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Expression, purification and crystallization of an extended-spectrum β-lactamase from *Klebsiella oxytoca*

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OXY-1a is an extended-spectrum β-lactamase from the conditional pathogenic bacterium *Klebsiella oxytoca*. OXY-1a is responsible for the antibiotic resistance of this pathogen. A soluble form of OXY-1a with a His tag at its C-terminus was overexpressed in *Escherichia coli*. The recombinant protein was purified and crystallized at room temperature using PEG 4000 as the main precipitant. Two crystal forms were obtained from the same growth conditions. One was orthorhombic, with crystals that diffracted to better than 1.9 Å, while the other was tetragonal, with crystals that only diffracted to about 3.0 Å. Complete data sets were collected from both crystal forms. The orthorhombic crystal belongs to space group $P2_12_12_1$, with unit-cell parameters a = 46.54, b = 73.43, c = 84.56 Å, while the tetragonal crystal has unit-cell parameters $a = b$ = 73.72, c = 96.81 Å. The asymmetric unit of the orthorhombic crystal is estimated to contain one OXY-1a molecule, giving a crystal volume per protein weight (V_M) of 2.25 Å^3 Da^{-1} and a solvent content of 45%.

1. Introduction

β-Lactam antibiotics are very useful chemotherapeutic drugs in the treatment of various infectious diseases, as they have high potency and low toxicity. However, the efficiency of the antibacterial chemotherapy of β-lactam antibiotics has been challenged by the emergence of bacterial strains that exhibit high levels of antibiotic resistance. This resistance is mostly caused by the production of bacterial β-lactamases, which inactivate the antibiotics by hydrolyzing the β-lactam amide bond. The diversity of the specificity profiles of β-lactamases and their high catalytic power are the most significant characteristics of β-lactamase-mediated antibiotic resistance. Elucidation of the three-dimensional structures of clinically relevant β-lactamases by X-ray crystallography has provided and will continue to provide precious insight into the catalytic mechanisms of β-lactamases and will help to design improved antibiotics that cannot be inactivated by β-lactamases. Furthermore, structural data can also help in the design of inhibitors to prevent β-lactamases from hydrolyzing β-lactam antibiotics.

In previous studies (Wu *et al.*, 1992, 1999), investigation of the resistance mechanism of *Klebsiella oxytoca* septicaemia isolates resulted in the identification of two extended-spectrum β-lactamases, OXY-1a and OXY-2a, which belong to the species-specific enzymes of the *K. oxytoca* OXY family (Fournier, Roy *et al.*, 1996; Fournier & Roy, 1997). The OXY β-lactamases are encoded by the chromosomal DNA of each isolate of the species *K. oxytoca* and are classified according to their amino-acid sequence into Ambler class A, most of the members of which are penicillinases, rather than the class C cephalosporinases commonly found in many other enterobacteria, even though the OXY β-lactamases do hydrolyze cephalosporines effectively (Ambler *et al.*, 1991). In wild-type *K. oxytoca*, the OXY enzymes are constitutively produced at low levels and confer resistance to aminopenicillins and carboxypenicillins (Livermore, 1995); nevertheless, overproduction of the OXY β-lactamases confers resistance to penicillins, cephalosporins and aztreonam (Fournier *et al.*, 1994). Additionally, overproducers of OXY enzymes are commonly resistant to all combinations of β-lactams with β-lactamase inhibitors (Livermore, 1995). Since the early 1980s, isolates of *K. oxytoca* have been recognized to be clinically significant and there have been increasing reports of clinical isolates of *Klebsiella* spp. that are resistant to newer β-lactam antibiotics (Wu, 1998). The chromosomal β-lactamase genes (*bla*) in *K. oxytoca*, bla_{OXY}s, may serve as a genetic reservoir of resistance genes because of their ubiquitous presence in the species and their high mutation rate in the promoter region (Fournier, Lagrange *et al.*, 1996). So far, it has been found about 10–15% of *K. oxytoca* strains overproduce their chromosomal β-lactamases (Buirma *et al.*, 1991; Livermore, 1995). The OXY β-lactamases may be widespread among pathogenic entero-

bacteria, as two plasmid-mediated OXY derivatives, MEN-1 (Barthelemy *et al.*, 1992) and Toho-1 (Ishii *et al.*, 1995), have also been reported. bla_{OXY} was named by combining *bla*, the general symbol for β-lactamase genes, with the first three letters in the species name *oxytoca*.

Although a number of crystal structures of extended-spectrum β-lactamases from *Escherichia coli* have been solved (*e.g.* Shimamura *et al.*, 2002; Minasov *et al.*, 2002; Tranier *et al.*, 2000), no structure data are available for *K. oxytoca* β-lactamases. Their unique characteristics in enzymology, such as their low level of activity to most penicillins and their high level of activity to both penicillins and cephalosporins upon induction (Fournier *et al.*, 1994; Wu, 1998), and their significance in clinical practice, require further understanding of the OXY enzymes. Thus, we carried out X-ray crystallographic analysis of the OXY-1a enzyme. Furthermore, the high-resolution crystal structure is required for structure-based drug design; we hope that more efficient and specific β-lactamase inhibitors can be designed and tested in combination with such structural studies. In this report, we describe the details of the protein preparation, crystallization screening and the preliminary crystallographic analysis of the OXY-1a crystals.

2. Experimental

2.1. Protein expression and purification

To construct a plasmid suitable for high-level expression and single-step purification, PCR primers OXY1AF (5′-TCG GGA TCC TTG AAA AGT TCG TGG CGT-3′) and OXY1AR (5′-CCC AAG CTT TCG GTG ACG ATA **GTG ATG GTG ATG GTG ATG** AAG CCC TTC GGT GAC GAT-3′) were used to amplify the DNA region encoding OXY-1a from the chromosomal DNA of *K. oxytoca* KH66 (Wu *et al.*, 1999). A *Bam*HI site (underlined) was incorporated in OXY1AF and a *Hin*dIII site (underlined) and a short DNA sequence coding for six histidines (bold) were included in OXY1AR. Thus, the resulting PCR product was expected to encode a protein OXY-1a-His_6 with a C-terminal His tag. The PCR product of OXY-1a-His_6 was digested with *Bam*HI and *Hin*dIII and ligated into the QIAexpress SRF vector pQE-8 (Qiagen Inc., Chatsworth, CA, USA) to generate the recombinant plasmid pQESW-9. The plasmid pQESW-9 was transferred into *E. coli* M15 containing the repressor plasmid pREP4 in order to overexpress OXY-1a-His_6.

The overexpression procedures were basically performed by following the steps described in the QIAexpressionist's guidelines (Qiagen Inc., Chatsworth, CA, USA). 1 l of Luria–Bertani (LB) medium containing 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin was inoculated with 20 ml of an overnight culture of *E. coli* M15 harbouring pQESW-9 and the culture was grown at 310 K with vigorous shaking. When the culture reached an OD_{600} of 0.6, OXY-1a expression was initiated by adding isopropyl β-thiogalactopyranoside (IPTG) to a final concentration of 1 m*M*. The induced bacterial cells were allowed to grow for 3–5 h. The culture was cooled and bacterial cells were harvested by centrifugation at 6000*g* for 15 min; the cell pellet was then resuspended in 50 ml sonication buffer (50 m*M* sodium phosphate pH 7.8, 300 m*M* NaCl) and stored overnight at 253 K. The cell suspension was thawed in water at 277 K and sonicated by applying eight 15 s bursts at 200 W with a 15 s cooling period between each burst (Model W-225 sonicator, Heat Systems Ultrasonics, Inc., Farmingdale, NY, USA). The cell lysate was centrifuged at 17 000*g* for 25 min to remove insoluble cell debris.

The OXY-1a-His_6 protein was purified by mmobilized-metal affinity chromatography (IMAC) on an Ni–NTA (Ni–nitrilotriacetic acid) agarose column (Qiagen Inc., Chatsworth, CA, USA). The lysate was loaded onto an Ni–NTA column equilibrated with ten column volumes of sonication buffer and a wash with 20 column volumes of wash buffer (50 m*M* sodium phosphate pH 6.0, 300 m*M* NaCl, 20 m*M* imidazole, 10% glycerol) was performed to remove impurities. OXY-1a-His_6 was eluted with a gradient of 20–500 m*M* imidazole in 200–250 ml wash buffer. The fractions containing OXY-1a-His_6 were pooled together, diafiltrated in a Stirred Ultrafiltration Cell (Amicon, Inc., Beverly, MA, USA) against 20 volumes of 10 m*M* sodium phosphate buffer pH 7.0 and eventually concentrated to a concentration of 10–20 mg ml^{-1}.

2.2. Crystallization

The initial crystallization conditions were screened by the sparse-matrix method using Hampton Crystal Screen kits (Hampton Research, USA). All crystallization experiments were performed at 296 K by the hanging-drop vapour-diffusion method in 24-well VDX plates (Hampton Research, USA). In each trial, a hanging drop of 1 μl of protein solution mixed with 1 μl of reservoir solution was equilibrated against a reservoir of 500 μl precipitant solution.

2.3. Data collection and processing

Crystals of OXY-1a-His_6 protein were mounted in nylon CryoLoops (Hampton Research, USA), immersed in cryoprotectant (5% glycerol in mother liquor) for a few seconds and then flash-cooled in a cold nitrogen stream (Oxford Cryosystems Cryostream Cooler). Diffraction data were collected at the crystallographic beamline BL711 at the MAX-II synchrotron, Lund, Sweden at 100 K using a MAR 345 image-plate detector and the oscillation method. The wavelength for data collection was 1.0292 Å and the crystal-to-detector distance was typically 200 mm, with an oscillation range of 1° per image. A typical exposure time was 60 s per frame. Data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

The purity of the OXY-1a-His_6 protein was estimated to be about 95% by SDS–PAGE (data not shown). About 10–20 mg of OXY-1a-His_6 could be produced from 1 l of culture.

The crystals were first observed in condition No. 20 of the Hampton Crystal Screen kit. The crystallization conditions (pH, PEG concentration, ammonium sulfate concentration *etc.*) were then optimized by finer screening around condition No. 20. As shown in Fig. 1, two kinds of crystals could

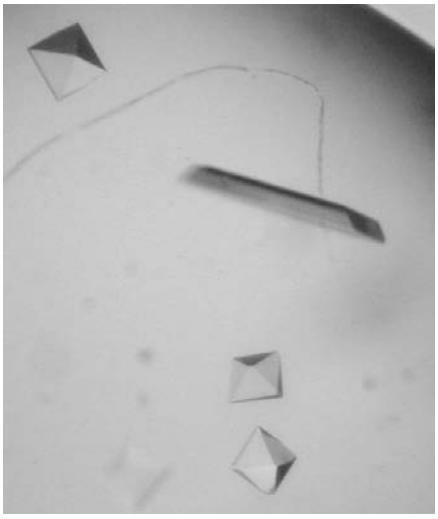

Figure 1
Both orthorhombic and tetragonal crystals of OXY-1a-His_6 were grown in 25%(*w*/*v*) PEG 4000 with 0.1 *M* sodium acetate pH 4.5 and 0.2 *M* ammonium sulfate. The approximate dimensions of the orthorhombic crystal are 0.7 × 0.2 × 0.2 mm.

Table 1
X-ray data-collection statistics.

Values in parentheses are for the last resolution shell.

	Orthorhombic crystal	Tetragonal crystal
Wavelength (Å)	1.0292	1.0292
Resolution (Å)	50–1.9 (2.0–1.9)	50–3.0 (3.1–3.0)
Completeness (%)	97 (90)	98 (99)
$R_{\rm merge}$† (%)	5.2 (12.9)	7.1 (29.1)
$I/\sigma(I)$	30 (10.5)	18.8 (4.2)
Space group	$P2_12_12_1$	$P4_122$
Unit-cell parameters (Å)	a = 46.54, b = 73.43, c = 84.56	a = 73.72, b = 73.72, c = 96.81
No. of unique reflections	22488	5635
Observed No. of reflections	210916	21312
$V_{\rm M}$ (Å^3 Da^{-1})	2.25	2.12
Molecules per asymmetric unit	1	1

† $R_{\rm merge} = \sum |I_{\rm obs} - I_{\rm avg}| / \sum I_{\rm obs}$, where the summation is over all reflections.

be obtained from the same drop: one had a long prismatic shape and dimensions of 0.7 × 0.2 × 0.2 mm; the others are cubic prism-shaped crystals with dimensions of about 0.4 × 0.4 × 0.4 mm. It was subsequently shown that the long prismatic crystals belong to the orthorhombic crystal system, while the cubic prism-shaped crystals belong to the tetragonal lattice. Both crystal forms grew in 25%(*w*/*v*) PEG 4000 with 0.1 *M* sodium acetate buffer pH 4.5 and 0.2 *M* ammonium sulfate. It took two to three weeks for the crystals to appear. However, crystal growth was not easily reproduced, particularly for the long prismatic crystals. It seemed that the freshness of the protein preparation and the addition of a further gel-filtration purification step were essential for obtaining good crystals. The type of crystals obtained thus depended on the protein batches: the cubic prism-shaped crystals have been relatively easy to obtain, but the long prismatic crystals have so far been rare.

The orthorhombic crystal diffracted to better than 1.9 Å resolution on beamline BL711, MAX-lab, Lund, Sweden. However, the tetragonal crystals only diffracted to about 3.0 Å. Complete data sets were collected from both crystal forms; the statistics of the data sets are listed in Table 1. The orthorhombic crystal can be unambiguously assigned to space group $P2_12_12_1$ from the systematic absences, with unit-cell parameters a = 46.54, b = 73.43, c = 84.56 Å. There is one molecule per asymmetric unit as estimated by the calculated value of $V_{\rm M}$ (2.26 Å^3 Da^{-1}; Matthews, 1968). The solvent content of the orthorhombic crystal is thus approximately 45%. Data statistics for both crystal forms are listed in Table 1. Structure solution by molecular-replacement methods using homologous known structures is under way and it is hoped that by comparing the structures from the two different crystal forms we will gain further insight into the catalytic mechanisms of OXY-1a and that the structures will help us to design better inhibitors against OXY-1a.

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