An Increased Content of Protease La, the lon Gene Product, Increases Protein Degradation and Blocks Growth in Escherichia coli*

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The lon gene product in Escherichia coli is an ATP-dependent protease (La) that plays an important role in the breakdown of abnormal proteins and certain normal polypeptides. Since transcription of the lon gene rises as part of the heat-shock response, we studied the physiological effects of increased levels of protease La. In cells carrying additional copies of the lon gene under the control of the lac or tac promoter, induction of the protease resulted in a rapid cessation of cell growth and in a loss of viability at stationary phase. Similarly, cells carrying a multicopy plasmid encoding the lon gene contained 2–5-fold more protease La and grew much more slowly than did control cells. In such cells, insertion sequences appeared spontaneously in the lon gene on the plasmid and prevented the excess protease production and allowed more rapid growth.

The cells with increased content of protease La (due to the lon plasmid or induction of the lon gene) exhibited severalfold higher rates of degradation of abnormal proteins containing amino acid analogs and of incomplete polypeptides containing puromycin. Also, a β-galactosidase fusion protein with enzymatic activity was relatively stable in control cells but unstable in the cells with high protease La content. In these cells, the overall degradation of normal proteins increased 2-fold, and certain cellular polypeptides appeared particularly sensitive to proteolysis. Thus, rates of protein degradation in vivo are limited in part by the cellular content of the ATP-dependent protease, and increases in transcription of the lon gene enhance proteolysis and can be deleterious to the cell.

In the past several years, considerable progress has been made in our knowledge of the mechanisms and regulation of protein degradation in Escherichia coli (1, 2). However, it is still unclear what factors determine the rates of degradation of a protein in vivo. One major determinant of a protein's stability is its conformation. For example, polypeptides encoded by nonsense or certain missense alleles (1–7) have been shown to be rapidly hydrolyzed to amino acids. Similarly, proteins with highly abnormal structures, such as those containing amino acid analogs or incomplete polypeptides containing puromycin (8, 9) are also rapidly degraded in E. coli.

Rates of intracellular protein degradation also must depend on the cell's content of proteolytic enzymes. Mutants with a reduced capacity to degrade various abnormal proteins (originally called deg) were isolated by Bukhari and Zipser (10) and subsequently were shown to map in the lon locus (11). lon mutants have a decreased rate of degradation of highly abnormal proteins (10–13) as well as certain native E. coli proteins (14, 15). These mutants show a variety of other phenotypic alterations (e.g. mucoidy or sensitivity to DNA-damaging agents) which seem to result from their decreased ability to degrade certain short-lived proteins (14, 15). The breakdown of abnormal proteins is also greatly reduced (16, 17) in htpR strains, in which lon gene expression is reduced (16).

The lon gene was shown by Chung and Goldberg (12) and Charette et al. (18) to code for an ATP-dependent protease previously designated protease La (19). The degradation of abnormal proteins in E. coli requires metabolic energy (9), and the ATP dependence of protease La appears to account, in large part, for this energy requirement. This enzyme is a new type of endoprotease, which hydrolyzes proteins and ATP in a coupled fashion (12, 18, 20). A variety of biochemical and genetic findings suggest that this protease catalyzes an initial rate-limiting step in the degradative pathway (1, 12, 13).

Although the consequences of reduced protease La function have been extensively studied, the physiological effects of high levels of this protein have not been defined previously. A rise in lon transcription does occur as part of the heat-shock response (16, 21, 22). This response is induced when cells produce large amounts of an abnormal protein, and it has been postulated that this adaptation may enhance the cell's capacity to degrade such aberrant and potentially harmful polypeptides (16, 21, 22). This argument makes the important assumption that the content of protease La in normal cells is rate-limiting for protein breakdown.

The present studies were undertaken to clarify whether an increase in the level of this protease enhances the rate of degradation of abnormal or normal polypeptides in vivo. To determine whether an increased content of protease La enhances proteolysis or influences cell viability, we have studied cells carrying a multicopy lon plasmid (23) and cells carrying a plasmid with the lon gene under control of an inducible promoter.

MATERIALS AND METHODS

Strains, Bacteriophage, and Plasmid Construction—Strains of E. coli and bacteriophage used in this study are listed in Table I. The derivatives of previously published strains were constructed by P1vir bacteriophage-mediated transduction, by lysogenization with a bacteriophage derivatives (10), by deletion of the transposon Tn10 as previously described (26), or by CaCl₂-mediated transformation with plasmid DNA (27). The lon-containing plasmid pJMC40 (Fig. 1A) was kindly provided by Dr. A. Markowitz (University of Chicago); it was derived from pBR322 as previously described (23, 28). The tac
Effects of Increased Protease La in E. coli

| Strain      | Reference or derivative |
|-------------|-------------------------|
| N5116       | F-arg::Tn10 his ilv    | 24 |
| SY327       | F-Δ(lac-prom) X11 recA65 argE(am) pSG10 | 25 |
| SG825       | F-Δ(lac-prom) X11 recA65 argE(am) pSG10 pUC18 | SY327 |
| SG826       | F-Δ(lac-prom) X11 recA65 argE(am) pSG10 pUC18 | 25 |
| SG827       | F-Δ(lac-prom) X11 recA65 argE(am) pSG10 pUC18 | 25 |
| SG840       | F-Δ(lac-prom) X11 recA65 argE(am) pSG10 pUC18 | 25 |
| SG841       | F-Δ(lac-prom) X11 recA65 argE(am) pSG10 pUC18 | 25 |
| SG1016      | F-Δ(arg) his ilv pUC40 | N5116 |
| SG1017      | F-Δ(arg) his ilv pUC40 | N5116 |

Table I

Bacterial strains

- **Strain**: N5116, SY327, SG825, SG826, SG827, SG840, SG841, SG1016, SG1017
- **Reference or derivative**: 24, 25, SY327, 25, 25, 25, 25, N5116, N5116

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**Fig. 1.** A, plasmids pJMC40 and pJMC40IS. pJMC40 was kindly provided by Dr. A. Markowitz (University of Chicago). pJMC40IS was isolated in this study as a lon+ derivative of pJMC40. Restriction mapping indicates the presence of an insertion of approximately 800 base pairs in the amino-terminal lon coding sequence. B, In an attempt to subclone the lon gene away from both flanking chromosomal sequences and the plasmid pHA105, pJMC40 was digested with EcoRI and re-ligated with T4 DNA ligase at low DNA concentrations to promote circularization. The resulting plasmid mixture was transformed into competent tetracycline-sensitive cells (SY327) and plated on rich broth agar plates with 10 μg/ml tetracycline. The resulting plasmids were analyzed by restriction digestion followed by 1% agarose gel electrophoresis, staining with ethidium bromide, and visualization with short-wave ultraviolet light. They were also tested for their ability to complement a chromosomal lon mutant. No lon+ plasmids were generated by this procedure. C, Shows the structure of pSG5 and pSG11. lon was placed under control of the lac promoter (pSG5) by inserting a promoterless lon restriction fragment into pUC18 or tac promoter (pSG11), by insertion of this lon fragment into the tac promoter vector pKK223.

**Generation of Promoter Deletions**—A series of deletions 5' to the lon structural gene was generated by Bal31 exonuclease treatment of linear DNA fragments. The extent of each deletion was verified by DNA sequencing, using the dideoxy method (32). To confirm the removal of the lon promoter, we assayed production of β-galactosidase from a lon-lacZ operon fusion carried on a pBR322-derived plasmid. The plasmids generated by this technique were used to construct a promoterless lon restriction fragment and plasmids carrying lon under control of an inducible promoter.

**Results**

Cells Carrying pJMC40 Have Increased Levels of Protease La—The plasmid pJMC40 containing the lon gene and the derivative plasmid pJMC40IS described below contain approximately 7 kilobase pairs of E. coli chromosomal DNA (23, 28). Approximately 2.4 kilobase pairs are required for the lon gene, which encodes the 87-kDa polypeptide 1 subunit of the promotor plasmid, pKK223, was provided by J. Brosius (Columbia University).

Plasmids and bacteriophage were constructed in vitro using standard recombinant DNA techniques. Plasmid and phage DNA were isolated according to methods previously described (29). Restriction enzymes were purchased from either New England Biolabs or Bethesda Research Laboratories.

**Enzyme Assays**—β-Galactosidase produced from the lon-lacZ protein fusion was assayed according to the method of Miller (30). Cells growing in rich media were centrifuged at 8000 × g for 1 min and resuspended in minimal media prior to assay. At each point, 0.1 ml of the culture was assayed in 0.9 ml of Z buffer. To measure protease La, cells with control and experimental plasmids were grown at 37 °C. After sonication, equal amounts of cell protein were applied to phosphocellulose columns at 4 °C, and the bound material was eluted with a salt step. The enzyme was dialyzed against assay buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA, 1 mM β-mercaptoethanol, 20% glycerol). Protease La was assayed with the specific fluorometric substrate, glutaryl-alanyl-alanyl-phenylalanine-methoxynaphthylamine (Enzyme Systems Products, Livermore, CA) in the presence or absence of ATP (1 mM), as described by Waxman and Goldberg (31).

**Degradation of Abnormal Proteins**—Amino-terminal polypeptide fragments were generated by exposure of cells growing on minimal medium to the antibiotic puromycin (100–400 μg/ml) for 20 or 30 min. During the final 5 min, the cells were exposed to [35S]methionine. Prior to assay of protein degradation, the cells were washed free of the antibiotic by filtration and were resuspended in media lacking puromycin (9) and containing large amounts of nonradioactive methionine to prevent reincorporation of [35S]methionine released from proteins.

To cause the incorporation of the arginine analog canavanine into cell proteins, an arginine auxotroph was exposed to media lacking arginine but containing canavanine (100 μg/ml) and [35S]methionine (5 μCi/ml) for 5 min. To produce azoleucine-containing proteins, a leucine auxotroph was exposed to azoleucine (100 μg/ml) for 15 min prior to the addition of [35S]methionine (5 μCi/ml) for 5 min. Prior to assay of protein degradation, the cells were filtered free of analog-containing media and resuspended in media supplemented with the normal amino acid (arginine or leucine) plus an excess of nonradioactive methionine. The rates of protein degradation were estimated by measuring the release of radioactive amino acids from previously labeled proteins (9).

1 Although previously identified as 82 or 94 kDa (12, 18, 20, 27) based on SDS gel electrophoresis, the protease subunits is in fact 87 kDa, based on its amino acid sequence (D. T. Chin, S. A. Goff, and A. L. Goldberg, manuscript submitted for publication).
Effects of Increased Protease La in E. coli

ATP-dependent protease La. Extracts of cells carrying this multicycoplasmid were reported to have 2-10-fold more protease La than extracts of cells without this plasmid (12). To confirm the elevation in the levels of this ATP-dependent protease in cells carrying pJMC40, crude extracts of these cells were subjected to phosphocellulose chromatography, and the bound fraction was eluted and assayed with the fluorometric substrate glutaryl-Ala-Ala-Phe-methoxy-naphthylamine (31).

Extracts of cultures with an active episomal lon gene exhibit 2-4-fold higher rates of hydrolysis of the fluorometric substrate than those of control cells carrying either no plasmid or a plasmid with an inactive lon allele (Table II). In addition, polyacrylamide gel electrophoresis of either crude extracts or phosphocellulose eluates in the presence of sodium dodecyl sulfate revealed 3-4-fold higher amounts of the 87-kDa polypeptide, corresponding to the protease subunit (Fig. 2).

Inhibition of Growth by pJMC40—E. coli transformed with the multicopy plasmid pJMC40 containing the lon gene (Fig. 2) were found to grow slowly and were difficult to maintain in a viable condition either in liquid or on solid growth media. Such cells frequently failed to form colonies following entrance into stationary phase or after storage on LB agar plates at 4°C for only a few days. Occasionally, the plasmid-carrying cells gave rise to colonies which grew faster and showed greater viability than the original pJMC40 transformants. Such colonies, however, remained resistant to tetracycline, and therefore still carried a pBR322-derived plasmid.

In an attempt to understand the basis for the faster growth and enhanced viability of these colonies, plasmids were isolated from these variants and from the original pJMC40 transformants. These plasmids were analyzed by restriction enzyme cleavage and gel electrophoresis using a variety of enzymes that recognize sequences within or flanking the lon gene (EcoRI, PstI, BamHI, and SalI). In 10 such cases analyzed, the rapidly growing variants carried an episome in which a restriction fragment from the lon gene contained an additional 800 base pairs. Further analysis positioned this inserted sequence within the EcoRI to PstI restriction fragment which contains the promoter and amino-terminal coding sequence for the lon gene (Fig. 1A). When these plasmids, designated pJMC40IS, were transformed into lon mutants, they failed to give a wild-type phenotype, unlike the original plasmid pJMC40 with an intact lon gene. When transformed into wild-type cells, the plasmid bearing an insert allowed approximately twice as much rapid growth as did the original pJMC40 plasmid (data not shown).

In subsequent studies on the effects of increased expression of the lon gene, we used the pJMC40 plasmid and as a control used the plasmid containing the insertion element within the lon gene (pJMC40IS). This approach controls for the presence of the several kilobase pairs of chromosomal DNA in addition to the lon gene on the plasmid pJMC40. When plasmids were constructed carrying either a 5' or 3' truncated end of the lon-coding sequence and were transformed into wild-type cells, we observed no effect on cell growth or viability (data not shown). Therefore, the cause of the poor growth and viability of cells carrying pJMC40 is probably the increase in the amount of the lon gene product, rather than of any controlling DNA sequences present on the lon plasmid.

Attempts to Subclone lon—In an attempt to construct a lon-containing plasmid with a minimal number of additional genes, pJMC40 was digested with the restriction enzyme EcoRI and re-ligated at low concentrations (Fig. 1B). This procedure should yield a much smaller plasmid carrying tetracycline resistance and the lon gene with only a few hundred base pairs of chromosomal DNA flanking lon. Although this relatively simple procedure was attempted several times, very low transformation frequencies of wild-type cells were obtained following re-ligation of the cut material. None of the resulting transformants were capable of complementing a chromosomal lon mutation when transformed into a lon- cell and assayed for the mucoid phenotype on minimal media agar plates (i.e. all colonies remained mucoid). A few tetracycline-resistant transformed colonies were obtained, and restriction analysis of the plasmids present in these colonies revealed the presence of an insertion within the lon gene indistinguishable from that described for pJMC40IS above.

Constructing lon under lac Promoter Control—Since cells carrying pJMC40 are difficult to use for either physiological

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**Table II**

Protease La activity measured in phosphocellulose eluates

| Plasmid present | Relative cleavage of substrate | pmol substrate hydrolyzed/h |
|-----------------|--------------------------------|---------------------------|
| Subclone        | Experiment 1 | Experiment 2 | Experiment 3 |
| pJMC40IS        | 82         | 25           | 55           |
| pJMC40          | 160        | 69           | 160          |
| Ratio           | 2          | 2.8          | 3.4          |

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**Fig. 2. Protease La in crude extracts.** Crude extracts of E. coli containing either the lon+ plasmid pJMC40 (SG1016), or the lon- derivative pJMC40IS (SG1017) were prepared by growing the cells at 37°C to an OD<sub>600</sub> of 0.5, centrifugation of cells at 4000 x g for 5 min, and resuspending the cell pellets in 100 mM potassium phosphate buffer, pH 7.0, 20% glycerol, 10 mM MgSO<sub>4</sub>, and 1 mM EDTA. The cells were disrupted by sonication and centrifuged to remove cellular debris. Protein concentrations were determined, and equal amounts of protein were subjected to electrophoresis in a 6% sodium dodecyl sulfate-polyacrylamide gel. The gel was stained, dried, and then photographed.
or genetic studies and often give rise to phenotypic revertants, it was desirable to have lon under the control of an inducible promoter. Such a construct was achieved by a series of steps involving removal of the original lon promoter and replacement of this promoter with either a lac promoter or a trp-lac (tac) hybrid promoter. The lon promoter was deleted in vitro using the double-stranded DNA exonuclease Bal31, as described under "Materials and Methods," and EcoRI restriction endonuclease sites were placed flanking the promoterless gene with the aid of synthetic oligonucleotide linkers (33). To place the lon gene under lac promoter control, the promoterless coding sequence was subcloned downstream from the lac promoter in pUC18 or into the tac promoter vector pKK223 (Fig. 1C) (33). Cells overproducing the lac repressor (the lacI gene product) carried on a compatible plasmid (pSG10) were transformed with either of these plasmids. The resulting transformants appear to retain viability and grow well provided lon transcription is repressed, in contrast to the cells transformed with the original lon plasmid pJMC40. However, growth of these transformants is very sensitive to induction of the lon gene. For example, when the lac inducer, isopropyl-1-thio-β-D-galactopyranoside (IPTG),2 and cells carrying this plasmid were cross-streaked on agar plates, these cells failed to grow in the vicinity of the IPTG.

As expected, induction of lon under lac or tac promoter control with IPTG results in preferential synthesis of a 94-kilodalton polypeptide as determined by pulse-labeling of induced cells with [35S]methionine, separation of proteins with polyacrylamide gel electrophoresis in the presence of SDS, and autoradiography of the dried gel (Fig. 3).

**Induction of Protease La Inhibits Growth**—To verify that overproduction of protease La is the cause of the decreased growth of cells containing pJMC40, cells with lon under lac promoter control (plasmid pSG5) or under tac promoter control (plasmid pSG11) were grown in rich media at 37 °C to mid-log phase. One half of the culture served as a control, whereas in the other, protease La synthesis was induced by the addition of IPTG. Within 15–30 min after the addition of the inducer, the cells decreased their rate of growth, and within 1–2 generations, no further increase in optical density was observed (Fig. 4). As discussed below, these effects are probably a consequence of the excessive degradation of certain critical cell proteins. These findings thus support the earlier conclusion from studies with pJMC40 that high rates of expression of protease La are detrimental to growth.

To test whether overproduction of protease La reduces the viability of non-growing cells, cells carrying lon under tac promoter control were grown in rich or minimal media to stationary phase. In half the culture, protease La was induced by the addition of IPTG to the medium. After different periods of incubation at 37 °C, colony-forming units were determined by dilution and plating onto rich-media agar plates without IPTG. Although no change was seen for several hours, after 17 or 24 h in the presence of inducer, the plating efficiency of the cells with high levels of protease La was reduced 90–99% relative to the uninduced controls (Table III) or to their initial cell number. These findings emphasize the importance of precise regulation of protease La content, especially in non-growing cells.

**Effects of High Protease Levels on Protein Degradation**—To test whether an increased content of the ATP-dependent protease leads to more rapid degradation of abnormal proteins, a leucine auxotroph carrying pJMC40 or pJMC40S was allowed to incorporate the amino acid analog azaleucine in place of leucine. The cultures were also exposed to [35S]methionine in order to follow the turnover of these proteins. In both cultures, proteins containing the analog were consistently degraded severalfold faster than ones containing leucine.

2 The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; SDS, sodium dodecyl sulfate.

FIG. 3. **Preferential synthesis of protease La.** Cells (SG84O and SG841) carrying a control plasmid (pKK223) or the lon structural gene under tac promoter control (pSG11) were grown in M9 minimal media at 30 °C to an OD600 of 0.3. Protein was labeled with 50 μCi of [35S]methionine for 30 min. Cells were disrupted by exposure to 15% trichloroacetic acid on ice for 30 min, and proteins were pelleted by centrifugation. Protein pellets were washed with 5% trichloroacetic acid and 1:1 ethanol/ether and dried. Pellets were then resuspended in SDS sample buffer by boiling for 4 min. Equal counts were added to a 9–15% linear gradient polyacrylamide gel and electrophoresed in the presence of SDS. The gel was fixed in 10% acetic acid, dried, exposed to x-ray film, and then autoradiograph photographed.

FIG. 4. **lon induction decreases growth.** Cells (SG480) carrying lon under tac promoter control (pSG11) were grown at 37 °C in Luria broth to an OD600 of 0.1 and split into two cultures. One served as the uninduced control, whereas the other was induced for excess protease synthesis by exposure to 500 μM IPTG (added at time indicated by arrow). Growth was estimated using optical density as determined by a Klett densitometer. Similar results were obtained in cells with lon under lac promoter control (pSG5).
TABLE III

Loss of viability of cells overproducing protease La in stationary phase

Cells (SG825) carrying pSG11 were grown in Luria broth at 37 °C to saturation (time 0). The culture was divided in half, and IPTG was added to 500 µM in one half, whereas the other half served as a control. The cells were incubated for an additional 5, 17, or 24 h and then serial diluted and plated for colony-forming units on Luria broth plates.

| Incubation | Survival -IPTG | Survival +IPTG |
|------------|----------------|----------------|
| h          | %              | %              |
| 5          | 96             | 49             |
| 17         | 83             | 4              |
| 24         | 81             | 1              |

![Graph](image)

Fig. 5. Increased degradation of azaleucine-containing proteins. Cells carrying pJMC40 (SG1016) and pJMC40IS (SG1017) were grown in minimal media, and azaleucine-containing proteins were generated, as described under “Materials and Methods.” Aliquots were taken over time, and the release of label from protein (trichloroacetic acid-soluble counts) was used to estimate the percentage of labeled protein degraded. Similar results were obtained in three independent experiments.

(Fig. 5), in accord with previous findings (8, 9). The cells with an inactivated lon allele on the multicopy plasmid (pJMC40IS) degraded approximately 10–20% of the azaleucine-containing proteins per hour, whereas cells with an active lon gene on the episome (pJMC40) exhibited 3–5-fold more rapid degradation of these abnormal polypeptides (Fig. 5).

Analogous results were observed in cells with lon under control of the tac promoter. An arginine auxotroph carrying the plasmid pSG11 was grown to mid-log phase in minimal media at 37 °C and washed free of arginine. These cells were resuspended in media containing canavanine, and the proteases synthesized with this analog were pulse-labeled with [35S]methionine. Following removal of the analog and the radioactive amino acid, the culture was split in two and resuspended in the original medium. One half served as a control, whereas the other half, protease La was induced with IPTG.

The induced cells consistently degraded the labeled abnormal proteins 30–50% more rapidly than did uninduced cells (Fig. 6). This effect was clearly evident within ½ h after addition of the inducer. In addition, the total amount of abnormal protein degraded was greater in the induced cells, presumably as a consequence of the higher levels of protease La. (This enhancement of the degradation of analog-containing proteins in the induced cells appeared smaller than that in cells carrying pJMC40. However, different amino acid analogs were used in different strain backgrounds, and the amount of protease La generated under these conditions may have also differed.)

Incomplete polypeptides are also degraded more rapidly in cells overproducing protease La. When cultures carrying the plasmid pJMC40IS were exposed to puromycin for 15 min at 37 °C, the resulting labeled polypeptides were degraded at a rate of approximately 10% per hour. The rate of proteolysis in cells with the intact lon gene on the plasmid pJMC40 was between 20 and 25% per hour (Table IV). Similarly, when the lon gene under the tac promoter control was induced following the synthesis of puromycyl polypeptides, both a greater rate and a greater amount of degradation of these protein fragments occurred than in uninduced controls (data not shown).

A Protein Fusion Is Rapidly Lost in Cells with Increased Protease La—To test whether the degradation of a particular abnormal protein is enhanced in cells carrying high levels of protease La, the stability of a β-galactosidase fusion protein was compared in cells carrying either pJMC40 or the control plasmid. Cells carrying a lon-lacZ protein fusion on an integrated bacteriophage were transformed with either pJMC40
Effects of Increased Protease La in E. coli

Fig. 7. Loss of activity of a protein fusion. Cells carrying either the lon+ plasmid pJMC40 or the lon- derivative pJMC40IS and a protease La-β-galactosidase protein fusion produced from an integrated bacteriophage λ derivative were used. Aliquots were taken over time as cells were incubated at 37 °C, and β-galactosidase activity from the fusion protein was measured according to the method of Miller (30). Similar results were obtained in three independent experiments.

(lon+) or pJMC40IS. To determine the stability of the fusion protein, chloramphenicol was added to prevent protein synthesis, and β-galactosidase activity was assayed at various times thereafter. At 37 °C, a rapid loss of this activity was observed in cells carrying pJMC40, but not in cells carrying either pJMC40IS (Fig. 7) or no plasmid (data not shown).

The data suggest that protease La, when present at high levels, will degrade a specific protein which would otherwise remain quite stable. In fact, in the control cells, this protein fusion decreased by 50% in approximately 4 h whereas in lon mutants no such loss of activity was seen. Furthermore, at 30 °C, this fusion appeared quite stable even in cells carrying pJMC40 (data not shown). Thus, the rate of degradation of this fusion protein appears to depend on both the growth temperature and the protease content of the cells.

Effects of Increased Amounts of Protease La on Degradation of Normal Cell Proteins—Unlike polypeptides containing amino acid analogs or puromycin, most proteins synthesized in growing cells are quite stable (1, 2, 33, 34), and their rate of breakdown appears similar in lon and wild-type strains. To test the effects of high levels of the lon gene product on the degradation of these “normal” proteins, cells carrying the lon+ plasmid pJMC40 or the control plasmid pJMC40IS were grown in minimal media at 37 °C, and the proteins were labeled for 30 min with [35S]methionine. After washing, the cells were resuspended in media without radioactivity and with excess methionine. The cells carrying pJMC40 degraded these normally stable proteins approximately twice as fast (6%/h) as cells carrying the inactivated lon allele on the plasmid pJMC40IS (3%/h) (Fig. 8A). Similar results were obtained when cell proteins were labeled for 90 min (data not shown).

Analogous experiments were performed with a strain containing the lon gene under tac promoter control. Cells grown without the inducer were exposed to [35S]methionine for 30 min; the culture was then divided in two halves, one of which received IPTG to induce synthesis of protease La. The in-

duced cultures consistently exhibited a 2-4-fold greater rate of hydrolysis of the prelabeled normal proteins (Fig. 9). The rate of degradation of these normal proteins did not rise further with time, even though more protease La was synthesized.

Although it is clear that protease La can degrade proteins present in normal cells, these experiments do not distinguish whether the enhanced proteolysis is due to slightly more rapid

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degradation of most cell proteins or whether certain polypeptides are selectively degraded under these conditions. To decide between these alternatives, extracts of cells carrying the lon gene under tac control were subjected to SDS-gel electrophoresis at different times (up to 3 h) after addition of IPTG to induce protease La. As protease content increased (i.e. the 87-kDa band in Fig. 10) and growth ceased, most polypeptide bands did not change significantly. However, several minor bands were found to decrease reproducibly in different experiments. It is interesting that the clearest examples of such polypeptides were ones associated with the fraction sedimenting at 100,000 × g (Fig. 10). In these extracts, there also was a clear accumulation of small polypeptides (i.e. less than 15 kDa), especially in the 100,000 × g pellet. Presumably, these bands represent protein fragments generated by endoproteolytic attack. At the same time, there was no obvious broadening of protein bands, as might occur upon exposure of extracts to exoproteases. Thus, the small rise in overall protein breakdown (Fig. 8A) seems to reflect the differential loss of certain polypeptides, whose disappearance may be related to the cessation of growth under these conditions.

The rate of degradation of these relatively stable polypeptides increases 2-4-fold upon starvation of E. coli for a variety of essential nutrients or ions (2, 34-36). It is unclear what proteolytic activities are responsible for this accelerated degradation of otherwise stable proteins. This process is prevented by various inhibitors of ATP production (34, 36).

Although this finding suggests that an ATP-dependent system is involved, lon mutants display enhanced protein degradation upon starvation for nitrogen or a carbon source like wild-type cells (2, 37). In an attempt to determine whether an increase in protease La enhances protein degradation during starvation, cells carrying either pJMC40 or pJMC40I5 were deprived of nitrogen, and the rates of degradation of prelabeled cell proteins were determined (Fig. 8B). Although starved cells containing pJMC40 exhibit a higher rate of protein degradation than starved cells carrying the control plasmid, the magnitude of this difference was indistinguishable from that seen in the growing cultures. In other words, the increment in overall protein breakdown during starvation was not affected by the increase in protease La, and presumably therefore involves a distinct proteolytic system, as suggested previously (2, 37, 38).

DISCUSSION

These studies indicate that a severalfold increase in the intracellular level of the ATP-dependent protease La can be highly deleterious. Cellular growth is reduced (Fig. 4), and in stationary phase such cells lose viability (Table III). Furthermore, there is a strong selection against cells with excess protease content and for cells in which the cloned gene is inactivated. Since no such selection was observed when fragments of the lon gene were carried on a multicopy plasmid, these detrimental effects must be due to the increased content of protease La and to the resulting enhancement of protein breakdown.

Interestingly, growth ceased even when the breakdown of average cell proteins had increased only by 1-2% /h (Figs. 8A and 10). In fact, the overall rate of protein degradation when protease La was induced was at most 4-6%/h, which represents only a small fraction of the rate of new protein synthesis in the growing cells. Therefore, it seems likely that the sharp decrease in cell growth and the loss of viability in stationary phase occur because certain critical cell proteins are being degraded selectively under these conditions. Following protease induction, several polypeptides, in particular membrane-associated or particulate components that sedimented in the 100,000 × g pellet, seem to be especially susceptible to proteolysis (Fig. 9). The identity of these proteins is unknown. It is unclear whether they are proteins that contain silent mutations or enzymes that normally turn over in growing E. coli. It is interesting that in mammalian cells, many polypeptides necessary for cell growth (i.e. rate-limiting enzymes in DNA and RNA synthesis) have short half-lives (2, 39). Perhaps in E. coli there are polypeptides with analogous functions which are particularly susceptible to protease La. Isolation of pheno- typic revertants which grow despite the presence of high levels of protease La should provide valuable information on the natural substrates of the lon-encoded protease and on the mechanism of the reduced viability.

These experiments provide direct evidence that protein stability in vivo depends on protease content. The presence of both the lon plasmid pJMC40 and induction of lon from an inducible promoter results in an increased degradation of various abnormal polypeptides (Figs. 5 and 6 and Table IV). Since both the initial rate of degradation and the total amount of protein hydrolysed were greater in these cells than in the controls, protease La content seems critical in determining whether a protein undergoes degradation. Thus, in normal cells, some proteins containing amino acid analogs or puromycin or protein fusions can escape being digested, apparently because the level of this protease is insufficient for their recognition and degradation. For example, the β-galactosidase
fusian protein that appears stable in control cells (tₚ of about 4 h) is rapidly inactivated in cells with high levels of protease La (Fig. 7). This fusion protein is apparently recognized as “abnormal” and is degraded only in cells with a high content of protease La. The structural difference between this fusion protein and wild-type β-galactosidase is unclear, and attempts to demonsstrate differences in their thermal stability or protease sensitivity in extracts have been unsuccessful.4

It is of particular physiological interest that cells with increased transcription of the lon gene degrade certain proteins which would otherwise be retained. Therefore, during the heat-shock response (16, 21, 22) when the levels of protease La rise, the cells’ capacity to degrade abnormal polypeptides which would otherwise be retained. Therefore, during the heat-shock response (16, 21, 22) when the levels of protease La rise, the cells’ capacity to degrade abnormal polypeptides should rise. These findings thus support our earlier suggestion (16, 21) that induction of lon, and perhaps other heat-shock genes, can help prevent the accumulation of highly abnormal and potentially harmful polypeptides.

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