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Degradation of Abnormal Proteins in *Escherichia coli*

(protein breakdown/protein structure/mistranslation/amino acid analogs/puromycin)

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ABSTRACT **Evidence is presented that *E. coli* contains a mechanism for selective degradation of abnormal proteins. Unfinished polypeptides containing puromycin, proteins containing frequent errors in translation, such as those synthesized by strains containing a *ram* mutation or a missense suppressor, and proteins containing amino-acid analogs were degraded more rapidly than were normal cell proteins. The degradation of analog- or puromycin-containing proteins appears to be an energy-dependent process. Unlike normal proteins, such proteins were degraded at similar rates by growing and by nongrowing cells.**

In nongrowing bacteria, 5–12% of the total cell proteins are degraded per hour (1, 2). This degradation provides starving cells with essential amino acids for new protein synthesis (3). Protein catabolism occurs several-times more slowly in growing cells (3), where its physiological significance is unclear (4). One function might be the degradation of abnormal proteins, such as might be produced by the denaturation of cell proteins, by errors in transcription or translation, or by genetic mutations. Several lines of evidence indicate that alterations in the primary structure of a protein can affect its rate of turnover. In reticulocytes (5) and in *Escherichia coli* (6), the incorporation of certain amino-acid analogs into proteins appears to promote their degradation. In addition, in *E. coli* a deletion mutant of the *lac* repressor (7) and certain nonsense fragments of β-galactosidase (8) are both rapidly degraded under conditions where the wild-type proteins are stable.

The present studies provide further evidence for the existence of a general mechanism for selective degradation of abnormal proteins. These experiments indicate that growing cells rapidly catabolize (*a*) unfinished polypeptide chains, (*b*) proteins containing frequent errors in translation, and (*c*) proteins containing amino-acid analogs. In addition, certain features of the degradative process are described.

MATERIALS AND METHODS

L-[4,5-^{3}H]leucine (55.2 μCi/mmol) and [methoxy-^{3}H]puromycin·2 HCl (1.0 μCi/mmol) were purchased from New England Nuclear corp. D-*threo*-α-amino-β-chlorobutyric acid and *S*-(β-aminoethyl)L-cysteine were generously provided by Dr. Marco Rabinowitz. Trifluoromethoxyphenylhydrazone carbonyl cyanide was a gift of Dr. E. C. C. Lin.

The following strains of *E. coli* were kindly provided by Dr. B. Davis: M55-25, a proline auxotroph; M26-26, a lysine auxotroph; M42-37, which requires both valine and isoleucine; and A33, (isolated by G. Stent), which requires arginine and tryptophan. The strains carrying *ram* or supressor mutations were a gift of Dr. L. Gorini. All cells were grown on glucose–minimal medium (9), with aeration, at 37°C. Required amino acids or analogs were provided at the concentrations indicated in Fig. 4.

The exact protocol to measure protein breakdown varied slightly in different experiments. In general, bacteria were grown for two generations and then provided L-[^{3}H]leucine (0.2 μCi/ml) for 5 min to label cell proteins. The cells were collected by filtration and washed twice with the final medium, which contained 75 μg/ml of [^{1}H]leucine to prevent reutilization of [^{3}H]leucine released from protein (2). The cells were then resuspended at 2–5 × 10^8 per ml. Protein breakdown was estimated by the rate of appearance of [^{3}H]leucine soluble in Cl_3CCOOH (2); radioactivity in the supernatant was measured by liquid scintillation counting (10). To determine the radioactivity present in protein, the pellet was washed, solubilized, and counted (11). The rate of protein breakdown is expressed as the amount of acid-soluble radioactivity relative to that originally in protein. Differences in quenching were corrected by an internal standard.

For studies of the effects of amino-acid analogs, exponentially growing auxotrophs were centrifuged, washed twice with medium lacking the required amino acids, and resuspended in medium containing either the required compound or equimolar amounts of the analog. After 12 min, [^{3}H]leucine was added; 5 min later the cells were filtered. The degradation of protein was then studied by resuspension of both cultures in medium containing the natural amino acids and 75 μg/ml of leucine. In experiments with [^{3}H]-puromycin, this compound was administered for one generation. After filtration, the cells were resuspended in medium lacking the drug. The release of [^{3}H]puromycin was assayed in the same manner as release of [^{3}H]leucine.

RESULTS

Pine (6) and Goldschmidt (8) have presented evidence that incomplete polypeptide chains are selectively degraded *in vivo*. To test this possibility, I have studied the stability of proteins made in the presence of puromycin, which is incorporated into the growing polypeptide chains and causes their premature release from the ribosome (12). Cells growing exponentially were administered puromycin (40 μg/ml) for 15 min; this treatment did not significantly inhibit growth. [^{3}H]leucine was then added for 5 min to label proteins in the puromycin-treated cells and in untreated controls. 5 Min later, both cultures were filtered, washed, and resuspended in medium lacking the drug and the labeled amino acid. Fig. 1*A* demonstrates that proteins made in the presence of the drug were degraded to free amino acid appreciably faster than proteins of treated cells. Even more rapid degradative rates were observed with higher concentrations of puromycin, which presumably cause a higher proportion of unfinished polypeptide chains. The drug, however, did not promote the degradation of proteins synthesized before its administration (Fig. 1*B*). Furthermore, puromycin amino-

nucleoside, which is not incorporated into polypeptide chains (14), did not increase the rate of protein degradation.

These findings strongly suggest that incorporation of puromycin into the incomplete peptide chain leads to its rapid hydrolysis. This conclusion was confirmed by treatment of growing cells with [^{3}H]puromycin. The growing cells incorporated labeled puromycin into acid-precipitable form (13). However, when [^{3}H]puromycin was removed from the medium, we observed the reappearance of the labeled puromycin in acid-soluble form (Fig. 2). These findings indicate continued intracellular hydrolysis of the puromycin-containing polypeptides. The net release of [^{3}H]puromycin overestimates the stability of puromycin-containing material because of the continuous reincorporation of released [^{3}H]-puromycin. Addition of chloramphenicol or nonradioactive puromycin to such cells (Fig. 2) markedly increased the release of [^{3}H]puromycin.

Mistakes in transcription or translation of the genetic information should also produce proteins with altered conformations. Rosset and Gorini (14) have isolated *E. coli* mutants (*ram*) that produce frequent errors in translation as a result of an alteration in the 30S ribosomal subunit. I tested whether these mutants also showed an increased rate of protein breakdown. As shown in Fig. 3, strain *ram1* degraded its proteins several times more rapidly than the wild type. This effect cannot be accounted for by the slightly lower growth rate of the mutant. In analogous experiments, an independent mutant *ram2* (14) was also found to degrade its proteins 2- to 3-times faster than the isogenic *ram*$^+$ strain. By contrast, a spontaneous reversion of *ram1* that did not make appreciable translational errors showed rates of breakdown indistinguishable from the wild type (unpublished observations).

Increased protein degradation was also observed in strains carrying missense suppressors. Such mutations affect the coding specificity of a single tRNA species and thus cause an increased rate of erroneous translation (15). Both a K-12 strain carrying Su23 and a B strain carrying Su78 suppressor mutations degraded proteins during growth at 2- to 3-times the rate of isogenic controls. These observations indicate an association between decreased fidelity of protein synthesis and increased protein breakdown.

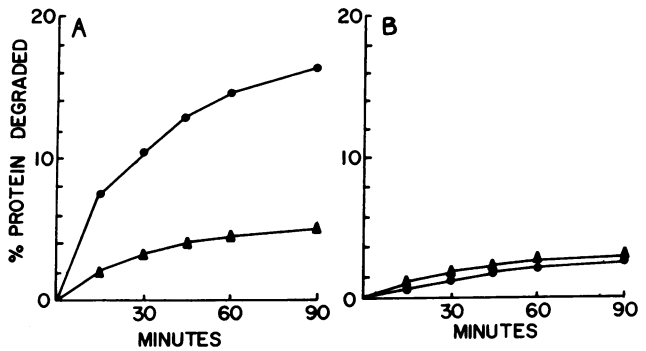

FIG. 1. (*A*) Degradation of proteins synthesized in the presence of puromycin. *E. coli* A33 was incubated with or without 40 μg/ml of puromycin for 12 min. [^{3}H]Leucine was then added for 5 min to label proteins. Both cultures were filtered to remove ^{3}H and drug; protein degradation was then measured. The puromycin inhibited [^{3}H]leucine incorporation by 20%, but both cultures subsequently grew at the same rate. ▲——▲, control; ●——●, puromycin.

(*B*) Effects of puromycin on the degradation of pre-existent proteins. *E. coli* A33 was supplied [^{3}H]leucine for 5 min; the breakdown of the labeled proteins was subsequently measured in the presence or absence of 40 μg/ml of puromycin. ▲——▲, control; ●——●, puromycin.

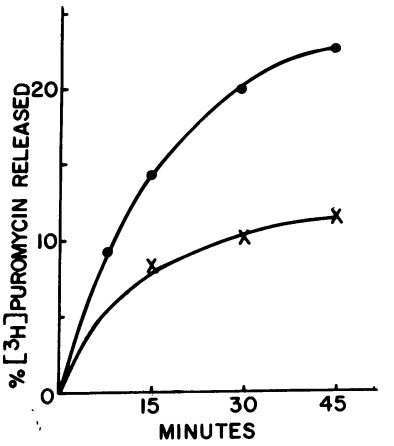

FIG. 2. Release of [^{3}H]puromycin from labeled proteins. *E. coli* A33 was treated with 1 μg/ml of [^{3}H]puromycin (2 μCi/μg) for 1 hr. The cells were then filtered and resuspended in medium lacking puromycin. The release of [^{3}H]puromycin into acid-soluble form was measured with (●——●) or without (×——×) 100 μg/ml chloramphenicol to block reincorporation of [^{3}H]-puromycin.

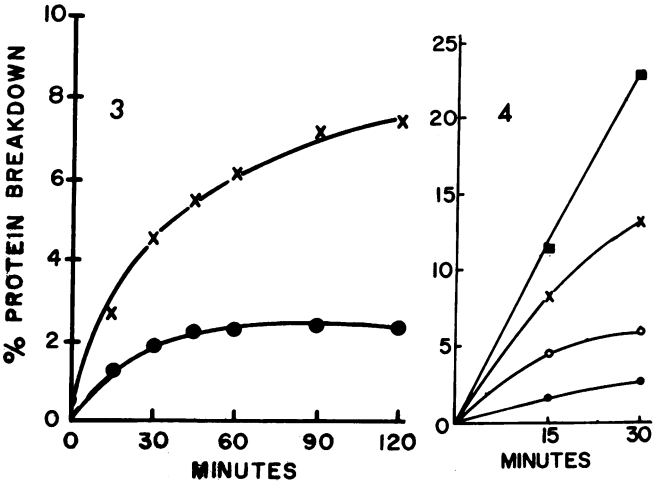

FIG. 3. Breakdown of proteins in *ram1* and *ram*$^+$ strains. Degradation of labeled protein was measured in the strains L1 (*ram1*, ×——×) and T9031 (*ram*$^+$, ●——●). Cells were grown in minimal media supplemented with tryptophan (20 μg/ml).

FIG. 4. Degradation of proteins containing amino-acid analogs. *E. coli* A33 was grown on glucose–minimal medium supplemented with 20 μg/ml arginine and 20 μg/ml of tryptophan. The cells were collected during exponential growth and resuspended either (*a*) in the original growth medium (●——●), or in media in which (*b*) 20 μg/ml of canavanine replaced arginine (■——■), or (*c*) 20 μg/ml of 7-azatryptophan replaced tryptophan (×——×), or (*d*) 5-fluorotryptophan (20 μg/ml) replaced tryptophan (O——O). After 12 min, all cultures were exposed to [^{3}H]lecuine for 5 min, then protein breakdown was measured in medium containing the natural amino acids. These analogs reduced [^{3}H]leucine incorporation by less than 15%.

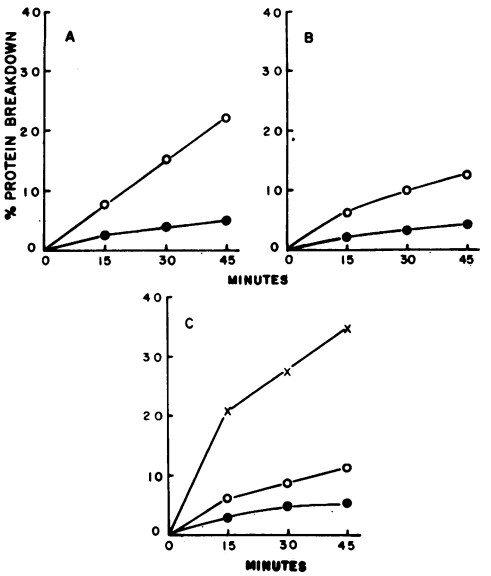

FIG. 5. Degradation of proteins containing amino-acid analogs. Auxotrophs were grown as in Fig. 4 in medium containing the required amino acids (20 μg/ml), then exposed to [^{3}H]leucine in the presence of the required compound or its analog (20 μg/ml). Degradation was measured in medium containing the normal amino acids. (*A*) catabolism of proteins containing lysine (●——●) or *S*-(β-aminoethyl)cysteine (O——O) in M2626, (*B*) proline (●——●) or azetidine carboxylic acid (O——O) in M55-25, and (*C*) isoleucine and valine (●——●), *O*-methylthreonine (an analog of isoleucine) and valine (×——×), or isoleucine and α-amino-β-chlorobutyric acid (a valine analog) (O——O), in M42-37. These analogs reduced incorporation of [^{3}H]leucine by less than 15%.

Although many analogs of the natural amino acids can be incorporated into proteins in place of their natural counterparts without marked effects, the incorporation of certain analogs prevents the appearance of normal enzymes (16–19). The stability *in vivo* of *E. coli* proteins containing various amino-acid analogs were examined systematically. Rabinowitz and Fischer (5) have shown that incorporation of the valine analog, D-*threo*-α-amino-β-chlorobutyric acid or the lysine analog, *S*-(β-aminoethyl) cysteine into proteins of reticulocytes causes their rapid hydrolysis. In addition, Pine (6) found that *E. coli* proteins made in the presence of norleucine, canavanine, fluorophenylalanine, or thienylalanine were degraded more rapidly than those containing normal amino acids. Further experiments with amino-acid analogs were undertaken to test the generality of these observations and to define further the degradative process.

A large number of different amino-acid analogs (Figs. 4 and 5; unpublished observations), specifically promoted the breakdown of proteins made in their presence. Typical experiments are shown in Fig. 4, which compare the effects of canavanine, an arginine analog, and 7-azatryptophan and 6-fluorotryptophan, analogs of tryptophan, on protein stability in strain A33. Results with canavanine were most marked, although azatryptophan also promoted breakdown severalfold. Under these conditions, the analog-treated and control cells grew at similar rates. Furthermore, administration of these analogs after exposure to [^{3}H]leucine did not affect the degradation of labeled proteins.

A number of additional analogs promoted protein breakdown, including 5-fluorotryptophan, *p*-fluorophenylalanine, selenomethionine, 5-fluorotyrosine, and azaleucine. These compounds differed appreciably in the magnitude of their effects on protein stability. Those analogs known to promote the degradation of hemoglobin in reticulocytes (5) also stimulated proteolysis in *E. coli*. Noteworthy was the finding that those analogs whose incorporation is known to lead eventually to cell death (18, 19)—canavanine, *O*-methylthreonine, azetidine carboxylic acid, and azatryptophan—had marked effects on protein breakdown. By contrast, those analogs that permit relatively normal growth of *E. coli* (16, 17) (e.g., fluorotyrosine, and 5- or 6-fluorotryptophan) increased proteolysis to a much-smaller degree.

In many cells (1, 2, 21), protein catabolism appears to be an energy-requiring process. Administration of carbonyl cyanide (22), sodium azide, or potassium cyanide markedly inhibited degradation of proteins containing amino-acid analogs and [^{3}H]puromycin (Table 1). The effects of azide and KCN were reversible.

This degradation of abnormal proteins occurs under conditions where the majority of cell proteins are stable. Normal proteins are degraded at an increased rate in cells deprived of a carbon or nitrogen source (1, 2). I tested whether this degradation of abnormal proteins also increases during starvation. Auxotrophs were allowed to incorporate an analog (Fig. 4) together with [^{3}H]leucine, and then were resuspended either in minimal medium or in medium lacking a nitrogen or carbon source. Proteins made in the presence of the analogs (Table 1) were degraded at similar rates in starving and in growing cells. By contrast, deprivation of carbon or nitrogen source (Table 2) stimulated breakdown of average cell proteins 2- to 4-fold (1, 2).

DISCUSSION

The present findings, together with other observations (5, 8), provide strong evidence for the existence of a general cellular mechanism for the degradation of abnormal proteins. Various experimental conditions that alter normal protein conformations cause selective degradation of the affected proteins. These include (*a*) incorporation of puromycin into

TABLE 1. *Effects of energy blockers on degradation of abnormal proteins*

Proteins synthesized in the presence of		% Breakdown in 45 min
Canavanine	Control	38
	+ Carbonyl cyanide	4
	+ Potassium cyanide	14
	+ Sodium azide	13
S-(Aminoethyl)-cysteine	Control	21
	+ Carbonyl cyanide	7
[^{3}H]Puromycin	Control	20
	+ Carbonyl cyanide	12

Effects of energy blockers on degradation of abnormal proteins. Protein breakdown was measured as in Figs. 4 or 5, in the presence of carbonyl cyanide (10 μg/ml), NaN_3 (50 mM), or KCN (1 mM). Release of [^{3}H]puromycin, was measured as in Fig. 2, in the presence of chloramphenicol (100 μg/ml).

growing polypeptide chains (Figs. 1 and 2), (*b*) frequent mistranslation of genetic information, induced by alterations in the ribosome (*ram1*, Fig. 3) or in tRNA specificity (Su23 or Su78), and (*c*) incorporation of various amino-acid analogs into cell proteins in place of the normal residue (Figs. 4 and 5).

These findings confirm the earlier suggestions of Pine (6). In similar studies, however, Pine failed to observe increased protein breakdown in suppressor strains, probably because his experiments used nonsense suppressors that should not cause generalized mistranslation (15). Pine also reported increased protein breakdown in cells treated with streptomycin or fluorouracil, which may affect the fidelity of gene translation or transcription, respectively. However, the finding of increased proteolysis under these conditions is open to alternative explanations, since these agents have various other physiological effects [e.g., streptomycin can cause release of unfinished polypeptides (20)].

The extent to which different amino-acid analogs promote proteolysis presumably is related to the extent to which they disrupt normal protein conformation. In related studies, I have found a simple negative correlation between the relative extent to which analogs promote protein breakdown and the extent to which they permit the formation of active enzymes, such as β-galactosidase. It is interesting that those analogs whose extensive incorporation into protein leads to cell death [e.g., canavanine (18), *O*-methylthreonine (19), azetidine carboxylic acid (19), and azatryptophan (19)] all stimulated protein breakdown much more than those analogs that permit relatively normal growth of *E. coli* (e.g., fluorotyrosine, 6- or 5-tryptophan). Also of interest was the observation that aminochlorobutyric acid and aminoethylcysteine, which stimulate protein catabolism in reticulocytes (5), had similar effects in *E. coli*.

The present results strongly suggest that normal proteins of *E. coli* share certain general conformational features that prevent their rapid hydrolysis. Deviations from these common characteristics might be expected to make the proteins more sensitive to the degradative system. It has long been known that proteins vary widely in their sensitivity to proteases and that denatured proteins are especially sensitive (23). The simplest explanation of the present findings would be that the alternatives in tertiary structure make the proteins appreciably more susceptible to hydrolysis by cell proteases. In related studies, I have demonstrated that the various conditions found here to promote proteolysis also increase the sensitivity of proteins to well-characterized endopeptidases (24).

Although the increased sensitivity of denatured proteins to proteases can account for the selective degradation of abnormal proteins, the findings in Table 1 indicate that the degradation of abnormal proteins is an energy-requiring process. This degradative process is thus clearly different from simple hydrolysis catalyzed by known proteolytic enzymes. In many types of cells (1, 2, 21), protein breakdown appears to require metabolic energy. These various observations, however, do not necessarily indicate a direct role of ATP in protein hydrolysis and may reflect an energy requirement for the activation of the proteolytic enzymes, for the intactness of a subcellular component, or even for the sequestering of the proteins into a specialized degradative site.

The hydrolysis of abnormal proteins in *E. coli* occurs at similar rates in growing and in nongrowing cells (Table 2). By contrast, degradation of average cell proteins increases severalfold during stationary phase or when cells are deprived of required nutrients (1, 2). This increase in proteolysis is essential for the cell to adapt to poor media (3), and it appears to be regulated by mechanisms similar to those controlling the accumulation of ribosomal RNA (2). These results thus suggest that two types of proteolysis are independently regulated. Moreover, it appears likely that these two types of degradation are catalyzed by distinct proteolytic systems (3), since I have found certain protease inhibitors that selectively inhibit protein breakdown in starving cells, but not the degradation of proteins containing amino-acid analogs.

TABLE 2. *Effects of carbon or nitrogen deprivation on degradation of proteins containing amino-acid analogs*

Proteins synthesized in the presence of		% Breakdown in 45 min
Azatryptophan	Control	14
	$-NH_3$	15
	−Glucose	15
Canavanine	Control	34
	$-NH_3$	35
	−Glucose	23
α-Amino-β-chloro-butyric acid	Control	19
	$-NH_3$	23
	−Glucose	22

Effects of starvation on degradation of proteins containing amino-acid analogs. Protein breakdown was studied as in Figs. 4 or 5 in cells growing either in minimal medium or in medium lacking glucose or NH_4Cl. In each strain, the breakdown of normal proteins increased 2- to 4-fold upon starvation.

There must exist strong selective pressure favoring the evolution of a mechanism to degrade abnormal proteins, since the intracellular accumulation of inactive or partially active enzymes should be highly deleterious. Accumulation of such proteinaceous "garbage" should constitute an especially serious problem in highly differentiated, nondividing cells, which unlike bacteria cannot dilute out abnormal components by division. However, the substrates for this degradative system in normal cells are difficult to identify. The frequency of errors in transcription and translation may be significant (15). In addition, cells may carry appreciable silent mutations, whose products are rapidly degraded. Perhaps the most important source of abnormal proteins is the intracellular denaturation of enzymes. Unfortunately, we know very little about this process or its frequency *in vivo*. Nevertheless, since denaturation would make proteins much more susceptible to degradation, it may be the rate-limiting step in the turnover of most cellular enzymes (25).

The implicit conclusion of these studies is that the stability of a protein is an inherent property of its conformation and thereby its primary structure (24). Direct evidence for a general relationship between protein structure and biological half-life has been lacking until now. The evidence gained thus far, however, concerns only abnormal proteins, and it remains to be established whether the variations in the half-life of normal cell proteins also reflect differences in their conformation (24). Nevertheless, these results demonstrate that alterations in primary sequence can influence protein

concentrations by altering their rate of catabolism (8, 9). In fact, it has frequently been observed that missense mutations reduce the concentration of the gene product. Such observations have generally been explained by hypothetical effects of the mutations on protein synthetic rates (26). These findings, however, may simply reflect the selective hydrolysis of the abnormal protein.

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