Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11528–11533, October 1996
Biochemistry

Mutant LexA proteins with specific defects in autodigestion

(self-processing reactions/LexA repressor/specific cleavage/proteolysis/RecA-mediated cleavage)

DONALD P. SHEPLEY[†] AND JOHN W. LITTLE[†‡§]

Departments of [†]Biochemistry and [‡]Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721

Communicated by Sydney Kustu, University of California, Berkeley, CA, July 24, 1996 (received for review April 4, 1996)

ABSTRACT **In self-processing biochemical reactions, a protein or RNA molecule specifically modifies its own structure. Many such reactions are regulated in response to the needs of the cell by an interaction with another effector molecule. In the system we study here, specific cleavage of the *Escherichia coli* LexA repressor, LexA cleaves itself *in vitro* at a slow rate, but *in vivo* cleavage requires interaction with an activated form of RecA protein. RecA acts indirectly as a coprotease to stimulate LexA autodigestion. We describe here a new class of *lexA* mutants, *lexA* (Adg⁻; for autodigestion-defective) mutants, termed Adg⁻ for brevity. Adg⁻ mutants specifically interfered with the ability of LexA to autodigest but left intact its ability to undergo RecA-mediated cleavage. The data are consistent with a conformational model in which RecA favors a reactive conformation capable of undergoing cleavage. To our knowledge, this is the first example of a mutation in a regulated self-processing reaction that impairs the rate of self-processing without markedly affecting the stimulated reaction. Had wild-type *lexA* carried such a substitution, discovery of its self-processing would have been difficult; we suggest that, in other systems, a slow rate of self-processing has prevented recognition that a reaction is of this nature.**

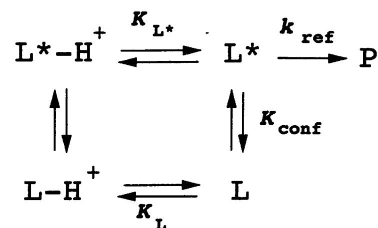
Self-processing biochemical reactions are those in which a protein or RNA molecule carries out a specific covalent modification of its own structure (1). In principle, the rates of self-processing reactions can be very high, because all the reactants are present at high local concentrations. Nonetheless, in some cases, the rates of self-processing reactions are slow and are greatly stimulated by an external agent, implying that these self-processing molecules are designed to undergo a slow intrinsic reaction but to be capable of large rate increases. Examples of regulated reactions include many reactions in two-component regulatory systems; LexA repressor self-cleavage, which is mediated by an activated form of RecA; and an "honorary" self-processing reaction, the GTPase activity of eukaryotic Ras proteins, which is stimulated by interactions with GTPase-activating proteins.

It is of interest in such systems to understand the relationship between the self-processing reaction and its stimulated counterpart. Are the mechanisms of these reactions the same? That is, does the effector increase the rate of the reaction, or does the stimulated reaction follow a different pathway? In the present system, we have explored the properties of mutant proteins to address the relationship between self-cleavage and its RecA-stimulated counterpart. In this work, we describe a new type of mutant: one that affects the rate of a self-processing reaction but not that of the reaction catalyzed by the outside effector.

The self-processing reaction we study here plays a central role in the SOS regulatory system of *Escherichia coli* (1–3). In this system, a set of ≈20 genes is derepressed following conditions that damage DNA or inhibit DNA replication. These SOS genes are under control of two regulatory proteins: the LexA repressor, which represses the SOS genes during normal growth; and the RecA protein, which is quiescent during normal growth but is promptly activated upon inducing treatments to a form that can facilitate the specific cleavage of LexA. Cleavage inactivates LexA, leading to derepression of the SOS regulon. Although RecA serves as a catalyst *in vivo* for LexA cleavage, RecA serves an indirect role in stimulating cleavage, and hence we term this function of RecA its coprotease activity.

As with any self-processing molecule, LexA has an active site and a substrate site. The cleavage site is analogous to the substrate in an enzyme-catalyzed reaction; the active site is composed of a catalytic center, which carries out the chemistry of bond breakage, and of a binding pocket, which binds the cleavage site and positions the peptide bond relative to the catalytic center. LexA (202 aa) has the following domain structure: the N-terminal domain is the DNA-binding domain; the C-terminal domain allows dimerization and contains the active site for cleavage; and the cleavage site (Ala-84/Gly-85) lies between the two domains. At a mechanistic level, LexA is almost certainly a serine protease; Ser-119 in LexA reacts with a serine protease inhibitor (4), and changes of Ser-119 to Ala completely block cleavage (5). Unlike classical serine proteases, however, the serine appears to be activated by a deprotonated lysine residue, Lys156 in LexA. It seems likely that RecA acts by somehow reducing the pK_a of Lys-156, allowing cleavage to proceed at neutral pH.

A model for the action of RecA was recently developed (6) based on the properties of a new type of *lexA* mutation, termed *lexA* (Ind^S) and here referred to as Ind^S. Ind^S mutant proteins display greatly increased rates of specific cleavage, both *in vivo* and *in vitro* (6–8). Biochemical analysis of the cleavage reaction for three of these proteins, all changing Gln-92 in LexA to an aromatic amino acid, led to the conclusion that these mutations mimic, to some extent, the role of RecA in promoting cleavage. Based on the properties of these mutant proteins, we developed a model (Scheme **I**) for the specific cleavage reaction.



Scheme **I**

In Scheme **I**, P represents the products of the cleavage reaction. According to this model, LexA can exist in two forms: the L form cannot autodigest, while the L* form autodigests with a first-order rate constant k_{ref}. L and L* interconvert, and the [L*]/[L] ratio is defined by the equilibrium constant K_{conf}.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.
[§]To whom reprint requests should be addressed. e-mail: jlittle@biosci.arizona.edu.

Both L and L* can be protonated on Lys-156, but the pK_a for protonation is considerably lower for L* than for L; pK_{L^*} is <6, while pK_L is $\approx$ 10.

One important tenet of this model is that the Ind^S mutations increase the value of K_{conf} and that RecA has the same effect, but to a far greater extent. If $K_{conf} \gg 1$, the equilibrium between LH^+ and L* is shifted toward L*, reducing the apparent pK_a of LexA. Activated RecA greatly stabilizes the L* form, even at neutral pH. We omit RecA from this diagram, since there may be multiple equilibria involving RecA and RecA:LexA complexes, and we do not know which species of LexA bind to RecA.

The model predicts that it may be possible to isolate LexA mutations that have specific defects in autodigestion but are largely unaffected for RecA-mediated cleavage. These mutations would be those that specifically reduce the value of K_{conf}. If RecA could overcome the effect of these mutations, it might still be able to exert its effect on cleavage, so that it could stabilize the L* form well enough to promote cleavage at neutral pH. Accordingly, we would expect a mutation with a reduced K_{conf} to be much more severely affected for autodigestion than for RecA-dependent cleavage; the difference would depend on the extent to which RecA could overcome the defect. Hence, we term this type of mutation a *lexA* (Adg^-) mutation, for autodigestion-defective, and refer to it here as Adg^- for the sake of brevity. We report the isolation of several Adg^- mutants.

MATERIALS AND METHODS

Chemicals and Reagents. Restriction enzymes were from New England Biolabs, DNA ligase was from Promega, dNTPs were from Perkin–Elmer, *Taq* and *Pfu* DNA polymerases were from Stratagene, and Sequenase (version 2.0) was from United States Biochemical. Oligodeoxynucleotides were made in the Macromolecular Structures Facility at the University of Arizona. RecA protein was from United States Biochemical. LexA and mutant derivatives were purified as described (9); chromatographic steps used step elutions, and the Affigel 501 (Bio-Rad) step was omitted.

Plasmids and Bacterial Strains. Bacterial strains used (with relevant genotype) were the following. JL468 was AB1157/F′ *lacI*^{q} (10). JL1752 *recA*Δ*306*::Tn*10 sulA211 lexA71*::Tn*5* (λ *sulA*::*lacZ imm*$^\lambda$ *cI*ind$^-$)/F′ lacI^q*I*^{s} (11) and JL1436 *recA*$^+$ *sulA211 lexA71*::Tn*5* (λ *sulA*::*lacZ imm*$^\lambda$ *cI*ind$^-$)/F′ lacI^q *lacZ*ΔM15::Tn*9* (12) were used as indicator strains for LexA function. JL2963 was a *recA*Δ*306*::Tn*10* Δ*lon510* derivative of JL1436 (7). Strain HMS174 (λDE3)/pLysS was used as a host for synthesis of LexA and mutant proteins from pJWL228 derivatives.

The following plasmids, based on pBR322, were used. pJWL184 carried an operon fusion of the *lac*UV5 promoter to the wild-type *lexA* gene (9, 11); this gene contains silent changes at codons for residues 65, 101, 152, and 180, creating unique *Bam*HI, *Sal*I, *Bst*EII, and *Bgl*II sites, respectively. pShep15 was made from pJWL184 in three steps. First, an *Ngo*MI site located in the M13 origin of replication was destroyed by treatment with *Ngo*MI, mung bean nuclease, DNA ligase, and *Ngo*MI, followed by transformation to give pShep8. Two successive steps of site-directed mutagenesis introduced silent changes at the codons for residues 85 and 166/167, making unique *Ngo*MI and *Sac*I sites, respectively. All changes were verified by sequencing. pJWL228 is a derivative of pET11a in which the wild-type *lexA* gene from pJWL184 is fused to the T7 promoter. Plasmids based on pJWL184 (or pShep15 in a few cases) and pJWL228 were made that contained one or more mutations affecting cleavage, as indicated; pJWL184 derivatives were used for *in vivo* assay of repressor function, and those based on pJWL228 were used for overproduction of LexA for biochemical studies. Primary isolates of pJWL228 derivatives were maintained in JL468, which lacks T7 RNA polymerase, to avoid selective pressure for loss of LexA function.

Assay of Cleavage Rate. Reaction conditions for LexA autodigestion and RecA-mediated cleavage were measured as described (9). Autodigestion was carried out at pH 10.0 or, where indicated, pH 8.7. In RecA-mediated cleavage reactions, activated RecA was at 1 μM and LexA was at 4 μM. Rates were estimated by visual inspection of Coomassie-stained gels, and have an uncertainty of ≈20%. In some experiments, we used Tricine gels (13), which give better resolution of the cleavage fragments than the Laemmli system. We estimated from mixing experiments that a sample containing 70% fragments and 30% intact LexA gave about equally intense staining for the intact protein and the C-terminal fragment.

***In Vivo* Assay for Repressor Function.** Derivatives of pJWL184 were introduced into strain JL1752, which is *recA*$^-$ *lexA* (Def), so that the only LexA function was provided by the plasmid, and cleavage could not result from the action of RecA. This strain also carried a *sulA*::*lacZ* operon fusion as a reporter of LexA repressor function, and β-galactosidase levels were detected on MacConkey indicator plates. Finally, the strain had an F′ with a *lacI*^{q}*I*^{S} mutation, rendering the Lac repressor insensitive to the presence of the inducer, lactose, in the indicator plates, ensuring that LexA was expressed at the basal level from the *lac* promoter. In this system, a cell with a plasmid coding for wild-type or Ind^- LexA protein formed a white colony, while one with a plasmid coding for QW92 made a deep red colony (data not shown).

To isolate suppressors of the Ind^S mutation QW92 (see *Results*), various regions of the *lexA* gene from residues 65 to 180 were mutagenized by PCR mutagenesis (14), followed by cloning into pJWL184 or pShep15. Ligation mixtures were transformed into JL1752, and aliquots were spread on MacConkey ampicillin plates. Cells carrying suppressors formed white or pink colonies. Plasmids were isolated from such variants; they were retransformed into JL1752 to map the suppressor to the plasmid. To screen for response to the SOS-inducing agent Mitomycin C, plasmids were transformed into the *recA*$^+$ strain JL1436, and colony colors on plates containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) were compared with and without 0.05 μg of Mitomycin C per ml. VA82 was isolated from a mutagenized *Bam*HI–*Sal*I fragment.

We isolated a large number of white or pink colonies among transformants of the QW92 plasmid. This approach yielded several isolates of VA82, the mutant described in the text; no other isolates gave the phenotype expected of an Adg^- mutation. However, these VA82 isolates may not be independent, since they arose by PCR mutagenesis of a single preparation of plasmid DNA; hence, we cannot be certain that we have saturated *lexA* for this type of mutant.

Miscellaneous. Routine cloning procedures were as described (15). Site-directed mutagenesis was carried out by PCR amplification using mutated primers, followed by subcloning of PCR products; the DNA sequences of all intervals arising from PCRs were determined. DNA was sequenced using Sequenase (United States Biochemical). Western blot analysis of cell extracts using a rabbit antibody against LexA (11, 16) was done using the enhanced chemiluminescence (ECL) Western Blotting Detection System (Amersham). β-Galactosidase was assayed as described (17), except that cells were grown in Luria broth (17) supplemented with the appropriate antibiotics. Computer analysis of protease superfamilies was carried out using the BLOCKS program (18); the trypsin and subtilisin superfamilies are denoted BL00134C and BL00136C, respectively, in the data base of this program.

RESULTS

Screen for Adg⁻ Mutants. Because the *in vivo* rate of LexA autodigestion is very slow, RecA is normally required for LexA cleavage; accordingly, previous genetic screens for mutant LexAs with cleavage defects would not isolate Adg⁻ mutants, since these by definition are not defective for RecA-mediated cleavage. We took advantage of the finding (7) that the Ind^S mutant protein QW92 rapidly autodigests *in vivo* in the absence of RecA, with a half-life of 1–2 min, so that it confers almost no repressor function. We reasoned that an Adg⁻ mutation would suppress the rapid rate of QW92 autodigestion, leading to increases in repressor function that could be detected with the use of a LexA-controlled *lacZ* reporter gene. Second-site intragenic suppressors of the QW92 mutation that reduce its rate of self-cleavage should result in formation of colonies that were less red than the QW92 parent on MacConkey indicator plates, due to reduced expression of β-galactosidase. Although many such isolates should be like the Ind⁻ mutants previously described, it was our hope that Adg⁻ mutants would also be included in the pool. As detailed in *Materials and Methods*, this screen yielded a second-site suppressor, VA82, that lies near the cleavage site.

Analysis of Mutations Near the Cleavage Site. The phenotype of the VA82-QW92 double mutant suggested that it might be an Adg⁻ mutant. First, it formed pink colonies on the initial indicator strain, indicating that VA82 partially suppressed the QW92 mutation. Second, a *recA*⁺ strain carrying the VA82-QW92 double mutation gave far higher levels of β-galactosidase upon treatment with mitomycin C, an SOS-inducing agent, than in the absence of mitomycin C, as judged by an increase in blue color on X-Gal plates (data not shown). This finding implied that the protein could undergo RecA-mediated cleavage.

To test whether VA82 allowed normal repressor function, it was separated from QW92, and its ability to repress the *sulA-lacZ* fusion was assessed in a *recA*⁻ strain (Table 1). The protein conferred repressor function almost equivalent to that of wild-type and a noncleavable mutant, SA119. Accordingly, VA82 did not appear to disrupt the repressor structure.

To test whether VA82 could undergo RecA-mediated cleavage *in vivo*, we assayed repressor function in a *recA*⁺ strain in the absence or presence of an SOS-inducing agent, Mitomycin C (Table 2). VA82, as well as the VA82-QW92 double mutant, gave levels of expression almost as high as that shown by wild-type after inducing treatment, suggesting that the rate of RecA-mediated cleavage under *in vivo* conditions was the same as that of wild-type or nearly so.

Table 1. Effects of Adg⁻ mutations on *in vivo* repressor function

lexA allele	β-Galactosidase-specific activity	Percent of full expression
None	6000	≡100
Wild type	47	0.8
SA119 (Ind⁻)	47	0.8
VA82	60	1.0
VG82	120	2.0
VS82	95	1.6
VT82	52	0.9
GA117	1700	28
GS117	100	1.7
GT117	250	4.1

Cultures of JL1752 carrying pJWL184 derivatives with the indicated mutations in *lexA* were grown exponentially in Luria broth plus ampicillin, and β-galactosidase activity was measured as described (17). The strain lacking LexA function carried a derivative of pJWL184, in which the *lexA* gene was deleted. Values given are the averages of duplicate assays; the results of a single representative experiment are given.

Table 2. RecA-dependent *in vivo* inactivation of VA82

	β-Galactosidase-specific activity		Percent of full expression	
lexA allele	− MitC	+ MitC	− MitC	+ MitC
None	3910	4004	≡100	≡100
Wild type	258	1190	6.6	29.7
SA119 (Ind⁻)	12	12	0.3	0.3
VA82	163	867	4.2	21.7
VA82-QW92	318	858	8.1	21.4

Derivatives of the *recA*⁺ strain JL1436 were grown as in Table 1; cultures were then split, and Mitomycin C was added to a portion (1 μg/ml final concentration). After shaking for an additional 60 min, both portions were assayed as in Table 1. In the absence of Mitomycin C, the wild-type showed a higher level of expression than the Ind⁻ control; this behavior has been seen previously in similar uncoupled systems (7, 11), and we attribute it to a low level of RecA-mediated cleavage in uninduced cells (16). We would expect the difference between wild-type and an Ind⁻ derivative to be far less when *lexA* is present on the chromosome, because the *lexA* gene is autoregulated (2). MitC, Mitomycin C.

To test directly if VA82 was an Adg⁻ mutation, we purified the VA82 mutant protein and characterized its rates of autodigestion and RecA-mediated cleavage (Table 3). We found that VA82 reduced the rate of autodigestion ≈10- to 20-fold, while having little or no effect on the rate of RecA-mediated cleavage. To test whether the defect in autodigestion resulted from exposure to pH 10 in the autodigestion assay, this assay was also carried out at pH 8.7; again, a slow rate of autodigestion was observed. We conclude that VA82 is an Adg⁻ mutation.

Table 3. Relative rates of *in vitro* cleavage of Adg⁻ mutant proteins

	Relative rate of autodigestion		
Protein	pH 8.7	pH 10	Relative rate of RecA-mediated cleavage
Wild type	≡1.0	≡1.0	≡1.0
VA82	0.05	0.05*	1.0
VS82	<0.01	<0.01	1.0
VG82	0.03	≈0.01	0.4
VT82	0.04	0.01–0.02	1.0
GA117	<0.01	<0.01	0.3
GS117	<0.01	<0.01	0.3
GT117	<0.01	<0.01	<.02
VM82 (Ind⁻)		<0.005†	0.07†
GE117 (Ind⁻)		<0.005†	0.06†

Rates of cleavage were measured as described. For autodigestion of wild-type LexA, the half-lives at pH 8.7 and 10 were 60 and 10 min, respectively. For RecA-mediated cleavage, half the wild-type LexA was cleaved in 4 min. In autodigestion reactions, we estimated that we could easily detect cleavage of 10% of the substrate. For a substrate cleaving at 0.01 times the wild-type rate, cleavage with first-order kinetics would give this amount in 17 and 2.5 hr at pH 8.7 and 10, respectively; when we saw no cleavage after 48 hr of incubation, we estimated conservatively an upper limit of 0.01 times the wild-type rate; for pH 10 reactions, this conservative limit was set because it is possible that this high pH might inactivate the protein during prolonged incubation.

*In some experiments, the time course of VA82 autodigestion appeared to be biphasic; ≈20% of the protein autodigested at 10-20% the rate of wild-type LexA, and the remainder broke down much more slowly. This pattern suggested that the VA82 protein contained two different forms that interconverted slowly and differed in their ability to autodigest; to test this idea, we carried out RecA-dependent cleavage at pH 10, the same pH as used for autodigestion. The rate of wild-type and VA82 cleavage was the same, and there was no sign of a biphasic rate for VA82. This finding suggests that VA82 does not exist in two forms, but it is plausible that RecA can catalyze the interconversion of such forms.

†Data taken from Lin and Little (12).

To test whether other changes at position 82 could confer an Adg$^-$ phenotype, we used site-directed mutagenesis to change Val-82 to Thr, Ser, and Gly, three other amino acids with small side chains. *In vivo*, VT82, VS82, and VG82 appeared to confer normal repression (Table 1), suggesting that they do not impair repressor structure. The mutant proteins were purified and the rate of cleavage was measured. These assays showed that the rate of autodigestion was also reduced to low values, ≈1% that of wild-type in the case of VS82 and VG82, while the rate of RecA-dependent cleavage was normal or nearly so (Table 3). We conclude that VT82, VS82, and VG82 are also Adg$^-$ mutations.

Analysis of Mutations Near the Active Site. In previous work (12), we found that several Ind$^-$ mutant proteins with defects in RecA-mediated cleavage were even more severely affected for autodigestion. These included VM82, at the same residue as the Adg$^-$ mutants described above, and GE117 (see Table 3). Gly-117 is of particular interest, because it is completely conserved in all the known cleavable proteins of the LexA type, and it lies two residues away from the active site Ser-119; accordingly, it probably lies in or very near the active site. To test whether substitutions in position 117 could also confer the Adg$^-$ phenotype, we changed it to Ala, Ser, and Thr, replacing the hydrogen of Gly-117 with a small side chain. Again, *in vivo* function was assessed with reporter genes; mutant proteins were purified and their *in vitro* cleavage rates were measured.

The pattern shown by these mutant proteins was more complicated than for those altered at position 82. The GA117 protein gave poor repressor function, as judged by the expression of the reporter gene *in vivo* (Table 1), but it appeared to be of the Adg$^-$ type when cleavage was assayed (Table 3). Preliminary Western analysis (data not shown) revealed that GA117 protein levels were low, presumably accounting for the defect in repressor function (see next section). The GT117 protein was partially functional as a repressor, but showed little or no cleavage in either type of cleavage reaction. Finally, the GS117 protein gave almost wild-type repressor function, and was of the Adg$^-$ type, being far more impaired for autodigestion than for RecA-mediated cleavage. We conclude that GS117 is an Adg$^-$ mutant protein with essentially normal structure; GA117 is also an Adg$^-$ mutant protein, but its structure may be abnormal (see next section).

Interaction of Adg$^-$ Mutations with Other Alleles Affecting Cleavage. We next tested whether Adg$^-$ mutations at positions 82 and 117 acted independently of one another and of other alleles affecting cleavage. We combined VA82 with each of the position 117 mutations, and then made many other multiple mutant proteins, and carried out the same assays as used above. This analysis showed that the two mutations did not act independently of each other nor of other mutations affecting cleavage. We focus primarily on the results for the VA82-GS117 combination.

In vivo, this combination did not give a wild-type level of repressor function (Table 4, line 6), a finding compatible either with effects on protein structure or on cleavage. *In vitro*, purified VA82-GS117 double mutant protein could not undergo specific cleavage, either in the autodigestion or RecA-mediated cleavage reaction (data not shown).

Addition of SA119, which suppresses repression defects of QW92 and other Ind^S mutations, did not have this effect on the VA82-GS117 combination; instead, it caused a further defect in repressor function. This finding strongly implies that the mutations did not act independently of one another, and that, at least for the triple mutant protein, the cumulative effect of the three changes was to disrupt the structure. Several other mutant combinations were also tested for repressor function. Adding SA119 to GS117 impaired repressor function (Table 4, line 5), but improved that of QW92-GS117 (line 7). Adding the Ind^S mutation QW92 to VA82-GS117, making the triple mutant protein VA82-QW92-GS117, yielded essentially no *in vivo* repressor function (line 8), and SA119 did not suppress this defect. In striking contrast, another Ind$^-$ mutation, KA156, completely restored repressor function to VA82-GS117, with or without QW92 (last column). Many other allele-specific effects are also evident in these data.

Preliminary Western analysis of extracts from cells of strains carrying many of these plasmids (data not shown) showed a correlation between the level of LexA and its capacity to repress the reporter gene; that is, cells showing defects in repression contained lower steady-state levels of LexA than those with normal repressor function. We also did Western analysis in a *recA$^-$ lon$^-$* strain, in which the C-terminal cleavage product is relatively stable (7), and which contained wild-type Lac repressor to allow isopropyl β-D-thiogalactoside (IPTG) induction; in the presence of IPTG, most proteins were present at high levels, and only QW92 showed high levels of C-terminal fragment, while intact protein levels were similar to those shown by wild-type for most of the mutant combinations tested. We infer tentatively that the reduced protein levels seen for many mutant proteins in the former set of strains probably resulted from nonspecific breakdown by a degradative system that could be saturated by high levels of LexA.

DISCUSSION

We have described a new class of lexA mutations that selectively impair the ability of LexA to autodigest, while leaving intact its ability to undergo RecA-mediated cleavage. Two diametrically opposed interpretations of these findings can be offered. One view is that these data support the idea that the

Table 4. Effects of multiple mutations on LexA function

	Residue at position			Percent gene expression for combination with		
Line	82	92	117	Wild type at S119 and K156	SA119	KA156
1	+	+	+	0.9	0.9	0.4
2	A	+	+	1.0	1.4	ND
3	+	W	+	75	2.0	ND
4	A	W	+	7.7	6.8	ND
5	+	+	S	1.5	3.1	ND
6	A	+	S	7.4	18	0.5
7	+	W	S	36	24	ND
8	A	W	S	46	60	0.8

Numbers in the last three columns represent the levels of β-galactosidase in strain JL1752 as a percentage of that seen in a host carrying a derivative of pJWL184 with a deletion of the *lexA* gene. Cultures were grown and assayed as in Table 1. In the absence of LexA function, the specific activity of β-galactosidase was 5100. One-letter codes for amino acids are used; the + indicates wild-type. ND, not done.

two cleavage reactions are not closely related, contrary to our working model for LexA cleavage. In this view, the ability to separate these reactions by mutation suggests that RecA-mediated cleavage involves a pathway distinct from that leading to autodigestion, and that the Adg^- mutations affect the latter pathway.

The alternative view is that the two cleavage reactions follow, for the most part, a common pathway, and that the Adg^- mutations act to impair autodigestion in a way that RecA is able to overcome. For several reasons, we favor this view. Many previous lines of evidence (1) argue for a close mechanistic relationship: the two types of cleavage reactions have the same specificity (that is, the same cleavage site); most Ind^- mutations that affect RecA-mediated cleavage also affect autodigestion; and several other proteins undergo both reactions. In addition, there is no evidence that RecA is a classical protease with an active site that carries out the chemistry of cleavage. Although we cannot rule out the former model completely, we can explain the properties of the Adg^- mutant proteins in terms of a conformational model for LexA cleavage (see Introduction and below), and will assume for the sake of the following discussion that our data do not disprove the existence of a close mechanistic relationship between the two cleavage reactions.

We suggest that the Adg^- mutant proteins are impaired in their ability to form a productive cleavage site–active site complex. These mutations affect residues near the cleavage site and active site, suggesting that some aspect of this interaction is affected. At the same time, the Adg^- mutant proteins show normal or near-normal rates for the RecA-mediated cleavage reaction, implying that their defects in autodigestion can be overcome by RecA. In terms of the conformational model (6) for LexA cleavage (Scheme **I**), we suggest that the value of K_{conf} is reduced in the Adg^- mutant proteins, but in a way that RecA can overcome. Perhaps the value of K_{conf} in the RecA-catalyzed reaction is reduced from that for the wild-type protein, but it should still be high enough to put essentially all the protein in the L* form.

The properties of the Adg^- mutants also have implications for structure–function relationships in LexA. It is not known what the relationship is between the cleavage site and the active site in LexA and related proteins. Two opposing models are the following. The cleavage site could normally lie within the binding pocket of the active site in a way that does not allow cleavage, and this reaction would occur only upon a relatively subtle realignment of groups within this complex. Alternatively, as embodied in the "dumbbell" structure often used to depict λ CI repressor (19), the cleavage site could be fully exposed to solvent, lying in an extended hinge region connecting two structural domains, and cleavage would result when the cleavage site bound to the active site. Various lines of evidence (4, 10, 19–23) have provided support for both points of view, and we do not regard the issue as resolved.

The present work does provide, however, additional support for the proposition that the cleavage site is bound in some way to the active site. We found a complicated and unpredictable pattern of interaction among mutations at positions 82, 92, 117, 119, and 156 (Table 4). This pattern suggests that these mutations are not acting independently of one another. One reasonable interpretation is that the side chains of these residues lie close together in the folded structure of the protein. In this view, the structure of a particular mutant protein depends on the exact arrangement of the side chains in a confined space; if the side chains do not fit together, perhaps the structure is destabilized, exposing the protein to degradative systems in the cell. We note that a recent x-ray crystal structure of the UmuD′ protein, which is homologous to LexA, shows that the residues corresponding to Ser-119 and Lys-156 do lie together (24); the cleavage site is not included in this structure.

Structural analysis may reveal whether the cleavage site is normally bound in some way to the active site. If it is, we infer that K_{conf} represents a slight rearrangement in such a complex. Whether the defect of the Adg^- mutant proteins is in the initial binding of the cleavage site to the active site or in a subsequent rearrangement is unclear.

Two final points deserve comment. The Adg^- mutations in Gly-117 affect an amino acid that is completely conserved among all cleavable proteins of the LexA type (25–28). Strikingly, a Gly is located two residues before the active site Ser in all members of the subtilisin superfamily and in >90% of the members in the trypsin superfamily. In the structures of both trypsin and subtilisin, this glycine is located in a turn; in trypsin, but not in subtilisin, the main-chain amide group of the Gly residue forms part of the oxyanion hole that stabilizes the tetrahedral intermediate (29). From the UmuD′ crystal structure, one would predict that changing the corresponding glycine would hinder access of the cleavage site to the active site catalytic residues (24) by filling up the binding pocket.

At the biological level, it is unclear whether an Adg^- mutation would affect the operation of the SOS regulatory system. If cleavage in the RecA-stimulated reaction is normal *in vivo*, as our data suggest, it seems likely that the SOS system would operate in a normal fashion in a mutant with VS82 in the chromosome. However, the natural *lexA* gene is autoregulated, unlike the uncoupled system we have used here, so that it is difficult to be confident of this conclusion. Moreover, Val-82 and Gly-117 are conserved in five other *lexA* genes (25, 30). We note in passing that if the wild-type *lexA* gene had Ser at position 82, the discovery of LexA autodigestion would have been more difficult. Indeed, it is possible that self-processing in many other systems has eluded discovery to date, because the rate of self-processing is too slow to allow ready detection.

To our knowledge, this is the first example of a mutation in a regulated self-processing reaction that impairs the rate of self-processing without markedly affecting the stimulated reaction. In cases where the stimulated reaction is normally the one that is effective *in vivo*, it might be difficult to isolate such a mutant without a search designed for this purpose, as was done here. It seems plausible, however, that such mutations should exist for other systems and that analysis of them should prove fruitful in understanding the mechanisms by which these self-processing reactions are regulated.

We are grateful to Liqun Zhang for making several site-directed mutational changes, to Tom Peat and Wayne Hendrickson for describing the UmuD′ structure to us before publication, to Jim Hu for useful information, and to Carol Dieckmann and Roy Parker for comments on the manuscript. This work was supported by Grant MCB-9305092 from the National Science Foundation.

1. Little, J. W. (1993) *J. Bacteriol.* **175**, 4943–4950.
2. Little, J. W. & Mount, D. W. (1982) *Cell* **29**, 11–22.
3. Friedberg, E. C., Walker, G. C. & Siede, W. (1995) *DNA Repair and Mutagenesis* (Am. Soc. Microbiol., Washington, DC).
4. Roland, K. L. & Little, J. W. (1990) *J. Biol. Chem.* **265**, 12828–12835.
5. Slilaty, S. N. & Little, J. W. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3987–3991.
6. Roland, K. L., Smith, M. H., Rupley, J. A. & Little, J. W. (1992) *J. Mol. Biol.* **228**, 395–408.
7. Smith, M. H., Cavenagh, M. M. & Little, J. W. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7356–7360.
8. Slilaty, S. N. & Vu, H. K. (1991) *Protein Eng.* **4**, 919–922.
9. Little, J. W., Kim, B., Roland, K. L., Smith, M. H., Lin, L.-L. & Slilaty, S. N. (1994) *Methods Enzymol.* **244**, 266–284.
10. Little, J. W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1375–1379.
11. Lin, L. L. & Little, J. W. (1988) *J. Bacteriol.* **170**, 2163–2173.
12. Lin, L. L. & Little, J. W. (1989) *J. Mol. Biol.* **210**, 439–452.
13. Schägger, H. & von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379.

14. Leung, D. W., Chen, E. & Goeddel, D. V. (1989) *Technique (Philadelphia)* **1,** 11–15.
15. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987) *Current Protocols in Molecular Biology* (Wiley Interscience, New York).
16. Little, J. W. (1983) *J. Mol. Biol.* **167,** 791–808.
17. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
18. Henikoff, S. & Henikoff, J. G. (1994) *Genomics* **19,** 97–107.
19. Ptashne, M. (1992) *A Genetic Switch: Phage λ and Higher Organisms* (Cell–Blackwell Scientific, Cambridge, MA).
20. Kim, B. & Little, J. W. (1993) *Cell* **73,** 1165–1173.
21. De Anda, J., Poteete, A. R. & Sauer, R. T. (1983) *J. Biol. Chem.* **258,** 10536–10542.
22. Oertel-Buchheit, P., Schmidt-Dörr, T., Granger-Schnarr, M. & Schnarr, M. (1993) *J. Mol. Biol.* **229,** 1–7.
23. Astromoff, A. & Ptashne, M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8110–8114.
24. Peat, T. S., Frank, E. G., McDonald, J. P., Levine, A. S., Woodgate, R. & Hendrickson, W. A. (1996) *Nature (London)* **380,** 727–730.
25. Garriga, X., Calero, S. & Barbé, J. (1992) *Mol. Gen. Genet.* **236,** 125–134.
26. Lewis, L. K., Harlow, G. R.; Gregg-Jolly, L. A. & Mount, D. W. (1994) *J. Mol. Biol.* **241,** 507–523.
27. Battista, J. R., Ohta, T., Nohmi, T., Sun, W. & Walker, G. C. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 7190–7194.
28. Little, J. W. (1996) in *Regulation of Gene Expression in Escherichia coli*, ed. Lin, E. C. (Landes, Austin, TX), pp. 453–479.
29. Fersht, A. (1985) *Enzyme Structure and Mechanism* (Freeman, New York).
30. Riera, J. & Barbé, J. (1993) *Nucleic Acids Res.* **21,** 2256.