Colicin E3 Induced Cleavage of 16S Ribosomal Ribonucleic Acid; Blocking Effects of Certain Antibiotics†

Albert E. Dahlberg,*‡ Elsebet Lund, Niels Ole Kjeldgaard, C. Michael Bowman, and Masayasu Nomura

ABSTRACT: Certain antibiotics have been found which block the colicin E3 mediated cleavage of 16S rRNA both *in vivo* and *in vitro*. This block, in the case of streptomycin, occurs with ribosomes from streptomycin-sensitive cells but not streptomycin-resistant cells. It is suggested that E3 requires a specific ribosome structure in order to function, and that this structure is altered by these antibiotics.

The primary action of colicin E3 on sensitive *Escherichia coli* cells is to inhibit protein synthesis (Nomura, 1963; Nomura and Maeda, 1965) by inactivating 30S ribosomal subunits (Konisky and Nomura, 1967). This inactivation involves a direct and specific cleavage of about 50 nucleotides ("E3 fragment") from the 3′ end of the 16S rRNA (Bowman *et al.*, 1971a; Senior and Holland, 1971). The demonstration of this reaction *in vitro* (Boon, 1971; Bowman *et al.*, 1971b) now permits a more precise investigation of the essential components and their specificity in the reaction. It has been shown that protein-free 16S RNA cannot be cleaved by E3 (Boon, 1971; Bowman *et al.*, 1971b) and that both the 30S and the 50S subunits must be present for the cleavage reaction to occur (Boon, 1972; Bowman, 1972). To examine the specificity of the requirements for the two subunits we attempted to modify the subunits by pretreatment with antibiotics (both 30S and 50S inhibitors) prior to treatment with colicin E3. Here we report that certain antibiotics do "protect" the 30S subunits from E3 cleavage of 16S rRNA both *in vitro* and *in vivo*.

Experimental Procedures

Escherichia coli K12 strains, W3110 and NO49 (a spontaneous streptomycin-resistant (Str-R) mutant derived from W3110), were used in these experiments as the colicin-sensitive cells. Colicin E3 was prepared from *E. coli* strain CA38 (Col E3,I) according to the method of Herschman and Helinski (1967) ("purified E3") or the method described by Konisky and Nomura (1967) ("crude E3").

In Vitro Methods. ^{32}P-Labeled ribosomes were obtained from both W3110 and NO49 strains as previously described (Bowman *et al.*, 1971b). The crude 70S ribosomes were salt-washed by pelleting them through 1 M NH_4Cl in TMAI (10^{-2} M Tris (pH 7.8)–10^{-2} M $MgCl_2$–3×10^{-2} M NH_4Cl–6×10^{-3} M 2-mercaptoethanol). The E3-induced *in vitro* cleavage assay using purified E3 was previously described (Bowman *et al.*, 1971b). Reaction mixtures contained about 0.3 A_{260} unit of salt-washed ^{32}P-labeled 70S ribosomes containing 2×10^6 cpm, antibiotics (when indicated) and 1.5 μg of colicin E3 (when used) in 10 μl of TMAI. After incubation for 30 min at 37°, samples were mixed with sodium dodecyl sulfate (final concentration 0.2%) and EDTA (final concentration 2 mM), incubated at 37° for 15 min, and applied to an 8% polyacrylamide slab gel in Tris–EDTA–borate buffer (pH 8.3) (Peacock and Dingman, 1967). Electrophoresis was carried out at 200 V for 4 hr at 0°. The RNA bands were visualized by autoradiography (Ikemura and Dahlberg, 1972[1]).

In Vivo Methods. E. coli cells were grown at 37° in Tris–minimal salts–glucose medium with 30 μg each of 20 amino acids/ml, plus adenosine and guanosine (25 μg/ml) (Lund and Kjeldgaard, 1972) to a cell concentration of about 3.0×10^7/ml. Aliquots of 25 ml were treated with antibiotics for 15 min (unless otherwise indicated) prior to the addition of colicin E3 (crude E3 preparations) for 10 min (multiplicity about 30–50 killing units/cell, see Konisky and Nomura (1967)). The reaction was stopped by cooling the cells rapidly on crushed ice with NaN_3 (10 mM) prior to lysis according to the method of Godson and Sinsheimer (1967). Membrane fragments were removed by centrifugation (6000*g* for 5 min at 4°). E3 cleavage of 16S rRNA was determined by gel electrophoresis of RNA extracted from the cell lysates. The extraction of RNA was done according to the method described previously (Dahlberg and Peacock, 1971). Samples of 5 μg were electrophoresed into 3.0% polyacrylamide–0.5% agarose slab gels in Tris–EDTA–borate buffer (pH 8.3) at 0° and 200 V for 6 hr as described previously (Dahlberg and Peacock, 1971). The gels were stained with Stains-All (Dahlberg *et al.*, 1969).

Tetracycline was purchased from Calbiochem; erythromycin was purchased from Sigma; gentamicin and kasugamycin were gifts from Dr. J. Davies; streptomycin was purchased from Pfizer.

Results and Discussion

In Vitro Effect of Streptomycin on E3 Cleavage of 16S rRNA. Since colicin E3 (Boon, 1971, 1972; Bowman *et al.*, 1971b; Bowman, 1972) and streptomycin (Cox *et al.*, 1964; Davies, 1964; Ozaki *et al.*, 1969) interact directly with *E.*

† From the Department of Molecular Biology, University of Aarhus, Aarhus, Denmark (A. E. D., E. L., and N. O. K.), and from the Institute of Enzyme Research and Departments of Genetics and Biochemistry, University of Wisconsin, Madison, Wisconsin (C. M. B. and M. N.). *Received October 20, 1972.* This work was supported in part by the College of Agriculture and Life Sciences, University of Wisconsin, and by grants from the National Institute of General Medical Sciences (GM-15422), the National Science Foundation (GB-31086X), and the Danish Science Research Council. This is paper no. 1583 from the Laboratory of Genetics, University of Wisconsin.

‡ Recipient of a Postdoctoral Fellowship from the European Molecular Biology Organization. Present address: Division of Biological and Medical Sciences, Brown University, Providence, R. I.

[1] T. Ikemura and J. E. Dahlberg (1972), submitted for publication.

TABLE I: Effect of Streptomycin on E3-Induced Cleavage of Str-S and Str-R Ribosomes *in Vitro:* Summary of Experimental Results.[a]

	Gel Number							
	1	2	3	4	5	6	7	8
Ribosomes Str-R	+	+	+	+				
Ribosomes Str-S					+	+	+	+
Streptomycin	−	−	+	+	−	−	+	+
Colicin E3	−	+	−	+	−	+	−	+
Production of E3 fragment	−	+	−	+	−	+	−	−

[a] This is a summary of the experiment described in Figure 1.

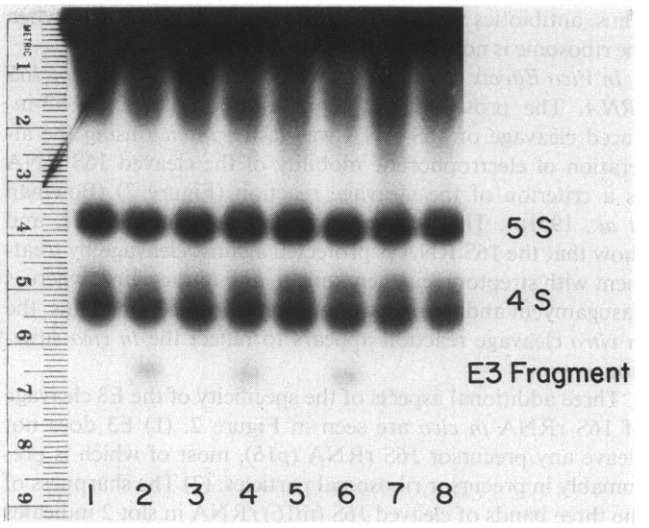

FIGURE 1: Effect of streptomycin on E3-induced cleavage of Str-S and Str-R ribosomes *in vitro*. Outline of the experiment is shown in Table I. Samples 1–4 contain ^{32}P-labeled 70S ribosomes from Str-R strain (NO49); samples 5–8 contain ^{32}P-labeled 70S ribosomes from Str-S strain (W3110). Streptomycin (35 μg/ml) was added to samples 3, 4, 7, and 8 just prior to the addition of 1.5 μg of colicin E3 to samples 2, 4, 6, and 8. The samples were then incubated and electrophoresed as described in Experimental Procedures. The figure is a photograph of the autoradiogram of the gel.

coli ribosomes, we treated 70S ribosomes first with streptomycin and then with colicin E3 to determine whether streptomycin might alter the ribosomal sensitivity to E3. As indicated in Figure 1 and Table I, there is no production of the E3-fragment from streptomycin-sensitive (Str-S) ribosomes in the presence of 6×10^{-5} M streptomycin (sample 8 compared to sample 6). Thus, streptomycin does "protect" 70S ribosomes of Str-S cells from E3-induced cleavage. However, the antibiotic shows no "protection" of ribosomes from Str-R cells (sample 4 compared to sample 2 in Figure 1 and Table I). It is clear that streptomycin, which binds specifically to ribosomes from Str-S cells (Kaji and Tanaka, 1968; Ozaki *et al.*, 1969) and alters normal ribosome function (for a review, see Gorini and Davies, 1968, and Pestka, 1971), somehow affects the ribosomes so as to "protect" them from E3-induced cleavage. The fact that streptomycin does not protect ribosomes of Str-R cells from the E3-induced cleavage is consistent with its lack of effect on ribosome function. Thus, the protection produced by the antibiotic is the consequence of its specific interaction with the ribosome.

Effects of Other Antibiotics on E3-induced Cleavage of 16S rRNA in Vitro. Other antibiotics were tested for their ability to protect ribosomes from E3-induced RNA cleavage *in vitro*. The concentrations of the antibiotics used were sufficient to cause a near-maximum inhibition in cell-free protein synthesizing systems (Pestka, 1971). Table II summarizes the results. In addition to streptomycin, tetracycline and gentamicin also protect the ribosome from E3-induced cleavage, while erythromycin and kasugamycin do not.

The three antibiotics which protect against E3 probably do not share a common binding site on the ribosome (for example, see Pestka's review, 1971). It thus seems unlikely, although not completely excluded, that a simple steric hindrance mechanism prevents the physical contact of the E3 molecule with the ribosome. Instead, it appears more likely that the binding of these antibiotics to 30S subunits causes some conformational alteration in the 30S subunits, rendering them insensitive to the E3-induced cleavage reaction. Under *in vitro* conditions no protein synthesis can occur since no mRNA, tRNA or enzyme factors are added to the system.

TABLE II: Summary of Antibiotic Effects on E3-Induced Cleavage in Strain W3110.

Antibiotic	Concn (μg ml)		Protection	
	In Vitro	*In Vivo*	*In Vitro*	*In Vivo*
Streptomycin	35	200	+	+
Gentamicin	10	100	+	+
Tetracycline	200	200	+	+
Erythromycin	3.5	200	−	−
Kasugamycin	100	200	−	−

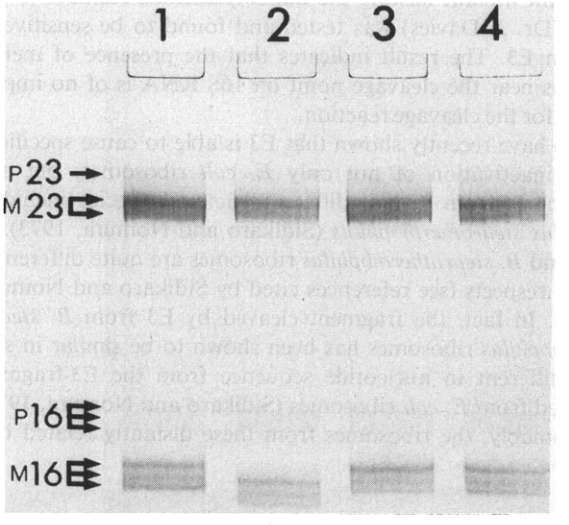

FIGURE 2: Effect of tetracycline on E3-induced cleavage of rRNA *in vivo*. Aliquots of a W3110 culture were treated with tetracycline and/or colicin E3 as indicated below. RNA was extracted and electrophoresed as described in Experimental Procedures. Slot 1, control (untreated); slot 2, colicin E3 for 10 min; slot 3, tetracycline (200 μg/ml of cells) for 15 min; slot 4, tetracycline for 5 min, then colicin E3 for 10 min. P16 and P23 = precursor 16S and 23S rRNAs, respectively. M16 and M23 = mature 16S and 23S rRNAs, respectively. The figure is a photograph of the stained gel.

Thus, antibiotics which protect the ribosome do so even when the ribosome is nonfunctioning.

In Vivo Effects of Antibiotics on E3-induced Cleavage of 16S rRNA. The protective action of antibiotics against E3-induced cleavage of 16S RNA was tested *in vivo* using the alteration of electrophoretic mobility of the cleaved 16S RNA as a criterion of the cleavage reaction (Figure 2) (Bowman *et al.*, 1971a). The results are summarized in Table II, and show that the 16S RNA is protected against cleavage by treatment with streptomycin, gentamicin, and tetracycline, whereas kasugamycin and erythromycin gave no protection. Thus, the *in vitro* cleavage reaction appears to reflect the *in vivo* situation.

Three additional aspects of the specificity of the E3 cleavage of 16S rRNA *in vivo* are seen in Figure 2. (1) E3 does not cleave any precursor 16S rRNA (p16), most of which is presumably in precursor ribosomal particles. (2) The sharpness of the three bands of cleaved 16S (m16) rRNA in slot 2 indicates that the cleavage site on the RNA is quite specific. The retention of the triplet band pattern suggests that the 50 nucleotides cleaved from the 3′ end of the 16S rRNA probably contribute little to the secondary structure of the molecule. The relationship between the triplet band pattern and the secondary structure of the 16S RNA has been described previously (Dahlberg and Peacock, 1971). (3) E3 does not cause any change in electrophoretic mobility of 23S rRNA. This is consistent with the known fact that E3 inactivates 30S subunits, but not 50S subunits (Konisky and Nomura, 1967).

While all of the drugs tested which protect ribosomes are classified as inhibitors of the 30S subunit (Pestka, 1971), the 30S inhibitor kasugamycin did not protect the ribosomes from E3-induced cleavage. This result is of particular interest since kasugamycin resistance is due to the lack of methylation of adenine residues located at the 3′ end of the 16S rRNA (Helser *et al.*, 1971, 1972), the region of the rRNA cleaved by E3. This suggests that kasugamycin may interact with a ribosome site which is close to the cleavage point. The present results show that such an interaction does not prevent the E3-induced cleavage reaction. Furthermore, a kasugamycin-resistant mutant lacking the dimethyladenine residues (a gift from Dr. J. Davies) was tested and found to be sensitive to colicin E3. The result indicates that the presence of methyl groups near the cleavage point on 16S RNA is of no importance for the cleavage reaction.

We have recently shown that E3 is able to cause specific *in vitro* inactivation of not only *E. coli* ribosomes, but also ribosomes from several different bacterial species including *Bacillus stearothermophilus* (Sidikaro and Nomura, 1973). *E. coli* and *B. stearothermophilus* ribosomes are quite different in many respects (see references cited by Sidikaro and Nomura, 1973). In fact, the fragment cleaved by E3 from *B. stearothermophilus* ribosomes has been shown to be similar in size, but different in nucleotide sequence from the E3-fragment cleaved from *E. coli* ribosomes (Sidikaro and Nomura, 1973). Presumably, the ribosomes from these distantly related bacterial species share a common structural feature required for susceptibility to E3-induced cleavage. It is probable that such a ribosomal structure needed for the E3 action is altered by certain antibiotics and that this is the basis of the protection of 16S RNA by these antibiotics against the E3 cleavage as reported in the present paper.

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