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Crystallization and preliminary X-ray diffraction analysis of the chloramphenicol acetyltransferase from Tn*2424*

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Crystals of chloramphenicol acetyltransferase B2, an enzyme encoded by the transposon Tn*2424* from *Escherichia coli*, have been obtained utilizing polyethylene glycol as a precipitant. The enzyme inactivates the antibiotic chloramphenicol and is a member of the xenobiotic acetyltransferase family. Two crystal forms were obtained and complete data sets have been collected at a synchrotron source: form I, which diffracted to 3.2 Å, and form II, grown in the presence of $NiCl_2$, for which crystals of the apoenzyme and of the enzyme–chloramphenicol complex have been obtained. For the form II crystals, complete data sets have been collected at 2.7 and 3.2 Å resolution, respectively. The space group of the above two crystal forms is $P2_13$, with unit-cell parameter a = 130 Å.

1. Introduction

The enzymatic inactivation of the antibiotic chloramphenicol (Cm) is a well understood mechanism of bacterial resistance. Chloramphenicol acetyltransferases (CATs) are the enzymes responsible for the inactivation of Cm by converting it to 1-acetoxy-Cm, 3-acetoxy-Cm and 1,3-diacetoxy-Cm. These Cm derivatives are no longer able to interrupt the activity of ribosomal peptidyltransferase and thus are unable to inhibit bacterial growth and survival (Gale *et al.*, 1981; Pongs, 1979).

The chloramphenicol acetyltransferase B group enzymes are encoded by Gram-negative bacteria (Tennigkeit & Matzura, 1991; Parent & Roy, 1992; Bunny *et al.*, 1995; White *et al.*, 1999). In a recent review (Murray & Shaw, 1997), chloramphenicol acetyltransferase Bs (CAT Bs) and paralogous acetyltransferases obtained from Gram-positive bacteria (Rende-Fournier *et al.*, 1993; Allignet *et al.*, 1988, 1993) have been classified as a novel protein family, the xenobiotic acetyltransferase (XAT) family. In common with the classical CATs, the proteins in the XAT family also form homotrimers with similar molecular masses. Both families mediate acetyl-transfer reactions, possibly by the use of a similar biochemical mechanism. However, the XATs have no primary sequence similarities with the classical CATs; some XAT proteins have a low affinity toward Cm (Tennigkeit & Matzura, 1991), while others prefer completely different substrates (Rende-Fournier *et al.*, 1993; Allignet *et al.*, 1993). Moreover, XATs contain the common $(IGxxxx)_n$ motif in their carboxyl moieties and thus can be classified within a large protein family called the hexapeptide-repeat-containing acyltransferases. Here, we report the purification, crystallization and preliminary X-ray diffraction analysis of CAT B2, which is an enzyme encoded by transposon Tn*2424* of *E. coli* (Parent & Roy, 1992). Compared with the classical CATs, it produces very little 1,3-diacetoxy-chloramphenicol and appears to only be able to use acetyl-CoA as an acetyl-group donor, not the alternate substrate butyryl-CoA.

2. Materials and methods

Polyethylene glycols (PEGs) and Crystal Screen kits I and II were purchased from Hampton Research Inc. (CA, USA); chloramphenicol–caproate resin, dithiothreitol (DTT), glycerol, phenylmethylsulfonyl fluoride (PMSF), sodium acetate, sodium cacodylate, nickel chloride, sodium chloride (NaCl) and triethanolamine (TEA) were purchased from Sigma; Centricon 30K was from Millipore Inc.

The *catB2* gene of Tn*2424* was amplified from plasmid pLQ161 (Parent & Roy, 1992) using oligonucleotide primers which provided a *Bsp*HI site at the initiation codon and a *Bam*HI site downstream of the gene. It was then cloned into pET3-d which was digested with *Nco*I and *Bam*HI. The resulting clone, pLQ460, was transformed into *E. coli* HMS174 for maintenance and freshly transformed into *E. coli* BL21 (DE3) just before overproduction. A 2 l culture in the late logarithmic phase was induced with 0.4 m*M* IPTG and kept at 289 K for 20 h so as to permit overproduction while minimizing the formation of inclusion bodies. The cells were harvested, treated with lysozyme and PMSF and then

Table 1
Data-processing statistics.

Values in parentheses refer to the highest resolution shell.

Crystal type	Form I	Form II	
	Apoenzyme	Apoenzyme	Enzyme–CM complex
No. of images	26	60	49
Space group	$P2_13$	$P2_13$	$P2_13$
Unit-cell parameters (Å) ($a = b = c$)	130.2	130.7	129.5
Mosaicity (°)	0.46	0.35	0.44
Resolution range (Å)	50–3.2	20–2.7	50–3.2
No. of measured reflections	69260	345209	125692
No. of unique reflections	12471	20756	12261
Redundancy	2.8	10.1	5.7
R_{sym}† (%)	9.5 (52.0)	8.7 (58.2)	14.9 (57.6)
Completeness (%)	95.3 (98.5)	100.0 (100.0)	99.5 (100.0)
$\langle I/\sigma(I)\rangle$	11.9 (2.3)	25.3 (3.7)	11.9 (3.0)

† $R_{sym} = \sum|I - \sigma(I)|/\sum\sigma(I)$.

sonicated. The extract was filtered through a 0.22 µm membrane and stored at 203 K.

CAT B2 was purified using a Cm-caproate column loaded with CAT B2 in TNM buffer (50 m*M* Tris–HCl pH 7.8, 1 *M* NaCl and 0.1 m*M* 2-mercaptoethanol), washed with TNM and eluted with TNM plus 5 m*M* Cm. The protein solution was desalted on a Centricon CF50A and rinsed with 10 m*M* TEA pH 7.7, 50 m*M* NaCl. It was then diluted fivefold with 10 m*M* TEA pH 7.7 and loaded onto a mono-Q HR5/5 column. CAT B2 was eluted using a 0–1 *M* NaCl gradient in 10 m*M* TEA pH 7.7. The peak fractions were pooled and stored in buffer *A* (20 m*M* TEA pH 7.7, 250 m*M* NaCl and 50% glycerol) at 253 K.

In the presence of buffer *A*, the purified CAT B2 is very stable at 253 K. Prior to crystallization, the buffer was changed to 10 m*M* TEA pH 7.7, 0.5 m*M* EDTA and 0.1 m*M* PMSF (buffer *B*) by centrifugal filtration with Centricon 30K.

With both screening kits I and II, crystals of CAT B2 were obtained from the first screening in hanging drops using the vapor-diffusion technique. The crystallization conditions were modified by using PEGs of varying sizes and concentrations, by using different salts at various concentrations and by varying the pH.

The X-ray diffraction analysis of the crystals of CAT B2 was performed using the X8C beamline at the National Synchrotron Light Source at Brookhaven National Laboratory.

3. Results

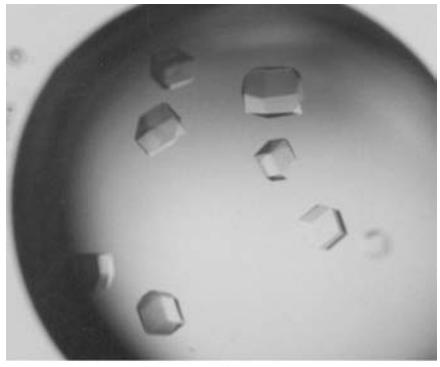
Crystals of CAT B2 were grown using the vapor-diffusion technique in hanging drops. Two distinct crystal forms were identified. As shown in Fig. 1(*a*), form I crystals were first obtained in a cubic habit. They were grown by mixing equal volumes of protein sample (14 mg ml^{-1}) and reservoir solution, the latter containing 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* sodium acetate and 22% PEG 8K. These crystals usually grow to dimensions of 0.8 × 0.8 × 0.8 mm within 2–4 d. The number of crystals in the drop can be reduced to one or two by varying the PEG concentration. The maximum diffraction from these primitive cubic crystals was 3.2 Å. The data for this form were collected on a Quantum-IV CCD imaging-plate detector at beamline X8C of the National Synchrotron Light Source, Brookhaven National Laboratory. Bipyramidal crystals (form II) were later obtained after adjusting the salt type and pH (see Fig. 1*b*). These crystals were grown by mixing protein solution containing 8 mg ml^{-1} CAT B2 in buffer *B* and well solution containing 12 m*M* $NiCl_2$, 6.5% PEG 8K, 1 m*M* NaN_3 in a buffer of 0.1 *M* MES at pH 6.7. When 4 m*M* chloramphenicol substrate was present in the protein sample, crystals of the CAT B2–chloramphenicol complex were obtained. At room temperature, the above crystals diffracted to low resolution (limited to 3.2 Å). Cryocrystallographic techniques (Garman & Schneider, 1997) were applied to improve the diffraction quality. Although the crystallization conditions allowed flash-cooling of the crystals, the use of additional glycerol to a final concentration of 30–35% led to improved diffraction from these crystals.

A complete data set to 2.7 Å resolution for the apoenzyme (form II crystals) was collected at beamline X8C of the National Synchrotron Light Source, Brookhaven National Laboratory. Using 1° oscillations, a 60° wedge of data was collected from a single crystal at 100 K. The wavelength was 1.009 Å and the crystal-to-detector distance was 220 mm. Another data set for the enzyme–chloramphenicol complex was also collected under similar conditions, but this crystal diffracted to only 3.2 Å resolution.

Data were processed using the *DENZO* and *SCALEPACK* package (Otwinowski & Minor, 1997). The results are summarized in Table 1. The space group of both form I and form II crystals was shown to be $P2_13$, based on the systematic absences of the reflections. V_M was calculated to be 2.6 Å^3 Da^{-1} (Matthews, 1968), assuming three subunits in the asymmetric unit.

4. Discussion

Several CAT genes from various bacterial species have been sequenced. The encoded proteins are divided into two classes: the classical CAT (CAT A) and the XAT (which includes CAT B) (Murray & Shaw, 1997). Although the members of the two families have considerable similarities, they also have very significant differences. The CAT B2 studied here belongs to the XAT family. Roderick and coworkers have reported the structure of PaXAT (Beaman *et al.*, 1998), another member of the XAT family, which shares 72.4% sequence identity and 92.4% sequence similarity with CAT B2. The report reveals a structural feature of the family, the left-handed LβH domain. However, it is not clear yet whether this left-handed LβH



(*a*)

(*b*)

Figure 1
CAT crystals were obtained in the space group $P2_13$. (*a*) Form I crystals (cubic) were obtained by mixing equal volumes of protein sample (14 mg ml^{-1}) and a reservoir solution containing 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* sodium acetate and 22% PEG 8K. These crystals yielded a maximum resolution of 3.2 Å. (*b*) Form II crystals (bipyramidal) were obtained by mixing equal volumes of the protein sample (8 mg ml^{-1}) and a reservoir solution containing 12 m*M* $NiCl_2$, 6.5% PEG 8K, 1 m*M* NaN_3 in a buffer of 0.1 *M* MES at pH 6.7. A complete data set to 2.7 Å resolution was collected at a synchrotron source.

domain is related to the low affinity of XATs for Cm.

How is the active site different from that of the classical CATs? Our structural study of this enzyme may contribute significantly to the understanding of the XAT family and even to the study of other hexapeptide-repeat-containing acyltransferases. CAT B2 could serve as a prototype for the XAT family, which includes SAT A (Rende-Fournier *et al.*, 1993) and VAT (Allignet *et al.*, 1993). These two enzymes mediate resistance to the streptogramin family of antibiotics and are thus becoming the last-resort antibiotics against vancomycin-resistant enterococci (VRE) and glycopeptide-intermediate *Staphylococcus aureus* (GISA).

Work is in progress to solve the structure of CAT B2 and of its complex with its substrate by the molecular-replacement method using the structure of PaXAT (PDB code 2xat) as the model.

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