Production, crystallization and X-ray diffraction analysis of the protease CT441 from *Chlamydia trachomatis*

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The prokaryotic obligate intracellular pathogen *Chlamydia trachomatis* is the most prevalent cause of preventable blindness, affecting approximately six million people worldwide. In addition, *C. trachomatis* is the most commonly reported sexually transmitted pathogen in Europe and the US, causing pelvic inflammation, ectopic pregnancy and infertility. As in other intracellular pathogens, proteases play crucial roles during most stages of the complex life cycle of *Chlamydia*. CT441 is a chlamydial protease that has been reported to interfere with oestrogen signalling of the host cell. Here, the recombinant production, purification and crystallization of an inactive variant of CT441, designated CT441[°] (active-site Ser455 replaced by Ala), are described. CT441[°] was crystallized in space group $P2_12_1$, with unit-cell parameters $a = 86.7$, $b = 184.0$, $c = 209.6$ Å. A complete diffraction data set was collected to a resolution of 2.95 Å.

1. Introduction

Chlamydiae are Gram-negative obligate intracellular prokaryotes with a wide host range including humans, animals, free-living amoebae and other eukaryotes. *C. trachomatis* is the most prevalent cause of preventable blindness (affecting six million people worldwide; Mariotti *et al.*, 2009) and the most commonly reported sexually transmitted pathogen in Europe (340 000 reported cases per year) and the US (1.3 million reported cases per year) (Centers for Disease Control and Prevention, 2011; European Centre for Disease Prevention and Control, 2011; Peipert, 2003). Although *C. trachomatis* has been recognized as a major healthcare problem worldwide, many aspects of the underlying virulence mechanisms are far from being well understood. During the replication phase, *C. trachomatis* depends on an extensive modification of host-cell signalling pathways, utilizing a number of virulence factors which are secreted into the host-cell cytosol. CT441 is a chlamydial tail-specific protease (Tsp) with a catalytic serine and a PDZ domain. It has been reported to interfere with the pro-inflammatory NF- κ B signalling pathway of the host cell, modulating antimicrobial and inflammatory responses (Lad, Li *et al.*, 2007; Lad, Yang *et al.*, 2007). However, in subsequent reports, the role of CT441 in the cleavage of NF- κ B has been questioned (Chen *et al.*, 2012; Christian *et al.*, 2010). The results presented by Borth *et al.* (2010) indicate that CT441 is able to modulate the oestrogen-

signalling pathway of the host cell by interacting with human SRAP1 (steroid receptor RNA activator protein 1), a co-activator of oestrogen receptor α (ER α). We have previously reported the biochemical and structural characterization of CT441 (Kohlmann *et al.*, 2015). Here, we report details of the production, purification, crystallization and X-ray diffraction analysis of an inactive variant of CT441.

2. Materials and methods

2.1. Macromolecule production

Genomic DNA from *C. trachomatis* L2/Bu/434 was isolated and *ct441* lacking the nucleotides encoding the N-terminal signal peptide was cloned into a pQE30 expression vector (Qiagen) modified to contain the *lacI* gene and a PreScission protease (GE Healthcare) cleavage site. Site-directed mutagenesis to replace the active-site Ser455 by Ala (CT441 $^\circ$) was performed using the QuikChange kit (Stratagene). CT441 $^\circ$ was produced at 22°C by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG; final concentration 1 mM) to *Escherichia coli* C43 (DE3) cells in 2 $\times$ YT medium at an OD₆₀₀ of 0.8–1.0. After 16 h, cells were harvested by centrifugation (5000g, 30 min), resuspended in buffer A [50 mM Na₂HPO₄, 500 mM NaCl, 20 mM imidazole, 5% (v/v) glycerol pH 8.0] and lysed by sonification. After centrifugation (27 000g, 277 K, 60 min), the supernatant was loaded onto a HisTrap column (GE Healthcare) and CT441 $^\circ$ was eluted with buffer A containing 500 mM imidazole. After buffer exchange to 20 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT pH 7.4, the His₆ tag was removed by incubation with PreScission protease (1:10 molar ratio of protease to CT441 $^\circ$, 24 h, 4°C).

Uncleaved CT441 $^\circ$, His₆ tag and PreScission protease were removed using connected GST and HisTrap columns (GE Healthcare). After dialysis into 20 mM Tris, 150 mM NaCl pH 7.4, the sample was subjected to size-exclusion chromato-

Table 1

Macromolecule-production information.

Source organism	<i>C. trachomatis</i> L2/Bu/434 (NC_010287.1)
DNA source	<i>C. trachomatis</i> L2/Bu/434 (NC_010287.1)
Forward primer	TATCAGCATGCGCAGAGCCTCTTCGACGAC
Reverse primer	CGGGGTACCTTATTGAGCTGGAGTTTGTGATATAG
Cloning vector	pGEM-T (Promega)
Expression vector	pQE30 (Qiagen), modified to include <i>lacI</i> and a PreScission protease cleavage site for removal of the His ₆ tag
Expression host	<i>E. coli</i> C43
Complete amino-acid sequence of the construct produced†	GPR SACAEPLRRQDVVRKTVDKLVEHHIDTQQISP-YILSRSLDYVRSFDSHKAYLTQDEVFSAFSEETHPLFKYQEDNFSSFKELDTCTIQQSISRAREWRSSWLTDSIRVIQDAMSHTIEKKPSAWASSIEEVQRQYDLLLSYASIVLEDAAKNRYQGKEHGLVKLCIRQIENHENPYIGINDHGYRMSP-EEEANSFHVRIKSIASLSLAHTAYFSQEEALSMRAQLEKMGCGIGVVLKEDIDGVVKEVLGAGPADKTGSLRVGDIIYRVNGKNIENTPFPGLVDSLGRSPGSSVTLDIRQNDHVIQLRREKILLDSRRVDVSYEPYNGIIGKITLHSFYEGENQVSSEQDLRKAIRELQEKNNLLGLVDIRENTGGFLSQAIVKVSGLFLTNGVVVSRVYADGSKVRYRTISPKFYDGPLAVLVSKSSAAAEIVAQTLQDYGVALLVGDQTYGKGTIQTITGNSQEDFFKVTVGRYSPSGKSTQLEGVKSDIVIPSRYAEDKLGERFLEYALPADQYDNDVINDNLGDLDIRPWFQKYSPHLLKPELVREMLPQLAHNSQERLEKNKNFEIFVQHLKKTNKQDRSFGSNDLQMEESVNIKDMILLKSIQTTPAQ

† A CT441 $^\circ$ variant without a His₆ tag was used for crystallization: additional N-terminal residues after the removal of the purification tag are shown in bold and the active-site S455A mutation is underlined.

Figure 1

Purification of the proteolytically inactive variant CT441 $^\circ$. (a) SEC elution profile. CT441 $^\circ$ eluted at the volume expected for a monomeric species (78 ml; 73 kDa). (b) SDS-PAGE analysis of purified CT441 $^\circ$ after SEC (fractions indicated by a green bar above the chromatogram). (c) Dynamic light-scattering profile indicating an average hydrodynamic radius of 7 nm for CT441 $^\circ$.

Table 2
Crystallization.

Crystallization experiments have also briefly been described in Kohlmann *et al.* (2015).

Method	Sitting-drop vapour diffusion
Plate type	Cryschem M plate
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	2.5
Buffer composition of protein solution	20 mM Tris, 150 mM NaCl pH 7.4
Composition of crystallization solution	100 mM MES pH 6.0, 100 mM MnSO ₄ , 5% (v/v) PEG 6000, 6% (v/v) ethylene glycol
Composition of reservoir solution	1.5 M NaCl
Volume and ratio of drop	5 µl protein solution + 5 µl crystallization solution
Volume of reservoir (µl)	500
Cryoprotection solution	70 mM MES pH 6.0, 140 mM MnSO ₄ , 3.5% (v/v) PEG 6000, 34.5% (v/v) ethylene glycol

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL14.2, BESSY II
Wavelength (Å)	0.9184
Temperature (K)	100
Detector	Rayonix MX225
Crystal-to-detector distance (mm)	350
Rotation range per image (°)	0.7
Total rotation range (°)	140
Exposure time per image (s)	5
Space group	<i>P</i> 2 ₁ 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	86.73, 183.99, 209.62
α , β , γ (°)	90, 90, 90
Mosaicity (°)	0.71
Resolution range (Å)	33.44–2.95 (3.11–2.95)
Total No. of reflections	294118 (38566)
No. of unique reflections	65713 (9182)
Completeness (%)	92.3 (89.3)
Multiplicity	4.5 (4.2)
$\langle I/\sigma(I) \rangle$	11.5 (2.1)
$R_{\text{r.i.m.}}$ †	0.078 (0.583)
Overall <i>B</i> factor from Wilson plot (Å ²)	76.5

† $R_{\text{r.i.m.}}$ is the redundancy-independent merging *R* factor (Weiss & Hilgenfeld, 1997); part of the data-collection statistics in this table have been described in the Supporting Information of Kohlmann *et al.* (2015).

graphy (HiLoad 16/60 Superdex 200; GE Healthcare; Fig. 1*a*). CT441° samples (Fig. 2*b*) were pooled, concentrated to 2.5 mg ml⁻¹, analyzed by dynamic light scattering (DLS; Fig. 2*c*) using a SpectroSize 300 instrument (Xtal Concepts, Germany) and stored at –80°C. Macromolecule-production information is summarized in Table 1.

CT441 variants 2, 7 and 8 shown in Fig. 2 and CT441 homologues from *C. pneumoniae* (CPn555), *Simkania negevensis* and *Protochlamydia amoebophila* were produced, purified and stored as described for CT441°. CT441 variants 3–6 were found to be insoluble under all expression conditions tested.

2.2. Crystallization

CT441° was crystallized using the sitting-drop vapour-diffusion technique in 24-well Cryschem plates (Hampton Research). Equal volumes (5 µl) of protein (2.5 mg ml⁻¹) and

crystallization solution were mixed and equilibrated against 500 µl reservoir solution (Table 2). Prior to diffraction experiments, crystals were transferred into cryoprotection solution, mounted in CryoLoops (Hampton Research) and flash-cooled in liquid nitrogen.

2.3. Data collection and processing

X-ray diffraction data for several crystal forms of CT441° which had initially been identified during screening were collected using synchrotron radiation on beamline BL14.2 operated by the Joint Berlin MX-Laboratory at the BESSY II electron-storage ring, Berlin-Adlershof, Germany (Mueller *et al.*, 2012), beamline P11 (DESY) at PETRA III, Hamburg, Germany, beamline ID29 at ESRF, Grenoble, France and beamlines PX I and PX III at SLS, Villigen, Switzerland. The best crystals diffracted X-rays to a resolution of 2.95 Å at 100 K in a nitrogen-gas stream at BESSY II. Diffraction data were indexed, reduced and scaled using *XDS* (Kabsch, 2010) and *SCALA* (Evans, 2006). Data-collection statistics are given in Table 3.

3. Results and discussion

To obtain structural information on chlamydial Tsps, plasmids for the high-yield production of mature Tsp proteins from the pathogenic *C. trachomatis* (CT441) and *C. pneumoniae* (CPn555), as well as from the chlamydia-like species *S. negevensis* and *P. amoebophila*, have been prepared. For each protein an active-site mutant replacing the nucleophilic Ser

Figure 2
Rational design of CT441 protein variants for structure determination and overview of the outcome. (a) Domain architecture of full-length CT441 (1) and truncated protein variants (2–8). All variants include a cleavable N-terminal His tag (yellow) and variants 1–6 carry the S455A mutation. Domain boundaries were assigned according to *CDD* (Marchler-Bauer *et al.*, 2011). (b) Overview of the outcome at crucial stages towards structure determination.

with an Ala was generated to prevent auto-degradation during prolonged crystallization trials. The expression of all constructs was individually optimized by rational variation of protein-production conditions such as the *E. coli* expression strain, broth medium and expression temperature. In addition, seven constructs for CT441 comprising a combination of predicted protein domains (Fig. 2) have been prepared and tested for expression in *E. coli*. Interestingly, the N-terminal residues 22–250 seem to play a crucial role in the structure or folding of CT441, as variants lacking this region were found to form insoluble inclusion bodies during production in *E. coli* (Fig. 2). For soluble proteins, large-scale production and purification was initiated. Large amounts of high-quality protein were obtained for CPn555 (10 mg), CT441 (19 mg) and the

Figure 3

CT441° crystals obtained by the sitting-drop vapour-diffusion technique in the presence of PEG 6000 and MnSO_4 . Crystals grew within 2–4 weeks to final dimensions of $0.13 \times 0.11 \times 0.08$ mm.

Figure 4

X-ray diffraction pattern to a resolution of $2.95 \text{ \AA}$ acquired on beamline BL14.2 at BESSY, Berlin.

S. negevensis and *P. amoebophila* homologues (>5 mg), as well as for the CT441° variants 1 (>100 mg), 2 (20 mg), 7 (30 mg) and 8 (10 mg) (Fig. 2). The monodispersity of all protein samples was confirmed by dynamic light scattering. Purified proteins were subjected to initial crystallization trials using 672 conditions from commercial crystallization screens (Hampton Research, Molecular Dimensions). Experiments were set up at 20°C using Intelli-Plate 96 plates (Art Robbins Instruments) and a Phoenix robot (Art Robbins Instruments). Out of all of the proteins tested, only full-length CT441° (variant 1) formed crystals in initial crystallization trials. The preliminary conditions were optimized by variation of the concentrations of buffers, divalent cations, polyethylene glycols and ethylene glycol, the temperature and the pH using 24-well Cryschem M plates (Hampton Research). Optimization of protein-production and crystallization conditions (Tables 1 and 2) yielded CT441° crystals with final dimensions of $0.13 \times 0.11 \times 0.08$ mm within 2–4 weeks at 293 K (Fig. 3). After the identification of a suitable cryoprotection condition (Table 2), a diffraction data set was collected to a resolution of $2.95 \text{ \AA}$ at BESSY, Berlin from a crystal belonging to space group $P2_12_1$, with unit-cell parameters $a = 86.7$, $b = 184.0$, $c = 209.6 \text{ \AA}$ (Fig. 4). The asymmetric unit contains four CT441° molecules with a Matthews coefficient (Matthews, 1968) of $2.97 \text{ \AA}^3 \text{ Da}^{-1}$ (estimated solvent content of 58.5%). Full structure determination, refinement and analysis of CT441° together with data on its biological function have been reported elsewhere (Kohlmann *et al.*, 2015).

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