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Functional Domains of *Brevibacillus thermoruber* Lon Protease for Oligomerization and DNA Binding

ROLE OF N-TERMINAL AND SENSOR AND SUBSTRATE DISCRIMINATION DOMAINS*

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Lon protease is a multifunctional enzyme, and its functions include the degradation of damaged proteins and naturally short lived proteins, ATPase and chaperone-like activities, as well as DNA binding. A thermostable Lon protease from *Brevibacillus thermoruber* WR-249 (Bt-Lon) has been cloned and characterized with an N-terminal domain, a central ATPase domain that includes a sensor and substrate discrimination (SSD) domain, and a C-terminal protease domain. Here we present a detailed structure-function characterization of Bt-Lon, not only dissecting the individual roles of Bt-Lon domains in oligomerization, catalytic activities, chaperone-like activity, and DNA binding activity but also describing the nature of oligomerization. Seven truncated mutants of Bt-Lon were designed, expressed, and purified. Our results show that the N-terminal domain is essential for oligomerization. The truncation of the N-terminal domain resulted in the failure of oligomerization and led to the inactivation of proteolytic, ATPase, and chaperone-like activities but retained the DNA binding activity, suggesting that oligomerization of Bt-Lon is a prerequisite for its catalytic and chaperone-like activities. We further found that the SSD is involved in DNA binding based on gel mobility shift assays. On the other hand, the oligomerization of Bt-Lon proceeds through a dimer ↔ tetramer ↔ hexamer assembly model revealed by chemical cross-linking experiments. The results also showed that hydrophobic interactions may play important roles in the dimerization of Bt-Lon, and ionic interactions are mainly responsible for the assembly of hexamers.

Lon protease (La), the first ATP-dependent protease purified from *Escherichia coli* (1, 2), is a member of the ATPases associated with diverse cellular activities (AAA^+)[1] superfamily (3). Lon consists of a variable N-terminal domain, a central ATPase domain, and a C-terminal protease domain on a single polypeptide (4, 5). The N-terminal domain with its still obscure function contains a potential coiled coil region built from α-helices and is proposed to be involved in binding and recognition of substrate proteins (5, 6). Lon is active as a homo-oligomer (7–10), which is distinct from other ATP-dependent proteases, Clp/HSP100 proteins, forming a hetero-oligomer. Nevertheless, the oligomeric state of Lon is still vague. Lon behaves as a tetramer or an octamer in *E. coli* (7), as a tetramer to a hexamer in *Mycobacterium* (5, 10), and as a hexamer or a heptamer in yeast mitochondria (8, 9). More recently, we have shown that *Brevibacillus thermoruber* Lon (Bt-Lon) is a hexameric structure (11). On the other hand, Clp/HSP100 proteins form a hexameric structure in the presence of ATP (12–15). Furthermore, the N-terminal domain of many Clp/HSP100 proteins, which is involved in binding of protein substrates (16–18), is not necessary for oligomerization (17–19). By analogy with Clp/HSP100 proteins, it is quite reasonable to predict that the regulation of oligomerization of Lon may occur through an N-terminal domain-independent and an ATP-dependent mechanism. However, the oligomeric state of Lon is not significantly affected by the absence of ATP (9–11). So far, not many studies about this phenomenon of Lon have been carried out or discussed. Although the oligomeric states of Lon are facilitated by $Mg^{2+}$and unfolded proteins (10), the detailed mechanism of its oligomerization is not determined.

Lon protease has been identified from Archaea to prokaryotes to mitochondria of eukaryotes (20–23) and has demonstrated that it acts as a DNA-binding protein (24–26). The phenotype of yeast strain lacking Lon is the presence of large deletions within the mtDNA (22, 27), suggesting that Lon is required for the stability and expression of the mitochondrial genome. Moreover, *in vivo* studies in bacteria and yeast suggest that Lon is involved in controlling gene expression, either by regulating the levels of transcription factors or by influencing the stability of mRNA transcripts (28, 29). Recently, Liu *et al.* (30) reported that ATP inhibits the binding of human Lon to DNA or RNA. However, the physiological role of DNA binding by Lon has still not been well understood. Furthermore, although two short polybasic regions of Lon are proposed to be involved in DNA binding (31), the DNA binding domain has not been identified so far.

Lon protease and Clp/HSP100 are proposed as members of the AAA^+ superfamily (3). The AAA^+ proteins participate in such processes as ATP-dependent proteolysis, DNA replication, recombination, vesicle transport, and organelle biogenesis (3, 32).

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[1] The abbreviations used are: AAA^+, ATPases associated with diverse cellular activities superfamily; Bt-Lon, *B. thermoruber* Lon protease; Bt-LonΔC, residues 1–605 of Bt-Lon; Bt-LonΔN, residues 317–779 of Bt-Lon; Bt-LonN316, residues 1–316 of Bt-Lon; Bt-LonATPase, residues 317–605 of Bt-Lon; Bt-Lon-(317–490), residues 317–490 of Bt-Lon; Bt-Lon-C289, residues 491–779 of Bt-Lon; Bt-LonSSD, residues 419–605 of Bt-Lon; BSA, bovine serum albumin; DTT, dithiothreitol; GMSA, gel mobility shift assays; Ms-Lon, *M. smegmatis* Lon protease; PBS, phosphate-buffered saline; SSD, domain sensor- and substrate-discrimination domain; CHES, 2-(cyclohexylamino)ethanesulfonic acid; WH, winged helix.

The crystal structure data (33, 34) showed that the conserved AAA^+ domain consists of two structural domains, a α-β-α-sandwich Rossmann fold seen in nucleotide-binding proteins and C-terminal, mostly helical, domain referred to as the sensor 2 (3), or "sensor and substrate discrimination" domain (35). The SSD contacts its own ATPase domain and that of adjacent subunits and has been shown to mediate nucleotide binding and hexamer formation in HslU (33) and in ClpB (17, 19). Moreover, it is proposed that the SSD is involved in recognizing protein substrates and guiding them into cavities inside the Clp or proteolytic domain hexamers (35). However, recently determined crystal structures of the AAA^+ proteases like HslU and ClpA do not support this SSD hypothesis. The structural data show that the SSD of each monomer faces either the outside of an adjacent monomer or the solvent (33, 34). Hence, the SSD may not be involved in the recognition and the transfer of protein substrates into the intra-hexamer cavity.

In this study, we represent the extensive analysis of the structure-function relationship of Bt-Lon to identify the functional role of each domain. Several Bt-Lon truncated mutants were used for analysis of ATP-independent oligomerization, ATP-dependent proteolysis, ATPase activity, chaperone-like activity, and DNA binding activity. We found that the N-terminal region of Bt-Lon was essential for oligomerization, proteolytic ATPase, and chaperone-like activities but not involved in interactions with DNA. The results further demonstrated that the SSD in Bt-Lon was involved in DNA binding. Additionally, the chemical cross-linking experiments revealed that the oligomerization of Bt-Lon proceeds through a dimer ↔ tetramer ↔ hexamer assembly model and that hydrophobic interactions play important roles in the formation of the dimer, whereas ionic interactions are mainly responsible for hexamers assembly.

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals—Restriction enzymes were purchased from New England Biolabs (London, UK). Fluorogenic peptide, glutaryl-Ala-Ala-Phe-methoxynaphthylamide (Glt-AAF-MNA) was purchased from Bachem (Bubendorf, Switzerland). Insulin from bovine pancreas, dithiothreitol (DTT), and glutaraldehyde were purchased from Sigma.

Plasmids—The DNA fragments encoding Bt-Lon residues 1–779 (full-length Bt-Lon), residues 1–605 (Bt-LonΔC), residues 317–779 (Bt-LonΔN), residues 1–316 (Bt-LonN316), residues 317–605 (Bt-LonATPase), residues 317–490 (Bt-Lon-(317–490)), residues 491–779 (Bt-Lon-C289), or residues 491–605 (Bt-LonSSD) were produced by PCR and subcloned between the NdeI and XhoI sites of pET-21a (Novagen).

Proteins—Bt-Lon and its truncated mutants were overexpressed in *E. coli* strain BL21(DE3) (Novagen) and purified with a procedure similar to that used preciously to obtain wild type Bt-Lon (11). *E. coli* cells were grown at 37 °C to $A_{600\ nm}$ ~0.6 in LB broth containing 50 μg/ml ampicillin. Protein expression was induced with 1.0 mM isopropyl-β-D-thiogalactoside. Cells were grown at 37 °C for 4 h after induction and were collected by centrifugation. Cell pellets were suspended in buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1% Triton X-100, 20% glycerol, 10 mM imidazole, and 10 mM β-mercaptoethanol. Following sonication, the recombinant proteins were purified by Ni^{2+}-chelate affinity chromatography as described by the manufacturer (Qiagen). The protein concentration of the purified Bt-Lon and its truncated mutants was determined with the Bradford method (Bio-Rad) using bovine serum albumin as standard, and the homogeneity of the purified proteins was analyzed by SDS-PAGE.

Analytical Gel Filtration Chromatography—The gel filtration experiments were performed using fast protein liquid chromatography on a Superose 6 HR 10/30 column (Amersham Biosciences) equilibrated with buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 150 mM NaCl, and 10% glycerol with a flow rate of 0.5 ml/min. Blue dextran was used for determination of the void volume (V_0). Several proteins of known molecular weight (thyroglobulin, 669 kDa; apoferritin, 443 kDa; β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; BSA, 66 kDa; and carbonic anhydrase, 29 kDa from Sigma) were used as the standards, and their elution volumes (V_e) were determined. The standard curve was plotted with the logarithm of molecular weight against V_e/V_0 of the standard protein.

Analytical Ultracentrifugation—Sedimentation velocity and sedimentation equilibrium experiments were performed by using a Beckman-Coulter XL-A analytical ultracentrifuge with an An60Ti rotor. Samples were dialyzed overnight against buffer containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, and 150 mM NaCl. The concentration of proteins was 1 mg/ml. Sedimentation velocity experiments were conducted at 40,000 rpm in two-channel aluminum centerpieces at 20 °C. The data were analyzed with the SedFit program (36). The observed sedimentation coefficients were normalized to $s_{20,w}$ by using measured values of partial specific volumes of the proteins and density and viscosity of the buffer. Sedimentation equilibrium experiments were carried out in Al-Epon six-channel centerpieces at 20 °C. The data were collected at rotor speeds of 4000, 10,000, and 12,000 rpm. The equilibrium data were analyzed using the Optima XL-A/XL-I analysis software (Beckman/Coulter) within Origin version 4.0 (Microcal). The partial specific volumes were calculated from the amino acid composition of the proteins using SEDNTERP (37). The solution density of 1.00248 g/cm^3 and the solution viscosity of 1.0274×10^{-2} centipoise were also calculated using SEDNTERP.

Peptidase and Protease Activities Assays—The peptidase activity of Bt-Lon and its truncated mutants was examined as described previously (11). In brief, peptidase assays contained 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1.0 mM ATP, 0.3 mM fluorogenic peptide, Glt-AAF, and 5 μg of proteins in a total volume of 200 μl. Reactions were incubated for 60 min at 50 °C and stopped by the addition of 100 μl of 1% SDS and 1.2 ml of 0.1 M sodium borate (pH 9.2). Fluorescence was measured in a Hitachi F4010 fluorescence spectrophotometer with excitation at 335 nm, and emissions were monitored at 410 nm.

Protease assays contained 10 mM $MgCl_2$, 1.0 mM ATP, 50 mM Tris-HCl (pH 8.0), 1.0 μg of Bt-Lon, and 10 μg of α-casein fluorescein isothiocyanate type I (Sigma) in a 200-μl total volume (38). Reaction mixtures were incubated for 60 min at 50 °C and reactions terminated by the addition of 10 μl of 10 mg/ml bovine serum albumin (Sigma) and 100 μl of 10% trichloroacetic acid. Mixtures for terminated reactions were incubated for 10 min on ice and centrifuged for 10 min at 13,000 rpm. Supernatants were transferred to fresh tubes, and 200 μl of 0.5 M CHES-Na (pH 12.0, Sigma) was added. Fluorescence was also measured in a Hitachi F4010 fluorescence spectrophotometer with excitation at 490 nm and emission at 525 nm.

ATPase Activity Assays—ATPase assays were performed using the method of Lanzetta *et al.* (39) for free inorganic phosphate detection. Reaction mixtures consisted of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1.0 mM ATP, and 2–5 μg of Bt-Lon in a total volume of 100 μl and were incubated for 30 min at 50 °C or at indicated temperatures. The color of reaction was developed with the addition of 800 μl of malachite/molybdate solution and terminated by addition of 100 μl of 34% sodium citrate. The optical density of the final reaction was determined at 660 nm. Optical densities were converted to phosphate concentrations using K_2HPO_4 standards. One unit of ATPase activity was defined as the amount of enzyme required to release 1 nmol of P_i per h. The background values of hydrolysis were subtracted in each assay.

Chaperone-like Activity Assays—The assay is based on preventing the aggregation of denatured insulin B chain. Bovine insulin was unfolded with 20 mM DTT at 37 °C and monitored by measuring the apparent absorption due to light scattering in a spectrophotometer. Bt-Lon protease and its truncated mutants were added to the target proteins indicated in the figure legends.

Gel Mobility Shift Assays (GMSA)—For plasmid mobility shift assays we routinely used plasmid pET21a in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 10 mM $MgCl_2$. Bt-Lon and its truncated mutants (4–5 μg) was incubated with plasmid DNA (500 ng) in a total volume of 25 μl for 30 min at 25 °C. The protein-DNA complexes were analyzed by gel electrophoresis in standard 0.8% agarose. DNA bands were visualized by ethidium bromide staining.

Antibody Preparation and Western Blot Analysis—The recombinant Bt-Lon was purified by Ni^{2+}-chelate affinity chromatography as described above. The recombinant Bt-Lon in phosphate-buffered saline (PBS) was used to immunize New Zealand White rabbits for polyclonal antiserum production. For Western blot analysis, the protein extracts from *B. thermoruber* were prepared by boiling in 2× Laemmli sample buffer. Approximately 10-μg samples of total protein extracts were fractionated by electrophoresis on 10% SDS-PAGE and then were transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore) by using a Bio-Rad Trans Blot Cell. The membrane was blocked for 1 h with blocking buffer (TTBS, 10 mM Tris-HCl (pH 7.5), 0.9% NaCl, and 0.05% Tween 20, supplemented with 5% dry skimmed milk), given two washes of 10 min in TTBS, and incubated for 1 h with anti-Bt-Lon polyclonal antibody at a 1:25,000 dilution in the blocking

buffer. The blot was washed five times for 10 min each in TTBS and then incubated for 30 min with a goat anti-rabbit IgG, alkaline phosphatase-conjugated secondary antibody (Jackson ImmunoResearch) at a 1:5000 dilution in the blocking buffer. After another two washes of 10 min each in TTBS, the blot was visualized by nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma).

Chemical Cross-linking Assays—Bt-Lon was dialyzed against buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 20% glycerol. Four μg of Bt-Lon (1 μM) was incubated in buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$ in the absence and the presence of increasing concentrations of NaCl or SDS in a total volume of 50 μl. Cross-linking reactions were started by addition of 0.2% glutaraldehyde and incubated at 30 °C for another 30 s or the indicated times. Reactions were stopped by addition of 1 M Tris (pH 7.5), and cross-linked products were analyzed by SDS-PAGE (4–12%) followed by Western blot analysis.

Homology Modeling of Bt-LonSSD—The three-dimensional model of Bt-LonSSD was generated with the MODELER program (40) encoded in InsightII (Accelrys, Inc.) by using *E. coli* SSD (Protein Data Bank code 1QZM) as the template structure. MODELER uses a spatial restraint method to build up the three-dimensions of the structure protein. For alignment of the Bt-LonSSD protein, MODELER yielded 10 models, each of which contains three optimizing loop structures. The coordinates of the high resolution structure of *E. coli* SSD were used to model the main chain conformation of Bt-LonSSD. The structure with the lowest violation score and lowest energy was chosen as the candidate. Several structural analysis softwares were adopted to check the model quality. The distribution of the backbone dihedral angles of the model was evaluated by the representation of Ramachandran plot using PROCHECK (41). The Prostat module of InsightII was used to analyze the properties of bonds, angles, and torsions. Profile three-dimensional program (42) was used to check the structure and sequence compatibility. The electrostatic potential of the modeled Bt-LonSSD was calculated by the program GRASP (43).

RESULTS

Design of Bt-Lon Mutants for Structure-Function Studies—Earlier studies of limited proteolysis have shown that Lon protease consists of an N terminus with its still unknown function, a central ATPase containing SSD, and C-terminal protease domains (4, 5) (as illustrated in Fig. 1*A*). To investigate the structure-function relationship of Bt-Lon, seven truncated mutants were constructed, overexpressed, and purified (Fig. 1, *A* and *B*). Regarding the role of the N-terminal domain, three truncated mutants, Bt-LonΔN (residues 317–779), Bt-LonΔC (residues 1–605), and Bt-LonN316 (residues 1–316), were constructed according to the limited proteolysis experiments (Fig. 1*A*). Bt-Lon and the three truncated mutants were characterized with respect to oligomerization, proteolytic, ATPase, and chaperone-like activities. In addition to the three mutants described above, four Bt-Lon truncated mutants, Bt-LonATPase (residues 317–605), Bt-Lon-(317–490) (residues 317–490), Bt-Lon-C289 (residues 491–779), and Bt-LonSSD (residues 491–605), were constructed and characterized regarding DNA binding activities to identify the DNA binding domain of Bt-Lon (Fig. 1*A*). Bt-Lon and its truncated mutants were purified, and their homogeneity was examined with SDS-PAGE (Fig. 1*B*).

Oligomerization State of Bt-Lon and Its Mutants—Previous studies (7, 9, 10) have revealed that Lon protease has a homo-oligomeric structure. Gel filtration analysis under nondenaturing conditions has shown that the purified Bt-Lon has a molecular mass of ~550 kDa, corresponding to a size of a hexamer of 88-kDa subunits (11). The size of Bt-Lon and its two truncated mutants, Bt-LonΔC and Bt-LonΔN, was estimated by gel filtration chromatography on a calibrated Superose 6 HR 10/30 column. The size of Bt-LonΔC and Bt-LonΔN estimated by gel filtration chromatography was ~422 and ~50 kDa (Fig. 2), suggesting that they exist as hexamer and monomer, respectively. These results revealed that oligomerization is strictly dependent on the presence of the N-terminal domain, whereas the C-terminal protease domain is dispensable. The isolated N-terminal fragment of Bt-Lon (Bt-LonN316) was also estimated by analytical gel filtration chromatography. The elution profile of Bt-LonN316 on the gel filtration column is shown by a major species of ~312 kDa (octamer) and a minor species of ~81 kDa (dimer) (Fig. 2). This indicates that Bt-LonN316 contributes to oligomerization and is a predominantly octameric structure in solution.

Analytical ultracentrifugation experiments were used as an additional approach to confirm the oligomeric structures of Bt-Lon and its three mutants. In a sedimentation velocity experiment, the shape of protein concentration profiles is related to homogeneity and diffusion properties of species in solution, whereas the rate of movement of a concentration boundary gives the sedimentation coefficient. Fig. 3 shows apparent distributions of the sedimentation coefficient ($g(s)$) for Bt-Lon and its mutants obtained from the analysis of raw data. The results indicated that the S values of Bt-Lon, Bt-LonΔC, and Bt-LonN316 are 15.11, 14.66, and 11.31, respectively (Fig. 3). Instead, Bt-LonΔN did not show a distinct peak and was displaced by low molecular weight species with S values ~3–6, suggesting that it was present as a monomer and dimer (data not shown). This is different from Ms-Lon mutant N-I278 that behaved as a higher multimeric state (5). These results confirmed the finding obtained by gel filtration analysis that the N-terminal domain is essential for oligomerization. Using the relation $(s_1/s_2)^3 = (M_1/M_2)^2$ (44), we obtained an apparent molecular mass of 301,576 Da for Bt-LonN316. This is consistent with that obtained from gel filtration analysis that Bt-LonN316 itself can form an octameric complex. In a supplementary approach we used sedimentation equilibrium experiments to examine the precise information about the molecular weight and states of oligomerization of Bt-LonN316. A representative data set from a sedimentation equilibrium experiment is shown in Fig. 4. The molecular mass of the Bt-LonN316 was calculated to be 301,392 Da as an octamer. The results obtained from analytical ultracentrifugation experiments are consistent with those of analytical gel filtration chromatography.

Proteolytic and ATPase Activities of Bt-Lon Mutants—To examine the catalytic activities of Bt-Lon and its two truncated mutants, Bt-LonΔC and Bt-LonΔN, we first conducted the protease, peptidase, and ATPase assays. Bt-LonΔC showed neither peptidase nor protease activity and retained 20 ± 2% of ATPase activity of Bt-Lon (Table I). This result indicated that the ATPase and proteolytic domains function independently but interact structurally. These findings parallel the facts that substitution in the active site Ser nucleophile abolishes the peptidase activities but not the whole ATPase activities of Lon proteases (45–47). By contrast, Bt-LonΔN retained less than 1% of ATPase activity and only 1 ± 0.5 and 2 ± 0.3% of protease and peptidase activities, respectively, compared with those of the wild type. (Table I). The combination of our results and others, Bt-LonΔN and Ms-Lon mutant N-I278 did not exhibit either peptidase or ATPase activity and behaved as incorrect multimeric states. These results suggested that correct oligomerization is essential for the proteolytic and ATPase activities of Bt-Lon.

Chaperone-like Activity of Bt-Lon Mutants—To examine the chaperone-like activity, three truncated mutants, Bt-LonΔC, Bt-LonΔN, and Bt-Lon-N316, were performed to compare their chaperone-like activities with the wild type Bt-Lon. As shown in Fig. 5, Bt-LonΔC prevented insulin from DTT-induced aggregation as efficiently as the wild type (Fig. 5, *curves 2* and *3*), whereas Bt-Lon-N316 also showed the prevention of the aggregation but in less efficient way (Fig. 5, *curve 5*). On the contrary, Bt-LonΔN had no ability in preventing the aggregation (Fig. 5, *curve 4*). In addition, the results of the above experi-

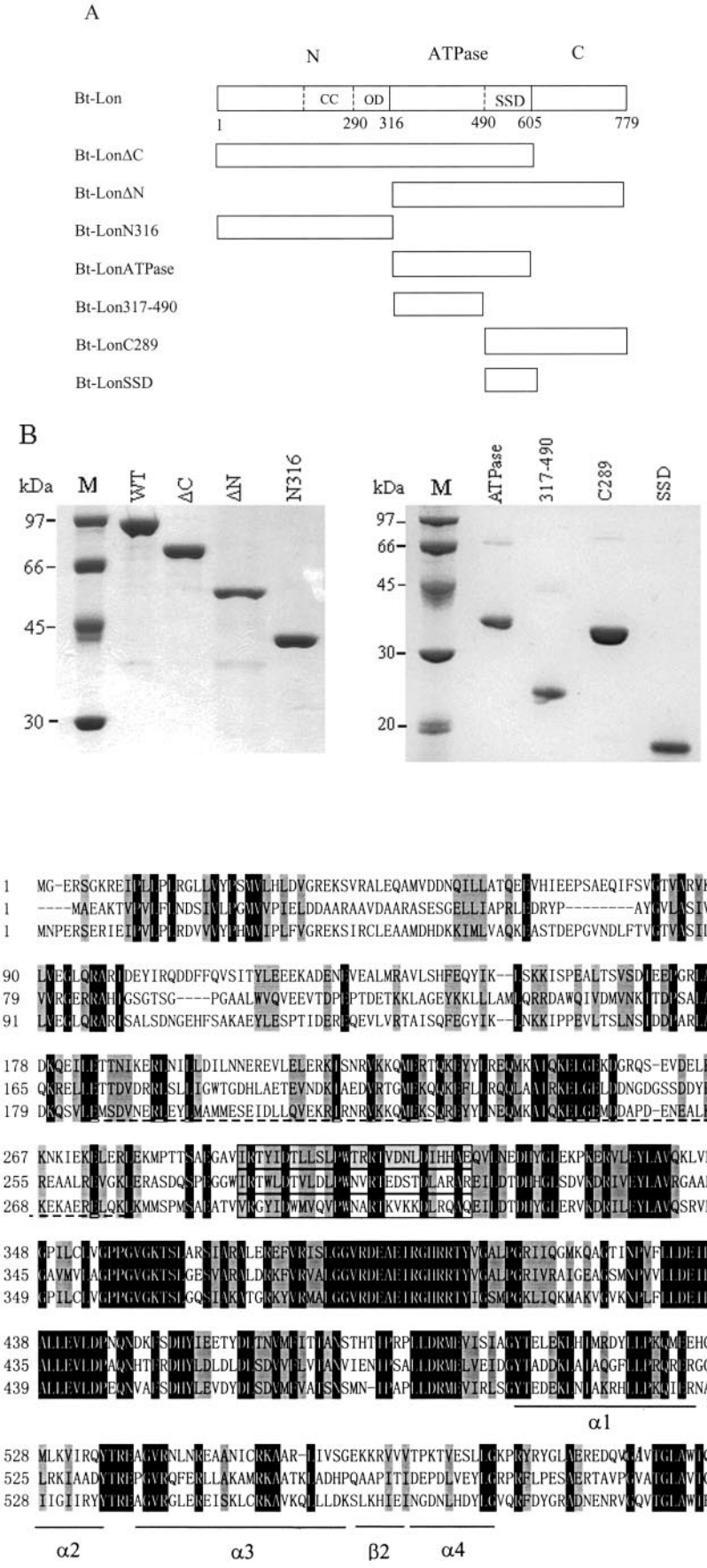

FIG. 1. *A*, Bt-Lon domain structure and schematic diagram of its truncated mutants. Shown are the N-terminal domain, the ATPase domain (AAA$^+$ module), and C-terminal protease domain. *CC*, *OD*, and *SSD* represent the predicted "coiled coil" region, "oligomerization domain," and "sensor and substrate discrimination," respectively. *B*, SDS-PAGE of the purified Bt-Lon and its mutants. The proteins were overexpressed in *E. coli* cells and purified by nickel-nitrilotriacetic acid affinity column. The purified proteins were analyzed by 10% SDS-PAGE followed by Coomassie

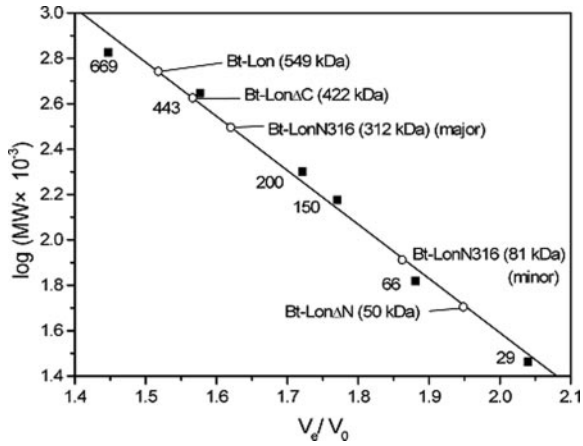

FIG. 2. **Estimation of the molecular mass of Bt-Lon and its truncated mutants by analytical gel filtration.** The semi-logarithmic plot of elution volume (V_e/V_0) *versus* log(M_r) of standard proteins (thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), and carbonic anhydrase (29 kDa)) was shown as the standard curve. The molecular mass of native Bt-Lon and its mutants (*open circles*) were estimated from the standard curve based on their elution volume and the molecular weights of the standard proteins (*filled squares*). The estimated sizes of the eluting species, shown in *parentheses*, were derived from the log(M_r) *versus* volume (V_e/V_0) standard curve. V_e, elution volume of the protein; V_0, column void volume.

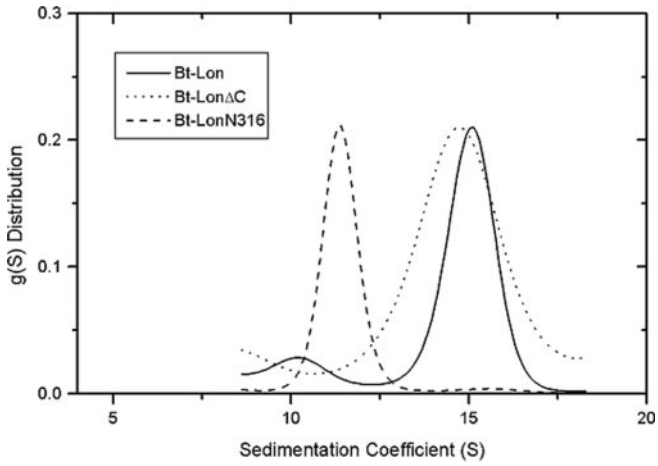

FIG. 3. **Apparent sedimentation coefficient distributions of Bt-Lon and its truncated mutants.** The experiment was performed as described under "Experimental Procedures." The sedimentation velocity profiles are shown for the full-length Bt-Lon, Bt-LonΔC, and Bt-LonN316. The apparent *S* values corresponding to the maxima of the peaks were 15.11, 14.66, and 11.31 S for Bt-Lon, Bt-LonΔC, and Bt-LonN316, respectively. The *lines* show apparent distribution functions *g*(*s*) *versus* the sedimentation coefficient in Svedberg units (S).

ments were not affected by the oligomeric state of Bt-Lon because the control experiment lacking insulin did not show any time-dependent changes of Bt-Lon in absorbance (data not shown). These results indicated that oligomerization is essential for the chaperone-like activity of Bt-Lon. Even the N-terminal segment of Bt-Lon, Bt-LonN316, can form an octamer and partially retains the chaperone-like activity.

The DNA Binding Activity of Bt-Lon and Its Mutants—To test for DNA binding activities of the purified Bt-Lon and its

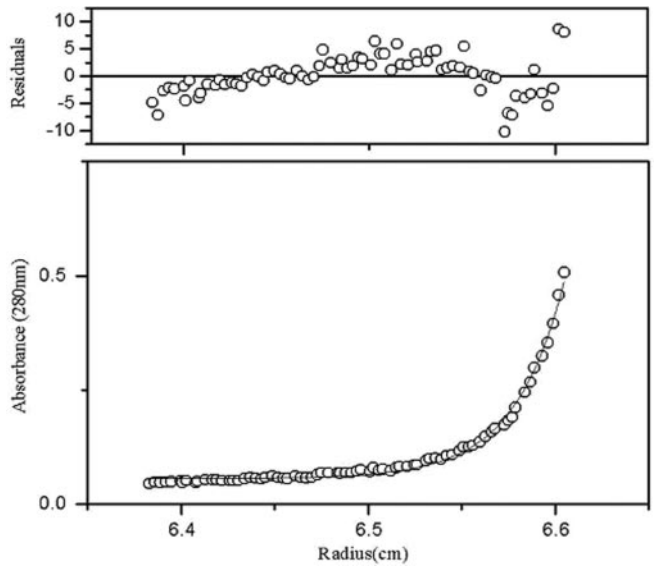

FIG. 4. **Sedimentation equilibrium distribution of Bt-LonN316.** The experimental details are described under "Experimental Procedures." The absorbance of the cell was measured at 280 nm at speeds of 4000, 10,000, and 12,000 rpm and at 20 °C. The *circles* in the *lower panel* are the data points, and the *solid line* is the model fit to a single species of 301,392 Da. The computed residuals of the fit ($A_{exp} - A_{model}$) are shown in the *upper panel*.

TABLE I
Activities of Bt-Lon and its truncated mutants

Activities of Bt-Lon and its truncated mutants were assayed under standard conditions (see "Experimental Procedures"). Activities of Bt-Lon wild type were set as 100%.

	Activity (mean ± S.D.)[a]		
	ATPase	Protease	Peptidase
	%	%	%
Bt-Lon	100 ± 5	100 ± 3	100 ± 3
Bt-LonΔN	<1	1 ± 0.5	2 ± 0.3
Bt-LonΔC	20 ± 2	<1	<1

[a] The results shown represent the average of at least three separate experiments.

truncated mutants, gel mobility shift assays (GMSA) were performed. The purified Bt-Lon was first assayed by a supershift experiment. To demonstrate the specific participation of Bt-Lon in the formation of the protein-DNA complex, a polyclonal antiserum was raised in rabbits against a full-length Bt-Lon. Specificity of this antiserum was evaluated by Western blotting using *B. thermoruber* WR-249 protein extracts and purified recombinant Bt-Lon, where it detected a single band of the expected molecular weight (~90 kDa; Fig. 6*A, right panel*). The presence of Bt-Lon in the protein-DNA complex was confirmed by the addition of this antiserum in the binding reaction. Fig. 6*B* shows no complex formation was observed when no protein or BSA alone was added (*lanes 1*and *2*); the migration of DNA was shifted upon the addition of Bt-Lon, producing a protein-DNA complex (*lane 4*). The addition of rabbit preimmune serum failed to supershift the protein-DNA complex (Fig. 6*B*, *lane 3*), and polyclonal antiserum directed against Bt-Lon caused a marked supershift (Fig. 6*B*, *lanes 5–7*). We also found that the supercoiled DNA is shifted and disappeared immedi-

Brilliant Blue staining. *Lane M*, molecular mass markers; *lane WT*, wild type; *lane ΔC*, Bt-LonΔC; *lane ΔN*, Bt-LonΔN; *lane N316*, Bt-LonN316; *lane ATPase*, Bt-LonATPase; *lane 317–490*, Bt-Lon-(317–490); *lane C289*, Bt-LonC289; *lane SSD*, Bt-LonSSD. *C*, partial sequence alignment of Lon proteases of *B. thermoruber, M. smegmatis*, and *E. coli*. Identical and similar amino acid residues are indicated by *black* and *gray* backgrounds, respectively. The sequences with *dashed lines below* indicate the coiled coil region. The *boxed* residues (amino acids 290–316, numbering in Bt-Lon) indicate the proposed oligomerization domain for Lon proteases. Secondary structure elements of SSD are displayed *below the underlined sequences*, which are based on the crystal structure of *E. coli* AAA^+ α domain (62).

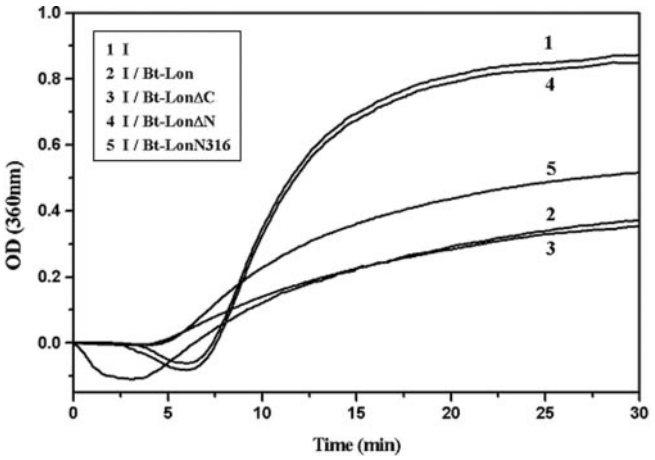

FIG. 5. **The chaperone-like activities of Bt-Lon truncated mutants.** The chaperone-like activities were measured based on the aggregation of denatured insulin B chain induced with the addition of 20 mM DTT in PBS buffer. *Curve 1*, insulin alone; *curve 2*, insulin + Bt-Lon; *curve 3*, insulin + Bt-LonΔN; *curve 4*, insulin + Bt-LonΔC; *curve 5*, insulin + Bt-LonN316. Protein concentration of Bt-Lon and its truncated mutants is 50 μg/ml in PBS buffer (pH 7.4), and that of insulin is 300 μg/ml in PBS buffer (pH 7.4). The experiments were not affected by the oligomeric state of Bt-Lon because the control experiment lacking insulin did not show any time-dependent changes of Bt-Lon in absorbance.

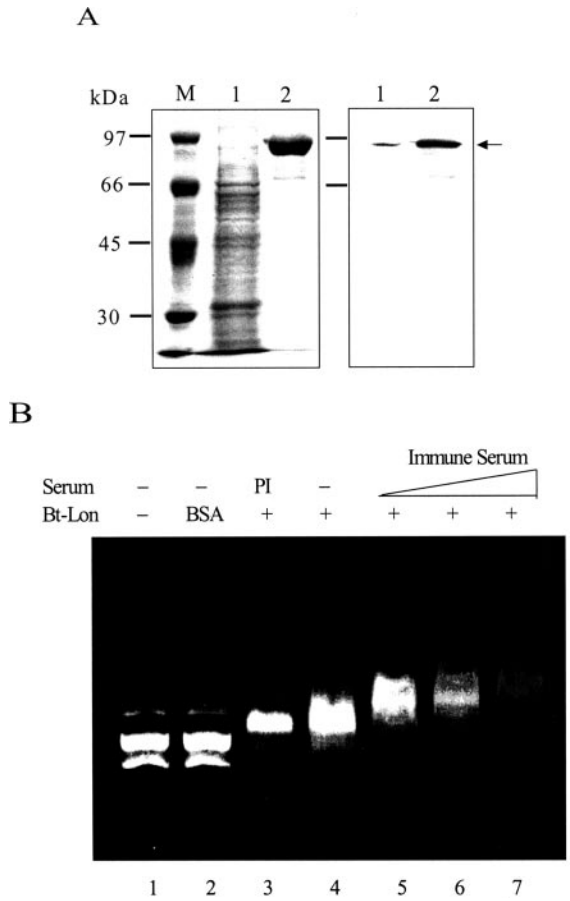

FIG. 6. **DNA binding activity of Bt-Lon analyzed by GMSA.** *A*, specificity of the polyclonal antiserum raised against Bt-Lon as assayed by Western blot analysis with *B. thermoruber* protein extracts. A replica gel stained with Coomassie Brilliant Blue is shown on the *left*. *Lane M*, molecular mass markers; *lane 1*, *B. thermoruber* protein extracts; *lane 2*, affinity-purified Bt-Lon. The *arrow* indicates the position of Bt-Lon. *B*, DNA binding activity of recombinant Bt-Lon in solution. Binding reactions contain 4 μg of affinity-purified protein and 500 ng of plasmid DNA. DNA binding activity of Bt-Lon was examined at the incubation with serum, 2 μl of preimmune serum (*PI*) (*lane 3*), and 2–4 μl of anti-Bt-Lon immune serum (*lanes 5–7*), or in the absence of serum (*lane 4*). *Lane 1*, no protein and serum; *lane 2*, 15 μg of bovine serum albumin (*BSA*) only. The protein-DNA complex was analyzed by agarose gel electrophoresis. Specific supershift of the protein-DNA complex was observed.

ately upon the addition of the protein (Fig. 6*B*, *lanes 1* and *4*), suggesting that the DNA binding activity of Bt-Lon possesses a marked preference for supercoiled DNA. This observation is consistent with the previous studies that supercoiled DNA is more effective in promoting ATPase activity than relaxed circles form (48). Together, these results show that Bt-Lon has DNA binding activity.

Previous studies proposed that predicted coiled-coil regions located at the N terminus and the SSD might be involved in the ability of Lon to bind DNA (6, 11, 31). To identify the DNA binding domain of Bt-Lon, we first examined the DNA binding ability of the three truncated mutants of Bt-Lon, Bt-LonΔC, Bt-LonΔN, and Bt-LonN316, using GMSA as described above. The Bt-LonΔC mutant binds to DNA less well than wild-type Bt-Lon (Fig. 7, *lanes 2* and *3*). Additionally, the Bt-LonΔN mutant that is defective in oligomerization appeared to react with DNA (Fig. 7, *lane 4*), whereas Bt-LonN316 fails to bind DNA (Fig. 7, *lane 5*). These results initially demonstrated that the domain of Bt-Lon required for DNA binding is localized between residues 317 and 779. Further definition of the DNA binding domain was obtained through four overlapping truncated mutants that were tested for DNA binding activity in GMSA (Fig. 7, *lanes 6–9*). The data showed that Bt-LonATPase, Bt-Lon-C289, and Bt-LonSSD mutants appear to bind DNA (Fig. 7, *lanes 6, 8* and *9*), whereas Bt-Lon-(317–490) fails to bind DNA (Fig. 7, *lane 7*). Taken together, these results showed that the SSD is required for DNA binding.

Oligomerization of Bt-Lon Under Various Conditions—To understand the process of Bt-Lon oligomerization, the method of chemical cross-linking was used at various conditions. The results of chemical cross-linking with the homobifunctional amino-reactive reagent glutaraldehyde are shown in Fig. 8. Chemical cross-linking of Bt-Lon (4 μg) with 0.2% glutaraldehyde for 30 s resulted in the formation from monomers to hexamers (Fig. 8*A*, *lane 2*). Increasing the protein concentration led to an increase in hexamers but a decrease in monomers, trimers, and pentamers (Fig. 8*A*, *lane 1*), suggesting that Bt-Lon behaved as a concentration-dependent equilibrium of hexamer formation. Similarly, increasing the cross-linkage time resulted in an increase in hexamers (Fig. 8*A*, *lanes 3* and *4*). In addition, the cross-linkage resulted in the predominance of dimers, tetramers, and hexamers, revealing that the monomer is not highly populated or that it does not participate in the formation of oligomers larger than dimers. Based on the above results, we propose that there is little monomer in the solution and that Bt-Lon initially exists as a tightly bound dimer, which is apparently prone to further oligomerization. To test this hypothesis, a cross-linking experiment was performed in the presence of increasing concentrations of NaCl. At a concentration of >0.2 M NaCl, bands representing the dimer and tetramer forms of Bt-Lon began to increase (Fig. 8*B*, *lanes 1–4*). At a concentration of 2.0 M NaCl, the hexamer form of the protein almost disappeared, and the predominance of the dimer form was detected (Fig. 8*B*, *lane 4*). These results suggested that the dimer form of Bt-Lon is an initiator for oligomerization and that the association of the dimer to form the hexamer is mainly mediated by hydrophilic interactions. To test whether hydrophobic interactions are responsible for dimer formation, a cross-linking experiment was performed in the presence of increasing concentrations of SDS. At a concentration of >0.01% SDS, bands representing the hexamer form of Bt-Lon began to

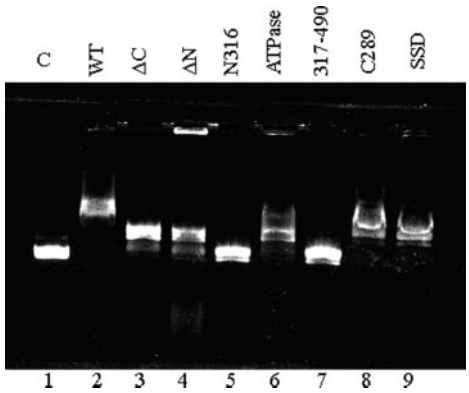

FIG. 7. **DNA binding activity of Bt-Lon truncated mutants.** 25 μl of the solution containing 4 μg of the affinity-purified proteins and 500 ng of plasmid DNA was incubated for 20 min and then subjected to a GMSA as described under "Experimental Procedures." The mutants proteins assayed are indicated *above* the gel as described in Fig. 1*B*. *C*, control experiment, indicates that the reaction solution is identical as above except it is lacking the protein.

decrease (Fig. 8*C, lanes 1–3*). Furthermore, increasing the SDS concentration led to an increase in monomers (Fig. 8*C, lanes 4–8*), and Bt-Lon hexamers were completely disassociated into monomers in the presence of 0.1% SDS (Fig. 8*C, lane 7*), suggesting that the hydrophobic interaction is responsible for the formation of the dimer.

DISCUSSION

The N-terminal Domain of Lon Proteases Is Essential for Oligomerization—The Lon proteases are members of the ATP-dependent proteases of AAA^+ superfamily and possess the oligomeric structure that is required for their activities. Regarding the N-terminal domain, Roudiak and Shrader (5) have demonstrated that three N-terminal truncations of Lon protease from *Mycobacterium smegmatis* (Ms-Lon) lacking 90, 225, and 277 residues (named Ms-Lon N-G91, N-E226, and N-I278, respectively) revealed different behaviors toward their functions and quaternary structures. For example, Ms-Lon N-G91 and N-E226 retained peptidase activities against small unstructured peptides and basal ATPase activities. In addition, their quaternary structures were not altered drastically. Ms-Lon N-I278, however, displayed neither peptidase nor ATPase activity and behaved as a higher multimeric state despite the fact that it was stable and soluble in solution. To investigate further the role of the N-terminal domain, we constructed a truncated mutant, Bt-LonΔN, with a deletion of 316 residues in the N terminus, corresponding to Ms-Lon N-E305 that has 27 amino acid residues less than N-I278 (refer to the alignment in Fig. 1*C*). Analytical gel filtration chromatography and sedimentation velocity experiments reveal that the mutant, Bt-LonΔN, has defects in oligomerization, whereas the other mutant, Bt-LonΔC (residues 1–605), can form a hexamer. These results indicated that the N-terminal domain is essential for oligomerization, and the C-terminal protease domain is dispensable. These findings are also consistent with the previous report (49) that the proteolytic domain of Lon protease does not undergo oligomerization. Moreover, Roudiak and Shrader (5) showed that the quaternary structure of Ms-Lon N-I278 is altered drastically. By aligning homologous regions of Bt-Lon, *E. coli* Lon, and Ms-Lon (Fig. 1*C*), we propose that the residues 290–316 within the Bt-Lon N-terminal domain, called oligomerization domain, is involved in the oligomerization of Bt-Lon. In order to support this model, we have presented our data that the N-terminal domain of Bt-Lon (Bt-LonN316) itself could form a stable octameric structure. Several observations also support this model. First, FtsH, a membrane-bound and ATP-dependent protease, has a homohexameric structure (50, 51). The N-terminal region of FtsH is also important for its oligomerization (50). Second, N-G91 and N-E226 mutants of Ms-Lon displayed that the interaction of inter-subunits was altered, but their quaternary structures remained the same (5). The deleted regions do not contain the oligomerization domain. Finally, despite Lon proteases belonging to the AAA^+ superfamily of ATPase, their oligomerization is not modulated by ATP binding (8–10) but is facilitated by Mg^{2+} and unfolded proteins (10). On the contrary, Clp/HSP100 proteins, also belonging to the AAA^+ superfamily (3), form a hexameric structure in the presence of ATP (12–15, 52). This is because the Arg residue in the GAR motif of the C-terminal domain acts as an Arg finger by contacting the ATP bound to an adjacent subunit (32). Thus the C-terminal domain of Clp/HSP100 proteins is essential for the oligomerization. The results presented here may explain why the oligomerization of Lon protease is ATP-independent.

Oligomerization of Bt-Lon Is a Prerequisite for Its Catalytic and Chaperone-like Activities—We have shown that Bt-LonΔN is unable to oligomerize and is devoid of proteolytic and chaperone-like activities, suggesting that a multimeric Bt-Lon is clearly required for the ability to bind unfolded proteins and then either prevent them from aggregation or degrade them. Bt-LonΔN with defects in oligomerization also exhibited little or no ATPase activity. What is the reason for the loss of the ATPase activity in Bt-Lon upon removal of the N-terminal domain? The crystal structure of the AAA domain of FtsH provides a possible explanation (53, 54). The ATPase activity of Bt-Lon is influenced by oligomerization, because ATP is bound at the interface of two neighboring subunits in the hexamer. Consistently, the peptidase and ATPase activities of Ms-Lon are inhibited by urea-induced dissociation of oligomer (10). On the other hand, the truncated mutant, Bt-LonΔC, retains ATPase activity but only exhibits 20% activity of the wild type. The decrease in ATPase activity of the Bt-LonΔC mutant may be due to the unstable quaternary structure of the mutant by the deletion of C-terminal domain, implying that the C-terminal domain maintains and stabilizes the quaternary structure of Bt-Lon. This explanation could be supported by previous reports described as follows. The *CapR9* mutant of Lon from *E. coli* retains the oligomeric structure but dissociates into dimers and monomers more readily than the wild type (55), which shows about 50% of the wild type in peptidase and ATPase activities (2). The *CapR9* mutant was later proved to carry a single amino acid mutation (E614K) on its C-terminal domain, which has a higher isoelectric point than that of the wild type (55). Judging from the three-dimensional structure of the proteolytic domain of *E. coli* Lon, the point mutation (E614K) is located at β-sheet 1 that plays an important role in forming the subunit interface (56). The crystal structure data also showed that the subunit interface is characterized by mostly hydrophilic interactions (56), implying that the hydrophilic interactions among subunit interfaces stabilize the oligomerization. In our experiments, Bt-LonN316 mutant lacking the C-terminal domain forms an octamer as the major species and a dimer as the minor one by analytical gel filtration chromatography. This result also implies that the C-terminal domain may stabilize the quaternary structure of Bt-Lon.

The N-terminal Domain May Possess Functions Other Than Oligomerization—Although we cannot rule out the possibility that the loss of the proteolytic and chaperone-like activities of Bt-LonΔN is due to the deletion of substrate binding regions, our findings show that these activities and oligomerization are tightly coupled. Indeed, two mutants, Bt-LonΔC and Bt-LonN316, have multimeric structures and retain the chaper-

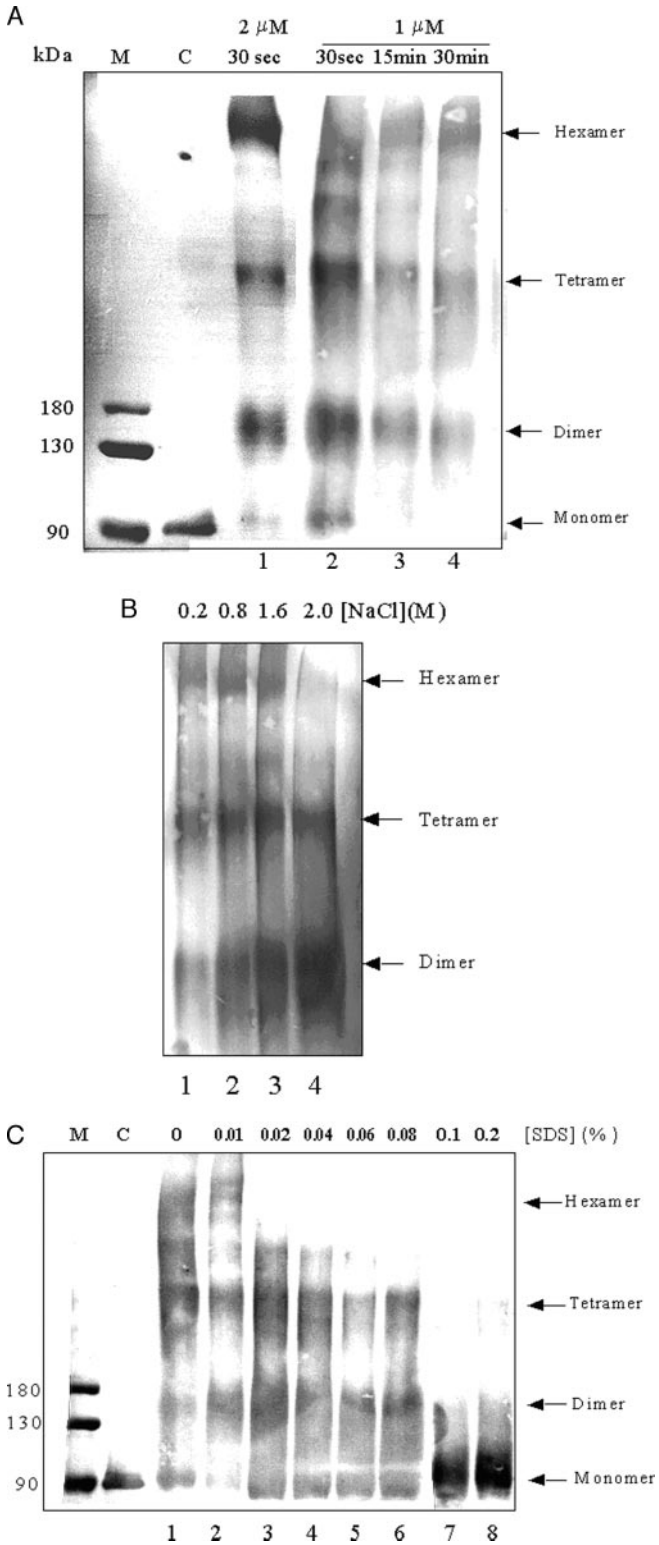

FIG. 8. **Oligomerization nature of Bt-Lon revealed by chemical cross-linking.** *A*, 2 (*lane 1*) or 1 μM (*lanes 2–4*) Bt-Lon were incubated in buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$. Cross-linking reactions were initiated by addition of 0.2% glutaraldehyde and proceeded at 30 °C for 30 s (*lanes 1* and *2*), 15 min (*lane 3*), or 30 min (*lane 4*). Cross-linked products were analyzed by SDS-PAGE (4–12%) followed by Western blot analysis. Incubation of Bt-Lon in the absence of cross-linker (glutaraldehyde) served as control (*lane C*). *Lane M*, molecular mass markers. Cross-linked proteins ran at the appropriate molecular weight for monomer, dimer, tetramer, and hexamer as shown by *arrows* in the figure. Positions of molecular mass markers are shown. *B*, Bt-Lon (4 μg) was incubated in buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$ in the presence of increasing concentrations of NaCl ranging from 0.15 to 2 M (*lanes 1–4*). Cross-linking reactions were performed as described above except they were incubated at 30 °C for 30 s. Cross-linked proteins ran at the appropriate molecular weight for dimer, tetramer, and hexamer as shown by *arrows* in the figure. *C*, Bt-Lon (4 μg) was incubated in the absence (*lane 1*) or the presence of increasing concentrations of SDS ranging from 0.01 to 0.2% (*lanes 2–8*). Cross-linking reactions were performed as described in *B*. Incubation of Bt-Lon in the absence of cross-linker served as control (*lane C*). *Lane M*, molecular mass markers. Cross-linked proteins ran at the appropriate molecular weight for monomer, dimer, tetramer, and hexamer as shown by *arrows* in the figure. Positions of molecular mass markers are shown.

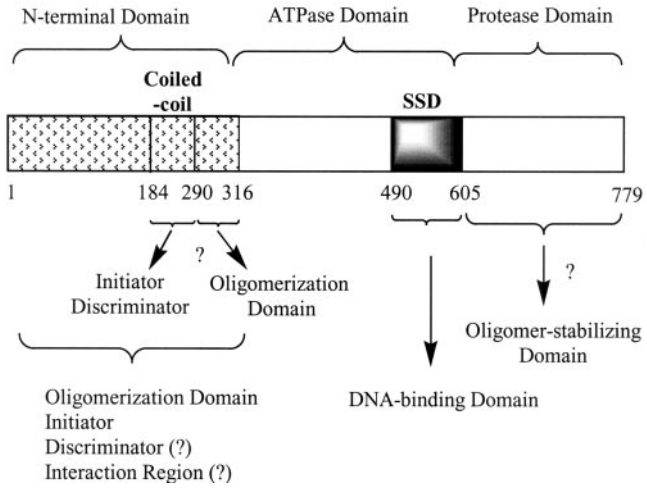

FIG. 9. **Model diagram for domain organization and related functions of Bt-Lon.** Bt-Lon consists of an N-terminal domain, an ATPase domain including an SSD, and a C-terminal protease domain. The amino acid position of each domain is indicated. The N-terminal domain appears to be essential for oligomerization and is the interaction site of unfolded nonspecific substrates. By aligning homologous regions of Bt-Lon and Ms-Lon (5) (Fig. 1*C*), residues 290–316 within the N-terminal domain are proposed as the oligomerization domain that is involved in the oligomerization of Bt-Lon. The C-terminal domain is assumed to maintain and stabilize the oligomeric complex of Bt-Lon. Lon protease has two substrate-binding sites, one for specific substrates (discriminator site) and one for nonspecific substrates (initiator site), as described previously (7, 57). In an expansion of this model, the N-terminal domain of Lon protease has at least four functions: initiator, discriminator, domain-domain interaction, and oligomerization. The initiator and discriminator regions may be located in the putative coiled-coil region of the N-terminal domain. Finally, the SSD is proved to be a DNA binding domain of Bt-Lon. The *question mark* indicates that the function of the domain has not yet been demonstrated.

one-like activity. This result indicates that the N-terminal domain of Bt-Lon itself partially retains the chaperone-like activity and should interact with unfolded nonspecific substrates. Goldberg and co-workers (7, 57) proposed a model for the mechanism of substrate degradation by ATP-dependent protease Lon (reviewed in Refs. 58 and 59). According to this model, Lon protease has two substrate-binding sites, one for specific substrates (discriminator site) and another for nonspecific substrates (initiator site). If Lon protease has two binding sites, then what do these sites occupy? On the one hand, it has been proposed that the substrate discriminator site is located at the N-terminal domain (5, 6). However, Smith *et al.* (35) demonstrated that a small α-domain, present in most AAA^+ modules, acts as a "sensor and substrate discrimination" domain. On the other hand, both peptidase and ATPase activities of Lon proteases are stimulated by unfolded proteins such as α-casein (47, 60). The existing working model of Lon proteases indicates that the α-casein interaction site also resides in the N-terminal domain (5, 61), which has been termed previously the allosteric site (or initiator site) (57, 59).

The previous studies by Roudiak and Shrader (5) showed that the peptidase activities of N-terminal truncation mutants

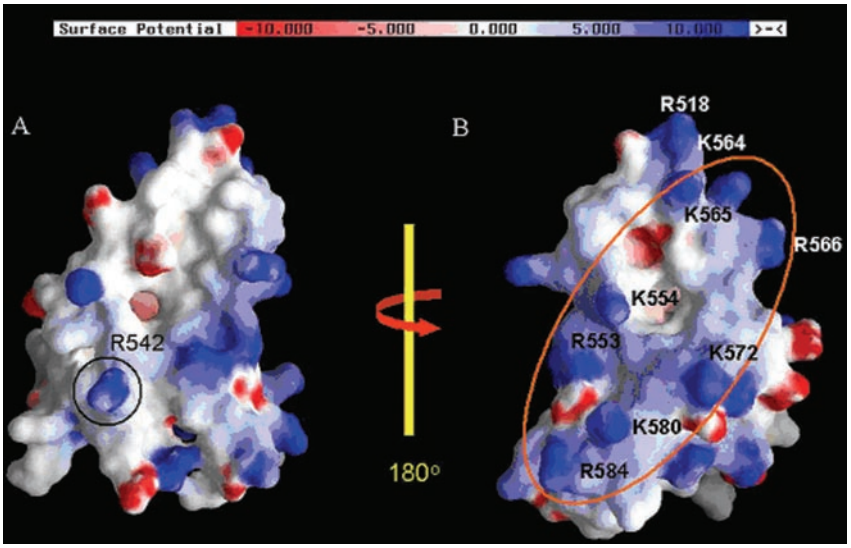

FIG. 10. **The electrostatic surface potential of Bt-LonSSD.** Interior surface view (*A*) and solvent-accessible surface view (*B*) of Bt-LonSSD are in the opposite orientation. Areas of positive potential are *blue* and those with negative potential are *red*. *A,* the conserved sensor-2 Arg residue, a putative nucleotide-binding site, is shown in the area highlighted with a *black circle*. *B*, the clustering of positively charged residues is also shown in the area highlighted with an *orange ellipse.* The diagram was generated by the program GRASP (43).

of Ms-Lon (N-G91 and N-E226) were stimulated by unfolded proteins but not ATPase activities. This finding implies that the N-terminal domain not only interacts with unfolded proteins but also communicates with other functional regions such as the AAA^+ module. This inference parallels the findings that conformational changes in Lon holoenzyme induced by nucleotides or protein substrates modulate the functional activities through domain-domain interactions (5, 8, 30, 46). Accordingly, we propose that the N-terminal domain of Lon proteases is the region for at least four functions as follows: initiator, discriminator, domain-domain interaction, and oligomerization (Fig. 9). The initiator is responsible for the first binding of unfolded proteins and the discriminator for the recognition of specific substrates. The initiator and discriminator regions may be located in the putative coiled coil region of the N-terminal domain (Fig. 9) (6). The interaction region interacts with the oligomerization domain, AAA module, or other regions. In response to substrate binding, these subdomains may undergo changes in conformation and orientation. Transduction of the mechanical motions within the N-terminal domain to bound substrates provides the driving force for various functions, including ATP/ADP exchange, unfolding of target proteins, translocation of proteins to a linked proteolytic domain, and coordinated stabilization of the oligomerization domain. Subsequently, conformational changes in the AAA^+ module may affect subunit interfaces within the proteolytic domain through the SSD (56). This model provides a possible explanation for how α-casein stimulates catalytic activities and oligomerization of Lon proteases. Finally, further studies such as three-dimensional structural information are required to identify the proposed regions of the N-terminal domain.

SSD and DNA Binding—The results presented here have shown that the SSD is a DNA-binding site for Bt-Lon. Several features in the SSD attracted our attention as motifs potentially involved in DNA interaction. A recently determined x-ray structure of SSD revealed that it consists of three intact α-helices, a partial α-helix and two β-strands, arranged in order H1-S1-H2-H3-W1-S2-H4, in which the second β-strand loops back to form a parallel β-sheet with the first strand, designated as W1 (wing, see below) (62). No obvious similarities to the canonical helix-turn-helix of known DNA-binding proteins were observed. Most interesting, we found that the winged helix (WH) motif, another DNA-binding motif, also has a similar topology, H1-S1-H2-H3-S2-W1-S3-W2. The WH family is a large and diverse family of the helix-turn-helix DNA-binding proteins. Each monomer of a WH protein consists of a helix-turn-helix motif followed by one or two β-hairpin wings (63, 64). In WH proteins, H3, the recognition helix, typically lies in the major groove and makes most of the sequence contacts with DNA. Similarly, the structural data showed that helix 3 of Bt-LonSSD, together with the beginning part of strand 1 and 2 and helix 4, forms a concave surface that is highly positively charged (Fig. 1*C* and Fig. 10). This highly positively charged surface is a cluster of basic residues including Arg-518, Lys-564, Lys-565, Lys-554, Arg-553, Arg-546, Arg-566, Lys-572, Lys-580, and Arg-584 (Fig. 9). A recently determined crystal structure of Lon proteolytic domain also showed that this highly positively charged surface faces the solvent (56) (Fig. 10). Consistently, several AAA^+ proteases like HslU and ClpA were found in which their small C-terminal domain of each monomer faces either the outside of an adjacent monomer or the solvent (33, 34). On the other hand, the WH domains of Cdc6 and RuvB were suggested to directly mediate DNA binding (32), which are linked to the C terminus of the AAA^+ modules. Therefore, the SSD may not be directly involved in the recognition of protein substrates, but it is involved in the DNA recognition for assistance in contacting those DNA-binding proteins as substrates.

Nature of Bt-Lon Oligomerization—As shown previously (11), analytical gel filtration and chemical cross-linking experiments have revealed that Bt-Lon is a hexameric structure, which is also strongly supported by the proteolytic domain of Lon in the crystal assembly into a hexameric ring (56). However, the exact nature for the formation of the Lon hexamer remains unclear. Recently, the studies by Shrader and coworkers (10) suggest that Mg^{2+}-linked oligomerization of Ms-Lon has two possible models: dimer ↔ tetramer ↔ hexamer and trimer ↔ hexamer. The present data suggest that the process of Bt-Lon oligomerization favors the dimer ↔ tetramer ↔ hexamer assembly model, based on the results from cross-linking experiments (see Fig. 8*A*). Our results also suggest that the Bt-Lon monomer is minimally present in solution and that the dimer of Bt-Lon is an initiator for oligomerization. In general, the two subunits in the dimer contact each other by helix-packing interactions involved in hydrophobic interactions between two adjacent helices. Indeed, the cross-linking experiment performed in the presence of an increasing concentration of SDS shows that the hydrophobic interaction is important for the dimerization of Bt-Lon. Taken together, these results show that ionic interactions are responsible for hexamer assembly and that hydrophobic interactions are major forces for the formation of the dimer. Finally, it is suggested that the equi-

librium between dimeric and hexameric forms of Bt-Lon may regulate its biological activities.

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