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DNA-binding specificity of the Lon protease α-domain from *Brevibacillus thermoruber* WR-249

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ABSTRACT

Lon protease has been well studied in many aspects; however, the DNA-binding specificity of Lon in prokaryotes has not been clearly identified. Here we examined the DNA-binding activity of Lon protease α-domains from *Brevibacillus thermoruber* (Bt), *Bacillus subtilis* (Bs), and *Escherichia coli* (Ec). MALDI-TOF mass spectroscopy showed that the α-domain from Bt-Lon binds to the duplex nucleotide sequence 5′-CTGTTAGCGGGC-3′ (ms1) and protected it from DNase I digestion. Surface plasmon resonance showed that the Bt-Lon α-domain binds with ms1 double-stranded DNA tighter than Bs- and Ec-Lon α-domains, whereas the Bt-Lon α-domain has dramatically lower affinity for double-stranded DNA with 0 and 50% identity to the ms1 binding sequence. Our results indicated that Bt-Lon α-domain plays a critical role with ms1 sequence in the DNA-binding specificity.

Introduction

The Lon protease, also known as CapR or La protease, is the first ATP-dependent proteolytic enzyme discovered [1], which is the most widespread family in prokaryotes and eukaryotic organelles with multiple functions including ATPase, protease and DNA-binding activities and has been classified as a member of the AAA^+ (ATPases associated with diverse cellular activities) superfamily [2,3]. For DNA-binding activity assay, the human Lon binds preferentially to a specific TG-rich single-stranded sequence and G-quartet DNA [4,5], but the interaction between other Lon proteins and DNA has generally been assumed to be nonspecific [6,7]. Since the entire Lon protease structure has not been determined, several studies have identified the structure–function relationship of the activities of protease, ATPase and chaperone with correlative domains [8–10]. Despite these extensive studies, little is known about the mechanism of the Lon and DNA interaction to date.

In our previous study, we constructed several truncated mutants of the Lon protein from *Brevibacillus thermoruber* WR-249 (Bt-Lon) and demonstrated that the α-domain, the sub-domain of ATPase domain of Bt-Lon, is the only domain involved in DNA binding [9]. Here, we used DNase I digestion combined with MALDI-TOF mass spectroscopy to identify the α-domain bound DNA sequence and used surface plasmon resonance (SPR) to verify the interactions of DNA with the α-domains of Bt-Lon, *Bacillus subtilis* Lon (Bs-Lon), and *Escherichia coli* Lon (Ec-Lon).

Materials and methods

Subclone, over-expression and purification of the α-domains. The preparation of Bs-, Bt- and Ec-Lon α-domains are described under Supplementary data. The primers used are listed in Table 1. The protein concentration was determined using the Bradford method (Bio-Rad), and the homogeneity of the purified α-domain proteins was analyzed by SDS–PAGE.

Circular dichroism. Circular dichroism spectra were recorded on a JASCO J-715 spectropolarimeter (Jasco, Japan) as described previously ([11] see Supplementary data).

Identification of the Bt-Lon α-domain DNA-binding sequence. For simplicity purposes, pET28a(+) plasmid DNA was used as the binding substrate of the Bt-Lon α-domain instead of the whole genome from *Br. thermoruber*. The interaction between α-domain and pET28a(+) plasmid was confirmed by gel mobility shift assay (see Supplementary data). DNase I was added to the α-domain-pET28a(+) complex and incubated for 2 min at 25 °C. The reaction was terminated by adding 1% SDS, 200 mM NaCl, 20 mM EDTA pH

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Table 1
Oligonucleotides used in this study.

Primers for cloning	
Bs-α-F	5′-CATATGGCAGGCTACACAGAAATAGAAAAACTTG-3′
Bs-α-R	5′-TTCAGCCTCGAGATATCTGAAAATGCGTTTTCCGAT-3′
Bt-α-F	5′-GGAGATATACATATGGGCGACTACACCGAG-3′
Bt-α-R	5′-CTCAGCCTCGAGGTAGCGATAGCGCGGTTTGCCCAG-3′
Ec-α-F	5′-GATCATATGAGCGGTTATACCGAAGATGAAAAACTG-3′
Ec-α-R	5′-CAGCAAGTCCTCGAGCACTTCCGTCCACGCCAGACC-3′
Hairpin DNA for SPR	
hp-ms1	5′-Biotin-CCTGTTAGCGGGCTTTTGCCCGCTAACAGG-3′
hp-ms2	5′-Biotin-CCCGCTGGTGAGTTTTTACTCACCAGCGGG-3′
hp-ms3	5′-Biotin-CTCACCGATAAATTTTTATTTATCGGTGAG-3′

8.0. Undigested DNA was extracted by phenol–chloroform method and re-dissolved in matrix solution [50 ng mL^{-1} 3-hydropicolinic acid in water/acetonitrile/50 mg mL^{-1} diammonium citrate (4:5:1 by vol)] then spotted onto a 96-well Teflon sample plate. MALDI-TOF-MS were acquired using a reflectron TOF mass spectrometer (Voyager-DE Pro, Applied Biosystems). Measurements were taken in linear, negative ion mode at 20 kV acceleration voltage and 380 nm delayed ion extraction. The DNA sequence was determined by MALDI-TOF mass combined with exonuclease III digestion pretreatment as described previously [12]. Known oligonucleotide primers were added as internal standards for mass calibration.

SPR measurements. The experiments were performed in a Biacore T100 (GE Healthcare) using Biacore control software, with a constant flow rate of 30 μl min^{-1} at 25 °C. The 5′-biotin labeled hairpin DNA ligand (Table 1, see Supplementary data) was immobilized on the surface of flow cell 2 of a SA chip by non-covalent biotin–streptavidin interaction. The α-domain protein concentration in the binding assay ranged from 10 to 100 nM. During each binding cycle, analyte protein was injected for 120 s and dissociation was monitored for 120 s. The analyte injection was followed by an additional wash command with HBS-P buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% surfactant P20) for another 120 s. The binding surface was regenerated with 0.5 M NaCl in HBS-P after each injection. Specific response was obtained by subtracting the blank flow cell 1 response from the flow cell 2 response. Biacore T100 Evaluation Software was used to fit the binding kinetics, and the processed data were fit to a simple Langmuir 1:1 binding model. The R_{max} was fitted locally, with the RI set to zero.

Homology model. The homology model of the Bt-Lon α-domain was built using the modeler module of Insight II. Averaged structure of the Bs-Lon α-domain and crystal structure of the Ec-Lon α-domain were selected as the templates and their sequences were aligned based on the known 3-D structures. Structurally conserved regions of the Bt-Lon α-domain were then generated according to the multiple sequence alignment. Atomic coordinates were transformed from the template structures, and loops were constructed using the automated methodology of the modeler module, which simultaneously satisfies spatial restraints and local molecular geometry. The homology model of the Bt-Lon α-domain was further refined by calculating the energy minimization using the steepest descent method with the force-field Discover CVFF (consistent valence force field).

Results and discussion

Br. thermoruber, B. subtilis, and E. coli Lon α-domains

Although the structure and function of the α-domain from the AAA^+ superfamily proteins have been studied extensively, the data obtained are not always applicable to Lon protease because of the diversity in the function compared with other protein members of the AAA^+ superfamily, despite the similarities in the structures [13]. Based on α-domain sequence alignment of AAA^+ superfamily proteins, arginine is conserved only in the sensor-2 residue [14]. However, an alignment of the α-domains from Lon proteases more specifically revealed that most arginine and lysine residues are comparatively conserved (Fig. 1A), even though the Bt-Lon α-domain sequence is only 57.5 and 48.2% identical to those of the Bs-Lon and Ec-Lon α-domains, respectively. For protein purification, SDS–PAGE analysis indicated that approximately 12-kDa proteins were >95% pure (Fig. 1B). The characteristics of the far-UV CD spectra of Bs-Lon, Bt-Lon, and Ec-Lon α-domains were very similar (Supplementary Fig. S1A). A negative band with two minima at 209 and 222 nm was observed. Based on the CD spectra analyzed by the popular programs: CONTIN, SELCON and CDSSTR [15], the estimation for these α-domains proteins showed mainly α-helical secondary structure. Close melting temperature (T_m) of the Bs-Lon, Bt-Lon, and Ec-Lon α-domains (53, 53, and 51 °C, respectively; Supplementary Fig. S1B) implied that the main tertiary structure of the three α-domains is assumed to be similar, as shown in the result of homology modeling (Fig. 1C). To gain insight into the DNA-binding characteristics of the α-domains, we compared the ribbon and surface structures of the three α-domains. Those arginine and lysine residues were not only conserved in the sequence alignment but also clustered together to form a positively charged region on the surface (Fig. 1C). This positively charged region may play an important role in DNA binding [16].

Bt-Lon α-domain preferred DNA sequence

In gel mobility shift assay, all three α-domains bound to DNA, as indicated by the slower mobility of linear pET28a(+) when mixed with increasing amounts of Bs-Lon, Bt-Lon, or Ec-Lon α-domain protein compared to linear pET28a(+) alone (Supplementary Fig. S2A). The Bs-Lon and Bt-Lon α-domains retarded the DNA mobility more than the Ec-Lon α-domain; BSA, the negative control, did not affect DNA mobility. Similar results were obtained when supercoiled pET28a(+) DNA was used (Supplementary Fig. S2B). The different degree of the retardation was not due to the secondary or tertiary structures since their CD spectra were indistinguishable. It suggests that the retardation of DNA migration was influenced by the distribution of polar residues on the protein surface.

To identify the specific binding sequence, the DNA fragments protected from digestion by the Bt-Lon α-domain were purified and analyzed by MALDI-TOF mass. Two single-stranded DNA molecules (m/z 3677.7 and 3615.5) which correspond to the complementary strands of the protected fragment were detected (Fig. 2A). Based on the molecular masses, each strand of the fragment contains 12 nucleotides. After exonuclease III digestion, cation-exchange resin purification and analysis by MALDI-TOF mass, the mass differences between each peak and its adjacent peak were evaluated, and the four bases of the allele were identified as 3′-CGGG (Fig. 2B). Based on the molecular masses of intact ions and partial sequences, we identified the candidate sequence as 5′-CTGTTAGCGGGC-3′ from pET28a plasmid DNA sequence map and labeled it ms1. In addition, another ionized peak (m/z = 3615.5) can be matched to the complementary sequence 5′-GCCCGCTAACAG-3′. In earlier Lon experimental reports, the use of long DNA sequences, e.g., polynucleotides of 1000 bases, calf thymus DNA, or plasmid DNA, probably caused the non-specific interactions and inconsistent results observed [6,17]. Therefore, a short DNA fragment containing only the binding site is more suitable for a binding study because additional flanked DNA might increase nonspecific protein–DNA interactions [18]. Consequently, we used this short, double-stranded dodecamer: ms1 DNA and its derivatives as the sequence-specific binding target in our SPR experiment.

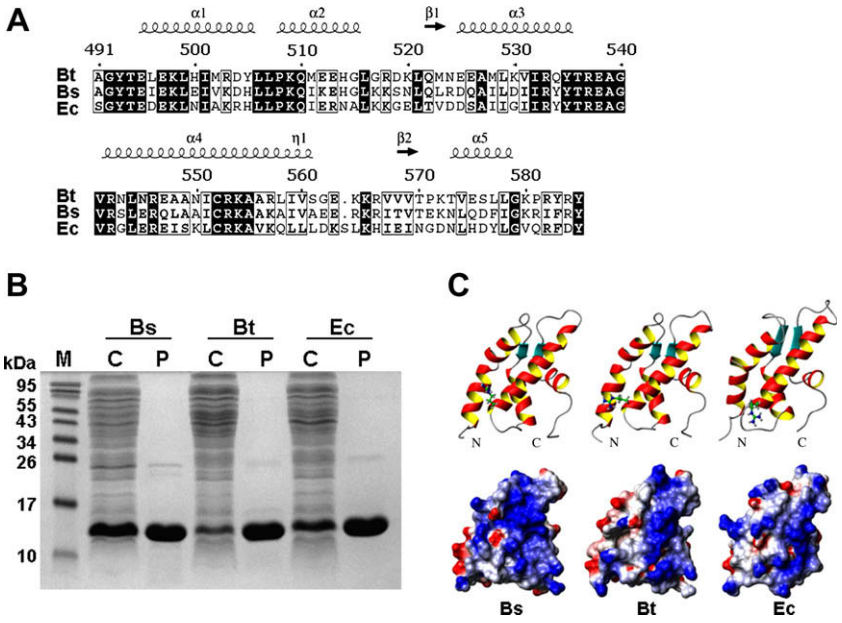

Fig. 1. (A–C) Comparison of Lon protease α-domains from *B. subtilis* (Bs), *Br. thermoruber* (Bt), and *E. coli* (Ec). (A) The amino acid sequence alignment was generated using the programs CLUSTAL-W and ESPript. Original sequences used in the analysis (GenBank/EMBL accession numbers): Bs, residues 490–584 (X76242); Bt, residues 491–585 (AY197372); and Ec, residues 490–585 (J03896). Identical residues in all three sequences are shown in white letters with a black background, and conserved residues are boxed. (B) SDS–PAGE of purified recombinant α-domains stained by Coomassie Blue. M, protein markers; C, crude extract from cell lysate; P, His-tagged α-domain purified from crude extract by Ni–NTA affinity agarose column (predicted molecular mass Bs: 12,318 Da, Bt: 12,267 Da, Ec: 12,292 Da). (C) Comparison of the surface structures of the Lon protease α-domains: Bs, PDB code 1X37; Bt, homology model; and Ec, PDB code 1QZM. Positive and negative charges are shown in blue and red, respectively.

Kinetics of the α-domain and DNA interaction

Synthetic palindromic oligonucleotide can be used to form a hairpin DNA to mimic the short double-stranded DNA for the SPR assay [19]. The oligonucleotide with ms1 sequence invert repeat was separated by four bases of thymine, labeled hp-ms1 (Table 1). Extra cytosine and guanine in the flanking sequences increase the stability of the hairpin. Four thymine bases in the center of the oligonucleotide with very low concentration (1 nM) for annealing decrease the probability of inter-oligonucleotide dimer formation (Supplementary Fig. S3) [20]. Constant baseline observed indicated that the immobilized hairpin DNA on the SA chip was very stable. Fig. 3A–C displays a typical set of sensorgrams presenting the different concentrations of each α-domain binding to hp-ms1. Previous studies demonstrated the α-domain remains monomeric in solution [9,21], therefore the SPR results of Bs-Lon and Bt-Lon α-domain were fitted by the Langmuir 1:1 binding model. The kinetic data indicated that the responses during the association are concentration dependent and the dissociation is slow, which results in a high affinity complex (Fig. 3A and B). The K_D of the Bt-Lon α-domain and hp-ms1 was 13.5 nM (Table 2). The K_D of the Bs-Lon α-domain and hp-ms1 DNA was 2.8-fold lower. Although the Ec-Lon α-domain interacted with hp-ms1, the K_D could not be well determined because the interaction was weak (Fig. 3C). Moreover, the slow dissociation rate reveals that the wild-type Bt-Lon has also high affinity to hp-ms1 and the lower R_{max} may be due to the steric hindrance of the bulky Bt-Lon protease (Fig. 3D).

DNA sequence specificity of Bt-Lon α-domain binding

We exchanged adenine and guanine, and cytosine and thymine to determine sequence specificity of Bt-Lon α-domain binding to DNA. The molecules designed had 50% (hp-ms2) and 0% (hp-ms3) identity to hp-ms1. Since the three hairpin DNA molecules have very similar molecular masses and similar surface immobilization levels, the capacity of the three different DNA molecules to bind the α-domain on the chip is expected to be approximate. The sensorgrams of Bt-Lon α-domain binding to each of the three different DNA molecules (Fig. 3B, E, and F) reveals that both hp-ms2 and hp-ms3 had lower association rates and higher dissociation rates than hp-ms1. The K_D of Bt-Lon α-domain binding with hp-ms2 and hp-ms3 displayed a 139- and 1910-fold loss in affinity, respectively. This distinct difference of K_D indicated that binding between the Bt-Lon α-domain and ms1 DNA is indeed sequence specific (Table 2).

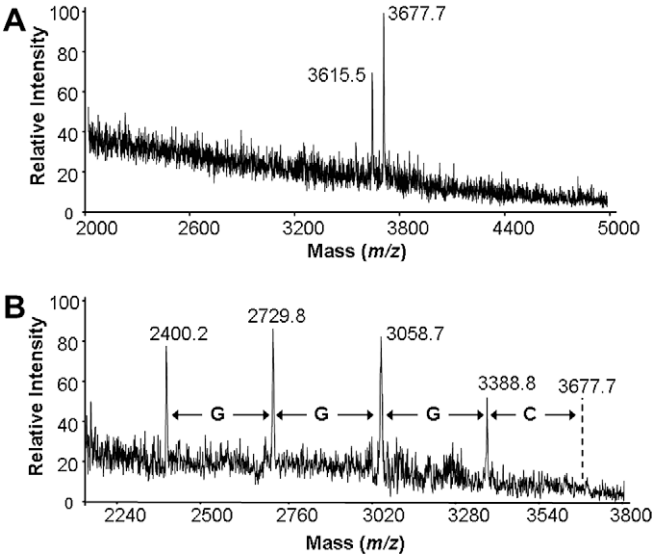

Fig. 2. (A,B) MALDI-TOF mass spectroscopy of Bt-Lon α-domain binding to DNA. Purified DNA fragments were mixed with 3-hydroxypicolinic acid and analyzed by MALDI-TOF mass spectroscopy. (A) The intact molecular mass of two complementary strands of one double-stranded DNA fragment. (B) Mass spectral data obtained on ion *m/z* = 3677.7 following digestion with exonuclease III showing the molecular mass intervals of the sequence (CGGG).

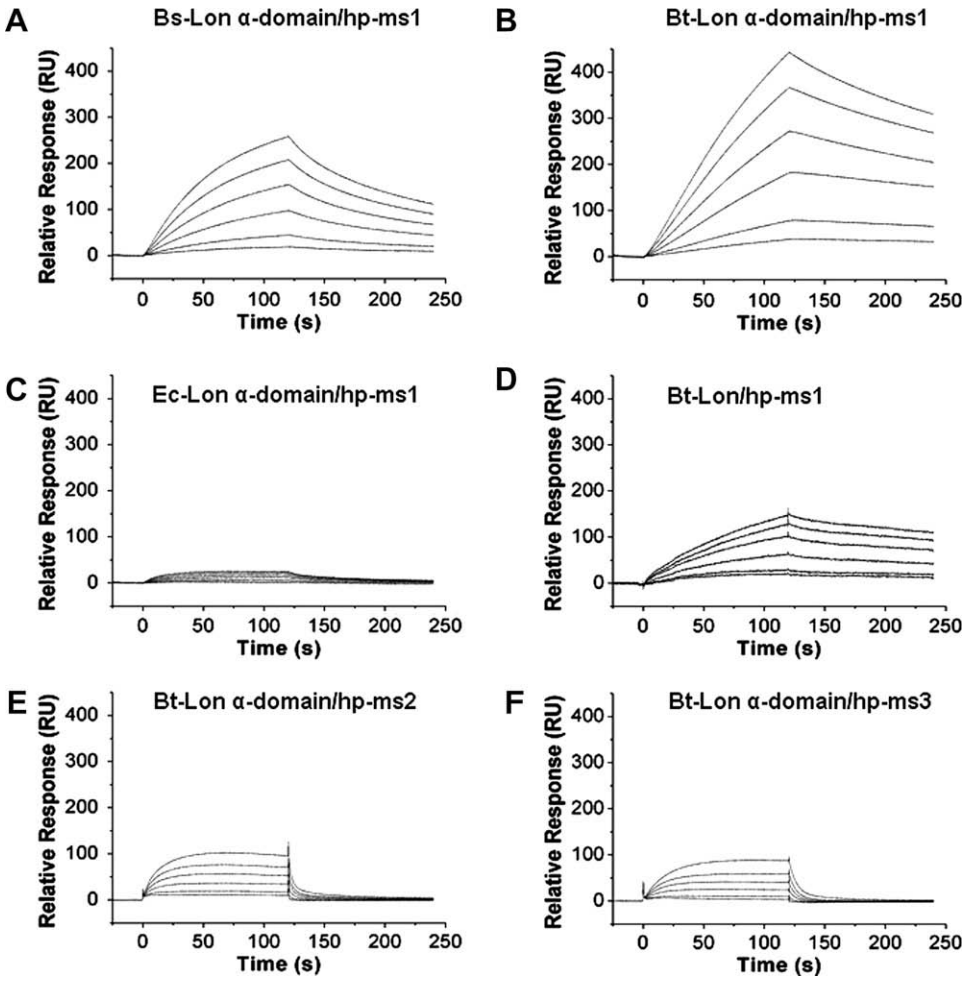

Fig. 3. (A–F) SPR sensorgrams of the binding of three α-domains or full-length Bt-Lon with hp-ms1, hp-ms2, or hp-ms3 DNA. The DNA-binding activity is given in response units (RU). (A) Bs-Lon α-domain/hp-ms1, (B) Bt-Lon α-domain/hp-ms1, (C) Ec-Lon α-domain/hp-ms1, (D) Bt-Lon/hp-ms1, (E) Bt-Lon α-domain/hp-ms2 and (F) Bt-Lon α-domain/hp-ms3. Each α-domain or full-length Bt-Lon protein was injected over the sensor chip at concentrations ranging from 10 to 100 nM.

In addition to degrading the misfolded proteins for protein quality control, Lon also plays an important role in regulating many biological processes in bacteria [22]. Some regulatory proteins which belong to DNA binding proteins were identified as Lon substrates [23]. Since the human LON can bind specifically to the critical regulatory region of mitochondria DNA and play an important role in the control of mitochondria gene regulation or DNA replication [4,24], it suggest that the bacteria Lon may have the same mechanism for action in the physiological regulation. When the Lon protease searches for its natural substrates, such as transcription factors for degradation, the DNA-specific binding process may help the Lon protease to localize and get close to the protein substrate for further proteolysis.

In conclusion, this work demonstrates that Bt-Lon α-domain binds specifically to a double-stranded oligonucleotide ms1: 5′-CTGTTAGCGGGC-3′ which was identified from pET28a(+) plasmid. The binding ability of full-length Bt-Lon to ms1 was also confirmed by SPR. Although *Br. thermoruber* WR-249 does not have this particular plasmid, it suggests that the Bt-Lon may bind to similar sequence sites to ms1 in its genomic DNA. Since all three α-domains from different bacteria retarded the mobility of plasmid or even interacted with sequence-specific oligonucleotide, we therefore concluded that the DNA binding ability of Lon through α-domain may exist widespread among all bacteria. The physiological function of the DNA-specific binding of Lon is still unclear since the α-domain is a sub-domain of ATPase domain without any independent enzy-

Table 2
Kinetic constants for the interaction of the α-domains from *B. subtilis* (Bs), *Br. thermoruber* (Bt), and *E. coli* (Ec) with DNA.

	K_{ass} (M^{-1} S^{-1})	Fold decrease[a]	K_{diss}(S^{-1})	Fold decrease	K_D	Fold decrease
Each α-domain and hp-ms1						
Bs-Lon α-domain	5.21×10^5	6.6	1.95×10^{-2}	2.4	37.4 nM	2.8
Bt-Lon α-domain	3.42×10^6	1	4.61×10^{-2}	1	13.5 nM	1
Ec-Lon α-domain	ND[b]	–	ND	–	ND	–
Bt-Lon α-domain and other DNA						
hp-ms2	5.35 × 104	63.9	1.00×10^{-1}	0.5	1.87 μM	139
hp-ms3	3.50×10^4	97.7	9.01×10^{-1}	0.05	25.7 μM	1910

[a] Fold decrease is given with respect to the Bt-Lon α-domain/hp-ms1 interaction.
[b] ND, not determined because binding was too weak to calculate the constants (raw data sensorgrams shown in Fig. 3C).

matic activities. Structural studies of the Bt-Lon α-domain and duplex ms1 complex are currently underway. Further experiments to characterize the functions of Bt-Lon binding by ms1 DNA will provide the physiological significance of this protease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.07.118.

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