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The N-terminal sequence after residue 247 plays an important role in structure and function of Lon protease from *Brevibacillus thermoruber* WR-249

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ABSTRACT

Previous studies on the N-terminal domain of Lon proteases have not clearly identified its function. Here we constructed randomly chosen N-terminal-truncated mutants of the Lon protease from *Brevibacillus thermoruber* WR-249 to elucidate the structure–function relationship of this domain. Mutants lacking amino acids from 1 to 247 of N terminus retained significant peptidase and ATPase activities, but lost ~90% of protease activity. Further truncation of the protein resulted in the loss of all three activities. Mutants lacking amino acids 246–259 or 248–256 also lost all activities and quaternary structure. Our results indicated that amino acids 248–256 (SEVDELRAQ) are important for the full function of the Lon protease.

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ATP-dependent protease Lon, consists of a variable N-terminal domain (N domain), a central ATPase domain (A domain), and a C-terminal protease domain (P domain) on a single polypeptide. The Lon protease was the first ATP-dependent protease from *Escherichia coli* (*Ec*-Lon) and most Lon studies have largely focused on all aspects of the *Ec*-Lon [1]. The sizes of each domain of various Lon proteases have been compared [2], and the domains of *Ec*-Lon have been identified [3–5]. Most studies of Lon function and structure have focused on the A and P domain [6–10]. N domain has not fully revealed its function due to few studies.

Previous reports indicated that the N domain is related to or involved in binding and recognition of substrate proteins [11–13]. Our previous study showed that the N-terminal domain is essential for oligomerization; failure of oligomerization can lead to the inactivation of the A and P domain functions [14]. The N domain is also thought to function in the discrimination and recognition of substrates and in domain–domain interactions [3,14,15]. Recently, the three-dimensional structure of the N-terminus of *Ec*-Lon was partially resolved [11], but the three-dimensional structure of a full-length Lon protease is lacking. On the basis of the partial structure, N domain appears to be a general protein- and polypeptide-interaction domain. In the present study, we investigated the role of the N domain of Lon protease from *Brevibacillus thermoruber* WR-249 (*Bt*-Lon) with constructed N-terminal truncated proteins, and identified nine amino acids (SEVDELRAQ; residues 248–256) in the N domain is essential for the full function and structure of *Bt*-Lon.

Materials and methods

Bacterial strains, enzymes, and chemicals. ECOS 101 [F (Φ80d lacZΔM15) Δ(lacZYA-argF)U169 hsdR17($r_k^-r_k^+$) recA1 endA1 relA1 deoRλ⁻], used in cloning experiments, and ECOS-21 [*E. coli* B F dcm, ompT, hsdS ($r_B^-m_B^-$), galλ (DE3)], used for expression, were purchased from ECOS (Taiwan). The substrates used for assays of ATPase, peptidase, and protease were purchased from Sigma: adenosine 5′-triphosphate (ATP), fluorogenic peptides, glutaryl-Ala-Ala-Phe-methoxynaphthylamide (Glt-AAF-MNA), and fluorescein isothiocyanate (FITC)-α-casein type I. The protein molecular weight standards for analytical gel filtration were purchased from Amersham Biosciences (GE Healthcare, USA). All other chemicals were analytical grade.

Construction of Bt-Lon mutants, protein production, purification, and activity assays. The mutants were constructed and then checked by DNA sequencing (see Supporting information). The genes encoding *Bt*-Lon and mutant proteins were over-expressed in *E. coli* strain BL-21. The proteins were purified as described previously [16]. Protein concentrations were determined using the Bradford method (Bio-Rad) and bovine serum albumin as the standard. The homogeneity of the purified proteins was analyzed by SDS–PAGE. ATPase, peptidase, and protease activities of *Bt*-Lon and mutant proteins were assayed as described previously [14]. The activities of *Bt*-Lon were considered as 100% activity; results given are the average of three experiments.

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Circular dichroism (CD) spectra of Bt-Lon, Δ246–259 and Δ248–256 mutants. CD spectra were recorded on a Jasco J-715 spectropolarimeter with 0.1 cm light path at protein concentrations of 1 μM (0.089 mg ml^{-1}) in 0.4 mM NaH_2PO_4, 0.08 mM KH_2PO_4, 0.12 mM KCl, 0.004 M NaCl, and 2% glycerol, pH 7.4. The far-UV CD spectra were the mean of three accumulations with a 1.0 nm bandwidth.

Analytical gel filtration chromatography. Purified proteins were applied to a Superose 10/300 GL column (GE Healthcare) equilibrated with 50 mM Tris–HCl (pH 8), 10 mM $MgCl_2$, 300 mM NaCl, 400 mM imidazole, and 10% glycerol; proteins were eluted at a flow rate of 0.3 ml min^{-1}. Blue dextran 2000 was used to determine the column void volume (V_0) and elution volumes (V_e) of the standards (GE Healthcare); the elution volumes were determined from the volume of the eluent at the point of application to the center of the elution peak detected at 280 nm. K_{av} values for the standards were calculated as $(V_e - V_0)/(V_t - V_0)$, where V_t is the total bead volume of the column. The standard curve was plotted as the logarithm of the molecular weight against the K_{av} of the standard proteins. The corresponding K_{av} for *Bt*-Lon and the mutant proteins Δ246–259 and Δ248–256 was calculated to determine their molecular weight from the calibration curve.

Results and discussion

The N domain of Bt-Lon dominates the enzyme's oligomeric state and influences ATPase, peptidase and protease activity

Previous sequence alignments of Lon proteases from various bacterial species have shown that this N domain is poorly conserved and of variable length [2,16], yet this domain is the most stable part of the Lon protease (*E. coli*), as shown by both limited proteolysis and autolysis [3–5]. For example, limited trypsin digestion of *Mycobacterium smegmatis* Lon (*Ms*-Lon) yields a stable N-terminal fragment, which indicates that this fragment of approximately 220 residues forms an independently folded domain [13]. Mutants of *Ms*-Lon lacking 90–225 N-terminal residues exhibit low-level peptidase activity, and a mutant lacking 277 N-terminal residues displays neither peptidase nor ATPase activity [13]. The previous studies have suggested that the N-terminal domain of Lon plays an important role in the full function of this protease.

In our earlier study, we showed that the N-terminal 316 amino acids of *Bt*-Lon play a role in oligomerization of the quaternary structure [14]. This fragment is, however, too large to determine which motif or which sequences form the key domain for the full function of Lon protease. In the present study, we therefore successively truncated the N-terminus of *Bt*-Lon (Δ1–129, Δ1–175, Δ1–219, Δ1–232, Δ1–247, Δ1–256, Δ1–265, and Δ1–279) and measured the ATPase, peptidase, and protease activities of the mutant proteins relative to those of full-length *Bt*-Lon (Fig. 1 and Table 1). Mutant proteins with deletions up to residue 247 had only <10% of the protease activity of full-length *Bt*-Lon, but the ATPase and peptidase activities of these mutant proteins were affected to a lesser extent. However, the larger deletions (Δ1–256, Δ1–265, and Δ1–279) led to loss of all activities. The quaternary structures of these mutant proteins also changed (dimer–trimer) as determined from analytical ultracentrifugation as compared with *Bt*-Lon (hexamer) (see Supporting information, Table S1). The results demonstrated that (1) region 1–247 of *Bt*-Lon probably is less involved in the ATPase and peptidase activities and these two activities can tolerate drastic changes in the structure, and (2) *Bt*-Lon requires residues 1–247 as the discriminator for specific substrates or as the initiator site to hold nonspecific substrates (α-casein as substrate in this case) with an affinity strong enough or a period long enough to activate ATP hydrolysis, which would cause a conformational change in *Bt*-Lon and make the active site accessible for subsequent hydrolysis of the protein substrate, a model proposed for Lon proteolysis by Goldberg and Waxman [17]. Our results on the protease activities of the truncated mutants of *Bt*-Lon are consistent with results reported for truncated mutants of *Ms*-Lon [13]. Although *Ec*-Lon mutant obtained by limited proteolytic digestion, lacking the N domain but still containing the A and P domain (235–784 residues), has significant protease activity against β-casein under several special conditions and in the prolonged incubation (about 16–24 h), AP fragment of *Ec*-Lon has no ability to hydrolyze β-casein under standard reaction conditions (such as in the presence of nucleotides) and in the relatively short time of incubation (about 2 h) [3].

The N-terminal sequence after residue 247 plays an important role in structure and function

Considering the above results and a postulation [11] derived from the crystal structure of the N-terminus of *Ec*-Lon, the N domain of *Bt*-Lon can be regarded as a general protein/polypeptide interaction domain that interacts with proteins targeted for degradation, i.e., the discriminator/initiator site is located at the N-terminus of *Bt*-Lon. Although Smith et al. [18] have shown that a sensor and substrate discrimination domain (SSD) is located in the α-domain of *Ec*-Lon and *Ec*-Clp family, we cannot rule out the possibility that the N domain of *Bt*-Lon plays a role in substrate discrimination. Interestingly, *Bt*-Lon mutants Δ1–256, Δ1–265, and Δ1–279 lacked ATPase, peptidase, and protease activities; the loss of activities by these constructs, in comparison with the forms lacking only the N-terminal sequences from 1–129 up to 1–247 confirms the importance of the fragment 248–256 for the activity of the enzyme. In addition, previous results indicated that coiled-coil regions in the N-terminus (residues 184–226 and 238–

```
1    MGERSGKREI PLLPLRGLLV YPSMVLHLDV GREKSVRALE QAMVDDNQIL
     ●
51   LATQEEVHIE EPSAEQIFSV GTVARVKQML KLPNGTIRVL VEGLQRARID
                                             129
101  EYIRQDDFFQ VSITYLEEEK ADENEVEALM RAVLSHFEQY IKLSKKISPE
                                            ▲
                                    175
151  ALTSVSDIEE PGRLADVIAS HLPLKMKDKQ EILETTNIKE RLNILLDILN
                                    ▲
                          219        232        247
201  NEREVLELER KISNRVKKQM ERTQKEYYLR EQMKAIQKEL GEKDGRQSEV
                          ▲          ▲          ▲
         256        265        279
251  DELRAQLEKS DAPERIKNKI EKELERLEKM PTTSAEGAVI RTYIDTLLSL
         ▲          ▲          ▲
                 316                                779
301  PWTRRTVDNL DIHHAE.... .......... .......... .....GETR
```

Fig. 1. N-terminal amino acid sequences of *Bt*-Lon (Accession No. AY197372) and each N-terminal truncated mutant was constructed from the beginning of Met (●) to the amino acid position pointed out by the symbol (▲) and numbered above each of the symbols.

Table 1
Relative ATPase, peptidase and protease activities of *Bt*-Lon and mutant proteins. The activities of *Bt*-Lon were set to 100%. Activities were measured in 50 mM Tris–HCl, pH 8, 1 mM ATP, and 10 mM $MgCl_2$ at 50 °C.

Enzyme/mutants	Activity (mean ± SD)		
	ATPase (%)	Peptidase (%)	Protease (%)
Bt-Lon	100 ± 2	100 ± 3	100 ± 5
Δ1–129	30 ± 5	78 ± 5	12 ± 3
Δ1–175	46 ± 4	81 ± 4	12 ± 2
Δ1–219	82 ± 4	112 ± 5	6 ± 2
Δ1–232	95 ± 4	130 ± 3	13 ± 3
Δ1–247	55 ± 3	80 ± 4	9 ± 2
Δ1–256	0	0	0
Δ1–265	0	0	0
Δ1–279	0	0	0
Δ246–259	0	0	0
Δ248–256	0	0	0

279) of *Bt*-Lon [16] might participate in the binding of Lon to proteins. Residues 248–256 are therefore probably part of the coiled-coil conformation, and these amino acids are probably involved in protein–protein interaction.

We tested this hypothesis by constructing two deletion mutants (Δ246–259, Δ248–256) within this sequence and assayed their activities (Table 1). Mutants Δ246–259 and Δ248–256 lost all activities. Moreover, these deletions slightly affected the protein conformation, as indicated by the CD spectra (Fig. 2), although the CD spectra revealed ellipticities at 222 nm, indicating that a helix was still the major component. To gain further insights into the differences in the structure of *Bt*-Lon and that of the two deletion mutants, we compared their quaternary structures using gel filtration chromatography (Fig. 3) and sedimentation velocity. The molecular weights were estimated to be 550 kDa (hexamer) for *Bt*-Lon and 180–190 kDa (dimer) for mutants Δ246–259 and Δ248–256. As compared with previous measured data [14], the predominant S value of *Bt*-Lon is shifted from 15S to 8.6S due to a different working condition (10% glycerol and 400 mM imidazole was added to buffer at this measurement for its stability and solubility in this case, see Supporting information, Fig. S1). In spite of the difference in working condition, the tendency in M_w based on size exclusion were consistent with those of sedimentation velocity, indicating the predominant peak of *Bt*-Lon was 8.6 S whereas it shifted to the interval of 1.5–4.0 S for two deletion mutants. These results indicated that deleting residues 248–256 of *Bt*-Lon not only changes its quaternary structure but also drastically affects all functions. These amino acids (SEVDELRAQ) therefore play a key role in the function and structure in subunit–subunit interactions.

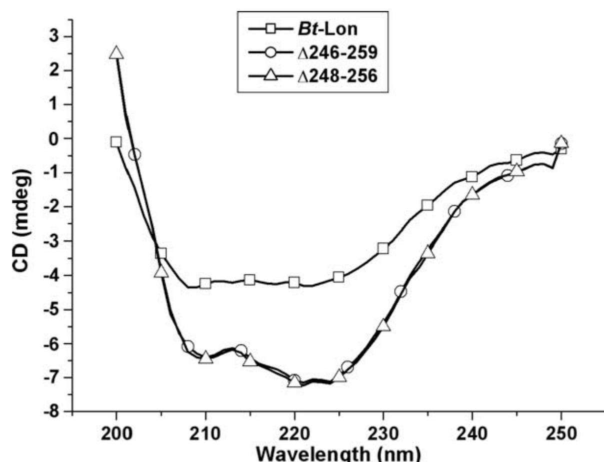

Fig. 2. Circular dichroism spectra of full-length *Bt*-Lon (□) and the deletion mutants Δ246–259 (○) and Δ248–256 (Δ) from 200 to 250 nm. Spectra were recorded on a Jasco-715 instrument at 25 °C. The far-UV CD spectra were the mean of three accumulations with a 0.1 cm light-path cell.

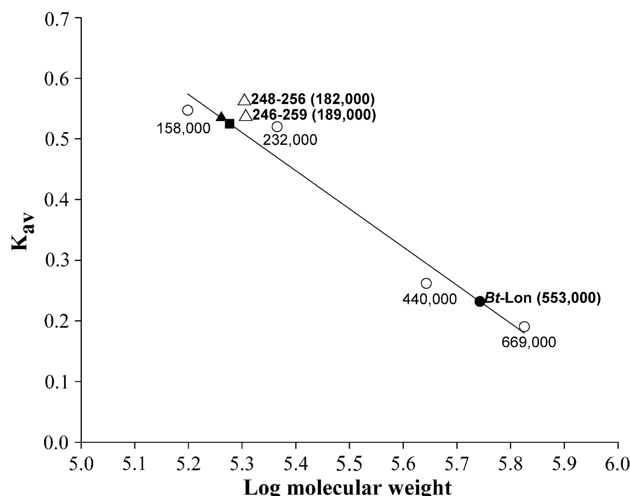

Fig. 3. Molecular weight of the *Bt*-Lon protease and mutants Δ246–259 and Δ248–256 estimated by analytical gel filtration. The standard calibration curve was obtained by plotting the K_{av} value of each protein standard (○) against the corresponding log standard molecular weight (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; and aldolase, 158 kDa). The molecular weights of *Bt*-Lon (●), mutant Δ246–259 (■), and mutant Δ248–256 (▲) were calculated from the standard curve based on their corresponding K_{av} values.

It has been reported that conformational changes in Lon holoenzyme induced by nucleotides or protein substrates modulate the functional activities through domain–domain interactions [15,19,20]. The domain–domain interactions of the *Bt*-Lon holoenzyme might be blocked by the deletion of 248-SEVDELRAQ-256. Although the partial structure of an N-terminal subdomain (residues 1–119) of *Ec*-Lon has been reported [11], the structure of residues 248–256 of *Bt*-Lon is unknown. We therefore cannot rule out the possibility that residues 248–256 play a role in domain–domain interactions within one subunit of *Bt*-Lon. However, our data revealed that these residues are crucial for the function and quaternary structure. The relationship between the quaternary structure and protease activity needs to be investigated further.

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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.2009.03.109.

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