

Binding and Cleavage of *E. coli* HU β by the *E. coli* Lon Protease

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ABSTRACT The *Escherichia coli* Lon protease degrades the *E. coli* DNA-binding protein HU β , but not the related protein HU α . Here we show that the Lon protease binds to both HU β and HU α , but selectively degrades only HU β in the presence of ATP. Mass spectrometry of HU β peptide fragments revealed that region K18-G22 is the preferred cleavage site, followed in preference by L36-K37. The preferred cleavage site was further refined to A20-A21 by constructing and testing mutant proteins; Lon degraded HU β -A20Q and HU β -A20D more slowly than HU β . We used optical tweezers to measure the rupture force between HU proteins and Lon; HU α , HU β , and HU β -A20D can bind to Lon, and in the presence of ATP, the rupture force between each of these proteins and Lon became weaker. Our results support a mechanism of Lon protease cleavage of HU proteins in at least three stages: binding of Lon with the HU protein (HU β , HU α , or HU β -A20D); hydrolysis of ATP by Lon to provide energy to loosen the binding to the HU protein and to allow an induced-fit conformational change; and specific cleavage of only HU β .

INTRODUCTION

The DNA-binding histone-like proteins HU α (HU2) and HU β (HU1) of *Escherichia coli* are encoded by two closely related genes, *hupA* and *hupB*, located at 90 and 9.8 min of the chromosome, respectively (1,2). The 90-amino-acid HU α and HU β proteins are 68.9% identical; the functional protein, HU, is isolated mainly as an $\alpha\beta$ heterodimer (3,4). HU is highly conserved among prokaryotes, but bacterial species other than *E. coli* and *Salmonella typhimurium* synthesize only one type of HU subunit, which forms a homodimer (5,6).

HU plays important pleiotropic roles in DNA replication (7), gene regulation (8,9), translation (10), DNA supercoiling (11,12), and other processes (13). A connection between HU and the ATP-dependent Lon protease is suggested by the role of both Lon and HU in the mucoid phenotype of *E. coli* (14,15). Lon is most likely involved in controlling gene expression in bacteria and yeast, either by regulating the levels of transcription factors (16,17) or by influencing the structural stability of genomic DNA (18,19). A *lon* mutation has a pleiotropic phenotype: ultraviolet sensitivity, mucoidy, deficiency for lysogenization by bacteriophage λ and P1, and lower efficiency in the degradation of abnormal proteins (15,20–24). Based on the results of in vivo experiments, it has been suggested that the regulation of HU in *E. coli* is Lon-dependent and that HU is degraded by Lon or a Lon-dependent protease (25).

Lon protease (EC 3.4.21.53) is a member of the AAA⁺ superfamily (ATPases associated with diverse cellular activities). This superfamily is characterized by a conserved segment of 220–250 amino acids, referred to as the AAA domain or nucleotide binding domain, which contains several conserved motifs for ATP binding and hydrolysis. Lon and other known members of this superfamily, e.g., FtsH, ClpAP, ClpXP, ClpC, and HslIVU are all ATP-dependent proteases (26). Lon and FtsH protease carry both the ATPase and the proteolytic active sites within a single polypeptide chain. In the Clp family, these functions are encoded by separate polypeptide chains. Lon protease functions as a homooligomer, in which each subunit consists of three domains: the amino-terminal domain, possibly involved in substrate recognition and binding (27); the ATPase domain (A domain), containing the ATP-binding motif (28); and the carboxy-terminal proteolytic domain (P domain) (29). Like a molecular chaperone, Lon recognizes a broad range of proteins, both misfolded and properly folded, and mediates their turnover. Through the degradation of various specialized proteins, Lon protease also regulates a number of biological functions (30). These specialized proteins include the λ N protein (31,32), the SulA cell division regulator (33,34), the RcsA positive regulator of capsule synthesis (35), the F factor addiction system protein CcdA (36), and the DNA-binding protein HU (25).

We have previously cloned and investigated the functional domains of the Lon protease from *Brevibacillus thermoruber* (37,38). Here, we elucidate in detail the substrate recognition of the *E. coli* Lon protease in the known cleavage of HU β and the lack of cleavage of HU α (25).

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MATERIALS AND METHODS

Preparation of Lon and HU

Lon protease from *Br. thermoruber* (*Bt*-Lon) and *E. coli* (*Ec*-Lon) were prepared as described previously (37–39). We sequenced the prepared *Ec*-Lon and found an E269G mutation. Proteolysis activity was measured in a peptidase assay. *E. coli hupA* and *hupB* were cloned from the commercial *E. coli* strain ECOS 101 (Yeastern Biotech, Taipei, Taiwan, Republic of China). The amino acid sequences of isolated HU α and HU β were the same as those reported previously (National Center for Biotechnology Information accession numbers BAB38346 and BAB33917). The concentration of the protein in the pooled fractions was determined using the Bradford method (BioRad Laboratories, Hercules, CA).

Identification of the initial cleavage sites

E. coli HU β (8.4 μ g), *E. coli* HU α (11.3 μ g), and *Bacillus subtilis* HU (*Bs*-HU) (10.2 μ g) were each incubated at 37°C with *Ec*-Lon (15.2 μ g) and at 50°C with *Bt*-Lon (10 μ g) in buffer containing 25 mM Tris-HCl (pH 8.0), 75 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, 5 μ M ATP, and 10% glycerol in a total volume of 200 μ l. After incubation, the reaction was stopped by adding 500 μ l 95% acetonitrile/5% H₂O/0.1% trifluoroacetic acid. The reaction mixture was centrifuged at 16,060 \times *g* for 10 min. The supernatant was dried, and the residue was dissolved in 5% acetonitrile/95% H₂O/0.1% trifluoroacetic acid. The solution was then applied to a Waters C18 column (5 μ m particle size, 250 \times 4.6 mm), and eluted by reverse-phase high-performance liquid chromatography (1100 series, Agilent, Santa Clara, CA) with a gradient of acetonitrile (5–95% in 30 min). Fractions were collected and lyophilized. Peptides of HU proteins were identified using a nanoESI-Q-TOF mass spectrometer (MicroMass, Cary, NC) (40) with a peptide mass tolerance of 0.25 Da and a tandem mass spectroscopy ion mass tolerance of 0.25 Da with up to one missing cleavage.

Model construction of the homodimeric HU, *E. coli* HU β ₂

The amino acid sequences of *E. coli* HU α and HU β were used for BLAST analysis and then aligned with identified homologous proteins using the program CLUSTAL-W (41). The high-resolution structure of HU α (Protein Data Bank code 1MUL) (42), incomplete due to a lacking electron density map, was used to model the main-chain conformation of HU β . For HU α , the MODELER program of InsightII (Accelrys, San Diego, CA) was used to generate the missing loop (Arg58-Ile72) in the DNA-binding arms (43). This allowed us to obtain a 3-D structure of HU α with a complete internal loop region. The homodimeric proteins were then assembled by superimposing the monomeric model with the crystal structure of HU α . The structure with the lowest violation score and lowest energy was chosen as the candidate. The distribution of the backbone dihedral angles of the model was evaluated using a Ramachandran plot and the program PROCHECK (44). The ProStat module of InsightII was used to analyze the properties of bonds, angles, and torsions. The Profile-3D program was used to check the structure and sequence compatibility (45).

Circular dichroism spectra of HU β and HU β -A20D

E. coli HU β was incubated at 37°C with *Ec*-Lon in 25 mM Tris-HCl (pH 8.0), 75 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, and 10% glycerol in a total volume of 25 μ l. After incubation, the reaction was stopped by adding 200 μ l of the same buffer and freezing at –80°C. The samples were melted before Circular dichroism (CD) measurements. CD spectra were obtained with a J-715 spectropolarimeter (JASCO, Tokyo, Japan). The far-ultraviolet CD spectra were the mean of five accumulations with a 0.1-cm light path. The final concentrations of *Ec*-Lon, HU β , HU β -A20D, and ATP were 3.0 μ g ml^{–1}, 23 μ g ml^{–1}, 23 μ g ml^{–1}, and 0.4 μ M, respectively.

Optical tweezers

A schematic diagram of the optical tweezers setup is shown in Fig. S3 in the Supporting Material. A linearly polarized laser beam ($\lambda = 1064$ nm, 300 mW, Nd:YVO₄ cw laser, LeadLight Technology, Taoyuan, Taiwan, Republic of China) was used for optical trapping. A half-wave plate and a polarizer were used to control the trapping-laser power by rotating the half-wave plate. A beam expander was used to expand and collimate the beam such that the beam diameter (~1 cm) slightly overfilled the back aperture of the microscope objective. A telescope (telescope 1, consisting of two identical lenses, each with focal length $f = 150$ mm) was used to change the trapping-beam pathways to manipulate the trapped particle by moving the first lens (in telescope 1). A second laser beam (wavelength 632.8 nm, 5 mW, He-Ne cw laser, Uniphase, Ottawa, Ontario, Canada) provided a tracking beam to track the position of the trapped particle. A spatial filter/beam expander unit was used to expand and collimate the beam to a beam diameter of ~1 cm. A second telescope (telescope 2) was used to adjust the relative positions of the focal points of the two laser beams in the sample chamber. The two laser beams were combined by a dichroic mirror (DM1, SRR-25.4, Lambda Research Optics, Littleton, MA), which is highly reflective at 632.8 nm and highly transmissive at 1064 nm, and injected into the back aperture of an oil-immersion microscope objective (NA 1.24, 100 \times , working distance 0.13 mm, Olympus, Melville, NY). The tracking beam, scattered by and diffracted off the trapped particle, was collected by a condenser (NA 0.42, 50 \times , working distance 20.5 mm, Mitutoyo, Tokyo, Japan) and projected onto a quadrant photodiode (QPD; S7479, Hamamatsu, Hamamatsu City, Japan) to track the motion of the particle in the transverse plane. A notch filter was used to block the trapping beam (1064 nm) from entering the QPD. The electrical signals from the QPD were recorded by a data acquisition system, and wide-field images of the trapped particle were captured by a charge-coupled device camera (WAT-120N, Watec, Vienna, Austria) for optical alignment of the trap and for image observation and analysis.

Immobilized proteins on beads

Polystyrene beads (10 μ l) were washed with buffer containing 25 mM Tris-HCl (pH 8.0), 75 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, and 10% glycerol. Large polystyrene beads (12.3 μ m in diameter) were incubated with Lon protease at 4°C for 18 h. Small polystyrene beads (2.88 μ m in diameter) were incubated at 4°C for 18 h with HU α , HU β , HU β -A20D, or bovine serum albumin (BSA). After incubation, the beads were washed three times with 1 ml of the same buffer. The beads were then incubated with 1 ml BSA (10 mg/ml in phosphate-buffered saline) before measurement with optical tweezers. Beads with or without protein coating were coated with 20 nm gold and inspected by scanning electron microscopy (S-2700, Hitachi, Tokyo, Japan) (Fig. S6), using 5000 \times magnification for large beads and 15,000 \times for small beads.

Rupture force measurements

Optical tweezers were used to measure the strength of the interaction between HU proteins and Lon protease coated on polystyrene beads (Fig. S5). One bead (2.88 μ m in diameter) coated with an HU protein was trapped by the optical tweezers. Its position was tracked by QPD. Another bead (12.3 μ m in diameter) coated with the Lon protease and adhered to a coverglass was placed near the trapped bead (Fig. S8). At the beginning of each experiment, the small bead was trapped by optical tweezers at the beam axis, and the displacement of the trapped bead, deduced from the calibrated QPD output signal, was set to 0 μ m by fine adjustment of the QPD lateral position. The Lon-protease-coated bead on the coverglass was moved toward the trapped bead semicontinuously, at 37 nm/step/s, by a piezoelectric-transducer (PZT)-driven sample stage until the trapped bead was pushed away by ~60 nm from the original equilibrium position, as deduced from the precalibrated QPD signal. When the two beads touched, as shown in Fig. S8 A, the stage was stopped for 3 min to allow the enzyme to interact

with the proteins. Then, the PZT-driven sample stage was moved semicontinuously (at 37 nm/step/s) away from the HU-protein-coated bead (Fig. S8 B). If the two beads are bound by a protein-protein interaction, the small bead will be gradually pulled away from the trapping center by the larger bead. Since the optical force on the small bead is proportional to its distance from the trapping center (within a linear range on the order of 100 nm), the optical force increased as the displacement of the bead increased. If the trapping force exceeds the enzyme-substrate binding force, the two beads would be pulled apart, and the 2.88- μm bead coated with the HU proteins would bounce back to the trapping center (Fig. S8 C). The maximum displacement of the small bead was measured by QPD and converted into force using Hooke's law. Data of noninteracting cases were not used. Only those cases which showed clear rupture force were taken into account. The force as a function of the displacement of the larger bead (12.3 μm), coated with the Lon protease, is shown in Fig. S9. The three stages described above are illustrated schematically in the force-versus-position plot in Fig. S8 D. In stage B, the optical force increased as the large bead moved away from the small bead, until the force was sufficient to rupture the binding force between the two beads. The maximum force in the plot appears at the boundary between stages B and C, when the two beads are pulled apart (Fig. S8 D). The rupture force that pulled the two beads apart was equal to the maximum force of the force-versus-position plot. The system was computer-controlled (via LAB VIEW) to maintain stability and to allow reproducibility.

Substrate interference assay

Protease activity was measured as described previously (46). The substrate, fluorescein isothiocyanate (FITC)- α -casein (10 μg ; Sigma, St. Louis, MO), was incubated with Lon protease (4.6 μg) in 5 mM Tris-HCl (pH 8.0), 1 mM MgCl_2 , and 1 mM ATP, with $\sim 2.3 \times 10^{-9}$ mol of the competing substrate BSA, casein, HU α , HU β , or HU β A20D in 200 μl . After 1 h incubation at 37°C, 100 μl BSA was added. To precipitate proteins, 100 μl of 10% trichloroacetic acid was added and the solution was placed on ice for 10 min. The solution was then centrifuged, and the pellet was discarded. The pH of the solution was adjusted with 0.2 ml CHES-Na (0.5 M, pH 12.0). Activity was assayed by measuring the fluorescence of the solution (excitation 490 nm, emission 525 nm).

RESULTS

Ec-Lon selectively degrades *E. coli* HU β in the presence of ATP

E. coli HU exists mostly as an HU $\alpha\beta$ heterodimer, and sometimes as a homodimer, and the composition of the dimer varies during growth (47). Exponentially growing *E. coli* contains a mixture of HU α_2 and HU $\alpha\beta$, but not HU β_2 . This indicates that HU β_2 is unstable and might therefore be susceptible to protease processing. To determine whether Lon is the responsible protease, we cloned and expressed HU and Lon from *E. coli*. After incubation of *E. coli* HU α or HU β with *Ec*-Lon, the samples were denatured and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). *E. coli* Lon degraded HU β in the presence of ATP (Fig. 1 A), but it did not degrade HU α (Fig. 1 B), even after overnight incubation (not shown). This is consistent with a previous *in vivo* study showing that HU α is not degraded by Lon or Lon-dependent protease (25).

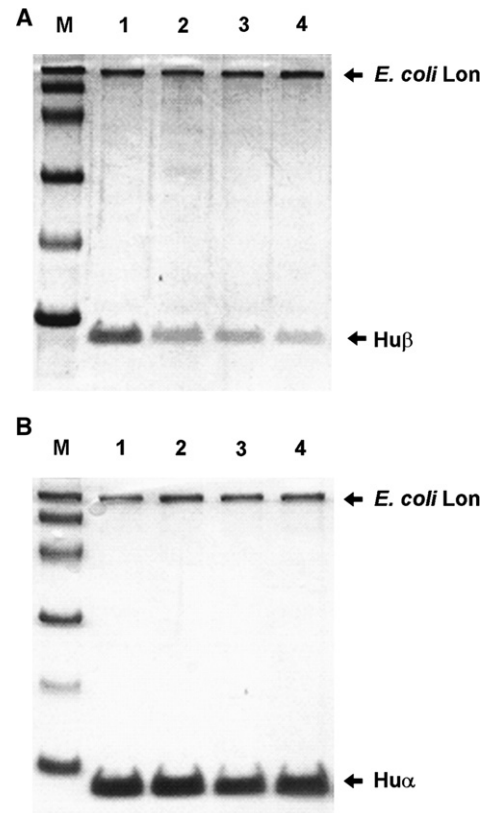


FIGURE 1 Analysis of the substrates of the *E. coli* Lon protease by SDS-PAGE. *E. coli* HU β (A) and *E. coli* HU α (B) were incubated with the Lon protease and 5 μM ATP for 0 (lane 1), 1 (lane 2), 2 (lane 3), and 3 h (lane 4). M, size markers (94, 66, 45, 30, 20, and 14 kDa). The reaction volume of the sample was 20 μl . The amount of Lon protease was 1 μg and that of the substrate HU β was ~ 3 μg .

Cleavage sites of the Lon proteases

After incubation of HU β with *Ec*-Lon protease and ATP for 30 min, the peptide fragments formed were separated by liquid chromatography and analyzed by nanoESI-Q-TOF mass spectrometry. One main peak at 793.86, 2+, was identified. Mass spectrometry of this peak yielded five major peaks (Fig. S3): 1*, 584.31; 2*, 655.34; 3*, 768.43; 4*, 881.52; and 5*, 952.57. Peaks of other peptide fragments were also observed. The mass spectra data were combined into a single-peak list file, which was searched using the program MASCOT. We found 29 peptides that matched; a significant hit with a Mowse score of 1189 indicated that HU β is a substrate of *Ec*-Lon. After 30, 10, and 5 min incubation of HU β with *Ec*-Lon protease and ATP, 21, 9, and 3 HU β peptide fragments, respectively, with a Mowse score >25 were formed (Table S1). The peptide fragment AGRALDAIIASVTESL (peptide 9, molecular weight 1585.80) was found in all reactions.

We identified two preferred cleavage sites and regions by aligning the peptide fragments K18-G22 (KAAAG) and L36-K37. The three peptide fragments formed after 5 min

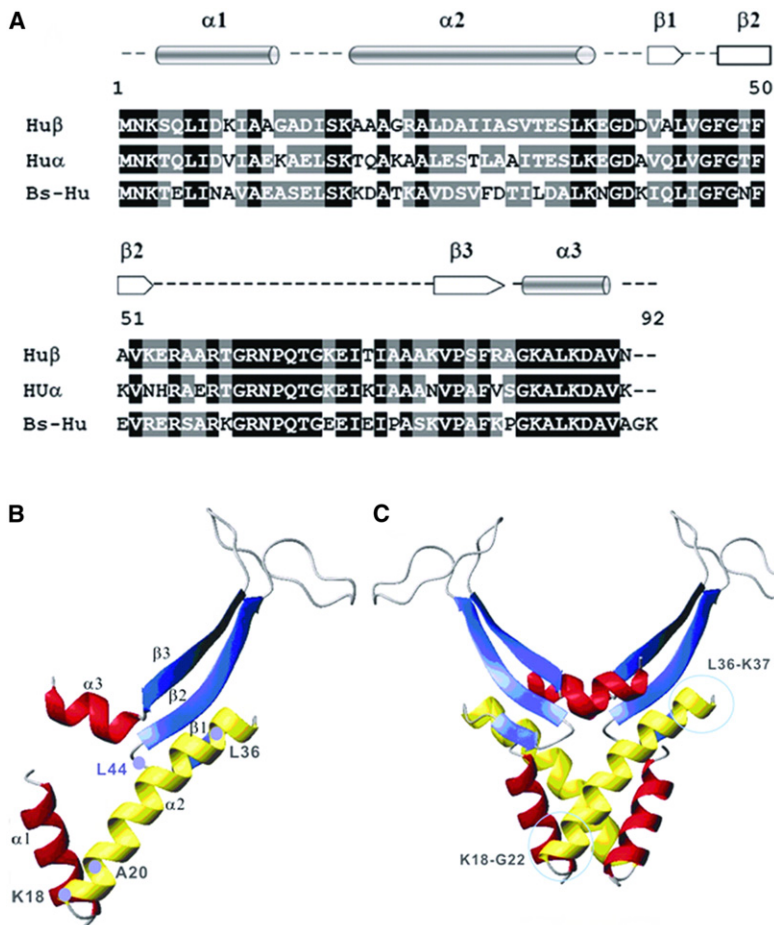


FIGURE 2 Structures of *E. coli* HU β , *E. coli* HU α , and *B. subtilis* HU. (A) Alignment of the sequences using Vector NTI Advance 9. Residues identical in all sequences are highlighted in black. Similar residues are highlighted in gray. The secondary structure of the sequence is shown above the primary sequence. (B) The tertiary structure of the *Ec*-HU β monomer composed of three α -helices and three β -sheets. Residues K18, A20, L36, and L44, which are part of the preferred cleavage sites, are indicated. The α 1-helix and the α 3-helix are shown in red, and the α 2-helix is shown in yellow. The β -sheets are shown in blue. (C) The quaternary structure of the HU β homodimer, which can be considered as the association of an α -subdomain and a β -subdomain (42). The DNA-binding domain is located in the β -subdomain. Peptide 9 is located in the α -subdomain.

incubation of *Ec*-Lon with HU β are cleaved at one or both of these sites. We therefore postulated that *Ec*-Lon preferentially cleaves at the C-terminal end of alanine or leucine, and cleavage occurs within region K18-G22 or region L36-K37 or both, at the beginning of the reaction.

***Ec*-Lon specificity**

We analyzed the specificity of *Ec*-Lon for HU β by comparing the amino acid sequences of *E. coli* HU α , *E. coli* HU β , and *Bs*-HU. HU α and HU β are 68.9% identical and 80% similar; HU α and *Bs*-HU are 56.5% identical and 67.4% similar, and HU β and *Bs*-HU are 52.2% identical and 75% similar. The sequence alignment of these three proteins (Fig. 2 A) reveals that they differ in amino acid residues 18–22, one of the preferred cleavage sites of *Ec*-Lon in HU β : HU β , KAAAG; HU α , KTQAK; and *Bs*-HU, KKDAT. HU α and *Bs*-HU, which are not degraded by *Ec*-Lon, lack the A-A bond cleaved by *Ec*-Lon in HU β .

Although HU α and *Bs*-HU contain the L36-K37 bond that is cleaved by *Ec*-Lon in HU β , *Ec*-Lon did not cleave either of these proteins, probably because of a difference between them and HU β somewhere else in the amino acid sequence. We analyzed this hypothesis by creating a homology-based model of HU β (Fig. 2 B). The structure of HU α (PDB

code 1MUL) (42) was used to model the main-chain conformation of HU β . The HU β peptide fragment A21GRALDAIIASVTESL36, which is formed by cleavage at both of the preferred cleavage sites, comprises the main part of the α 2-helix, with A20-A21 and L36-K37 at the N- and C-terminal ends, respectively, of this helix. The sequence between G13 and K18 is a loop that links the α 1- and α 2-helices. In the HU α monomer, the tertiary fold is stabilized by intrahelical salt bridges between K22 and E26 (42). In HU β , however, these amino acid residues differ, and G22 cannot interact with D26. Therefore, the α 2-helix of HU β is weaker than that of HU α . This might explain why *Ec*-Lon can degrade HU β , but not HU α .

The main *Ec*-Lon cleavage site in HU β

We further tested the main *Ec*-Lon cleavage site at A20-A21 by constructing HU β proteins with an A20Q or an A20D mutation. After incubation of HU β or HU β -A20Q with *Ec*-Lon and ATP for 7 h, SDS-PAGE analysis of the products showed that HU β was completely degraded and that the same concentration of HU β -A20Q was degraded only to a small extent (Fig. S4). The minor bands that appeared are only Lon protease autodegradation products (48). These results indicated that the A20Q mutation reduced the

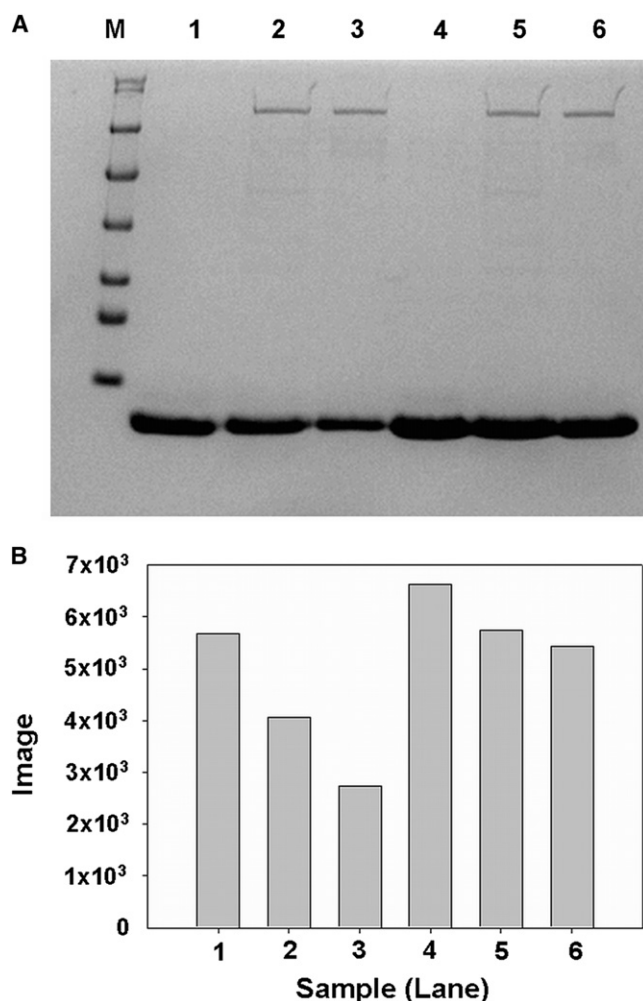


FIGURE 3 Importance of residue 20 in the cleavage of HU β by Lon. (A) HU β -A20D (0.08 mg ml⁻¹) and HU β (0.08 mg ml⁻¹) were each incubated with *Ec*-Lon (0.1 mg ml⁻¹) with or without ATP (5 μ M) for 6 h. Samples were then denatured by heating and analyzed by SDS-PAGE. Amounts loaded were 3 μ g HU β , 3 μ g HU β -A20D, and 1 μ g *Ec*-Lon. *M*, Size markers (116, 66, 45, 35, 25, 18.4, and 14.4 kDa); lane 1, HU β ; lane 2, *Ec*-Lon and HU β without ATP; lane 3, *Ec*-Lon and HU β with ATP; lane 4, HU β -A20D; lane 5, *Ec*-Lon and HU β -A20D without ATP; and lane 6, *Ec*-Lon and HU β -A20D with ATP. (B) The bands of HU β and HU β -A20D were analyzed by IQuant software (Molecular Dynamics).

degradation rate of HU β by *Ec*-Lon. We also analyzed the reaction of *Ec*-Lon and HU β , as well as that of *Ec*-Lon and HU β -A20D, using SDS-PAGE (Fig. 3) and CD spectrometry (Fig. 4). SDS-PAGE indicated that HU β -A20D is resistant to degradation by *Ec*-Lon (Fig. 3). The CD spectrum of *Ec*-Lon incubated with HU β revealed two negative peaks at 205 and 222 nm and was similar to the spectrum of HU β at the same concentration. After incubation of *Ec*-Lon, HU β , and ATP for 6 h, the spectrum showed that HU β was degraded by *Ec*-Lon, and a random coil signature was evident (Fig. 4 A). In contrast, after incubation of *Ec*-Lon, HU β -A20D, and ATP for 6 h, the spectrum indicated an α -helix as a major secondary structure (Fig. 4 B),

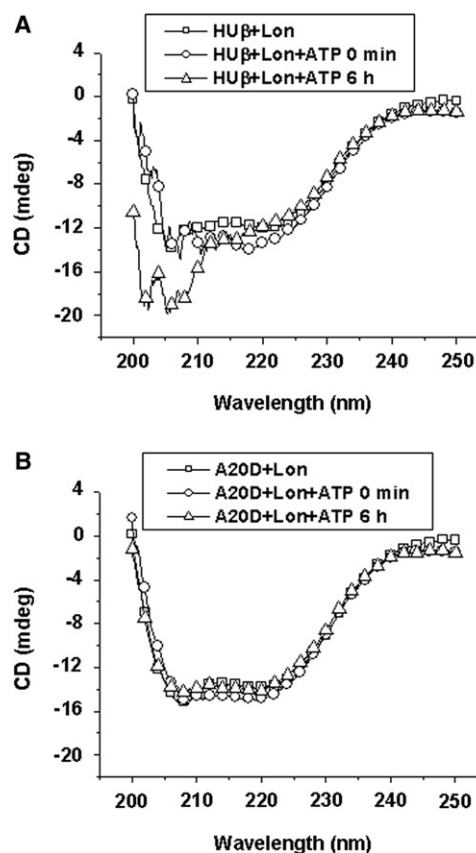


FIGURE 4 (A) Degradation of HU β by Lon protease. (B) Lack of degradation of mutant HU β -A20D by Lon protease. The change in the CD spectrum of HU β incubated with Lon and ATP for 6 h indicates the formation of a random coil, i.e., degradation.

i.e., Lon protease degraded HU β -A20D more slowly than it degraded HU β . These results indicate that residue A20 has a powerful impact on the degradation of HU β by Lon protease. Recently, synthetic peptides were used to evaluate the interaction of amino acid residues in peptide substrate with the proteolytic site of *Ec*-Lon (49). The degradation rate of the substrate peptide with glutamic acid was significantly slower compared to the rate with serine under the same substrate concentration, which implies that negative charge is not favored at the P1' site of the *Ec*-Lon substrate. Our data support this point of view.

Force measurements

We determined the rupture force of HU-Lon protein-protein binding using HU-coated polystyrene beads and optical tweezers. The force was calculated from the displacement of the small bead when the force-versus-position curve peaked and the rupture force broke the binding (see Fig. S8 D). The average rupture forces in the presence and absence of ATP were determined (Fig. 5). Without ATP, the average rupture forces of *Ec*-Lon with HU α , HU β , and HU β -A20D were 24 \pm 6, 28 \pm 5, and 31 \pm 4 pN,

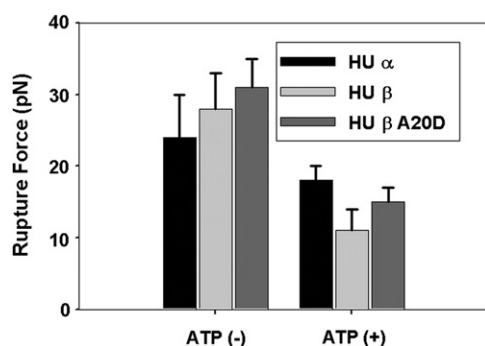


FIGURE 5 Results of rupture-force measurements. The average rupture forces of HU α , HU β , and A20D-HU β without ATP were 24 ± 6 , 28 ± 5 , and 31 ± 4 pN, respectively. The average rupture forces of HU α , HU β , and A20D-HU β became smaller in the presence of ATP and they were 18 ± 2 , 11 ± 3 , and 15 ± 2 pN, respectively.

respectively, i.e., the binding forces between *Ec*-Lon and each of the three different HU proteins are similar. In the presence of ATP, the average rupture forces of *Ec*-Lon with HU α , HU β , and A20D-HU β were 18 ± 2 , 11 ± 3 , and 15 ± 2 pN, respectively, i.e., all of the rupture forces decreased in the presence of ATP. The average and the standard deviation were obtained by repeating the experiments with ~ 10 pairs of samples for each case (see Table S3 for details).

Substrate interference assay

We further studied Lon protease in a substrate interference assay using FITC- α -casein in competition with equimolar concentrations of unlabeled HU α , HU β , A20D-HU β , casein, or BSA (Fig. 6). The fluorescence of cleaved FITC- α -casein was measured at 525 nm. Cleavage of the other, unlabeled substrate decreased the amount of FITC- α -casein cleaved, and therefore lowered the fluorescence. All proteins except BSA competed similarly with FITC- α -casein as substrates

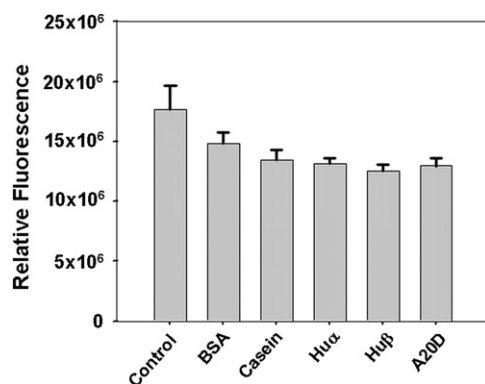


FIGURE 6 Competition of FITC- α -casein with BSA, unlabeled casein, HU α , HU β , and HU β -A20D as substrates of the Lon protease. In the control sample, there was no competing protein. The activity was assayed by measuring the fluorescence of released FITC (excitation, 490 nm; emission, 525 nm).

of *Ec*-Lon. In keeping with these results, it has been reported previously that native BSA is nondegradable by Lon protease (50), and casein and HU β are both substrates of Lon protease. HU α , however, is not cleaved by Lon protease in the presence of ATP, yet still competed with FITC- α -casein. In addition, similar interference effects of A20D-HU β and HU β may indicate that position 20 is not involved in the substrate binding of Lon protease.

DISCUSSION

A protease in *E. coli* and other bacteria must select its substrates from ~ 4300 different proteins. Cleavage of the wrong proteins could be fatal, and recognition of the correct substrates is therefore critical. In eukaryotes, the 26S proteasome recognizes and degrades proteins modified by the attachment of ubiquitin. In bacteria, the Clp and Lon proteases interact directly with their protein targets. Even though a recent report indicated that the Lon protease degrades tmRNA-tagged proteins (51), accumulated evidence suggests that the sensor and substrate-discrimination domain, also named the α -domain, of Clp and Lon proteases interacts with polypeptide regions with a hydrophobic patch (52,53). For example, the C-terminus of the Lon substrate Sula is sufficient for recognition, but not for degradation (54). Recognition of a degradation tag can be the sole determinant of targeted proteolysis. The interaction between protease and substrate can be considered as the initial step. Once the substrate is recognized, it will be transferred to the proteolytic active site. The degradation progresses, and the fragments are released.

Lon protease has been shown to cut native proteins including RcsA (55), ribosomal S2 protein (56), and Sula (34,57). Earlier results indicated that Lon or a Lon-dependent protease degrades HU (25), and our results here showed that *Ec*-Lon degrades *Ec*-HU β , but not *Ec*-HU α . In addition, the heterodimeric HU $\alpha\beta$ is stable in the presence of *Ec*-Lon and ATP (see Fig. S1). Our in vitro findings are in line with those of previous in vivo studies (25).

The *Ec*-Lon cleavage sites of various peptides and proteins have been reported. *Ec*-Lon cleaves Sula, which is synthesized only as part of the SOS response to DNA damage (58,59). Cleavage occurs mainly at sites where L and S are in the P1 and P1' positions; the other cleavage sites have various residues in the P1 position, such as A, V, M, T, S, L, F, Q, and G (57). *Ec*-Lon cleaves the ribosomal S2 protein at 45 sites, whose P1 and P3 positions are dominantly occupied by hydrophobic residues (56). In our present and past studies, both *Ec*-Lon and *Bt*-Lon cleaved at sites with A or L in the P1 position and in most cases with an uncharged amino acid, but also with a positively charged amino acid (e.g., K) in the P1' position.

Our alignments of the HU β sequence with the peptide fragments revealed the major *Ec*-Lon cleavage sites in HU β . One major cleavage site is located at K18-G22,

specifically at A20-A21 within an AAA cluster. In an earlier study of fluorogenic peptide substrates of *Ec*-Lon (60), the best substrates were those with an A-A bond (glutaryl- or succinyl-A-A-F-methoxynaphthylamine); substitution of A-A with G-G generated a very poor substrate. *Ec*-Lon was unable, however, to degrade glutaryl-A-A-A-methoxynaphthylamine. Another favored cleavage site of *Ec*-Lon in HU β is L36-K37. Our results showed that the cleavage within K18-G22 and at L36-K37 occurs early in the degradation of HU β by *Ec*-Lon (Table S1). Since fragments 12, 14, 18, 21, 22, and 23 have an intact K18-G22 and fragments 17, 19, 20, and 22 have an intact L36-K37 (Table S1), there might be more than one initial cleavage site in HU β . Since all these fragments were found after 10 min of incubation, it is reasonable to postulate that one subunit of the dimer is degraded first, which leads to more cleavage sites being exposed on the other subunit. In such a scenario, large fragments would be present even after longer incubation.

The sequence of HU β differs from that of HU α and *Bs*-HU particularly in the K18-G22 region, i.e., the initial *Ec*-Lon cleavage site in HU β (Table S1). HU α and *Bs*-HU are not degraded by *Ec*-Lon and the mutants HU β -A20Q and HU β -A20D are degraded at a lower rate than HU β . We therefore propose that region K18-G22 is involved in the *Ec*-Lon recognition of HU β . The other favored *Ec*-Lon cleavage site in HU β is L36-K37. Based on the results of MS analysis, L36-K37 became more accessible for cleavage after the first cleavage at K18-G22 near the N-terminus of HU β . The cleavage mechanism of the Lon protease may be similar to that of ClpP (61). The active sites in the Lon protease that catalyze peptide-bond cleavage are sequestered in a hollow interior chamber formed by the subunits. The loop near K18-G22 of HU β enters this chamber through axial channels. Since there is more steric hindrance near L36-K37, K18-G22 is the major cleavage site.

Taken together, we propose a mechanism for the degradation of a HU β homodimer. The model of the homodimer (Fig. 2) can be considered as the association of two subdomains. In the cleavage mechanism, *Ec*-Lon recognizes K18-G22 at the end of the α 2-helix of one HU β subunit and cleaves in this region, thereby exposing the α 2-helix so that L36-K37 can be cleaved. The loss of the structural integrity of HU β would then enable cleavage at the hydrophobic core of HU β , so that the fragment with cleavage site F47-G48 can be accessed. The degradation of the first subunit of the HU β homodimer leads to the cleavage of more sites in the second subunit.

The heterodimer HU $\alpha\beta$, on the other hand, is not degraded by *Ec*-Lon in the presence of ATP, even though K18-G22 is present in the HU β subunit. This lack of cleavage could be explained by the greater thermodynamic stability of the heterodimeric form over that of the homodimeric forms (42). HU $\alpha\beta$ forms a spiral structure, residues L6, I7, I10, A11, A21, and L25 of HU β interact with each other to generate a V-shape between the α 1-helix and the α 2-helix, and the

amino acids in the turn between these helices could interact with another protein (62). It might therefore be difficult for *Ec*-Lon to unfold the heterodimeric HU $\alpha\beta$, and hence degradation by Lon is arrested.

It appears that only a few research findings are available to date concerning the substrate recognition of Lon protease (54,63). Our attempts to study the substrate recognition using surface plasmon resonance and isothermal titration calorimetry failed, because the interaction between the Lon protease and HU β were too weak. Recent developments with protein-coated polystyrene beads and optical tweezers (64) enabled us to measure this interaction. Our results indicated that both HU α and HU β can interact with the Lon protease. It is very unlikely that the rupture force we measured was the result of a single molecular-pair interaction. The force measured will thus depend on the number of molecules present in the contact area of the two beads, and this can fluctuate significantly from sample to sample. This is evident from the relatively large error bars in the results (Fig. 5) and from the statistics shown in Table S3. Despite this deficiency, the comparison of the relative average values of the rupture force under different conditions is still quite informative. It reveals not only that HU α , HU β , and HU β -A20D can interact with the Lon protease, but also that the interactions become weaker in the presence of ATP. There is an assertion that approximately equal rupture force in all three cases (i.e., for the binding of *Ec*-Lon with HU α , HU β , or HU β -A20D) implies random and unknown interaction sites. However, our current data indicate that in the presence of ATP, the rupture forces in all three cases were significantly reduced compared to the corresponding one without ATP. Therefore, it is more likely that such interaction is site-specific.

Our results indicate that *Ec*-Lon recognizes and interacts with both HU α and HU β , but only degrades HU β in the presence of ATP. *Ec*-Lon also binds HU β -A20D, which has a negative charge at position 20, but does not cleave the mutated protein well. HU α , which has glutamine at this position, is also not cleaved. Alanine 20 is therefore not involved in the binding to *Ec*-Lon, but is involved in the recognition for cleavage by *Ec*-Lon. In a comparative study, the substrate competition assay also revealed that HU α , HU β , and HU β -A20D show similar competitive ability with α -casein (Fig. 6). In other words, Lon protease cannot distinguish HU α from HU β by interaction with these two proteins. It is obvious that appropriate substrates of Lon protease should be recognized and should have special cleavage sites. BSA is not recognized by Lon protease and therefore cannot be further degraded by Lon protease (Fig. S11). HU α can be recognized by Lon protease but does not have special cleavage sites.

The selection of appropriate substrates by proteases is a critical step in regulatory degradation. Once the substrate is recognized, it is transferred to the proteolytic active site. For the Clp and FtsH proteases, short sequence motifs serve as recognition tags (61,65–67). In this study, the recognition

tags have not been found. Here, we must emphasize the difference between initial recognition tags and the initial cleavage site. The region K18-G22 is the initial cleavage site of HU β by *Ec*-Lon, but is not involved in substrate binding of *Ec*-Lon. The Lon protease interacts with certain specific recognition elements within HU proteins. It has been proposed that ATP hydrolysis is necessary to unfold and/or translocate the substrate before its degradation, thereby weakening the binding force between enzyme and substrate and facilitating substrate translocation (68–70). We showed here that the rupture forces do weaken in the presence of ATP. Our results indicate that Lon protease cleaves HU β in three stages. In the first, less specific step, Lon binds the HU protein (HU β , HU α , or HU β -A20D). Lon then hydrolyzes ATP, providing energy to loosen the binding, conformationally changes the substrate, and translocates the substrate to the active site if the sequence of the N-terminus of the substrate allows. Finally, the translocated substrate, HU β , but not HU α or HU β -A20D, is cleaved. Substrates with high affinity will replace unfavorable, bound substrates. With such a mechanism, the Lon protease has at least two checkpoints to ensure that appropriate substrates are degraded.

SUPPORTING MATERIAL

Additional text, a table, and 12 figures are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(09\)01566-5](http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)01566-5).

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