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Crystallization and preliminary X-ray diffraction analysis of the complex between a human anti-alpha toxin antibody fragment and alpha toxin

Staphylococcus aureus alpha toxin (AT) has been crystallized in complex with the Fab fragment of a human antibody (MEDI4893). This constitutes the first reported crystals of AT bound to an antibody. The monoclinic crystals belonged to space group $P2_1$, with unit-cell parameters $a = 85.52$, $b = 148.50$, $c = 93.82$ Å, $\beta = 99.82^\circ$. The diffraction of the crystals extended to 2.56 Å resolution. The asymmetric unit contained two MEDI4893 Fab–AT complexes. This corresponds to a crystal volume per protein weight (V_M) of $2.3 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 47%. The three-dimensional structure of this complex will contribute to an understanding of the molecular basis of the interaction of MEDI4893 with AT. It will also shed light on the mechanism of action of this antibody, the current evaluation of which in the field of *S. aureus*-mediated diseases makes it a particularly interesting case study. Finally, this study will provide the three-dimensional structure of AT in a monomeric state for the first time.

1. Introduction

Staphylococcus aureus is a leading cause of mortality and morbidity worldwide, causing a wide array of infections such as pneumonia, dermonecrosis, endocarditis and sepsis (Bayer *et al.*, 1997; Lowy, 1998; Bubeck-Wardenburg *et al.*, 2007; Kleven *et al.*, 2007). In particular, *S. aureus* alpha toxin (AT) has been shown to be a key virulence determinant (Bubeck-Wardenburg & Schneewind, 2008; Kennedy *et al.*, 2010; Powers *et al.*, 2012). AT is a cytolytic pore-forming toxin and lyses cells in a multistep process whereby an AT monomer binds to a specific cell membrane receptor (ADAM-10) and oligomerizes into a heptameric transmembrane pore leading to cell lysis and tissue damage (Bhakdi & Tranum-Jensen, 1991; Song *et al.*, 1996; Bartlett *et al.*, 2008; Craven *et al.*, 2009; Wilke & Bubeck-Wardenburg, 2010). At sublytic concentrations, AT also triggers signalling events leading to a disruption of the epithelial and endothelial barriers (Inoshima *et al.*, 2011, 2012; Powers *et al.*, 2012). These observations make AT an attractive target to reduce the severity of some *S. aureus*-mediated diseases. In particular, potent inhibitory anti-AT monoclonal antibodies (mAbs) have recently been described, which inhibit AT heptamer formation and significantly reduce lesion size in a murine dermonecrosis model (Tkaczyk *et al.*, 2012). This attractive targeting potential led to the development by MedImmune of MEDI4893, a human anti-AT mAb.

We set out to solve the X-ray crystal structure of the Fab fragment of MEDI4893 bound to monomeric AT and report here diffracting crystals of the corresponding complex. This work will allow us to better understand the molecular basis of the MEDI4893–AT interaction and the mAb mechanism of action. It is also worth noting that although the X-ray crystal structure of AT in a heptameric state has previously been determined (Song *et al.*, 1996), that of the corresponding monomer has yet to be elucidated. Therefore, the MEDI4893 Fab–AT complex structure should also reveal for the first time that of AT in a monomeric state.

2. Materials and methods

2.1. Expression and purification of AT

AT was expressed and purified from the *S. aureus* Wood strain according to a modification of a previously described procedure (Walker *et al.*, 1992). Briefly, the supernatant from an overnight culture of *S. aureus* in Tryptic Soy Broth (VWR, Radnor, Pennsylvania, USA) was harvested by centrifugation and brought to 75% saturation using solid ammonium sulfate. After stirring for 3 h at 277 K, the precipitate was collected by centrifugation at $\sim 12\,000g$ for 45 min at 277 K, then resuspended in SP Buffer A (25 mM sodium acetate pH 5.2, 20 mM NaCl, 1 mM EDTA). Following dialysis against this buffer for 21 h at 277 K, the insoluble portion was removed by centrifugation at $\sim 27\,000g$ for 30 min at 277 K. The soluble dialysate was then filtered using a 0.2 μm filter (PALL Corporation, Port Washington, New York, USA) and loaded onto a 10 ml SP Sepharose FF column (GE Healthcare, Piscataway, New Jersey, USA) previously equilibrated with SP Buffer A. Following washes to baseline with SP Buffer A, native AT was eluted using a linear gradient of 0–0.3 M NaCl. Fractions containing AT were pooled and dialyzed overnight at 277 K against phosphate-buffered saline (PBS) pH 7.2 containing 1 mM EDTA. Finally, the dialysate was loaded onto a HiPrep Sephacryl S-200 High Resolution column (GE Healthcare) at a flow rate of 1.3 ml min⁻¹ in PBS pH 7.2 containing 1 mM EDTA. Fractions containing AT were pooled, dialyzed against 50 mM Tris–HCl pH 8.0 overnight at 277 K and concentrated to approximately 5 mg ml⁻¹ (as measured by the absorbance at 280 nm) using a Vivaspın ultrafiltration device (10 kDa cutoff, Vivascience AG, Hanover, Germany). This procedure allowed us to obtain over 95% homogeneous AT as judged by SDS–PAGE (Fig. 1).

2.2. Production and purification of MEDI4893 Fab

MEDI4893 Fab was obtained directly from the enzymatic cleavage of the corresponding human monoclonal antibody (known as MEDI4893). Digestion was carried out using immobilized papain according to the manufacturer's instructions (Pierce, Rockford, Illinois, USA). Briefly, MEDI4893 was buffer-exchanged in 20 mM

Figure 1

SDS–PAGE profile of MEDI4893 Fab, AT and a dissolved crystal of the MEDI4893 Fab/AT complex under reducing (R) and/or non-reducing (NR) conditions. Lanes 1, 4 and 7, Magic Mark protein standard (labelled in kDa); lanes 2 and 3, MEDI4893 Fab (R and NR, respectively); lanes 5 and 6, AT (R and NR, respectively); lane 8, crystal of the MEDI4893 Fab–AT complex (NR). The 4–12% NuPAGE gradient gel (Invitrogen, Carlsbad, California) was stained with Simply Blue Stain (Invitrogen). It shows MEDI4893 Fab migrating at around 25 and 50 kDa under R and NR conditions, respectively, owing to the presence of an expected interchain disulfide bond between its light and heavy chain. AT migrated at around 35 kDa under both R and NR conditions, consistent with a predicted molecular weight of around 33 kDa. AT present in the complex seemed to run slightly faster than when alone. Though we do not believe this difference to be significant, it is possible that the crystal's buffer composition or the clipping of a few AT amino acids affected the corresponding migration pattern. Magic Mark Protein Standard was purchased from Invitrogen.

sodium phosphate pH 7.0, 10 mM EDTA, 20 mM L-cysteine using Zeba Spin desalting columns (40 kDa cutoff, Pierce), then incubated with immobilized papain resin for 6 h at 310 K. The resin was then removed by centrifugation and the resulting supernatant dialyzed against 10 mM Tris–HCl pH 8.0 overnight at 277 K. Fc and undigested IgG were removed by passing the mixture through a 1 ml HiTrap Q HP column (GE Healthcare) previously equilibrated in 10 mM Tris–HCl pH 8.0. MEDI4893 Fab was found in the flow-through, which was then concentrated using a Vivaspın ultrafiltration device (30 kDa cutoff, Vivascience AG). The protein was further applied onto a 5 ml HiTrap SP HP column (GE Healthcare) previously equilibrated with 50 mM sodium acetate pH 5.2 and eluted in a 0–1 M NaCl gradient. The purified Fab was then concentrated to approximately 5 mg ml⁻¹ (as measured by the absorbance at 280 nm). This procedure allowed us to obtain over 95% homogeneous MEDI4893 Fab as judged by SDS–PAGE (Fig. 1).

2.3. Complex preparation and crystallization

Previously purified MEDI4893 Fab and AT were mixed at a 1:1.1 molar ratio. The resulting mixture was concentrated using a Vivaspın concentrator (10 kDa cutoff, Vivascience AG) to approximately 10 mg ml⁻¹ as measured by the absorbance at 280 nm. Purification and buffer-exchange then followed using an ÄKTA purifier (GE Healthcare) fitted with a Superdex S200 column (GE Healthcare) pre-equilibrated with 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.02% NaN₃. Molecular-weight analysis was carried out using an Agilent 1200 system (Agilent Technologies, Santa Clara, California, USA) fitted with a DAWN-Heleos multi-angle laser light scattering system (MALLS; Wyatt, Santa Barbara, California, USA) and TSKgel column (Tosoh Bioscience, King of Prussia, Pennsylvania, USA). Representative size-exclusion chromatograms (SEC) of MEDI4893 Fab, AT and MEDI4893 Fab/AT complex are shown in Fig. 2. The purified complex was then concentrated to 15 mg ml⁻¹ using a Vivaspın concentrator (10 kDa cutoff, Vivascience AG) and subjected to crystallization trials as described in the following paragraphs.

Sitting-drop crystallization experiments were initially set up in 96-well Intelli-plates (Art Robbins Instruments, Sunnyvale, California, USA) using a Phoenix crystallization robot (Art Robbins Instruments). Favorable conditions were first identified using the following commercially available crystallization screens: PEG/Ion (Hampton Research, Aliso Viejo, California, USA), Cryo I and II (Emerald BioSystems, Bainbridge Island, Washington, USA) and JCSG-plus (Molecular Dimensions, Apopka, Florida, USA). The well and drop

Figure 2

Superimposition of the SEC profiles of MEDI4893 Fab, AT and MEDI4893 Fab/AT complex. The retention volume of AT was consistent with the molecular weight of the corresponding monomer (~ 33 kDa). Likewise, the retention volume of MEDI4893 Fab was consistent with its expected molecular weight of ~ 50 kDa. Finally, MALLS analysis of the MEDI4893 Fab/AT complex yielded a molecular weight of 81 kDa, very close to that of the expected 1:1 complex. Taken together, our data suggest that AT was added and crystallized in a monomeric form.

Table 1

X-ray data-collection statistics.

Values in parentheses correspond to the highest-resolution shell.

Wavelength (Å)	1.0000
Resolution (Å)	148.5–2.56 (2.56–2.57)
Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 85.52$, $b = 148.50$, $c = 93.82$, $\beta = 99.82$
Total reflections	421349 (4493)
Unique reflections	74008 (763)
Completeness (%)	97.5 (98.6)
$R_{\text{merge}}^{\dagger}$	0.103 (0.825)
Mean $I/\sigma(I)$	11.5 (1.3)
Mosaicity (°)	0.2
Multiplicity	3.3 (3.3)

$$^{\dagger} R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$$

compartments of the 96-well plates were filled with 50 and 0.3 μl , respectively, of the various screen solutions. We then added 0.3 μl of the MEDI4893 Fab/AT complex at a concentration of 15 mg ml^{-1} in 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.02% NaN_3 to the drop compartment and let the resulting mixture equilibrate against the well solution.

Based on initial screening results, the C4 screen solution from JCSG-plus [10% (w/v) PEG 6000, 100 mM HEPES, pH 7.0] was selected for further optimization in hanging drops. We used 24-well VDX plates (Hampton Research) and 300 μl of C4 solution in the reservoir, and tested varying ratios of protein/reservoir solution in the drop. This step included seeding using Seed Bead (Hampton Research).

2.4. X-ray data collection and processing

One diffraction data set was collected from a single crystal on the IMCA-CAT 17ID beamline of the Advanced Photon Source (APS) of the Argonne National Laboratory (University of Chicago, Chicago, Illinois, USA) equipped with a PILATUS 6M detector (Paul Scherrer Institute, Villigen, Switzerland). Cryoprotection was achieved by soaking the selected crystals in 10% (w/v) PEG 6000, 100 mM HEPES pH 7.0, 20% glycerol. Crystals were then flash-cooled in liquid nitrogen. We collected 1530 consecutive images using an oscillation range of 0.2°. A crystal-to-detector distance of 300 mm

Figure 3

Crystal of the MEDI4893 Fab/AT complex. The crystal shown grew to a size of up to 0.15 $\times$ 0.15 $\times$ 0.05 mm in a hanging drop in which 3 μl of a 15 mg ml^{-1} MEDI4893 Fab/AT complex solution in 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.02% NaN_3 was mixed with 2 μl of reservoir solution 10% (w/v) PEG 6000, 100 mM HEPES pH 7.0. This and similar crystals grew within a week at room temperature.

was used along with an exposure time of 0.5 s. The diffraction images were reduced and scaled using the XDS suite (Kabsch, 2010; Vornrhein *et al.*, 2011).

3. Results and discussion

Out of three commercial screens used, only JCSG-plus produced crystals within the first week under the A8, B5, C4, D6, G1, G6, G9, G10, H3 and H7-11 conditions. Screen solutions B5, C4, D6 and G6 were further used for optimization as they allowed production of plate-like crystals (as opposed to thin needles in all other conditions). Optimization using the B5 screen solution did not yield diffraction-quality crystals. The other three conditions (C4, D6 and G6) yielded similar results.

Diffraction-quality crystals grew in about a week in hanging drops in which 3 μl of the MEDI4893 Fab/AT complex at a concentration of 15 mg ml^{-1} in 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.02% NaN_3 was mixed with 2 μl of 10% (w/v) PEG 6000, 100 mM HEPES pH 7.0 and left to equilibrate against 300 μl of 10% (w/v) PEG 6000, 100 mM HEPES pH 7.0 (Fig. 3). These crystals belonged to monoclinic space group $P2_1$ according to POINTLESS from the CCP4 suite (Winn *et al.*, 2011) and reached a diffraction limit down to 2.56 Å. SDS–PAGE analysis of these crystals confirmed that they did indeed contain the expected complex formed by MEDI4893 Fab and AT (Fig. 1). Data statistics are shown in Table 1.

The asymmetric unit is expected to contain two MEDI4893 Fab/AT complexes based on the V_M of 2.3 Å³ Da^{−1} and solvent content of 47% (Matthews, 1968). For molecular replacement, the AT model (PDB entry 7ahl; Song *et al.*, 1996) was modified by removing extended portions of the polypeptide involved in interactions between molecules in the heptameric state (corresponding to amino acids 1–20 and 103–152). The remaining molecule was used as a template for Phaser (McCoy *et al.*, 2005). The model of an unpublished Fab structure (MedImmune) with complementarity determining regions removed was used as a template for MEDI4893 Fab. Phaser found two complexes in the asymmetric unit with a final Z-score above 20. Electron density calculated with phases generated by Phaser showed very clean electron density for most of the molecules in the asymmetric unit. Positive difference density showed missing portions of the model. Model refinement is under way.

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