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Crystallization and preliminary X-ray diffraction analysis of the α subdomain of Lon protease from *Brevibacillus thermoruber*

DNA-binding ability has previously been reported as a novel function for the thermostable Lon protease from *Brevibacillus thermoruber* WR-249 (Bt-Lon), and the α subdomain (amino acids 491–605) of Bt-Lon has been identified as being responsible for DNA binding. However, the physiological role and DNA-recognition mode of Bt-Lon still remain unclear. In this study, the crystallization and preliminary crystallographic analysis of the Bt-Lon α subdomain are presented. Native diffraction data to 2.88 Å resolution were obtained from a vitrified crystal at 100 K on the BL13C1 beamline at the NSRRC (National Synchrotron Radiation Research Center), Taiwan. The crystals belonged to space group *P*23, with unit-cell parameters $a = b = c = 94.28$ Å. Solvent-content calculations and molecular-replacement results suggest that there are two molecules of Bt-Lon α subdomain per asymmetric unit.

1. Introduction

ATP-dependent Lon protease has been identified in bacteria and archaea, as well as in the mitochondria of eukaryotes (Wang *et al.*, 1993; van Dyck *et al.*, 1994; Gottesman, 1996; Fukui *et al.*, 2002). It has been assigned to the AAA⁺ (ATPases associated with diverse cellular activities) protein family (Neuwald *et al.*, 1999). As for other ATP-dependent protease families, Lon protease is a bifunctional enzyme consisting of a protein-unfolding chaperone coupled to a broad-specificity protease; it forms a ring-shaped homo-oligomer (Goldberg *et al.*, 1994; Stahlberg *et al.*, 1999; Rudyak *et al.*, 2001). In many families of ATP-dependent proteases, the AAA⁺ unfolding/translocation ring and the self-compartmentalized peptidase are encoded by distinct subunits; however, the chaperone and protease components of Lon are encoded in tandem in a single polypeptide. Lon protease degrades multiple regulatory and physiological targets including the cell-division inhibitor Sula in enteric bacteria (Ishii & Amano, 2001) and the steroid acute regulatory protein in human mitochondria (Granot *et al.*, 2007). In addition, Lon protease has DNA-binding ability (Zehnbauer *et al.*, 1981), suggesting that it is involved in gene regulation by controlling the levels of transcription factors (Schmidt *et al.*, 1994). However, the DNA-recognition mode and DNA-binding ability for the physiological role of Lon protease remain unclear.

Lon protease consists of an N-terminal domain, a central ATPase domain and a C-terminal protease domain (Vasilyeva *et al.*, 2002; Lee *et al.*, 2004; Fig. 1). The central ATPase domain includes a large α/β subdomain and a small α subdomain which participates in nucleotide binding and oligomerization with the α/β subdomain on the adjacent subunit. In addition, the α subdomain is also referred to as the sensor- and substrate-discrimination domain (SSD; Smith *et al.*, 1999) since it has been reported to carry the substrate-recognition site. Our previous studies demonstrated that the Lon protease from *Brevibacillus thermoruber* (Bt-Lon) is a DNA-binding protein and that the α subdomain of Bt-Lon is responsible for its DNA-binding ability (Lee *et al.*, 2004; Lin *et al.*, 2009). Furthermore, the DNA sequence of the binding motif for Bt-Lon α subdomain has been identified by a DNase I protection assay and matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectrometry (Lin *et al.*, 2009). In an effort to elucidate the DNA-recognition mode of Bt-Lon α subdomain, protein crystallography was employed to determine its structure.

Figure 1

Schematic diagram showing the domain composition of Bt-Lon. The N-terminal domain, the ATPase domain including a large α/β subdomain and a small α subdomain, and the protease domain are indicated with their respective starting and ending amino acids.

2. Experimental methods

2.1. Expression and purification of the Bt-Lon α subdomain

The 345 bp DNA fragment encoding the Bt-Lon α subdomain (residues 491–605) flanked by *Nde*I and *Xho*I sites was amplified from the full-length *lon* DNA template using the following primers: forward primer, 5'-GGAGATATACATATGGGCGACTACACCG-AG-3'; reverse primer, 5'-CTCAGCCTCGAGGTAGCGATAGCG-CGGTTTGCCAG-3'. The PCR product with restriction-enzyme cutting sites was ligated with pGEM-T Easy Vector (Promega, USA); recombinant plasmids were introduced into *Escherichia coli* JM109 by transformation. Plasmid DNA was isolated from the transformants and digested with *Nde*I and *Xho*I; the fragment was cloned into pET-21a (Novagen) linearized with the same enzymes.

Bt-Lon α subdomain with a C-terminal hexahistidine tag was expressed in *E. coli* BL21(DE3) cells as follows. A 10 ml aliquot of an overnight culture of the recombinant bacterial colony was inoculated into 1 l Luria–Bertani medium containing 50 mg l⁻¹ ampicillin; the culture was grown at 310 K with shaking at 200 rev min⁻¹ to an OD₆₀₀ of 0.6. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression at 310 K. 5 h after induction, the cells were harvested by centrifugation at 9000g for 30 min. The cell pellet was resuspended in resuspension buffer (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 20 mM imidazole) and disrupted by ultrasonication. Cell debris was removed by centrifugation at 20 000g for 20 min at 277 K. The supernatant was added to Ni Sepharose 6 Fast Flow resin (GE Healthcare, USA) and gently mixed for 20 min at 298 K. The resin mixture was packed into an Econo-Pac column (Bio-Rad, USA) and washed with 20 column volumes of resuspension buffer. The protein was eluted with five

column volumes of the same buffer containing 200 mM imidazole. Fractions containing Bt-Lon α subdomain were pooled and dialysed against 50 mM Tris–HCl pH 8.0 containing 100 mM NaCl at 277 K (Fig. 2). Prior to crystallization screening, the purified protein was concentrated to 6.7 mg ml⁻¹ using an Amicon Ultra-15 device (5 kDa molecular-weight cutoff, Millipore, USA). No attempt was made to remove the His tag.

2.2. Crystallization

Initial crystallization experiments were set up using the screening kits Crystal Screen and Crystal Screen 2 (Hampton Research, California, USA) as well as Wizard I and II (Emerald BioSystems, Washington, USA) according to a previously described protocol (Chen *et al.*, 2010). The protein solution (1 μ l, 6.7 mg ml⁻¹) was manually mixed with an equal volume of reservoir solution to screen for crystallization conditions using the sitting-drop vapour-diffusion method. Crystals were obtained as shown in Fig. 3 from several conditions, which were then optimized to obtain diffraction-quality crystals. The crystals for data collection grew in one week at 283 K using the optimal condition consisting of 0.1 M sodium/potassium phosphate buffer pH 6.6, 0.2 M sodium chloride, 12.5% (w/v) polyethylene glycol 8000. The approximate dimensions of the crystals were 80 $\times$ 80 $\times$ 80 μ m.

2.3. X-ray data collection and processing

Data were collected using a synchrotron-radiation X-ray source on the protein crystallography beamline BL13C1 equipped with an ADSC Q315r detector at the National Synchrotron Radiation Research Center (NSRRC) in Taiwan. The crystal was transferred

Figure 2

15% SDS–PAGE analysis of Bt-Lon α subdomain stained with Coomassie Brilliant Blue. Lane M, protein markers (labelled in kDa); lane 1, concentrated His-tagged Bt-Lon α subdomain after dialysis.

Figure 3

Crystals of Bt-Lon α subdomain obtained in (a) 0.2 M sodium chloride, 0.1 M sodium/potassium phosphate pH 6.2, 10% (w/v) polyethylene glycol 8000, (b) 0.1 M *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) pH 9.5, 20% (w/v) polyethylene glycol 8000, (c) 0.1 M CHES pH 9.5, 30% (w/v) polyethylene glycol 3000, (d) 0.2 M sodium chloride, 0.1 M *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) pH 10.5, 20% (w/v) polyethylene glycol 8000 by the sitting-drop vapour-diffusion method.

Table 1Data-collection statistics for Bt-Lon α subdomain crystals.

Values in parentheses are for the outermost resolution shell.

X-ray source	BL13C1, NSRRC, Taiwan
Wavelength (Å)	0.97622
Detector	ADSC Q315r
Data-collection temperature (K)	77
Crystal-to-detector distance (mm)	350
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	60
Resolution range (Å)	29.82–2.88 (2.98–2.88)
Space group	<i>P</i> 23
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = <i>c</i> = 94.28
Mosaicity (°)	0.301
Total No. of measured intensities	133457 (14102)
Unique reflections	6542 (641)
Multiplicity	20.4 (22.0)
Completeness (%)	99.8 (100.0)
$R_{\text{merge}}^{\dagger}$ (%)	6.6 (50.4)
Average $I/\sigma(I)$	38.0 (9.0)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ are the intensities of the individual replicates of a given reflection hkl and $\langle I(hkl) \rangle$ is the average intensity over all replicates of that reflection.

into mother liquor containing 20% (v/v) glycerol as a cryoprotectant for 1 min, mounted in a 0.2–0.3 mm nylon loop (Hampton Research) and then flash-cooled in liquid nitrogen to 100 K. Diffraction images were indexed, integrated and scaled using *DENZO* and *SCALE-PAK* from the *HKL-2000* program suite (Otwinowski & Minor, 1997). On the basis of the diffraction pattern (Fig. 4), the Bt-Lon α subdomain crystal belonged to the cubic space group *P*23, with unit-cell parameters $a = b = c = 94.28$ Å. Assuming the presence of two monomers of 14 kDa protein in the asymmetric unit, the calculated Matthews coefficient (V_M ; Matthews, 1968) was $2.49 \text{ Å}^3 \text{ Da}^{-1}$, with a solvent content of 50.69%. A complete data set was obtained to

Figure 4

Typical diffraction image obtained from crystals of the Bt-Lon α subdomain collected at a synchrotron-radiation source (beamline 13C1, NSRRC, Taiwan). Diffraction extends to 2.88 Å resolution.

2.88 Å resolution, corresponding to an R_{merge} of 6.6%. Details of the data-collection statistics are summarized in Table 1.

3. Results and discussion

Structure determination was attempted by the molecular-replacement method as implemented in the program *Phaser* (McCoy *et al.*, 2007) using a theoretical Bt-Lon α subdomain model derived from the *Bacillus subtilis* Lon protease structure (PDB entry 3m6a; Duman & Löwe, 2010) as a model. Preliminary structure refinement using *REFMAC5* (Murshudov *et al.*, 2011) and *CNS* (Brünger *et al.*, 1998) resulted in a model with an R_{work} and an R_{free} of 31.39 and 32.96%, respectively. Further model building and structural refinement are currently in progress. Finally, in an attempt to understand how the α subdomain recognizes DNA, cocrystallization experiments of the Bt-Lon α subdomain with different dsDNA sequences are under way.

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