Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray studies of the protease domain of the heat-shock protein HtrA from *Thermotoga maritima*

Dong Young Kim and Kyeong Kyu Kim*

Department of Molecular Cell Biology, Center for Molecular Medicine, SBRI, Sungkyunkwan University School of Medicine, Suwon 440-746, South Korea

Correspondence e-mail: kkim@med.skku.ac.kr

Received 22 August 2001
Accepted 29 October 2001

HtrA (high-temperature requirement A) is a widely distributed heat-shock protein which has both molecular-chaperone and proteolytic activities. It is composed of two PDZ domains essential for oligomerization and a protease domain. To understand the molecular basis of the dual function of HtrA, the protease domain of *T. maritima* HtrA has been crystallized. X-ray diffraction data have been collected to 2.7 Å resolution using a synchrotron-radiation source. Crystals belong to the cubic space group $P2_13$, with unit-cell parameters $a = b = c = 120.55$ (8) Å. The asymmetric unit contains two protease domains, with a corresponding V_M of 2.80 Å^3 Da^{-1} and a solvent content of 56.1%.

1. Introduction

HtrA (also called DegP or protease Do) is a heat-shock protease located in the periplasmic space of bacteria (Strauch & Beckwith, 1988; Pallen & Wren, 1997). From its proteolytic activity, HtrA is known to play a role in the degradation of heat-denatured protein in periplasmic space (Pallen & Wren, 1997). Therefore, its activity is essential for bacterial thermotolerance and for cell survival at high temperature (Lipinska *et al.*, 1989). Recently, it was found that bacterial HtrA has a dual role, acting as a molecular chaperone at normal temperature and as a protease at elevated temperature (Spiess *et al.*, 1999). This functional switch of HtrA is considered to be necessary for controlling protein stability as well as eliminating denatured proteins to maintain cellular viability (Spiess *et al.*, 1999). In addition, HtrA is known to be involved in bacterial virulence (Pallen & Wren, 1997) and is identified as a vaccine-candidate antigen (Cates *et al.*, 2000). Two human homologues of bacterial HtrA are also expected to be involved in mammalian stress-response pathways (Zumbrunn & Trueb, 1996; Gray *et al.*, 2000; Faccio *et al.*, 2000).

Bacterial HtrA is a serine protease and exists as a dodecameric complex consisting of two stacks of hexameric rings (Kim *et al.*, 1999). Each subunit is composed of one protease domain in the amino-terminus and two PDZ (named after three proteins, PSD-95, DLg and ZO-1) domains in the carboxyl-terminus, while human HtrA has only one PDZ domain (Hu *et al.*, 1998; Gray *et al.*, 2000; Faccio *et al.*, 2000). The amino-terminal protease domain contains a well conserved catalytic triad that is characteristic of serine proteases. In addition, it is known that the protease domain is sufficient to show full molecular-chaperone activity in *Escherichia coli* (Spiess *et al.*, 1999). The carboxyl-terminal PDZ domains are known to be involved in protein–protein interactions and assumed to be essential for oligomerization of HtrA. The PDZ domains, also found in the Clp/Hsp100 family of heat-shock proteins (Clp, caseinolytic protein; Hsp, heat-shock protein), are suggested to play a role in substrate recognition (Levchenko *et al.*, 1997). However, unlike other proteases in the Clp/Hsp100 family, HtrA does not have a regulatory component or ATP-binding domain as it is an ATP-independent protease.

In order to better understand the dual role of HtrA and its functional switch at atomic resolution, a structural study is indispensable. As a first step in this approach, we report here the overexpression, purification, crystallization and preliminary crystallographic data of the protease domain of HtrA from *T. maritima*, which has 38.2 and 31.0% sequence identities to *E. coli* HtrA and human HtrA2, respectively.

2. Materials and methods

2.1. Protein expression and purification

The protease domain has been determined to be within amino-acid residues 24–262 of the full-length *T. maritima* HtrA (Gene Bank ID AAD35656) by deleting the two PDZ domains in the C-terminus and the signal peptide in the

N-terminus. The DNA encoding the protease domain was amplified by the polymerase chain reaction (PCR) using the *T. maritima* gemomic DNA as template. The forward (5′-CATACGTCCATATGGATT-ACGAAAGTCCGATC-3′) and reverse (5′-GGTCAAGCTTCACACGCCGACGTAG-GCTTT-3′) oligonucleotide primers were synthesized. PCR products were digested by *Nde*I and *Hin*dIII and inserted into *Nde*I/*Hin*dIII-digested expression vector pET-22b (Novagen). The plasmid encoding the protease domain of HtrA was transformed into *E. coli* strain BL21(DE3) which harbours RIG plasmid encoding the rare codon t-RNAs. The cells were grown in Luria–Bertani (LB) medium containing 50 µg ml^{-1} ampicillin and induced with 1 m*M* isopropyl β-D-thiogalactopyranoside (IPTG) at 303 K. After 4 h induction, the cells were harvested by centrifugation at 4200*g* (Sorvall GS3 rotor) for 10 min at 277 K. The cell pellet was resuspended in buffer *A* (20 m*M* Tris–HCl pH 7.5) and disrupted by sonication. The homogenate was clarified by centrifugation at 27 000*g* (Beckman JA 25.50 rotor) for 15 min at 277 K. The supernatant was kept between 348 and 353 K for 7 min and placed on ice for 10 min to remove heat-labile *E. coli* proteins. The sample was clarified by ultracentrifugation at 120 000*g* (40 000 rev min^{-1}; Beckman TYPE 70 Ti rotor) for 30 min at 293 K. The supernatant was loaded onto a Q-Sepharose anion-exchange column (Amersham-Pharmacia) which was equilibrated with buffer *A*. After washing with 20 m*M* phosphate pH 6.0, the protein was eluted with a linear gradient of 0.0–1.0 *M* NaCl. The fractions containing the protease domain of HtrA were pooled and loaded onto a HiLoad XK16 Superdex 75 prep-grade gel-filtration column (Amersham Pharmacia) previously equilibrated with buffer *A*. The protein was concentrated for crystallization. Purity was checked by SDS–gel electrophoresis.

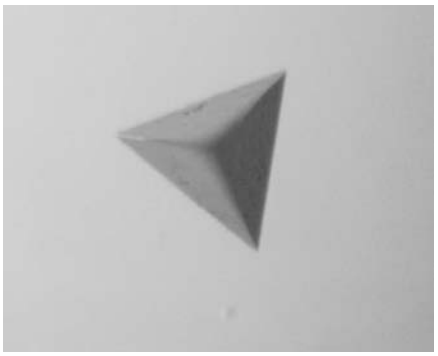

Figure 1
A crystal of recombinant HtrA protease domain. Crystal dimensions are about 0.3 × 0.3 × 0.2 mm.

2.2. Crystallization and X-ray diffraction experiment

Crystallization was achieved by the hanging-drop vapour-diffusion method at 295 K using 24-well tissue-culture plates. Each hanging drop, prepared by mixing 1 µl of the reservoir solution and 1 µl of the protein solution (10 mg ml^{-1}), was equilibrated against 0.5 ml of reservoir solution. Initial crystallization conditions were tested using WIZARD crystal screen kits (Emerald Structures, Bainbridge Island, USA). Tetrahedral crystals (Fig. 1) were grown using polyethyleneglycol (PEG) 1000 as precipitant. Crystals suitable for diffraction experiments were obtained from protein solution with 100 m*M* phosphate–citrate pH 4.4, 110 m*M* Li_2SO_4 and 5%(*w*/*v*) PEG 1000 after optimizing the initial crystallization conditions. Crystals were transferred into a cryosolvent consisting of 100 m*M* phosphate–citrate pH 4.4, 110 m*M* Li_2SO_4 and 30%(*w*/*v*) PEG 1000 for a few seconds before being flash-frozen in a cold nitrogen stream. X-ray diffraction data were collected at 100 K with a MacScience 2030b area detector at beamline 6B of the Pohang Accelerator Laboratory, South Korea. The wavelength of the synchrotron radiation was 1.0079 Å. A total of 25 frames of 1.2° oscillation were measured with the crystal-to-detector distance set to 340 mm. Data were processed and integrated using *DENZO* and scaled using *SCALEPACK* from the *HKL* program suite (Otwinowski & Minor, 1997).

3. Results and discussion

The protease domain of HtrA from *T. maritima* was overexpressed and purified with an approximate yield of 30 mg of homogeneous protein from 1 l culture. The protein is more than 95% pure judged from SDS–PAGE analysis. The molecular weight of the HtrA protease domain is about 26 kDa from SDS–PAGE, which is in agreement with the calculated molecular weight of 25.907 kDa, indicating that the protein exists as a monomer in solution. The purified protease domain showed molecular-chaperone and proteolytic activities at 358 K (data not shown). The crystals grew to dimensions of 0.3 × 0.3 × 0.2 mm within 2 d. The diffraction data from a cryogenic crystal were collected to 2.7 Å resolution at 100 K using a synchrotron-radiation source. A total of 94 435 measured reflections were merged into 15 460 unique reflections with an R_{merge} (on intensity) of 6.9%. The merged data set is 95.1% complete to 2.7 Å resolution. The crystals belong to the cubic space group $P2_13$, with unit-cell parameters $a = b = c = 120.55$ (8) Å. Assuming the presence of two monomers in the asymmetric unit and based on the molecular weight of 25 907 Da per monomer, the value of the crystal packing parameter V_M is 2.80 Å^3 Da^{-1}, with a corresponding solvent content of 56.1%, which falls within the range commonly observed for protein crystals (Matthews, 1968). Data statistics for the synchrotron data collection and processing are summarized in Table 1. Based on the sequence alignment and arrangement of the residues in the catalytic triad, the protease domain of HtrA is expected to share a similar structural fold with the trypsin family of serine proteases. However, an attempt to solve the structure by molecular replacement using the known structures of trypsin homologues as search models was not successful. Therefore, multiple isomorphous replacement (MIR) or multi-wavelength anomalous dispersion (MAD) methods will be used for phasing. The crystal structure of the protease domain of *T. maritima* HtrA will represent the first domain structure of the ATP-independent heat-shock protease. In addition, it will elucidate the molecular mechanism of chaperone activity and structural architecture of the protease domain and provide a clue for understanding the functional switch of HtrA.

Table 1
X-ray data-collection and processing statistics.

Values in square brackets refer to the highest resolution shell.

Space group	$P2_13$
Unit-cell parameters (Å, °)	$a = b = c = 120.55$ (08), $\alpha = \beta = \gamma = 90$
Resolution range (Å)	30–2.7 [2.8–2.7]
No. of observed reflections	94435
No. of unique reflections	15460
Data completeness (%)	95.1 [96.2]
R_{sym}† (%)	6.9 [37.1]
$I/\sigma(I)$	11.6 [2.7]

† $R_{sym} = \sum_{hkl} |I - \langle I \rangle| / \sum_{hkl} I$.

We thank Drs H. S. Lee and G.-H. Kim for assistance during data collection at beamline 6B of Pohang Accelerator Laboratory, Korea. We are grateful to Dr H. D. Yun for providing *T. maritima* genomic DNA. This work was supported by a Korea Research Foundation Grant (KRF-2000-015-FS0003).

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