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Crystallization and preliminary X-ray diffraction studies of bleomycin-binding protein from bleomycin-producing *Streptomyces verticillus*

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Abstract

A bleomycin-binding protein (BLMA) produced by bleomycin-producing *Streptomyces verticullus* was crystallized in a form suitable for X-ray diffraction analysis using the vapor-diffusion method. Crystals were grown at pH 5.7, in 0.2 *M* NH_4 actate and 0.1 *M* Na acetate, using 30% PEG 4000 as a precipitant. They belong to the orthorhombic system, with space group $P2_12_12$, cell dimensions $a = 54.90$, $b = 67.94$, $c = 35.60$ Å, and one BLMA molecule in the asymmetric unit. The crystals diffract X-rays well and the diffraction intensity data was collected up to 1.5 Å resolution with a merging R value of 0.054 at beamline 6B of the Photon Factory. The diffraction data set is 94% complete.

1. Introduction

Bleomycin (Bm), produced by *Streptomyces verticullus* (Umezawa, 1974), is an important anti-tumor antibiotic which causes cell death as a result of multiple strand scissions by direct interaction with tumor and bacterial DNA's. The Bm-producing microorganism must be protected from the lethal effect of its own product. We have cloned and sequenced a gene, designated *blmA*, which confers resistance to Bm from Bm-producing *S. verticullus* ATCC15003 (Sugiyama *et al.*, 1994). The gene *blmA* encodes a Bm-binding protein (BLMA) with a molecular mass of 13 197 (Sugiyama *et al.*, 1994, 1995). Another gene encoding a Bm-binding protein, designated Sh*ble*, has been cloned from *Streptoalloteichus hindustranus* the microbe producing tallysomycin which is a Bm analogue (Gatignol, Durand & Tiraby, 1988). This microorganism belongs to the family Actinoplanaceae which is taxonomically distinguished from *Streptomyces*. We have generated a monoclonal antibody against BLMA and examined the immunological homology between the BLMA and the Sh*ble* proteins, showing that Sh*ble* protein does not cross react with the monoclonal antibody against BLMA (Sugiyama *et al.*, 1995). In addition, a polyclonal antibody raised against the Sh*ble* protein did not cross react with BLMA (Sugiyama *et al.*, 1995). Thus, although these Bm-binding proteins are functionally the same, they differ in immunological response. We have compared the translated protein sequences from the genes encoding *blmA* and Sh*ble* protein and shown that the homology between these proteins is close to 60% (Sugiyama *et al.*, 1994).

The Sh*ble* protein has been crystallized (Rondeau, Cagnon, Moras & Masson, 1989) and its X-ray structure has been determined at 2.3 Å resolution (Dumas, Bergdoll, Cagnon & Masson, 1994). However, the successful crystallization of the binding complex between the Sh*ble* protein and Bm has not been achieved, alternatively, a Sh*ble* protein–antibiotic complex model, based on a proposal three-dimensional model of the Sh*ble* protein and the chemical structure of Bm molecule, has been proposed.

BLMA is a small acidic protein which binds to positively charged Bm under physiological conditions presumably by electrostatic interactions. If a crystallographic analysis of BLMA could be performed at higher resolution than for the Sh*ble* protein, more detailed information about the mode of antibiotic binding could be obtained. In the present study, we obtained crystals of BLMA that give diffraction intensity data at the high resolution necessary for studying the mode of Bm binding.

2. Materials, methods and results

To obtain large amounts of BLMA purified to homogeneity, the following scheme for production and purification of the Bm-binding protein was developed: *Escherichia coli* HB101 harboring the plasmid pKM10 (Sugiyama *et al.*, 1995), which contains *blmA* under the control of the *trp* promoter, was used for the production of the protein. The bacteria were cultured in M9 casamino acids medium (Sambrook, Fritsch & Maniatis, 1989). At the exponential phase of growth, 3-indoleacrylic acid (IAA) was added to the medium to induce gene expression under the control of the *trp* promoter. The bacterial cells from 20 l of culture broth were harvested and washed with buffer I (5 m*M* Na phosphate, pH 7.2). The washed cells were ground with quartz sand and extracted with buffer II (10 m*M* sodium phosphate pH 7.2, 1 m*M* EDTA, 1 m*M* phenylmethyl sulfonyl fluoride and 5% glycerol). Cell debris and quartz sand were removed by centrifugation at 16 000*g* for 20 min. The supernatant was dialyzed against 50 m*M* Na acetate buffer (pH 5.0) and loaded on a CM-Sepharose column equilibrated with the same buffer. The fraction containing BLMA was collected and dialyzed against buffer I and then solid ammonium sulfate was added to 20% saturation. The solution was kept for 1 h in an ice bath with gentle shaking and then centrifuged at 25 000*g* for 20 min. The concentration of ammonium sulfate in the supernatant was increased to 80% and the precipitate was collected by centrifugation at 25 000*g* for 20 min and dissolved in buffer III (10 m*M* sodium phosphate pH 7.2, 1 m*M* EDTA and 10% glycerol). The solution was then loaded on a DEAE-Sepharose column equilibrated with the same buffer. Elution was with a 0–0.5 *M* NaCl linear gradient in buffer III and the

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fractions containing BLMA were concentrated by ultrafiltration with CENTRICUT-10 (Kurabo, Japan). The concentrated solution was loaded on a Sephadex G-75 column equilibrated with buffer III and eluted with the same buffer. The fractions containing BLMA were concentrated by ultrafiltration with ULTRACENT-10 (Tosoh, Japan) and the protein concentration assayed by the method of Bradford (1976) using bovine serum albumin as a standard. The protein was judged to be pure and homogeneous by the observations of a single bond on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and a single peak on high-pressure liquid chromatography (HPLC).

Crystals of BLMA were grown by vapor diffusion at 298 K using the hanging-drop method (McPherson, 1982). Crystallization droplets of 10 µl initial volume were prepared on siliconized glass cover slips suspended over 1.0 ml reservoirs containing 0.2 m*M* NH_4 sodium acetate (pH 5.7) and 30% PEG 4000. The crystallization droplets consisted of 5 µl (19 mg ml^{-1}) protein solution and 5 µl well solution. After one week, crystals of up to 0.2 × 0.2 × 0.5 mm were obtained.

For the crystallographic characterization of the crystal mounted in 0.7 mm diameter glass capillary with 10 µm wall thickness, an imaging-plate (IP) data-collection system, R-AXIS IIc, on a rotating-anode X-ray generator, RU-200 (Rigaku Co. Ltd, Tokyo), was used. Two still photographs were taken with Cu $K\alpha$ radiation for auto-indexing. The unit-cell dimensions of the crystal were determined to be $a = 54.90$, $b = 67.94$, $c = 35.60$ Å and $\alpha = \beta = \gamma = 90°$ by a least-squares fit of diffraction spots recorded on the two still photographs. After checking the diffraction intensity distribution of the whole data set preliminary collected to 2.0 Å resolution with the R-AXIS IIc, the crystal system and the space group were identified as being orthorhombic and $P2_12_12$, respectively. Assuming that the asymmetric unit contains one molecule, the V_m value is calculated to be 2.5 $Å^3$ Da^{-1}, corresponding to a solvent content of 51% (Matthews, 1968).

For crystal structure analysis, a high-resolution data set was collected with synchrotron radiation (1.0 Å wavelength) at beamline 6B of the Photon Factory, the National Laboratory for High Energy Physics, Tsukuba, Japan. A detailed description of the beamline will be reported elsewhere. A BLMA crystal of size 0.29 × 0.34 × 1.1 mm (Fig. 1) was mounted on a newly developed Sakabe camera (Sakabe, 1983, 1991; Sakabe *et al.*, 1995) with its *c* axis aligned along the rotation axis of the camera. Three Weissenberg photographs were taken for the data collection. Each photograph was recorded on a large area of 800 × 800 mm, composed of a pair of large-format image plates (IPs) (Fuji Film Co. Ltd) with dimensions 400 × 800 mm. The X-ray images recorded on the IPs were read out with the corresponding reader for the large-format IPs (Sakabe *et al.*, 1995). The diffraction intensities were evaluated and processed within 1.5 Å resolution by the *WEIS* (Higashi, 1989) and *CCP*4 program suites (Collaborative Computational Project, Number 4, 1994), respectively. A total of 75 016 reflections were measured and 20 649 independent reflections were obtained with a merging R value of 0.054 ($R_{\rm merge} = \sum_{ih}\sum_j |I_{hj} - \langle I\rangle_h| / \sum_h \sum_j I_{hj}$, where $\langle I\rangle$ is the mean intensity of a reflection h and I_{hj} is the jth measurement of reflection h). The diffraction data set is 94% complete.

The X-ray structure determination of the BLMA protein is in progress.

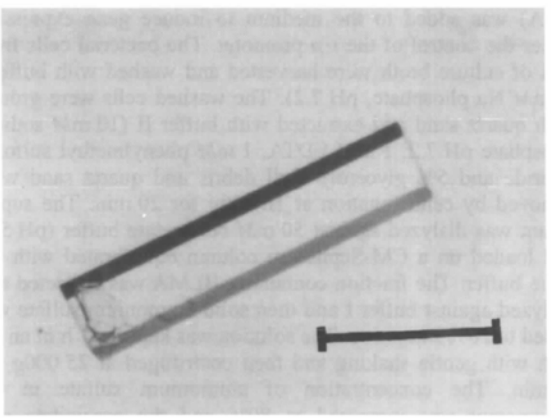

Fig. 1. A typical crystal of BLMA. The scale bar is 0.5 mm.

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