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Crystallization of *Escherichia coli* CdtB, the biologically active subunit of cytolethal distending toxin

Jill S. Hontz, Maria T. Villar-Lecumberri,‡ Lawrence A. Dreyfus and Marilyn D. Yoder*

Division of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri-Kansas City, 5007 Rockhill Road, Kansas City, MO 64110-2499, USA

‡ Current address: University of Kansas Medical Center.

Correspondence e-mail: yoderm@umkc.edu

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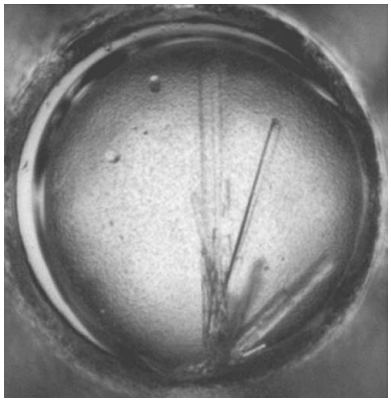


Cytolethal distending toxin (CDT) is a secreted protein toxin produced by several bacterial pathogens. The biologically active CDT subunit CdtB is an active homolog of mammalian type I DNase. Internalization of CdtB and subsequent translocation into the nucleus of target cells results in DNA-strand breaks, leading to cell-cycle arrest and apoptosis. CdtB crystals were grown using microbatch methods with polyethylene glycol 8000 as the precipitant. The CdtB crystals contain one molecule of MW 30.5 kDa per asymmetric unit, belong to space group $P2_12_12_1$ and diffract to 1.72 Å.

1. Introduction

Cytolethal distending toxin (CDT) is a DNA-damaging protein toxin produced by several strains of Gram-negative bacterial pathogens including *Actinobacillus actinomycetemcomitans*, *Campylobacter jejuni*, *Escherichia coli*, *Haemophilus ducreyi* and *Helicobacter* spp. (Dreyfus, 2003). In all species characterized thus far, CDT is encoded by an operon of three adjacent genes, *cdtA*, *cdtB* and *cdtC*, the products of which are secreted proteins with molecular weights of approximately 27, 29 and 20 kDa, respectively (Pickett & Whitehouse, 1999). The CDT holotoxin is a heterotrimer composed of the three CDT subunits CdtA, CdtB and CdtC in a 1:1:1 molar ratio (Lara-Tajero & Galan, 2001; Saiki *et al.*, 2001; Shenker *et al.*, 2004). The holotoxin acts as an canonical AB toxin in which the biologically active A subunit, CdtB, associates with the target-cell surface and is internalized by virtue of the B, or binding, component composed of CdtA and CdtC (Lee *et al.*, 2003). Cell-surface binding is mediated by CdtA and CdtC, which recognize carbohydrate moieties on the target-cell membrane (McSweeney & Dreyfus, 2005; Mise *et al.*, 2005). Following binding and internalization by receptor-mediated endocytosis, CdtB traffics through the Golgi apparatus to the nucleus (Cortes-Bratti *et al.*, 2000; Guerra *et al.*, 2005). Translocation of CdtB across the nuclear envelope is facilitated by nuclear-localization sequences at the carboxy-terminus of CdtB from *E. coli* strain 9142–88 (EcCdtB-II; McSweeney & Dreyfus, 2004) and the amino-terminus of *A. actinomycetemcomitans* CdtB (AaCdtB; Nishikubo *et al.*, 2003). CdtB is a mammalian type I DNase homolog and enzymatic activity is required for toxicity (Elwell & Dreyfus, 2000; Lara-Tajero & Galan, 2000; Hassane *et al.*, 2001). CdtB-mediated chromosomal strand breakage results in activation of an ATM-dependent checkpoint leading to cell-cycle arrest at the G_2/M boundary (Cortes-Bratti *et al.*, 2001; Sert *et al.*, 1999; Escalas *et al.*, 2000; Alby *et al.*, 2001; Whitehouse *et al.*, 1998). Thus, the effect of CDT on cells is similar to that of ionizing radiation (Chaturvedi *et al.*, 1999).

Nesic *et al.* (2004) recently reported the X-ray crystallographic structure of the *H. ducreyi* CDT holotoxin (HdCDT; PDB code 1sr4). The HdCDT structure reveals a trimer, with each subunit sharing an interface with the other two. The CdtAC dimer possesses two functional regions, one of which serves as a platform for CdtB and the second of which contains a putative cell-surface binding site. Although cell-surface binding and internalization of CDT is dependent upon the intact CDT holotoxin, CDT-subunit dissociation is thought to precede and facilitate CdtB nuclear localization and

chromatin degradation. The CdtBs characterized thus far all share a reasonably high degree of sequence identity and similarity (for example, EcCdtB-II shows 49.7% identity with the HdCdtB subunit); however, notable sequence variations exist between these two enzymes and other CdtBs. Moreover, the enzymatic activity of the free CdtB subunit appears to be significantly greater than that of the holotoxin-bound form (Nesic *et al.*, 2004). Thus, assessing the structural differences between CdtBs of different species of origin and the holotoxin *versus* the free subunit form is critical to our understanding of CdtB function.

2. Materials and methods

2.1. Expression construct

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich Chemical. The protein was expressed from the *cdtB* gene from *E. coli* strain 9142-88 and is designated EcCdtB-II to differentiate this toxin from the three forms produced by various *E. coli* strains (Dreyfus, 2003). The *cdtABC* operon was originally cloned from an *E. coli* 9142-88 chromosomal DNA gene library by conventional techniques (Aragón *et al.*, 1997). Subsequent sequencing of the operon identified a two-base variation in cdtB compared with the published sequence (Pickett *et al.*, 1994). This variation results in a change in two CdtB amino acids, T57R and L58V (numbering from mature protein minus signal peptide). The variation in CdtB amino-acid sequence is likely to represent a DNA-sequencing error in the published *E. coli* 9142-88 CDT sequence.

The EcCdtB-II gene was subcloned into pET-45b (Villar-Lecumberri *et al.*, 2005) and the vector transformed into *E. coli* BL21 Star One Shot (DE3) (Invitrogen) cells. CdtB expressed from this vector contains residues 19–269, which represents the mature protein minus the signal peptide of EcCdtB-II and the N-terminal sequence 5′-MAHHHHHHVGT-CdtB$_{19–269}$.

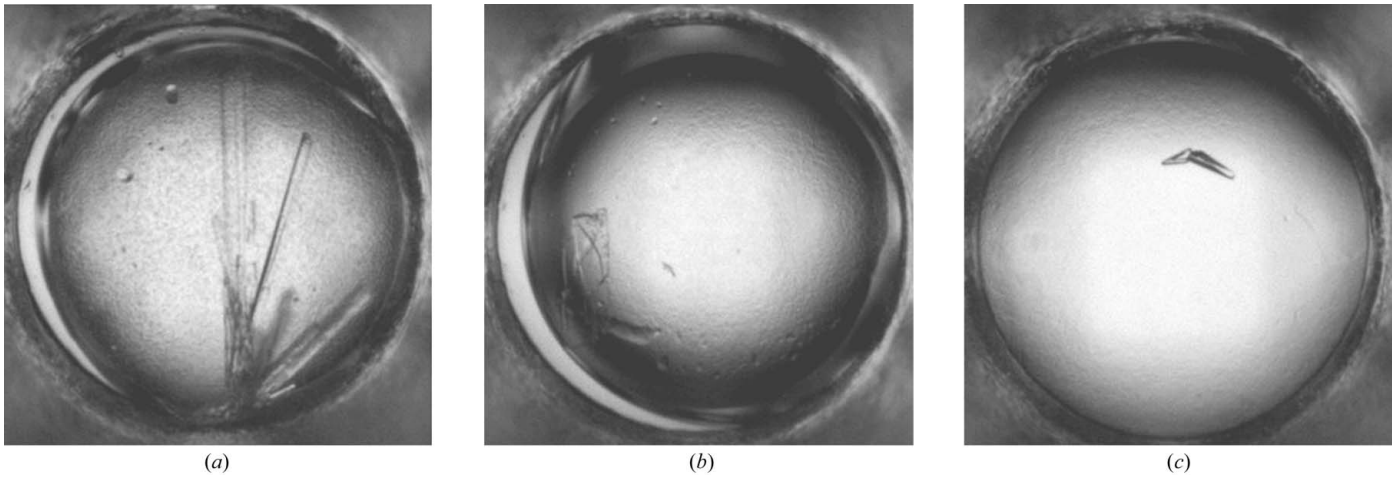
2.2. Protein purification

Expression and purification of EcCdtB-II was performed as reported previously (Elwell *et al.*, 2001). Briefly, 40 ml of an overnight culture of the *E. coli* cells harbouring the expression vector was used to inoculate 2 l cultures of LB media with 100 µg ml^{-1} ampicillin contained in 6 l Erlenmeyer flasks. Cultures were grown at 310 K with vigorous shaking until the $A_{600\,nm}$ reached 0.6. Recombinant protein expression was induced by the addition of 1 m*M* isopropyl-1-thio-β-D-galactopyranoside followed by incubation for 3.5 h at 310 K. Cells were harvested at 277 K by centrifugation at 6000*g* for 15 min. Bacterial cells were washed in ice-cold 25 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl and then suspended in the same buffer containing Protease Inhibitors Cocktail Set I (Calbiochem). Cell lysis was achieved by a single pass through a Watts FluidAir Model 110-L microfluidizer according to the manufacturer's recommendations (Watts FluidAir, Richland, MI, USA). The disrupted cell suspension was centrifuged at 100 000*g* to pellet insoluble material. Recombinant EcCdtB-II was purified from the soluble fraction by Ni–NTA–agarose affinity chromatography (Qiagen) under native conditions. EcCdtB-II was recovered from the affinity matrix by stepwise elution with 250 m*M* imidazole. The EcCdtb-II fractions were dialyzed against 20 m*M* sodium phosphate pH 6.0 and 50 m*M* NaCl and concentrated to 12 mg ml^{-1} by ultrafiltration using a 10 kDa molecular-weight cutoff membrane (Amicon). Protein concentration was determined with the BCA protein-assay kit (Pierce) using bovine serum albumin as a standard. The purity of the sample was estimated at >95% as judged by visualization by SDS–PAGE. A typical yield from 2 l of culture was approximately 10 mg EcCdtB-II. The biological activity of purified EcCdtB was assessed as previously described (Elwell & Dreyfus, 2000) by measuring the cell-cycle block imposed on cultured HeLa cells following combination of this subunit with CdtA and CdtC. The CDT-imposed cell-cycle blockade at the G_2/M boundary, characteristic of CDT intoxication, was measured by flow cytometry using a FACSCalibur (BD Biosciences, Santa Jose, CA, USA).

2.3. Crystallization and X-ray data collection

EcCdtB-II used for crystallization was assayed for activity using cell-cycle distribution analysis by flow cytometry (Elwell & Dreyfus, 2000). Purified EcCdtB-II showed complete activity, causing G_2/M cell-cycle arrest after 24 h intoxication.

Crystallization experiments used microbatch methods. For final microbatch setups, 4 µl protein drops were overlaid with 0.2 ml paraffin oil and sealed with transparent Scotch packing tape (3M). Microbatch experiments were conducted in 96-well round-bottomed microtitre plates (Nunclon). Crystals were mounted in cryoloops (Hampton Research) and the mother liquor removed by dragging through paraffin oil. Looped crystals were flash-cooled by rapid submersion into liquid nitrogen. No additional cryprotectant was used. X-ray diffraction data was collected on a MAR Research 225



(*a*) (*b*) (*c*)

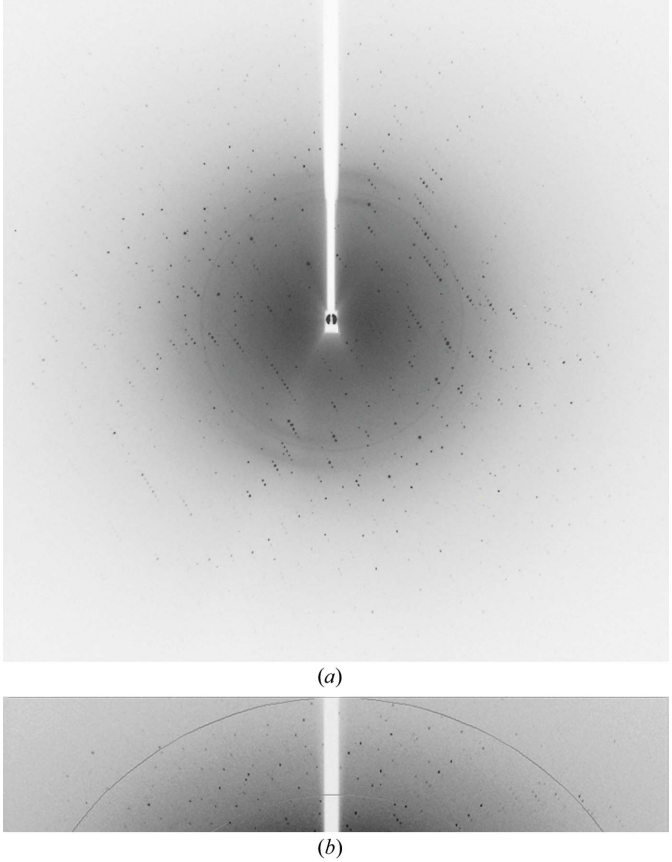
Figure 1
Crystals of CdtB from *E. coli*. (*a*) Crystal form 1; (*b*) crystal form 2; (*c*) crystal form 3.

CCD detector using synchrotron radiation at the Advanced Photon Source (APS) at beamline 22-BM. Exposure time was 2 s, with 0.5° oscillation angle, 175 mm crystal-to-detector distance and a crystal temperature of 100 K. X-ray data were indexed, processed and scaled using *HKL*2000 (Otwinowski & Minor, 1997).

3. Results and discussion

Crystallization of EcCdtB-II using Hampton Research Index I and II screens was unsuccessful, so the protein was submitted to the Hauptman–Woodward Institute for high-throughput crystallization screening. Three morphologically distinct crystal forms were obtained within two weeks (Fig. 1). Crystal form 1 consisted of long thin rods radiating from a single epicentre and was grown from 0.1 *M* ammonium thiocyanate, 0.1 *M* sodium citrate pH 4.0 and 40%(*w*/*v*) polyethylene glycol (PEG) 8000. Growth continued for 30 d to typical dimensions of 0.85 × 0.05 × 0.05 mm. Crystal form 2 consisted of very thin rectangular plates grown from 0.1 *M* calcium chloride dihydrate, 0.1 *M* HEPES pH 7.0 and 20%(*w*/*v*) PEG 4000. Crystals grew to full size in 14 d and measured 0.3 × 0.4 mm in length; the third dimension was too thin to measure accurately. Crystal form 3 was grown from 0.1 *M* calcium chloride dihydrate, 0.1 *M* HEPES pH 7.0 and 80%(*w*/*v*) PEG 400. Crystals grew in 14 d and with length and width dimensions comparable to those of crystal form 2.

Crystal form 1 was most readily reproducible and was the crystal form from which X-ray data were ultimately obtained. Final crystals were grown at 292 K by microbatch under paraffin oil using a protein solution of 6 mg ml^{-1} CdtB, 0.05 *M* ammonium thiocyanate, 0.05 *M* sodium citrate pH 4.0 and 20%(*w*/*v*) PEG 8000.



(*a*)

(*b*)

Figure 2
X-ray diffraction image from crystal form 1 of EcCdtB-II. (*a*) Single frame of 0.5° oscillation with a crystal-to-detector distance of 175 mm. (*b*) Top area of image shown in (*a*), but contoured to show reflections at high resolution. Resolution rings drawn at 1.8 and 2.4 Å.

Table 1
Crystal parameters for *E. coli* CdtB.

Values in parentheses indicate the highest resolution shell (1.73–1.79 Å).

Crystal parameters	
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 39.815, b = 47.058, c = 114.494
Unit-cell volume (Å^3)	2.1 × 10^5
V_M (Å^3 Da^{-1})†	1.87
Molecules per ASU	1
Estimated solvent content (%)	34.1
Diffraction limit (Å)	1.72
X-ray data statistics	
Wavelength (Å)	1.0
Temperature (K)	100
Resolution (Å)	1.72
Reflections collected	102947
Unique reflections	23348
Completeness‡ (%)	99.1 (91.8)
$\langle I/\sigma(I)\rangle$	4.7
$I > 3\sigma(I)$‡ (%)	16.4 (43.6)
R_{sym}§ (%)	0.066 (0.212)
Mosaicity (°)	0.42

† Estimated by $V_{prot} = (\rho/V_M)$ where ρ = 1.23 g cm^{-3} (Matthews, 1968). ‡ Percentage of data with $I > 3\sigma(I)$. § $R_{sym} = \sum|I_{hkl} - \langle I_{hkl}\rangle|/\sum I_{hkl}$ (single measurements excluded).

The X-ray diffraction pattern shows good intensity to a resolution of 1.72 Å (Fig. 2). The crystals belong to space group $P2_12_12_1$. A complete X-ray data set was collected from a single native EcCdtB-II crystal; crystal parameters and X-ray data statistics are listed in Table 1. EcCdtB-II shows 49.7% identity with the HdCdtB subunit. A successful molecular-replacement solution was determined with *AMoRe* (Navaza, 1994) using the HdCdtB subunit from the HdCdt holotoxin (PDB code 1sr4). A final model of EcCdtB-II is nearing completion.

Data were collected at Southeast Regional Collaborative Access Team (SER-CAT) 22-BM beamline at the Advanced Photon Source, Argonne National Laboratory. Use of the Advanced Photon Source was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. W-31-109-Eng-38. This work was supported by a Public Health Services Grant from the National Institutes of Health AI47999 (to LAD).

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