Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization of the mosquito-larvicidal binary toxin produced by *Bacillus sphaericus*

Alex W. Smith, Ana Camara-Artigas‡ and James P. Allen*

Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona 85287, USA

‡ Present address: Departamento de Quimica-Fisica, Bioquimica y Quimica Inorganica, Universidad de Almeria, Carretera Sacramento, Almeria 04120, Spain.

Correspondence e-mail: jallen@asu.edu

Received 22 January 2004
Accepted 22 March 2004

The *Bacillus sphaericus* binary toxin is expressed during the early stages of sporulation and is composed of two separately encoded polypeptides: BinA (41.9 kDa) and BinB (51.4 kDa). The binary toxin forms microcrystalline inclusions inside the mother cell that, once ingested, are solubilized in the alkaline pH of the larval gut. *B. sphaericus* cultures were grown to complete sporulation, harvested and washed. The binary toxin was solubilized with 50 m*M* NaOH, purified using column chromatography and crystallized. Crystals were grown using the sitting-drop vapor-diffusion method in a glycine-buffered solution containing 0.5 *M* NaCl and 19.5% PEG 4000 as the precipitant. Native data were collected to a resolution of 2.7 Å.

1. Introduction

Bacillus sphaericus is a Gram-positive spore-forming aerobic bacterium, common to soil and aquatic environments, that produces a binary toxin active against the larvae of *Culex* and *Anopheles* mosquitoes. The binary toxin is composed of two polypeptides: BinA (41.9 kDa) and BinB (51.4 kDa). BinA and BinB are encoded by two highly conserved chromosomal genes, *binA* and *binB*, which are expressed in equimolar amounts during the early stages of sporulation (Baumann & Baumann, 1991). Spores of active *B. sphaericus* strains contain the binary toxin in the form of crystalline parasporal inclusions (Payne & Davidson, 1984). Once ingested by the larval mosquito, the binary toxin is solubilized in the alkaline pH of the larval midgut and activated by larval proteases (Broadwell & Baumann, 1987; Davidson *et al.*, 1987).

Previous studies have shown that both BinA and BinB are necessary for full toxicity (Broadwell *et al.*, 1990; Nicolas *et al.*, 1993; Oei *et al.*, 1990). However, BinA alone is toxic to *C. quinquefasciatus* cells at high doses (Baumann & Baumann, 1991). The binary toxin is specifically bound to a unique receptor at the surface of the midgut epithelium of *Culex* and *Anopheles* larvae (Davidson, 1988; Silva-Filha *et al.*, 1997). BinA and BinB are both responsible for receptor binding in *Anopheles* sp., whereas only BinB is responsible for receptor binding in *Culex* sp. (Charles *et al.*, 1997). The binary-toxin receptor has been identified as a 60 kDa glycosyl-phosphatidylinositol-anchored α-glucosidase (Cmp1) present in the brush-border membranes of susceptible *C. pipiens* larvae (Darboux *et al.*, 2002).

Although the binding and toxicity of the binary toxin have been studied extensively, the mechanism of pore-formation is unknown. Sequence and mutational analysis suggest that BinA and BinB are bound together by their N-terminal and C-terminal regions, respectively, leaving the N-terminal region of BinB to bind to Cmp1 and the C-terminal region of BinA to facilitate internalization and pore-formation (Yuan *et al.*, 2001). However, without a detailed three-dimensional structure, the mechanism of binary-toxin pore formation will remain unclear. The crystallization of the binary toxin produced by *B. sphaericus* is the first step in determining the three-dimensional structure of the binary toxin.

2. Materials and methods

Spores of *B. sphaericus* 2362 were streaked onto an NYSM (Myers & Yousten, 1978) agar plate and grown for 12 h at 303 K. Single colonies from the plate were used to inoculate six 5 ml NYSM starter cultures. Starter cultures were grown for approximately 7 h in a 303 K shaker set at 300 rev min^{-1} and used to inoculate six Fernbach flasks containing 1.0 l NYSM media. These cultures were placed in a 303 K shaker set at 300 rev min^{-1} and allowed to grow for approximately 72 h in order to ensure complete sporulation. Spores were harvested at 3000*g* for 10 min. The spore pellet was washed once with 1 *M* NaCl and 10 m*M* EDTA pH 7, twice with 10 m*M* EDTA pH 7 and twice with double-distilled water. The average yield of washed spores was approximately 25 g per 6 l of culture. The washed spores were stored at 253 K.

Washed *B. sphaericus* spores (~25 g) were extracted with 100 ml 0.05 *M* NaOH while stirring on ice for 3 h. The suspension was centrifuged at 15 000*g* for 20 min. The supernatant containing the solubilized binary toxin, initially at pH 12.0, was dialysed against phosphate-buffered saline pH 7.0 until the pH reached a value of 9.5. A 1% solution of poly(ethyleneimine) pH 7 was then added dropwise until a heavy white precipitate was visible. The precipitate was removed by centrifugation (20 min at 15 000*g*) and the supernatant containing the binary toxin was dialysed against 5 m*M* glycine, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, 2.7 m*M* KCl and 1 m*M* 2-mercaptoethanol pH 9.5 (buffer *A*). The binary-toxin solution was loaded onto a diethylaminoethyl (DEAE) Toyopearl 650M (Supelco) column (30 × 2.5 cm) equilibrated with buffer *A* and the binary toxin was eluted using an NaCl gradient (0.0–0.5 *M* NaCl). Protein fractions were collected based on A_{278}. Fractions containing the binary toxin were pooled and dialysed extensively against buffer *A*. After dialysis, the pooled fractions were concentrated to ~3 mg ml^{-1} and activated with trypsin (1.7 mg ml^{-1}) at 310 K for 4 h. Trypsin digestion of the binary toxin has been shown to increase the activity of the toxin and is intended to mimic the activity of larval proteases, which cleave the N- and C-terminal ends of both BinB and BinA (Davidson *et al.*, 1987). In addition, the trypsin-digested binary toxin is more soluble than the non-digested toxin. Small peptides generated by trypsin digestion were removed using a 100 × 2.5 cm gel-filtration column (Sephacryl HR-200, Amersham Biosciences) equilibrated with buffer *A*. Protein fractions were collected based on A_{278}. All chromatography fractions were analysed using SDS–PAGE (12% acrylimide). Fractions containing the binary toxin were pooled and dialysed against 5 m*M* glycine and 1 m*M* 2-mercaptoethanol pH 9.5.

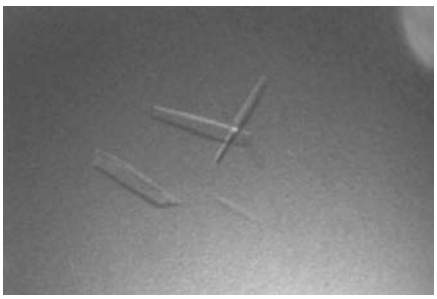

Figure 1
Crystals of the binary toxin produced by *B. sphaericus*. Crystals grew to a maximal dimension of 0.1 mm.

Table 1
Native data statistics of the binary toxin.

Values in parentheses are for the outer shell.

Space group	*P*1
Unit-cell parameters	
a (Å)	27.92
b (Å)	73.21
c (Å)	88.71
α (°)	101.39
β (°)	99.11
γ (°)	90.01
Resolution limits (Å)	25.0–2.7 (2.85–2.70)
No. observations	21455
Unique reflections	14062
Completeness (%)	75.4 (75.4)
$R_{\rm merge}$† (%)	6.5 (46.0)
$I/\sigma(I)$	10.7 (3.3)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_i I_i(hkl)$.

Solutions of purified trypsin-activated binary toxin were concentrated to approximately 20 mg ml^{-1} in 5 m*M* glycine and 1 m*M* 2-mercaptoethanol pH 9.5. Crystallization solutions consisted of 100 m*M* glycine pH 9.5, 0.5 *M* NaCl, 1 m*M* 2-mercaptoethanol and 19.5% polyethylene glycol 4000. The concentrated binary toxin solution was mixed with equal parts of reservoir solution to form 8 µl drops. All crystallization trials were conducted at 295 K using the sitting-drop vapor-diffusion method.

Diffraction data were collected at 295 K on a Rigaku R-AXIS IV^{++} image-plate area detector using Cu $K\alpha$ radiation from a Rigaku RU-200HB rotating-anode X-ray generator (50 kV, 100 mA). The X-ray source was equipped with an Osmic confocal mirror assembly. The diffraction data were integrated with *MOSFLM* (Leslie, 1999) and scaled with *SCALA* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

After mixing the protein solution with the reservoir, a microcrystalline precipitate was observed. Crystals grew from within this precipitate and reached a maximal size of 100 µm after 12–16 d (Fig. 1). To verify their composition, the crystals were washed extensively with precipitant, dissolved in 5 m*M* glycine pH 9.5 and visualized using SDS–PAGE. Stained SDS–PAGE showed two bands migrating to ~45 and 43 kDa. These bands confirm that the crystals are composed of the truncated form of the binary toxin.

The binary-toxin crystals were mounted in glass capillaries and diffraction data were collected. The crystals belong to the triclinic *P*1 space group, with unit-cell parameters $a = 27.92$, $b = 73.21$, $c = 88.71$ Å, $\alpha = 101.39$, $\beta = 99.11$, $\gamma = 90.01°$. On the basis of the reduced molecular weight of the activated binary toxin (83 kDa) and one copy of the binary toxin per unit cell, the Matthews coefficient (Matthews, 1968) was determined to be 2.1 Å^3 Da^{-1}, with a solvent content of 41%. Native binary-toxin data were collected to a resolution of 2.7 Å (Table 1). The completeness of the data was limited to 75.4% as a result of radiation damage and the triclinic space group. Data have also been collected from platinum and mercury derivatives. Experiments to determine the initial heavy-atom phases and optimal cryoconditions are in progress.

We thank E. Davidson for supplying the *B. sphaericus* cultures and helpful advice. ACA is supported by Grant No. BIO-2003 04274 from the Dirección General Científica y Técnica, Ministerio de Ciencia y Tecnología, Spain.

References

Baumann, L. & Baumann, P. (1991). *Curr. Microbiol.* **23**, 51–57.
Broadwell, A. H. & Baumann, P. (1987). *Appl. Environ. Microbiol.* **53**, 1333–1337.
Broadwell, A. H., Clark, M. A., Baumann, L. & Baumann, P. (1990). *J. Bacteriol.* **172**, 4032–4036.
Charles, J. F., Silva-Filha, M. H., Nielsen-LeRoux, C., Humphreys, M. J. & Berry, C. (1997). *FEMS Microbiol. Lett.* **156**, 153–159.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Darboux, I., Pauchet, Y., Castella, C., Silva-Filha, M. H., Nielsen-LeRoux, C., Charles, J. F. & Pauron, D. (2002). *Proc. Natl Acad. Sci. USA*, **99**, 5830–5835.
Davidson, E. W. (1988). *J. Med. Entomol.* **25**, 151–157.
Davidson, E. W., Bieber, A. L., Meyer, M. & Shellabarger, C. (1987). *J. Invertebr. Pathol.* **50**, 40–44.
Leslie, A. G. W. (1999). *Acta Cryst.* D**55**, 1696–1702.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Myers, P. & Yousten, A. A. (1978). *Infect. Immun.* **19**, 1047–1053.
Nicolas, L., Nielsen-Leroux, C., Charles, J. F. & Delecluse, A. (1993). *FEMS Microbiol. Lett.* **106**, 275–279.
Oei, C., Hindley, J. & Berry, C. (1990). *FEMS Microbiol. Lett.* **72**, 265–273.
Payne, J. M. & Davidson, E. W. (1984). *J. Invertebr. Pathol.* **43**, 383–388.
Silva-Filha, M. H., Nielsen-Leroux, C. & Charles, J. F. (1997). *Eur. J. Biochem.* **247**, 754–761.
Yuan, Z. M., Rang, C., Maroun, R. C., Juarez-Perez, V., Frutos, R., Pasteur, N., Vendrely, C., Charles, J. F. & Nielsen-LeRoux, C. (2001). *Eur. J. Biochem.* **268**, 2751–2760.