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

**Karen Van de Water,[a,b] Sameh H. Soror,[a,b,c] Alexandre Wohlkonig,[a,b]* Nico A. J. van Nuland[a,b] and Alexander N. Volkov[a,b]**

[a]Department of Molecular and Cellular Interactions, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium, [b]Structural Biology Brussels, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium, and [c]Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, Helwan University, Cairo, Egypt

Correspondence e-mail: awohlkon@vub.ac.be

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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 $OD_{600}$ of 0.6 was reached. At this point, BlaKr expression was induced with IPTG (final concentration of 1 m*M*) 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 m*M* MES pH 5.0 followed by the addition of 2.5 volumes of deionized $H_2O$. 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 m*M* 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 m*M* MES, 100 m*M* NaCl pH 5.5 (Fig. 1). Fractions containing pure BlaKr were pooled, exchanged into 20 m*M* Bis-Tris–HCl pH 6.6 containing 0.02% $NaN_3$ as a preservative and concentrated to 9 mg ml$^{-1}$. The final protein concentration was estimated by UV–Vis spectroscopy using the extinction coefficient ($\varepsilon_{280}$ = 28.21 m*M*$^{-1}$ 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 m*M* Bis-Tris–HCl pH 6.6) and was incubated at 295 K for 30 min before use.

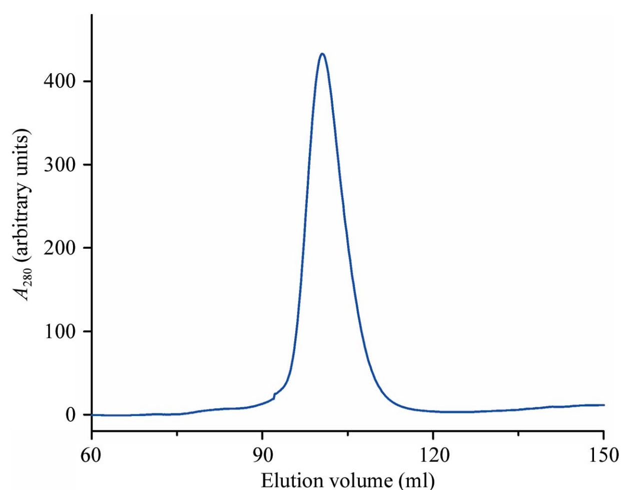


**Figure 1**
Elution profile of BlaKr from a Superdex 75 16/90 gel-filtration column in 20 m*M* MES pH 5.5, 100 m*M* NaCl.

### 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*

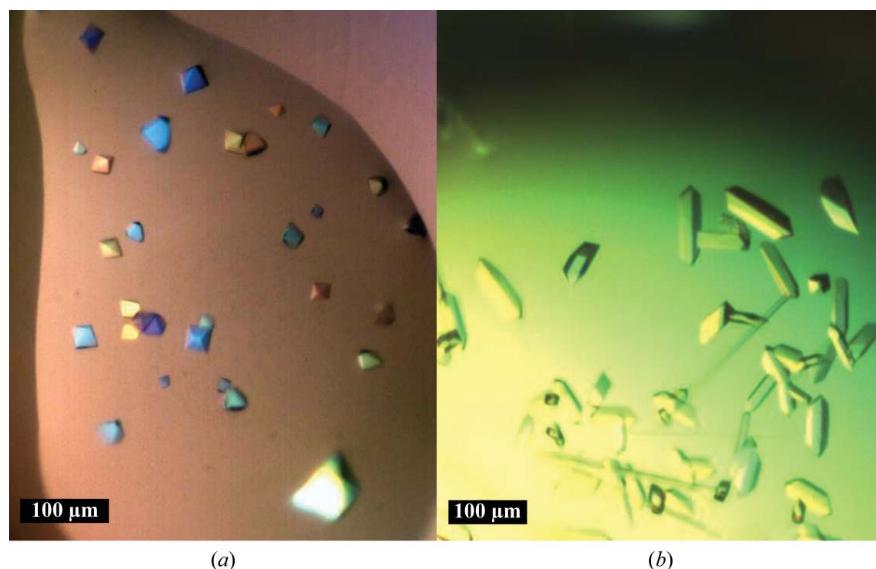


**Figure 2**
Crystals of BlaKr (*a*) from screening plate condition A12 (PACT premier) and (*b*) from a seeded hanging drop.

$ZnCl_2$, 0.1 *M* sodium acetate pH 5.0 and 20%(*w*/*v*) PEG 6000] and B12 [0.01 *M* $ZnCl_2$, 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. 2*a*). 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* $ZnCl_2$). 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. 2*b*). A data set was collected from a crystal grown in 16% PEG 6000, 0.1 *M* sodium acetate pH 5.25, 0.01 *M* $ZnCl_2$.

### 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 *CCP*4 package (Winn *et al.*, 2011).

The crystal was found to belong to the orthorhombic space group $P2_12_12_1$, 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 $R_{\rm merge}$ of 11.6% and mean $I/\sigma(I)$ values of 10.8 for all reflections and 2.1 for the highest resolution bin. The calculated Matthews coefficient ($V_{\rm M}$) of 2.12 Å$^3$ 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 2v1z; Volkov *et al.*, 2011) as the search model.

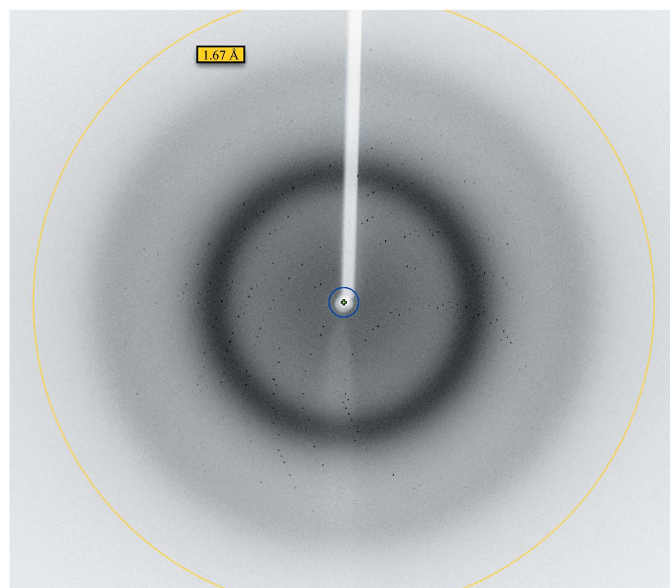


**Figure 3**
A representative 1° oscillation image of data collected from a crystal of BlaKr using synchrotron radiation. The image was collected from a crystal vitrified at 100 K using a MAR Mosaic CCD detector on the PXIII beamline at SLS, PSI, Switzerland.

**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 $V_{\rm M}$ (Å$^3$ Da$^{-1}$) | 2.12 |
| Solvent content (%) | 42.14 |
| Crystal system | Orthorhombic |
| Space group | $P2_12_12_1$ |
| Unit-cell parameters (Å, °) | *a* = 47.01, *b* = 72.33, *c* = 74.62, $\alpha = \beta = \gamma = 90$ |
| 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) |
| $\langle I/\sigma(I)\rangle$ | 10.8 (2.1) |
| $R_{\rm merge}$† (%) | 11.6 (87.2) |

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

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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