Proc. Nat. Acad. Sci. USA
Vol. 69, No. 9, pp. 2640–2644, September 1972

Correlation Between Rates of Degradation of Bacterial Proteins *In Vivo* and Their Sensitivity to Proteases

(protein conformation/abnormal proteins/aminoacid analogs/mistranslation)

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Communicated by Elkan R. Blout, June 26, 1972

ABSTRACT **Experiments were undertaken to test whether the relative rates of degradation of proteins *in vivo* might correlate with their sensitivity to proteases. Various experimental conditions that promote degradation of proteins in *Escherichia coli* increased sensitivity of average cell proteins to trypsin or other endoproteases, including incorporation of several aminoacid analogs into the proteins, incorporation of puromycin into the polypeptide chain, or frequent errors in translation. Those abnormal proteins that were degraded most rapidly within the cell appear responsible for the increased protease sensitivity of the cell extract. Normal *E. coli* proteins that rapidly turn over in growing cells are, on the average, more sensitive to trypsin or Pronase than cell proteins that turn over more slowly. The inherent sensitivity of a protein to proteolytic digestion is thus a major determinant of protein half-lives *in vivo*.**

Various proteins within mammalian (1, 2) or bacterial (3) cells differ appreciably in their stability. In rat liver, for example, the half-life of the average protein is about 3 days, although the half-lives of individual enzymes range between 11 min and many days (1, 2). It appears likely that these different rates of protein degradation *in vivo* reflect inherent differences in protein conformations. Recently several groups have demonstrated in *Escherichia coli* that alterations in the primary structure of a protein can affect its rate of catabolism (4–7). For example, in *E. coli* a mutant *lac* repressor with an altered carboxyl terminal (6) and certain nonsense fragments of β-galactosidase (7) are both rapidly catabolized even though the wild-type proteins are stable. In addition, the incorporation of puromycin (4, 5) or various aminoacid analogs into proteins leads to the rapid hydrolysis of the abnormal proteins (4, 5, 8). Finally, increased mistranslation of the genetic message (e.g., in bacterial strains carrying either a ribosomal mutation or missense suppressor) also is associated with an increased rate of protein breakdown (4).

The manner in which such alterations in the primary sequence of proteins affect their half-lives remains to be elucidated. The simplest possibility is that in the altered conformation a protein becomes more sensitive to proteolytic enzymes normally found in the cell. The present studies were undertaken to determine whether a general correlation might exist between the rate of protein catabolism *in vivo* and the sensitivity of cell proteins to proteolytic digestion. We demonstrate that the various conditions that promote protein breakdown in *E. coli* also increase the sensitivity of cell proteins to attack by pancreatic proteases *in vitro*. In addition, we have found that those normal proteins that turn over rapidly in growing *E. coli* appear inherently more sensitive to proteases than proteins that turn over more slowly.

METHODS AND MATERIALS

E. coli auxotrophs were grown on glucose–minimal medium supplemented with required amino acids for two generations, as described (4). For the study of the effects of incorporation of aminoacid analogs, growing cells were centrifuged, washed, and suspended for 12 min in the presence of either the required amino acids or equimolar concentrations of the appropriate analogs (4) before receiving radioactive leucine. To obtain polypeptides containing puromycin (4, 5), I added puromycin to growth medium for 12 min. [^{3}H]Leucine (2 μCi/ml, 50 Ci/mmol, Schwartz/Mann Biochemical Co.) was then added to such cultures or to normally growing controls for 5 min to label cell proteins. The cells were then filtered, washed, and suspended in the original growth medium, supplemented with 75 μg/ml of leucine to prevent reincorporation of [^{3}H]leucine.

For measurements of protein breakdown *in vivo*, the labeled cells were allowed to grow in the presence of the required amino acids, and proteolysis was estimated from the release of [^{3}H]leucine into Cl_3CCOOH-soluble form (4). For the study of the protease sensitivity of cell proteins, the labeled cells were centrifuged at 4° and lysed by freezing and thawing in the presence of lysozyme (9). The disrupted cells were suspended in cold buffer containing 50 mM Tris·HCl (pH 7.5)–10 mM magnesium sulfate–0.2 M KCl. The supernatants were treated with proteases immediately after centrifugation, since repeated freezing and thawing appeared to increase protease sensitivity.

Extracts were incubated with trypsin (Boehringer–Soehne), chymotrypsin (Sigma, Type II), Pronase (Worthington), or subtilisin (Sigma) in the buffer. The actual concentration of the enzyme used varied in the different experiments, but in each case the proteases were present in sufficiently high concentration that the rate of protein hydrolysis was much faster than the rate found in the intact cells, and that changes in extract concentration did not affect apparent protease sensitivity. Percentage of protein breakdown was expressed as the amount of trichloroacetic acid-soluble radioactivity relative to the amount initially found in proteins.

RESULTS

Incorporation of various aminoacid analogs into cell proteins increases their rate of degradation *in vivo* (4, 5, 8). To compare the protease sensitivity of proteins containing analogs with normal proteins, *E. coli* auxotrophs were allowed to

TABLE 1. *Effects of different proteases on proteins containing aminoacid analogs or puromycin*

Exp.	Proteins synthesized in presence of	Extracts incubated with Trypsin	Chymotrypsin	Subtilisin	Pronase
		(% Protein degradation per 45 min)			
1	Arginine (20 μg/ml)	72	43	56	62
	Canavanine (20 μg/ml)	92	58	73	89
2	Tryptophan (60 μg/ml)	49	51	62	58
	7-Azatryptophan (60 μg/ml)	65	58	76	73
3	Required amino acids	49	51	62	58
	+ Puromycin (300 μg/ml)	61	62	80	68

Extracts of *E. coli A33* were incubated for 45 min in the presence of 20 μg/ml trypsin, 20 μg/ml subtilisin, 20 μg/ml Pronase, or 40 μg/ml chymotrypsin. In all experiments, the cells were exposed to [^{3}H]leucine in the presence of the indicated amounts of amino acid or analog. Experiment 1: Extracts containing canavanine contained 0.61 mg/ml of protein; the control, 0.57 mg/ml. Experiment 2: extracts containing 7-azatryptophan contained 0.50 mg/ml of protein, the control, 0.46 mg/ml. Experiment 3: the puromycin-treated extract contained 0.46 mg/ml protein; the control, 0.50 mg/ml.

incorporate [^{3}H]leucine in the presence of analogs or required amino acids (4). Extracts from such cells were then incubated with proteolytic enzymes. Fig. 1 compares the trypsin sensitivity of cell proteins synthesized in the presence of arginine or canavanine, proline or azetidine carboxylic acid, and valine or D,L-threo-α-amino-β-chlorobutyric acid. In each case, proteins containing the analog were hydrolyzed more rapidly than the normal proteins. Differences in protease sensitivity were observed repeatedly, although the magnitude of the differences varied in individual experiments and with different incubation conditions. In other experiments, lower amounts of trypsin were found to produce greater differences in rates of hydrolysis of the cell extracts than those reported here. However, the present experiments used high protease concentrations in order to insure that the rate of protein hydrolysis in cell extracts greatly exceeded that found in intact cells and thus to avoid possible complications in interpretation (see below). Analogous findings were obtained in experiments with other analogs whose incorporation into cell proteins also promotes protein catabolism (4), such as azatryptophan (Table 1) or 5-fluorotryptophan in place of tryptophan, fluorotyrosine in place of tyrosine, and (β-aminoethyl)-cysteine in place of lysine. The observed differences (Fig. 1) in protease sensitivity must result from incorporation of the analogs into the labeled proteins, since addition of canavanine or azetidine carboxylic acid to control cells after exposure to [^{3}H]leucine did not alter the trypsin-sensitivity of the preformed (labeled) proteins. In addition, when an arginine auxotroph incorporated both [^{14}C]canavanine and [^{3}H]-arginine, the proteins containing the analog, which are degraded more rapidly *in vivo*, were also found to be more sensitive to trypsin *in vitro* (unpublished observations).

In addition to proteins containing aminoacid analogs, *E. coli* selectively degrade incomplete polypeptides, which had incorporated puromycin and were prematurely released from the ribosome (4, 5). Fig. 1*D* demonstrates that polypeptides made in the presence of puromycin are also more sensitive to trypsin than normal cell proteins. In addition,

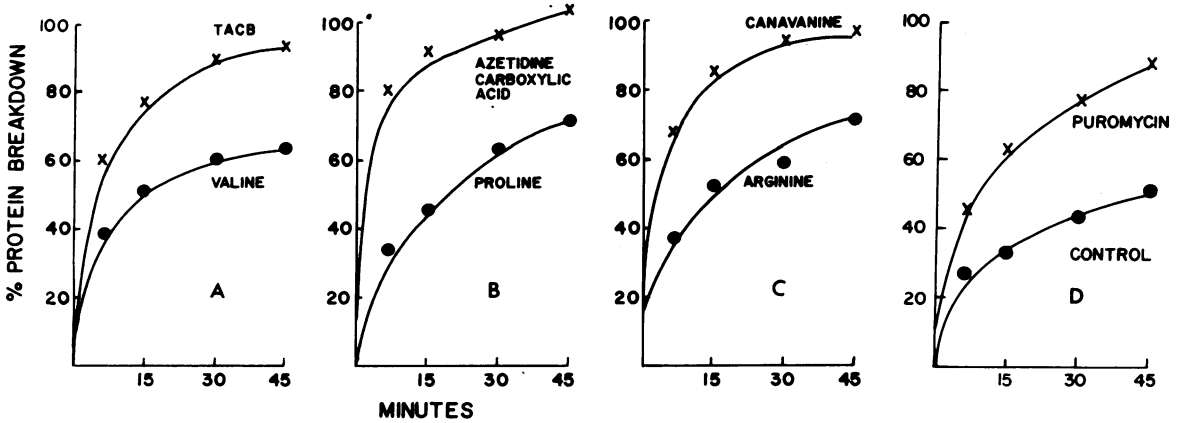

FIG. 1. Effect of trypsin on proteins containing aminoacid analogs. Extracts from different strains of *E. coli* were incubated with trypsin (100 μg/ml) for the indicated times. (*A*) *M* 48–62, a valine auxotroph, was exposed to [^{3}H]leucine and 50 μg/ml of either valine or D,L-threo-α-amino-β-chlorobutyric acid (TACB). Protein content of valine-containing extract was 0.40 mg/ml; that of TACB-containing extract, 0.35 mg/ml. (*B*) *M* 55–25, a proline auxotroph, was exposed to [^{3}H]leucine and 20 μg/ml of either proline or azetidine carboxylic acid. Protein content of both extracts was 0.4 mg/ml. (*C*) *A33*, an arginine and tryptophan auxotroph, was exposed to [^{3}H]leucine, tryptophan, and 20 μg/ml of either arginine or canavanine. The protein content of the canavanine-containing extract was 0.59 mg/ml; that of arginine-containing extract, 0.56 mg/ml. (*D*) *A33* was exposed to [^{3}H]leucine in the presence or absence of puromycin (300 μg/ml). This concentration inhibited incorporation of [^{3}H]leucine into protein by 76%. The protein content of puromycin-treated extract was 0.46 mg/ml; that of the control, 0.52 mg/ml.

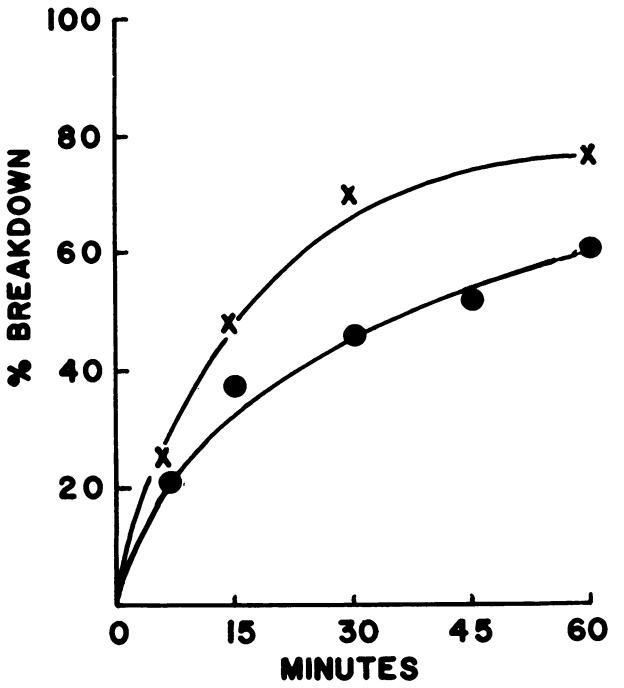

FIG. 2. Effect of trypsin on cell proteins in isogenic *ram 1* and *ram*$^+$ strains. Strains L1 (*ram 1*) (×) and T9031 (*ram*$^+$) (●) were given [^{3}H]leucine, and extracts containing 0.55 and 0.51 mg protein per ml, respectively, were incubated with 100 μg/ml of trypsin.

protein breakdown occurs more rapidly in strains that produce frequent errors in translation, such as those carrying ribosomal ambiguity mutations (*ram*), than in control cells (4). Fig. 2 indicates that the average proteins made in a *ram 1* strain are more sensitive to tryptic digestion than average proteins from an isogenic wild-type strain. Similar observations have also been made in comparisons of the trypsin-sensitivity of isogenic *ram 2* and *ram*$^+$ strains.

These data suggest a general correlation between rates of intracellular catabolism and sensitivity to trypsin. Analogous results were also obtained with other endoproteases of different specificities. As shown in Table 1, proteins containing canavanine or azatryptophan were more sensitive to chymotrypsin, subtilisin, and Pronase than those containing normal amino acids. Similarly, puromycin-containing proteins were more sensitive to Pronase and chymotrypsin than average cell proteins (Table 1).

Experiments were also undertaken to determine whether the abnormal proteins that are rapidly degraded by cells are the ones responsible for the increased protease-sensitivity observed *in vitro*. After exposure to canavanine and [^{3}H]-leucine, half of the culture was frozen. The other half was permitted to grow in medium containing arginine and non-radioactive leucine. After one or two generations (1 or 2 hr), these cells were frozen. By this time, they had degraded 48% and 78% of the labeled analog-containing proteins, respectively. Concomitantly, these cells appeared to have lost their most easily digested proteins since the remaining radioactive proteins were significantly less sensitive to the protease than the proteins of the cells frozen at earlier times (Fig. 3). In fact, by 2 hr after exposure to canavanine, the intracellular rates of protein breakdown had decreased to levels similar to those seen in control cells exposed only to arginine. At this time the remaining radioactive proteins in the extracts did not differ in trypsin sensitivity from labeled proteins of control cells. Thus there was a very strong correlation between rates of protein degradation *in vivo* and trypsin sensitivity *in vitro*.

Normal proteins

Normal cell proteins in *E. coli* also vary appreciably in their rates of degradation (3), and experiments were undertaken to determine whether such differences in protein half-lives can also be explained by differences in sensitivity to protease. Growing cells received [^{3}H]leucine for 5 min. The cells were then either frozen or permitted to grow in the presence of non-radioactive leucine for 2 hr. Immediately after the radioactive pulse, the apparent rate of degradation of labeled proteins exceeded that observed at the later times (i.e., the measured rate of proteolysis decreased as the cells lost their more labile components). The radioactive proteins remaining in the cells at later time were found to be less sensitive to trypsin than those synthesized 1 hr earlier (Fig. 4). Thus the proteins that are degraded most rapidly *in vivo* appear to be most sensitive to proteases.

Although small, such differences in protease sensitivity were observed consistently. This experimental approach necessarily yields only relatively small differences, since it can only examine the changes in trypsin sensitivity upon loss of the most labile proteins. This fraction represents at most about 7–9% of all the cell proteins (i.e., the amount of labeled proteins degraded by the cell during the 2-hr incubation). Growth of cells for an additional 2 hr to select for radioactive proteins with even longer half-lives further reduced the trypsin sensitivity. Analogous results were obtained upon treatment of cell extracts with Pronase at different times after exposure to [^{3}H]leucine (unpublished observations).

DISCUSSION

The present experiments provide strong evidence for a general correlation between rates of degradation of proteins in bacteria and their sensitivity to attack by endopeptidases. Various experimental treatments that promote protein catabolism *in vivo* also were found to increase the sensitivity of average cell proteins to trypsin or other proteases, including (*i*) incorporation of several aminoacid analogs, such as canavanine, *S*-(β-aminoethyl)-cysteine, α-amino-β-chlorobutyric acid, azatryptophan, fluorotyrosine, and fluorophenylalanine (Fig. 1, Table 1, and unpublished observations); (*ii*) premature termination of the polypeptide chain, resulting from incorporation of puromycin (Fig. 1); or (*iii*) frequent errors in synthesis resulting from a mutation affecting ribosomal structure (*ram 1*) (Fig. 2).

These studies thus offer a simple mechanism through which conformation may determine the half-life of cell proteins *in vivo*. Several findings strongly suggest that the increased protease sensitivity observed under these conditions is causally related to the changes in intracellular degradation. When the bacteria degraded the abnormal analog-containing proteins, the cell-free extracts decreased concomitantly in their sensitivity to proteases (Fig. 2). Eventually the degradative rate in the cells that had been exposed to the analogs returned to control levels, presumably as a result of the selective loss of the abnormal components. Extracts prepared at this time no longer appeared more sensitive to trypsin than

extracts from control cells (Fig. 2). Thus those proteins that were degraded most rapidly by the cells appear to have been responsible for the increased protease sensitivity. In related studies, we have found that those aminoacid analogs whose incorporation promoted intracellular proteolysis to the greatest extent also appear to have the greatest effects on sensitivity to trypsin.

In addition to these observations with abnormal proteins, evidence has been presented that normal proteins that turn over rapidly in growing cells are on the average more sensitive to trypsin or Pronase than more stable cell constituents (Fig. 4). As was observed with the abnormal proteins, the loss of the most labile proteins through degradation *in vivo* coincided with the loss of those proteins most easily digested by trypsin and Pronase. These findings strongly suggest that the variations in half-lives of normal proteins in *E. coli* are also determined by their different tertiary conformations, which presumably determine protease sensitivity. In fact, in recent experiments, I have found that treatment of extracts with mild denaturing agents can reduce the observed differences in protease sensitivity between the labile and stable cell constituents.

One possible trivial explanation for the observed correlation between degradative rates in intact cells and protease sensitivity of extracts could be that extracts from cells more active in catabolizing proteins would contain greater amounts of partially digested proteins. Such "slightly chewed" molecules are likely to be more senstitive to further attack by endoproteases. If they accumulated to an appreciable extent *in vivo*, they might influence the overall protease sensitivity of the extracts. However, analysis of cell extracts with acrylamide gel electrophoresis have not indicated any measurable differences in average protein size after incorporation of a normal amino acid or an analog. Furthermore, all experiments reported here were done with such

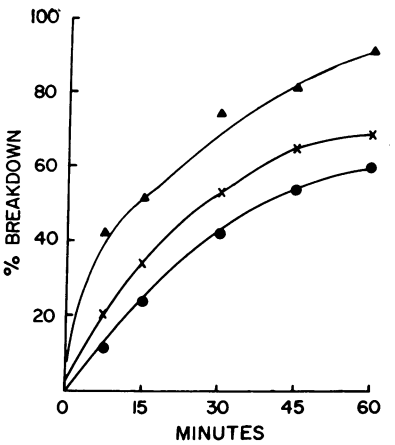

FIG. 3. Effect of degradation of canavanine-containing proteins *in vivo* on protease sensitivity of cell extracts. *E. coli A33* was exposed to [^{3}H]leucine in the presence of canavanine (see Fig. 1). Half the culture was frozen, and the other half grew in the presence of arginine and nonradioactive leucine for 1 or 2 generations (1 or 2 hr), during which the cells degraded, respectively, 48 and 78% of the radioactive proteins synthesized in the presence of canavanine. Before lysis, the cells frozen initially were combined with nonradioactive cells to give extracts containing 0.61 mg/ml protein. Extracts from incubated culture contained 0.65 mg/ml protein. ▲, 0 time; ×, 1 hr; ●, 2 hr.

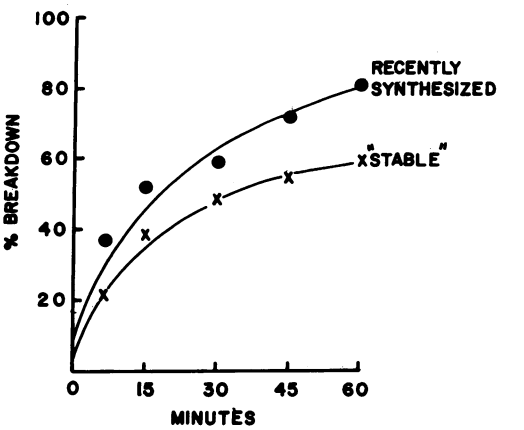

FIG. 4. Comparison of trypsin sensitivity of *E. coli* proteins with different intracellular stabilities. *E. coli A33* was exposed to [^{3}H]leucine for 5 min. Half the culture was frozen (*recently synthesized*), and the other half (*"stable"*) grew for two generations (2 hr) in the presence of nonradioactive leucine, during which time the cells degraded 8% of the labeled proteins to acid-soluble form. These cells, which had lost the more-labile cell proteins, were then frozen. Before preparation of the extracts and enzyme treatment, the cells frozen initially were combined with nonradioactive cells to give comparable amounts of protein. In this experiment, *recently synthesized* extract contained 0.56 mg/ml protein and the *stable* extract, 0.67 mg/ml. Trypsin concentration was 100 μg/ml.

high protease concentration that the rate of protein hydrolysis in the extracts was far more rapid that that which occurred *in vivo* [e.g., in normal cells 5–7% of the labeled proteins were degraded per hr, while in our experiments with trypsin most of the radioactive proteins in the extract were hydrolyzed within 45 min (Fig. 4)]. As a result, any partially digested polypeptides present in the extracts could not constitute a significant fraction of the proteins being hydrolyzed.

Evidence for a general correlation between intracellular half-lives and protease sensitivity has been previously lacking, although this possibility has been suggested (1, 4, 10). In related studies, I have also found that a mutant *lac* repressor (L-1), which is rapidly degraded by the cell, is far more easily digested by several proteases than the wild-type repressor, which is stable *in vivo*. Marked differences in protease sensitivity were found, even though these proteins do not differ in their ability to bind inducer or in their temperature sensitivity. In related experiments analogous to Fig. 4, I have demonstrated a correlation between protease sensitivity and average rates of protein catabolism in rat liver and rat kidney. In both tissues, those proteins turning over most rapidly appear most sensitive to trypsin and Pronase. These latter findings are in general accord with recent experiments of of Bond (10), who demonstrated that the relative sensitivity of five enzymes in rat liver to inactivation by trypsin or chymotrypsin correlated with their relative rates of degradation. However, in her experiments, sensitivity of these five enzymes to Pronase did not correlate simply with the *in vivo* data.

Substrates or cofactors can protect enzymes from proteolytic attack, and this effect has frequently been suggested as a possible mechanism through which such factors might influence intracellular enzyme levels (10–12). This possibility

appears especially likely in light of my findings that the average stability of cell proteins correlates with their protease sensitivity. However, it is unlikely that this simple correlation by itself can explain all the specificity in the regulation of of protein degradation. For example, in certain physiological states (13) cells may selectively degrade proteins that are quite stable under other conditions, possibly through highly specialized proteolytic systems (14). In addition, a specific polypeptide can have very different half-lives in different body tissues (15), possibly because of differences in tissue proteases.

Denatured proteins are more sensitive to proteolytic attack than native enzymes (16). This difference may provide cells with a simple mechanism for the selective degradation of abnormal proteins, such as those arising by mutation, mistakes in translation, intracellular denaturation, etc. Presumably, normal cell proteins share certain conformational features that protect them from rapid hydrolysis under most conditions. In the present studies, differences in protease sensitivity were observed with several endopeptidases of different specificities (e.g., trypsin and chymotrypsin) or even low specificity (e.g., subtilisin and Pronase). Because of these similar findings with such different enzymes, it is likely that rather marked differences in conformation or thermodynamic stability exist between normal and abnormal proteins. Presumably the structural differences that distinguish short-lived normal enzymes from more stable ones are similar in character although perhaps more subtle. The identification of the structural features responsible for such differences in protease sensitivity may prove of appreciable import for understanding the regulation of enzyme levels.

I am indebted to Mrs. Susan Martel, Mrs. Elizabeth Howell, and Miss Patricia Ritchie for their expert assistance with these experiments. These studies have been supported by a grant from the Air Force Office of Scientific Research.

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