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Streptomycin-Induced Synthesis of Abnormal Protein in an *Escherichia coli* Mutant

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To determine directly the effects of streptomycin on translational fidelity in intact cells, we studied the synthesis of β-galactosidase and of the coat protein of bacteriophage R17 in an *Escherichia coli* mutant in which the bactericidal effects of streptomycin are delayed. After the addition of streptomycin to exponentially growing mutant cells, protein synthesis continues at an undiminished rate for approximately an hour; however, as measured by enzyme assays, little functional protein is produced. Serological assays designed to detect β-galactosidase and bacteriophage R17 coat protein show that substantial amounts of the protein synthesized can react with antisera prepared against active β-galactosidase and phage R17, indicating the aberrance of the protein produced in the presence of the antibiotic. The polypeptides synthesized in the presence of streptomycin are degraded in the cell to a much greater extent than protein synthesized in the absence of the antibiotic. The proteolytic attack on this protein is not affected by inhibitors of serine proteases, suggesting that enzymes other than those involved in "normal turnover" of cellular protein are responsible. In this strain, certain of the multiple effects of streptomycin are separated in time and the production of abnormal protein (enzymatically inactive and susceptible to proteolytic attack) could be studied in the absence of the lethal effect of the drug.

Streptomycin, in wild-type sensitive bacteria, causes rapid inhibition of protein synthesis in growing cells and in cell-free systems programmed with natural messenger ribonucleic acid (mRNA) (18). Inaccurate translation of the genetic code has been clearly demonstrated in vitro in response to synthetic mRNAs and has been suggested in vivo by the action of streptomycin as an external suppressor of nonsense and missense mutations (7) and by the apparent production of a cross-reacting protein in response to neomycin (2). Studies of the miscoding phenomenon in intact cells are difficult because concentrations of the antibiotic that induce errors in translation also cause rapid cessation of protein synthesis and a dramatic loss of viability. To separate the lethal effects of streptomycin from the induction of translational errors, we examined protein synthesis in a strain of *Escherichia coli* that continues to synthesize protein long after cell division has been inhibited.

A number of *E. coli* mutants have been reported (3, 8) that continue to incorporate amino acids into peptides for some time in the presence of streptomycin, although no increase in the number of cells is found. Preliminary studies (3) showed that although there was a net increase in protein per cell in the presence of the drug there was little or no increase in functional protein as measured by enzyme activity assays. This observation suggested one of the following alternatives: (i) selective inhibition of protein synthesis; (ii) synthesis of inactive forms of protein as a result of incorporation of incorrect amino acids or premature chain termination; and (iii) increased general or specific degradation.

The synthesis and fate of several proteins were studied in this mutant, and the conclusion was reached that protein serologically related to the functional protein was synthesized in the presence of streptomycin. The protein made in streptomycin-treated cells was not only nonfunctional but particularly susceptible to attack by proteolytic enzyme(s).

MATERIALS AND METHODS

Bacterial strains and medium. The strain used was a streptomycin-independent mutant that arose spontaneously from a streptomycin-dependent parent. The dependent was derived from *E. coli* Hfr1, a

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methionine-requiring strain which can serve as host for bacteriophage R17 and which is inducible for β-galactosidase (EC 3.2.1.23) synthesis. The strain carries a mutation in the *strA* locus, as well as a second mutation which suppresses the requirement for streptomycin for growth and results in an alteration in 30*S* ribosomal protein S4.

The cells were grown in tris(hydroxymethyl)aminomethane (Tris)-maleate medium (12) supplemented with methionine (20 μg/ml), Casamino Acids (Difco; 0.05%), and glycerol (0.4%). When the cultures were infected with phage R17, $CaCl_2$ (5×10^{-3} M) was added immediately prior to the phage inoculum.

Immunological assay for β-galactosidase CRM. The assay for cross-reacting material (CRM) was based on the method of Nakada and Magasanik (11). A constant volume of anti-β-galactosidase serum was added to each of a series of tubes. Increasing amounts of the enzyme-containing extracts and a mixture of phage R17 and its homologous antiserum, which facilitated precipitation, were combined with the antiserum. The reaction tubes were incubated for 1 h at 37 C and then maintained at 4 C for several days. The antigen-antibody complex was removed by centrifugation, and the supernatant fractions were assayed for nonprecipitated β-galactosidase activity.

Preparation of antisera. Rabbits weighing 2 to 4 kg were injected in the marginal ear vein with 0.1 ml of phage R17 suspension containing approximately 2×10^{12} plaque-forming units. After 3 weeks, the rabbits were given a booster shot and blood was collected 1 week later. The serum was treated with bacterial lysate to neutralize any host cell antibodies. The resulting precipitate was removed by centrifugation, and the supernatant fluid was assayed for antiviral activity. The *K* value (velocity constant expressing antiviral neutralization activity) was 4,600. β-Galactosidase antiserum was purchased from Difco Laboratories.

Protein catabolism. Either 2.0 μCi of [^{3}H] phenylalanine (specific activity 6.2 Ci/mmol) per ml of culture medium or 0.2 μCi of [^{14}C] arginine (specific activity 316 mCi/mmol) per ml of culture medium was added to an exponentially growing culture of mutant cells. Immediately after addition of the labeled amino acid, streptomycin (100 μg/ml) was added to half the culture. After various periods of exposure to the labeled phenylalanine or arginine, incorporation was stopped by the addition of 100-fold excess of the unlabeled amino acid and chloramphenicol (100 μg/ml). The amount of labeled amino acid in each culture that was precipitable with 5% cold trichloroacetic acid at the time of chloramphenicol addition was considered 100% (zero degradation). At intervals after chloramphenicol addition, samples were removed into cold 5% trichloroacetic acid, and the precipitates were collected on glass-fiber filters, washed with a 5% trichloroacetic acid solution containing Casamino Acids, dried, and counted. Protein degradation was measured as loss of radioactivity from the acid-precipitable into the acid-soluble fraction.

Reagents. *O*-nitrophenyl-β-D-galactopyranoside, isopropyl-β-D-thiogalactopyranoside, and phenylmethane sulfonyl fluoride were purchased from Mann Research Laboratories; streptomycin was from Sigma Chemical Co. [^{3}H] phenylalanine (6.2 Ci/mmol) and [^{14}C] arginine (316 mCi/mmol) were purchased from New England Nuclear Corp.

RESULTS

Effects of streptomycin on protein synthesis. The mutant used in these studies has properties similar to other revertants described previously (3, 9). It grows exponentially in media containing up to 10 μg of streptomycin per ml. At high concentrations, no colonies appear on solid medium; in liquid culture, there is no increase in the number of viable cells. In contrast to wild-type sensitive cells, however, no loss of viability is noted for at least one generation. For approximately 1 h after addition of streptomycin (100 μg/ml) to growing cultures, the viable cell number remains constant and the mass of the culture doubles. Protein synthesis, as measured by a change in adsorption at 420 nm or by incorporation of a radioactive amino acid, continues in the presence of this streptomycin concentration (Fig. 1). For the first 30 min the rate of synthesis is the same in the drug-treated culture and the nontreated control. After one generation, the amount of protein synthesized in the streptomycin-treated culture is >80% of that in the control. Assays for β-galactosidase (Fig. 1) indicate little increase in enzyme activity with cessation of active enzyme synthesis occurring between 5 and 10 min after addition of the antibiotic. Assays for other enzymes, both induced and constitutive (serine deaminase, ribonuclease, alkaline phosphatase, and malate dehydrogenase), showed a similar pattern; in no case was there more than 20% increase in any enzyme activity when total protein had increased by 100%.

To determine the nature of protein synthesized in the presence of streptomycin, we used a serological assay technique for detecting the synthesis of proteins related to β-galactosidase and to a structural protein, the coat protein of phage R17.

Synthesis of β-galactosidase in streptomycin-treated cells. The number of enzyme units that must be used to cause a fixed quantity of antiserum to reach the equivalence point (the point of maximal aggregation of antigen and antibody) is constant. If enzymatically inactive but serologically CRM is present in an extract, the equivalence point for that particular quantity of antiserum will be reached

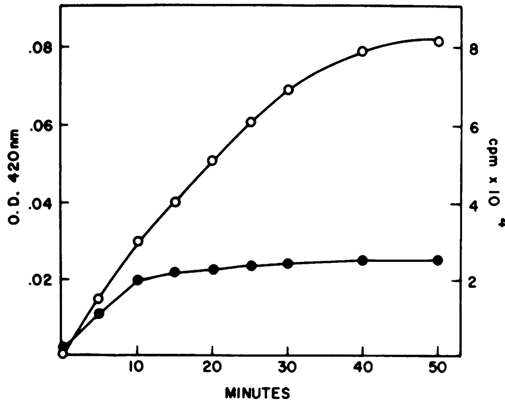

FIG. 1. *Effect of streptomycin on β-galactosidase and on protein synthesis. Isopropyl-β-D-thiogalactopyranoside (10^{-3} M) was added to cells growing exponentially. Streptomycin (100 μg/ml) and [^{14}C]arginine (0.2 μCi/ml) were added after 1 min. Total protein synthesis was determined by measuring the incorporation of [^{14}C]arginine into acid-precipitable material (O). β-Galactosidase activity (●) was assayed by the method of Pardee et al. (13). A unit of enzyme activity is equivalent to 130 mmol of o-nitrophenol. In untreated controls, the enzyme synthesized by 30 min is equivalent to 0.08 optical density (420 nm) units.*

with a lower number of active enzyme units. In comparing the capacity of the crude enzyme extracts from streptomycin-treated and control cells to effect immunological precipitation with a constant volume of anti-β-galactosidase serum, a significant shift in the equivalence point was noted when the extract from the drug-treated culture was used (Table 1). Equivalence occurred with approximately 35 enzyme units in the streptomycin-treated extract as compared with 60 units in the control, suggesting that a substantial amount of serologically CRM was present in the former.

When studies on CRM synthesis continued for more than 1 h, a decrease in the amount of CRM per cell was often noted, suggesting possible degradation of the inactive protein. The enhanced susceptibility of abnormal protein to proteolytic attack has been documented by Pine (14), Goldberg (4), and Platt et al. (15). To demine whether the protein synthesized in streptomycin-treated cells was unusually susceptible to proteolysis, studies were performed on the stability of this protein.

Stability of protein synthesized in presence of streptomycin. The results observed in monitoring the degradation of protein synthesized in the presence of streptomycin are illustrated in Fig. 2 and 3. The protein synthesized during

TABLE 1. *Precipitin reaction of β-galactosidase and β-galactosidase cross-reacting material with anti-β-galactosidase serum*[a]

Enzyme units added	Enzyme activity after removal of Ab-Ag Complex	
	−SM	+SM
8	+	+
17	+	+
26	+	+
34	+	−
43	+	+
52	+	+
60	−	+
69	+	+
78	+	+

[a] β-Galactosidase synthesis was induced in an exponentially growing culture. At 1 min after addition of isopropyl-β-D-thiogalactopyranoside (10^{-3} M), streptomycin (100 μg/ml) was added to half of the culture. Incubation of both cultures was continued for 40 min. Cells were collected and disrupted in a French pressure cell. The levels of active β-galactosidase in the extracts from the streptomycin-treated and nontreated cultures were determined by the method of Pardee et al. (13), and the level in the non-antibiotic-treated extract was adjusted with phosphate buffer (0.1 M, pH 7) to that observed in the streptomycin-treated extract to achieve equivalent numbers of active enzyme units in both extracts. β-Galactosidase was immunologically precipitated, and after the antibody-antigen complex was removed the supernatant fractions were assayed for nonprecipitable β-galactosidase activity. +, Enzyme activity; −, no detectable enzyme activity in the supernatant fraction.

either a 20- or 40-min exposure to the antibiotic was degraded appreciably faster than that synthesized in the nontreated controls. One hour after termination of synthesis in the sample exposed to streptomycin for 40 min (Fig. 2), the decrease in acid-precipitable, labeled protein was more than 20% as compared to approximately 2% in the control. The degradation observed in the cultures treated with streptomycin for 40 min was considerably higher than that occurring in the 20-min sample, indicating that production of aberrant protein susceptible to degradation increased with time of exposure to the antibiotic.

Effects of streptomycin on synthesis and stability of a structural protein. To determine whether the production of abnormal protein and the susceptibility of such protein to proteolysis were general phenomena, we studied the effects of streptomycin on the synthesis of the viral coat protein in phage R17-infected mutant cells. Although no infectious phage or phagelike

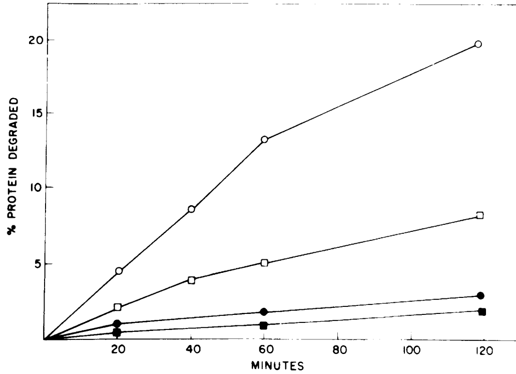

FIG. 2. *Degradation of protein synthesized in the presence and absence of streptomycin. [³H]phenylalanine (2 μCi/ml) was added to exponentially growing cultures in early log phase. Streptomycin (100 μg/ml) was added to half of the culture immediately after addition of labeled amino acid. After 20 or 40 min, chloramphenicol (100 μg/ml) and excess unlabeled phenylalanine were added to the cultures to stop further synthesis. Counts in the acid-precipitable material were determined and considered to represent zero degradation. At intervals, the percentage of counts precipitable with trichloroacetic acid were determined. Open symbols, Protein synthesized in the presence of streptomycin: 20 min (□), 40 min (○). Closed symbols, Protein synthesized in the absence of the drug: 20 min (■); 40 min (●). Appearance of counts in acid-soluble form parallelled loss of counts from precipitates.*

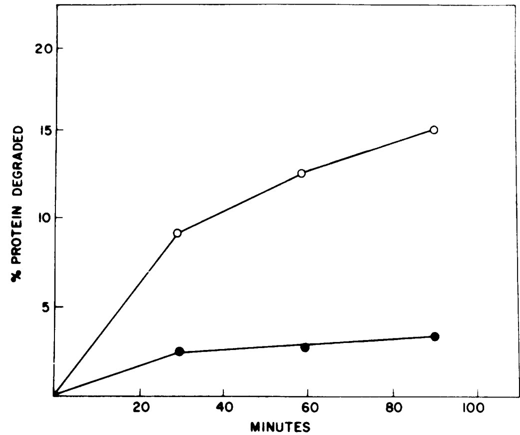

FIG. 3. *Degradation of protein synthesized in phage-infected cells in the presence and absence of streptomycin. An exponentially growing culture was infected with phage R17 (10 phage/bacterium). After 15 min streptomycin (100 μg/ml) was added to half. Thirty minutes after infection, [¹⁴C]arginine (0.2 μCi/ml) was added to each culture and the cultures were incubated an additional 15 min. Cells were collected, washed, and suspended in medium containing chloramphenicol (200 μg/ml) and unlabeled arginine, and samples were taken to determine the radioactivity in the acid-soluble and acid-insoluble fractions at zero time. Similar samples were taken at 30-min intervals. Data are expressed as the percentage of radioactivity lost from acid-precipitable fraction with reference to time. Symbols: ○, streptomycin; ●, no streptomycin.*

particles are produced when the infected cells are exposed to streptomycin (drug added at least 10 min after infection to allow for penetration of phage ribonucleic acid and the synthesis of phage replicase), protein that can be precipitated with serum made against the phage is produced. In the absence of an assay for the functionality of this protein, it is not possible to tell whether the protein made is identical to normal coat protein or is CRM. The protein produced is highly susceptible to degradation. Figure 3 shows the loss of acid-precipitable material synthesized in the presence of streptomycin in infected cells. At the time of incorporation of the labeled amino acid, more than 50% of the protein being synthesized in the control culture was coat protein.

Analysis of the tryptic peptides of coat protein synthesized in the presence of streptomycin and precipitated with antiphage serum (M. O. Pinkett, Ph.D. thesis, Temple University, Philadelphia, Pa., 1972) showed that no peptide was missing, but small amounts of material were detected as new peaks in the chromatogram of peptides isolated from streptomycin-treated cultures. This suggested that at least some of the protein in the immune precipitate was aberrant. It is very possible that the more grossly aberrant protein is most susceptible to proteolysis and is not precipitated with the antiphage serum.

Addition of phenylmethane sulfonyl fluoride (400 to 500 μg/ml), an inhibitor of serine endopeptidases, had no effect on protein degradation in either the uninfected or R17-infected cells. This protease inhibitor at these concentrations has been reported to prevent the breakdown of protein in cells starved for a carbon or nitrogen source, but not in cells which have accumulated abnormal protein as a result of incorporation of amino acid analogues (16). Apparently the enzyme(s) responsible for the degradation of protein produced in this mutant in the presence of streptomycin is not the same as that active in catabolism of protein in nutrient-deprived cells.

DISCUSSION

Several lines of evidence have demonstrated that *E. coli* proteins which are distinctly aberrant are highly prone to catabolism (4, 5, 14,

15). Pine (14) and Goldberg (4) have shown the vulnerability to proteolytic attack of proteins synthesized in the presence of amino acid analogues including canavanine, thienylalanine, fluorophenylalanine, and norleucine. Both investigators have shown that labeled amino acid incorporated in the presence of these agents was exceptionally sensitive to release into the acid-soluble form. Experiments of Goldschmidt (5), in which potentiated catabolism of a fragment of β-galactosidase was demonstrated, and Platt (15), illustrating the increased susceptibility of an abnormal *lac* repressor to proteolysis, further indicate a mechanism in *E. coli* for the selective degradation of atypical proteins.

Our evidence indicates that a substantial portion of the protein synthesized in the presence of streptomycin in a mutant in which the lethal effect has been delayed is highly susceptible to degradation. By analogy with the forementioned studies, this protein undoubtedly is abnormal. Although the nature of the aberrancy cannot be determined directly from these data it seems likely that the degree of error ranges considerably. Proteins with few changes in amino acid sequence may be assayed as active enzyme. Recent findings of Gorini and associates (1) demonstrate low levels of β-galactosidase activity in *lac* frameshift mutants, suggesting that proteins with some level of inaccurate translation can be active. Substantial cross-reacting, but apparently enzymatically inactive, material is also found in these cells. Such material could be either miscoded so as to affect the structure of the active site of the enzyme but still leave antigenically reactive sites intact or could, in the case of β-galactosidase, be large fragments resulting from premature chain termination. In the case of the coat protein of R17, it seems unlikely that fragments of this small protein (molecular weight approximately 14,000) would be precipitated with antiphage serum. Thus, the possibility of premature chain termination cannot be ruled out but seems less likely in light of the amount of CRM found particularly with the small structural protein.

It is interesting to note that in the flow of labeled amino acid into protein there was a greater association of newly synthesized peptide with the membrane fraction in streptomycin-treated cells than in the control. This could reflect an effect of streptomycin on the integrity of the membrane or could be related to the recent finding of Prouty and Goldberg (17) that certain proteins destined for degradation are found in aggregate form in a rapidly sedimenting fraction of cell lysates.

It also seems clear that the production of abnormal protein (enzymatically inactive and susceptible to proteolysis) can be separated from the lethal effect of streptomycin by the use of mutants with ribosomal alterations. The bactericidal effect appears linked to an event in the protein synthesis cycle leading to cessation of amino acid incorporation. This event occurs rapidly in wild-type streptomycin-sensitive cells, probably before much (or any) abnormal protein has accumulated, but is delayed in mutants, such as the one in this study, until substantial amounts of abnormal protein has been synthesized. Although the possibility that miscoding is involved in cell death in wild-type cells cannot be ruled out, the studies with mutants indicate that the streptomycin-induced production of abnormal protein does not cause an immediate loss of viability.

All of the mutants, isolated in our laboratory, which show this separation of the miscoding and lethal effects result from alterations in the gene coding for ribosomal protein S4; other changes in S4 are responsible for the *ram* phenotype, resulting in ambiguity in translation (19). Although little is known of the structural relationship of proteins S12 (the product of the *strA* locus) and S4 (the product of the *ram* locus), there is clearly a functional interaction between them, with mutations in the gene for S4 resulting in altered responses to streptomycin. By using various combinations of mutant S12 and S4, it may be possible to separate what appear to be the multiple effects of streptomycin, such as errors in translation, interference with one or more steps in the ribosome cycle leading to cessation of protein synthesis, and effects on cell division.

The possibility of such separable sites was suggested by Gorini (6), who proposed that S4 may be at the site of codon recognition and be involved in misreading and that protein S12 may be at a "control" site. Such mutants might also be used to further investigate the proposal of Modolell and Davis (10) that streptomycin interacts with or affects both the "A" site and the "P" site of bacterial ribosomes.

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