

Crystallization and preliminary X-ray diffraction analysis of kanamycin-binding β -lactamase in complex with its ligand

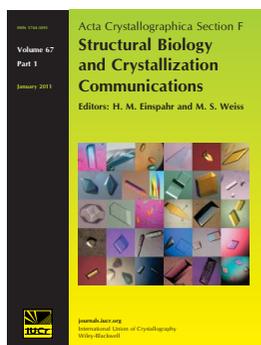
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Crystallization and preliminary X-ray diffraction analysis of kanamycin-binding β -lactamase in complex with its ligand

TEM-1 β -lactamase is a highly efficient enzyme that is involved in bacterial resistance against β -lactam antibiotics such as penicillin. It is also a robust scaffold protein which can be engineered by molecular-evolution techniques to bind a variety of targets. One such β -lactamase variant (BlaKr) has been constructed to bind kanamycin (kan) and other aminoglycoside antibiotics, which are neither substrates nor ligands of native β -lactamases. In addition to recognizing kan, BlaKr activity is up-regulated by its binding *via* an activation mechanism which is not yet understood at the molecular level. In order to fill this gap, determination of the structure of the BlaKr–kan complex was embarked upon. A crystallization condition for BlaKr–kan was identified using high-throughput screening, and crystal growth was further optimized using streak-seeding and hanging-drop methods. The crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 47.01$, $b = 72.33$, $c = 74.62$ Å, and diffracted to 1.67 Å resolution using synchrotron radiation. The X-ray structure of BlaKr with its ligand kanamycin should provide the molecular-level details necessary for understanding the activation mechanism of the engineered enzyme.

1. Introduction

TEM-1 β -lactamase (Bla) is a highly efficient enzyme that is involved in bacterial resistance against β -lactam antibiotics such as penicillin, ampicillin and some cephalosporins (Matagne *et al.*, 1998). Antibiotic resistance conferred by Bla-producing bacteria presents an acute clinical challenge (Rice, 2009; Petrosillo *et al.*, 2010), and considerable effort has been devoted to the search for novel Bla inhibitors (Pérez-Llarena & Bou, 2009). Apart from its clinical importance, this enzyme has proven to be a popular biotechnological target. It has been shown that Bla is a robust scaffold protein, tolerating insertions of 3–12 amino acids into three of its surface loops (Mathonet, Deherve *et al.*, 2006). Randomization of the grafted peptides and selection for binding to a target of interest by molecular-evolution techniques, such as phage display, allows the isolation of Bla clones that not only interact with the target but also exhibit binding-induced changes in enzymatic activity (Soumillion & Fastrez, 2001; Fernandez-Gacio *et al.*, 2003). This opens the possibility of creating sensitive biosensors, *e.g.* for homogenous immunoassays (Legendre *et al.*, 1999). To date, a number of Bla variants regulated by the binding of monoclonal antibodies (Legendre *et al.*, 1999), non-immunoglobulin proteins (*e.g.* streptavidin, ferritin and β -galactosidase; Legendre *et al.*, 2002) and transition metals (Mathonet, Barrios *et al.*, 2006) have been reported.

Very recently, a Bla mutant that is regulated by aminoglycoside antibiotics, which are neither substrates nor ligands of the native β -lactamases, has been engineered (Volkov *et al.*, 2011). The constructed enzyme (BlaKr) is up-regulated by the binding of kanamycin (kan) and other aminoglycosides *via* an activation mechanism involving the expulsion of an aminosulfonate inhibitor bound to an additional fortuitous site. Except for the engineered loop regions, the BlaKr structure solved by X-ray crystallography is very similar to that of wild-type Bla (Jelsch *et al.*, 1993). Using NMR chemical shift perturbation analysis, the protein surface involved in kan binding has been delineated (Volkov *et al.*, 2011); however, the technique used does not provide molecular-level details of BlaKr–kan interactions,



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which are crucial to understanding the mechanism of enzyme activation. Here, we report the crystallization conditions and preliminary crystallographic analysis of BlaKr in the presence of its ligand kanamycin.

2. Experimental procedures and results

2.1. Protein expression and purification

The gene coding for BlaKr (His26–Trp290; numbering according to Ambler *et al.*, 1991), a 30 kDa protein, was optimized for *Escherichia coli* expression and synthesized by GENEART, and cloned into a pET24(ompA) vector allowing extracellular expression (Sosa-Peinado *et al.*, 2000). The resulting construct, pET24-BlaKr(ompA), was transformed into *E. coli* BL21 (DE3) and grown overnight at 310 K and 180 rev min⁻¹ agitation in 10 ml LB medium containing 25 µg ml⁻¹ kanamycin (LB-kan). The next day, a larger culture (1 l LB-kan in 2 l Erlenmeyer flasks) was inoculated with the overnight pre-culture (200-fold dilution) and incubated at 310 K with

180 rev min⁻¹ shaking until an OD₆₀₀ of 0.6 was reached. At this point, BlaKr expression was induced with IPTG (final concentration of 1 mM) and the cultures were grown at 293 K for a further 24 h. The cells were centrifuged at 8000 rev min⁻¹ for 15 min at 277 K and the BlaKr-containing supernatant was collected. After the addition of protease inhibitors (AEBSF–HCl, 0.1 mg ml⁻¹; leupeptin, 1 µg ml⁻¹) the supernatant was diluted twofold with 20 mM MES pH 5.0 followed by the addition of 2.5 volumes of deionized H₂O. The protein solution was filtered through a 5 µm syringe filter (Millipore, Belgium) and loaded onto a 30S Source anion-exchange column (GE Healthcare, The Netherlands) pre-equilibrated with 20 mM MES pH 5.0 and the protein was eluted with a linear gradient of 0–1 M NaCl. Fractions containing BlaKr (as judged by 15% SDS–PAGE) were pooled and concentrated to ~2 ml in an Amicon ultracentrifugal filter (10 kDa cutoff; Millipore, Belgium). The protein was further purified on a Superdex 75 gel-filtration column (GE Healthcare, The Netherlands) pre-equilibrated with 20 mM MES, 100 mM NaCl pH 5.5 (Fig. 1). Fractions containing pure BlaKr were pooled, exchanged into 20 mM Bis-Tris–HCl pH 6.6 containing 0.02% NaN₃ as a preservative and concentrated to 9 mg ml⁻¹. The final protein concentration was estimated by UV–Vis spectroscopy using the extinction coefficient ($\epsilon_{280} = 28.21 \text{ mM}^{-1} \text{ cm}^{-1}$) determined in previous work (Volkov *et al.*, 2011). The BlaKr–kan complex used for screening was prepared by mixing 100 µl BlaKr solution (9 mg ml⁻¹) with 3 µl kan stock (0.1 M in 20 mM Bis-Tris–HCl pH 6.6) and was incubated at 295 K for 30 min before use.

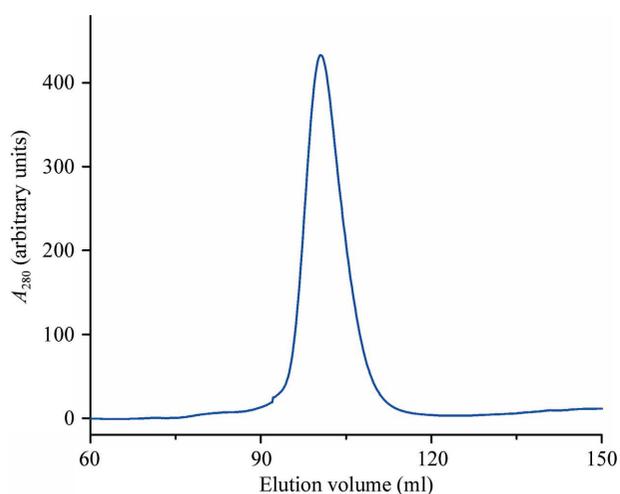


Figure 1 Elution profile of BlaKr from a Superdex 75 16/90 gel-filtration column in 20 mM MES pH 5.5, 100 mM NaCl.

2.2. Protein crystallization

A large screening of crystallization conditions was performed using eight commercial screens, each consisting of 96 conditions (Index, Crystal Screen, Crystal Screen 2 and Natrix from Hampton Research, USA, JB Screen Classic 1–4 HTS, JB Screen Classic 5–8 HTS and JB Screen Basic HTS from Jena Bioscience, Germany and PACT premier and JCSG-plus from Molecular Dimensions, UK) in 96-well Intelli-Plates (Art Robbins Instruments). The screening was performed using a Phoenix crystallization robot (Art Robbins Instruments). The sitting-drop vapour-diffusion method was used, with 100 nl protein sample (9 mg ml⁻¹) mixed with an equal volume of the reservoir screening solution. Two related conditions, A12 [0.01 M

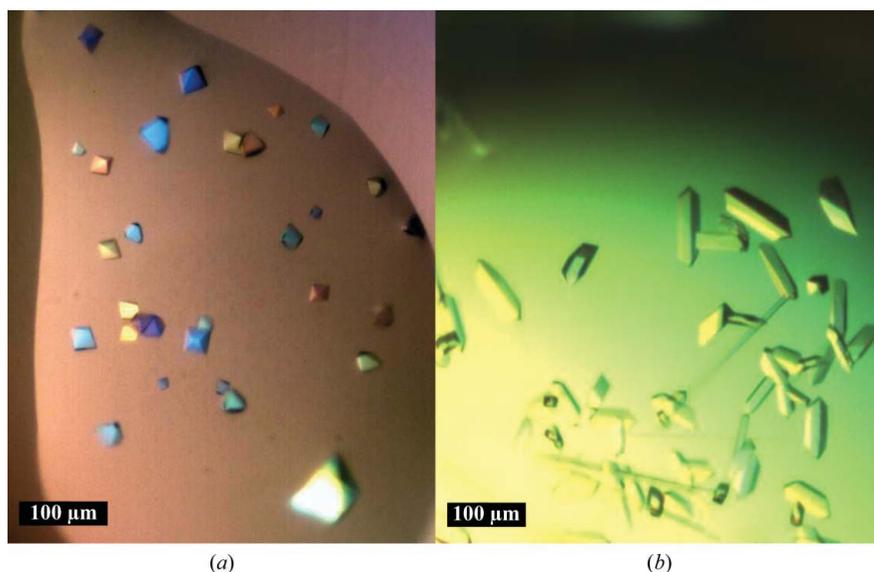


Figure 2 Crystals of BlaKr (a) from screening plate condition A12 (PACT premier) and (b) from a seeded hanging drop.

ZnCl₂, 0.1 M sodium acetate pH 5.0 and 20% (w/v) PEG 6000] and B12 [0.01 M ZnCl₂, 0.1 M MES pH 6.0 and 20% (w/v) PEG 6000], from the PACT premier screen (Molecular Dimensions, UK) produced crystals after 5 d at 293 K (Fig. 2a). Crystallization was optimized by the hanging-drop method in BD Falcon 24-well multi-well plates (catalogue No. 353047; BD Biosciences, USA), in which the PEG 6000 concentration was varied from 12 to 22% in 2% increments while the buffer and salt concentrations were kept constant (0.1 M sodium acetate pH 5.25, 0.01 M ZnCl₂). 1 µl of the protein batch used for screening (9 mg ml⁻¹) was mixed with 1 µl well solution; this was followed by streak-seeding of the drops (using a cat whisker) with pulverized crystals from the screening plates. The seeded crystals grew in 24 h and were larger in size (0.15 × 0.05 × 0.03 mm; Fig. 2b). A data set was collected from a crystal grown in 16% PEG 6000, 0.1 M sodium acetate pH 5.25, 0.01 M ZnCl₂.

2.3. Data collection

A complete X-ray diffraction data set was collected from a single crystal of BlaKr, which was cryocooled in liquid nitrogen using reservoir solution containing 20% glycerol as a cryoprotectant. The X-ray diffraction data were collected on a MAR Mosaic CCD detector using synchrotron radiation on beamline PXIII at the Swiss Light Source (SLS), Paul Scherrer Institute (PSI), Switzerland. The data-collection strategy was as follows: 120 images were collected with an oscillation step of 1° and 1 s exposure time. The crystal-to-detector distance was 112.94 mm.

2.4. Preliminary X-ray analysis

The data set extended to 1.67 Å resolution (Fig. 3). Indexing was performed with *iMOSFLM* (Battye *et al.*, 2011) and scaling and merging were performed using the *CCP4* package (Winn *et al.*, 2011).

The crystal was found to belong to the orthorhombic space group *P*₂₁₂₁, with unit-cell parameters *a* = 47.01, *b* = 72.33, *c* = 74.62 Å (Table 1). A total of 30 269 unique reflections were measured. The merged data set is 100% complete to 1.67 Å resolution, with an *R*_{merge}

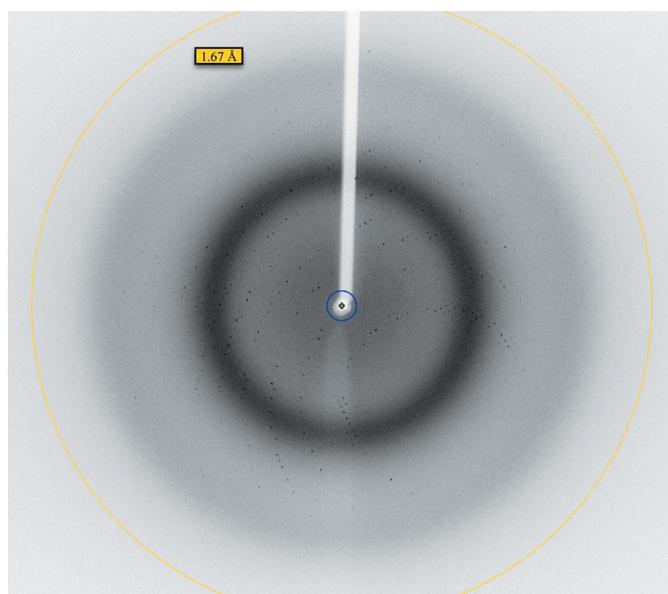


Figure 3
A representative 1° oscillation image of data collected from a crystal of BlaKr using synchrotron radiation. The image was collected from a crystal vitrified at 100 K using a MAR Mosaic CCD detector on the PXIII beamline at SLS, PSI, Switzerland.

Table 1

Data-collection and structure-solution statistics.

Values in parentheses are for the outer shell.

Crystal dimensions (mm)	0.150 × 0.050 × 0.030
Matthews coefficient <i>V</i> _M (Å ³ Da ⁻¹)	2.12
Solvent content (%)	42.14
Crystal system	Orthorhombic
Space group	<i>P</i> ₂ ₁ ₂ ₁
Unit-cell parameters (Å, °)	<i>a</i> = 47.01, <i>b</i> = 72.33, <i>c</i> = 74.62, α = β = γ = 90
No. of molecules in asymmetric unit	1
Wavelength (Å)	1.000
Temperature (K)	100
Resolution range (Å)	29.22–1.67 (1.76–1.67)
No. of unique reflections	30269 (4348)
No. of observed reflections	144890 (20849)
Completeness (%)	100 (100)
Multiplicity	4.8 (4.8)
<i>I</i> /σ(<i>I</i>)	10.8 (2.1)
<i>R</i> _{merge} [†] (%)	11.6 (87.2)

[†] $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where *I*_{*i*}(*hkl*) and ⟨*I*(*hkl*)⟩ are the observed intensity of the *i*th measurement and the mean intensity of the reflection with indices *hkl*, respectively.

of 11.6% and mean *I*/σ(*I*) values of 10.8 for all reflections and 2.1 for the highest resolution bin. The calculated Matthews coefficient (*V*_M) of 2.12 Å³ Da⁻¹ indicates the presence of one BlaKr molecule in the asymmetric unit, with a solvent content of about 42.14% (Winn *et al.*, 2011; Matthews, 1968). The structure will be determined by molecular replacement using free BlaKR (PDB entry 2vlz; Volkov *et al.*, 2011) as the search model.

We believe that the crystal structure of BlaKr with its ligand kanamycin will provide the molecular-level details necessary for understanding the activation mechanism of the engineered enzyme.

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