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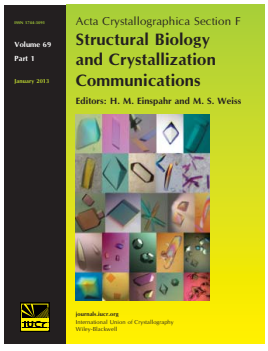

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Preliminary X-ray crystallographic study of staphylococcal α-haemolysin monomer

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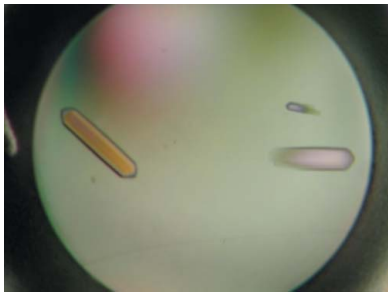

Staphylococcal α-haemolysin is a β-barrel pore-forming toxin expressed by *Staphylococcus aureus*. α-Haemolysin is secreted as a water-soluble monomeric protein which binds to target membranes and forms membrane-inserted heptameric pores. Although the crystal structures of the heptameric pore and monomer bound to an antibody have been determined, that of monomeric α-haemolysin without binder has yet to be elucidated. Previous mutation studies showed that mutants of His35 retain the monomeric structure but are unable to assemble into heptamers. Here, α-haemolysin H35A mutants were expressed, purified and crystallized. Diffraction data were collected to 2.90 Å resolution. The crystals belonged to space group $P6_1$, with unit-cell parameters $a = b = 151.3$, $c = 145.0$ Å. Molecular replacement found four molecules in an asymmetric unit. The relative orientation among molecules was distinct from that of the pore, indicating that the crystal contained monomeric α-haemolysin.

1. Introduction

Staphylococcal α-haemolysin is a β-barrel pore-forming toxin (PFT) expressed by *Staphylococcus aureus*. α-Haemolysin is secreted as a water-soluble monomeric protein with a molecular mass of 34 kDa which binds to target membranes and forms membrane-inserted heptameric pores. On the appearance of the pore on the membrane, the cells are killed through leakage. The structure of the heptameric pore has been determined and each protomer was shown to assemble along a noncrystallographic sevenfold axis (Galdiero & Gouaux, 2004; Song *et al.*, 1996; Tanaka *et al.*, 2011). Moreover, the crystal structure of the α-haemolysin monomer in complex with an antibody has recently been reported (Oganesyan *et al.*, 2013; Foletti *et al.*, 2013). Although α-haemolysin has a strong propensity to spontaneously form heptameric pores (Tanaka *et al.*, 2011), the bound antibody inhibited pore formation. The overall structure of α-haemolysin is divided into three domains: the cap, rim and stem domains. The stem region (Pro103–Asp152) that is folded beside the cap domain in the monomeric form protrudes out from the cap domain to form the transmembrane β-barrel in the pore form. Although the monomeric structure was determined in an antibody-bound form, that without binder has not yet been determined.

Since the gene of the toxin was identified, a number of mutational studies have been carried out in order to clarify the mechanism of pore formation (Cheley *et al.*, 1997; Jayasinghe *et al.*, 2006; Walker & Bayley, 1995; Walker *et al.*, 1992). In the present study, we focus on the substitution of His35 among the reported mutants. Substitution of His35 caused marked decreases in oligomerization and lysis activities instead of sufficient cell-binding activity (Walker & Bayley, 1995). His35 is located at the interface between protomers in the heptameric pore structure. It is likely that the His35 mutant retains the monomeric structure but is unable to assemble into heptamers owing to an inability to form the correct interprotomer interactions because of the substitution. Therefore, this mutant is probably the most suitable for obtaining crystals of monomeric α-haemolysin. In the present study, we expressed, purified and crystallized the α-haemolysin H35A mutant. Diffraction data were collected at a resolution of 2.90 Å. The

results of molecular replacement showed that the obtained crystal consisted of monomeric α-haemolysin.

2. Materials and methods

2.1. Cloning, expression and purification

An α-haemolysin H35A mutant expression vector was prepared by inverse PCR using the expression vector for wild-type α-haemolysin (Tanaka *et al.*, 2011) as a template with the following synthesized primers: H35A-S (5′-AATGGCATG**GCA**AAAAAAGTATTTTA-TAG-3′) and H35A-AS (5′-TACTTTTTTT**TGC**CATGCCATTTTC-TTTATC-3′) (mutation sites are shown in bold). In the resultant expression vector, α-haemolysin excluding the signal sequence from Met1 to Ala26 was cloned between the *Nco*I and *Xho*I sites of the pET28b vector. A His_6 tag was fused at the C-terminus to facilitate purification. The recombinant plasmid was introduced into *Escherichia coli* strain B834 (DE3). The transformed cells were grown at 310 K in 1000 ml LB medium containing 25 µg ml^{-1} kanamycin until the optical density at 600 nm reached 0.6–0.8. After the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 m*M*, bacterial culture was continued at 298 K for an additional 24 h. The cells were collected and suspended in 35 ml sonication buffer (20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl). The cells were then disrupted by sonication and centrifuged at 40 000*g* for 30 min at 283 K. The supernatant was loaded onto a HisTrap affinity column (GE Healthcare, Buckinghamshire, England). After washing the column with 35 ml sonication buffer, the adsorbed protein was eluted with a linear gradient of 35–200 m*M* imidazole in sonication buffer. Fractions containing the target protein were collected and dialysed against the sonication buffer at 277 K for 20 h. The collected fractions were further purified by gel filtration on HiLoad 26/60 Superdex 200 columns (GE Healthcare). The homogeneity of the purified protein was confirmed by SDS–PAGE.

2.2. Crystallization

For crystallization, the purified protein was concentrated to approximately 10 mg ml^{-1} using an Amicon Ultra-15 ultrafiltration device (Millipore, Billerica, Massachusetts, USA). The protein concentration was determined from the absorption at a wavelength of 280 nm using a molar extinction coefficient of 63 440 M^{-1}. The absorption was measured with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Crystal screening of α-haemolysin was performed with The JCSG Core Suites I–IV and The Cryos, PACT, $AmSO_4$, Nucleix, Cations and Anions Suites (Qiagen, Valencia, California, USA), giving a total of 960 conditions, using the sitting-drop method by mixing 0.2 µl protein solution (approximately 10 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl) with 0.2 µl reservoir solution at 293 K. The crystals of α-haemolysin that were most suitable for further diffraction experiments (Fig. 1) were grown from a buffer consisting of 0.05 *M* MES pH 6.5, 0.01 *M* magnesium chloride, 2.1 *M* lithium sulfate, 5%(*w*/*v*) ethylene glycol.

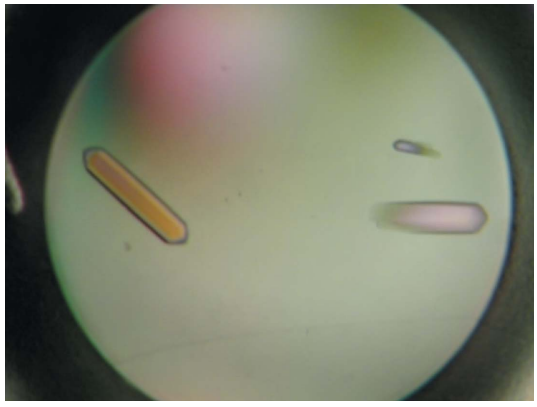

Figure 1
Crystals of the α-haemolysin H35A monomer

Table 1
X-ray data-collection statistics.

Values in parentheses are for the outermost resolution shell.

Beamline	BL5A, Photon Factory
Space group	$P6_1$
Unit-cell parameters (Å)	$a = b = 151.3$, $c = 145.0$
Wavelength (Å)	1.0
Resolution (Å)	48.68–2.90 (3.09–2.90)
Total No. of reflections/No. of unique reflections	446636/41677 (58111/6679)
R_{merge}† (%)	13.5 (70.0)
R_{meas}‡ (%)	14.2 (74.4)
Completeness (%)	99.7 (99.4)
Multiplicity	10.72 (8.70)
Average $I/\sigma(I)$	13.48 (2.38)
No. of molecules in asymmetric unit	4
Solvent content (%)	65.3

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of a set of equivalent reflections. ‡ $R_{meas} = \sum_{hkl}\{N(hkl)/[N(hkl)-1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ and $N(hkl)$ are the mean intensity of a set of equivalent reflections and the multiplicity, respectively.

2.3. Data collection and molecular replacement

An X-ray diffraction data set for α-haemolysin was collected on beamline BL5A of the Photon Factory, Tsukuba, Japan using a Quantum 315r detector (ADSC, Poway, California, USA) under cryogenic conditions at 100 K. Crystals were soaked in mother liquor containing 10%(*w*/*v*) ethylene glycol and were flash-cooled under a stream of liquid nitrogen. The distance between the crystal and detector was set to 452.6 mm. A total range of 180° was covered with 0.5° oscillation per frame. The diffraction data were indexed, integrated and scaled using *XDS* (Kabsch, 2010). Data-collection statistics are summarized in Table 1. Molecular replacement was carried out with *Phaser* (McCoy *et al.*, 2007) using the structure of the α-haemolysin pore protomer (PDB entry 3anz; Tanaka *et al.*, 2011) as a model.

2.4. Size-exclusion chromatography

A 1 ml aliquot of the purified α-haemolysin H35A mutant was loaded onto a HiLoad 26/60 Superdex 200 column (GE Healthcare) pre-equilibrated with 20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl. To estimate the molecular weight in the solution, gel-filtration standards (Bio-Rad, Hercules, California, USA) dissolved in 1 ml of the same buffer were loaded onto the same column.

3. Results and discussion

α-Haemolysin H35A mutant was expressed in *E. coli* and purified as a soluble protein. The monomeric state of the purified protein was confirmed by size-exclusion chromatography (data not shown).

The diffraction data set was collected to a resolution of 2.90 Å. The crystal belonged to space group $P6_1$ or $P6_5$, with unit-cell parameters $a = b = 151.3$, $c = 145.0$ Å (Table 1). Molecular replacement was carried out using a protomer of the heptameric α-haemolysin pore in which the N-terminal amino latch (Ala1–Thr22) and the stem region

(Pro103–Asp152) of which had been deleted as a search probe. Four molecules were found by *Phaser* on the assumption of space group $P6_1$. Positive electron density corresponding to the stem region was observed beside the cap domain for all molecules. Further model building and structure refinement are currently under way. Intermolecular interactions similar to those observed in the pore form were not observed among the molecules, showing that the crystal is composed of monomeric α-haemolysin H35A mutant. To our knowledge, this is the first report of a crystal of monomeric α-haemolysin without binder. The structure will provide insight into the pore-forming mechanism of α-haemolysin.

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