Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

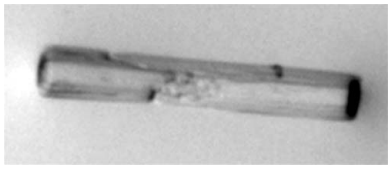
Crystallization of truncated hemolysin A from *Proteus mirabilis*

Luke Bailey,[a] Sean Agger,[b] Luke Peterson,[c] James Thompson[d] and Todd Weaver[e]*

[a]Department of Biochemistry, University of Wisconsin–Madison, Madison, Wisconsin, USA, [b]Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, St Paul, Minnesota, USA, [c]Department of Pediatrics, University of Wisconsin–Madison, Madison, Wisconsin, USA, [d]Department of Physiology and Biomedical Engineering and Mayo Proteomics Research Center, Mayo Clinic, Rochester, Minnesota, USA, and [e]Chemistry Department, University of Wisconsin–La Crosse, La Crosse, Wisconsin, USA

Correspondence e-mail: weaver.todd@uwlax.edu

Received 18 February 2004
Accepted 25 March 2005
Online 1 April 2005



Proteus species are second only to *Escherichia coli* as the most common causative agent of Gram-negative bacteria-based urinary-tract infections and many harbor several virulence factors that provide inherent uropathogenicity. One virulence factor stems from a two-partner secretion pathway comprised of hemolysin A and hemolysin B; upon hemolysin B-dependent secretion, hemolysin A becomes activated. This system is distinct from the classic type I secretion pathway exemplified by the hemolysin system within *Escherichia coli*. In order to describe the mechanism by which hemolysin A is activated for pore formation, an amino-terminal truncated form capable of complementing the non-secreted full-length hemolysin A and thereby restoring hemolytic activity has been constructed, expressed and purified. A room-temperature data set has been collected to 2.5 Å resolution. The crystal belongs to the orthorhombic space group $P2_12_12$, with unit-cell parameters $a = 34.47$, $b = 58.40$, $c = 119.74$ Å. The asymmetric unit is expected to contain a single monomer, which equates to a Matthews coefficient of 1.72 Å^3 Da^{-1} and a solvent content of 28.3%.

1. Introduction

Proteus species have been implicated in a number of nosocomial and opportunistic infections. They present most often as urinary-tract infections in patients with urologic abnormalities or catheters, but even in a normal host *Proteus* species are second to *Escherichia coli* as the leading cause of urinary-tract infections caused by Gram-negative rods (Bahrani & Mobley, 1994; Bauernfeind *et al.*, 1987). *Proteus* and *E. coli* are both members of the *Enterobacteriaceae* and each species expresses a distinct battery of virulence determinants including urease, pili, swarming motility, iron acquisition, IgA protease and hemolysin. Collectively, these factors provide *Proteus* with inherent uropathogenicity (Mobley & Belas, 1995).

There are numerous examples of cytolytic/hemolytic toxins, synthesized by a variety of Gram-negative bacteria, that are associated with diseases in humans (Bhakdi & Tranum-Jensen, 1988; Braun & Focareta, 1991; Chakraborty *et al.*, 2004). One such system has been shown to be part of the two-partner secretion (TPS) pathway in which a large exoprotein is secreted into the environmental milieu. There are three general model systems described by the TPS pathway: (i) the filamentous hemagglutinin adhesion system of *Bordetella pertussis*, (ii) the high-molecular-weight adhesions 1 and 2 of *Haemophilus influenzae* and (iii) the hemolysin A (HpmA) and hemolysin B (HpmB) system of *Proteus mirabilis* (Jacob-Dubuisson *et al.*, 2001). This system differs significantly from the classic type I hemolysin system found within *E. coli*, a system that harbors four distinct protein components: (i) HlyA, the cytolysin/hemolysin, (ii) HlyB, the inner membrane trafficking ATPase, (iii) HlyC, an internal protein acyltransferase and (iv) HlyD, an inner membrane protein. Complete activation of HlyA requires post-translational acylation *via* HlyC prior to extracellular secretion by the HlyB–HlyD complex (Stanley *et al.*, 1998).

All TPS pathways utilize an amino-terminal module termed the 'secretion domain' within the large exoprotein (termed the A-component) and a channel-forming β-barrel transporter protein (termed the B-component; Könninger *et al.*, 1999). HpmA and HpmB from *P. mirabilis* are analogous to the A- and B-components in the TPS system. The secretion of the A-component has been described as

a two-phase process: (i) translocation into the periplasmic space *via* the Sec pathway followed by (ii) specific activation and secretion into the external environment by the B-component. The amino-terminal secretion domain directs the exoprotein to the β-channel-specific transporter for coupled secretion and activation into the external environment (Jacob-Dubuisson *et al.*, 2001). Activated in this fashion, HpmA has been shown to disrupt both human and sheep red blood cells and display cytotoxic effects towards other cell lines (Swihart & Welch, 1990).

More recently, the structure of truncated filamentous hemagglutinin (FHA) from the whooping cough agent *B. pertussis* has been reported (Chakraborty *et al.*, 2004). While FHA harbors minimal overall amino-acid sequence identity, there were two more highly conserved regions noted between the TPS A-component proteins, including HpmA from *P. mirabilis* and FHA from *B. pertussis*. The structure of FHA comprises a 30 kDa amino-terminal fragment and it is noted that this TPS domain folds into a β-helix-type structure. This motif is thought to provide a scaffolding to fold the TPS A-components as they are secreted into the extracellular milieu. In the present study, we have cloned, expressed, purified and collected an initial native data set for a truncated form of HpmA (HpmA358) from *P. mirabilis*. This form has been shown to activate a full-length non-activated form of HpmA (data not shown) and the structure would be the first TPS-related hemolysin.

2. Methods and results

2.1. Construction, overexpression and purification of HpmA358

PCR was performed with 55.7 ng *hpmA*/*hpmB* template (pWPM140), 2.5 units Pfu (Promega, Madison, WI, USA), 10× Pfu buffer (Promega, Madison, WI, USA) and 100 pmol HpmA-forward and HpmA-reverse primers (GibcoBRL, Carlsbad, CA, USA). The HpmA-forward primer contained an *Nde*I restriction site, an ATG start site and a 21-base sequence complementary to *hpmA*. The HpmA-reverse primer contained a *Hin*dIII restriction site, a stop codon, an in-frame coding region for a five histidines to start after base pair 1074 and a 19-base sequence complementary to hpmA. Standard molecular-cloning techniques were used to directionally clone the 1089 bp PCR fragment of hpmA, incorporating the amino-terminal 358 amino acids of HpmA and an in-frame carboxy-terminal five-histidine sequence, into pET-24a(+) using the *Nde*I and *Hin*dIII restriction sites (Novagen, Madison, WI, USA). The resulting plasmid (pHPMA358) was transformed into a recombinant strain containing pWMP109 (*hpmB* on pACYC184) to generate the expression strain SAA38.

The rationale for constructing a truncated version of HpmA stems from past investigations, in which the first 358 amino acids of HpmA were shown (i) to be sufficient for HpmB-dependent secretion across the outer membrane and (ii) to complement an inactive full-length HpmA during the activation of hemolytic activity (Uphoff, 1991). From these two observations, HpmA358 was postulated to form a functionally folded product amenable to crystallization studies.

E. coli B834 (DE3) (Novagen), harboring plasmids for both *hpmA358* and *hpmB*, was used to express HpmA358. Cells were grown in 2.8 l Fernbach flasks containing 1.4 l Luria–Bertani broth supplemented with kanamycin (30 µg ml^{-1}) and chloramphenicol (34 µg ml^{-1}) to an optical density (at 600 nm) between 0.4 to 0.8 and induced with 1 m*M* isopropyl-β-D-thiogalactoside (IPTG) for 5 h. The cells were harvested by centrifugation at 5000*g* for 25 min. The resulting supernatant was saved and the pH was adjusted to 7.8 through the addition of 1 *M* NaOH. The supernatant was loaded onto an Ni^{2+}–NTA column (Qiagen) equilibrated with buffer *A* (50 m*M* sodium phosphate, 300 m*M* sodium chloride pH 7.8). Extraneous proteins were removed by the addition of buffer *B* (50 m*M* sodium phosphate, 300 m*M* sodium chloride, 15 m*M* imidazole pH 7.8). Truncated HpmA was eluted from the column with buffer *C* (50 m*M* sodium phosphate, 300 m*M* sodium phosphate, 400 m*M* imidazole pH 7.8). Purity was confirmed using SDS–PAGE (Laemmli, 1970) and mass-spectroscopic analysis. HpmA358 was dialyzed three times against 4 l 10 m*M* Tris–HCl pH 7.5 and concentrated to 15 mg ml^{-1} using a Savant Speed Vac SC110A (Global Medical Instrumentation Inc). HpmA358 was dialyzed once more against 4 l 10 m*M* Tris–HCl pH 7.5. All chemicals were purchased from Fisher Scientific.

2.2. Crystallization and X-ray data collection

The initial crystallization conditions were obtained using Crystal Screen (Hampton Research) using the hanging-drop vapor-diffusion method with a 10 µl drop size (5 µl HpmA358 and 5 µl mother liquor) in 1.7 × 1.6 cm wells at 291 K. The initial conditions were modified to include 0.1 *M* HEPES pH 7.1, 0.1 *M* NH_4NO_3 and 8–10% PEG 8000. Typical crystals appeared within two weeks and reached maximum size (0.2 × 0.1 × 0.075 mm) over the course of two months (see Fig. 1).

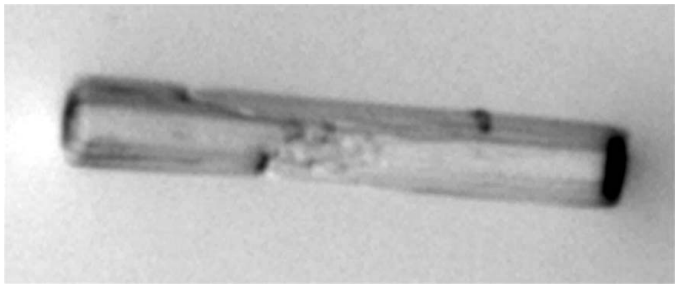


Figure 1
Native crystal of truncated hemolysin A from *P. mirabilis*.

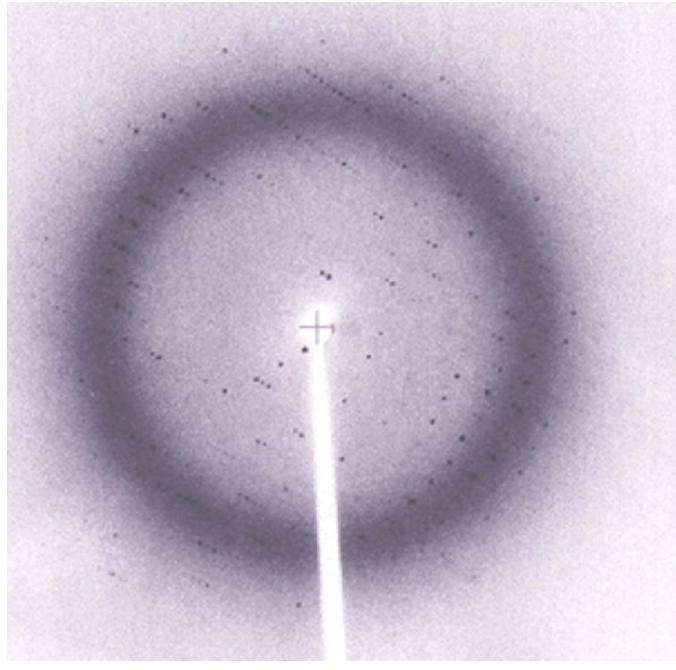


Figure 2
X-ray diffraction image from a truncated hemolysin A crystal. The edge of the detector corresponds to 1.6 Å resolution.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution bin.

Wavelength	1.54
Resolution range (Å)	30–2.50 (2.59–2.50)
Measured reflections	26398
Unique reflections	8863
Completeness	99.7 (99.9)
Mean $I/\sigma(I)$	7.0 (2.5)
$R_{\rm merge}$ (%)	11.5 (41.5)

X-ray diffraction data were collected using 0.5° oscillations with a crystal-to-detector distance of 90 mm on a Rigaku/MSC 007 microfocus generator with VariMax optics and an R-AXIS IV^{++} image-plate detector. The crystals were mounted in a 0.5 mm glass capillary tube and diffracted to 2 Å resolution. A complete data set was collected to 2.5 Å at room temperature (a diffraction image is shown in Fig. 2). The diffraction data were indexed and scaled using *d*TREK* with the *CrystalClear* interface (Rigaku/MSC) and the dataset statistics are listed in Table 1. The crystals belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 34.47, b = 58.40, c = 119.74 Å. The asymmetric unit is expected to contain a single monomer, which equates to a Matthews coefficient of 1.72 Å^3 Da^{-1} and a solvent content of 28.3%. Attempts to solve the HpmA358 structure are under way using molecular replacement with the FHA model (PDB code 1j8f).

TW acknowledges Dean Michael Nelson and the Dean's Summer Fellowship Program within the College of Science and Allied Health at the University of Wisconsin–La Crosse for their continued support of undergraduate research. The research was supported through both an NIH–AREA grant (1R15AI057437-01) from the National Institutes of Health and a Faculty Research Award from the University of Wisconsin–La Crosse to TW.

References

Bahrani, F. & Mobley, H. (1994). *J. Bacteriol.* **176**, 3412–3419.
Bauernfeind, A., Naber, K. & Sauerwein, D. (1987). *Eur. Urol.* **1**, 9–12.
Bhakdi, S. & Tranum-Jensen, J. (1988). *Prog. Allergy*, **40**, 1–43.
Braun, V. & Focareta, T. (1991). *Crit. Rev. Microbiol.* **18**, 115–158.
Chakraborty, T., Kathariou, S., Hacker, J., Hof, H., Hohle, B., Wagner, W., Clantin, B., Hodak, H., Willery, E., Locht, C., Jacob-Dubuisson, F. & Villeret, V. (2004). *Proc. Natl Acad. Sci. USA*, **101**, 6194–6199.
Jacob-Dubuisson, F., Locht, C. & Antoine, R. (2001). *Mol. Microbiol.* **40**, 306–313.
Könninger, U., Hobbie, S., Benz, R. & Braun, V. (1999). *Mol. Microbiol.* **32**, 1212–1225.
Laemmli, U. (1970). *Nature (London)*, **227**, 680–685.
Mobley, L. T. & Belas, R. (1995). *Trends Microbiol.* **3**, 280–284.
Stanley, R., Koronakis, V. & Hughes, C. (1998). *Microbiol. Mol. Biol. Rev.* **62**, 309–333.
Swihart, K. & Welch, R. (1990). *Infect. Immun.* **58**, 1861–1869.
Uphoff, T. (1991). PhD thesis. University of Wisconsin–Madison, Madison, WI, USA.