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Crystallization and preliminary crystallographic studies of the butyrolactone autoregulator receptor protein (BarA) from *Streptomyces virginiae*

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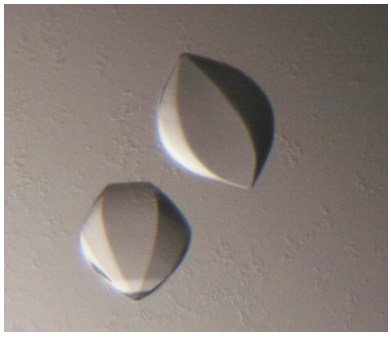

The *Streptomyces* butyrolactone autoregulator receptor protein (BarA) is a DNA-binding protein that regulates the biosynthesis of the antibiotic virginiamycin. In this study, BarA from *S. virginiae* was overexpressed in *Escherichia coli*, purified and crystallized. Crystals of purified protein have been grown that diffracted to beyond 3.0 Å resolution at 100 K using synchrotron radiation. The protein crystals belonged to the hexagonal space group $P6_522$, with unit-cell parameters $a = b = 128.0$, $c = 286.2$ Å. With four molecules per asymmetric unit, the crystal volume per unit protein mass (V_M) was 3.2 Å^3 Da^{-1} and the solvent content was 62%.

1. Introduction

In the Gram-positive bacterial genus *Streptomyces*, γ-butyrolactone autoregulators act as microbial hormones together with their specific γ-butyrolactone receptors to control antibiotic production, morphological differentiation such as aerial mycelium formation or both (Hinrichs *et al.*, 1994; Ramos *et al.*, 2005). *S. virginiae* produces two types of antibiotic, virginiamycins M1 and S, which are induced by nanomolar concentrations of γ-butyrolactone autoregulators called virginiae butanolides (VBs; Suzuki *et al.*, 1998; Pulsawat *et al.*, 2007). The VBs bind to the butyrolactone autoregulator receptor BarA and this transmits the signal to the cell. BarA binds to the upstream regulatory regions of the *barA* and *barB* genes and complex formation of VBs with BarA *in vitro* has been shown to cause dissociation of BarA from these regulatory regions (Kinoshita *et al.*, 1999).

BarA is a 232-amino-acid protein that has been shown to form a dimer. BarA belongs to the TetR family of transcriptional repressors, which have a helix–turn–helix DNA-binding motif at the N-terminus (Kisker *et al.*, 1995; Kinoshita *et al.*, 1997). CprB from *S. coelicolor* A3(2) has 29% identity to BarA and its structure has been solved, although the ligand that it binds to is unknown (Natsume *et al.*, 2004). An amino-acid sequence alignment comparing BarA and CprB suggests that BarA is composed of two domains: an N-terminal DNA-binding domain and a C-terminal regulatory domain. The N-terminal regions of the proteins show stronger similarity. Of the final 19 amino-acid residues at the C-terminus of BarA, 11 are alanine residues. In this study, we constructed plasmids to express full-length BarA and BarA truncated at the C-terminus (BarA residues 1–213, 1–219 and 1–225). However, the truncated proteins showed reduced expression and were prone to aggregation, which prevented crystallization. Full-length BarA was expressed and purified for crystallization in order to further understand the mechanism of this autoregulator–receptor regulatory system.

2. Material and methods

2.1. Cloning and expression

The gene encoding full-length *S. virginiae* BarA was amplified by polymerase chain reaction (PCR) and cloned into a pET-28b(+) derivative (Novagen) modified to incorporate a tobacco etch virus (TEV) protease cleavage site between an N-terminal 6×His tag and the multiple cloning site (MRGSHHHHHHGSENLYFQG). The

Table 1
Data-collection statistics for BarA.

Values in parentheses are for the highest resolution shell.

Resolution range (Å)	50.0–3.00 (3.11–3.00)
Space group	$P6_522$
Unit-cell parameters (Å)	$a = b = 128.1$, $c = 286.2$, $\alpha = \beta = 90$, $\gamma = 120$
$\langle I/\sigma(I)\rangle$	18.3
No. of measured/unique reflections	217102/26674
Completeness (%)	93.0 (79.8)
$R_{\rm merge}$† (%)	5.5 (40.4)
Redundancy	8.2 (5.2)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of $I(hkl)$ and $\langle I(hkl)\rangle$ is the mean value for this reflection.

primers used for PCR were as follows: forward, 5′-GGG CCC GGA TCC ATG GCA GTG CGA CAC GAA CGG GTG-3′; reverse, 5′-GGG CCC CTC GAG CTA CTC GTC GGA GGC GGC CTC GGA-3′. The amplified DNA was digested using *Bam*HI and *Xho*I endonucleases and inserted into modified pET-28b(+) to generate pET-28b(+)6×His-TEV-BarA. The construct was then transformed into BL21 (DE3)/pLysS (Novagen). Cells were grown in 2 l Luria–Bertani (LB) medium containing 40 µg ml^{-1} kanamycin and 35 µg ml^{-1} chloramphenicol at 310 K until the optical density (600 nm) reached ∼0.5; this was followed by induction with 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside at 293 K and overnight culture with shaking at 200 rev min^{-1}.

2.2. Purification

The cultured cells were harvested by centrifugation at 3000*g* for 30 min at 277 K. The cell pellet was resuspended in 50 ml lysis buffer [50 m*M* Tris–HCl pH 8.0, 300 m*M* KCl, 10 m*M* imidazole and 1 m*M* phenylmethylsulfonyl fluoride] and disrupted by sonication at 277 K. The crude lysate was centrifuged at 25 000*g* for 1 h at 277 K. The supernatant was loaded onto a 20 ml Ni^{2+}–NTA (Qiagen) column which had been pre-equilibrated with binding buffer (50 m*M* Tris–HCl pH 8.0, 300 m*M* KCl, 10 m*M* imidazole). The protein was eluted from the column with elution buffer (50 m*M* Tris–HCl pH 8.0, 300 m*M* KCl and 500 m*M* imidazole) using a linear gradient (10–500 m*M*) of imidazole. After Ni–NTA chromatography, the histidine tag was removed by digestion with TEV protease, leaving three N-terminal residues (Gly-Gly-Ser) attached to BarA. The peak fractions were incubated overnight with His-tagged TEV protease at room temperature while dialysing against Ni–NTA binding buffer. After complete cleavage, the sample was again loaded onto an Ni–NTA column to remove the His tag, His-tagged TEV protease and minor protein contaminants. The peak fractions were pooled and concentrated for gel-filtration chromatography. After concentration, the protein was applied onto a Superdex 200 prep-grade (16/60) gel-filtration column (GE Healthcare) equilibrated in 50 m*M* Tris–HCl pH 8.0 and 300 m*M* KCl. The purified BarA was concentrated to 4 mg ml^{-1} using a Centricon YM-10 filter (Millipore) and the homogeneity of the purified protein was examined by 12% SDS–PAGE and determined to be >95%. No further buffer exchange was carried out before crystal screening. The gel-filtration elution time suggests that BarA forms a dimer in solution as expected.

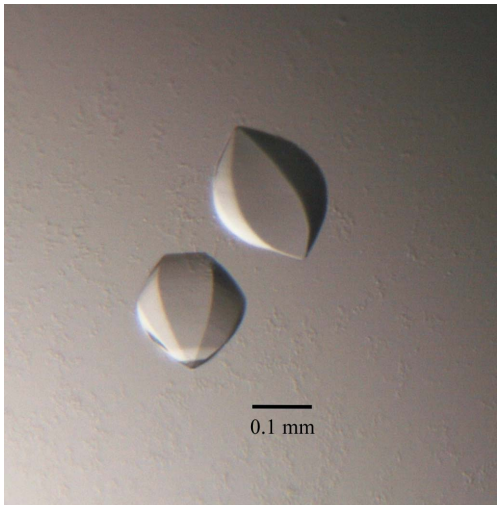

Figure 1
A reservoir solution containing 0.6 *M* sodium phosphate, 0.12 *M* potassium phosphate pH 5.6 was found to be optimal for obtaining crystals of BarA. The approximate dimensions of the crystal shown are 200 × 150 × 150 µm.

2.3. Crystallization

Initial crystallization screening of BarA was performed at 293 K by the sitting-drop vapour-diffusion technique using The Classics Lite Suite, Classics II, PEGs, pHClear and pHClear II commercial screening kits (Qiagen). Drops containing protein and precipitant solution were produced using a Hydra II e-drop automated pipetting system (Matrix Technologies Ltd, UK) in 96-well sitting-drop Intelli-Plates (Art Robbins Instruments, USA); the drops consisted of 0.3 µl protein solution and 0.3 µl well solution and were equilibrated over 60 µl well solution. After 6 d, BarA crystals were observed in reservoir conditions consisting of 0.1 *M* sodium acetate pH 4.6, 2.0 *M* sodium formate and 1.26 *M* sodium phosphate, 0.14 *M* potassium phosphate. The optimal crystallization buffer was found to be 0.6 *M* sodium phosphate, 0.12 *M* potassium phosphate pH 5.6. Crystallization of the BarA protein was performed using the hanging-drop vapour-diffusion method (with a drop consisting of 2 µl protein solution and 2 µl reservoir solution equilibrated against 0.5 ml

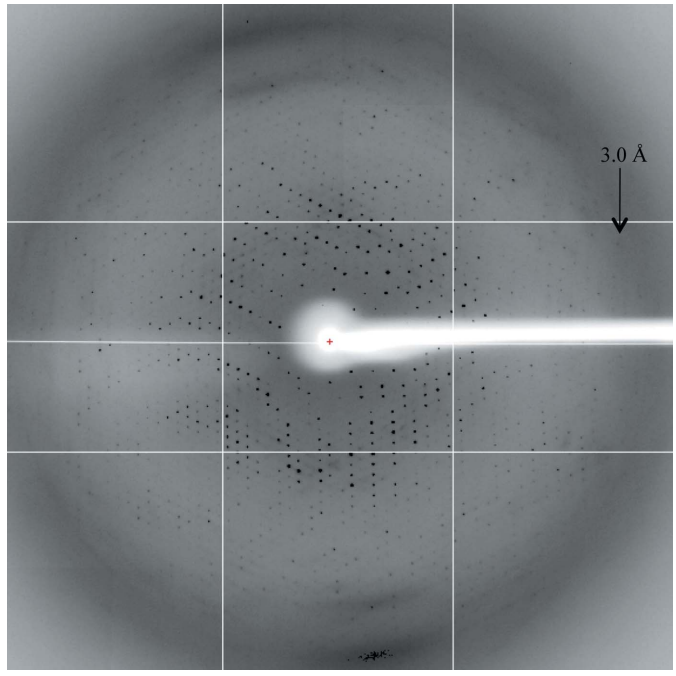

Figure 2
Diffraction image of the BarA crystal. The resolution limit (3.0 Å) is indicated.

reservoir solution) at 293 K. Crystals grew to maximal dimensions (50 × 400 µm in length) within one week (Fig. 1).

2.4. Data collection

The crystal was flash-cooled to 100 K in nitrogen gas after washing with cryoprotectant solution [reservoir solution containing 25%(*v*/*v*) glycerol]. X-ray diffraction data were collected using synchrotron radiation (wavelength 1.0 Å) and an ADSC Quantum 210 mm CCD detector on beamline AR-NW12A at Photon Factory, KEK, Japan. Data were processed using *HKL*-2000 (Otwinowski & Minor, 1997). All images were collected at 100 K using a 0.5° oscillation angle per frame (Fig. 2). A total of 26 674 unique reflections were obtained with an $R_{\rm merge}$ of 5.5%. The data-collection statistics are summarized in Table 1.

3. Results and discussion

BarA from *S. virginiae* was overexpressed in *E. coli*, purified and crystallized for structural study. X-ray diffraction data from the crystal indicated that the space group of the crystal was $P6_522$. The refined unit-cell parameters were $a = b = 128.0$, $c = 286.2$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. Assuming a molecular weight of 25 kDa, this gives a solvent content of 62% and a crystal volume per unit protein mass ($V_{\rm M}$) of 3.2 Å^3 Da^{-1} for four molecules in the asymmetric unit. BarA has 29% amino-acid sequence identity to CprB, which was used as a search model for molecular replacement with the program *MOLREP* (Collaborative Computational Project, Number 4, 1994). *MOLREP* found two rotation–translation solutions in space group $P6_522$, which were rigid-body-refined using the program *CNS* (Brünger *et al.*, 1998). The best MR solution was obtained using one monomer of the dimeric structure of CprB (PDB code 1ui5; Natsume *et al.*, 2004) as a template, giving an *R* factor of 44.6% for data in the resolution range 20–3.0 Å. We are now attempting to determine the structure of BarA using native data.

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