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A Conserved Domain in *Escherichia coli* Lon Protease Is Involved in Substrate Discriminator Activity

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Lon protease of *Escherichia coli* regulates a diverse set of physiological responses including cell division, capsule production, plasmid stability, and phage replication. Little is known about the mechanism of substrate recognition by Lon. To examine the interaction of Lon with two of its substrates, RcsA and SulA, we generated point mutations in *lon* which affected its substrate specificity. The most informative *lon* mutant overproduced capsular polysaccharide (RcsA stabilized) yet was resistant to DNA-damaging agents (SulA degraded). Immunoblots revealed that RcsA protein persisted in this mutant whereas SulA protein was rapidly degraded. The mutant contains a single-base change within *lon* leading to a single amino acid change of glutamate 240 to lysine. E240 is conserved among all Lon isolates and resides in a charged domain that has a high probability of adopting a coiled-coil conformation. This conformation, implicated in mediating protein-protein interactions, appears to confer substrate discriminator activity on Lon. We propose a model suggesting that this coiled-coil domain represents the discriminator site of Lon.

Energy-dependent Lon (La) protease, first isolated from *Escherichia coli* (7, 9), has been identified in every organism examined thus far, including gram-positive and gram-negative bacteria, yeast, plants, and humans. Lon consists of four identical subunits (63; reviewed in references 19 and 20) each having an N-terminal highly charged domain (spanning amino acids [aa] 211 to 271), a centrally located ATP binding domain (aa 351 to 421), and a C-terminal proteolytically active domain (1, 8, 16). Mutations in serine 679 suggest that it is the catalytically active residue (2). Mutations in residues H665, H667, and D676, but not D743, also appear to be essential for Lon's proteolytic activity, yet it is unclear whether these residues belong to a catalytic triad (55). A K362A change in motif A of the ATP-binding domain was found to affect the catalytic efficiency and peptidase activity of Lon (14). To date no physiological function has been defined for the conserved charged domain located at the N terminus.

The characteristic phenotypes of Δ*lon* mutant cells (mucoidy, sensitivity to DNA-damaging agents, and defectiveness in bacteriophage λ and P1 lysogenization and in the degradation of abnormal proteins) (3, 5, 34, 42, 44; reviewed in references 24 and 26) can be directly attributed to the stabilization of Lon substrates with regulatory roles in these pathways. The mucoidy phenotype (overproduction of colanic acid capsular polysaccharide) of Δ*lon* cells is mediated through the stabilization of RcsA (58; reviewed in references 23 and 26). RcsA is a transcriptional activator of the capsular polysaccharide genes (*cps*): in *lon*$^+$ cells RcsA is highly unstable and *cps* transcription is barely detectable, whereas in Δ*lon* cells RcsA stability increases, resulting in a high level of *cps* transcription (28, 56, 58; reviewed in references 23 and 26). Increasing the amount of RcsA by stabilization of RcsA through the removal of Lon, overexpression of *rcsA*, or better protection of RcsA by its partner, RcsB, results in expression of the *cps* genes (27, 56; reviewed in references 23 and 26).

Treatment of cells with DNA-damaging agents (e.g., UV light or methyl methanesulfonate [MMS]) activates expression of SOS genes (reviewed in reference 61) including that encoding SulA, a cell division inhibitor (17, 35–37). UV or MMS treatment of *lon*$^+$ cells gives rise to short filaments which are eventually resolved into individual cells when normal cell division resumes (25, 35). *lon*$^+$ cells are resistant to UV or MMS, and SulA is highly unstable in these cells (46; reviewed in reference 26). UV or MMS treatment of Δ*lon* cells gives rise to long, nonseptated filaments. Δ*lon* cells are sensitive to UV or MMS, and SulA is stabilized in these cells (46; reviewed in reference 26). Δ*lon* cells fail to recover after removal of the DNA-damaging agent because the stabilization of SulA renders filamentation irreversible and cell death inevitable. Analogous to the situation for RcsA, increasing the amount of SulA by stabilization of SulA through the removal of Lon, overexpression of *sulA*, or better protection of SulA by its partner, SulB, results in the formation of lethal filaments (38, 41).

Lon's function and its substrates vary from organism to organism, and for some Lon enzymes neither function nor substrates have been identified. Very little is known as to how Lon discriminates its substrates from among the hundreds of other nonsubstrate proteins in a cell. In this study we provide genetic and biochemical evidence defining a potential discriminator activity for the conserved charged domain located at the N terminus of Lon.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains are described in Table 1. All strains are derivatives of MC4100 unless otherwise indicated. Liquid medium used in this study was LB broth except where noted, and solid media used were LB agar, TB agar, and MacConkey's lactose agar (Difco). Antibiotics were used at 25 (tetracycline) and 100 (ampicillin) μg/ml. P1*vir* transductions were performed as described by Silhavy et al. (51).

Phenotypic assays. Mucoidy was assayed on TB agar containing ampicillin, MMS sensitivity on LB agar containing 0.05% MMS, and mitomycin C sensitivity on LB agar containing 0.3 μg of mitomycin C per ml. The UV phenotype was assessed as follows. Overnight cultures were diluted in LB containing ampicillin, grown to an optical density at 600 nm (OD_{600}) of 0.3, pelleted, resuspended in 0.1 volume of 0.01 M $MgSO_4$, and irradiated with UV light (λ = 254) at a dose of 5 mJ/cm^2. Cells were diluted, plated on LB agar containing ampicillin, and incubated in the dark.

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TABLE 1. Bacterial strains and plasmid used in this study

Strain[a] or plasmid	Relevant genotype	Reference(s), source, or construction
E. coli strains		
CAG12017	*lon*$^+$ *zba-3054*::Tn*10*	52
DDS90	*lon*$^+$ *rcsA*$^+$ *rcsA90*::*lacZ*	D. Sledjeski; 47, 54
JM12	*lon* E240K (class III) *zba-3054*::Tn*10*	This study
JM64	*lon* G384D (class II) *zba-3054*::Tn*10*	This study
JM98	*lon* G374R D483N (class I) *zba-3054*::Tn*10*	This study
JT1900	*lon* E240K *zba-3054*::Tn*10 cpsB10*::*lacZ*	SG20781 + P1(JM12)
JT1916	*lon* G384D *zba-3054*::Tn*10 cpsB10*::*lacZ*	SG20781 + P1(JM64)
JT1920	*lon* G374R D483N *zba-3054*::Tn*10 cpsB10*::*lacZ*	SG20781 + P1(JM98)
JT2029	*proCYA221 zaj*-403::ΔTn*10 rcsA*$^+$ *rcsA90*::*lacZ*	DDS90 + P1(SG1030)
JT2036	*lon* E240K *zba-3054*::Tn*10* Δ*gal-165*	SG21020 + P1(JM12)
JT2037	*lon* G384D *zba-3054*::Tn*10* Δ*gal-165*	SG21020 + P1(JM64)
JT2038	*lon* G374R D483N *zba-3054*::Tn*10* Δ*gal-165*	SG21020 + P1(JM98)
JT2046	Δ*lon-510 rcsA*$^+$ *rcsA90*::*lacZ*	JT2029 + P1(SG4144)
JT4000	Δ*lon*-510	SG1030 + P1(SG4144)
MS100	*lon* E240K *zba-3054*::Tn*10 rcsA*$^+$ *rcsA90*::*lacZ*	DDS90 + P1(JM12)
MS101	*lon* G384D *zba-3054*::Tn*10 rcsA*$^+$ *rcsA90*::*lacZ*	DDS90 + P1(JM64)
MS102	*lon* G374D D483N *zba-3054*::Tn*10 rcsA*$^+$ *rcsA90*::*lacZ*	DDS90 + P1(JM98)
SG1030	F$^-$ Δ*lac araD proCYA221 zaj*-403::ΔTn*10*	60
SG4144	Δ*lon-510*	44
SG20250	*lon*$^+$ Δ*lac*	29
SG20780	Δ*lon-510 cpsB10*::*lacZ*	4
SG20781	*lon*$^+$ *cpsB10*::*lacZ*	4
SG21020	*lon*$^+$ Δ*gal-165*	S. Gottesman
SG21155	Δ*lon* Δ*gal-165*	S. Gottesman
Plasmid		
pATC400	pBR322-*rcsA*$^+$	58

[a] All strains except CAG12017 and SG4144 are derived from MC4100 (Δ*lacU169 araD flbB rel*).

Mutagenesis. *E. coli* CAG12017, which carries a Tn*10*Tet^r 98% linked to the *lon*$^+$ gene, was mutagenized with nitrosoguanidine (51). P1*vir* was grown on the mutagenized culture and used to infect a wild-type, nonmucoid *lon*$^+$ strain (SG20250). Tet^r transductants were selected and then screened for acquisition of a mucoid phenotype. These potential *lon* mutants were moved by P1 into a *lon*$^+$ *cpsB10*::*lacZ* indicator strain (SG20781). Tet^r transductants were again selected and screened for lactose utilization as an indication of *cps* expression. Lac$^+$ transductants, indicating expression of *cpsB10*::*lacZ*, were saved.

β-Galactosidase assay. Three independent candidates of all strains examined were assayed. β-Galactosidase activities were determined by the method of Miller (45). Values reported represent the mean of three assays.

Assessment of filament formation. Cells were grown to OD_{600} of 0.2. Nonirradiated cell samples were removed at 0-, 2-, 4-, and 8-h time points, sonicated, pelleted, resuspended in phosphate-buffered saline containing 1% formaldehyde, incubated for 30 min on ice, and then evaluated in light microscopy. Remaining cells in the original 0-h culture were pelleted, resuspended in 0.1 volume of 10 mM $MgSO_4$, and irradiated with UV light (λ = 254) at a dose of 5 mJ/cm^2. Irradiated cell samples were removed at 0, 2, 4, and 8 h, fixed in formaldehyde, and evaluated by light microscopy.

RcsA and SulA protein detection. To examine RcsA, cells were grown to an OD_{600} of 0.6 and processed as described below. To examine SulA, cells were grown to an OD_{600} of 0.3, pelleted, resuspended in 0.1 volume of 10 mM $MgSO_4$, and irradiated with UV light (λ = 254) at a dose of 5 mJ/cm^2. Cells were diluted and incubated 30 min in foil-lined flasks. Samples were removed, washed twice in 10 mM $MgSO_4$, resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (49), and boiled. Protein concentrations were determined by the bicinchoninic acid protein assay method (Pierce). Equal amounts of total cellular protein, resuspended and boiled in SDS-PAGE sample buffer, were fractionated by tricine-SDS-PAGE (50) (16.5% gel for SulA; 14% gel for RcsA). Fractionated samples for SulA analysis were transferred to a polyvinylidene difluoride (PVDF) membrane (Dupont) in 20 mM Tris (pH 8.3)–20% methanol–150 mM glycine. Fractionated samples for RcsA analysis were transferred to a PVDF membrane in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (pH 11) with 20% methanol (12, 57). After transfer, all PVDF membranes were blocked in Tris-buffered saline (20 mM Tris [pH 7.4], 125 mM NaCl) containing 0.1% Tween 20, incubated with preabsorbed antiserum specific to *E. coli* RcsA or SulA protein, washed in Tris-buffered saline containing 0.1% Tween 20, and incubated with an appropriate dilution of goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase. Immunoreactive protein bands were visualized on autoradiography film (Hyperfilm; Amersham) by using enhanced chemiluminescence (Amersham).

λOts stability assessment. Stability of the bacteriophage λO temperature-sensitive mutant protein (λOts) was measured as previously reported (11, 30). Briefly, *E. coli* cells were grown in LB medium, wild-type λ and λOts phages were diluted, and the dilutions were spotted on TB agar plates containing the *E. coli* cells to be examined in an overlay. Duplicate plates were prepared; one set was incubated at the permissive temperature (32°C), and the other set was incubated at the nonpermissive temperature (39°C). At the nonpermissive temperature in the *E. coli lon*$^+$ strain, λO is unstable and thus no plaques form. At either the nonpermissive or permissive temperature in the *E. coli* Δ*lon* strain, λOts is stable and thus plaques form.

DNA sequencing and analysis. PCR was used to amplify two overlapping fragments of each *lon* mutant, encompassing the complete open reading frame (ORF) and regulatory region. DNA from this region was amplified directly from the chromosome as previously described (13), the DNA was purified on Qiagen columns, and both strands were sequenced with appropriate primers on an ABI 377 automated sequencer (Center for Gene Research and Biotechnology, Central Services Laboratory, Oregon State University). All oligonucleotide primers used for PCR and DNA sequencing were provided by the Biopolymer Unit of the University of Maryland Medical School, Baltimore County. PCR oligonucleotide primers used were 5′ AGCCTGCCAGCCCTGTTT 3′, 5′ AGTATCTTGCGG TTCAAA 3′, 5′ GGCGTGAAGCACCGTCGTGT 3′, and 5′ GCATAGAACC GATGTAAGTA 3′. Mutations were identified by comparing the newly generated *lon* mutant sequences to the wild-type *lon* sequence (GenBank accession no. 146642, 146644, 1773123, and 1786643), using the Genetics Computer Group program Bestfit.

RESULTS

Lon-substrate interactions. In an attempt to define the substrate specificity of Lon, Dervyn et al. in the laboratory of O. Huisman overexpressed *sulA* in *lon*$^+$ cells and observed that the cells became mucoid, suggesting that high levels of SulA protected RcsA from Lon-dependent degradation (11). Mutations in SulA which abolished this ability to saturate Lon, evident by the loss of the mucoid phenotype, could be identified; however, the mutant SulA protein was still degraded in a Lon-dependent fashion. These observations led them to hypothesize that Lon protease contains different substrate rec-

TABLE 2. High-level expression of RcsA in *lon*$^+$ cells does not protect SulA

Relevant genotype (strain[a])	Phenotype			
	Mucoidy[b]	MMS[c]	UV[c]	Mitomycin C[c]
lon$^+$ (SG20250/pBR322)	−	R	R	R
lon$^+$ (SG20250/pATC400)	+	R	R	R
Δ*lon* (JT4000/pBR322)	+	S	S	S
Δ*lon* (JT4000/pATC400)	+	S	S	S

[a] All strains are MC4100 derivatives.
[b] Assessed visually. −, nonmucoid; +, mucoid.
[c] Determined as described in Materials and Methods. R, resistant; S, sensitive.

ognition sites: a high-affinity site for specific substrates such as SulA and RcsA, and a low-affinity site for nonspecific substrates such as abnormal or mutant proteins (11, 22, 26). Presumably, both RcsA and SulA are recognized by the high-affinity site, while the mutant SulA is no longer recognized by this high-affinity site but rather interacts with the low-affinity site. We extended these studies by overexpressing RcsA in *lon*$^+$ cells and then examining their response to UV light, MMS, and mitomycin C (Table 2). We predicted that if RcsA and SulA had equivalent affinities for Lon, then by overexpressing RcsA to such a level that *lon*$^+$ cells were mucoid, SulA should be protected from Lon-dependent degradation. This protection would be observed phenotypically as sensitivity to UV, MMS, and mitomycin C. We observed that under conditions of high levels of RcsA, *lon*$^+$ cells were very mucoid yet still resistant to UV, MMS, and mitomycin C, suggesting that SulA was not protected from Lon-dependent degradation (Table 2, line 2). Failure to express high enough levels of RcsA to protect SulA was ruled out because enough intact and functional RcsA was available to activate *cps* expression (mucoid phenotype), the level of *cps* activation (as monitored by a *cpsB10*::*lacZ* fusion) was higher than that observed in Δ*lon* cells (data not shown), and RcsA was readily detected by immunoblot analysis (data not shown). Several possibilities exist to explain these results, including that specific substrates, although presumably acting at a high-affinity site, may have different affinities for Lon, thus creating a hierarchical order with respect to substrate selection.

If RcsA and SulA interact at a high-affinity site but their interaction is dictated by a preference order, we predicted that *lon* mutations which defined the difference in this interaction for these two substrate classes could be isolated. To test this prediction, a strain carrying a Tn*10* 98% linked to *lon*$^+$ was mutagenized with nitrosoguanidine. P1*vir* was grown on the pool mutant cells, and this lysate was used to infect a *lon*$^+$ strain (SG20250). Tet^r transductants were selected and subsequently screened for the Lon$^-$ phenotype, mucoidy, indicating accumulating RcsA. Of the 100,000 Tet^r transductants screened in this approach, 0.05% were mucoid. The increase in RcsA levels was verified by transducing the *lon* mutations in to a strain containing *cpsB10*::*lacZ* (SG20781). This fusion reports the expression of the *cps* genes as a function of the available RcsA protein (4). High-level expression of *cpsB10*::*lacZ* fusion correlates with high levels of RcsA protein (26, 56). All *lon* mutants examined showed an increase in *cps* expression compared to *lon*$^+$, indicating an increased availability of intact and functional RcsA. The mucoid mutants were screened for sensitivity to the DNA-damaging agents UV light, MMS, and mitomycin C and assayed for filament formation. As shown in Table 3, three distinct classes of *lon* mutants were identified. Class I mutants were mucoid, had high-level *cpsB10*::*lacZ* expression, were completely sensitive to DNA-damaging agents, and had extremely long filaments 8 h after UV exposure. Class II mutants were mucoid, had low-level *cpsB10*::*lacZ* expression, were partially sensitive to DNA-damaging agents, and had medium-length filaments 8 h after UV exposure. Class III mutants were mucoid, had medium- to high-level *cpsB10*::*lacZ* expression, were completely resistant to DNA-damaging agents, and exhibited no filaments.

Previously we reported that *rcsA* expression was 100-fold higher in Δ*lon* mutant cells than in *lon*$^+$ cells (13). In these studies, expression of *rcsA* was measured by using a *rcsA*::*lacZ* fusion at the λ*att* site (54), creating partial diploid strains (*rcsA90*::*lacZ* at the λ*att* site and *rcsA* at its normal position in the chromosome). The 100-fold increase in *rcsA* expression in a Δ*lon* strain was due to both an accumulation of functional RcsA protein in the absence of Lon protease and *rcsA* expression activated by RcsA protein (13). We predicted that if RcsA activated its own expression, then a difference in the level of *rcsA* activation would be observed between the three *lon* mutant classes. As shown in Table 4, expression of the *rcsA90*::*lacZ* fusion was highest in the class I and class III *lon* mutants and was approximately 380- to 420-fold higher than in *lon*$^+$ cells. Expression of the *rcsA90*::*lacZ* fusion in the class II *lon* mutant was somewhat less than in Δ*lon* cells and in the class I and class III *lon* mutants, yet *rcsA* expression in the class II *lon* mutant was still 100-fold higher than in the *lon*$^+$ strain. These results provide further support that functional and intact RcsA is available to activate *rcsA* expression in all three classes of *lon* mutants, with functional RcsA levels highest in the class I and III mutants.

Results described thus far indicate that phenotypically, class I *lon* mutants behave most like the previously reported Δ*lon* mutant (44), and class II *lon* mutants behave phenotypically as

TABLE 3. Phenotypic descriptions of the effects of three classes of *lon* mutation on the stability of RcsA and SulA protein

Relevant genotype (strains[a])	RcsA		SulA	
	Mucoidy[b]	*cpsB10*::*lacZ* (β-galactosidase activity)[c]	MMS phenotype[d]	Filament formation 8 h after UV exposure[e]
lon$^+$ (SG20250 or SG20781)	−	5	R	No filaments (single cells)
Δ*lon* (JT4000 or SG20780)	+	418	S	Extremely long filaments
Class I *lon* (JM98 or JT1920)	+	575	S	Extremely long filaments
Class II *lon* (JM64 or JT1916)	+/−	117	S/R	Medium-length filaments
Class III *lon* (JM12 or JT1900)	+	230	R	No filaments (single cells)

[a] All strains are MC4100 derivatives. Strains assayed for β-galactosidase activity contain *cpsB10*::*lacZ*.
[b] Assessed visually. −, nonmucoid; +, mucoid.
[c] β-Galactosidase assays were carried out as described by Miller (45). Values represent the average of three independent assays.
[d] Determined on LB agar containing 0.05% MMS. R, resistant; S, sensitive; S/R, intermediate phenotype.
[e] Assayed as described in Materials and Methods.

TABLE 4. Effects of class I, II, and III *lon* mutations on *rcsA* expression as measured by *rcsA90*::*lacZ* activity

Relevant genotype (strain[a])	β-Galactosidase activity[b]	Fold change from:	
		lon$^+$	Δ*lon*
lon$^+$ (DDS90)	12		0.0045
Δ*lon* (JT2046)	2,641	220	
Class I *lon* (MS102)	5,137	428	2.0
Class II *lon* (MS101)	1,220	100	0.46
Class III *lon* (MS100)	4,658	388	1.8

[a] All strains are MC4100 derivatives carrying a *rcsA90*::*lacZ* fusion integrated at the λ*att* site.

[b] β-Galactosidase assays were carried out as described by Miller (45). Values represent the average of three independent assays.

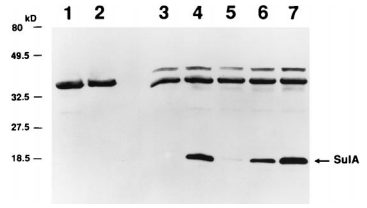

FIG. 2. Immunodetection of SulA in *lon* mutants. Cultures were treated with UV light as described in Materials and Methods. Samples were treated as described for Fig. 1 except that antiserum specific to SulA was used. Lanes: 1, SG20780 (Δ*lon*), no UV; 2, SG20781 (*lon*$^+$), no UV; 3, SG20781 (*lon*$^+$), with UV; 4, SG20780 (Δ*lon*), with UV; 5, JT1900 (class III *lon*), with UV; 6, JT1916 (class II *lon*), with UV; 7, JT1920 (class I *lon*), with UV.

if protease activity toward RcsA and SulA has been impaired. The most interesting class, the class III *lon* mutants, behave phenotypically as if they are Δ*lon* with respect to RcsA (mucoid) yet *lon*$^+$ with respect to SulA (resistant to DNA-damaging agents). To determine if the phenotypes were a result of decreased levels of Lon protein or truncated Lon, immunoblot analyses of whole-cell extracts of the *lon* mutants were performed. Intact Lon protein was expressed at normal levels in the three classes of *lon* mutants (data not shown), indicating that the observed phenotypes were not due to an absent or truncated Lon. A representative from each mutant class was chosen for further analysis.

RcsA and SulA protein levels correspond to the *lon* mutant phenotypes. Changes in the level of *cps* and *rcsA* expression and in the response to DNA-damaging agents is consistent with changes in RcsA and SulA protein levels. To determine if the protein levels vary as predicted, levels of RcsA (Fig. 1) and SulA (Fig. 2) were evaluated by immunoblotting. RcsA protein was detected in representative extracts from each *lon* mutant class (Fig. 1, lanes 3 to 5). The level of RcsA in the class I and class III *lon* mutants (lanes 3 and 5) was comparable to the level detected in the Δ*lon* mutant (lane 2), whereas the level of RcsA in the class II *lon* mutant was reduced (lane 4). RcsA half-life was measured in the three classes of *lon* mutants in vivo (data not shown); RcsA half-life in the class I and class III *lon* mutants was similar to that reported for Δ*lon* cells (greater than 30 min [56]), whereas RcsA half-life in the class II *lon* mutant appeared to be less than 10 min.

In contrast, amounts of detectable SulA varied among the three classes of mutants (Fig. 2). SulA protein is readily detected in the class I *lon* mutant (Fig. 2, lane 7) at levels comparable to that observed in Δ*lon* cells (lane 4). SulA protein was not detected in the class III *lon* mutant (lane 5) and was detected at a reduced level in the class II *lon* mutant (lane 6). Analysis of filament formation (Table 3) corresponds with SulA levels observed by immunoblotting. SulA half-life was measured in the three classes of mutants in vivo (data not shown); SulA half-life in the class I *lon* mutant was similar to that reported for Δ*lon* cells (20 to 30 min [44, 46, 59]), whereas SulA half-life in the class II *lon* mutant appeared to be only 10 to 15 min. SulA half-life in the class III *lon* mutant was similar to that reported for *lon*$^+$ cells (less than 3 min) (46, 59).

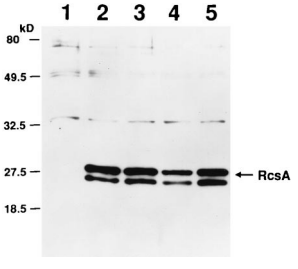

FIG. 1. Immunodetection of RcsA in *lon* mutants. Equal amounts of protein from whole-cell extracts were boiled in sample buffer, fractionated on a 14% tricine-SDS-polyacrylamide gel, and analyzed by immunoblotting with preabsorbed polyclonal antiserum specific to RcsA protein. Lanes: 1, SG20781 (*lon*$^+$); 2, SG20780 (Δ*lon*); 3, JT1900 (class III *lon*); 4, JT1916 (class II *lon*); 5, JT1920 (class I *lon*).

λOts, an abnormal protein, is not affected by the *lon* mutations. RcsA and SulA proteins are normal physiological proteins degraded in a Lon-dependent fashion in *E. coli* and thus are considered specific Lon substrates. Abnormal proteins such as nonsense peptides, protein fragments, missense proteins, and damaged proteins also are substrates for Lon (5, 18, 30, 31, 44). Thus, it seemed reasonable to test the activity of the *lon* mutants against an abnormal protein. Wild-type λO protein, involved in the replication of phage λ, is not a substrate for Lon, and a λ phage expressing wild-type O protein forms plaques on *lon*$^+$ (SG21020) and Δ*lon* (SG21155) cells with equal efficiency (10^{10} plaques/ml). A temperature-sensitive mutation in λOts causes the protein to be abnormal at high temperatures and makes it a substrate for Lon. The degradation of λOts in a Lon-dependent fashion prevents λ phage replication, and thus no plaques form on *lon*$^+$ (SG21020) cells. In Δ*lon* (SG21155) cells, however, the abnormal λOts protein is stabilized and thus can function, allowing λ phage to replicate and form plaques (efficiency of 10^{10} plaques/ml). Plaquing efficiencies on the *lon* mutants JT2036 (class III), JT2037 (class II), and JT2038 (class I) were similar to that seen in the *lon*$^+$ strain, indicating that λOts protein was still a substrate for these mutant Lons.

Sequence analysis reveals changes at highly conserved residues. Sequencing the full *lon* ORF and its regulatory region from the three classes of *lon* mutants revealed unique changes in each class. In the class I *lon* mutant, there were two nucleotide changes: a G-to-A transition resulting in a glycine-to-arginine change at position 374 (G374R), and a G-to-A transition resulting in an aspartate-to-asparagine change at position 483 (D483N). In the class II *lon* mutant there was a single-nucleotide G-to-A transition, resulting in a glycine-to-aspartate change at position 384 (G384D). In the class III *lon* mutant there was a single-nucleotide G-to-A transition, resulting in a glutamate-to-lysine change at position 240 (E240K).

Amino acid substitutions G374 and G384 are located in the region between the A and B motifs comprising the ATP binding domain (Fig. 3). Both amino acid substitutions represent a change of a small aliphatic residue to a charged residue. Comparison of Lon sequences from other organisms revealed that

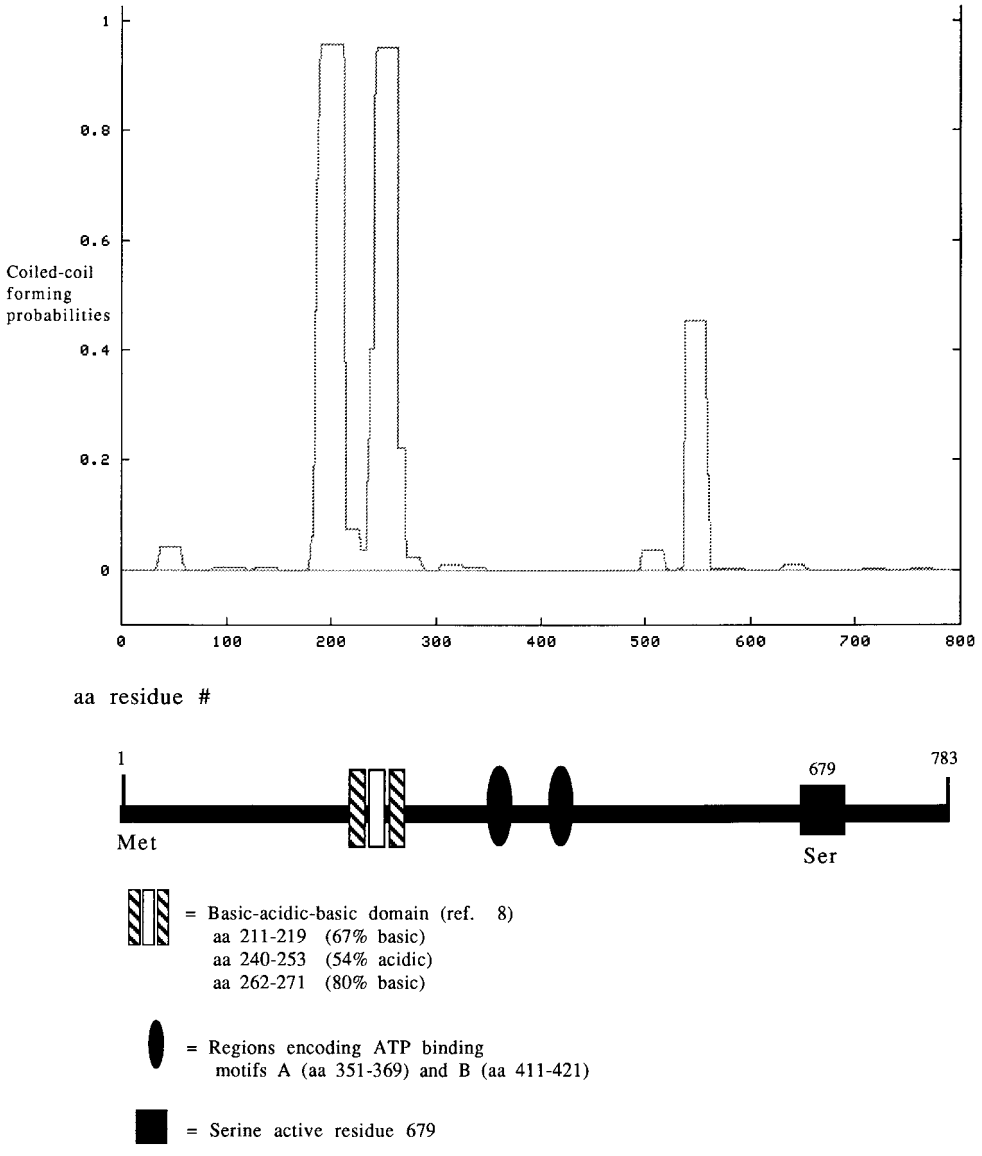

FIG. 3. Comparison of the predicted coiled-coil structure (generated with COILS version 2.2 [39, 40, 48]) to the predicted domain structure of *E. coli* Lon protease.

glycine 374 is conserved in Lon sequences from gram-positive and gram-negative bacteria and *Arabidopsis*. In *Saccharomyces cerevisiae* and *Homo sapiens*, an asparagine residue is found at this position. A G384D substitution (class II) resulted in reduced catalytic behavior toward both RcsA and SulA. The G384 residue is conserved in all known Lon sequences; it is positioned 15 residues downstream from the last residue comprising motif A of the ATP binding domain. The sequence surrounding the G384 residue (boldface) of Lon is highly conserved among bacterial species: X(S/A)G**G**VRDE (where X = M, I, or L). Lon sequences from *Arabidopsis*, *S. cerevisiae*, and *H. sapiens* also have similar residues in this region, with residues G383, G384, and D387 being identical.

The class I *lon* mutant, which has no proteolytic activity toward RcsA and SulA, has an additional amino acid substitution, D483N, which represents a change of a charged residue to a noncharged residue. The D483 residue is conserved in all Lon sequences. As in the case of the G384 residue, the residues surrounding the D483 residue (boldface) are highly conserved in bacterial species, with a P(A/G)PLX**D**RME(V/I) consensus (where X = L, P, Q, or R) evident. *Arabidopsis*, *S. cerevisiae*, and *H. sapiens* Lon sequences also contain similar residues in this region, with residues P478, P480, L481, D483, R484, M485, and E486 being identical.

The Lon sequence of the class III mutant revealed an amino acid substitution (E240K) in the proposed acidic region of the conserved charged domain located at the N terminus which substitutes an acidic residue with a basic residue. Glutamate 240 is conserved among all Lon isolates. The residues surrounding the E240 residue (boldface) are highly conserved in bacterial species, with a (K/Q)AIQK**E**LG(D/E) consensus evident. *Arabidopsis*, *S. cerevisiae*, and *H. sapiens* Lon sequences contained similar residues in this region, with residues I237, E240, L241, and G242 being identical.

Coiled-coil analysis. Coiled-coil regions are frequently solvent-exposed regions believed to be involved in protein-pro-

tein interactions (39, 40, 48). The COILS program (39, 40, 48), which is specific for solvent-exposed, left-handed coiled coils, was used to analyze the primary amino acid sequence of Lon and to subsequently make a prediction as to the likelihood of a particular domain adopting a coiled-coil conformation. Analysis of the *E. coli* Lon sequence identified a region spanning residues 185 to 228 and 237 to 280 at the N terminus (Fig. 3) as a region with a high probability (ca. 95%) of adopting coiled-coil structures. In this region, approximately 50% of the residues are charged, with an equal distribution of acidic and basic residues. The residues comprising the A and B motifs of the ATP binding domain and the residues surrounding the proposed catalytic active residue (S679) had little probability of forming coiled coils (Fig. 3). Interestingly, the region spanning residues 538 to 558 (approximately 120 residues upstream of the active site S679 residue) is predicted to adopt a coiled-coil structure but at a much lower probability than the N-terminal region. Approximately 48% of residues 538 through 558 are charged, with two-thirds of the charged residues basic. Because the prediction of coiled-coil regions is biased toward hydrophilic, highly charged sequences, the analysis was performed with a weighted and an unweighted matrix, which did not reveal any differences between the two types of analysis. A similar analysis was performed on all known Lon sequences, revealing very similar coiled-coil profiles for all Lon isolates.

DISCUSSION

Goldberg and Waxman (21, 62–64) proposed a model for Lon proteolysis which accounted for the energy requirements and the processive nature of this enzyme (reviewed in references 19, 26, and 43). In this model, ATP hydrolysis manipulates the conformation of Lon, controlling the accessibility of Lon's active site. In an expansion of this model (26), a substrate capture function that has two binding sites, one for nonspecific substrates (initiator site) and one for specific substrates (discriminator site), was proposed. If a putative substrate had a low affinity, it would bind the initiator site and be partially degraded; if it had no affinity, it would be released from the initiator site without being cleaved. On the other hand, the discriminator site would bind specific substrates (those with very high affinity) and hold them long enough to activate ATP hydrolysis, resulting in a conformational change and accessibility of Lon's active site. The substrate would be retained under these conditions and subsequently cleaved.

If Lon protease has two binding sites, a discriminator site for specific substrates, such as RcsA and SulA, and an initiator site for nonspecific substrates, such as abnormal proteins, then where do these sites reside? Dervyn et al. in the laboratory of O. Huisman demonstrated that saturating Lon with SulA protects RcsA from degradation (11), suggesting that these two substrates compete for the same binding site. In contrast, we observed that overexpressing RcsA did not appear to protect SulA from degradation, possibly indicating a hierarchy among these substrates. We propose that if RcsA and SulA interact with different affinities at the same site, then this site would be identifiable by mutations which discriminated between these two substrates. In this study, we provide evidence supporting the hypothesis that discriminator activity can be assigned to a domain located at the N terminus. An E240K substitution in this domain abolished Lon's activity on RcsA but had no affect on Lon's activity on SulA. This E240 residue resides in a domain that has a high probability of adopting a coiled-coil conformation conducive to protein-protein interactions, and this conformation and its location are highly conserved among all Lons reported to date. Furthermore, this domain is a strong candidate for protein-protein interactions by virtue of its highly charged, thus potentially sticky, "velcro" behavior. Clearly, these studies provide direct support for the interaction of RcsA with Lon at the velcro domain. Observations made in overexpression studies with both RcsA and SulA provide indirect support for interaction of SulA with this domain. Direct evidence supporting SulA's interaction with Lon's velcro domain will come from mutations in this region that impede Lon-SulA interaction. Further studies are under way to identify this class of mutation.

Our study also uncovered other novel mutations, for example, G384D (class II), which resides between motifs A and B of the ATP binding domain and which affects the catalytic behavior of Lon toward both RcsA and SulA but not λOts. In support of this, RcsA and SulA levels of detection and half-life were between the values obtained for *lon*$^+$ and Δ*lon* cells. Furthermore, *rcsA*::*lacZ* expression was not as high in this mutant as in the other *lon* mutants, indicating that reduced amounts of functional RcsA were available to activate the expression of *rcsA*. These results suggest that Lon with a G384D substitution can still affect the stability of RcsA and SulA but not as efficiently as wild-type Lon activity. The combination of G374R and D483N substitutions (class I) completely abolishes proteolytic activity toward RcsA and SulA but, like the G384 substitution, does not affect degradation of λOts. Possibly, proteolytic activity toward certain nonspecific abnormal proteins is retained with the G384 or G374R and D483N substitutions, yet Lon-dependent degradation of specific substrates such as RcsA and SulA is abolished due to an inability of Lon to hydrolyze ATP or to assemble into a functional multimeric enzyme, suggesting that catalytic-proteolytic efficiency rather than discriminator activity has been impaired.

If discriminator activity resides in the N-terminal velcro domain of Lon, then where does the initiator site, or the site for nonspecific substrate binding, reside? Functional analysis of the abnormal protein λOts demonstrated that an E240K, a G384D, or a D483N in combination with a G374R substitution in Lon had no effect on λOts stability. These results suggest that Lon's interaction with λOts protein was at a site distinct from that of specific substrates and that reducing the catalytic behavior toward specific substrates had no apparent affect on λOts stability. While these studies were not designed to identify the binding site for abnormal proteins, they have provided a testable hypothesis for where this domain may reside, namely, the highly conserved residues 538 to 558 predicted to adopt a coiled-coil structure, to which no function has been assigned.

Goldberg and coworkers (8, 10) proposed that the basic regions (aa 211 to 219 and aa 262 to 271) of the velcro domain might be involved in Lon's ability to bind DNA (6, 65) whereas the acidic region (aa 240 to 253) of this domain might be involved in its activation by polybasic peptides (8). Sequence specific binding of *E. coli* Lon protease to the peri-Ets site of the human immunodeficiency virus type 2 enhancer was recently reported (15). This information has led several investigators to hypothesize that DNA binding of Lon protease might be involved in its substrate recognition. The velcro domain of Lon does not display characteristics common to DNA binding proteins. Whether DNA binding is specific or nonspecific and whether DNA binding is involved in substrate recognition remain to be determined.

Interestingly, the E240K substitution does not significantly alter the predicted coiled-coil conformation of the velcro domain. However, this substitution was sufficient to abolish Lon's ability to recognize and degrade RcsA, without affecting the Lon-SulA interaction. Numerous possibilities exist to account

for this observation. The velcro domain, a region which spans approximately 100 residues, may have multiple binding sites for different specific substrates, and only the site which interacts with RcsA was affected by the E240K substitution. Alternatively, both substrates may bind at the same site, and thus a more drastic substitution at position 240 is needed to impede SulA binding. In support of this, overexpression studies suggest that RcsA and SulA bind at the same site, yet there may be a hierarchy defining these interactions. Furthermore, coiled-coil analysis of RcsA and SulA revealed a striking difference between these two substrates. SulA has a high probability (60%) of adopting a coiled-coil at the C terminus (residues 124 to 137), and residues in this region are predominately acidic (85%). In contrast, RcsA has a low (10%) probability of adopting a coiled-coil structure (C-terminally located, residues 172 to 188), and the residues in this region are predominately basic. Are the residues comprising the predicted coiled-coils of SulA and RcsA involved in Lon-substrate interactions? If protease-substrate discriminator activity can be assigned to residues involved in coiled-coil conformations, then it remains to be determined if substrate affinity is a reflection of the overall charge of the coiled-coil region of these proteins.

We propose that the simplest way Lon recognizes and prioritizes its interactions with specific physiological substrates would be through the discriminator activity of the velcro domain. The charge interactions occurring here would define the substrate's affinity for Lon and thus dictate a hierarchy for substrates as they were being positioned for cleavage at the proteolytic active site located at the C terminus. Proteolysis of nonspecific substrates, such as abnormal proteins, would not require the discriminator activity of the velcro domain. Correspondingly, specific inhibitors, such as T4 PinA (32, 33, 53), may interact at the velcro domain, thus preventing the capture of specific substrates. Many more intriguing questions as to how Lon selects its substrates from among other nonsubstrate proteins in the cell remain unanswered. Answering these questions may provide a means by which to identify new biological pathways controlled by Lon-dependent degradation in organisms other than *E. coli*.

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