Defining the crucial domain and amino acid residues in bacterial Lon protease for DNA binding and processing of DNA-interacting substrates

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Lon protease previously has been shown to interact with DNA, but the role of this interaction for Lon proteolytic activity has not been characterized. In this study, we used truncated *Escherichia coli* Lon constructs, bioinformatics analysis, and site-directed mutagenesis to identify Lon domains and residues crucial for Lon binding with DNA and effects on Lon proteolytic activity. We found that deletion of Lon's ATPase domain abrogated interactions with DNA. Substitution of positively charged amino acids in this domain in full-length Lon with residues conferring a net negative charge disrupted binding of Lon to DNA. These changes also affected the degradation of nucleic acid-binding protein substrates of Lon, intracellular localization of Lon, and cell morphology. *In vivo* tests revealed that Lon-DNA interactions are essential for Lon activity in cell division control. In summary, we demonstrate that the ability of Lon to bind DNA is determined by its ATPase domain, that this binding is required for processing protein substrates in nucleoprotein complexes, and that Lon may help regulate DNA replication in response to growth conditions.

Many proteins exhibit their activity while forming complexes with nucleic acids. The formation of nucleoprotein complexes is important for instance for DNA replication, transcription, and DNA repair (1–4). It has been postulated that formation of nucleoprotein complexes by proteases can influence their activity (5–9). However, there is very limited data concerning the structural basis and molecular mechanisms involved in the interactions of proteases with DNA.

One of the most abundant proteases, with homologues present in both prokaryotic and eukaryotic cells, is Lon. It is responsible for degradation of damaged or misfolded proteins as well as influencing many cellular processes through proteolysis of natively folded regulatory proteins. Lon protease is considered as a proteasomal quality control protease, and it affects such processes as DNA replication, DNA methylation, cell division, virulence, sporulation, motility, and biosynthetic pathways in bacteria (10) and mitochondrial DNA maintenance (11), aging, neurodegenerative diseases (12), and cancerogenesis (11, 13, 14) in eukaryotes. It is composed of three domains: an N-terminal domain (domain N) responsible for oligomerization and interactions with the substrate (15–17), a central ATPase domain (domain A) containing an AAA+ module with α/β- and α-subdomains (18), and a C-terminal proteolytic domain (domain P) engaged in substrate degradation (19). Lon protease functions as an oligomer. Electron microscopy of *Escherichia coli* Lon (*Ec*Lon)[5] (20) and analytical ultracentrifugation of *Mycobacterium smegmatis* Lon (21) showed that bacterial Lon is a hexamer, whereas analysis of the *Saccharomyces cerevisiae* Lon homologue Pim1 revealed it to be a heptamer (22). Recent studies of *Ec*Lon showed that it can also form dodecameric structures; however, those, when compared with the *Ec*Lon hexamers, have been proposed to be less active in degradation of large substrates (23). Oligomerization of Lon protease was shown to be dependent on Mg^{2+} (20, 21), and recent data obtained for *Meiothermus taiwanensis* Lon indicated a role for Mg^{2+} in substrate-binding loop remodeling and activation of Lon (24).

Protein degradation by Lon is dependent on energy obtained from ATP hydrolysis. It can be stimulated by the presence of unfolded proteins (*e.g.* apomyoglobin, glucagon, and α-casein) (25, 26) as well as inorganic polyphosphate accumulated during amino acid starvation (7). The Lon protease was also found to form nucleoprotein complexes with DNA (8, 17, 27–29) and RNA (human Lon) (30). The binding of *Ec*Lon to DNA is sequence-unspecific (8); however, there is a set of biochemical

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[5] The abbreviations used are: *Ec*, *E. coli*; *Ms*, *M. smegmatis*; SPR, surface plasmon resonance; SUMO, small ubiquitin-like modifier; EIA, enzyme immunoassay; TMB, tetramethylbenzidine; MBP, maltose-binding protein.

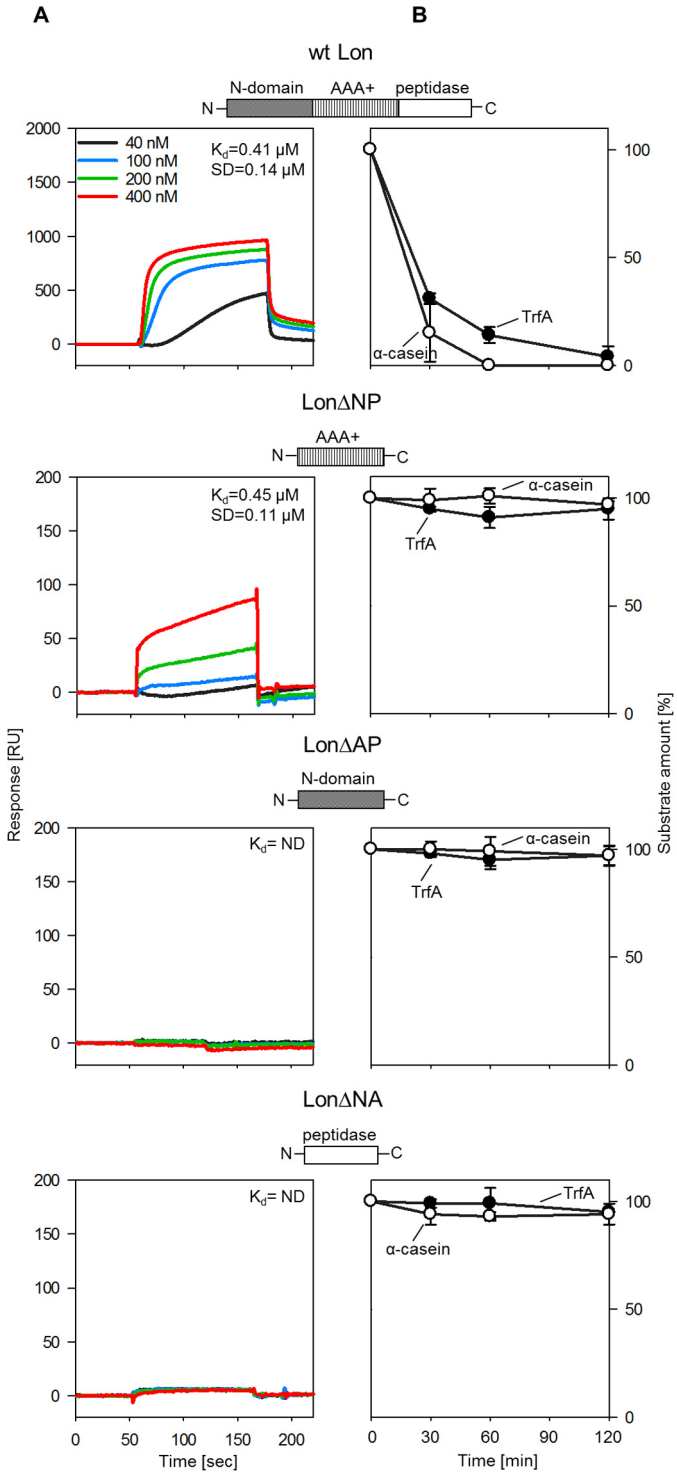


Figure 1. ATPase domain of *E. coli* Lon protease interacts with DNA. *A*, the results of SPR analysis of DNA binding by WT Lon and truncated forms of Lon protease containing single domains, LonΔNP accounting for ATPase domain, LonΔAP accounting for N domain, and LonΔNA accounting for peptidase domain. Sensorgrams show the results of binding of each depicted Lon variant to a double-stranded DNA fragment containing nonspecific sequence of pUC19 plasmid. Injections contained the indicated concentrations of Lon variants in running buffer supplemented with 10 mM $Mg(OAc)_2$ and 2 mM ATP. The assay was performed in triplicate, and representative sensorgrams are presented. The results were analyzed using BIAevaluation software version 3.2. Kinetic (K_d) measurements were performed using a separate K_a/K_d fit algorithm (local fitting algorithm). For LonΔAP and LonΔΔNA, K_d was undetermined (*ND*) with the software used. *B*, TrfA and α-casein *in vitro* proteolysis by the noted Lon variants. Each substrate (1.5 μg) was incubated in the reaction buffer (total reaction volume of 25 μl) with each Lon variant (1.5 μg) for 2 h at 32 °C. TrfA-containing reaction mixtures were supplemented with 250 ng of supercoiled plasmid pKD19L1. Reactions were stopped at certain time points by the addition of Laemmli buffer. Samples of 10 μl were run on SDS-PAGE followed by Coomassie staining and densitometric analysis of the amount of TrfA and α-casein. Each experiment was repeated three times, and the mean values with standard deviations (*error bars*) are presented as graphs. *RU*, response units.

assays showing that *Ec*Lon might bind preferentially to TG-rich DNA promoter elements (28). Similar sequence preferences (TG-rich) were observed for eukaryotic Lon proteases with the only difference being that mouse and human Lon bind to single-stranded DNA (27, 31), whereas the bacterial proteases interact with double-stranded DNA (dsDNA). The formation of nucleoprotein complexes with DNA can stimulate ATPase activity (8, 29) of bacterial Lon, and this interaction can also influence substrate degradation. It was shown that protease binding to DNA can stimulate substrate degradation (6, 9).

Despite years of research concerning Lon-DNA interaction, there are still little data showing the region and particular amino acid residues of Lon engaged in nucleoprotein complex formation. Different subdomains for DNA interaction were proposed in *Ec*Lon (32) and in *Brevibacillus thermoruber* Lon (17, 33, 34). The role of this interaction for degradation of substrates is also elusive.

In the presented work, by performing *in silico*, biochemical, and *in vivo* mutant analyses, we identified amino acid residues in *Ec*Lon engaged in interaction with DNA. Lon mutants, defective in DNA interaction, revealed that protease interaction with DNA is required for degradation of substrates that interact with DNA but not for α-casein or *E. coli* IbpB. Moreover, we determined that *E. coli* strains expressing Lon mutants defective in DNA interaction have the same phenotype as Lon-deficient strains, thus showing the essentiality of Lon interaction with DNA.

Results

EcLon ATPase domain interacts with DNA

We previously demonstrated that the replication initiator protein TrfA of RK2 plasmid is degraded by Lon protease only in the presence of DNA (6). Although we have shown that TrfA-DNA interaction is essential for proteolysis by Lon, it is not clear whether and how Lon-DNA interaction affects the processing of nucleoprotein complexes formed by Lon substrates. To address these questions, we decided to search for DNA-binding sites within the *Ec*Lon protease structure and to construct Lon mutants defective in DNA binding.

Lon protein from *E. coli* consists of three functional domains. To narrow down the range of potential DNA-binding sites within the protein, we constructed and purified three truncated variants of Lon protease, LonΔNP, LonΔAP, and LonΔNA, each containing only a single full-length domain: ATPase domain, N domain, and peptidase domain, respectively. The analysis of isothermal circular dichroism spectra indicated the maintenance of secondary structures of the purified truncated mutants (supplemental Fig. S1A). Surface plasmon resonance (SPR) was used to analyze the interaction of truncated Lon variants with a 129-bp linear double-stranded DNA fragment con-

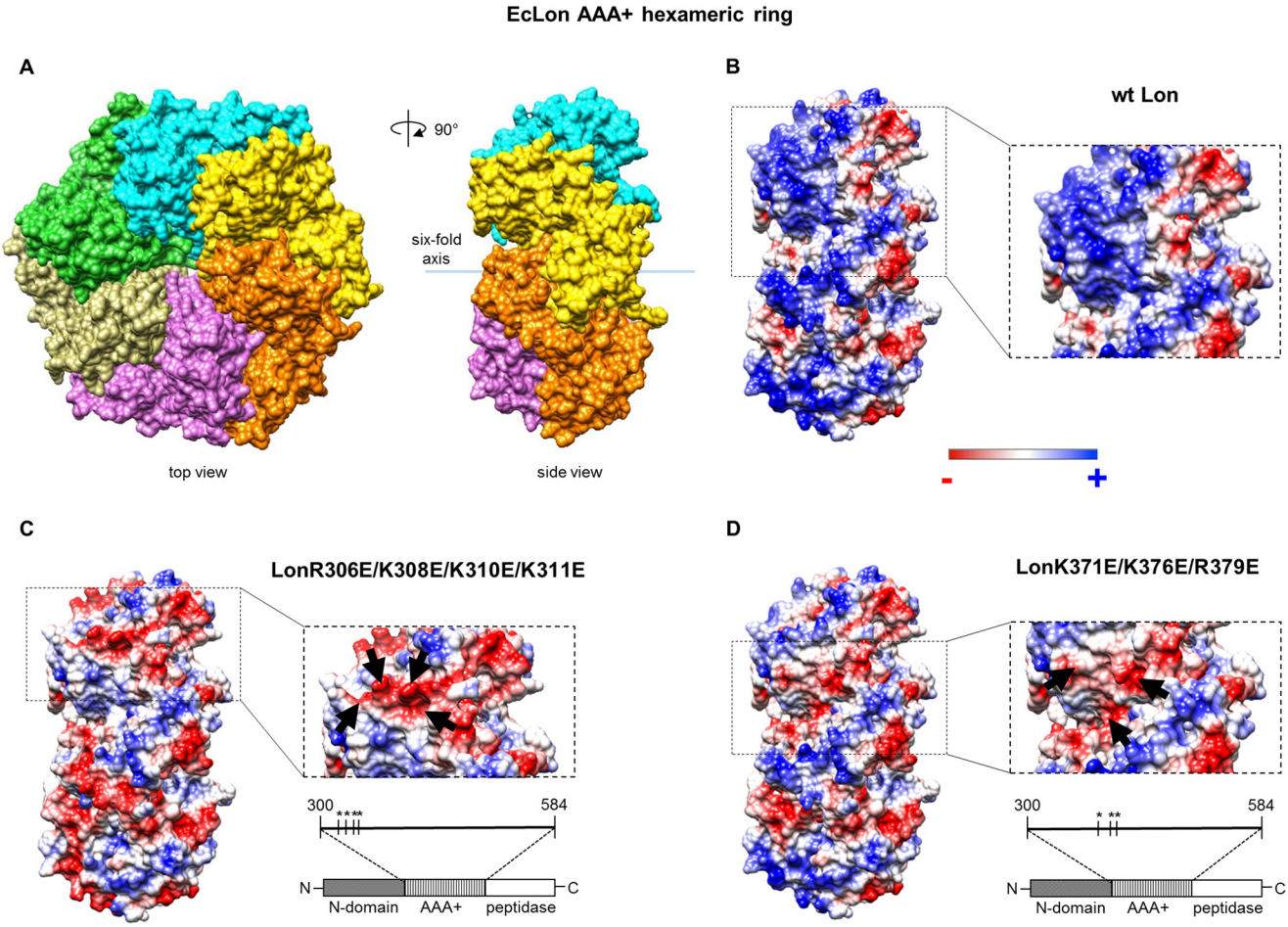


Figure 2. Surface representations of the hexameric ATPase domain of *Ec*Lon protease. A model of the hexameric ring of the ATPase domain of *E. coli* Lon protease was created as described under "Experimental procedures." Surface representation of the ATPase domain was rendered in UCSF Chimera. Electrostatic potential surfaces of Lon variant proteins are depicted with positive charge in *blue*, negative charge in *red*, and neutral charge in *white*. *A*, surface representation showing the top and side views of the hexameric ATPase domain of the *E. coli* Lon protease model. Each monomer is shown in a different color. Shown are side views of the surface charge representation of the ATPase hexameric ring of WT Lon (*B*) and quadruple (*C*) and triple (*D*) mutant variants of *E. coli* Lon protease containing the indicated substitutions, which replace positively charged amino acids with negative glutamic acid residues (*asterisks*).

taining a nonspecific sequence of pUC19 plasmid (Fig. 1*A*). As a control, we used full-length Lon protein. The results demonstrated that Lon and LonΔNP proteins were able to interact with DNA immobilized on a sensor chip (with use of a local fitting algorithm, K_d values were 0.41 ± 0.14 μM for Lon and 0.45 ± 0.11 μM for LonΔNP, and with use of a global fitting algorithm, K_d values were 0.14 ± 0.03 and 0.05 ± 0.05 μM, respectively), whereas we did not observe any binding response after the injections of LonΔAP and LonΔNA proteins (Fig. 1*A*). This implies that the DNA-binding site is located within the ATPase domain. Binding responses in the SPR technique are proportional to the increase of molecular mass on the sensor chip surface. Therefore, in the performed experiment (Fig. 1*A*), the binding response for LonΔNP protein is lower than for the same amount of wild-type Lon due to its lower molecular weight and possible perturbations in the oligomeric state.

In addition to the analysis of nucleoprotein complex formation, we also tested degradation of TrfA in the presence of DNA and the truncated Lon variants. In a control reaction, TrfA was incubated with full-length Lon protease in the presence of DNA. Samples taken at specific time points were analyzed electrophoretically, and the quantity of TrfA protein was estimated by densitometry and presented in graphs (Fig. 1*B*, *closed circles*). As we demonstrated previously (6), when wild-type Lon was present in the reaction mixture, we observed a substantial decrease in the amount of TrfA after 2 h of incubation. None of the truncated Lon variants were able to process either TrfA or α-casein (Fig. 1*B*) during the course of our *in vitro* test.

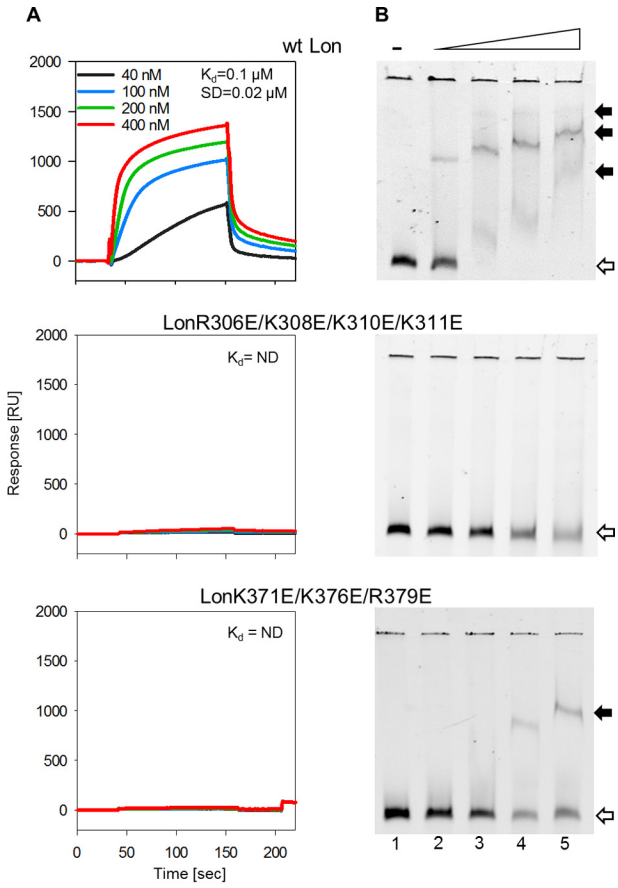
Surface charge of the ATPase domain of Lon affects interaction with DNA

Knowing that the DNA-binding site of the *E. coli* Lon protease is located within the ATPase domain but lacking crystallographic data on the full domain structure, we constructed a model of hexameric ATPase domain of *Ec*Lon protease using the comparative modeling approach (35) by combining the crystal structure of the *Ec*Lon C-terminal domain with a model of the central domain based on Lon structures from other organisms (see "Experimental procedures" for details).

The electrostatic surface potential of the ATPase hexamer model is shown in Fig. 2*B*. We hypothesized that the positively

charged patches over the surface of the Lon ATPase domain could potentially serve as the binding site for DNA. Based on the constructed model, we selected positively charged amino acid residues on the ATPase domain surface and, by means of site-directed mutagenesis, constructed full-length Lon mutants containing arginine/lysine-to-glutamic acid substitutions (Fig. 2, *C* and *D*; all of the constructed mutants are listed in supplemental Table S2). These substitutions change the charge from positive to negative on the surface of the Lon protein. Lon mutant proteins were purified and tested for DNA-binding ability. SPR analysis revealed the most pronounced effect on DNA interaction for the quadruple and triple Lon mutants R306E/K308E/K310E/K311E and K371E/K376E/R379E, respectively (K_d for Lon was 0.1 ± 0.02 μM with use of a local fitting algorithm and 0.14 ± 0.03 μM with use of a global fitting algorithm; for Lon mutants, K_d was undetermined) (Fig. 3*A*). In comparison with the wild type, both mutant Lon proteins were severely impaired in nucleoprotein complex formation with DNA immobilized on a sensor chip. To further confirm this result, we used an electrophoretic mobility shift assay (EMSA) with a 420-bp-long dsDNA probe. In the control reaction with wild-type Lon, we observed several shifted bands corresponding to the nucleoprotein complexes formed by Lon with the analyzed DNA fragment (Fig. 3*B*). Under the same experimental conditions, both LonR306E/K308E/K310E/K311E and LonK371E/K376E/R379E demonstrated significantly reduced binding capabilities. We did not detect any retarded bands in experiments with LonR306E/K308E/K310E/K311E (Fig. 3*B*). CD spectra analysis did not indicate substantial differences in the secondary structure content of the mutant proteins and wild-type Lon (supplemental Fig. S1B). Additionally, we introduced substitutions R306E, K308E, K310E, and K311E into the truncated Lon variant (LonΔNP) containing only the ATPase domain. We tested the purified mutant protein for the DNA-binding ability using SPR and EMSA analyses. As expected, the mutant ATPase domain was unable to form a nucleoprotein complex with DNA (supplemental Fig. S2), although it retained the ability to hydrolyze ATP (supplemental Fig. S2C). Because the effects of triple substitutions in the full-length Lon protein on the protease interaction with DNA in EMSA tests were not as pronounced as for the quadruple mutant (Fig. 3*B*), LonΔNP with triple mutations was not investigated.

Under normal growth conditions, Lon is localized in the nucleoid (7, 36). Because our *in vitro* experiments demonstrated that both Lon mutants R306E/K308E/K310E/K311E and K371E/K376E/R379E were incapable of efficient interaction with DNA, we examined the localization of the Lon protein variants *in vivo*. We used a cellular fractionation assay (see "Experimental procedures") using the Lon-deficient *E. coli* strain BL21 carrying pLon plasmid coding for wild-type Lon or its derivatives coding for mutant Lon proteins. The results of the assay are shown in Fig. 3*C*. Up to 95% of the entire cellular content of wild-type Lon was localized within the DNA-containing fraction. The content of the Lon variants in DNA-containing fractions was reduced by ~40 and 30%, respectively, for LonR306E/K308E/K310E/K311E and LonK371E/K376E/R379E. The results indicate that these Lon mutants are defective in proper cellular localization, most probably due to their impaired ability to form a nucleoprotein complex.



C

Localization of Lon *in vivo*

Lon variant	Cellular fraction		SD
	DNA-containing	Cytoplasmic	
wt Lon	0.95	0.05	0.03
LonR306E/K308E/K310E/K311E	0.56	0.44	0.06
LonK371E/K376E/R379E	0.62	0.38	0.01

Figure 3. Mutations within ATPase domain of Lon influence the ability of the protease to interact with DNA. Analysis of nucleoprotein complexes formation involving DNA and WT Lon or mutant proteases containing the depicted substitutions was performed by real-time SPR analysis (*A*) and EMSA (*B*) in the manner described under "Experimental procedures." Increasing amounts of proteins (40, 100, 200, and 400 nM) were run over the surface of sensor chip SA with an immobilized dsDNA fragment of pUC19 (*A*) or incubated with 1 pmol of fluorescently labeled dsDNA and separated electrophoretically (*B*). Results of SPR analysis are presented as sensorgrams. The SPR results were analyzed using BIAevaluation software version 3.2. Kinetic (K_d) measurements were performed using a separate K_a/K_d fit algorithm (local fit). For Lon mutants, K_d was undetermined (*ND*) with the software used. Visualization of EMSA results was performed with the ChemiDoc MP system. *Black arrows* indicate nucleoprotein complexes, and *white arrows* indicate unbound DNA probe. Both assays were performed in triplicate, and representative results are shown. *C*, analysis of *in vivo* localization of Lon variants. *E. coli* BL21(DE3) cells carrying pLon plasmid or its derivatives coding for the noted Lon protein mutants were grown in LB medium supplemented with 0.02% (w/v) arabinose to an A_{600} of 0.6. Cytoplasmic and DNA-containing cellular fractions were collected as described under "Experimental procedures" and analyzed by SDS-PAGE followed by Western blotting and densitometric analyses. The value of 1 corresponds to total content of tested protein within cells. The mean values and standard deviations from three repeats of each experiment are presented. *RU*, response units.

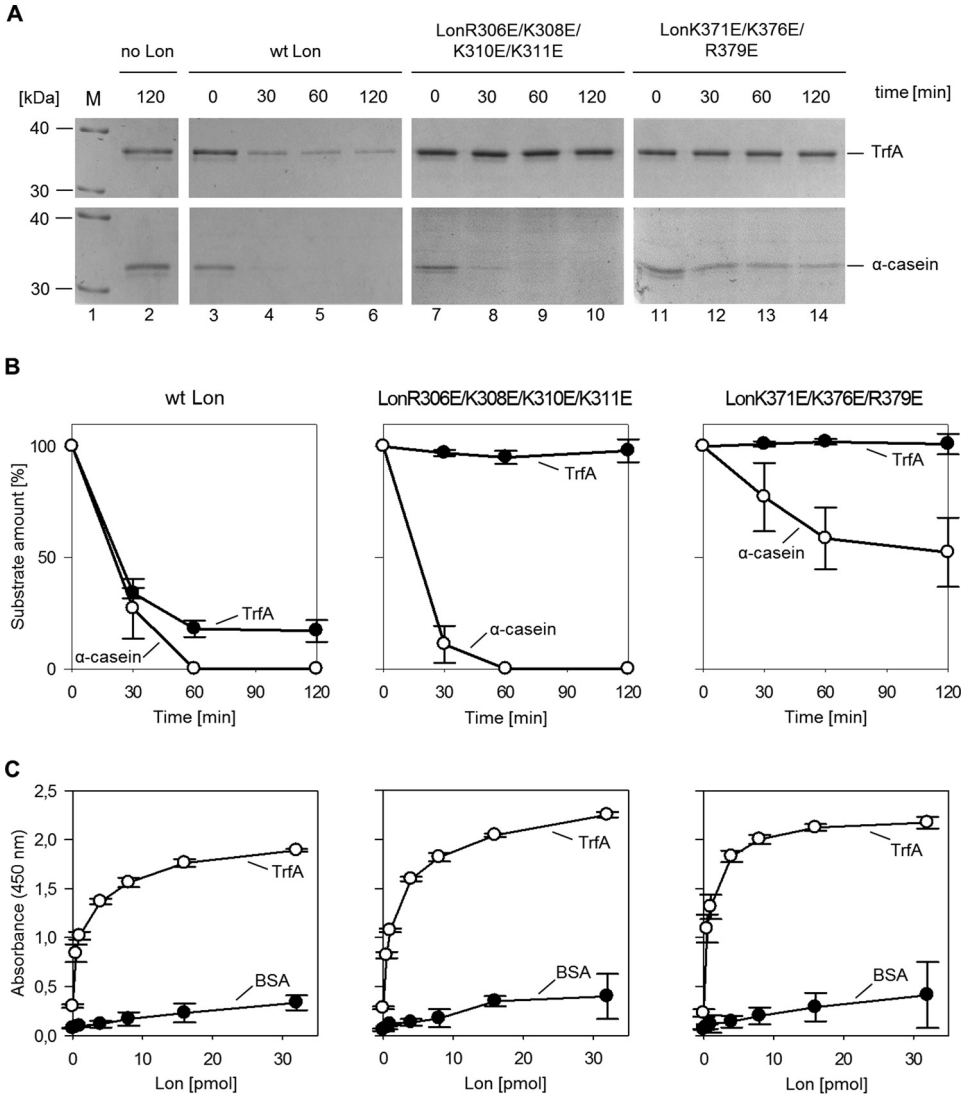


Figure 4. The DNA-binding defect of Lon protease affects TrfA proteolysis. *A* and *B*, the *in vitro* proteolysis of TrfA (in the presence of DNA) and α-casein by the noted Lon mutant proteins defective in DNA binding. Proteolysis was carried out as described under "Experimental procedures." The assay was performed in triplicate, and the results shown are representative gel images (*A*) and graphs plotted from the mean values with standard deviations (*B*) obtained after densitometric analysis of the amount of TrfA and α-casein at particular time points. *C*, TrfA binding by Lon variants. Interaction of WT Lon and mutant Lon variants with TrfA was analyzed by ELISA as described under "Experimental procedures." TrfA was immobilized on the plate, and increasing amounts of Lon variants were added. BSA was used as a negative control. Anti-Lon primary antibodies, goat anti-mouse IgG HRP-conjugated secondary antibodies, and TMB Peroxidase EIA Substrate kit were used to perform colorimetric detection. The graphs present the mean values with standard deviations (*error bars*) from three experiments.

Lon mutants defective in DNA binding are inefficient in degradation of Rep proteins

To test whether or not the DNA-binding ability of Lon protease affects its enzymatic activity, we analyzed TrfA degradation *in vitro* by LonR306E/K308E/K310E/K311E and LonK371E/K376E/R379E in the presence of DNA. The amount of TrfA remained constant after 2 h of incubation with both Lon mutants defective in DNA binding (Fig. 4, *A* and *B*, *closed circles*). In the control experiment, wild-type Lon was able to efficiently degrade TrfA (Fig. 4, *A* and *B*). We also tested the proteolytic activity of the Lon mutants toward α-casein, which itself does not bind DNA. Interestingly, LonR306E/K308E/K310E/K311E degraded α-casein as efficiently as wild-type Lon (Fig. 4, *A* and *B*, *open circles*), and it is characterized by even higher V_{max} and K_m values for FITC-casein degradation (supplemental Fig. S3). Proteolysis by the LonK371E/K376E/R379E mutant was less efficient, but still a decrease in the amount of α-casein in the reaction mixture was evident, and the V_{max} of FITC-casein degradation by the triple mutant was comparable with degradation by wild-type Lon (supplemental Fig. S3). Because α-casein has relatively little secondary or tertiary structure and is not a natural substrate for Lon protease, we decided to investigate the stability of IbpB protein from *E. coli* in the presence of the Lon variants. IbpB is a small heat-shock protein that assembles into large oligomers (37) and was reported to be a substrate for Lon protease (38). Our *in vitro* proteolysis experiments demonstrated that the efficiency of IbpB degradation by the mutant Lon proteins was comparable with the degradation of α-casein (Fig. 5). We observed that the LonR306E/K308E/K310E/K311E mutant degraded IbpB with

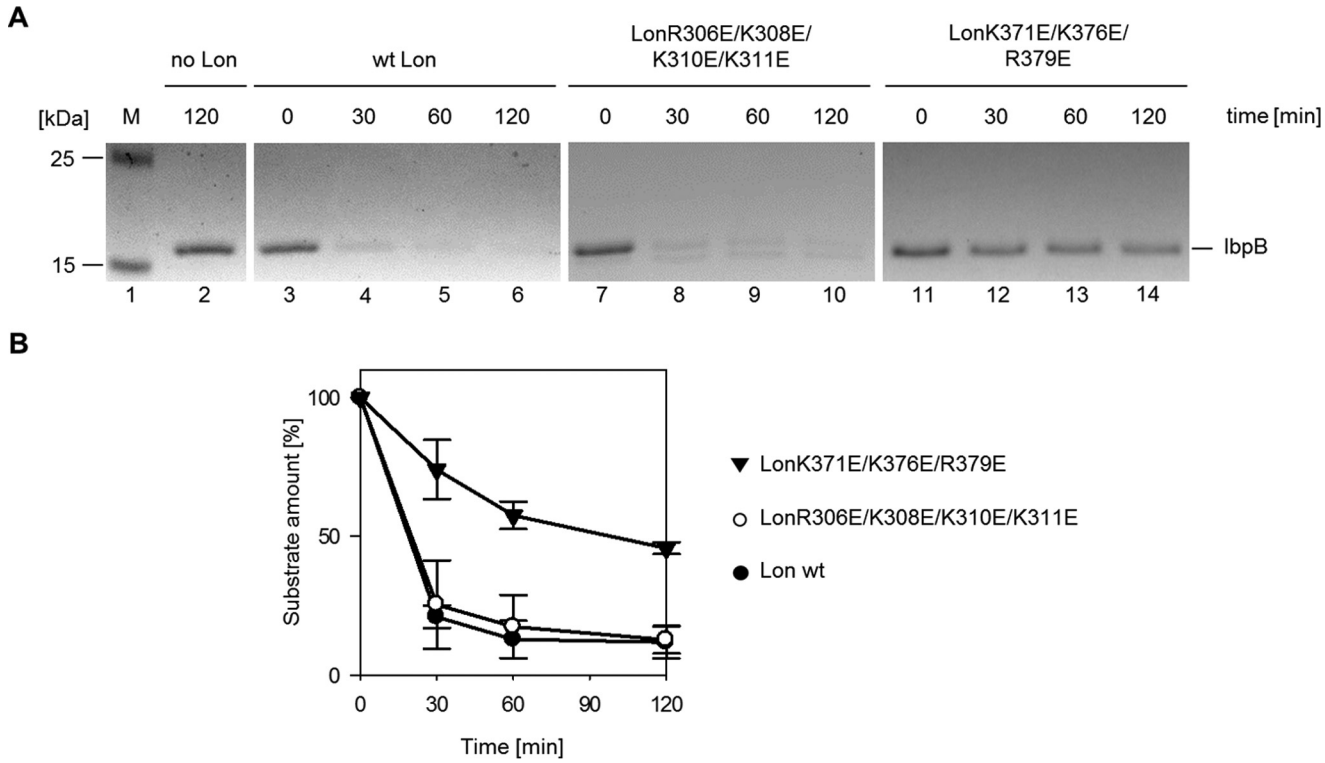


Figure 5. Lon protease variants containing substitutions within the ATPase domain retain the ability to degrade IbpB. A proteolytic assay utilizing the small heat-shock protein IbpB from *E. coli* as the substrate for Lon variants was performed as described under "Experimental procedures" and in the legend to Fig. 1*B*. The assay was repeated three times; the results shown are representative gel images (*A*) and graphs plotted from the mean values with standard deviations (*error bars*) (*B*) obtained after densitometric analysis of the amount of IbpB in particular time points.

an efficiency similar to that of wild-type Lon. LonK371E/K376E/R379E also degraded IbpB although with a lower efficiency.

To investigate whether the lack of TrfA degradation by the two Lon mutant proteins was due to disruption of the substrate-enzyme interaction, we used an ELISA (see "Experimental procedures"). We found that both LonR306E/K308E/K310E/K311E and LonK371E/K376E/R379E bind TrfA protein as efficiently as wild-type Lon (Fig. 4*C*). Therefore, the *in vitro* stability of TrfA in the presence of the Lon mutants was not the result of a deficiency in the interaction between TrfA and the analyzed Lon variants.

Because TrfA was resistant to degradation by the DNA binding-defective Lon mutants in the presence of DNA, we decided to test the stability of two other replication initiators, λO of bacteriophage λ and RepE of plasmid F. In the control reactions, we observed the stimulatory effect of DNA on degradation of the Rep proteins by wild-type Lon (Fig. 6). In contrast, in assays with either LonR306E/K308E/K310E/K311E or LonK371E/K376E/R379E, regardless of the presence of DNA in the reaction mixture, proteolysis of λO and RepE was not detected (Fig. 6). These results indicate that the mutant Lon proteins, deprived of DNA-binding ability, exhibit no stimulation of proteolytic activity by DNA toward the tested replication initiator proteins.

Stimulation of ATPase activity in Lon mutants

Our previously published experiments (6) showed that the ATPase activity of wild-type Lon was significantly stimulated in the presence of both TrfA substrate and DNA. The Lon mutants constructed in the current work do not interact with DNA and fail to degrade TrfA but retain the interaction with the substrate. Therefore we further tested whether DNA and substrate affect the ATPase activity of the Lon variants. We performed the ATPase activity tests using a coupled enzymatic assay (see "Experimental procedures"). In the control reaction, we observed substantial stimulation of the ATPase activity of wild-type Lon when both TrfA and DNA were present in the reaction mixture (Table 1). In contrast, the ATPase activity of neither of the mutant Lon proteins was stimulated by DNA and TrfA substrate under conditions used in the experiment.

Phenotypic effects of Lon defectiveness in interaction with DNA

To further analyze the significance of the Lon-DNA interaction, we investigated the phenotypic properties of *lon*-deficient *E. coli* cells harboring plasmids coding for wild-type Lon, LonR306E/K308E/K310E/K311E, or LonK371E/K376E/R379E. The main phenotype of Lon-deficient strains is the production of filamentous cells as the result of cell division arrest (39, 40). Lon protease affects cell division by regulating the half-life of SulA protein, a cell division inhibitor in *E. coli* cells (41). Thus functional Lon enzyme is necessary to ensure the proper length of bacterial cells. The average wild-type *E. coli* cell is ~2 μm long (Fig. 7*A*, *panel i*). In the *E. coli lon*$^-$ strain, we observed elongated cells with a length of ~3.5 μm due to compromised cell division. When wild-type Lon was expressed from a plasmid in the *E. coli lon*$^-$ strain, the typical length of the cells was

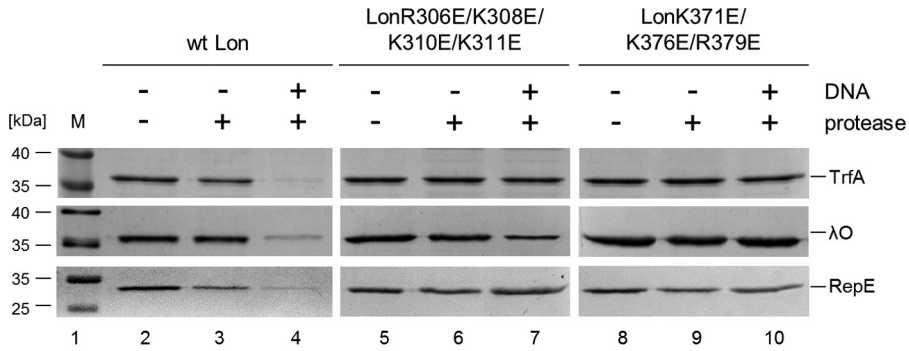


Figure 6. Substitutions within the ATPase domain of Lon decrease the *in vitro* proteolysis of replication initiators TrfA, λO, and RepE. The results of SDS-PAGE analysis of TrfA, λO, and RepE proteins *in vitro* proteolysis by Lon variants with or without DNA in reaction mixtures are shown. The assay was performed as described under "Experimental procedures" and in the legend to Fig. 1*B* with the following modifications. 1.5 μg of TrfA, λO, and RepE were used as substrate proteins. Only reactions in *lanes 4*, *7*, and *10* contained 250 ng of supercoiled pKD19L1 plasmid. Reactions were stopped after 2 h of incubation at 32 °C by the addition of Laemmli buffer. The assay was performed in triplicate, and the results shown are representative gel images.

Table 1
Effects of Lon protease ability to interact with DNA on stimulation of ATPase activity by DNA and substrate

Lon variant	ATPase activity[a]		
	+DNA	+TrfA	+DNA, TrfA
	%		
WT Lon	123 ± 14	151 ± 8	342 ± 3
LonK371E/K376E/R379E	111 ± 10	87 ± 3	101 ± 10
LonK371E/K376E/R379E	114 ± 5	93 ± 1	117 ± 10

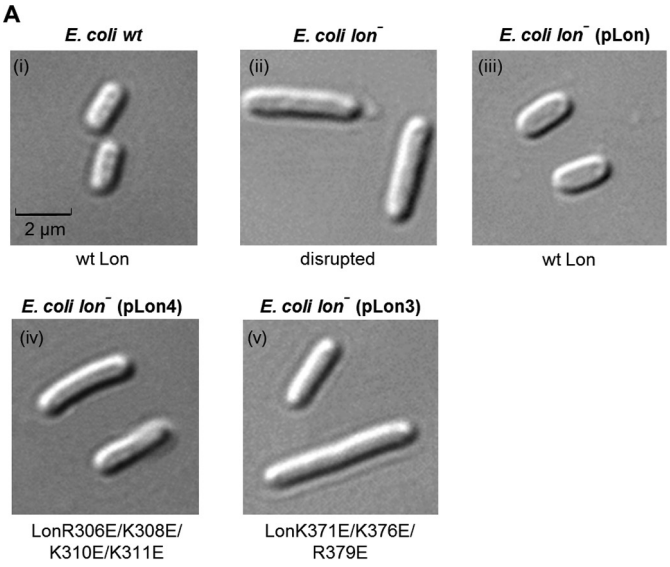
[a] The rate of ATP hydrolysis was estimated from the slope of dA_{340}/dt as described previously (67). The results were normalized to the activity of each protease variant alone and are presented as mean values with the S.D. from three experiments.

restored (Fig. 7*A*, *panels ii* and *iii*). Plasmids expressing either of the Lon mutants, LonR306E/K308E/K310E/K311E or LonK371E/K376E/R379E, did not restore cell length in the *lon*$^-$ strain; in both cases, cells remained filamentous with an approximate length of 3.5 μm (Fig. 7*A*, *panels iv* and *v*). The average cell length of the strains tested is summarized in a table in Fig. 7*B*. These results demonstrated that the phenotype of the DNA binding-defective Lon mutants is similar to that observed for Lon-deficient strains. This indicates that Lon interaction with DNA is crucial for its activity associated with cell division.

Discussion

Interaction of proteins with nucleic acids can change their activities. From the first identification of Lon protease, it was known as a DNA-binding protein (29). Various studies showed the influence of nucleoprotein complex formation by Lon on proteolysis of proteins (5–9). Although Lon is a very extensively studied enzyme, likely because of its structural and functional complexity, the region of the protease engaged in interaction with DNA as well as the role of this interaction for Lon proteolytic activity is still elusive.

The construction of truncated mutants of *Ec*Lon containing only a single full-length domain, domain N (LonΔAP), ATPase domain (LonΔNP), or domain P (LonΔNA), allowed us to identify the ATPase domain of *E. coli* Lon protease as the one interacting with DNA. Then, using bioinformatics tools, we mapped the electrostatic surface potential on a model of *Ec*Lon hexameric ATPase domain to predicted surface-exposed residues



B

E. coli strain	Plasmid	Plasmid-encoded Lon variant	Avarage cell length [μm]
wt	-	-	2.3 ± 0.5
lon$^-$	-	-	3.5 ± 1.0
	pLon	wt Lon	2.8 ± 0.6
	pLon4	LonR306E/K308E/ K310E/K311E	3.5 ± 0.8
	pLon3	LonK371E/K376E/ R379E	3.4 ± 0.8

Figure 7. Perturbations in Lon protease interaction with DNA affect the length of bacterial cells. *A*, bacteria were grown in LB medium at 30 °C, immobilized on glass slides, and observed and analyzed by microscopic imaging as described under "Experimental procedures." Representative cells of the tested *E. coli* strains, C600 WT (*panel i*) and ATC12017 (C600 derivative) deprived of functional Lon protease (*panel ii*), ATC12017 strain cells complemented with pLon plasmid variant coding for WT Lon (*panel iii*), LonR306E/K308E/K310E/K311E (*panel iv*), and LonK371E/K376E/R379E (*panel v*), are presented. *B*, a table showing average cell length (μm) of control strains (C600 and ATC12017) and ATC12017 strain carrying plasmid pLon or its derivatives coding for the noted Lon protease mutants. For each strain, ~100 cells were measured. The assay was performed in triplicate, and the mean values with standard deviations are shown. *Dashes* in the table represent lack of plasmids and lack of plasmid-encoded Lon variant.

engaged in DNA binding. Clear positively charged patches on the surface of the ATPase domain of the Lon protein were identified. We selected a set of arginine and lysine residues within this positively charged surface region and substituted them to change the side chain charge to negative. Some of the variants with substitutions in the α-subdomain of the ATPase module (LonR533E/R537E and LonK563E/K566E) retained the ability to interact with DNA (supplemental Table S2). Selected point mutants in the α/β-subdomain, LonK371E, LonK376E, and LonR379E, exhibited slightly reduced ability to interact with DNA (supplemental Table S2), but when these three mutations were combined to create the triple Lon mutant, a significant reduction in the formation of a nucleoprotein complex was observed. Even more noticeable effects were observed when the charge of amino acids was changed at the very end of the N domain and within the ATPase α/β-subdomain. The single point mutation in residues 306, 308, 310, and 311 reduced the ability of the mutant protease to interact with nucleic acid, but introduction of glutamic acid in all these positions and construction of the quadruple Lon mutant abolished Lon-DNA interaction. The effects of mutations were observed for both full-length *Ec*Lon protein and the truncated mutant LonΔNP. The involvement of Lon residues within the ATPase domain in interaction with DNA was also shown for Lon from *B. thermoruber* (17, 33, 34). In *B. thermoruber* Lon, electrostatic interaction between the protein α-subdomain and DNA was proposed. Interestingly, our results demonstrate that the *Ec*Lon residues located within the ATPase α-subdomain do not play as crucial a role for nucleoprotein complex formation as those located within the α/β-subdomain and at the intersection of N and ATPase domains. Our data clearly indicated that patches between amino acids 306–311 and 371–379 of *Ec*Lon interact with DNA. Consistent with our results, based on a DNA-binding assay with truncated MBP-Lon mutants, Nomura *et al.* (32) proposed that a 166-amino-acid fragment of *Ec*Lon, from amino acid residues 272 to 437, interacts with DNA. This region contains 6- and 9-amino-acid patches that we identified in the current work. Because *Ec*Lon functions as a hexamer, the identified patches in each of the six monomers form a characteristic pattern of positive charge on the ATPase domain surface accommodated by DNA. Interestingly, we found that the formation of similarly located positively charged patches in the ATPase domain can also be observed for the hexamer of Lon from *Bacillus subtilis* (supplemental Fig. S4B). The interaction of an ATPase domain with DNA might also occur in the case of proteases other than Lon. We previously showed that proteolysis by the *E. coli* ClpAP protease is also stimulated by the presence of DNA, and the protease subunit, unfoldase ClpA, can interact with DNA (6). The involvement of the ATPase domain in interaction with DNA was also shown for other proteins containing an AAA+ module, *e.g.* DnaA (42), DnaC, Orc1, and IstB (43). However, in contrast to Lon protease, most of them involved amino acids on the surface of the central pore formed by the AAA+ module (43, 44) or other amino acids within the initiator/loader-specific motif (43). This motif has not been identified in Lon protease.

Proteolysis of a substrate by Lon requires recognition of the target. Our ELISA test showed that both the triple and quadruple mutants of Lon interact with TrfA as efficiently as the wild-type Lon. However, even in the presence of DNA in the reaction mixtures, this substrate was not processed by either Lon mutant, whereas the wild-type protease degraded TrfA protein efficiently. We observed the same inability of the Lon mutants to degrade other replication initiation proteins, RepE protein from plasmid F and λO protein from bacteriophage λ. No depletion was observed when either IbpB, α-casein, or FITC-casein protein were substrates. However, some differences in FITC-casein proteolysis kinetics were observed when comparing wild-type Lon and its mutants. These dissimilarities may arise from some changes in quaternary structure of the protease variants. It was shown that hexamers and dodecamers of Lon may vary in activity toward the same substrate (23). In the case of the triple and quadruple mutants of Lon, further investigations are needed to resolve this matter. Nevertheless, α-casein, FITC-casein, and IbpB are proteins that do not interact with DNA as TrfA, RepE, and λO proteins do. We previously showed that substrates forming nucleoprotein complexes are efficiently degraded when both Lon and the substrate interact with DNA (6). In the current work, we observed that changing the charge of selected amino acids on the Lon ATPase domain surface that influence Lon protease interaction with DNA affects only the proteolysis of substrates interacting with DNA. It seems that the loss of Lon ability to bind DNA does not impair degradation of Lon substrates that do not form nucleoprotein complexes in the cytoplasm; however, it abolishes degradation of Lon substrates interacting with DNA. This effect could be a result of loss of the Lon ATPase activity stimulation by substrate and DNA as observed for LonK371E/376E/R379E and LonR306E/K308E/K310E/K311E. It could be considered that the processing of proteins complexed with DNA requires more energy and that the disturbed Lon-DNA interaction restrains the ability of the protease to degrade these specific substrates. It is known that a different demand for energy is observed when Lon processes different substrates. It was shown that degradation of the same protein in a folded form required more energy from ATP hydrolysis compared with the protein less structured or partially unfolded, the processing of which required only ATP binding (45).

Degradation of some substrates may also require specific adaptors. It was shown that the ClpS protein is an adaptor of the ClpA subunit of the ClpAP protease (46–48), and the SspB (49, 50), UmuD (51), and RssB (52, 53) proteins modify substrate recognition by ClpX, a component of the ClpXP protease. So far, for Lon protease, there has been only one adaptor identified, the *B. subtilis* SmiA protein (54). Canonical adaptors bind the substrate of the protease; however, recent data showed that adaptors that do not strongly bind the substrate protein might exist. In *Caulobacter crescentus*, the CpdR adaptor was described as a protein that, through binding to ClpX protein, prepares the protease ClpXP for degradation of specific substrates (55). This mechanism was named "priming" because it prepares the protease for binding the substrate or other adaptors. Although all known adaptors of proteases are proteins, DNA may also be a type of a priming adaptor for Lon. The interaction of Lon with DNA might prepare the protease for the degradation of specific substrates. This preparation could cor-

respond to a conformational change similar to that observed during the binding of nucleotide (56) and Mg^{2+} ions (24) as shown for *Meiothermus taiwanensis* Lon. Binding of Mg^{2+} by *M. taiwanensis* Lon results in the formation of a non-secluded hexameric barrel with openings that allow entrance and ATP-independent processing of unstructured substrates; additional binding of nucleotide results in a secluded chamber conformation and ATP-dependent proteolysis (24). Recently, with the use of cryoelectron microscopy, conformational changes dependent on nucleotide binding were also shown for human mitochondrial Lon protease (57).

It is also possible that binding of Lon to DNA enables the protease to efficiently find the substrate complexed with nucleic acid. Because binding of bacterial Lon to DNA is thought to be sequence-nonspecific, Lon protease might slide on DNA until it reaches the substrate protein. In such a scenario, the Lon protease should bind the same molecule of DNA as its substrate protein (in *cis*). If DNA only plays a role of Lon adapter, then it is possible that the protease interacts with DNA both in *cis* and in *trans*. Then it is possible that the activated Lon releases the DNA when it recognizes and binds the substrate. The reduction of DNA binding after the addition of an unfolded substrate was shown for MBP-*Ec*Lon fusion protein (58). Future investigations are required on this important issue.

In vivo analysis of phenotypes of Lon mutants constructed in this work clearly demonstrated that the impairment of Lon interaction with DNA results in cell filamentation. Probably the improper intracellular localization of Lon mutants, defective in DNA binding, and/or the lack of their activation by DNA influences the proteolysis of SulA or other substrates important for cell division. It is intriguing that SulA was not reported as a DNA-binding protein. A detailed study is required to elucidate why Lon interaction with DNA is required for this protease's role in cell division.

In bacterial cell physiology, two major functions provided by Lon protease can be distinguished. The first function concerns protein quality control that is mainly connected with stress conditions, *e.g.* heat shock and nutritional downshift. The second function involves specific degradation of a substrate to control its amount in normal growth conditions. Under non-stress conditions, Lon is localized in the nucleoprotein complex (7, 36), and *in vitro* tests revealed that at high temperatures the DNA-binding ability of a MBP-Lon fusion protein is reduced (58), indicating the link between formation of a nucleoprotein complex by Lon with growth conditions. In this study, it was speculated that the Lon-DNA interaction is important to prevent uncontrolled protein degradation (58). Lin *et al.* (34) suggested that DNA binding may help Lon protease localize close to a target protein. Our data are consistent with this hypothesis. In the presented work, we demonstrate that the DNA-binding ability of Lon is an essential feature of this enzyme to process substrates engaged in nucleoprotein complexes, *e.g.* replication initiation proteins. We speculate that Lon may play an essential role in the regulation of DNA replication depending on growth conditions.

Experimental procedures

Bacterial strains, oligonucleotides, and plasmids

E. coli strains used in this study were BL21(DE3), C600, and its derivative ATC12017 (C600 *lon510*) (59, 60). Sequences of oligonucleotides used in this work are listed in supplemental Table S1: 1–6, primers for obtaining truncated mutants of Lon protein; 7–12, primers for site-directed mutagenesis to introduce substitutions in the ATPase domain of Lon protease; 13 and 14, primers for preparation of the EMSA probe; 15 and 16, complementary single-stranded oligonucleotides (Thermo Scientific) for obtaining the 5′-terminally biotinylated dsDNA fragment used for the SPR analysis. Plasmid RK2 was used for preparation of the EMSA probe. Plasmid pKD19L1, a minireplicon of the RK2 plasmid containing the origin of replication *oriV* (61), was used for the *in vitro* proteolysis assay and ATPase activity assay. Plasmid pAT30 (62) was used for purification of TrfA protein. Linearized pET SUMO vector (Invitrogen) was used for cloning and purification of truncated single-domain variants of Lon protein fused with the hexahistidine tag and SUMO protein at the N terminus of the polypeptide chain. Plasmid pBADLon (in this study named pLon) (6) was used for purification of Lon protease and as a template for PCRs, amplification of fragments containing truncated variants of the *lon* gene, and site-directed mutagenesis. Plasmid pZZ36 (63) was used for purification of RepE protein.

Protein purification

Proteins described in this study were of high purity (95% or higher). All histidine-tagged protein preparations including TrfA, RepE, LonΔAP, LonΔNP, and LonΔNA were purified with nickel-nitrilotriacetic acid resin (Macherey-Nagel) essentially as described previously (62, 73). Purified proteins LonΔAP, LonΔNP, and LonΔNA were digested with SUMO (ULP-1) protease to remove the SUMO tag. Full-length Lon protease variants were purified following a published protocol (64). The *E. coli* IbpB protein and bacteriophage λ O protein were kindly provided by Dr. Krzysztof Liberek (Intercollegiate Faculty of Biotechnology of the University of Gdansk and Medical University of Gdansk, Gdansk, Poland). Commercially available proteins were α-casein and BSA (Sigma-Aldrich).

Surface plasmon resonance analysis

Standard SPR analyses using BIAcore 2000 were performed as described previously (6) with the following modifications. DNA binding by Lon variants was studied using a biotinylated double-stranded DNA fragment containing a nonspecific sequence of pUC19 plasmid immobilized on a streptavidin-coated matrix on sensor chip SA. Running buffer used was HBS150 (150 mM NaCl, 10 mM HEPES, pH 7.4, 3 mM EDTA, 0.005% (v/v) Surfactant P20, 10 mM $Mg(OAc)_2$, 2 mM ATP). The results were analyzed using BIAevaluation software version 3.2. Kinetic (K_d) measurements were performed using separate K_a/K_d fit algorithms: local (calculations based on interaction analysis of each protein concentration separately) and global (based on analysis of each titration experiment as entirety). Each experiment was repeated three times, and the representative sensorgrams are presented.

In vitro proteolysis assay

The *in vitro* proteolysis assay was performed essentially as described previously (6) with the following modifications. The reaction contained 1.5 μg of each substrate protein (TrfA, α-casein, IbpB, RepE, or λO) and 1.5 μg of each Lon variant. Additionally, 250 ng of supercoiled plasmid pKD19L1 was included in the reactions containing TrfA, RepE, and λO. To determine the amount of substrates in each reaction, densitometric analysis was performed with the ChemiDoc MP system (Bio-Rad) and Image Lab software (Bio-Lab). Each experiment was repeated three times, and the mean values with standard deviations are presented as graphs. For substrate dependence of FITC-casein (type III) (Sigma) degradation, FITC fluorescence was detected with the ChemiDoc MP system. The V_{max} and K_m values were calculated with GraphPad Prism software. Values are means ± S.E. ($n = 3$). Data were presented on a graph, and the solid lines were fit to the Michaelis-Menten equation.

EMSA

To analyze the formation of nucleoprotein complexes, EMSA was used. The DNA probe was prepared by DNA labeling with Alexa Fluor 555 (Invitrogen) as described previously (65). The 420-bp-long dsDNA fragment was obtained by PCR with primers oriV1 and oriV2 (see supplemental Table S1 for oligonucleotide sequences) from RK2 plasmid template. Reactions containing 1 pmol of the labeled DNA and increasing concentrations of proteins (40, 100, 200, and 400 nM) in proteolysis buffer were incubated at 32 °C for 20 min and electrophoretically separated in a 4% polyacrylamide Tris borate-EDTA gel. Visualization of fluorescently labeled DNA was performed using the ChemiDoc MP system. Each experiment was repeated three times, and the representative gel images are shown.

In vivo localization of Lon

E. coli BL21(DE3) cells carrying pLon plasmid or its derivatives coding for Lon variants were used for analysis of Lon localization *in vivo*. Overnight cultures were diluted 1:50 in fresh LB medium supplemented with ampicillin (100 μg/ml) and 0.02% (w/v) arabinose and grown at 30 °C to an A_{600} of 0.6. 2 ml of culture were harvested by centrifugation, washed with PBS, and fixed with a 0.1% (v/v) formaldehyde solution for 15 min at room temperature with gentle shaking. After washing with PBS, cells were incubated with mild lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM EDTA, pH 8.0, 1 mM DTT, 40 μg/ml lysozyme) for 20 min at room temperature. After subsequent centrifugation (4 °C, 21,130 × *g*, 15 min) cells were divided into cytoplasmic (soluble) and DNA-containing (insoluble) fractions. Collected fractions were analyzed using SDS-PAGE followed by Western blotting and densitometric analysis. Each experiment was repeated three times, and the results are presented as the mean values with standard deviations. The value of 1 corresponds to the total cellular content of tested protein.

ELISA

Binding of Lon WT, LonK371E/K376E/R379E, and LonR306E/K308E/K310E/K311E to TrfA was analyzed by ELISA as described previously (66) with the following modifications. 200 nM TrfA and 1% (w/v) BSA solution (negative control) were immobilized on the 96-well ELISA plate (Costar) for 90 min. Increasing amounts of each Lon variant (0, 1, 2, 4, 8, 16, and 32 pmol) were incubated with the immobilized proteins. Buffer B contained 10 mM MgAc instead of $MgCl_2$. Anti-Lon antibodies were used to detect Lon-TrfA interaction. Goat anti-mouse IgG HRP-conjugated secondary antibodies and TMB Peroxidase EIA Substrate kit (Bio-Rad) were used to perform colorimetric detection. Each experiment was repeated three times, and the mean values with standard deviations are presented as graphs.

ATPase activity assay

The ATPase activity of Lon WT, LonK371E/K376E/R379E, and LonR306E/K308E/K310E/K311E was measured by utilizing a coupled enzymatic assay as described previously (6, 67). Concentrations of Lon protease variants used in the reaction were 700 nM WT Lon and LonK371E/K376E/R379E and 70 nM LonR306E/K308E/K310E/K311E, and where indicated 900 nM TrfA protein substrate and/or 2 μg of pKD19L1 plasmid were added. Reaction mixtures and the ATP-regenerating system were separately preincubated (32 °C, 2 min), mixed together, and immediately transferred to the Jasco V-650 spectrophotometer for real-time measurement of the decrease in absorbance at 340 nm. The ATPase activity of each Lon variant alone was set as 100%. Each experiment was repeated three times, and the mean values with standard deviations are presented.

Cell length measurements

Cell length measurements were obtained using optical microscopy. Strains used for the assay were *E. coli* C600 and its Lon-deficient derivative ATC12017 (C600 *lon510*) carrying pLon vectors enabling inducible expression of Lon WT, LonK371E/K376E/R379E, and LonR306E/K308E/K310E/K311E proteins. Overnight cultures were diluted 1:50 in fresh LB medium supplemented with ampicillin (100 μg/ml) and 0.02% (w/v) arabinose and allowed to grow at 30 °C to an A_{600} of ~0.1. The cells were immobilized on a poly-L-lysine-treated glass slide and observed using an Olympus BX51 microscope with Nomarski differential interference contrast prism and F-View II charge-coupled device camera. Image analysis and measurements of ~100 cells of each strain were performed by AnalySIS software (Soft Imaging System). Each experiment was repeated three times. The results are presented as representative microscopic images and mean values with standard deviations.

Protein structure prediction and modeling

Modeling of the hexameric ATPase domain of *E. coli* Lon involved the following steps: 1) initial structure prediction by matching the *E. coli* Lon to experimentally determined structures using the GeneSilico MetaServer (68), 2) modeling of the monomeric structure based on the output from the MetaServer by comparative modeling using the "FRankenstein's Monster" approach (35, 69), and 3) the assembly of subunits into a hexameric structure followed by minor adjustments of side chains to relieve steric clashes between the subunits.

According to the sequence alignments generated by the GeneSilico MetaServer (supplemental Fig. S5), the *Ec*Lon ATPase structure was generated by combining the known crystal structure of the *Ec*Lon C domain (amino acids 585–784; Protein Data Bank code 1rre (19)) and the *Ec*Lon N domain (amino acids 1–245; Protein Data Bank code 3ljc (70)) with a comparative model of the central domain based on the structures of Lon from *Thermococcus onnurineus* NA1 (Protein Data Bank code 3k1j (71)) and from *B. subtilis* (Protein Data Bank code 3m6a (72)), which were detected as the potentially closest homologues in the fold recognition step.

Author contributions—A. K., K. W., and J. M. B. designed Lon mutants. A. K., K. W., M. G., D. K., and M. R. constructed and purified Lon mutants and performed experiments. J. M. B. and M. S. performed computational modeling. A. K., K. W., and I. K. designed experiments and wrote the manuscript.

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