

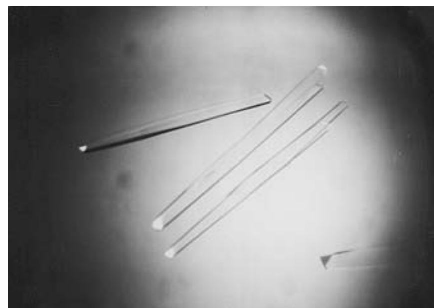

Figure 2
Photograph of *S. enterica* RmlB crystals of dimensions 1.5 × 0.15 × 0.15 mm.

5. Data collection

Data from a single frozen crystal were collected on station 7.2 at Daresbury SRS at a wavelength of 1.488 Å using a MAR345 image-plate detector. Prior to data collection, the crystal was soaked for 180 s in cryoprotectant (20% glycerol, 0.1 *M* MES pH 6.3, 1.5 *M* lithium sulfate). The data were collected at 110 K to a resolution of 2.8 Å. The crystal-to-detector distance was 220 mm, with data being recorded as 214 non-overlapping 90 s 1° rotation exposures. Data were indexed and integrated with the program *DENZO* (Otwinowski & Minor, 1996) and merged with *SCALEPACK* (Otwinowski & Minor, 1996). A total of 210 905 measurements were recorded, with 51 729 unique reflections. RmlB crystals were assigned to space group $P2_1$ following the observation of a 2*n* condition along the *k* axis. For four molecules in the asymmetric unit, the V_M value (Matthews, 1968) was 3.27 Å^3 Da^{-1}, giving a solvent content (V_S) of 62%. For two and six molecules in the asymmetric unit, the V_M values were 6.53 and 2.18 Å^3 Da^{-1}, respectively. Given these V_M values, it is conceivable that either four or six molecules could exist in the asymmetric unit, corresponding to two or three dimers of RmlB, respectively.

6. Structure determination

The structure of RmlB from *S. enterica* was solved by molecular replacement using *AMoRe* (Navaza, 1994) as implemented in the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The search model used was the refined 1.9 Å structure of RmlB from *E. coli* (PDB code 1bxk). The amino-acid sequence shows a 70.7% identity with that of *S. enterica* RmlB. All residues in *S. enterica* RmlB that differed from those of *E. coli* RmlB were mutated to alanine and insertions in two loop regions were removed. Using a dimer search model and data in the resolution range 10–3.3 Å, a clear solution for two dimers in the asymmetric unit was obtained. This solution had a correlation coefficient of 62.6%.

The structure is being refined using *CNSsolve* (Brunger *et al.*, 1998). A random subset of data (5%) was omitted from all refinement calculations to provide an assessment of the progress of refinement. The starting working *R* factor and free *R* factor were 42 and 45%, respectively. Following restrained positional and thermal factor refinement of this alanine model, the working *R* factor and free *R* factor improved to 32 and 35%, respectively, confirming the correctness of our solution. We are currently fitting the side chains of the model using the graphics program *O* (Jones *et al.*, 1991). We will report the full structure shortly.

This project is supported by a grant from the Wellcome Trust (JHN, 056851) and a Wellcome Trust international travel grant (JHN and CW). We thank Michael Graninger for helpful suggestions and discussions. The use of beamline 7.2 at the CCLRC Daresbury Laboratory, UK is gratefully acknowledged. We thank Hazel Holden and Jim Thoden for access to unpublished data.

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