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Cubic crystals of chloramphenicol phosphotransferase from *Streptomyces venezuelae* in complex with chloramphenicol

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Chloramphenicol 3-*O*-phosphotransferase (CPT) from *Streptomyces venezuelae* ISP5230, a novel chloramphenicol-inactivating kinase, has been overexpressed and purified using *Escherichia coli* as the heterologous host. Crystals of CPT in complex with its substrate chloramphenicol (Cm) were obtained which were suitable for X-ray diffraction. The crystals belong to the cubic space group $I4_132$ with unit-cell dimension $a = 200.0$ Å. The initial CPT crystals diffracted to 3.5 Å and the diffraction was improved significantly upon adding acetonitrile and Cm to the crystallization drop. The CPT–Cm crystals diffract to at least 2.8 Å resolution.

1. Introduction

Chloramphenicol (Cm), a broad-spectrum antibiotic produced by the soil bacterium *Streptomyces venezuelae*, is a potent inhibitor of bacterial growth (Miyamura, 1964). Cm binds to the peptidyltransferase centre of 50S prokaryotic ribosomes, thereby inhibiting protein biosynthesis. Resistant strains of eubacteria have become widespread, largely owing to inactivation of the antibiotic by acetylation of the primary (C3) hydroxyl of Cm, mediated by the enzyme chloramphenicol acetyltransferase (CAT; Shaw & Leslie, 1991). However, CAT is not present in strains of *S. venezuelae* known to produce Cm (Shaw & Hopwood, 1976), which are at risk of autoinhibition by their own secondary metabolite. In addition to a putative membrane-associated efflux protein to facilitate the export of Cm (Mosher *et al.*, 1995), it was recently shown that the resistance of the producing organism to Cm may be a consequence of modification of the same functional group by phosphorylation rather than acetylation (Mosher *et al.*, 1995). In over four decades of clinical use, phosphorylation has never been reported as a Cm-resistance mechanism.

The enzyme responsible for this novel reaction, chloramphenicol 3-*O*-phosphotransferase (CPT), is active as a homodimer with a subunit molecular weight of 18920 Da. CPT uses ATP as phosphoryl donor, transferring the γ-phosphate to the primary (C3) hydroxyl of Cm (Campopiano *et al.*, 1999, in preparation). The 3-phosphoryl-Cm product is biologically inactive as an antibiotic.

Phosphorylation is commonly used to confer resistance to other antibiotics (Cundliffe, 1992) and is perhaps best exemplified by the aminoglycoside kinases, which catalyse the phosphorylation of a wide range of important aminoglycoside antibiotics including neomycin B and kanamycin (Shaw *et al.*, 1993). However, the similarity between these aminoglycoside phosphotransferases and CPT appears to extend no further than their functional roles within the bacterial cells, since an inspection of the primary structure of CPT shows no significant sequence similarity with such proteins.

By way of contrast, CPT bears the most significant sequence identity to the enzyme shikimate kinase type II (SKII) from *Escherichia coli*, having an 18% sequence identity across its entire 178 residues, including a Walker A-motif (G*xxxx*GKT/S; Walker *et al.*, 1982) near the N terminus, often considered to be the signature of a phosphate-binding loop. The high-resolution (1.9 Å) structure of SKII from *Erwinia chrysanthemi* reveals the core of the structure to be a classical mononucleotide-binding fold, common to such diverse proteins as myosin, p21-*ras* and elongation factor EF-Tu. The overall fold of SKII resembles that of yeast adenylate kinase (Krell *et al.*, 1998).

2. Methods and results

To obtain substantial amounts of CPT purified to homogeneity, the following protocol was developed. *E. coli* HMS174(DE3), harbouring an expression vector containing the *cpt* gene from *S. venezuelae* ISP5230 under the control of the T7 protomer (Campopiano *et al.*, in preparation), were cultured in 1 l of 2YT medium (containing 16 g tryptone, 10 g yeast extract and 5 g NaCl per litre). The cells were grown to high density at 310 K overnight, then were diluted with 400 ml of fresh medium and allowed to grow for a further 15 min before induction with 0.5 m*M* isopropyl-1-thio-β-D-galactopyranoside (IPTG). After a further 3 h growth, the cells were harvested and resuspended in 50 m*M* Tris–HCl pH 7.5 prior to lysis

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by sonication. Cell debris was removed by ultracentrifugation at 45000 rev min^{-1} at 277 K for 1 h. CPT was precipitated from crude cell extract between 25 and 42% ammonium sulfate saturation and collected by centrifugation. The pellet was dissolved in 50 m*M* Tris–HCl pH 7.5 and dialysed into the same buffer containing 0.4 *M* ammonium sulfate. The protein was loaded onto a Hi-Load phenyl Sepharose HP FPLC column (Pharmacia) equilibrated in the same buffer. Contaminant proteins were washed through the column and CPT eluted with a gradient of decreasing ammonium sulfate concentration. Fractions were assayed for CPT activity by pyruvate kinase/lactate dehydrogenase coupled assay (Campopiano *et al.*, in preparation) and assessed to be greater than 98% pure by SDS–PAGE. Protein concentration was estimated by Biorad Protein Assay.

A typical yield of 60 mg of purified CPT may be obtained from a 1 l culture medium of *E. coli* HMS174(DE3) cells carrying the plasmid.

The purified protein was concentrated to 7.5 mg ml^{-1} at 277 K in an Amicon stirred-cell concentrator (30 kDa molecular weight cut-off). CPT was buffer-exchanged into 10 m*M* Tris–HCl pH 7.5 and briefly centrifuged to remove insoluble material. Initial crystals of native CPT were obtained using vapour diffusion by equilibrating 5 µl of the protein preparation against 5 µl of a reservoir solution containing 0.1 *M* Tris–HCl (pH 7), 1.6 *M* ammonium sulfate. The largest dimension of these cubic crystals was 80 µm (Fig. 1*a*), and they diffracted to about only 8 Å resolution on a Cu $K\alpha$ rotating-anode generator. The large number of crystals and high speed of crystal growth (within 6 h) was reduced by including ammonium sulfate in the protein solution, concomitant with the ammonium sulfate concentration in the reservoir being reduced to 1.14 *M* (Fig. 1*b*). The subsequent use of protein in the storage buffer (50 m*M* Tris–HCl pH 7.5 containing 0.4 *M* ammonium sulfate) produced a smaller number of much larger crystals in each hanging drop. Crystal size was further improved by the inclusion of 4% acetonitrile and 5 m*M* Cm in the crystallization medium. Under these conditions, 4–5 crystals of CPT in complex with Cm appeared in each drop after 2–4 d and reached a maximum size of 0.4 mm (Fig. 1*c*) after about two weeks. These crystals diffracted to 3.5 Å resolution on a Cu $K\alpha$ rotating-anode generator.

Crystals were cryoprotected by including glycerol to 30% in the mother liquor. X-ray data from CPT-Cm crystals were collected at beamline X12-C of the National Synchrotron Light Source, Brookhaven National Laboratory. A Brandeis CCD detector was used to record the images. Frozen crystals (100 K) showed diffraction beyond 2.8 Å resolution. The X-ray data were collected at a crystal-to-detector distance of 330 mm using 0.5° oscillations per image.

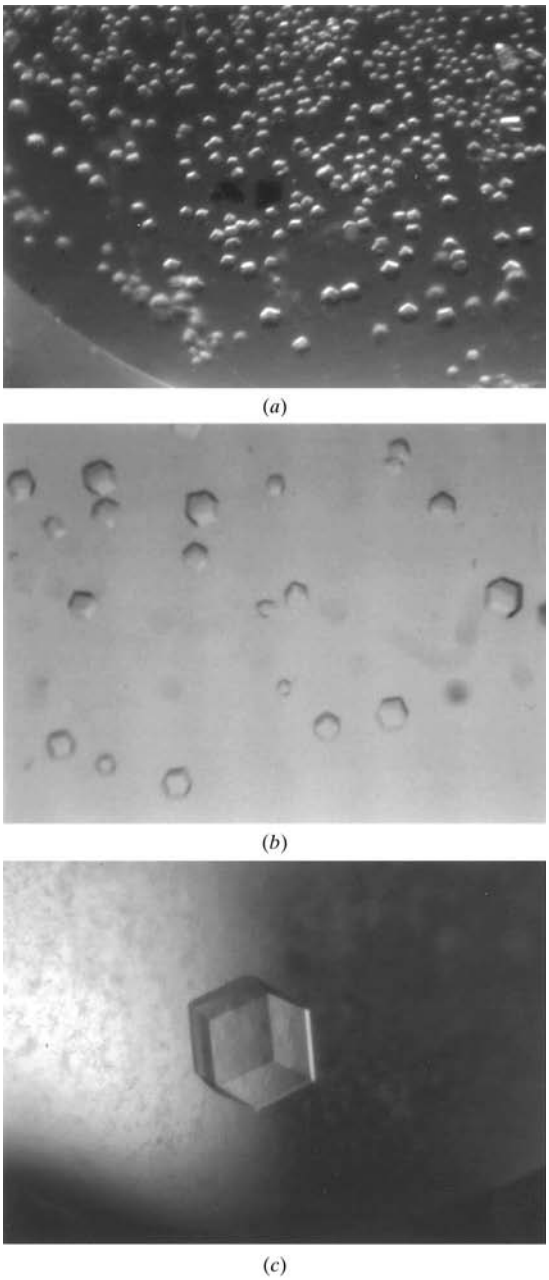

Figure 1
Crystals of CPT. (*a*) Initial native CPT crystals. The largest crystal dimension is <80 µm. (*b*) Improved crystallization conditions resulted in a largest crystal dimension of ~0.1 mm. (*c*) CPT–Cm crystals with dimensions up to 0.4 mm.

Table 1
Data reduction statistics for CPT–Cm.

Wavelength (Å)	1.1
Resolution (Å)	2.8
Total data	279639
Unique data	17120
Redundancy	16.3
$F^2 > 3\sigma(F^2)$ (100–2.8 Å) (%)	83.3
$F^2 > 3\sigma(F^2)$ (2.9–2.8 Å) (%)	49.9
Average $F^2/\sigma(F^2)$	14.6

Resolution range (Å)	$R_{\rm merge}$†	Completeness
100.00–6.03	0.027	0.992
6.03–4.79	0.042	0.999
4.79–4.18	0.046	0.999
4.18–3.80	0.057	1.000
3.80–3.53	0.079	0.998
3.53–3.32	0.100	0.999
3.32–3.15	0.154	0.999
3.15–3.02	0.195	0.998
3.02–2.90	0.276	0.999
2.90–2.80	0.371	0.997
100.00–2.80	0.068	0.998

† $R_{\rm merge} = \sum_{\rm unique\ reflections}(\sum_{i=1}^{N}|I_i - \bar{I}|)/\sum_{\rm unique\ reflections} \times (\sum_{i=1}^{N} I_i)$.

All data were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Statistics are given in Table 1. Autoindexing with *DENZO* determined that the crystals belong to the cubic body-centred crystal system, with unit-cell dimension a = 200.0 Å. The $h00$ reflections were only present for $h = 4n$, consistent with space group $I4_132$.

The self-rotation function calculated with either the program *GLRF* (Tong & Rossmann, 1990) or *POLARRFN* (Collaborative Computational Project, Number 4, 1994) showed no significant peaks above the noise level and therefore no indication of a non-crystallographic twofold axis. An assumption of one protomer in the asymmetric unit leads to a packing density V_m (Matthews, 1968) of 8.8 Å^3 Da^{-1}, corresponding to a solvent content of 0.86, which seems unlikely. However, assuming one (or two) dimers in the asymmetric unit results in the more plausible packing density and solvent content of 4.4 Å^3 Da^{-1} and 0.72 (or 2.2 Å^3 Da^{-1} and 0.44), respectively. In this case, any internal symmetry must be parallel to a crystallographic axis.

Repeated attempts to solve the structure by molecular replacement, using the SKII

crystal structure as a search model (Krell *et al.*, 1998), were unsuccessful. The cross-rotation function implemented in *AMoRe* (Navaza, 1994) gave no outstanding solution from any modifications to the search model, although a wide range of search radii and resolution cut-offs were tried. Moreover, the top 50 peaks from the various cross-rotation function peak lists did not reveal a significant peak in a translation search with acceptable crystal packing. A search for heavy-atom derivatives for a structure solution *via* isomorphous replacement has been initiated, therefore.

Diffraction data for this study were collected at Brookhaven National Laboratory at the Biology Department single-crystal diffraction facility on beamline X12-C at the National Synchrotron Light Source. This facility is supported by the United States Department of Energy Offices of Health and Environmental Research and of Basic Energy Sciences and by the National Science Foundation. We thank Bob Sweet (BNL) for his invaluable assistance during data collection. We are grateful to Bob Liddington (Leicester) for his continuous support and Peter Moody (Leicester) for fruitful discussion. TI acknowledges a Wellcome Trust Travel Grant. JE is supported by the Wellcome Trust (046351/Z/96) awarded to W. V. Shaw, whom we thank for critical reading of the manuscript.

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