Studies of the Protein Encoded by the Lon Mutation, CapR9, in Escherichia coli

A LABILE FORM OF THE ATP-DEPENDENT PROTEASE La THAT INHIBITS THE WILD TYPE PROTEASE

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The product of the lon (capR or deg) gene in Escherichia coli is protease La, an ATP-dependent protease with a linked ATPase activity. Unlike most lon mutations, CapR9 is dominant over the wild type under certain conditions. When protease La was isolated from R9 cells and from a recessive capR strain using DEAE-cellulose chromatography, the mutant enzymes showed about 5% of the wild type activity. Unlike the wild type, the R9 and R' proteases were inhibited by addition of NaCl (<0.1 M). In addition, the R9, but not the R', material inhibited protein synthesis by normal protease La, and this effect may account for its dominant phenotype.

When isolated by phosphocellulose chromatography, the R9 protein lost proteolytic activity but still inhibited the wild type enzyme. This inhibitory activity was purified to near homogeneity using DEAE-cellulose and heparin-agarose chromatography, and corresponded to the 94,000-dalton R9 gene product. At different concentrations, it inhibited ATP-dependent casein degradation and casein-stimulated ATP hydrolysis to a similar extent. Thus, rates of ATP and protein cleavage remained proportional. Similar inhibition of the wild type protease was observed in the presence of DNA which stimulates both protein and ATP hydrolysis. Half-maximal inhibition was observed with approximately a 1:1 ratio of the R9 to the wild type protein.

The subunit sizes of the R9 and the wild type protease were indistinguishable but they differed in isoelectric points. Upon gel filtration, both eluted as tetramers (450,000 daltons) in the absence of salt. However, with 0.1 M NaCl, the wild type protease La remained as a tetramer, but the R9 protein dissociated into dimers and monomers and became a more effective inhibitor. After mixing with R9 protein, 3H-labeled protease La remained tetrameric, though it had lost activity. These findings suggest that tetramer formation between the wild type and defective R9 subunits is responsible for the inhibition of the proteolytic and ATPase activities.

Mutations in the lon gene in Escherichia coli cause a variety of phenotypic alterations including a reduced ability to degrade abnormal proteins (1-4), reduced lysogenization of phage λ (5) and P1 (6), defective cell division and filament formation (7, 8), overproduction of capsular polysaccharides (9), and increased sensitivity to UV or other DNA-damaging agents (10, 11). Recently, the lon gene product has been purified as a DNA-binding protein (12) and shown to be an ATP-dependent protease (13, 14), identical to protease La (13, 15-19). This enzyme appears to catalyze the rate-limiting endoproteolytic steps in the breakdown of abnormal proteins in vivo (3, 17, 20). Protein degradation by this novel enzyme requires concomitant ATP cleavage (13, 17-20) and the protease contains an unusual substrate-activated ATPase activity (14, 18, 19). Both proteolysis and protein-dependent ATP hydrolysis are also markedly stimulated by DNA (19). This enzyme is normally a tetramer (13) comprised of identical 94,000-dalton subunits (12, 13).

Although most lon mutants are recessive, Markovitz and Rosenbaum (21) and Zehnbauer and Markovitz (22) have described an unusual lon mutant, called capR9,1 that is dominant over the lon (i.e. capR7) allele under certain conditions (21, 22). For example, when a lon- cell carried the R9 allele on high multiplicity plasmids, the lon+ phenotype was evident as shown by overproduction of capsular polysaccharides and increased sensitivity to UV (9-11, 22). They suggested that this phenotypic dominance may result from mixing of normal and R9 subunits to form a mixed oligomer in which the defective components prevent the function of the wild type subunits (9, 22).

The product of the R9 mutation has recently been isolated as a DNA-binding protein (12). Although R9 was reported to be an ochre mutation (9, 23), its gene product appeared indistinguishable in size (94,000 daltons) from the wild type protease La (12-14). Compared to wild type cells, the R9 or other lon mutants have reduced activity (e.g. 40-50% of normal) to carry out the energy-dependent degradation of abnormal proteins (13). In extracts of mutant cells, protease La activity was either decreased or absent depending on the initial purification steps used (13). When DEAE-cellulose and gel filtration were used, ATP-dependent casen-degrading activity of R9 was found, although its activity was about half that in the wild type strain in accord with the observations on intact cells (13). By contrast, when phosphocellulose chromatography was used, ATP-dependent proteolytic activity could not be demonstrated (13, 14). Thus, the R9 allele appears to code for a labile protease.

The present studies were undertaken to characterize further the R9 gene product in an attempt to learn more about the mechanism of action of protease La and to clarify the biochemical basis of the phenotypic dominance of the R9 allele.

MATERIALS AND METHODS

Bacterial Strains— E. coli K12 strains, RGC121/pIMC40 and

1 In previous literature, (9, 12-14, 19-23), this mutant was referred to as capR9. Because of the identity of the capR and lon mutations and to avoid further confusion, we refer to it as the R9 allele or R9 protein at the lon locus.
RESULTS

Lability of Protease La in Mutants—We have previously shown that the products of R9 and a recessive lon mutation contain ATP-dependent proteolytic activity which resembles the wild type protease La in its elution on DEAE-cellulose chromatography and gel filtration (13). However, the amount of this ATP-dependent activity recovered in extracts was approximately half that of the wild type strain (13). Fig. 1 compares further the protease La activities obtained by chromatography on DEAE-cellulose of the mutant and wild type extracts. With increasing NaCl concentration, the casein-degrading activities from R9 and R* cells decreased sharply, and at 0.1 M NaCl about 70% of their initial activity was lost. At this NaCl concentration, little or no loss of activity was observed with the wild type protease La (Fig. 1).

This difference in the effects of salt on the activity of the mutant and wild type enzymes may be related to the selective inactivation of the mutant protease observed previously upon chromatography on phosphocellulose (13, 14 and see below). However, the inhibition of the R0 protease by salt was reversed upon dialysis, unlike by phosphocellulose chromatography. In related studies, we compared the temperature stability of the mutant and wild type activities at 42°C but failed to demonstrate any significant difference (13, 17). Thus, the greater sensitivity of the R9 enzyme to salt and its lability with phosphocellulose chromatography, but not with heat, suggest that the defect in the R9 protease may be due to some alteration in ionic interactions within the enzyme (see below).

Effects of R9 Protein on Wild Type Protease La—Because of its unusual dominant phenotype, (9, 12), we tested if the mutant forms of the protease La obtained by DEAE-cellulose chromatography could inhibit the ATP-dependent casein-hydrolysis by the wild type enzyme (isolated by this same approach). As shown in Table I, the peak from the R9 strain inhibited the wild type protease La, but that from the R* did not. These data may account for the finding that the R9 mutation can be dominant in the expression of certain lon phenotypes, while the R* mutation is recessive (22). Therefore, the mechanism of this inhibition by the R9 extract and by the mutant forms of protease La obtained by DEAE-cellulose chromatography may be due to some alteration in ionic interactions within the enzyme (see below).

PARTIAL PURIFICATION OF PROTEASE LA—As judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the purified protease La was at least 97% pure.

In addition, partially purified R9 and R* proteins and wild type protease La were purified by chromatography on phosphocellulose columns as described by Zehnbauer et al. (12). As we reported previously (13), this treatment destroyed ATP-dependent proteolytic activity of both the R9 and R* proteins but not that of the wild type enzyme. The wild type protease La was further purified as described previously (13). As judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the purified protease La was at least 97% pure.

After we found that the partially purified R9 proteins from both purification approaches were capable of inhibiting proteolysis by the wild type protease La (Table I), we proceeded to purify this inhibitory activity by a procedure corresponding to the R9 gene product preparation from phosphocellulose was further fractionated using DEAE-cellulose chromatography under the same conditions used for the purification of the wild type protease La (13). The fractions containing high inhibitory activity from the last step were pooled and dialyzed against 50 mM Tris-HCl, pH 7.8, containing 0.1 mM EDTA and 100 mM NaCl (total 180 ml). Fractions of 1.5 ml were collected at a flow rate of 10 ml/h. The peak of R9 protein (i.e., the inhibitory activity) was eluted at about 0.45 M NaCl concentration.

Assays—Proteolytic activity against [3H]casein was measured in the presence of 1 mM ATP (13, 15, 16). ATPase activity was assayed with [3H]ATP as described previously (13). Under these conditions, purified enzyme hydrolyzes less than 10% of casein and less than 20% of ATP. These preparations of protease La showed typically K, values of 31 pg/ml for casein and 8 pg/ml for ATP (9). The R9 protein was assayed by its ability to inhibit the proteolytic activity of the wild type protease La. All reaction mixtures contained 0.85 mg of the purified protease La and were incubated for 30 min at 37°C unless otherwise mentioned.

Protein was assayed by the method of Bradford (24). Polynucleotide concentrations were measured by their absorbance at 260 nm.

Electrophoresis—Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed as described by Laemmli (25). Isoelectric focusing of purified protease La and R9 protein was carried out on 10-cm long slab gels composed of 5% acrylamide, 0.2% bisacrylamide, 8% urea, 1.0% amphotolysis, pH 5-8, and 0.4% ampholytes, pH 3-10. The anode solution was 100 mM phosphoric acid and the cathode solution 20 mM sodium hydroxide. Proteins were dissolved in 8 M urea solution containing 1 mM 2-mercaptoethanol and then loaded from the cathode end. The gels were run at 85°C for 12 h at 500 V, fixed in 4% sulfosalicylic acid plus 10% trichloroacetic acid, stained with Coomassie blue, and destained by diffusion in 12% isopropanol plus 7% acetic acid. The final pH gradient was estimated by cutting the gel run in parallel into 0.5-cm pieces and eluting them for 2 h into 1.5 ml of degassed H2O. Measurements of pHe were performed on a Radiometer pHM62 pH meter.

Materials—'Cal thrus DNA was obtained from Sigma, poly(dT) from Pharmacia, phosphocellulose (P11) and DEAE-cellulose (DE52) from Whatman, Sepharose 300 from Pharmacia, heparin-agarose from Bethesda Research Laboratories, [3H]ATP and [3H]formaldehyde from New England Nuclear, polyethyleneimine (PEI) cellulose plates from J. T. Baker Chemical Co., and amphotolines from LKB Instruments Inc. [3H]Casein was prepared as described (26). The wild type protease La was labeled with [3H]formaldehyde by the method of Jentoft and Dearborn (27). After this treatment, the activity and the subunit size of the protease were unchanged (data not shown).

FIG. 1. Effect of different concentrations of NaCl on protease La activity obtained from R9, R*, and wild type strains. Partially purified preparations of protease La from wild type, R9, and R* cells were obtained by pooling the ATP-stimulated proteolytic activity obtained from DEAE-cellulose chromatography of crude extracts. The ATP-dependent proteolytic activity against [3H]casein was measured by addition of various amounts of NaCl to reaction mixtures containing R9 (270 pg) (A), R* (270 pg) (B), or wild type protease La (135 pg) (C), and ATP (1 mM). Incubations were performed for 30 min at 37°C. Proteolysis was measured in the presence and absence of ATP, and the amount of activity seen without ATP was subtracted. For each enzyme preparation, these values were then compared to those obtained in the absence of NaCl which were normalized to 100% activity. Typical values for the casein hydrolysis obtained in this experiment were shown in Table I. Additions of salt showed little or no effect on the ATP-independent proteolytic activity (data not shown).
wild type material was added, either with partially purified R9- and R- proteins obtained by phosphocellulose chromatography and purified protease La. In mixing experiments, the activity seen in the absence of ATP was subtracted from that with ATP to calculate the ATP-dependent proteolytic activity (i.e., protease La).

The properties of the responsible protein were studied further. When the R9 and R- proteins were partially purified using phosphocellulose chromatography as an initial step, they showed no ATP-dependent proteolytic activity against [H]casein (13). However, when the fractions from the mutant strains, which should contain the protease, were mixed with ATP, independent proteases in E. coli (15), the activity in the absence of ATP was subtracted from that with ATP to calculate the ATP-dependent proteolytic activity (i.e., protease La).

Since the experiments in Table I used the partially purified R9 protein, it was unclear whether this protein by itself or some contaminating polypeptide was responsible for the inhibition of proteolysis. Therefore, the inhibitory activity obtained from the phosphocellulose column was further purified by sequential chromatography on DEAE-cellulose and heparin-agarose. Fractions from the heparin-agarose column were assayed for their ability to inhibit casein degradation by purified wild type protease La. The peak of inhibitory activity was eluted at approximately 0.45 M NaCl (Fig. 2) and contained a single band at 94,000 daltons on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 3). This molecular weight corresponds to that of the 94,000-dalton polypeptide from heparin-agarose column (see Fig. 2).

To test if this inhibition was indeed due to the 94,000-dalton polypeptide encoded by the R9 gene, the levels of this protein in the fractions under the peak were assayed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As shown in Fig. 3, the degree of inhibition of proteolysis in the various fractions (A) was proportional to the intensity of the 94,000-dalton protein band after staining with Coomassie blue (B). Thus, R9 protein (rather than a contaminant) appears responsible for the inhibition of wild type protease La, and no other proteins appear necessary for this effect.

Effects of R9 Protein on the ATPase Activity of Wild Type Protease La—It was recently shown that protease La contains an inherent ATPase activity, which is required for proteolysis (13, 16–18). Hydrolysis of protein substrates and ATP appears coupled, since ATPase inhibitors (e.g., vanadate) cause a proportional reduction in proteolysis (17, 18). The purified R9 protein from the heparin-agarose column showed a small amount of ATPase activity compared to the wild type enzyme.

**TABLE I**

| Protease peaks from | Extracts used | Casein hydrolyzed | Inhibition of Wild type |
|---------------------|---------------|-------------------|------------------------|
|                     |               | -ATP +ATP         | Difference              |
| DEAE-cellulose      |               | %                 | %                      |
| column              |               |                   |                        |
| Wild type           | 1.21          | 4.33              |                         |
| R9                  | 1.57          | 4.22              |                         |
| R-                  | 1.48          | 2.14              |                         |
| Wild type, R9 mixed | 2.99          | 4.84              | 38                     |
| Wild type, R- mixed | 2.76          | 7.01              | 10                     |
| Phosphocellulose    |               |                   |                        |
| column              |               |                   |                        |
| Wild type           | 0.00          | 8.75              | 8.75                   |
| R9                  | 0.30          | 0.21              | 0.09                   |
| R-                  | 0.24          | 0.23              | 0.00                   |
| Wild type, R9 mixed | 0.21          | 2.71              | 2.50                   |
| Wild type, R- mixed | 0.26          | 9.17              | 8.91                   |

**Fig. 1.** Inhibition of Protease La by R9 Protein

**Fig. 2.** Elution profile of R9 protein from heparin-agarose column. The inhibitory activity obtained from phosphocellulose chromatography and then from DEAE-cellulose chromatography was fractionated on a heparin-agarose column (1.0 x 5.8 cm) as described in the text. The ability of the column fractions to inhibit casein hydrolysis by the wild type enzyme was assayed. Reaction mixtures contained 5 μl of each fraction and 0.85 μg of the purified wild type protease La. Incubations were performed for 30 min at 37 °C. Every other fraction under the bar was dialyzed against 10 mM Tris-HCl, pH 7.8, containing 1 mM MgCl₂ and 10% (w/v) glycerol and used in subsequent studies (Fig. 3).

**Fig. 3.** Comparison of the elution profiles of inhibitory activities and levels of the 94,000-dalton polypeptide from heparin-agarose chromatography. The dialyzed fractions from heparin-agarose column (see Fig. 2, bar) were assayed for inhibitory activity (A) and also for their content of the 94,000-dalton polypeptide (B) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Reaction mixtures contained 5 μl of each fraction and 0.85 μg of the purified wild type protease La. The per cent inhibition of proteolysis was measured under conditions where the proteolytic process was linear with time and was proportional to the amount of inhibitor added.
but it was not stimulated by casein or DNA added either separately or together (Table II). It is presently uncertain whether this ATPase activity is inherent in the R9 protein or due to trace contamination by some other E. coli ATPase (28). In fact, the R9 preparation obtained from earlier purification steps was heavily contaminated with ATPases (28), which were active in the absence of casein and were removed in the flow-through fractions of the heparin-agarose column (Fig. 2 and data not shown).

To learn more about the mechanism of the inhibition of proteolysis, we tested if the R9 protein either inhibited or uncoupled the ATPase activity of the wild type enzyme. As shown in Table II, the purified R9 protein inhibited the casein-stimulated ATPase activity of the purified protease La. Furthermore, the extent of the inhibition of casein degradation and ATP cleavage both increased with increasing concentrations of the R9 protein (Fig. 5, A and B). At each concentration tested, the per cent inhibition of proteolysis was nearly identical to the per cent inhibition of ATP hydrolysis (Fig. 5C). These findings are in accord with the earlier data from our laboratory suggesting a stoichiometric relationship between peptide and ATP cleavage (18).

Half-maximal inhibition of both proteolysis and ATP hydrolysis was observed with approximately a 1:1 molar ratio of the wild type protease to R9 protein (Fig. 5, A and B). Thus, the R9 is not acting catalytically to inhibit protease La; instead, it is likely that there exists some stoichiometric interaction between protease La and R9 protein for the inhibitory effects.

Recently, DNA has been shown to stimulate both the proteolytic and casein-stimulated ATPase activities of protease La (19). When similar experiments were carried out in the presence of single stranded calf thymus DNA and casein, the total ATPase activity increased but the R9 protein still caused a similar per cent inhibition of ATP hydrolysis as in the absence of DNA (Table II and Fig. 5B). Addition of double-stranded calf thymus DNA, plasmid pBR322 DNA, or poly(dT) also stimulated both proteolysis and ATP hydrolysis, but R9 protein caused a similar inhibition of these processes. In each case, DNA seemed to enhance the amount of protein degraded to acid-soluble form per ATP cleaved, while

**TABLE II**

| Additions         | ATP hydrolyzed by | Inhibition of protease La |
|-------------------|-------------------|--------------------------|
|                   | Protease La | R9 Protein | La plus R9 |                   |
| None              | 2.9         | 0.8        | 3.2        | 14                   |
| Casein            | 9.8         | 0.8        | 6.7        | 37                   |
| DNA               | 2.9         | 0.8        | 2.5        | 32                   |
| Casein + DNA      | 17.3        | 1.1        | 9.0        | 51                   |

**Fig. 5. Effect of increasing concentrations of the purified R9 protein on ATP-dependent casein degradation and ATPase activities by the purified wild type protease La in the presence and absence of DNA.** Reaction mixtures for both proteolysis and ATP cleavage contained 0.85 pg of the purified wild type protease La, various amounts of the purified R9 protein and 25 pg of casein. Incubations were performed for 30 min at 37 °C with (O) and without (C) denatured calf thymus DNA (2.5 pg). In accord with our previous data (19), addition of DNA stimulated casein hydrolysis 3.0-fold and total ATP hydrolysis 2.1-fold (data not shown). The per cent inhibition is expressed relative to the activity seen without R9 protein, either in the presence or absence of DNA. Typical values for the ATP hydrolysis obtained in this study are shown in Table II.

R9 protein decreased both processes (Ref. 19 and data not shown).

**Comparison of R9 and Wild Type Proteins—**R9 has been reported to be a nonsense mutation which encoded a polypeptide that is indistinguishable in size (94,000 daltons) from the wild type subunit (Refs. 9 and 14). Therefore, it was suggested that the mutation occurred very near to the carboxyl terminus of the protein (22).

Isoelectric focusing gel electrophoresis in the presence of 8 M urea showed that the subunit of protease La had an isoelectric point of 6.0 while that of R9 protein was 6.2 (Fig. 4, lanes a and b). When a mixture of the two proteins was focused in the presence of urea and stained with Coomassie blue, two distinct bands were detected that were separated by about 0.2 pH units (Fig. 4, lane c). When we attempted to focus a mixture of these proteins in the absence of urea to test for the formation of mixed complexes, the protein failed to migrate into the gels (data not shown). In any case, the R9 gene apparently encodes a defective protein that has an altered charge from the wild type enzyme.

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1. C. H. Chung, L. Waxman, and A. L. Goldberg, unpublished observations.
Inhibition of Protease La by R9 Protein

Previously, we have shown that the wild type protease La was very labile upon incubation at 42 °C and that ATP completely prevented this loss of activity (13). Similarly, the inhibitory activity of the purified R9 protein was also very labile (Table III). Within 1 h at 42 °C, all inhibitory activity disappeared, but ATP (1 mM) completely prevents this effect. This result indicates that the purified R9 protein still contains an ATP binding site and shares other structural features with the proteolytically active form of the molecule.

To compare the multimeric sizes of the R9 protein and the wild type protease La, gel filtration was performed on a Sephacryl S-300 column both following DEAE-cellulose chromatography or following purification of R9 protein. In the absence of salt, both the wild type protease and R9 protein (assayed either as a protease (13) or as the inhibitor) eluted as sharp peaks corresponding to a tetrameric size of 450,000 daltons (Fig. 6, A and B). However, when a similar column was run in the presence of 0.1 M NaCl, the inhibitory activity of the R9 protein eluted as a much broader peak that corresponded in size to monomers, dimers, and tetramers (Fig. 6D), while the wild type protease remained as a tetramer (Fig. 6C). It is noteworthy that under this salt condition, only the R9 enzyme loses proteolytic activity (see Fig. 1). When the fractions containing the larger, probably tetrameric form of R9 protein (Fig. 6D, bar) were pooled, concentrated, and rechromatographed on the same column, the inhibitory activity again eluted as a broad peak (Fig. 6D). Thus, the R9 protein apparently consists of tetramers that readily dissociate to dimers of the 94,000-dalton subunit as well as monomers. At much higher salt concentrations (e.g. 0.35 M NaCl), both the R9 protein and the wild type enzyme appeared to be completely dissociated to dimers and monomers (i.e. the center of the broad nearly symmetric peak corresponded to the size of about 140,000 daltons as judged by elution profiles of protein standards) (data not shown).

In order to determine if the wild type enzyme and R9 protein interact in a large multimeric complex, in a mixed tetramer, or as heterodimers, we labeled the wild type protease La with [3H]formaldehyde so that its size might be determined even after enzymatic activity was lost. The [3H]-labeled material eluted from a Sephacryl S-300 column as a tetramer that consists of 94,000-dalton subunits (Fig. 6 and data not shown). The [3H]protein was then incubated with

Table III
Heat stability of the purified R9 protein

| Preincubation temperature | Inhibition of protease La | +ATP | -ATP |
|---------------------------|--------------------------|------|------|
| 4 °C                      | 41.2                     | 0.7  | 39.8 |
| 42 °C                     | 15                       | 0.35 | 4.6  |

% Inactivation by heat

98.3  4.6

Fig. 6. Elution profiles of the purified wild type protease La and R9 protein upon gel filtration on Sephacryl S-300. Top, the purified wild type protease La (170 µg in 300 µl) was loaded on a Sephacryl S-300 column (1 x 18 cm) equilibrated with 50 mM Tris-HCl, pH 7.8, containing 5 mM MgCl2 and 10% (v/v) glycerol, in the absence (A) and presence (B) of 0.1 M NaCl. Fractions of 0.5 ml were collected at a flow rate of 8 ml/h. Casein degradation was measured with 25-µl aliquots of each fraction. Incubations were performed at 37 °C for 30 min. Arrows indicate the peaks of markers eluted: a, β-galactosidase (469,000 daltons); b, catalase (240,000 daltons); and c, alkaline phosphatase (82,000 daltons). Bottom, the purified R9 protein (192 µg in 300 µl) was chromatographed as above in the absence (C) and presence (D) of 0.1 M NaCl. Inhibitory activity of the column fractions (C—C) was assayed as described in Fig. 2 by additions of 25 µl of each fraction and 0.85 µg of the wild type enzyme. To examine whether this broad elution profile reflected dissociation of multimers into subunits, fractions under the solid bar (12 to 15) that corresponded to tetramers were pooled, concentrated, and rerun on the same column. In the second run, the inhibitory activity of the R9 protein (Δ—Δ) was assayed by addition of 120 µl of each fraction. In the absence of 0.1 M NaCl, both wild type and R9 protein chromatographed as tetramers as we reported earlier (13).
different amounts of the R9 protein, and the mixture was run on the same column. Under all conditions, the radioactive protein eluted as a sharp peak indistinguishable in size from the product of wild type enzyme (Figs. 1 and 6). These findings raise the possibility that ATP-dependent proteinase by salt (Table I) occurred under conditions which inhibited by causing dissociation of wild type enzyme into dimers and monomers or by causing enzyme aggregation.

Since R9 tetramers are more readily dissociated into dimers and monomers with increasing salt (Fig. 6), we have tested whether the tetramer or its subunits are better able to inhibit wild type protease La. As shown in Table IV, increasing the salt concentration enhanced the inhibition by R9 protein of wild type protease La. This increase in inhibitory activity by salt is in contrast with the ability of salt to inhibit differentially the ATP-dependent proteolytic activity of the R9 protein demonstrable in crude extracts (Fig. 1). At these concentrations, NaCl had little or no effects on the proteolytic activity (Table IV) or the size (Fig. 6) of the wild type enzyme. These data suggest that the salt-induced dissociation of the R9 protein into dimers and monomers enhances subunit exchange with the wild type tetramers and thus increases the degree of inhibition. Therefore, the inhibited form of the protease La is probably a tetramer, composed of R9 and wild type subunits.

**Discussion**

These studies have demonstrated certain unique properties of the R9 gene product that can explain this mutation's unusual phenotypic effects (22). Our earlier observations (13) and the present findings (Table I and Fig. 1) indicate that this protein, like wild type protease La, is capable of degrading proteins by an ATP-dependent mechanism but that the R9 enzyme is much more labile than the wild type (13). The amount of ATP-dependent proteolysis seen in R9 cells (13) or extracts (Table I, Fig. 1, and Ref. 13) was approximately half that of the wild type, even though the R9 polypeptide may be synthesized in much higher quantities than the normal gene product (22). Furthermore, the addition of NaCl inhibited, and chromatography on phosphocellulose (which also involves high salt concentrations) inactivated irreversibly, the proteolytic activity of the mutant enzyme, but these treatments did not affect the wild type protease (Fig. 1 and Table I). This instability of the mutant protease can explain the conclusion of Charette et al. (14) that the R9 protein lacked proteolytic activity, since these workers isolated it only by phosphocellulose chromatography. The differential inhibition of the R9 protease by salt (Table I) occurred under conditions which readily dissociated R9 tetramers into dimers and monomers, but do not affect the size of the wild type enzyme (Figs. 1 and 6). These findings raise the possibility that ATP-dependent proteolytic activity requires tetramer formation.

Of particular interest was the finding that the R9 extract also could inhibit the wild type protease La (Table I). In fact, following phosphocellulose chromatography, this polypeptide lost all its proteolytic activity but retained its ability to inhibit the wild type protease (Table I). By themselves these observations alone might suggest that the labile protease La and the inhibitory activity were distinct components of the extract, but our subsequent purification of this inhibitory activity confirmed that inhibition was also due to the 94,000-dalton R9 polypeptide (Fig. 3) which is encoded by the R9 gene (12). Further strong evidence for this conclusion was the marked sensitivity of the R9 inhibitory activity to heat (42 °C) and its stabilization by ATP, which paralleled the effects seen earlier with the wild type protease (Table III and Refs. 13 and 17). Since the R9 polypeptide inhibited the pure wild type protease La and since it appeared free of other polypeptides (Figs. 3 and 4), no other component of the E. coli extract (e.g. an ATPase or protease) was necessary for this effect. Inhibition, therefore, must result from direct interaction between the mutant and wild type proteins in accord with earlier suggestions (9, 22).

The crude extracts of R9, and presumably intact R9 cells, thus contain both protease La activity and inhibitory activity against wild type protease La (both encoded by the same gene) (Table I). It is unclear if these two opposing functions reside simultaneously within the same oligomer, or if the R9 polypeptide exists in the extracts either of two forms, i.e. enzymatically active tetramers or inhibitory molecules that have lost their proteolytic function. The factors influencing the relative proportions of these two activities in vivo are unknown although the high ionic strength within E. coli (29) should by itself favor accumulation of the inhibitory form.

Mutations in the lon locus have a number of phenotypic consequences (1-4, 9-14). Like other lon mutations, R9 leads to a reduction in the ability of the cells to carry out the degradation of abnormal proteins (1-4), and the reduced protease La activity in the R9 mutants can account for this effect. In a different lon (R-) mutation, we also found a decreased amount of protease La activity, and it is also differentially inhibited by salt (Table I, Fig. 1, and Ref. 13). These results are consistent with its decreased ability to degrade abnormal proteins in vivo (12, 13, 22). The unique feature of the R9 allele is that it can show a dominant lon phenotype, including increased sensitivity to UV light and enhanced capsular polysaccharide synthesis when present on an episome or a high copy number plasmid (22). The finding that the R9 protein in vitro can inhibit both ATPase and proteolytic activities of the wild type enzyme provides a biochemical mechanism for the transdominance of the R9 gene over the wild type (Fig. 5). In fact these data would suggest that overproduction of R9 might be a useful approach to reducing rapid degradation of cloned polypeptides in vitro. By contrast, we found that the partially purified R- gene product, obtained by DEAB-cellulose, is not capable of inhibiting the wild type protease La (Table I and Fig. 1), and this lack of inhibition is consistent with its recessive phenotype (22).

However, it is not yet clear how decreased function of protease La can account for all the other physiological consequences of lon mutations, aside from the reduction in protein degradation. Possibly the various cellular processes affected involve short-lived proteins that are degraded by protease La. In fact, there is strong evidence that the defect in lysogeny of phage λ (30) and the increased UV sensitivity (31) of lon mutants result from the reduced ability of these cells to degrade rapidly certain regulatory proteins. It is also possible as suggested by Zehnbauer et al. (12) that the lon gene

| R9 added (μg) | NaCl 0% | NaCl 50 mM | NaCl 100 mM |
|---------------|---------|------------|-------------|
| Protease La activity remaining (%) | 99 | 99 | 99 |
product can act as genetic repressor. Direct evidence for this possibility is lacking, although the wild type protein, as well as R9, both bind to DNA (Refs. 12 and 22).²

The enzymatic processes coupling ATP and protein hydrolysis in the functioning of protease La are not understood (17, 18). Therefore, the mechanism by which the R9 polypeptide inhibits the purified wild type protease La is of particular interest. It is noteworthy that the purified R9 protein decreased both the proteolytic and casein-stimulated ATPase activities of protease La concomitantly (Fig. 5, A and B). Furthermore, at each R9 concentration tested, the extent of inhibition of ATP hydrolysis and of proteolysis were strikingly similar (Fig. 5C) even in the presence of DNA, which by itself activates the wild type enzyme (19). Thus, the fall in proteolysis and ATP cleavage appear closely linked. In related studies, we have demonstrated that ATPase inhibitors, such as vanadate, reduce proteolysis, while protease inhibitors (e.g., diisopropyl fluorophosphatase) also reduce ATP breakdown (13, 17, 18) and we have presented evidence for a stoichiometric relationship between these processes (18). It is interesting that after mixing protease La with R9 protein, the ratio of peptide to ATP hydrolysis (i.e. the slope of Fig. 5C) was unaltered. Possibly, the association with R9 protein causes an all-or-none inactivation of the enzyme such that the pure wild type tetramers are the only active molecules in the mixture.

Half-maximal inhibition of both ATP and protein breakdown occurred when R9 and wild type proteins were present in a one to one molar ratio (Fig. 5, A and B). Thus, the inactivation of protease La seems to involve a stoichiometric interaction of R9 and wild type subunits rather than a catalytic action by the R9 protein. The precise stoichiometry is clearly of interest, but our attempts to separate inactive molecules containing both subunits from the R9 tetramer and the active protease were unsuccessful, probably because of their very similar sizes and similar chromatographic behavior. Therefore, direct evidence about the interaction of the wild type and R9 subunits is lacking. Nevertheless, we were able to show that the inhibited form of protease La and the enzymatically active one were the same size (Fig. 7). The most likely explanation of these findings is that the R9 and wild type subunits must interact to form mixed tetramers which lack enzymatic activity. In accord with this conclusion, dissociation of the R9 tetramer into subunits with NaCl was found to increase their ability to inhibit proteolytic activity of the wild type enzyme (Table IV).

This conclusion about the mode of inhibition of protease La resembles the explanation for the dominant mutations in the lac repressor (32). In this case, the presence of a mutant subunit prevents the interaction of the wild type polypeptides which are essential for normal function of the repressor (32, 33). We, therefore, suggest that a similar mechanism is responsible for the inhibition of protease La by the R9 polypeptide. By analogy, our findings also suggest that some interaction between the normal subunits of protease La is required for both proteolytic and ATPase activity.

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