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Crystallization and preliminary X-ray characterization of arylamine *N*-acetyltransferase C (BanatC) from *Bacillus anthracis*

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The arylamine *N*-acetyltransferase (NAT) enzymes are xenobiotic metabolizing enzymes that have been found in a large range of eukaryotes and prokaryotes. These enzymes catalyse the acetylation of arylamine drugs and/or pollutants. Recently, a *Bacillus anthracis* NAT isoform (BanatC) has been cloned and shown to acetylate the sulfonamide antimicrobial sulfamethoxazole (SMX). Subsequently, it was shown that BanatC contributes to the resistance of this bacterium to SMX. Here, the crystallization and the X-ray characterization of BanatC (Y38F mutant) are reported. The crystals belong to the tetragonal space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 53.70$, $c = 172.40$ Å, and diffract to 1.95 Å resolution on a synchrotron source.

1. Introduction

Arylamine N-acetyltransferases (NATs) are phase II xenobiotic metabolizing enzymes that catalyse the acetyl-CoA-dependent N- and O-acetylation of several arylamines, hydrazines and their N-hydroxylated metabolites (Riddle & Jencks, 1971). NATs play an important role in the detoxication and/or bioactivation of numerous drugs and xenobiotics (Hein *et al.*, 2000; Dupret & Rodrigues-Lima, 2005). Initially identified in humans and other vertebrates (mouse, rabbit), NAT enzymes have now been identified in a range of prokaryotic species (Sim *et al.*, 2007). To date, the X-ray structures of four bacterial NATs have been published (Sinclair *et al.*, 2000; Sandy *et al.*, 2002; Holton *et al.*, 2005; Westwood *et al.*, 2005). Recently, the human NAT1 and NAT2 isoform structures were released in the Protein Data Bank. All structures indicate that NAT enzymes possess a conserved three-domain fold whose active sites contain a triad of residues (cysteine–histidine–aspartate) that catalyse substrate acetylation (Sim *et al.*, 2007).

Bacillus anthracis is a Gram-positive bacterium that causes anthrax in animals and humans (Mock & Fouet, 2001). We have recently shown that this bacterium possess three NAT homologues (BanatA, BanatB and BanatC; Pluvinage *et al.*, 2007). Interestingly, BanatC was found to efficiently acetylate the sulfonamide antimicrobial SMX and to afford higher than normal resistance to sulfamethoxazole (SMX) in recombinant *Escherichia coli* cells (Pluvinage *et al.*, 2007).

Because this enzyme could contribute to the resistance of *B. anthracis* to SMX, we focused on structural studies of this NAT isoform. Whereas wild-type BanatC proved difficult to crystallize, a BanatC mutant (in which the Tyr38 residue was engineered to a Phe residue) displaying the same enzymatic characteristics as wild-type BanatC was found to crystallize and to diffract to 1.95 Å. Here, we describe the preliminary X-ray study of this NAT enzyme isolated from *B. anthracis*.

2. Materials and methods

2.1. Cloning, expression and purification

The BanatC open reading frame was cloned into the pET28a vector (Novagen) and expressed in *E. coli* strain BL21 (DE3). Details of cloning and expression have been published elsewhere (Pluvinage *et al.*, 2007). The Y38F mutant of BanatC was created by PCR using the mutagenesis primer 5′-ATTCTCCCCTTCGAAAATCTT-3′ and

Table 1
Data-collection statistics for Y38F BanatC crystal.

Values in parentheses are for the highest resolution shell

X-ray source	ID23-1, ESRF Grenoble
Wavelength (Å)	1.07225
Temperature (K)	100
Space group	$P4_12_12$ or $P4_32_12$
Unit-cell parameters (Å, °)	a = 53.70, b = 53.70, c = 172.40, $\alpha = \beta = \gamma = 90$
Resolution (Å)	50–1.95 (2–1.95)
No. of observations	184066 (11894)
No. of unique reflections	19041 (1336)
$R_{\rm merge}$† (%)	11.5 (58.2)
Completeness (%)	98.7 (96.3)
Average $I/\sigma(I)$	16.56 (4.13)

† $R_{\rm merge} = \sum |I - \langle I \rangle| / \sum I$.

reverse primer 5′-AAGATTTTCGAAGGGGAGAAT-3′. Recombinant BanatC and Y38F BanatC were purified using HIS-select nickel resin (Sigma) as described in Pluvinage *et al.* (2007). The protein products contain an N-terminal hexahistidine tag followed by a linker (sequence SSGLVPRGSHMASMTGGQQMGRGS) which is fused directly to Met1 of the BanatC sequence.

Purity of protein samples (in 25 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA) was assessed by SDS–PAGE and Western blotting as described in Pluvinage *et al.* (2007). NAT activity assays were also carried out as described in Pluvinage *et al.* (2007). Recombinant proteins were then concentrated to 8 mg ml^{-1} using Amicon ultracentrifugation concentrators (10 kDa molecular-weight cutoff, Millipore).

2.2. Crystallization

Crystallization trials were performed at 294 K with the AmSO4 Suite (Qiagen) and CrystalQuick microplates (Greiner) using the hanging-drop vapour-diffusion method by mixing 1.3 µl concentrated protein solution with 0.7 µl reservoir solution. Microcrystals were observed after two weeks with several solutions (Nos. 23, 31, 37, 45). Using a mixture of 2 µl protein solution and 1 µl reservoir solution, the crystallization conditions were optimized on EasyXtal Tools X-Seal (Qiagen) with 1.8 *M* ammonium sulfate and 0.17 *M* potassium nitrate. Crystals suitable for X-ray analysis grow in 5–7 d to maximal dimensions of 120 × 120 × 50 µm. For cryoprotection, the crystal was fished from the drop, dunked directly in sodium formate (7 *M*), mounted in a cryoloop (Hampton Research) and immersed in liquid nitrogen prior to X-ray diffraction analysis.

2.3. Data collection

Diffraction data were collected at 100 K on beamline ID23-1 at the ESRF using a MAR CCD detector. The parameters used were an exposure time of 0.1 s with a transmission of 16%. 130 images were collected with 1° oscillation per image at a wavelength of 1.07225 Å. The data set was then processed using *XDS* (Kabsch, 1993).

3. Results and discussion

BanatC has been reported to be a NAT enzyme that contributes to the resistance of *B. anthracis* to the sulfonamide antibiotic SMX (Pluvinage *et al.*, 2007). This NAT isoform could therefore be a possible drug target, as has been suggested for other bacterial NAT enzymes such as the *Mycobacterium tuberculosis* isoform (Upton *et al.*, 2001). However, so far crystallization trials of the BanatC isoform have been unsuccessful. The presence in large-scale preparations of a proteolytic fragment (30 kDa) of the BanatC enzyme could explain, at least in part, the difficulties in obtaining crystals. Although several different culture conditions were tested for the production of BanatC, significant quantities of the 30 kDa fragment were found in the purified fractions. Therefore, we tested whether single-point mutants of the BanatC enzyme could be used for crystallization trials. We took advantage of a mutation study carried out in our laboratory aimed at characterizing the importance of the catalytic residues in bacterial NAT enzymes (Atmane *et al.*, 2007). One BanatC mutant (Y38F) was found to display identical enzymatic properties (data not shown) to those of the wild-type enzyme and based on this was used as a candidate for crystallization trials. As shown in Fig. 1, SDS–PAGE (Fig. 1*a*) and Western blot (Fig. 1*b*) analysis of purified BanatC and the Y38F mutant indicated that both proteins display a molecular weight of 34 kDa that is consistent with predicted values. Moreover, the proteolytic fragment of 30 kDa was found in the purified samples of wild-type and Y38F BanatC. However, both amido-black staining and Western-blot analysis showed that much less proteolysis occurred with Y38F BanatC compared with the wild-type enzyme. In contrast to BanatC, most eukaryotic and prokaryotic NAT enzymes have a Phe residue instead of a Tyr residue at this highly conserved position (Westwood *et al.*, 2005). So far, we have no clear explanation as to

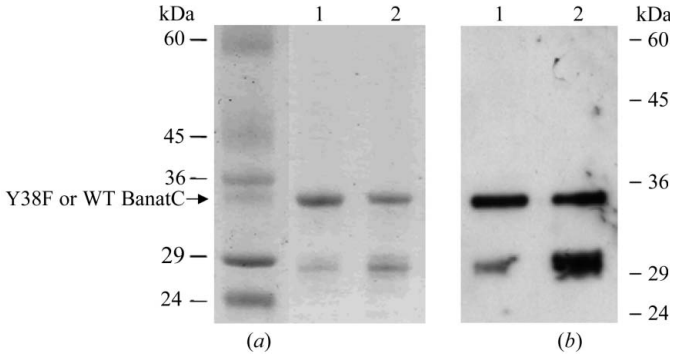

Figure 1
Expression of recombinant BanatC WT and mutant (Y38F). (*a*) SDS–PAGE analysis. 2 µg purified recombinant protein was subjected to SDS–PAGE under reducing conditions and stained with amido-black 10B (BioRad). Lane 1, Y38F BanatC mutant; lane 2, wild-type BanatC. (*b*) Western blot analysis. 0.5 µg purified protein was subjected to SDS–PAGE under reducing conditions and Western blot analysis was conducted using a polyclonal antibody raised against *S. typhimurium* NAT (1:50 000 dilution; lane 1, Y38F BanatC mutant; lane 2, wild-type BanatC).

Figure 2
Crystals of Y38F BanatC grown by the hanging-drop vapour-diffusion method in 1.8 *M* ammonium sulfate, 0.17 potassium nitrate. Dimensions are approximately 100 × 100 × 30 µm.

why replacement of the Tyr residue of BanatC by a Phe residue leads to decreased proteolysis when recombinantly expressed in *E. coli*.

As shown in Fig. 2, crystals suitable for X-ray analysis were observed within one week using optimized conditions (1.8 *M* ammonium sulfate and 0.17 *M* potassium nitrate). Crystals grew to maximum dimensions of 120 × 120 × 50 µm. A complete X-ray diffraction data set was collected to 1.95 Å resolution. Data-collection statistics are presented in Table 1. The crystals had tetragonal symmetry, with unit-cell parameters a = 53.70, b = 53.70, c = 172.40 Å, and belonged to space group $P4_12_12$ or $P4_32_12$. Molecular replacement using the structures of other bacterial NAT enzymes (Sinclair *et al.*, 2000; Sandy *et al.*, 2002; Holton *et al.*, 2005; Westwood *et al.*, 2005) will be used to attempt to solve the structure of the BanatC isoform.

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