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Purification, crystallization and preliminary structural studies of dTDP-4-keto-6-deoxy-glucose-5-epimerase (EvaD) from *Amycolatopsis orientalis*, the fourth enzyme in the dTDP-L-epivancosamine biosynthetic pathway

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The vancomycin class of antibiotics is regarded as the last line of defence against Gram-positive bacteria. The compounds used clinically are very complex organic molecules and are made by fermentation. The biosynthesis of these is complex and fascinating. Its study holds out the prospect of utilizing genetic engineering of the enzymes in the pathway in order to produce novel vancomycin analogues. In part, this requires detailed structural insight into substrate specificity as well as the enzyme mechanism. The crystallization of one of the enzymes in the chloroeremomycin biosynthetic pathway (a member of the vancomycin family), dTDP-3-amino-4-keto 2,3,6-trideoxy-3-*C*-methyl-glucose-5-epimerase (EvaD) from *Amycolatopsis orientalis*, is reported here. The protein is fourth in the pathway which makes a carbohydrate essential for the activity of chloroeremomycin. The crystals of EvaD diffract to 1.5 Å and have unit-cell parameters $a = 98.6$, $b = 72.0$, $c = 57.1$ Å with space group $P2_12_12$. Data to this resolution were collected at the European Synchrotron Radiation Facility.

1. Introduction

Chloroeremomycin (Fig. 1) is an important member of the vancomycin family of glycopeptide antibiotics, all members of which consist of an identical crosslinked heptapeptide scaffold to which carbohydrates are attached. Vancomycin is synthesized by *Amycolatopsis orientalis* and the genes for its synthesis have been sequenced (van Wageningen *et al.*, 1998). All vancomycin derivatives have glucose attached to the oxygen of the 4-hydroxyphenylglycine side chain of residue 4. Diversity arises from the addition of further carbohydrates to the peptide ring and to the glucose. These sugars contribute to the antibacterial efficacy by promoting antibiotic dimerization, which is essential in the mode of action of this group of compounds (Barna & Williams, 1984). Recent studies have suggested that the disaccharide itself is bactericidal (Ge *et al.*, 1999). Chloroeremomycin contains 4-epi-vancosamine (3-amino-2,3,6-trideoxy-3-*C*-methyl-L-arabino-hexopyranose), which is bound to the glucose 2′O as well as the β-OH-Tyr6 of the glycopeptide. The natural donor of 4-epi-vancosamine is deoxythymidine disphosphate (dTDP)-L-epivancosamine, which is synthesized from the dTDP-6-deoxy-4-keto-D-glucose precursor using five separate enzymes (encoded in the genes orf14 and orf23-26; van Wageningen *et al.*, 1998; Kirkpatrick *et al.*, 2000; Chen *et al.*, 2000). dTDP-6-deoxy-4-keto-D-glucose is produced during dTDP-L-rhamnose biosynthesis (product of RmlB), a biosynthetic pathway which has recently been completely structurally characterized (Blankenfeldt *et al.*, 2000, 2002; Allard *et al.*, 2002; Giraud *et al.*, 2000)

The precise sequence of reactions for the biosynthesis of dTDP-epivancosamine is disputed in the literature. Three of us (CTW, HCL and MDB) have proposed the scheme shown in Fig. 2 (Chen *et al.*, 2000) based on detailed biochemical studies. An alternative pathway has also been proposed (Kirkpatrick *et al.*, 2000). The structures of the enzymes involved will establish the mechanistic steps along the biosynthetic pathway. Although the total synthesis of the vancomycin has been reported (Nicolaou *et al.*, 1999), the quantities required for therapeutic use of this class of antibiotics are made by fermentation. This means that modification of the antibiotic to generate higher affinity analogues which may help overcome or manage resistance will either arise from synthetic modification of the fermented product or genetic engineering to produce altered product. The latter route is particularly attractive as it may allow considerable diversity in the structure and will utilize existing technology in the field. A rational

programme at such engineering requires a detailed knowledge of the structural basis of substrate recognition at each step of the pathway.

We report the successful crystallization and data collection to 1.5 Å of one of the enzymes in the pathway, EvaD, the dTDP-3-amino-4-keto-2,3,6-trideoxy-3-*C*-methyl-glucose-5-epimerase. The protein belongs to the RmlC class of epimerases for which the structure is known and the protein shares a 41% identity with the *Salmonella enterica* serovar *Typhimurium* enzyme (Giraud *et al.*, 2000).

2. EvaD overexpression and purification

The proposed gene for EvaD, orf26 from *Amycolatopsis orientalis*, was cloned into a pET vector (pET22b). Protein expression in this vector is controlled by the IPTG-inducible T7 promoter using the ribosome-binding sites conferred by the vector and the natural orf26 initiation codon ATG. The protein is expressed with a His_6 tag at the N-terminus of each chain.

BL21(DE3) cells were transformed with the plasmid and grown at 310 K in Luria broth (Maniatis *et al.*, 1982) containing 50 µg ml^{-1} carbenicillin until the OD_{600} reached 0.9–1.1. Overexpression was induced by addition of 1 m*M* IPTG. After 4 h, the cells were harvested by centrifugation (15 min, 6000 rev min^{-1}, 277 K), suspended in lysis buffer (100 m*M* NaCl, 2 m*M* DTT, 5 m*M* PMSF, 100 µg ml^{-1} lysozyme, 20 µg ml^{-1} DNAase, 20 m*M* Tris–HCl pH 7.5) and incubated at 277 K for 1 h and then at 298 K for 20 min to activate the DNAase. The resuspended cells were disrupted by a French press at 138 MPa, the lysate centrifuged (20 000 rev min^{-1}, 10 min) and the supernatant dialysed against three changes of 20 m*M* Tris–HCl pH 7.6. The filtered solution was passed through a DEAE Sepharose HPLC column (Amersham Pharmacia Biotech) using a linear 0–1 *M* NaCl gradient to elute the proteins. Fractions containing EvaD were identified by SDS–PAGE, pooled and dialysed against three changes of 20 m*M* Tris–HCl pH 8.0. EvaD was completely purified by nickel-affinity chromatography using Chelating Sepharose Fast Flow media (Amersham Pharmacia Biotech). Elution was performed using a linear imidazole gradient (0.5–300 m*M*); the protein was eluted at 100 m*M* imidazole. The purity of the 22.4 kDa protein was confirmed by SDS gel electrophoresis and its integrity was confirmed by tryptic digestion mass spectroscopy.

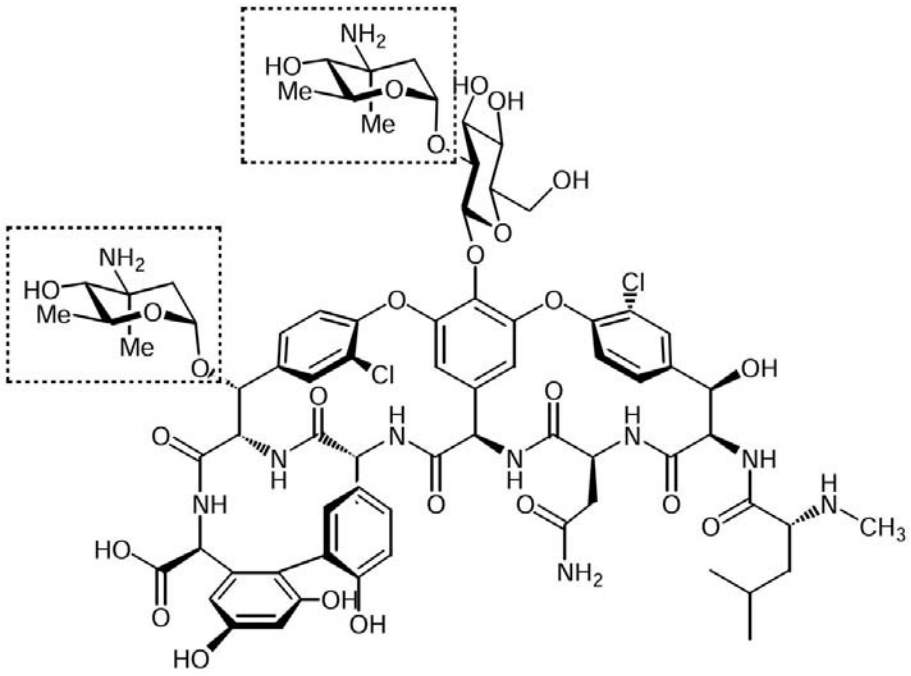

Figure 1
Chloroeremomycin, a member of the vancomycin family of antibiotics. The epivancosamine sugar is highlighted in the dotted boxes.

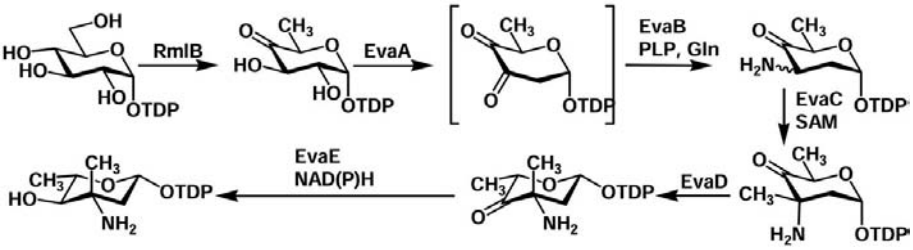

Figure 2
The pathways for the biosynthetic conversion of dTDP-6-deoxy-4-keto-D-glucose into TDP-L-epivancosamine as proposed by CTW, HCL and MDB. An alternative route has also been proposed (Kirkpatrick *et al.*, 2000).

Table 1
Data collection.

Values in parentheses refer to the highest resolution shell (1.54–1.5 Å).

Wavelength (Å)	0.933
Resolution (Å)	37.27–1.50
Space group	$P2_12_12$
Unit-cell parameters (Å, °)	a = 98.57, b = 72.00, c = 57.10, $\alpha = \beta = \gamma$ = 90
V_M† (Å^3 Da^{-1})	2.3
Solvent (%)	46
Total measurements	292414
Unique reflections	65259
$I/\sigma(I)$	7.3 (2.6)
Average redundancy	4.5 (4.1)
Data completeness (%)	100 (100)
R_{merge}‡ (%)	5.6 (26.3)

† Two molecules per asymmetric unit. ‡ $R_{merge} = \sum\sum I(h)_j - I(h)/\sum\sum I(h)_j$, where $I(h)$ is the measured diffraction intensity and the summation includes all observations.

3. EvaD crystallization and data collection

The fractions containing the pure protein were pooled, dialysed against three changes of 20 m*M* Tris–HCl pH 8.0 and concentrated to a final concentration of 10 mg ml^{-1}. Crystals were grown at 277 K using the sitting-drop vapour-diffusion method with 3 µl protein and 3 µl precipitant under the following conditions: 100 m*M* Tris–HCl pH 8.0, 100 m*M* $MgCl_2$, 25%(*w*/*v*) PEG 4000. Leaf-shaped crystals grew within 7–8 days.

A 0.5 × 0.3 × 0.1 mm crystal was equilibrated in a cryoprotectant solution consisting of 20%(*w*/*v*) glycerol in mother liquor, mounted in a loop and flash-frozen in a liquid-nitrogen gas stream. A native X-ray data set to 1.5 Å resolution was recorded at the ESRF BM14.2 beamline at a wavelength of 0.933 Å using an ADSC Q4 CCD area detector. The beam size was 50 µm and the

crystal-to-detector distance was 130 mm. Data were recorded as 110 non-overlapping 15 s 1° oscillations.

The reflections were indexed in an orthorhombic space group (unit-cell parameters a = 98.57, b = 72.00, c = 57.10 Å, $\alpha = \beta = \gamma = 90°$) and were integrated and merged in Laue group $P222$ using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1996). The space group was identified as $P2_12_12$ by the observation of $n = 2$ systematic absences along the a and b axis but no such absences on the c axis. A total of 292 414 measurements were recorded of which 65 259 were unique reflections. Calculation of the Matthews volume (Matthews, 1968) gave V_M = 2.3 Å^3 Da^{-1} and a solvent content of 46% assuming two molecules (*i.e.* one dimer) per asymmetric unit and a molecular weight of 22 000 Da. Table 1 summarizes the data set. We expect to solve the structure by molecular replacement using the RmlC structure from our earlier studies. We will then progress to substrate and substrate-analogue complexes in order to properly map out the mechanism of EvaD and provide a rational basis for redesign of the substrate profile.

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