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Crystallization and preliminary X-ray diffraction studies of aclacinomycin-10-methyl esterase and aclacinomycin-10-hydroxylase from *Streptomyces purpurascens*

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Two enzymes participating in the biosynthesis of anthracyclines in *Streptomyces purpurascens*, aclacinomycin-10-methyl esterase (RdmC) and aclacinomycin-10-hydroxylase (RdmB), have been crystallized. RdmB is a *S*-adenosyl-methionine-dependent hydroxylase and RdmC hydrolyses the carboxymethyl group of the aglycone skeleton of aclacinomycin. Crystals of RdmB obtained in the presence of *S*-adenosyl-L-methionine were orthorhombic, space group $C222_1$, with unit-cell parameters $a = 63.2$, $b = 92.2$, $c = 115.3$ Å; diffraction data were collected to 2.1 Å resolution. RdmC was crystallized as a complex with the substrate, aclacinomycin T. These crystals diffracted to 1.45 Å resolution and belonged to space group $P2_1$, with unit-cell parameters $a = 38.2$, $b = 84.7$, $c = 44.3$ Å, $\beta = 99.9^\circ$.

1. Introduction

Anthracyclines are an important group of aromatic antibiotics that are synthesized in the polyketide pathway by different *Streptomyces* species (Strohl, 2001). These secondary metabolites exhibit extraordinary cytotoxicity and the anthracyclines doxorubicin and daunorubicin are amongst the most used anti-tumour drugs in cancer chemotherapy (Grein, 1987). The biosynthesis of these compounds has attracted considerable interest, not least because of the potential for producing novel improved anthracyclines either by combinatorial biosynthesis or through rational design of the enzymes involved, or by a combination thereof (Hutchinson, 1992; Hopwood *et al.*, 1985; Strohl *et al.*, 1989; Strohl, 2001; Niemi *et al.*, 1994; Kast & Hilvert, 1997).

The biosynthesis of anthracyclines starts with the formation of the polyketide backbone, which is catalyzed by a polyketide synthase, with subsequent cyclization of the polyketide chain (Shen & Hutchinson, 1996). These steps lead to the formation of aklavinone, which is a common intermediate in the synthesis of most anthracyclines. At this stage, the biosynthetic pathways diverge and the polyketide aglycone is further modified through hydroxylation, glycosylation, methylation, reduction, decarboxylation and/or oxidation by the action of tailoring enzymes (Connors *et al.*, 1990; Madduri *et al.*, 1993; Dickens *et al.*, 1997; Walczak *et al.*, 1999). It is at this stage that most of the diversity of these secondary metabolites is generated.

The genes for several of the tailoring enzymes in rhodomycin biosynthesis in *S. purpurascens* are located in the *rdm* gene cluster (Niemi & Mäntsälä, 1995; Niemi *et al.*, 1994). Two of these enzymes, aclacinomycin esterase (RdmC) and aclacinomycin hydroxylase (RdmB), catalyze the conversion of aclacinomycins to 11-deoxy-β-rhodomycins (Fig. 1). RdmC, which is a monomer in solution, consists of 298 amino acids with a molecular mass derived from gel chromatography of 32 000 Da (the predicted M_r from the amino-acid sequence is 31 729; Wang *et al.*, 2000). It acts as an aclacinomycin methylesterase and exhibits significant sequence similarity to other esterases from different *Streptomyces* strains, for instance DnrP from *S. peucetius* and DauP from *Streptomyces* sp. C5 (Dickens *et al.*, 1995; Madduri & Hutchinson, 1995). The product of the reaction catalyzed by RdmC, 15-demethoxyaclacinomycin, is hydroxylated at carbon 10 to produce 11-deoxy-β-rhodomycin (Fig. 1). This reaction is catalyzed by RdmB, an enzyme of 374 amino acids. Gel chromatography gives a molecular weight of 152 000 Da, suggesting that the enzyme forms a homotetramer (the predicted M_r from the amino acid sequence is 41 287; Wang *et al.*, 2000). RdmB shows significant sequence similarities to a class of *O*-methyltransferases from *Streptomyces*, for instance carminomycin-*O*-methyltransferase (50% identity; Connors & Strohl, 1993). In spite of this sequence relation to methyltransferases and the dependence of RdmB on *S*-adenosyl-methionine, this enzyme does not act as a methyltransferase but as a hydroxylase. It is noteworthy that the oxygen-dependent hydroxylation reaction occurs in the absence of spin-delocalizing devices such as metal ions or flavins.

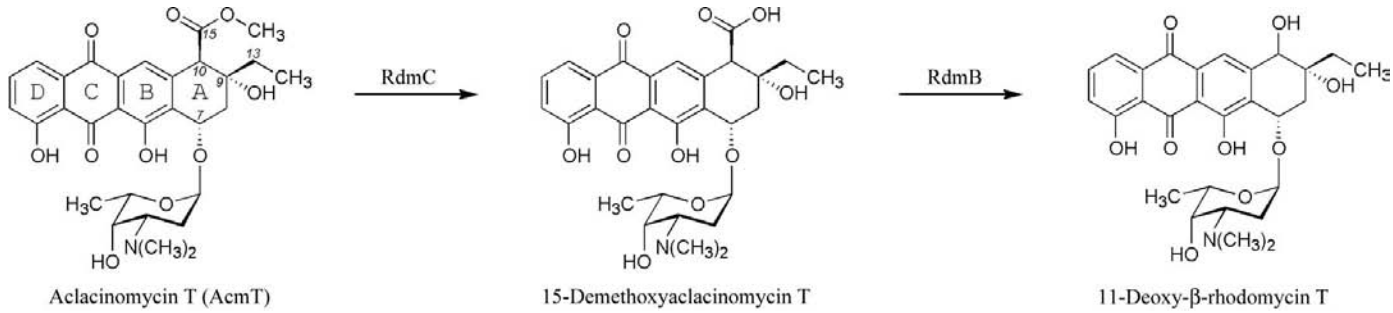

Figure 1
Reactions catalyzed by aclacinomycin esterase (RdmC) and aclacinomycin hydroxylase (RdmB). In aclacinomycin A and derivatives, the glycosylation is rhodosamine-deoxyfucose-cinerulose instead of the rhodinose in AcmT.

2. Material and methods

2.1. Protein purification and crystallization

Recombinant RdmC produced in *S. lividans* was purified as described elsewhere (Wang *et al.*, 2000). A solution of RdmC in 50 m*M* Tris buffer pH 7.5 containing 1 m*M* DTT was mixed with aclacinomycin T (AcmT) in a 1:10 protein:substrate ratio and incubated on ice for 1 h. The mixture was concentrated to 10 mg ml^{-1} protein and 1.2 m*M* AcmT and then used for crystallization. Crystallization trials were performed by the hanging-drop vapour-diffusion technique at 277 and 293 K using Hampton Reseach Crystal Screen kits. Each drop was prepared by mixing 2 µl of the protein solution with an equal amount of mother liquor and was allowed to equilibrate with 1 ml of the reservoir solution.

RdmB was produced as a recombinant GST-fusion protein in *Escherichia coli*, in which two amino acids from the thrombin cleavage site were added to the N-terminus; the protein thus starts with the sequence GSVSSSPGE (Wang *et al.*, 2000). Purification followed the protocol described previously (Wang *et al.*, 2000). The enzyme (6.7 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.5, 1 m*M* DTT) was incubated with 8 m*M* SAM for approximately 1 h before setup of the crystallization experiments. Drops (2 µl of protein solution mixed with 2 µl of mother liquid) were allowed to equilibrate against 1 ml of mother liquid. Hampton Research crystallization kits were used for initial screening and the experiments were carried out at temperatures of 277 and 293 K.

2.2. Data collection and processing

RdmC–AcmT crystals were quickly soaked in a cryoprotective buffer containing mother liquor and 20% PEG 400 prior to flash-freezing in a gaseous nitrogen stream at 100 K. X-ray diffraction data were collected at a wavelength of 0.8094 Å (oscillation angle 0.5°, crystal-to-detector distance 125 mm) at beamline X11 at the European Molecular Biology Laboratory (EMBL), DESY, Hamburg.

Diffraction data from native RdmB crystals were collected at beamline B711, MAX II Laboratory, Lund, Sweden. The crystals were flash-frozen at 100 K using paraffin oil as a cryoprotectant. Before transfer into the cryostream, crystals were soaked in the oil for about 5 s. Data were collected at a wavelength of 0.8410 Å with a crystal-to-detector distance of 350 mm and an oscillation angle of 2°. Indexing, processing and scaling were performed using *DENZO* and *SCALEPACK* from the *HKL* suite (Otwinowski, 1993). Space-group determination was performed using *DENZO* in combination with pseudo-precession images, which were generated using the program *PATTERN* (Lu, 1999).

3. Results

3.1. RdmC

A number of amorphous precipitates and small needles were obtained under various crystallization conditions and some of these conditions were further optimized by systematic exploration of the influence of the pH and the precipitant concentration. Needle-like crystals with a bright yellow colour, indicating binding of the substrate, were obtained at 293 K overnight using 1.6 *M* ammonium sulfate, 2% PEG 400 and 0.1 *M* HEPES pH 7.5. These crystals were further optimized by streak-seeding. Seeds from the crystals were transferred into freshly made drops of mother liquor with the same precipitant concentration as used previously. This was achieved by gently touching the crystals and transferring the seeds into the newly prepared drops with a needle. After 2 d, very thin plate-like crystals appeared in the drop. The average size of the crystals in two dimensions was 0.15 × 0.15 mm, whereas the third dimension was too thin to be determined. RdmC crystals exposed to the X-ray beam were found to be very mosaic. Annealing the crystal by back-transfer into the cryosolution for 1 min and then flash-freezing again reduced the mosaicity and increased the resolution significantly, for instance from 2 to 1.45 Å resolution with a final crystal mosaicity of 0.3°. Indexing of the diffraction data suggested that the crystals were monoclinic and a pseudo-precession image revealed systematic absences along 0*k*0. The space group was $P2_1$, with unit-cell parameters a = 38.2, b = 84.7, c = 44.3 Å, β = 99.9°. The V_M value (Matthews, 1968) of 2.22 Å^3 Da^{-1} suggested one monomer per asymmetric unit and 45% solvent content. This packing density is consistent with the absence of NCS-related peaks in the self-rotation function and in the native Patterson map.

Table 1
Data-collection statistics for RdmC and RdmB.

Values in parentheses are for the highest resolution shell.

	RdmC	RdmB
Space group	$P2_1$	$C222_1$
Unit-cell parameters		
a (Å)	38.2	63.2
b (Å)	84.7	92.1
c (Å)	44.3	115.3
β (°)	99.9	
Resolution (Å)	20.0–1.45	30.0–2.1
Wavelength (Å)	0.8094	0.8410
No. of observations	234728	67648
No. of unique reflections	34733	19452
R_{sym}† (%)	4.1 (27.3)	9.5 (35.5)
Completeness (%)	84.8 (63.5)	97.1 (92.5)
$I/\sigma(I)$	15.1 (2.0)	11.3 (2.4)

RdmC is produced in *S. lividans*, which has so far not allowed production of selenomethionine-substituted enzyme. We therefore have to rely upon heavy-atom compounds for phase determination and screening for derivatives is well under way.

3.2. RdmB

Thin plate-like crystals of 0.3 × 0.3 × 0.1 mm in size appeared within 2 d in wells containing 30% PEG 4000, 0.1 *M* ammonium acetate and 0.1 *M* sodium acetate pH 5.0. Further optimization gave the best crystals when increasing the ammonium acetate concentration to 0.2 *M*.

The crystals diffract to 2.1 Å at a synchrotron source (Table 1). Autoindexing and pseudo-precession images suggested an orthorhombic *C*-centred lattice. Systematic extinctions along 00*l* are consistent with space group $C222_1$. The unit-cell parameters were a = 63.2, b = 92.2, c = 115.3 Å. The asymmetric unit is most likely to contain one molecule, as indicated by the Matthews coefficient of 2.11 $Å^3\ Da^{-1}$. Selenomethionine-substituted protein has been produced and the incorporation of six selenomethionine residues was confirmed by mass spectroscopy. Availability of fully substituted enzyme samples should allow structure determination by multiple-wavelength anomalous diffraction methods.

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