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Purification, crystallization and preliminary X-ray analysis of *Bacteroides fragilis* Zn^{2+} β-lactamase

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Abstract

The Zn^{2+} β-lactamase from *Bacteroides fragilis* (E.C. 3.5.2.6) was overexpressed in *Escherichia coli* using an isopropylthiogalactoside-inducible T7 RNA polymerase expression system. Crystallization trials by the hanging-drop vapour-diffusion method have yielded two different crystal forms from two slightly different conditions. Crystals of form I belong to the monoclinic space group $C2$ with unit-cell dimensions $a = 56.03$, $b = 43.98$, $c = 105.32$ Å, $\beta = 112°$ and diffracted only up to 4.0 Å. Crystals of form II are orthorhombic, space group $P2_12_12_1$ with unit-cell dimensions $a = 48.10$, $b = 98.05$, $c = 111.76$ Å, diffract to at least 2.0 Å and are suitable for high-resolution structural analysis.

1. Introduction

β-lactamases are bacterial enzymes which hydrolyze antibiotics of the β-lactam family. Based on sequence analyses, these proteins are grouped in four different classes. Classes A, C and D enzymes have reactive serine residues, whereas those of class B are metalloenzymes that require a Zn^{2+} ion for activity.

Recently, several clinical isolates of *Bacteroides fragilis*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa* were reported to produce Zn^{2+} β-lactamases. The ability of the class B enzymes to hydrolyze carbapenems (third generation penicillins), compounds which generally escape the activity of the active serine enzymes, and the acquisition of this gene by some noxious species has increased the concern about this group of enzymes (Payne, 1993).

We had previously solved the X-ray structure of a Zn^{2+} β-lactamase from *Bacillus cereus* (569/H/9), which we denote as BCII, defined a sequence alignment for all available class B enzymes, and proposed a model for the interaction of a cephalosporin molecule with BCII, prior to catalysis based on this structure (Carfi *et al.*, 1995). Now we report the purification, crystallization, and a preliminary X-ray analysis of the Zn^{2+} β-lactamase of *B. fragilis* (BF). Recently, the crystal structure of BF in a different crystal form has been published (Concha, Rasmussen, Bush & Hertzberg, 1996).

Since BF has 35% sequence identity with BCII it should be possible to solve its structure by molecular replacement methods. This structure should increase our understanding of the enzymatic mechanism of the Zn^{2+} β-lactamases at the atomic level and, explain the large specificity differences between BCII and BF.

2. Materials and methods

2.1. *Cloning of the gene*

In order to obtain the large quantities required for crystallization, *B. fragilis* Zn^{2+} β-lactamase was overexpressed in *E. coli* using an isopropylthiogalactoside (IPTG) inducible T7 RNA polymerase expression system (Studier, Rosenburg, Dunn, & Dubendorff, 1990). The genetic construction was similar to that described (Yang, Rasmussen & Bush, 1992) for the overexpression of the CcrA β-lactamase. The source of the *CfiA* gene from *B. fragilis* TAL 2480 was the pJST241 plasmid, a kind gift of Dr M. H. Malamy (Thompson & Malamy, 1990).

A fragment of the *CfiA* gene, including nucleotides 573–700 according to the conventional numbering scheme (Thompson & Malamy, 1990), was amplified by the polymerase chain reaction (PCR) using the following oligonucleotide primers, which were purchased from Eurogentec (Seraing, Belgium).

5′-TCGCACATATGGCACAGAAAAGCGTAA-3′
*Nde*I

5′-CCCGTTGGAAGGTACCATACCCC-3′
*Kpn*I

The first oligonucleotide carries two mismatched bases designed to generate an *Nde*I restriction site upstream of the structural gene, thus deleting its signal sequence. These mutations also introduce an additional alanine residue at the N-terminus. The second oligonucleotide corresponds to an internal sequence of the gene and includes an *Kpn*I restriction site. The sequence of the PCR fragment, as determined on an ALF DNA sequencer (Pharmacia) as previously described (Ansorge, Sproat, Stegemann, Schwager & Zenke, 1987), confirmed the presence of only the desired mutations. The recombinant gene was cloned in a pET9a plasmid between the *Nde*I and *Bam*HI restriction sites of its multiple cloning site. The resulting construct, pBF14, was used to transform the BL21 strain of *E. coli*.

2.2. *Large-scale purification*

An overnight preculture (250 ml) of *E. coli* BL21/pBF14 was used to inoculate 15 l. of Luria broth medium at 310 K containing 50 mg l^{-1} kanamycin. When the culture reached the mid-log phase ($A_{600} = 0.6$), IPTG was added at a final concentration of 1 m*M*. After an additional 2 h the cells were harvested by centrifugation at 5 000 rev min^{-1} for 10 min at 277 K and the pellet resuspended in 1 l. of TEP buffer [10 m*M* Tris–HCl, pH 8.0, 1 m*M* EDTA, 0.1 m*M* phenylmethanesulfonyl fluoride (PMSF)]. Lysozyme was added (final concentration 0.5 mg ml^{-1}) and the suspension incubated for 30 min at 295 K. The cells were then broken with a cell disrupter (Constant System Ltd, England) and the resulting lysate adjusted to 0.5 *M* NaCl, 0.5% Nonidet, 5 m*M* $MgCl_2$. Benzonase (0.5 μg ml^{-1}) was added and the mixture was incubated at 277 K for 1 h. The insoluble proteins were collected by centrifugation at 9 000 rev min^{-1} for 30 min at 277 K and washed three times with TEP buffer. The pellet was

finally dissolved in 2 l of buffer *A* (50 m*M* Tris–HCl, pH 8.0, 10% glycerol, 50 m*M* $ZnSO_4$) containing 8 *M* urea, 0.1 m*M* PMSF and 1 m*M* 1,4-dithiothreitol (DTT). The urea and NaCl were removed later by three successive dialysis steps, each against 4 l. of 1 m*M* DTT and 0.1 m*M* PMSF in buffer *A*. The precipitate formed during dialysis was removed by centrifugation. The treatment of the inclusion bodies with DTT was necessary to prevent the formation of non-native disulfide bonds during the folding of the protein.

Complete purification was achieved by anion-exchange chromatography using Q-Sepharose Fast Flow (Pharmacia). Several runs were performed with a 4.6×30 cm column equilibrated with buffer *A*. The β-lactamase was eluted by a linear salt gradient (0–0.3 *M*). The flow rate was 5 ml min^{-1}. The chromatogram showed the presence of one single peak displaying β-lactamase activity. The final preparation contained about 350 mg of protein and exhibited a specific activity of 5.6 μmol s^{-1} mg^{-1} with nitrocefin as substrate.

The p*I* of the purified β-lactamase was 5.3 as shown by polyacrylamide gel isoelectric focusing. A mass spectrum revealed an M_r of 25 335±1 Da as expected from the amino-acid sequence (25 334 Da). The purity of the enzyme was estimated as being > 99%. The first seven N-terminal residues, as determined by gas-phase sequencing, were identical to those of the original protein, with the exception of the N-terminal as Ala expected from the genetic construction. The protein was concentrated to 12 mg ml^{-1} in 50 μ*M* Tris–HCl buffer, 15% glycerol, 100 μ*M* $ZnAc_2$ and stored at 193 K.

2.3. *Mass spectrometry*

The mass spectrum was obtained on an API III+ triple-quadrupole mass spectrometer (Perkin-Elmer Sciex) equipped with a nebulizer-assisted electrospray source (ion spray) operating at atmospheric pressure. A 5 kV voltage was applied to the electrospray needle. The reconstructed molecular mass profile was determined by using a deconvolution algorithm (PE/Sciex). For analysis, the protein (4 mg ml–1) was dialyzed overnight in a 10 m*M* $(NH_4)Ac$ buffer solution.

3. Results and discussion

3.1. *Crystallization of Zn^{2+} β-lactamase*

The Zn^{2+} β-lactamase was crystallized by the hanging-drop vapour-diffusion method. The thawed protein solution was dialyzed overnight against 50 m*M* Tris buffer pH 7.5, 100 μ*M* $ZnAc_2$ and 100 μ*M* DTT at 277 K. Crystallization trials using polyethylene glycol (PEG) 6000 and 10000 in the pH range 5.5–9.5 were performed at 281 and 288 K. Droplets consisting of 2.0 μl of the dialyzed protein solution at a concentration of 8.0 mg ml^{-1} mixed with an equal volume of buffered precipitating solution were suspended from siliconized glass coverslips over 500 μl of the same precipitant solution. In the initial experiments, plate-like crystals (form I) were obtained against reservoir solutions containing 25–28%(*w*/*v*) PEG 10 K, Hepes 100 m*M* pH 8.5, 100 μ*M* $ZnAc_2$, 10% glycerol. Diffraction symmetry and systematic absences were compatible with the monoclinic space group *C*2 with $a = 56.03$, $b = 43.98$, $c = 105.32$ Å and $\beta = 112°$. These crystals diffracted to a resolution limit of only 4 Å and, therefore, were not used for further crystallographic analysis.

Changing from Hepes to Bicine buffer resulted in thin, long crystals (form II). The thickest crystals ($0.35 \times 0.35 \times 0.8$ mm) were obtained at 288 K in 22–24%(*w*/*v*) PEG 8K, 100 μ*M* DTT, pH 8.6–9.0, 100 m*M* Bicine, 0.2–1 m*M* $ZnAc_2$, 2–5% glycerol (see Fig. 1). The crystals appeared in 3 d and grew to their final dimensions in one week. The new crystals diffracted to a resolution limit of 2.0 Å and had the orthorhombic space group

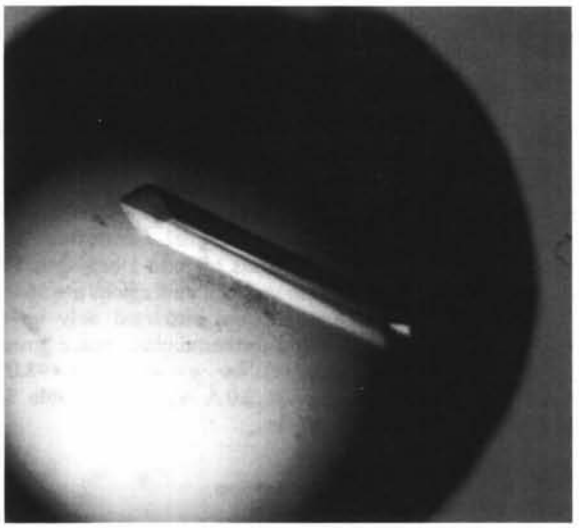

Fig. 1. Orthorhombic crystal of *B. fragilis* Zn^{2+}β-lactamase obtained by hanging-drop experiments.

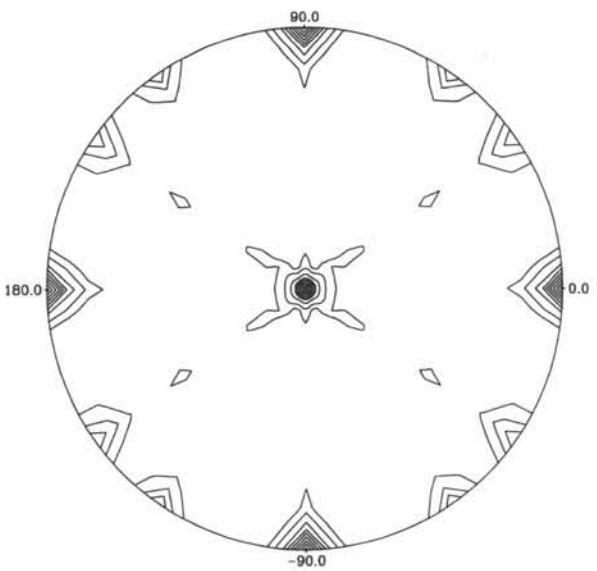

Fig. 2. Stereographic projection of the rotation function for $\kappa = 180°$. The map is contoured at levels of 10 units with a maximum value of 100 units (arbitrary). ω is equal to 0 or 180° at the centre and 90° at the edge; φ is marked at the periphery. The non-crystallographic rotation axis (at $\psi = 35°$) relates the two molecules in the asymmetric unit of the crystal.

$P2_12_12_1$ with cell dimensions $a = 48.086$, $b = 98.052$, $c = 111.764$ Å. Assuming two molecules per asymmetric unit, Matthews coefficient (Matthews, 1968) (V_m) is 2.4 Å^3 Da^{-1} corresponding to a solvent content of 40%(v/v).

3.2. *X-ray analysis*

Form II crystals were mounted in a quartz capillary and diffraction data were collected on a FAST/Enraf–Nonius area detector mounted on a Cu rotating-anode generator (FR5H). The data were processed using the *MADNES* program (Messerschmidt & Pflugrath, 1987). The *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) was used for further processing.

A set of diffraction data to a resolution limit of 2.8 Å (30 250 observed reflections, 11 666 unique reflections, $R_{merge} = 10\%$, completeness 86.3%) was collected for the form II crystals and a self-rotation function (*POLARRFN* written by Kabsch, in the *CCP*4 program suite) was computed in order to identify the relationship between the two molecules in the asymmetric unit. This function uses polar angles (ω, φ, κ). The self-rotation function was calculated for different resolution ranges and an integration radius of 20 Å. Fig. 2 is a $\kappa = 180°$ section calculated with intensity data between 15 and 3.5 Å resolution (data 98% complete) and shows peaks located at $\omega = 90°$ and $\psi = 35, 55°$, ... This figure confirms that there are two molecules in the asymmetric unit and indicates that they are related by a 180° rotation around a non-crystallographic rotation axis which is perpendicular to the c axis.

Attempts to solve the structure by the molecular replacement method are under way. The presence of two molecules in the asymmetric unit will facilitate the phase determination.

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