COLICIN E3 INHIBITS RABBIT GLOBIN SYNTHESIS

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1. Introduction

Colicin E3 is a bacteriocin which inhibits protein synthesis by cleaving about 50 nucleotides from the 3′-terminus of 16 S rRNA [1,2]. The role of 16 S rRNA in protein synthesis has been studied by using colicin E3 or cloacin DF13 [3–10]. If colicin E3 similarly inactivates mammalian ribosomes, colicin E3 becomes a good tool to study the structure and function of mammalian ribosomes. In vitro inactivation of mammalian ribosomes by colicin E3 was first reported [11], and this may be the only work that observed the effect of colicin E3 on mammalian ribosomes. The rabbit reticulocyte lysate system has been widely used to study protein synthesis, and much information has accumulated. Therefore, we studied the effect of colicin E3 on our lysate system.

This paper reports inhibition of rabbit globin synthesis in the rabbit reticulocyte lysate system. Globin synthesis exponentially decreased with the time of preincubation of the lysate with colicin E3. Both α- and β-globin synthesis were inhibited similarly. By preincubation of the lysate with colicin E3, the formation of the 80 S initiation complex was inhibited, but not that of the 40 S initiation complex.

2. Materials and methods

2.1. *Materials*

Rabbit reticulocyte lysate was prepared as in [12]. The ribosome concentration of the lysate was 26.1 A_{260} units of ribosomes/ml. Highly purified colicin E3 was prepared as in [13] and kindly supplied by Mr Y. Manabe, Dept Agricultural Chemistry, University of Tokyo. L-[U-^{14}C]Leucine (308 mCi/mmol), L-[^{35}S]-methionine (424.81 Ci/mmol) and Aquasol-2 were from New England Nuclear. Ca-Leucovorin for the preparation of f[^{35}S]Met–tRNA$_f^{Met}$ was from Nippon Lederle Co. Sparsomycin (NSC 59729) was kindly supplied by Dr Douros, the Developmental Therapeutics Program, Chemotherapy, National Cancer Institute, USA. L-f[^{35}S]Met–tRNA$_f^{Met}$ was prepared from unfractionated rabbit reticulocyte tRNA [14] as in [15]. *E. coli* aminoacyl tRNA synthetases and transformylase for the preparation of f[^{35}S]Met–tRNA$_f^{Met}$ were the same preparation as in [16]. L-f[^{35}S]Met–tRNA$_f^{Met}$ was identified to be more than 95% pure by the method in [17].

2.2. *Preparation of rabbit globin mRNA*

The 105 000 × *g* pellet of rabbit reticulocyte lysate was obtained as described [14]. The pellet was suspended in 5 mM Tris–HCl, pH 7.4 and 0.5% sodium dodecyl sulfate (SDS), and the polysomal RNA was extracted twice with an equal volume of 90% aqueous phenol. The aqueous phase was collected and 2 vol. ethanol were added. After 12 h at –20°C, precipitates were collected by centrifugation and dissolved in 5 mM Tris–HCl, pH 7.4 and 0.5% SDS.

About 20 A_{260} units of the polysomal RNA were applied on 32 ml 5–20% sucrose containing 5 mM Tris–HCl, pH 7.4 and 0.1% SDS in a centrifuge tube. The sucrose gradient was centrifuged at 25 000 rev./min in a Hitachi RPS25 rotor for 20 h at 5°C. After centrifugation, the sucrose gradient was pumped from the bottom through an ISCO ultraviolet monitor UA-4. Fractions containing globin mRNA were collected and recentrifuged through a similar gradient. The RNA thus prepared efficiently synthesized α- and β-globin chains in the lysate system [18].

2.3. *Amino acid incorporation experiments*

Each 45 μl incubation mixture contained the following: 20 μl lysate, 33.3 mM Tris–HCl, pH 7.4, 77.8 mM KCl, 1.89 mM magnesium acetate, 3 μg creatine kinase, 3.33 mM mercaptoethanol, 0.11 mM each of 19 L-amino acids minus leucine, 33.3 μM hemin, 0.96 mM GTP, 5.56 mM creatine phosphate, 0.4 mM ATP, various concentrations of colicin E3. After given times at 30°C, 5 μl [^{14}C]Leu (0.125 μCi) were added and the mixture was incubated for 30 min at 30°C. Aliquots of 5 μl of the incubation mixture were taken to count ^{14}C-incorporation into hot trichloroacetic acid-insoluble material as in [12]. The rest of the mixture was used to determine ^{14}C-incorporations into α- and β-globin chains [12,19].

2.4. *Effect of colicin E3 on initiation complex formation*

As rabbit reticulocyte Met–tRNA$_f^{Met}$ deacylase [20,21] does not deacylate fMet–tRNA$_f^{Met}$ [20], f[^{35}S]Met–tRNA$_f^{Met}$ was used to study initiation complex formation.

Aliquots of 40 μl lysate were preincubated with colicin E3 at a concentration of 130 μg colicin E3/A_{260} unit of ribosomes in the preincubation mixture. The total volume of the mixture was 90 μl. The mixture contained the components for globin synthesis as above, except that 0.11 mM each of 19 L-amino acids minus methionine were added. After 30 min at 30°C, 5 μl 53 A_{302} units/ml of sparsomycin were added to inhibit the elongation of nascent chains. After 2 min at 30°C, 5 μl f[^{35}S]Met–tRNA$_f^{Met}$ (1.18×10^6 cpm/A_{260}, 1.1×10^6 cpm) were added and incubation pursued for 5 min at 30°C. Reactions were stopped by chilling in ice-cold water and by adding 120 μl buffer A (10 mM Tris–HCl, pH 7.4, 10 mM KCl, 1.5 mM magnesium acetate). The mixture was analyzed by centrifugation through 15–30% sucrose containing buffer A as in [19].

3. Results

3.1. *Effect of colicin E3 on globin synthesis*

The rabbit reticulocyte lysate was incubated with colicin E3 in the presence of 0.125 μCi [^{14}C]Leu at 30°C. Figure 1 shows the effect of time on the incorporation of [^{14}C]Leu into hot trichloroacetic acid-insoluble material. Incorporations of [^{14}C]Leu were lower with colicin E3 than without colicin E3. To see the effect of preincubation of lysate with colicin E3, the lysate was preincubated for different times with various concentrations of colicin E3, then [^{14}C]Leu was added to measure globin synthesis as in section 2. As fig.2a shows, ^{14}C-incorporation decreased with longer times of preincubation with colicin E3 and with higher concentrations of colicin E3. When ^{14}C-incorporations at a concentration of 130 μg/A_{260} unit of ribosomes were plotted against preincubation time (fig.2b), the ^{14}C-incorporation decreased exponentially with the preincubation time. This suggests that a rate-limiting component of globin synthesis was inactivated by colicin E3. Figure 2c shows the effect of colicin E3 on the α/β ratio of globin synthesis. The ratios were almost constant at various concentrations of colicin E3 for a given preincubation time.

To see which component of globin synthesis was impaired by colicin E3, the lysate was preincubated with colicin E3 in the incubation mixture for 30 min at 30°C. The mixture contained the optimum concentration of components for globin synthesis. The poly-

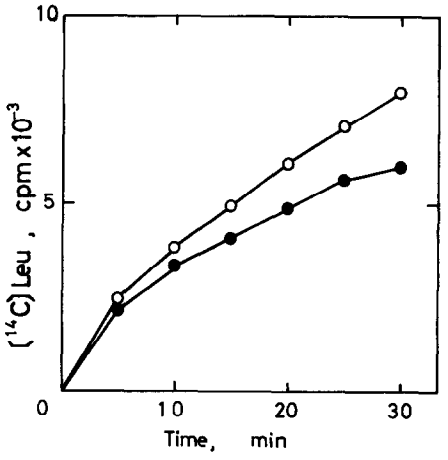

Fig.1. Effect of time on the incorporation of [^{14}C]Leu into hot trichloroacetic acid-insoluble material at a given concentration of colicin E3. Incubation conditions were as in section 2. Incubation mixture was total vol. 50 μl. At given times, 5 μl incubation mixture were taken to count the ^{14}C-incorporation into hot trichloroacetic acid-insoluble material. (o——o) Without added colicin E3; (•——•) with 130 μg colicin E3/A_{260} unit of ribosomes.

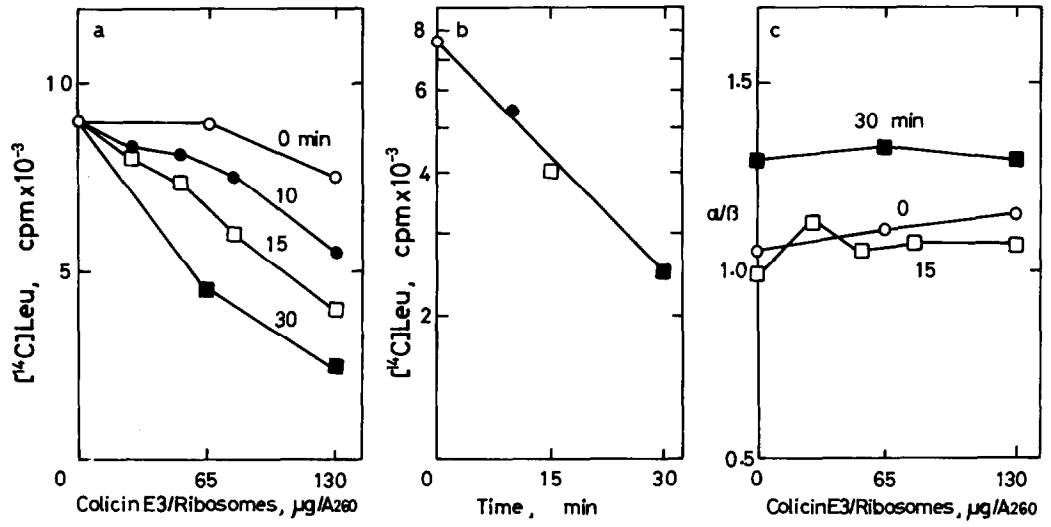

Fig.2. Effect of various concentrations of colicin E3 and of various times of preincubation with colicin E3 on globin synthesis. Aliquots of 20 μl lysate were preincubated with colicin E3 as in section 2. Each incubation mixture contained 0.552 A_{260} units of ribosomes. After 0 min (○), 10 min (●), 15 min (□) and 30 min (■) at 30°C, 5 μl [^{14}C]Leu were added and the mixture incubated for 30 min at 30°C. (a) Aliquots of 5 μl reaction mixture were taken to count the incorporation of [^{14}C]Leu into hot trichloroacetic acid-insoluble material; (c) the rest (45 μl) of the reaction mixture was used to determine the α/β ratio of globin synthesis. (b) The incorporation of [^{14}C]Leu at 130 μg colicin E3/A_{260} unit of ribosomes in fig.2a was plotted against the preincubation time.

Table 1
Action of colicin E3 on rabbit reticulocyte polysomes

	Expt 1 (cpm)	Expt 2 (cpm)
Control polysomes	2740	3231
Colicin E3-treated polysomes	1349	1739
Colicin E3-treated polysomes + globin mRNA	–	1650

Aliquots, 60 μl, of lysate were incubated with colicin E3 at a concentration of 130 μg colicin E3/A_{260} unit of ribosomes for 30 min at 30°C. The incubation mixture was total vol. 150 μl. The incubation mixture contained the optimum amount of each component for globin synthesis. The incubation mixture was centrifuged through 30% sucrose-containing buffer A for 3 h at 50 000 rev./min in a Hitachi RP65T rotor and at 5°C. The polysomes were suspended in 60 μl postribosomal supernatant of the original lysate and the activity for globin synthesis was measured by using 20 μl suspension in total vol. 50 μl incubation mixture. After 30 min at 30°C, 10 μl incubation mixture were taken to count incorporation of [^{14}C]Leu into hot trichloroacetic acid-insoluble material. Control experiments were done without colicin E3 in the preincubation mixture. Globin mRNA added was 0.20 A_{260} units

somes were obtained as in [19] and suspended in the postribosomal supernatant of the original lysate. The lysate containing polysomes was incubated with [^{14}C]Leu in the incubation mixture for 30 min at 30°C. As shown in table 1, low incorporations were

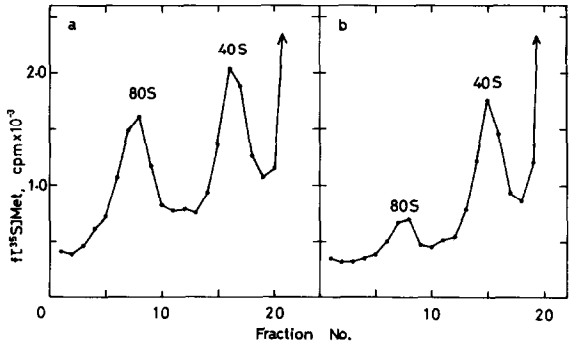

Fig.3. Effect of colicin E3 on initiation complex formation. Aliquots, 40 μl, of lysate were preincubated with (a) 0 μg and (b) 130 μg colicin E3/A_{260} unit of ribosomes. Each preincubation mixture contained 1.04 A_{260} unit of ribosomes. The preincubation mixture was total vol. 90 μl. After 30 min at 30°C, 5 μl sparsomycin, then 5 μl f[^{35}S]Met–tRNA$_f^{Met}$ were added and incubated for 5 min at 30°C. Other experimental conditions were as in section 2.

observed with colicin E3-treated polysomes and the addition of globin mRNA did not increase the ^{14}C-incorporation. These data clearly show that colicin E3 impaired ribosomes, but not the globin mRNA.

3.2. *Effect of colicin E3 on the formation of both a 40 S/fMet–tRNA$_f^{Met}$ complex and an 80 S/fMet–tRNA$_f^{Met}$ complex*

Aliquots of 40 μl lysate were preincubated with colicin E3. Then sparsomycin and f[^{35}S]Met–tRNA$_f^{Met}$ were added as in section 2. As fig.3 shows, the ^{35}S-radioactivities at 80 S ribosome region decreased with the preincubation of lysate with colicin E3. But the ^{35}S-radioactivities in the 40 S ribosome region did not change so much.

4. Discussion

This work has shown that colicin E3 inhibits rabbit globin synthesis in the rabbit reticulocyte lysate system, and that the inhibition is due to the inactivation of ribosomes. The formation of an 80 S/fMet–tRNA$_f^{Met}$ complex was shown to be impaired by the colicin E3 treatment of lysate.

Rabbit reticulocyte lysate required colicin E3 at a concentration of 65 μg colicin E3 per A_{260} unit of ribosomes for the 50% inactivation (fig.2). However, *E. coli* ribosomes are inactivated by colicin E3 at concentrations under 5 μg/A_{260} unit of ribosomes [3,6,22,23]. Therefore, it is worth considering why so much colicin E3 was required to inactivate rabbit reticulocyte ribosomes. Contamination by an RNase in our colicin E3 preparation is not possible, since an RNase, if any, must hydrolyze globin mRNA in the lysate but the addition of globin mRNA did not increase globin synthesis of colicin E3-treated polysomes (table 1). One possibility that would account for the above differences is that rabbit reticulocyte lysate was incubated at 3–30-times lower concentration of ribosomes and at 6–7°C lower temperature than *E. coli* ribosomes [3,6,22,23]. Another possibility is that since ascites ribosomes were inactivated by colicin E3 at a concentration of 30 μg colicin E3/A_{260} unit of ribosomes and at 37°C [11], mammalian ribosomes are more resistant to colicin E3 than bacterial ribosomes.

The α/β ratio of globin synthesis did not change at various concentrations of colicin E3 for a given preincubation time (fig.2c). The formation of an 80 S/fMet–tRNA$_f^{Met}$ complex decreased, but that of a 40 S/fMet–tRNA$_f^{Met}$ complex was not changed much by the colicin E3 treatment (fig.3). Considering the previous works on the initiation of globin synthesis [24–27], these data suggest that colicin E3 inactivated a rate-limiting component after mRNA binding. As colicin E3 inactivated ribosomes (table 1), one possibility that would account for the findings is that the 60 S ribosomal subunit was inactivated by colicin E3. This is the case for ascites ribosomes [11], though an 80 S/Met–tRNA$_f^{Met}$ complex formation was not inhibited by the colicin E3 treatment of ascites ribosomes [11]. Another possibility is that some site(s) of the 40 S ribosomal subunit for the formation of 80 S initiation complex was impaired by colicin E3. Further experiments are required to clarify these possibilities.

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