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Received 9 November 2012
Accepted 2 January 2013

Crystallization and preliminary X-ray crystallographic analysis of the functional form of BinB binary toxin from *Bacillus sphaericus*

The binary toxin from *Bacillus sphaericus* consists of two proteins, BinA and BinB, which work together to exert toxicity against mosquito larvae. BinB is proposed to be a receptor-binding domain and internalizes BinA into the midgut cells, resulting in toxicity *via* an unknown mechanism. The functional form of BinB has been successfully crystallized. The crystals of BinB diffracted to a resolution of 1.75 Å and belong to space group $P6_222$, with unit-cell parameters $a = b = 95.2$, $c = 154.9$ Å. Selenomethionine-substituted BinB (SeMetBinB) was prepared and crystallized for experimental phasing. The SeMetBinB crystal data were collected at a wavelength of 0.979 Å and diffracted to a resolution of 1.85 Å.

1. Introduction

A binary toxin is produced as crystalline inclusions during sporulation by the Gram-positive bacterium *Bacillus sphaericus* (*Bs*). This toxin is highly toxic to mosquito larvae after being ingested; hence it becomes an alternative mosquito-control agent. The binary toxin consists of two polypeptides, 42 kDa BinA and 51 kDa BinB (Baumann *et al.*, 1991; Charles *et al.*, 1996), and both of them are required for maximum toxicity against the larvae of *Culex* and *Anopheles* mosquito species (Berry *et al.*, 1993). The binary toxin components are synthesized as inactive protoxins that are solubilized under alkaline conditions upon ingestion by susceptible mosquito larvae, and are subsequently converted by larval gut proteases into their active forms of 40 and 45 kDa for BinA and BinB, respectively (Broadwell & Baumann, 1987; Nicolas *et al.*, 1990). The activated BinB has been demonstrated to bind to a specific receptor on the larval gut epithelial cells and mediates the regional binding and internalization of BinA (Oei *et al.*, 1992). The receptor of binary toxin has been identified as a Cpm1- α -glucosidase (*Culex pipiens* maltase 1) (Silva-Filha *et al.*, 1999; Darboux *et al.*, 2001).

The binary toxin has been shown to induce channel and pore formation in cultured *C. quinquefasciatus* cells and in a mammalian epithelial cell line (MDCK) expressing the binary toxin Cpm1 receptor (Cokmus *et al.*, 1997; Pauchet *et al.*, 2005). The ability of the binary toxin to insert into receptor-free lipid membranes and permeabilize phospholipid vesicles has also been reported (Schwartz *et al.*, 2001; Boonserm *et al.*, 2006; Kunthic *et al.*, 2011). Moreover, several cytopathological alterations were observed on intoxicated *C. quinquefasciatus* larvae including the formation of cytoplasm vacuoles, mitochondria swelling and microvilli destruction (Silva-Filha & Peixoto, 2003). The above evidence seems to indicate a complex mechanism for binary toxin activity. In the absence of a detailed three-dimensional structure, its toxicity mechanism remains unclear. Moreover, both BinA and BinB proteins show low amino-acid sequence similarity to proteins with known structures; hence their three-dimensional structures cannot be reliably predicted. Although there are reports describing the crystallization of BinB (Chiou *et al.*, 1999) and the binary toxin from a native *Bs* strain (Smith *et al.*, 2004), their structures have not been reported. As BinB plays a key role in the specificity of binary toxin, its protein fragments and amino-acid residues involved in the receptor binding have been investigated (Singkhamanan *et al.*, 2010; Tangsongcharoen *et al.*, 2011; Romão *et al.*, 2011). However, the lack of structural information

limits the usefulness of these functional data. To gain insight into the structure–function correlation and provide guidelines for engineering the protein with higher potency, we report the crystallization and preliminary X-ray crystallographic analysis of the functional form of BinB protein.

2. Materials and methods

2.1. Expression, purification and activation of native and selenomethionine-substituted BinB

Detailed cloning, expression and purification protocols for the trypsin-activated BinB have been described previously (Srisucharitpanit *et al.*, 2012). Briefly, the *binB* gene from *B. sphaericus* strain 2297 (GenBank accession No. AJ224478) was cloned by fusion with a hexahistidine tag at the N-terminus and expressed as a soluble protein in *Escherichia coli*. BinB protein was purified by using Ni-affinity and size-exclusion chromatography. To convert BinB into its active form, purified BinB protoxin was digested with trypsin enzyme (Sigma) at a trypsin:protoxin ratio of 1:20 (*w:w*) at 310 K for 2 h to remove some amino acids from both the N- and C-termini. The trypsin-activated reaction was stopped by adding 10 mM phenylmethylsulfonyl fluoride (PMSF, Sigma). The trypsin was removed by size-exclusion chromatography which was equilibrated with 50 mM Tris–HCl pH 9.0 and 1 mM dithiothreitol (DTT). The purified activated BinB was further concentrated to 5 mg ml^{−1} with an ultra-centrifugal 30 K cutoff filter (Amicon, USA) prior to crystallization.

(a)

(b)

Figure 1
Crystals of native (a) and SeMet-substituted (b) BinB used for X-ray diffraction.

Table 1

Summary of crystallographic data.

Values in parentheses correspond to the highest-resolution shell.

	Native	SeMet
Wavelength (Å)	1	0.979
Radiation source	BL41XU, SPring-8	BL41XU, SPring-8
Space group	<i>P</i> 6 ₂ 22	<i>P</i> 6 ₂ 22
Unit-cell parameters (Å)	<i>a</i> = 95.2, <i>b</i> = 95.2, <i>c</i> = 154.9	<i>a</i> = 95.0, <i>b</i> = 95.0, <i>c</i> = 154.5
Resolution range (Å)	28.99–1.75 (1.84–1.75)	28.99–1.85 (1.95–1.85)
No. of unique reflections	42536 (6069)	35916 (5127)
Multiplicity	10.6 (10.7)	15 (15)
Completeness (%)	100 (100)	100 (100)
$\langle I/\sigma(I) \rangle$	4.4 (1.8)	4.7 (2.4)
<i>R</i> _{meas} † (%)	12.6 (45.5)	11.9 (32.9)

† $R_{\text{meas}} = \sum_{hkl} [N(hkl)/[N(hkl) - 1]]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where I_h is the mean intensity of symmetry-equivalent reflection *h* and *N* is redundancy.

The activated BinB is composed of 390 amino acids with seven methionine residues; therefore, the selenomethionine-substituted BinB (SeMetBinB) was prepared for phasing. The recombinant plasmid, pET 28b-BinB, was introduced into *E. coli* B834 (DE3) methionine (Met) auxotrophic strain (Leahy *et al.*, 1992; Wood, 1966). The transformant was pre-cultured in 100 ml Luria-Bertani (LB) broth containing 50 µg ml^{−1} kanamycin at 310 K for 18 h and cultured cells were harvested by centrifugation. The cell pellets were washed twice with PBS (phosphate-buffered saline) buffer before being resuspended in 1 l of M9 medium containing 40 µg of each amino acid except methionine, 1 µg of each vitamin mixture supplement (riboflavin, pyridoxine monohydrochloride, thiamine and nicotinamide) and 40 µg of SeMet (Sigma). Expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the culture reached an OD₆₀₀ of 0.6 and cultured cells were further grown at 303 K for 24 h. SeMetBinB was purified and activated following the same protocols as those for the native protein.

2.2. Crystallization and X-ray diffraction data collection

Crystallization was performed by hanging- and sitting-drop vapour-diffusion methods in 96- and 24-well plates at 295 K (Molecular Dimensions, UK and Qiagen, Germany). Initial screening was performed using Hampton Research Crystal Screen kits (Hampton Research, USA) and positive hits were then optimized. Drops were prepared by mixing 1 µl of 5 mg ml^{−1} protein solution (50 mM Tris–HCl pH 9.0, 1 mM DTT) with an equivalent volume of reservoir solution and were equilibrated against 500 µl of reservoir solution. For the SeMetBinB, initial screening was done using the PEGs Suite crystallization screening kit (Qiagen, Germany). Both native and SeMetBinB crystals were briefly soaked in a cryoprotecting solution consisting of 25% (*v/v*) glycerol dissolved in their corresponding mother liquors before being cryocooled in a nitrogen steam at 100 K.

Preliminary diffraction data were collected in-house (SLRI Thailand) using a Cu rotating-anode generator ($\lambda = 1.54$ Å; Microstar, Bruker). Higher resolution data were collected on the BL41XU beamline of the SPring-8 synchrotron (Hyogo, Japan). The diffraction data set of the native crystal was collected at 1 Å wavelength. The single-wavelength anomalous dispersion (SAD) data of the SeMetBinB crystal were collected at 0.979 Å based on the fluorescence spectrum of the Se *K* absorption edge (Rice *et al.*, 2000). A total of 180 frames of native and SAD data were collected with an oscillation angle of 0.5° and an exposure time of 0.3 s for each image. The diffraction images of both crystals were recorded on the Rayonix MX-225HE CCD detector. All diffraction data were indexed and integrated using *iMOSFLM* software (Battye *et al.*, 2011), and scaled

Figure 2

Harker sections ($u = 0$, $w = 0$ and $w = 0.33$) of the anomalous difference Patterson maps of SeMet-substituted BinB (space group $P6_222$), calculated at 1.9 Å resolution with data collected at $\lambda = 0.979$ Å (see Table 1). Maps are drawn with a minimum contour level of 1.0σ , with 1.0σ increments. The seven Se peaks are labelled (A–G).

and merged with *SCALA* in the *CCP4* program suite (Winn *et al.*, 2011). Anomalous difference Patterson maps for SeMetBinB were calculated by the *CCP4* program package.

3. Results and discussion

As reported previously, the expression of recombinant BinB protein as a soluble form in *E. coli* was achieved by fusing the protein with a hexahistidine tag at the N-terminus and modifying the strategy of cell culture by reducing temperature (Srisucharitpanit *et al.*, 2012). The *in vitro* activation of the recombinant BinB was performed by trypsin digestion to generate a resistant fragment of 45 kDa which is fully active against *C. quinquefasciatus* larvae. Hence, the trypsin-activated product is a good candidate for the atomic structural study of BinB in its functional form.

During initial crystallization screening, microcrystals appeared in conditions containing PEG 4000 and some salts with divalent cation such as magnesium chloride, lithium sulfate and calcium chloride. All favourable conditions were further optimized by adjusting the concentrations of protein, precipitants and salts, and by varying pH from 6.5 to 10. The best native BinB crystals were grown in a condition consisting of 0.1 M Tris–HCl pH 8.0, 0.2 M lithium sulfate and 12% (w/v) PEG 4000. The crystals grew to their maximal dimensions of $250 \times 250 \times 50$ µm within 1 week (Fig. 1a).

Since a limited sequence identity is observed between BinB and other proteins with known structures, SeMetBinB was prepared to solve the phase problem. SeMetBinB was prepared with high purity comparable to native protein (data not shown). Furthermore, SeMetBinB still retained high toxicity against the *C. quinquefasciatus* larvae (data not shown). SeMetBinB crystals were obtained from a

condition consisting of 0.1 M Tris–HCl pH 8.0, 0.2 M magnesium acetate and 16% (w/v) PEG 3350, which is very similar to that used for the native protein. The crystals grew to their maximal dimensions of $100 \times 100 \times 50$ µm after 4 d (Fig. 1b).

X-ray diffraction data of the native BinB crystal were collected to 1.75 Å resolution using 25% glycerol in the mother liquor as a cryoprotectant. The crystal belongs to space group $P6_222$, with unit-cell parameters $a = b = 95.2$, $c = 154.9$ Å. The SAD data of a SeMetBinB crystal were collected to 1.85 Å resolution at the Se K absorption edge (wavelength 0.979 Å). The SeMetBinB crystal was found to be isomorphous with the native crystal, with unit-cell parameters $a = b = 95.0$, $c = 154.5$ Å. The Matthews coefficient (V_M) was estimated to be $2.35 \text{ Å}^3 \text{ Da}^{-1}$ with one molecule in the asymmetric unit, corresponding to a solvent content of 48% (Matthews, 1968). The seven selenium sites of SeMetBinB were found with the *SHELXD* program (Sheldrick, 2008) (Fig. 2), suggesting the usefulness of these data for phasing using the SAD method. Details of the data-collection statistics are summarized in Table 1. Structure determination and refinement are in progress.

We are grateful to the Synchrotron Light Research Institute (SLRI Public Organization, Thailand) for providing the in-house X-ray machine (MX end station) and the staff at BL41XU, SPring-8, for data collection. This work was supported by the Thailand Research Fund (TRF) and the Commission on Higher Education (CHE-PhD-SW).

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