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Crystallization and preliminary X-ray analysis of the vWA domain of human anthrax toxin receptor 1

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The Gram-positive spore-forming bacterium *Bacillus anthracis* causes anthrax by secreting anthrax toxin, which consists of protective antigen (PA), lethal factor and oedema factor. Binding of PA to receptors triggers the multi-step process of anthrax toxin entry into target cells. Two distinct cellular receptors, ANTXR1 (also known as tumour endothelial marker 8; TEM8) and ANTXR2 (also known as capillary morphogenesis protein 2; CMG2), for anthrax toxin have been identified. Although the crystal structure of the extracellular von Willebrand factor A (vWA) domain of CMG2 has been reported, the difference between the vWA domains of TEM8 and CMG2 remains unclear because there are no structural data for the TEM8 vWA domain. In this report, the TEM8 vWA domain was expressed, purified and crystallized. X-ray diffraction data were collected to 1.8 Å resolution from a single crystal, which belonged to space group $P1$ with unit-cell parameters $a = 65.9$, $b = 66.1$, $c = 74.4$ Å, $\alpha = 63.7$, $\beta = 88.2$, $\gamma = 59.9^\circ$.

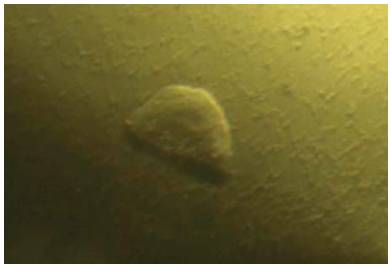
1. Introduction

Anthrax toxin is composed of a single 83 kDa protective antigen (PA) and two catalytic moieties: lethal factor and oedema factor (Liu *et al.*, 2003; Young & Collier, 2007). The binding of PA to receptors triggers the multi-step process of anthrax toxin entry into target cells. The PA-binding receptors play a major role in toxin activity by directing the assembly of a PA heptamer prepore on the cell surface and the subsequent internalization of the tripartite toxic complex into the endosomal compartments (Abrami *et al.*, 2003). In endosomes, the acidic pH triggers the insertion of the PA heptamer into the endosomal membrane to form a pore that translocates lethal factor and oedema factor into the cytosol (Abrami *et al.*, 2003).

Two human cellular receptors for PA have been identified: anthrax toxin receptor 1 (ANTXR1) and anthrax toxin receptor 2 (ANTXR2), which are also known as tumour endothelial marker 8 (TEM8) and capillary morphogenesis protein 2 (CMG2), respectively (Bradley *et al.*, 2001; Rainey *et al.*, 2005). These receptors are type I transmembrane proteins that possess an extracellular integrin-like I domain, which is a member of the larger family of von Willebrand factor A (vWA) domains (Bradley *et al.*, 2001). However, the two receptors show distinct differences in toxin binding and cell entry (Scobie & Young, 2006; Scobie *et al.*, 2007) which may indicate their roles in anthrax pathogenesis.

CMG2, which was the second anthrax receptor to be identified, is vigorously induced during capillary-tube formation in collagen gels and is expressed in a more ubiquitous pattern than TEM8 (Bradley *et al.*, 2001; Scobie *et al.*, 2003). Compared with the prevalent distribution of CMG2 in normal adult tissues (*e.g.* in lung, brain, kidney and muscle), TEM8 is only weakly detected in these tissues. However, TEM8 is abundant in tumour endothelial cells and in the vasculature during embryonic development (Bradley *et al.*, 2001; Hotchkiss *et al.*, 2005; Nanda *et al.*, 2004; Scobie *et al.*, 2003).

TEM8 was first identified as an anthrax receptor in 2000 and is encoded by the *TEM8* gene (St Croix *et al.*, 2000). TEM8 is one of



the few tumour endothelial marker (TEM) proteins believed to be expressed specifically in the tumour endothelium and is likely to be involved in tumour angiogenesis (Bradley *et al.*, 2001; Seaman *et al.*, 2007). Owing to its specific location on the cell surface and its highly conserved amino-acid sequence across species, TEM8 has been the focus of more research than other TEMs. Furthermore, TEM8 plays a positive role in endothelial cell behaviour relevant to angiogenesis, such as migration, adhesion and tube formation (Hotchkiss *et al.*, 2005; Rmali *et al.*, 2005). In addition, the extracellular vWA domain of TEM8 can bind to collagen type 1 and gelatin (Hotchkiss *et al.*, 2005) and co-immunoprecipitates with the C5 domain of collagen α3 (VI) (Nanda *et al.*, 2004). This evidence supports interaction between TEM8 and components of the extracellular matrix (ECM). Together, all of these characteristics make TEM8 a particularly attractive target for antitumour agents.

The crystal structures of CMG2 and the CMG2–PA complex have been solved (Lacy *et al.*, 2004; Santelli *et al.*, 2004) and shed light on the differences in the behaviour of the two types of receptor at the stage of toxin binding and cell entry. It was hypothesized that the interaction of the β4–α4 loop in CMG2 with domain 2 in PA is the main determinant underlying the higher affinity of CMG2 for PA and the requirement of a more acid pH for PA pore formation than for that of TEM8 (Scobie & Young, 2006; Scobie *et al.*, 2007). However, direct evidence should rely on a comparison of the crystal structures of the two receptors and their complexes with PA, which may lead to the elucidation of finer details. Furthermore, efforts have been made to engineer PA through random mutation and screening to selectively bind TEM8 as a tumour-targeting drug carrier (Chen *et al.*, 2007) and such studies would benefit from a TEM8–PA crystal structure.

Unlike the CMG2 vWA domain, which can be overexpressed in bacteria to obtain functional protein (Bell *et al.*, 2001), production of soluble and functional ATR/TEM8 vWA domain is relatively difficult because the TEM8 vWA domain forms inclusion bodies in *Escherichia coli* and requires the use of mammalian expression systems, preventing structural analysis (Bradley *et al.*, 2001, 2003). As reported by Ding *et al.* (2006), the expression of recombinant TEM8 vWA domain was achievable by using glutathione *S*-transferase (GST; Smith & Johnson, 1988) to facilitate protein expression in bacteria and applying the strategy of cell culture at reduced temperatures.

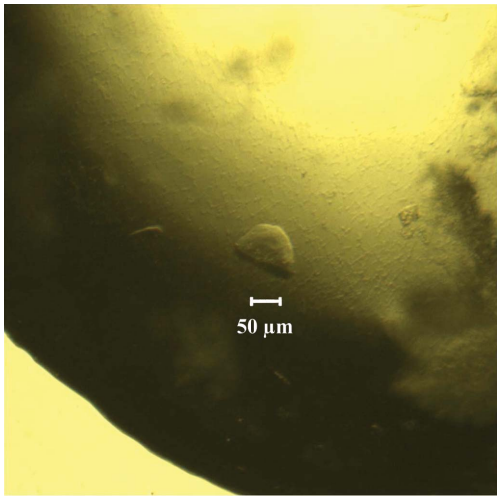


Figure 1
Typical crystals of the TEM8 vWA domain. Maximal dimensions are ~0.1 × 0.1 × 0.02 mm.

To gain insight into the differences between the CMG2 and TEM8 vWA domains, we modified the TEM8 vWA domain expression and purification procedure by using a C177A mutant to robustly express soluble TEM8 vWA domain protein in *E. coli*. The cysteine at position 177 in TEM8 (homologous to residue 175 in CMG2, which was buried inside the crystal structure) was not believed to interact with PA and may result in folding problems and incorrect intermolecular disulfide-bond formation (data not shown). The C175A mutation in CMG2 did not significantly attenuate the binding affinity to PA, nor did the C177A mutation affect the protection ability of TEM8 against PA challenge in cell assays (data not shown). However, the TEM8 C177A mutant displayed reduced dimer formation. We purified the C177A mutant and verified its identity by both N-terminal amino-acid sequencing and mass spectrometry. Furthermore, the TEM8 vWA domain was also crystallized for further analyses.

2. Materials and methods

2.1. Expression and purification

The primers 5′-CT **CCA TGG** CCT GCT ACG GCG GAT TT-3′ and 5′-CC **GGA TCC** TTA GCA GGA CTT CTT CA-3′ were used to amplify the TEM8 vWA domain sequence (corresponding to amino acids 38–220). The primers included *Nco*I and *Bam*HI restriction sites (shown in bold). The gene encoding the TEM8 vWA domain was amplified by PCR and cloned into the pHAT2 vector (EMBL Protein Expression and Purification Facility), which adds an N-terminal 6×His tag to the protein product without other additional residues. The identity of the insert was verified by sequencing and the recombinant plasmid was transformed into *E. coli* strain BL21 (DE3). Transformed cells were then cultured at 310 K in 1 l LB medium containing 50 mg ml^{-1} ampicillin.

After 4 h of incubation, the culture reached an OD_{600} of 0.6–0.7. Recombinant protein expression was then induced with 0.4 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubation continued for a further 16 h at 289 K. The cells were then harvested by centrifugation at 5000 rev min^{-1} for 10 min, resuspended in 1× phosphate-buffered saline (PBS) consisting of 140 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4 and 1.8 m*M* KH_2PO_4 pH 7.3 and homogenized by sonication. The lysate was centrifuged at 17 000 rev min^{-1} for 30 min to remove cell debris.

The fusion protein was isolated from the crude protein mixture by nickel-affinity chromatography (Qiagen, Holland) and dialyzed in buffer *A* (50 m*M* NaCl, 1 m*M* $MgCl_2$, 20 m*M* Tris–HCl pH 7.5) to reduce the salt concentration and remove imidazole. The protein was further purified using a Resource Q anion-exchange chromatography column (GE Healthcare, USA) pre-equilibrated in buffer *A* and was eluted by increasing the NaCl concentration gradually to 1 *M* using buffer *B* (1 *M* NaCl, 1 m*M* $MgCl_2$ and 20 m*M* Tris–HCl pH 7.5). Gel-filtration chromatography was carried out using a Superdex 75 column (GE Healthcare, USA) in buffer *C* (150 m*M* NaCl, 1 m*M* $MgCl_2$ and 20 m*M* Tris–HCl pH 7.5). Finally, the purity of the recombinant TEM8 protein was estimated to be >95% by SDS–PAGE.

2.2. Crystallization

The purified TEM8 vWA domain was concentrated to 5 mg ml^{-1} in 20 m*M* Tris–HCl pH 7.5 and 150 m*M* $CaCl_2$. Crystallization was performed by the hanging-drop vapour-diffusion method at 295 K in 16-well plates. Each drop contained 1 µl protein solution and 1 µl reservoir solution, with 200 µl reservoir solution in the well. Initial screening was carried out using Hampton Research Crystal Screen

kits (Hampton Research, USA) and positive hits were then optimized. The optimized reservoir solution consisted of 0.1 *M* sodium citrate trihydrate pH 5.6, 0.2 *M* ammonium acetate and 20%(*w*/*v*) polyethylene glycol (PEG) 4000 with 0.1 *M* hexammine cobalt(III) chloride as an additive reagent. Plate-shaped crystals with maximum dimensions of 0.1 × 0.1 × 0.02 mm were obtained within 7 d (Fig. 1).

Diffraction data were collected at 100 K using an ADSC Q270 detector on beamline BL17A at a wavelength of 0.9798 Å at the Photon Factory, Japan. The crystal was mounted on a nylon loop and flash-cooled in a cold nitrogen-gas stream at 100 K. Because the PEG 4000 in the well buffer is an effective cryoprotectant, no additional reagents were added. A total of 523 frames of data were collected with an oscillation angle of 1.0°. The data were indexed, integrated and scaled with the *HKL*-2000 software package (Otwinowski & Minor, 1997).

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	*P*1
Unit-cell parameters (Å, °)	*a* = 65.9, *b* = 66.1, *c* = 74.4, *α* = 63.7, *β* = 88.2, *γ* = 59.9
Wavelength (Å)	0.9798
Resolution (Å)	50.00–1.80 (1.90–1.80)
Completeness (%)	93.1 (84.9)
Total No. of reflections	452119 (31328)
No. of unique reflections	82369 (10462)
Average $I/\sigma(I)$	14.2 (3.1)
$R_{\rm merge}$† (%)	9.1 (26.1)
Matthews coefficient (Å^3 Da^{-1})	1.98

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean of the observations $I_i(hkl)$ of reflection *hkl*.

3. Results and discussion

Expression of the TEM8 vWA domain was optimal when induced with 0.4 m*M* IPTG and expressed at 289 K. The protein eluted as a single asymmetric peak from the Superdex 75 gel-filtration column which corresponded to the expected molecular weight of the TEM8 vWA domain (22.44 kDa). The purity of the recombinant protein was estimated to be >95%, with a sharp protein band at ∼22 kDa on SDS–PAGE.

Initial crystallization-condition screening was carried out using Hampton Research Crystal Screen kits and several positive hits were obtained. The initial crystals had poor diffraction quality, only diffracting to 5 Å resolution, which is not suitable for X-ray diffraction data collection. Crystal optimization was then carried out by slightly adjusting the protein concentration, precipitant concentration and crystallization temperature and by testing different additives. The addition of 0.1 *M* hexammine cobalt(III) chloride greatly improved the crystal quality, and crystal diffraction was optimal when using a reservoir solution consisting of 0.1 *M* sodium citrate trihydrate pH 5.6, 0.2 *M* ammonium acetate and 20%(*w*/*v*) PEG 4000 precipitant at 295 K. Notably, it has been reported that 100 m*M* Co^{3+} ions can replace 1 m*M* Mg^{2+} ions in several structures. For instance, cobalt was used to cocrystallize the integrin α_2 I domain with a collagen helix, where it bound to MIDAS. Therefore, the possibility of cobalt binding should be further validated by anomalous difference Fourier mapping.

X-ray diffraction data were collected to 1.8 Å resolution from a single flash-cooled (100 K) crystal (Fig. 2). The crystal belonged to space group *P*1, with unit-cell parameters *a* = 65.9, *b* = 66.1, *c* = 74.4 Å, *α* = 63.7, *β* = 88.2, *γ* = 59.9°. Details of the data-collection statistics are summarized in Table 1.

According to amino-acid sequence alignment, the CMG2 and TEM8 vWA domains are 54% identical. Therefore, the CMG2 vWA domain structure (PDB code 1shu; Lacy *et al.*, 2004) was used as a search model to determine the initial phase. The molecular-replacement method was performed using the *Phaser* program (McCoy *et al.*, 2007) and resulted in a distinct solution based on a clear rotation-function *Z* score of 11.9 and translation-function *Z* score of 100.0. Six molecules were found in one asymmetric unit, which is consistent

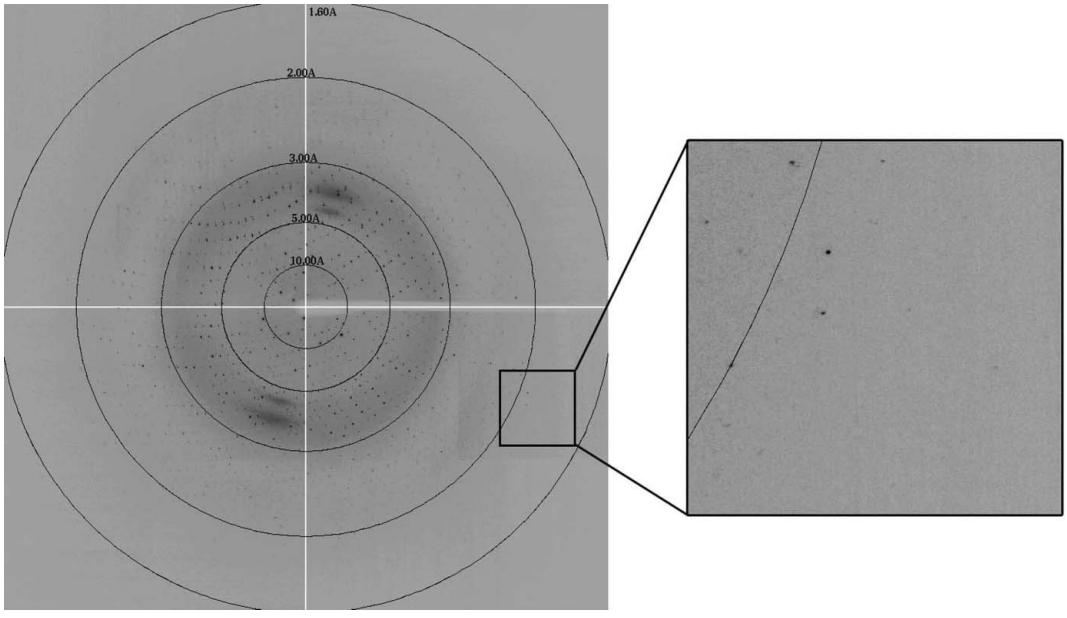


Figure 2
Typical diffraction pattern of the TEM8 vWA domain. The exposure time was 2 s, the crystal-to-detector distance was 183.0 mm and the oscillation range per frame was 1°. The diffraction image was collected on an ADSC Q270 detector. An enlarged image is shown on the right.

with a Matthews coefficient of 1.98 Å^3 Da^{-1} (Matthews, 1968) and a solvent content of 38%. Model building and further positional refinement is currently under way.

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