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Crystallization of CprB, an autoregulator-receptor protein from *Streptomyces coelicolor* A3(2)

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CprB, an autoregulator-receptor protein from *Streptomyces coelicolor* A3(2), was crystallized by the hanging-drop vapour-diffusion method using polyethylene glycol 6000 as a precipitant. Three crystal forms (I, II and III) were obtained; crystal forms I and II were useful for structure determination. Form I crystals belong to an orthorhombic system with space group $P2_12_12_1$ and diffracted to better than 2.4 Å. Form II crystals belong to a tetragonal system with space group $P4_{1(3)}2_12$.

1. Introduction

The Gram-positive bacteria genus *Streptomyces* employs γ-butyrolactones and their specific receptors as autoregulators or microbial hormones to regulate antibiotic production and/or morphological differentiation (Horinouchi & Beppu, 1994; Horinouchi, 2002). The A-factor (2-isocaprylolyl-3*R*-hydroxymethyl-γ-butyrolactone) is a representative of the γ-butyrolactone autoregulatory factors. It is essential for aerial mycelium formation, streptomycin production, streptomycin resistance and yellow-pigment production in *Streptomyces griseus*. The A-factor-binding protein (ArpA) shows high specificity for A-factor and has a repressor-type function (Onaka *et al.*, 1995). The A-factor–ArpA complex dissociates from the promoter region of its target genes, resulting in transcriptional activation of the particular gene (Onaka & Horinouchi, 1997; Ohnishi *et al.*, 1999). In other species, a pair of genes for the biosynthesis of γ-butyrolactone autoregulators and their specific receptors are contained in a number of biosynthetic gene clusters for secondary metabolites (Horinouchi & Beppu, 1994). They control the biosynthesis of the respective metabolites by activating pathway-specific regulatory genes within the gene cluster. A-factor/ArpA-like systems are considered to be a common regulatory mechanism controlling secondary metabolism and/or morphogenesis of *Streptomyces* spp. (Horinouchi, 2002).

To reveal the molecular basis of the autoregulator-receptor regulatory system in *Streptomyces*, we attempted to solve the crystal structure of ArpA. However, as ArpA is very difficult to crystallize, readily aggregating in solution (Onaka *et al.*, 1995), we initiated crystallization of CprB, an ArpA homologue from *S. coelicolor* A3(2) (Onaka *et al.*, 1998). CprB shows approximately 30% amino-acid sequence identity to ArpA and controls antibiotic production and aerial mycelium formation in this strain. As a first step towards understanding the mechanism of the autoregulator-receptor regulatory system, we initiated a structural study of CprB. Here, we report the expression, purification and crystallization of CprB.

2. Results and discussion

2.1. Protein expression and purification

The *cprB* gene was subcloned from a pUC19 plasmid carrying the *cprB* gene into a pET26b(+) expression vector for overexpression. *Escherichia coli* BL21(DE3) pLysS was transformed by the expression vector and cultured at 310 K in LB medium containing 20 μg ml^{-1} kanamycin and 33 μg ml^{-1} chloramphenicol. For selenomethionyl-CprB (Se-CprB) expression, *E. coli* B834(DE3) pLysS was used; the cells were cultured at 310 K in LeMaster medium (LeMaster & Richards, 1985) containing 20 μg ml^{-1} kanamycin and 33 μg ml^{-1} chloramphenicol. CprB expression was induced by the addition of 1.0 m*M* IPTG at a cell density OD_{600} of 0.5–0.6. The cells were harvested by centrifugation 2.5 h after induction. The bacterial cell pellet was resuspended in lysis buffer [50 m*M* sodium phosphate buffer, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) pH 7.0] and homogenized by sonication. The lysate was centrifuged at 20 000*g* for 2 h to remove the cell debris. The supernatant was applied to a SP-Sepharose column equilibrated with buffer *A* (50 m*M* sodium phosphate buffer pH 7.0) and washed with buffer *A* containing 0.33 *M* sodium chloride. CprB was eluted with buffer

A containing 0.7 *M* sodium chloride. The fractions containing CprB were diluted with an equal volume of buffer *A* and applied to a heparin Sepharose CL6B column equilibrated with buffer *A*. The proteins were eluted with a linear sodium chloride gradient (0.0–1.0 *M*). The CprB fractions were diluted with an equal volume of buffer *A* and applied to a Mono S column equilibrated with buffer *A*. The proteins were again eluted with a linear sodium chloride gradient (0.0–1.0 *M*). The purified protein was almost homogenous on SDS–PAGE. Because the concentration of CprB solutions with a membrane filter led to aggregation of the protein, we used a Resource S column for this process. Therefore, the CprB fractions, diluted with an equal volume of buffer *A*, were applied to a Resource S column equilibrated with buffer *A*. The protein was eluted with buffer *A* containing 1.0 *M* sodium chloride. The eluted CprB was dialyzed against buffer *A*. The concentration of the dialysate was 5–10 mg ml^{-1}. Protein concentrations were estimated spectroscopically by measuring the absorbance at 280 nm, assuming an A_{280} of 0.760 for a 1.0 mg ml^{-1} solution. All the columns used for purification of CprB were purchased from Amersham Biosciences.

2.2. Crystallization of CprB

Crystallization was performed using the hanging-drop vapour-diffusion method at 293 K in 24-well plates (Hampton Research). For the initial crystallization trials, we employed a factorial approach (Jancarik & Kim, 1991) using CprB at a concentration of 5 mg ml^{-1}. A hanging drop was prepared by mixing equal volumes (2.0 µl each) of the protein solution and reservoir solution and was equilibrated against 0.5 ml of the respective reservoir solution. Crystals grew under the following reservoir conditions: (*a*) 8%(*w*/*v*) polyethylene glycol (PEG) 6000, 0.1 *M* Tris–HCl pH 8.5, (*b*) 20%(*w*/*v*) PEG 6000, 50 m*M* dipotassium hydrogen phosphate pH 9.1, (*c*) 2%(*w*/*v*) PEG 6000, 0.2 *M* lithium sulfate, 0.1 *M* Tris–HCl pH 8.6 and (*d*) 15%(*w*/*v*) PEG 6000, 0.2 *M* lithium sulfate, 0.1 *M* Tris–HCl pH 8.6. As these crystals were very small (the maximal dimension was approximately 0.1 mm) and diffracted only to 4 Å resolution, the initial conditions were optimized by varying the pH of the Tris–HCl buffer (8.0–8.7) and the concentrations of PEG 6000 (1–20%), protein (2–8 mg ml^{-1}) and lithium sulfate (0–0.3 *M*). The mixing ratios of the protein solution and reservoir solution were also examined.

The reservoir solution that gave the best result was 2%(*w*/*v*) PEG 6000, 0.2 *M* lithium sulfate, 0.1 *M* Tris–HCl pH 8.5. Droplets were prepared by mixing 3 µl reservoir solution and 6 µl protein solution (4 mg ml^{-1} CprB in buffer *A*). Under these conditions, crystal forms I, II and III were obtained. To solve the crystal structure using the multi-wavelength anomalous diffraction (MAD) method, Se-CprB was also crystallized under the same conditions as the native protein.

Form I crystals of both Se-CprB (form Ia) and native protein (form Ib) were of truncated rectangular shape and grew to maximum dimensions of 0.5 × 0.3 × 0.3 mm within one month (Fig. 1*a*). Preliminary crystallographic studies showed that form I crystals diffracted to better than 2.5 Å resolution and belonged to space group $P2_12_12_1$. Form II crystals, which were of truncated pyramidal shape (Fig. 1*b*), belonged to the tetragonal space group $P4_{1(3)}2_12$. Although the form II crystals tended to be very small, one Se-CprB crystal grew to dimensions of 0.15 × 0.1 × 0.05 mm after two months and was used for data collection. Form III crystals, which were rod-shaped (Fig. 1*c*), did not yield diffraction suitable for X-ray studies.

Table 1
Summary of data collection.

Statistics for the highest shell are given in parentheses.

	Form Ia				
	Peak	Edge	Remote	Form Ib	Form II
Space group	$P2_12_12_1$			$P2_12_12_1$	$P4_{1(3)}2_12$
Unit-cell parameters (Å)					
a	38.15			37.79	111.95
b	68.06			69.56	111.95
c	145.42			148.93	43.44
Wavelength (Å)	0.9792	0.9795	0.9710	1.0	0.9780
Resolution (Å)	29.9–2.4 (2.69–2.4)	29.2–2.4 (2.69–2.4)	29.9–2.4 (2.69–2.4)	29.8–2.0 (2.11–2.0)	39.5–3.0 (3.16–3.0)
R_{merge}† (%)	7.7 (13.4)	8.2 (14.8)	8.9 (16.6)	4.3 (18.5)	8.9 (45.5)
$I/\sigma(I)$	4.5 (4.8)	4.0 (4.2)	3.2 (3.6)	10.6 (4.0)	5.8 (1.6)
No. measured reflections	91001	90761	90828	108697	97157
No. unique reflections	15478	15469	15476	25274	5938
Completeness (%)	99.8 (99.8)	99.7 (99.7)	99.8 (100)	92.5 (89.0)	100 (100)
Multiplicity	5.9 (6.0)	5.9 (6.0)	5.9 (6.0)	4.3 (3.2)	16.4 (17.1)

† $R_{merge} = \sum_h \sum_i |I(h) - I(h)_i| / \sum_h NI(h)$.

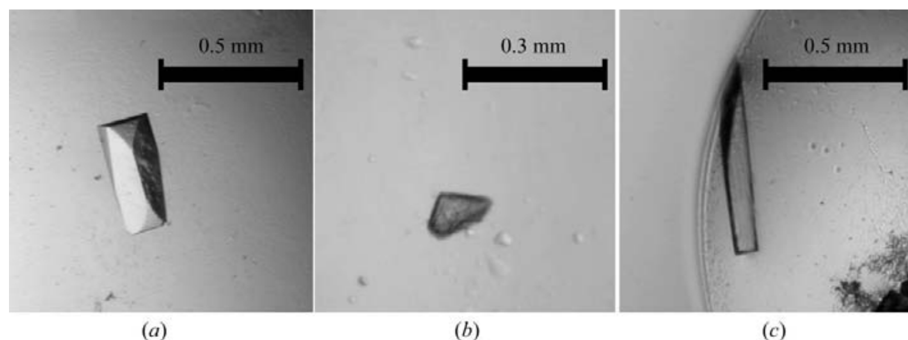

Figure 1
Three crystal forms of CprB. Forms I (*a*), II (*b*) and III (*c*) are shown.

2.3. Data collection and processing

All data collections were carried out at 100 K. The crystals were transferred to a cryoprotectant solution [25%(*w*/*v*) D-glucose, 4%(*w*/*v*) PEG 6000, 0.2 *M* lithium sulfate, 0.1 *M* Tris–HCl pH 8.5] and flash-cooled in a cold N_2 stream from a liquid-nitrogen cryostat (Rigaku). The MAD data collection from the Se-CprB crystal (form Ia) at 2.4 Å resolution was carried out at BL41XU, SPring-8 (Harima, Japan) using a MAR CCD area detector (Table 1). Based on the X-ray absorption fluorescence spectroscopy of the Se atoms in the Se-CprB crystal, three wavelengths, 0.9792 Å (peak), 0.9795 Å (edge) and 0.9710 Å (remote), were chosen for the MAD data collection. In addition to the MAD data set, diffraction data from crystal forms Ib and II were collected at BL6A, Photon Factory (Tsukuba, Japan) using an ADSC Quantum CCD detector. All data were processed using *MOSFLM* (Leslie, 1992) and scaled with *SCALA* from the *CCP*4 program suite

(Collaborative Computational Project, Number 4, 1994) (Table 1). On the basis of their molecular weights and unit-cell parameters, crystals of forms Ia and Ib are likely to contain two subunits in the asymmetric unit (Ia, V_M = 2.02 Å^3 Da^{-1}, ~39% solvent content; Ib, V_M = 2.09 Å^3 Da^{-1}, ~41% solvent content; Matthews, 1968). On the other hand, the form II crystal contains only one subunit in the asymmetric unit (V_M = 2.91 Å^3 Da^{-1}, ~58% solvent content). As it has been proposed that most autoregulator-receptors function as dimers (Horinouchi, 2002), the crystallographic twofold axis of the form II crystal is likely to coincide with the twofold axis of the dimeric CprB molecule. The anomalous Patterson maps calculated with the diffraction data of the form Ia crystal clearly showed several peaks in the Harker sections. Structure determination using the MAD method is in progress.

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