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Substrate sequestration by a proteolytically inactive Lon mutant

(SulA/RcsA/serine protease/ATPase/protease La)

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Contributed by Susan Gottesman, April 6, 1999

ABSTRACT **Lon protein of *Escherichia coli* is an ATP-dependent protease responsible for the rapid turnover of both abnormal and naturally unstable proteins, including SulA, a cell division inhibitor made after DNA damage, and RcsA, a positive regulator of transcription. Lon is a multimer of identical 94-kDa subunits, each containing a consensus ATPase motif and a serine active site. We found that overexpressing Lon, which is mutated for the serine active site (LonS679A) and is therefore devoid of proteolytic activity, unexpectedly led to complementation of the UV sensitivity and capsule overproduction of a *lon* deletion mutant. SulA was not degraded by LonS679A, but rather was completely protected by the Lon mutant from degradation by other cellular proteases. We interpret these results to mean that the mutant LonS679A binds but does not degrade Lon substrates, resulting in sequestration of the substrate proteins and interference with their activities, resulting in apparent complementation. Lon that carried a mutation in the consensus ATPase site, either with or without the active site serine, was no longer able to complement a Δ*lon* mutant. These *in vivo* results suggest that the pathway of degradation by Lon couples ATP-dependent unfolding with movement of the substrate into protected chambers within Lon, where it is held until degradation proceeds. In the absence of degradation the substrate remains sequestered. Comparison of our results with those from a number of other systems suggest that proteins related to the regulatory portions of energy-dependent proteases act as energy-dependent sequestration proteins.**

Intracellular protein degradation is responsible for the rapid turnover of specific, unstable regulatory proteins and the clearing of abnormal proteins from the cytoplasm. The high molecular weight, ATP-dependent proteases that are primarily responsible for this degradation must recognize and select their protein substrates from among an enormous pool of other proteins that should not be degraded. Recent studies suggest that binding of substrate is followed by unfolding of the substrate or, at least of a portion of it, sufficient to gain access to and be cleaved by the proteolytic sites (1, 2). Processive degradation of the substrate then proceeds, leading to the release of short peptides of 6–15 aa.

Escherichia coli contains at least five of these ATP-dependent proteases; the same protease families found in prokaryotes also are found in mitochondria and chloroplasts of eukaryotes (see ref. 3 for a review). The first discovered of these proteases and probably the most intensively studied biochemically is Lon (also called La) (4, 5). Genes encoding Lon protease have been found in a large variety of prokaryotes, where they have been implicated in developmental pathways, as well as in the degradation of specific regulatory proteins (see below) (6–17). In eukaryotes, Lon is found in mitochondria and has been shown to be essential for mitochondrial function in *Saccharomyces cerevisiae* (18–21).

The 94-kDa polypeptide chain contains all Lon activities within a single polypeptide chain (Fig. 1), in contrast to the ClpAP and ClpXP proteases, which contain two different subunits, one (ClpA or ClpX) encoding the nucleotide hydrolysis/substrate recognition activities and another (ClpP) encoding the peptide bond cleavage activity (22–25). In Lon, a consensus site for ATP binding and hydrolysis has been shown by mutation to be necessary for proteolysis and ATPase activity; mutations in the active site serine (Fig. 1) block proteolysis but do not fully abolish ATPase activity (14, 26–30). How and where substrate binding takes place and how substrates are presented to the active site, however, have not been clarified, and very few mutations other than those in the serine or ATPase sites have been described.

We began the studies described here to take advantage of the well-defined *in vivo* assays for Lon activity to investigate essential elements within Lon. We find that a *lon* mutant that has lost the proteolytic active site complements *lon* deletion phenotypes *in vivo*, apparently by recognizing and stably binding substrates.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. LVM781 (Δ*lon*-510) and LVM 783 (*lon*$^+$) are derived from SG20780 and SG20781 (31), respectively. Both strains carry a *cpsB*::*lacZ* transcriptional fusion, are deleted for the chromosomal *lac* genes, and are also *ara*$^+$. *ara*$^+$ was introduced by Plvir transduction from LVM100 (*leu*::*Tn*10 *ara*$^+$). LVM100 was constructed by transduction of the *leu*::*Tn*10 from LMG194 into KS272 (32), selecting tetracycline resistance and screening for Ara$^+$. LVM781 and LVM783 are also tetracycline resistant. A parallel set of Δ*ara* tetracycline-sensitive strains were made by transducing Δ*ara* *leu*::*Tn*10 from LMG194 (32) into SG20781 and selecting spontaneous Leu$^+$ derivatives. The *lon*$^+$ version, LVM788, was the starting strain for the construction of SG22542 (*lon*$^+$ *cpsB*::*lacZ* Δ*ara* *malP*::*lacI*^{Q}) and SG22569 (Δ*lon*-510 *cpsB*::*lacZ* Δ*ara* *malP*::*lacI*^{Q}). *malP*::*lacI*^{Q} was introduced into LVM788 as described (33) to create SG22542. The Δ*lon* allele was introduced into SG22542 by first introducing a *proC* linked to *Tn*10 and then selecting a Pro$^+$ tetracycline-sensitive derivative that had inherited the linked *lon* deletion. LVM806

Abbreviation: TCA, trichloroacetic acid.

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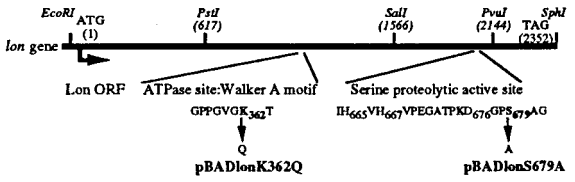

FIG. 1. Schematic of the *lon* gene and localization of *lon* mutations. The heavy black line represents the *lon* gene. Numbering nucleotides from the A of the starting ATG, the positions of the relevant restriction sites are indicated above the line. The ATPase Walker A motif and the site-directed mutation in this site (K362Q), as well as the region around the proteolytic active site, including the S679A mutation in that site, are shown. Other residues known to be important for proteolysis activity (H665, H667, and D676) (28) also are shown.

(Δ*lon*-510 *cpsB*::*lacZ* Δ*ara malP*::*lacI*^{Q}*sfiB* leu*::*Tn*10) was derived from SG22569. The *sfiB** mutation has been described (33). P1*vir* transductions were carried out as described by Silhavy *et al*. (34). We discovered after much of this work was done that many of our strains, including all of those derived from SG20780 and SG20781, were also lysogenic for ϕ80. Assays of the *cpsB*::*lacZ* fusions were repeated in strains without ϕ80, with similar results to those presented here. As far as we can tell, the presence or absence of ϕ80 does not change the nature of the conclusions reached in the paper, but may affect the degree of UV sensitivity. Transformations with appropriate plasmids were performed according to Miller (35) and most routine manipulations of plasmids as described by Maniatis *et al.* (36).

pBADlon^{+}, a derivative of pBAD24 (32) expressing Lon from the pBAD arabinose-controlled promoter, was constructed by first cloning the *Sac*I–*Eco*RV fragment of plon500 (37) into pUC19, deleting the natural promoter of the *lon* gene, to create pUClon. The *Eco*RI–*Sph*I fragment from pUClon was then cloned into the *Eco*RI–*Sph*I sites of pBAD24. pBADlon derivatives carrying mutations in either the ATPase consensus site or the active site serine were constructed by PCR amplification from mutant plasmids supplied by T. Rotanova (Russian Academy of Sciences, Moscow) and recloning of the appropriate fragment into the full-length pBADlon^{+} plasmid. For construction of pBADlonS679A, carrying a mutation at the serine active site (Fig. 1), the *Sal*I–*Sph*I fragment at the 3′ end of the *lon* gene was amplified from pBR327-S679A (26), by using primers Oli1 (5′-GCTGACCGTCGACGATAG) and Oli2 (5′-ACATGCATGCGGTCACTATTTTG). On sequencing, an unexpected *Sph*I site was present 23 bp from the stop codon of *lon*, leading to a frame-shift mutation at the carboxyl-terminal end of the S679A Lon. This site was removed by replacement of the 1,300-bp *Pvu*I–*Pvu*I fragment from that construct by the corresponding wild-type fragment from the pBADlon^{+} to give rise to pBADlonS679A. For pBADlonK362Q, containing a mutation in the ATP-binding motif (Fig. 1), the internal *Pst*I–*Sal*I fragment of pBADlon^{+} was replaced by the *Pst*I–*Sal*I fragment from pBR327K362Q (29). A double mutant carrying both S679A and K362Q, pBADlonK362Q-S679A, was constructed by cutting pBADlonS679A with *Sal*I and *Sph*I, purifying the resulting *Sal*I–*Sph*I fragment containing the mutated serine codon, and introducing it into pBADlonK362Q cut with the same enzymes, to replace the corresponding wild-type *Sal*I–*Sph*I fragment at the 3′ end of *lon*. Correct recombinants were detected by screening with *Pst*I. The serine mutant fragment introduces a *Pst*I site not present in the K362Q construct. All of the final pBAD constructs were partially sequenced to confirm the cloning junction and presence of the expected mutations.

β-Galactosidase Assays. Strains were grown in minimal M63 medium (35) supplemented with 0.2% glycerol, 0.0001% vitamin B1, 0.1% casamino acids, and various arabinose concentrations at 32°C. For *lon*$^{+}$ strains, β-galactosidase assays were performed according to Miller (35). For *lon* mutant strains, β-galactosidase assays were performed in a SpectraMax 250 spectrophotometer as described by Zhou and Gottesman (38). The conversion factor between the microtiter plate reader unit and Miller unit was calculated to be 25 from assays done in parallel with both protocols.

SulA Turnover and Solubility *in Vivo*. Cells were grown in LB supplemented with 1% arabinose and 100 μg/ml ampicillin to an OD_{600} of 0.2–0.3 at 32°C. Cells were collected by centrifugation, suspended in 1/2 vol of 0.01 M $MgSO_4$, and exposed to UV light (15–20 J/m^2). After centrifugation and resuspension in the starting volume of prewarmed LB supplemented with 1% arabinose and 100 μg/ml ampicillin, cells were grown for an additional 20 min to allow expression of SulA. A zero-time sample was removed, and spectinomycin was added to a final concentration of 150 μg/ml to block further protein synthesis. Samples were removed at various times and precipitated with trichloroacetic acid (TCA).

To determine the solubility of SulA, cells were induced as indicated above, and sodium azide was added to cultures to give a final concentration of 5 mM. A 1-ml sample was removed and TCA-precipitated. The rest of the cells were collected by centrifugation and resuspended in 2 ml of 50 mM Tris (pH 8), 1 mM EDTA, and 100 mM KCl. Cells were broken in a French Pressure cell at 20,000 psi, protease inhibitors were added (Calbiochem Protease Inhibitor set), and 1 ml of the extract was centrifuged for 6 min at 3,500 rpm. The low-speed pellet was frozen in dry ice; the low-speed supernatant was centrifuged for 15 min at 14,000 rpm and separated into a high-speed pellet and supernatant. The low- and high-speed pellets were resuspended in 1 ml of 50 mM Tris (pH 8), 1 mM EDTA, and 100 mM KCl. Samples from each fraction were analyzed by SDS/PAGE and Western blot.

Sample Preparation by TCA Precipitation, Gel Electrophoresis, and Western Blot. One-milliliter samples were removed from cultures to tubes containing 50 μl of cold 100% TCA. After centrifugation, pellets were washed twice with 500 μl of cold 100% acetone, air dried, and resuspended in SDS-gel loading buffer. Equal quantities of protein were separated on 10% (detection of Lon) or 15% (detection of SulA) SDS/PAGE and transferred onto nitrocellulose filters. Filters were incubated with polyclonal anti-Lon (37) or anti-SulA (16) antibodies. Immunoblots were developed by using horseradish peroxidase-conjugated goat anti-rabbit antibody, followed by enhanced chemiluminescence (Amersham Pharmacia). Blots were quantified by scanning films with the Eagle Eye II gel imager (Stratagene) or with the National Institutes of Health IMAGE software.

RESULTS

Conditional Expression of Lon from a pBAD Promoter. Previous studies with multicopy *lon* genes have demonstrated that the overproduction of Lon kills cells and that plasmids carrying *lon* tend to accumulate insertions and other disruptions in the gene (39). To avoid the problems of secondary mutations and allow analysis of mutant forms of Lon that might be even more detrimental to cell growth than the wild-type protein, the *lon* gene was inserted into pBAD24, where it is expressed from the pBAD promoter, which is inducible by arabinose and has a very low level of basal expression in the absence of arabinose (32). Lon expression was modulated in pBADlon^{+} transformants of Δ*lon* host cells by varying the arabinose concentration in the culture medium. In the absence of arabinose, no Lon protein was detected (Fig. 2*A*). At 0.001% arabinose, Lon expression was close to the level detected from a single copy *lon* gene in the chromosome and levels increased up to 0.1% arabinose. At concentrations above 0.1% arabinose, the induction system was probably

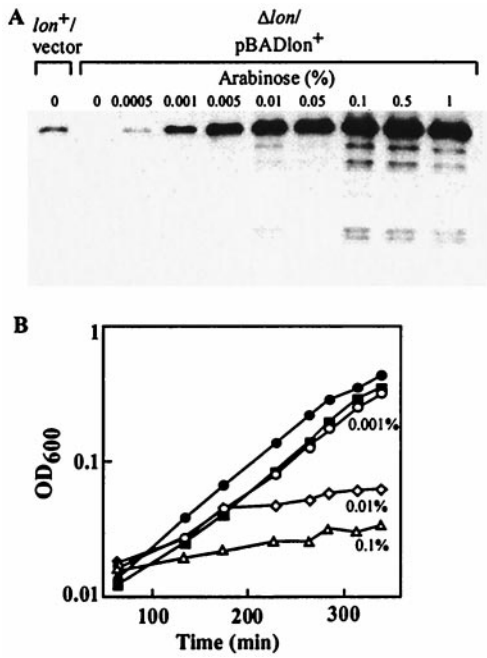

FIG. 2. Regulated synthesis of Lon. (*A*) Increasing levels of Lon produced with increasing arabinose concentrations. LVM781 (Δ*lon*)/pBADlon$^+$ and LVM783 (*lon*$^+$)/pBAD24 were grown in LB at 32°C to an OD_{600} of 0.2. Cultures were split, and arabinose was added at the final concentration indicated at the top of the panel. After 2 hr of induction, 1 ml of culture was removed and TCA-precipitated. Proteins were separated by SDS/PAGE, and Lon was detected by Western blot, as described in *Materials and Methods*. (*B*) Arrest of growth caused by Lon overproduction in minimal medium. Strains were grown at 32°C in minimal medium (M63) supplemented with glycerol (0.2%), vitamin B1 (0.0001%), and casamino acids (0.1%), containing arabinose as indicated below. The graph represents OD_{600} as a function of time. Strains used were: LVM781 (Δ*lon*)/pBADlon$^+$ grown in the presence of 0.001% (○), 0.01% (◇), or 0.1% (△) arabinose; LVM781/pBAD24 with 0.1% arabinose (●); and LVM783 (*lon*$^+$)/pBAD24 with 0.1% arabinose (■).

saturated; neither Lon nor the lower molecular weight Lon-related bands (probably breakdown products) increased further.

As seen previously with plasmids expressing Lon either from the native or other promoters (39) (S.G., unpublished observations), cells grew poorly or rapidly lost the plasmid after induction of high levels of Lon, particularly in minimal media (Fig. 2*B*) or at high temperature (data not shown). Efficiency of plating of cells on rich media decreased by 4 orders of magnitude at 42°C, in the presence of ≥ 0.5% arabinose (data not shown). It is possible that one or more proteins essential for growth have low affinity for Lon but are targeted inappropriately by excess Lon (39). Differential sensitivity to Lon overproduction under particular conditions (high temperature or minimal media) may reflect a change in Lon protease efficiency or specificity or an increased susceptibility to degradation or increased requirement for these substrates under these conditions.

In vivo activity of Lon expressed from the pBADlon$^+$ plasmid can be measured by using two well-characterized phenotypes of *lon* mutants. At normal cellular levels, Lon has been shown to degrade two regulatory proteins, RcsA and SulA (reviewed in ref. 40). RcsA is a positive regulator of capsule synthesis; its *in vivo* activity can be measured by expression of a *cpsB*::*lacZ* transcriptional fusion (*cps* for capsular polysaccharide synthesis). In a *lon*$^+$ cell, RcsA is unstable and the fusion is expressed poorly (17, 41); in a *lon* mutant the fusion is expressed well. SulA is an inhibitor of cell division synthesized during the SOS response to DNA damage. *lon* mutants are UV sensitive because the SulA induced after UV treatment blocks cell division by interacting with FtsZ, an essential cell division protein (42, 43). When Lon is present, SulA is rapidly degraded and cells are able to grow after repairing UV-induced DNA damage (16). We used these phenotypes (UV sensitivity and expression of the *cpsB*::*lacZ* fusion) to monitor the ability of the pBADlon plasmid and mutant derivatives to complement a *lon* deletion.

Both the UV sensitivity and the high levels of *cpsB*::*lacZ* expression observed in a *lon* mutant were complemented by the pBADlon$^+$ plasmid. UV sensitivity was tested by growing cells in rich medium, spotting serial dilutions of the culture on plates containing various amounts of arabinose, and exposing the plates to UV light. Under these conditions, the *lon*$^+$ host carrying a control vector plasmid has an efficiency of plating of 0.2, whereas the Δ*lon* host carrying the vector control has an efficiency of plating of 4×10^{-5}. The pBADlon plasmid restored full UV resistance to Δ*lon* cells at concentrations of arabinose of 0.1% and higher. Even without arabinose, under conditions where we were unable to detect Lon in a Western blot (estimated at less than 10% of the wild-type level of Lon) (Fig. 2*A*), the efficiency of plating of the *lon* mutant carrying pBADlon$^+$ was 0.01, which is 1,000-fold higher than for the control Δ*lon* strain.

In assays of the *cps* phenotype, expression of the *cpsB*::*lacZ* fusions was below 1 unit in a *lon*$^+$ host and about 45 units in the Δ*lon* host. Δ*lon* cells carrying the pBADlon$^+$ plasmid and grown without arabinose gave 40 units of activity, indicating very little complementation. At 0.001% arabinose, the concentration necessary to produce Lon protein levels equivalent to the single-copy level (Fig. 2*A*), almost full complementation was seen (5 units of activity). We were unable to measure *cpsB*::*lacZ* expression at higher arabinose concentrations because cells carrying the pBADlon$^+$ plasmid failed to grow well in the minimal medium (Fig. 2*B*).

***In Vivo* Complementation by a Lon S679A Mutant.** Site-directed mutations in the proteolytic active site (S679A), in the conserved ATPase motif (K362Q), or the double mutant (K362Q-S679A) (Fig. 1) were compared with the wild-type plasmid for ability to inhibit cell growth and to complement for *lon* phenotypes. Neither the S679A nor the K362Q mutant expressed from the pBAD plasmid caused cell death when overexpressed at 42°C (data not shown). Lon protein amounts produced from the different pBAD24 constructs, measured by Western blot, were comparable to that seen from the pBADlon$^+$ plasmid (data not shown).

Unexpectedly, the plasmid encoding Lon mutated in the active site serine, pBADlonS679A, was able to complement the UV sensitivity phenotype of a Δ*lon* strain. Because complementation was seen at high but not at low arabinose concentrations, whereas the pBADlon$^+$ plasmid showed partial complementation even in the absence of arabinose, overexpression of the mutant protein seems to be necessary for complementation (Fig. 3). Lon derivatives carrying the K362Q mutation, either in an otherwise wild-type *lon* gene or a double mutant carrying K362Q-S679A, gave no complementation for UV sensitivity even when induced with the highest arabinose concentrations (Fig. 3).

A similar pattern was observed for complementation of expression of a *cpsB*::*lacZ* fusion. At the lowest arabinose concentration tested, the wild-type protein expressed from pBADlon$^+$ gave some complementation; at higher concentrations, cells did not grow (Table 1). However, the plasmid expressing the *lon* S679A mutant complemented well (Table 1). K362Q and K362Q-S679A were not able to complement the capsule overproduction phenotype, consistent with their inability to complement for UV sensitivity.

Mutant Complementation by Sequestration. The assays described above indicate that overproduction of the Lon serine mutant S679A, expected to lack proteolytic activity, is able to

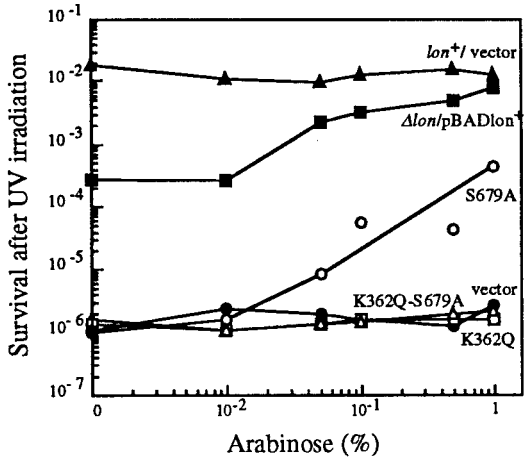

FIG. 3. Partial complementation of the UV sensitivity phenotype of a Δ*lon* strain by *lon* mutants. The graph represents the efficiency of plating after UV exposure as a function of arabinose concentration. Strains were grown in LB medium overnight at 32°C. After serial dilution, cells were spotted on LB plates containing arabinose at the final concentration indicated in the graph. Plates were UV irradiated (15–20 J/m^2). After overnight incubation at 32°C, colonies were counted and compared with the titer without UV irradiation. Strains were SG22542 (*lon*$^+$)/pBAD24 (▲), SG22569 (Δ*lon*) containing the pBAD24 vector (●), pBADlon$^+$ (■), pBADlonK362Q (□), pBADlonS679A (○), or pBADlonK362Q-S679A (△)

block SulA activity (leading to partial UV resistance) and RcsA activity (leading to low levels of capsule synthesis). It seemed unlikely that these effects on SulA and RcsA resulted from direct degradation of these substrates by the mutant Lon. Two classes of explanations were considered. The mutant Lon might have facilitated degradation of RcsA and SulA by another protease, either through regulatory effects on that protease or by direct or indirect modification of the substrates. Alternatively, interactions of Lon with the substrate, in the absence of degradation, might have been sufficient to sequester the substrates, thereby interfering with their normal activity.

To test whether Lon substrates were degraded in cells expressing the Lon mutants, we measured the half-life of SulA. In wild-type cells, SulA has a half-life of less than 2 min, and this half-life is increased to 20–30 min in a Δ*lon* strain, suggesting that Lon is the major protease responsible for SulA degradation (16). If the Lon mutants suppress UV sensitivity by increasing proteolysis, directly or indirectly, we would expect the SulA half-life to decrease. If SulA, however, is being

Table 1. Complementation of capsule synthesis by mutant Lon

		Arabinose concentration in medium,			
		0%	0.001%	0.01%	0.1%
Host	Plasmid	Expression of a *cpsB*::*lacZ* fusion (specific activity)			
Δ*lon*	pBAD24 (vector)	46	18	12	11
	pBADlon$^+$	40	5	N.D.	N.D.
	pBADlonS679A	36	8	3	4
	pBADlonK362Q	42	17	14	10
	pBADlonK362Q-S679A	45	15	12.5	20
lon$^+$	pBAD24	0.9	N.D.	N.D.	0.4

N.D., not done. Cells carrying the pBADlon$^+$ plasmid ceased growth or lost the plasmid at the higher arabinose concentrations. Δ*lon* strain is SG22569; the *lon*$^+$ host is SG22542. Lower β-galactosidase levels for strains carrying the pBAD24 vector at high arabinose concentrations were reproducibly observed; the basis for this is not known.

sequestered or inactivated but not degraded, SulA should be at least as stable as in the Δ*lon* strain itself.

The turnover of UV-induced SulA in the presence of overproduced mutant Lon was measured by treating cells with the protein synthesis inhibitor spectinomycin and following the decay of SulA as a function of time. SulA was visualized by Western blotting. In the presence of the wild-type Lon protein expressed from either the single copy gene or from pBADlon$^+$, SulA was not detectable in Western blots, consistent with rapid degradation. In the uncomplemented Δ*lon* cells, SulA was easily detected and the half-life was estimated at 30 min (Fig. 4*A*). When the S679A mutant was expressed in a Δ*lon* host, SulA was not degraded any faster than in the Δ*lon* strain without the mutant Lon. In fact, SulA appeared to be significantly more stable in the presence of S679A (half-life greater than 60 min) (Fig. 4*A*, ○). These results are most consistent with the S679A mutant leading to sequestration of substrates such as SulA and RcsA. The sequestration is apparently sufficient to keep SulA from interacting with its normal target, FtsZ, and to protect SulA from other proteases that degrade it slowly in the absence of Lon. The K362Q mutant and the K362Q-S679A double mutant, which did not show any complementation, neither protected nor degraded SulA, suggesting that an intact ATPase is necessary for sequestration (Fig. 4*A*).

For a more stringent test of the extent to which a mutant Lon protein could protect substrates from degradation, we measured SulA half-life in a strain carrying the FtsZ mutant *sfiB**. FtsZ, an essential cell division protein that forms a ring at the septum of dividing cells (44, 45), is the target of SulA. The *sfiB** mutation blocks the interaction of FtsZ with SulA, leading to resistance to SulA (42, 45, 46). We and others previously had noted that FtsZ can partially protect SulA from degradation, and that mutations that affect the interaction of FtsZ and SulA lead to more rapid degradation of SulA, even in cells lacking Lon activity (46, 47, 61). In the Δ*lon sfiB** strain, we estimated the half-life of SulA to be 10–15 min, compared with 30 min in the Δ*lon* background (Fig. 4*B*; note the difference in time scale between Fig. 4 *A* and *B*). Even in this host, expression of the S679A mutant led to stabilization of SulA for more than 40 min after the chase with spectinomycin, showing that SulA was completely protected from other proteases. SulA was not

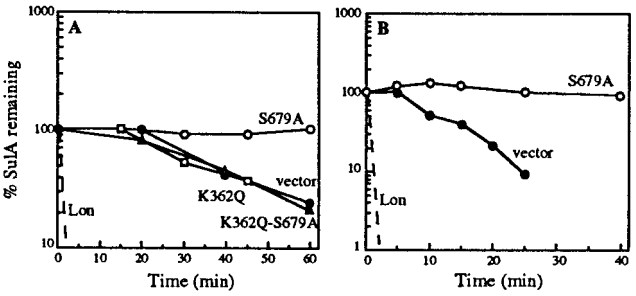

FIG. 4. SulA turnover in a Δ*lon* strain in the presence of mutant Lon protein. Strains were grown in LB medium supplemented with 1% arabinose to an OD_{600} of 0.2–0.3. Cells were UV irradiated at 15–20 J/m^2. After 20 min of growth to allow SulA induction, spectinomycin was added at a final concentration of 150 μg/ml. One milliliter of culture was removed at the times indicated and TCA-precipitated. Proteins were separated by SDS/PAGE, and Lon was detected by Western blot, as described in *Materials and Methods*. (*A*) SulA turnover in a Δ*lon ftsZ*$^+$ host. Percent of SulA remaining as a function of time after blocking protein synthesis with spectinomycin. Strains were SG22569 (Δ*lon ftsZ*$^+$) containing either pBAD24 (●), pBADlonK362Q (□), pBADlonS679A (○), or pBADlonK362Q-S679A (▲). The dotted line shows the previously observed degradation of SulA in a *lon*$^+$ host (16, 33). SulA was not detected in the *lon*$^+$ host in these experiments. (*B*) SulA turnover in a Δ*lon sfiB** host. As for *A*, but strains were LVM806 (Δ*lon sfiB**) containing either pBAD24 (●) or pBADlonS679A (○).

detectable in the Δ*lon sfiB** strain when wild-type Lon was expressed from the pBADlon$^+$ plasmid (data not shown). Protection of SulA from degradation strongly supports a model in which the mutant Lon protein promotes sequestration of SulA.

What is the sequestered state of SulA? We considered two possibilities. First, SulA could become aggregated in the presence of mutant Lon protein. Alternatively, SulA could bind tightly to the mutant Lon protein and be protected. We previously have observed that SulA does become insoluble in *lon* mutant strains when the DnaJ/K/GrpE chaperone system is impaired (33). SulA solubility was assayed under the same conditions as used in the turnover experiment. In the presence of the S679A and K362Q mutants, as well as in the *lon* deletion strain, SulA was found in the "high-speed supernatant" fraction, indicating that aggregation of SulA does not explain the protection from degradation in the presence of S679A (Fig. 5).

Requirement for an Intact Lon ATP Binding Site for SulA Sequestration. Both the K362Q protein and the K362Q-S679A protein, both mutated in the ATPase site, showed no evidence of complementation (Table 1, Fig. 3) and very little or no evidence for sequestration of SulA from other proteases (Fig. 4*A*). We have interpreted this to mean that an intact ATPase activity is necessary for movement of substrates into a sequestered state. A less interesting alternative for the failure of Lon ATPase mutants to sequester would be that these mutants are misfolded and/or unstable. However, the levels of the K362Q and K362Q-S679A proteins made from the pBAD promoter were comparable to the level of the wild-type Lon, and this particular ATPase mutation led to retention of low ATPase activity *in vitro* (unpublished data) (29). Also, the K362Q mutant has a dominant negative phenotype over the wild-type Lon (unpublished observations), suggesting it is folded sufficiently to multimerize, at least with a wild-type molecule. Thus sequestration appears to depend on an intact ATPase site, suggesting that an energy-dependent activity is necessary to change the mode of binding between Lon and its substrates.

DISCUSSION

Energy-dependent proteases, such as Lon, select abnormal and unstable proteins from the cytoplasm, and then use an ATP-dependent mechanism to transfer them to an arrayed set of active sites capable of cutting peptide bonds. Degradation provides a mechanism for regulating protein availability, eliminates unwanted proteins, and recycles amino acids, although at the expense of a significant amount of ATP. How selection of substrates is carried out and how ATP use is coupled to proteolysis are active areas of research. In the work described here, we find striking parallels between the ways in which Lon and the independent family of Clp proteases interact with and dispose of substrates. These parallels lead us to propose similar reaction pathways for all energy-dependent proteases and possibly for analogous ATP-dependent chaperones not associated with proteolytic active sites.

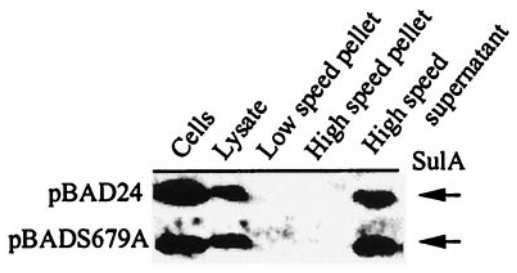

FIG. 5. SulA solubility. Differential centrifugations were performed as described in *Materials and Methods*. Samples of LVM781 (Δ*lon*) containing pBADlon$^+$ or pBADlonS679A were probed with anti-SulA antibody. Cells: Total extract prepared by TCA precipitation. Lysate: Total extract after French press treatment. Low-speed pellet: Resuspended pellet from centrifugation for 6 min at 3,500 rpm. High-speed pellet: Resuspended pellet from centrifugation for 15 min at 14,000 rpm. High-speed supernatant: From centrifugation for 15 min at 14,000 rpm.

We find that overproduction of a proteolytically inactive Lon, mutated in the serine active site (S679A), leads to partial complementation of the UV sensitivity and the capsule overproduction phenotypes associated with a *lon* deletion. Lon mutated in the ATPase consensus sequence (K362Q), however, shows no complementation, and introduction of the K362Q mutation into LonS679A abolished the suppression seen with the serine active site mutant. Normally, complementation by Lon is associated with increased degradation of RcsA (for capsule synthesis) and SulA (for UV sensitivity). However, complementation observed here did not result in rapid SulA degradation. In fact, the LonS679A mutant protected SulA from proteolytic destruction by proteases other than Lon. Based on these observations, we propose that the S679A Lon mutant binds substrates about as tightly as does the wild-type protease (Fig. 6 *A* and *B*). With wild-type Lon, substrates are passed to the proteolytic sites and the products of degradation are released (Fig. 6 *D* and *E*). However, in the S679A mutant in which degradation does not occur, substrates appear not to be released (Fig. 6*F*), suggesting that substrate passes from the initial binding sites through the proteolytic chamber, from which the peptide products normally would be released. In the absence of degradation of the substrate, no mechanism for release is available, and the substrate remains sequestered by the protease. Such sequestration of substrates by mutant Lon prevents the normal activity of SulA and RcsA, simultaneously making them inaccessible to other proteases. Overproduction of the mutant Lon was necessary to see significant complementation, which is consistent with a sequestration model in which mutant Lon would bind a stoichiometric quantity of substrate.

An alternative explanation for the ability of mutant Lon to protect substrates from degradation would be that Lon releases substrates in a form that is both inactive and resistant to proteolysis. One such inactive but protease-resistant form could be insoluble aggregates. However, we found that SulA extracted from cells in which it was inactive and protected from proteolysis was completely soluble (Fig. 5). Although a released, but soluble, protease-resistant and inactive state cannot yet be ruled out, we believe it is unlikely, especially because formation of such a species would not be likely to require high levels of mutant Lon.

Sequestration by ClpAP Protease Resembles Lon Sequestration. Our observations suggesting a tight-binding intermediate in the absence of proteolysis by Lon are intriguingly similar to recent *in vitro* results with the very different two-component ClpAP protease (48). For the Clp proteases, initial substrate recognition and binding is a function of the ATPase domain, which is contained within a subunit separate from the proteolytic domain. In *E. coli*, ClpA is one of two regulatory ATPases that can interact with the ClpP proteolytic subunit; substitution of the other ATPase subunit, ClpX, changes substrate specificity of the protease (22, 49). *In vitro*, in the absence of ClpP, ClpA and ClpX possess ATP-dependent chaperone-like (remodeling) activity with the same substrate specificity as that observed for degradation (2, 50, 51). Recent findings by Sue Wickner and coworkers (48) have shown that substrates translocated from ClpA to ClpP in an ATP-dependent reaction remain bound in the absence of ClpA when ClpP is proteolytically inactive. Therefore, for both Lon and ClpAP, initial substrate binding is a property of the ATPase domains, but ATP hydrolysis allows the substrate to be unfolded and translocated to a site where it will be sequestered if proteolysis is blocked.

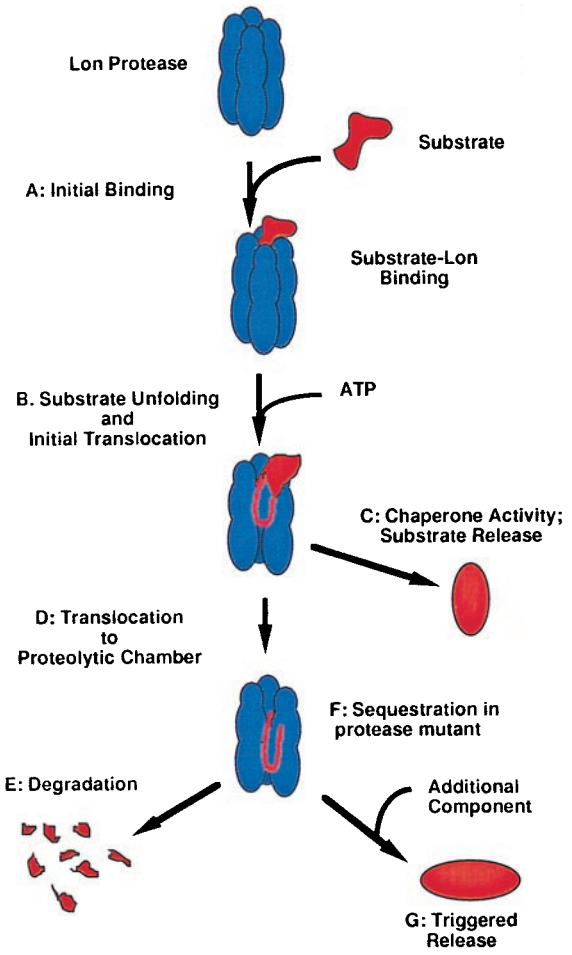

FIG. 6. Model for alternative activities of ATP-dependent proteases and related proteins. After initial recognition and binding of a substrate (*A*), an energy-dependent step is required for unfolding and translocation of substrates through the ATPase domain (*B*). In the case of ATPases devoid of a protease domain such as the Clp ATPases, or when degradation is otherwise bypassed, translocation may be followed by release of the protein; in this case the ATPase functions as a chaperone, allowing refolding or remodeling of substrates (*C*). When a protease-competent domain is present, translocation usually will result in rapid and processive degradation followed by release of products (*D* and *E*). If the proteolytic site is inactive, as in the Lon mutants described here or inactivated ClpP (48), or possibly when the protease domain is naturally absent (see *Discussion*), sequestration of the substrate may occur (*F*). We propose that in some cases, a triggered release of sequestered substrate would provide a way of delivering an unfolded substrate protein to a new environment (*G*).

A functional Lon ATPase was necessary for sequestration, as it is for rapid protein degradation. We know from previous work that ATP hydrolysis is also necessary for the unfolding associated with degradation of structured substrates by Lon (1). It seems likely that this unfolding is associated with translocation of substrates toward the proteolytic chamber, where they become stably bound when proteolysis cannot proceed. In sequestration of substrates by Clp, ATP was also necessary to see translocation of the substrate from ClpA to ClpP (48). The crystal structure of ClpP suggests that folded polypeptide chains are unlikely to be able to fit through the entry pore at the top of the ClpP chamber (52). Thus, unfolding of the substrate and possibly changes in the conformation of the ClpP entry pore may be required to allow access; ATP may be necessary for both steps. Nothing is yet known about the structure and accessibility of the Lon proteolytic active sites, except that, as for Clp, only short peptides or relatively disordered small proteins are capable of being rapidly degraded without ATP hydrolysis, consistent with folded polypeptides having access to the proteolytic chamber only after energy-requiring unfolding reactions catalyzed by the ATPase domain.

Chaperone Activity vs. Sequestration. Classical chaperones bind substrates and use ATP to remodel and release them. The activity of the Lon S679A mutant we have described here moves substrates into a sequestered state, presumably an intermediate stage on the way to degradation. For a protease with both chaperone activity and the ability to sequester substrates, *in vivo* evaluation of the function of degradation-deficient proteases may be misleading. The Clp ATPases, ClpA and ClpX, have both been shown to be able to remodel proteins that are otherwise degraded when ClpP is present (2, 50, 51). For Lon, with the proteolytic site on the same polypeptide chain as the substrate recognition and ATPase sites, expression of a chaperone-like activity would require inactivating or bypassing the proteolytic active site. It has been argued that *in vivo* function for the proteolytically inactive mutant form of proteases may be evidence that the protease is acting as a chaperone. Modest complementation of a Δ*lon* phenotype by the S675A mutant form of the Mycobacterial Lon (Ms-Lon) in *E. coli* was observed and the authors proposed either sequestration or a chaperone-like unfolding and release of the substrate in a protease-sensitive state as possible mechanisms (14); our results lead us to favor the sequestration model. In yeast mitochondria, Lon can suppress mutations in two other proteases (Afg3 and Rca1); the suppression, while requiring an intact ATPase, is helped when the conserved serine in the active site of Lon is mutated (53). Although these functions were attributed to a chaperone activity, our experiments suggest that sequestration by a mutant protease can substitute equally for degradation to functionally inactivate a normally unstable substrate.

In addition to a sequestration activity, we think it highly likely that Lon does contain an intrinsic chaperone activity. The requirement for ATP hydrolysis to degrade a relatively folded protein (CcdA), while a shorter, more disordered derivative (CcdA41) can be degraded without ATP hydrolysis is most easily explained by energy-dependent unfolding, an activity consistent with the ability to remodel (1). In addition, recent experiments by N. Craig and coworkers have implicated Lon chaperone activity in Tn7 transposition (C.A.S. Johnson and N. Craig, personal communication). Finally, observations made during this work and in earlier studies suggest that Lon degradation of naturally unstable substrates such as SulA and RcsA may reflect a chaperone-like dissociation of these substrates from their partners as part of the degradation pathway. We outline this argument below.

Consider the degradation of SulA in the presence or absence of an interaction of SulA with FtsZ, seen in the experiments presented here and in other work. In the absence of an interaction of SulA with FtsZ, examined by using the *sfiB** mutant host, SulA is turned over very rapidly in a *lon*$^+$ host. It is not detectable in Western blots even after induction, and in previous work was found to have a half-life of <5 min (54). More surprisingly, SulA is degraded with a 10- to 15-min half-life in a *sfiB** mutant even when Lon is absent (Fig. 4*B*). Clearly, because *lon* is deleted, other proteases must be responsible for this rapid degradation. In other work, we found that ClpYQ (also called HslVU) was responsible for the major part of this degradation (61). When SulA is capable of interaction with FtsZ, Lon degradation of SulA is still very rapid (16), but now the half-life of SulA in a *lon* deleted host is about 30 min (Fig. 4*A*), suggesting that secondary proteases are no longer able to degrade SulA rapidly.

What is the basis for this striking difference in SulA sensitivity to secondary proteases dependent on the ability of FtsZ to interact with SulA? We suggest that SulA structure may be significantly different in the presence and absence of FtsZ, and

the secondary proteases are unable to degrade it in the presence of FtsZ. This hypothesis also implies that normally most of the SulA in the cell is in a complex with FtsZ or is in such a complex enough of the time so that other proteases do not degrade it. It is known that SulA can be degraded from cells in which is it inhibiting cell division, allowing division to resume (55). Either Lon differs from other proteases because it can recognize and capture SulA in the short periods it is not in a complex with FtsZ or Lon can remove and degrade SulA from a FtsZ/SulA complex, whereas the other proteases do this poorly. If this is the case, it means that at least a portion of the substrate specificity of Lon for SulA is not caused by Lon recognition of SulA per se but by the ability of Lon to remove SulA and/or recognize it in a complex, whereas many of the cellular proteases recognize it only when it is not associated with FtsZ.

At least one other Lon substrate, RcsA, also has some slow Lon-independent degradation that is sensitive to the interaction of RcsA both with itself and with its partner, RcsB. Lon degrades RcsA rapidly whether or not RcsB is present (41). In the absence of Lon, RcsA tends to form aggregates that are relatively resistant to secondary proteases (33). However, when RcsB is absent, RcsA is turned over much more rapidly by these secondary proteases, before aggregates can form (Y. Jubete, M. Maurizi, and S.G., unpublished work) (41). It is not known what other proteases are responsible for degradation of RcsA, but they are apparently extremely active, because single-copy RcsA cannot be detected in the absence of RcsB. Although no structural information is available for SulA or RcsA, a third Lon substrate, lambda N protein, recently has been examined by NMR. Like RcsA and SulA, lambda N is degraded rapidly by Lon and slowly by proteases other than Lon (33). In this case, N interacts with at least two partners, a specific RNA sequence, called nut (N utilization), and the essential NusA protein. It is not yet known whether interaction with the partners affects N turnover. However, it is striking that N has been found to be relatively unstructured in solution, in the absence of NusA or the RNA (56). It is only in the context of these partners that N becomes structured. Possibly a lack of structure in the absence of the appropriate partner may be a characteristic of most of the Lon substrates. In such a case, substrate recognition by Lon reflects the ability of Lon to either bind and degrade a protein quickly enough when it is not bound to partners or the ability to remove the protein from its partners, rather than recognition of a specific motif or structure. The recognition of unstructured protein is consistent with the role of Lon in degradation of abnormal proteins; essentially all unstable proteins carrying canavanine in place of arginine are stabilized in Lon mutants, suggesting some general characteristic of improperly or relatively unfolded proteins is recognized by Lon (57).

Extending the Model: Is Sequestration a General Property of the ATPases Associated with Energy-Dependent Proteases? The observations that proteolytically inactive Lon or ClpAP proteases sequester substrates raise the possibility that similar sequestration activities might exist naturally. For instance, proteins with sequence similarity to the Lon ATPase, but without the proteolytic domain have been noted in the *E. coli* genome (YifB) and elsewhere, although their function has not been determined (58). The AAA ATPases, the ATPase domains of both the FtsH protease of *E. coli* and the 26S eukaryotic protease, had been identified initially associated with a variety of membrane fusion activities (see ref. 59 for recent review). These proteins do not contain obvious protease sites and proteolysis has not been associated with their function, but proteins such as NSF, a member of this family, are known to promote ATP-dependent disassembly of specific protein complexes in response to other protein and membrane signals. In *Bacillus subtilis*, ClpC appears to act like a sequestration protein for ComK, the competence transcription factor, via the MecA protein. ATP-dependent formation of the ternary complex of ClpC-MecA-ComK prevents ComK from activating transcription of the competence genes. ComS, a small protein synthesized in response to high cell density or nutritional stress, interacts with this ternary complex and causes the release of active ComK (60).

We propose that ATPases such as Lon, Clp, and the AAA ATPases may act as natural sequestration proteins, differing from chaperones in that, after binding and unfolding specific protein substrates, substrates will be held in a sequestration site until release is triggered by other protein–protein or protein–membrane interactions (Fig. 6 *C* and *G*). As in the case we examined here, the substrate will be held in a form where it cannot act. However, sequestration, in contrast to proteolysis, generally will be a reversible reaction. As a result, the unfolded or disassembled substrate would be protected from inappropriate interactions or aggregation until it was delivered to the correct environment. Possibly under some conditions the ATP-dependent proteases naturally abort or are inhibited for degradation and serve themselves as sequestration proteins.

SUMMARY

Our observations here on substrate sequestration by Lon coupled with similar observations for the Clp protease suggest a common pathway by which substrates, once recognized and processed by the ATPase domain, are transferred to the proteolytic active site. Transfer appears to occur in a protected environment, such that, if the proteolytic active sites are not active or available to degrade the unfolded substrate, the substrate can remain in a sequestered state for some period of time. Similar sequestration activities by related ATPases, in the absence of proteolysis, might allow release under specific, triggered conditions. The exact details of the coupling between ATP degradation and release of substrate for proteolysis are likely to be specific for each case, but structures and the initial steps of the pathway may be unexpectedly similar.

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