Note

In Vitro Inactivation of Rabbit Reticulocyte 60S Ribosomal Subunits by Colicin E3

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We have shown that colicin E3 inhibits rabbit globin synthesis in a rabbit reticulocyte cell-free system and suggested that the inhibition is due to the inactivation of either the 60S ribosome subunits or some site(s) of 40S subunits for the formation of 80S initiation complexes.¹⁾ The aim of this work is to find out which of these possibilities is correct.

Rabbit reticulocytes lysate was prepared as described²⁾ and the ribosome concentration of the lysate was 24.5 A_{260} units/ml. Highly purified colicin E3 was prepared by the method described³⁾. L-[Ala-2,3-³H]-Phe (24.0 Ci/mmol) was from New England Nuclear Corp. Poly(U) was from P-L Biochemicals.

The pH 5 enzyme was prepared from rabbit liver by the method of Falvey and Staehelin⁴⁾ with minor modifications. The liver (15 g) was homogenized in 40 ml of ice-cold buffer (0.3 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM mercaptoethanol, 5 mM magnesium acetate). The homogenate was centrifuged at 12,000 rpm for 20 min in a Hitachi rotor RPR20. The supernatant was centrifuged at 60,000 rpm for 1.5 hr in a Hitachi rotor RP65. Approximately two-thirds of the supernatant was then diluted with two volumes of ice-cold distilled water containing 1 mM mercaptoethanol and the pH was adjusted to 5.1 with 1 M acetic acid. The precipitates were collected by centrifugation and dissolved in ice-cold buffer (20 mM Tris-HCl (pH 7.4), 1 mM mercaptoethanol, 5 mM magnesium acetate). Undissolved material was removed by centrifugation and the supernatant was kept in small vials at -70°C .

To prepare control and colicin E3-treated ribosomal subunits, 0.55-ml portions of reticulocyte lysate were preincubated with various concentrations of colicin E3 in 1 ml of a mixture containing 30 mM Tris-HCl (pH 7.4), 70 mM KCl, 40 μM each of 19 L-amino acids minus

methionine, 1 mM mercaptoethanol, 50 μg creatine kinase, 5 mM creatine phosphate, 0.86 mM GTP, 0.36 mM ATP, 2.5 mM magnesium acetate, and 30 μM hemin. After 1 hr at 30°C , the incubation mixture was chilled in ice-cold water and 4 M KCl was slowly added to give the final concentration of 0.35 M. The mixture was put on 30 ml of a convex sucrose gradient ranging from 0.35 to 1.1 M sucrose containing 20 mM Tris-HCl (pH 7.4), 0.35 M KCl, 3.5 mM magnesium acetate, and 5 mM mercaptoethanol. The gradient was centrifuged at 24,000 rpm in a Hitachi rotor RPS25 for 22 hr at 5°C . After centrifugation, the gradient was pumped from the bottom of the centrifuge tube through an ISCO UV monitor, model UA-5. Fractions corresponding to the 40S and 60S peaks were collected according to the absorbance profile at 254 nm. The subunits were collected by centrifugation at 30,000 rpm for 22 hr in a Hitachi rotor RP65 at 5°C . Ribosomal pellets were suspended in ice-cold buffer (20 mM Tris-HCl (pH 7.4), 0.1 M NH_4Cl , 2 mM magnesium acetate, 1 mM mercaptoethanol) and used for the poly(U)-directed poly(Phe) synthesis.

FIG. 1. Sedimentation profiles of control (A, C) and colicin E3-treated lysates (B, D). Portions of 0.55 ml of lysate were preincubated for 1 hr at 30°C with 0 μg (A, C), 62 μg (B), and 130 μg of colicin E3 (D)/ A_{260} unit of ribosomes in a final volume of 1 ml. The incubation mixture was centrifuged through the sucrose gradient and the gradient was fractionated from the bottom of the centrifuge tube. Fractions indicated by a bar were collected and used for the poly(U)-directed poly(Phe) synthesis as shown in Table I. The control incubations for profiles B and D are shown as profiles A and C, respectively. A scale of absorbance at 254 nm per 1 cm path length is also shown (0.2).

TABLE I. ACTIVITY OF HYBRID RIBOSOMES RECONSTITUTED FROM CONTROL AND COLICIN E3-TREATED RIBOSOME SUBUNITS

Ribosome subunits		Poly(Phe) synthesis		
40S	60S	cpm (observed)	cpm (corrected for 40S and 60S alone)	%
<i>Expt. 1</i>				
Control		171		
	Control	90		
Colicin E3		23		
	Colicin E3	105		
Control	Control	1287	1026	100
Colicin E3	Colicin E3	935	807	79
Control	Colicin E3	971	695	68
Colicin E3	Control	1318	1205	117
<i>Expt. 2</i>				
Control		108		
	Control	171		
Colicin E3		186		
	Colicin E3	386		
Control	Control	2265	1986	100
Colicin E3	Colicin E3	1420	848	43
Control	Colicin E3	1165	671	34
Colicin E3	Control	2614	2257	114

Ribosomal subunits used were the preparations obtained by the experiments shown in Fig. 1. Expts. 1 and 2 were done using the ribosomal subunits obtained from the fractions shown in Fig. 1A, B and Fig. 1C, D, respectively. Amounts of ribosomal subunits (A_{260} unit) in each experiments: (*Expt. 1*), control 40S, 0.116; control 60S, 0.309; colicin E3-treated 40S, 0.117; colicin E3-treated 60S, 0.293. (*Expt. 2*), control 40S, 0.168; control 60S, 0.450; colicin E3-treated 40S, 0.173; colicin E3-treated 60S, 0.480.

Poly(U)-directed poly(Phe) synthesis was measured in 100 μ l of incubation mixture containing 40 mM Tris-HCl (pH 7.4), 5 mM magnesium acetate, 0.86 mM GTP, 0.36 mM ATP, 5 μ g creatine kinase, 5 mM creatine phosphate, 10 μ l of pH 5 enzyme, 10 μ l of postribosomal supernatant of rabbit reticulocytes lysate, 0.13 A_{260} units of poly(U), 1.25 μ Ci [3 H]Phe, and various amounts of 40S and 60S ribosomal subunits. After 60 min at 30°C, 50 μ l of the incubation mixture was taken into 1 ml of ice-cold water and 2 ml of 9% trichloroacetic acid were added. After boiling for 20 min, the mixture was kept in ice-cold water for 1 hr, the precipitates were collected, and the radioactivity was measured as described.²⁾

Control and colicin E3-treated ribosome subunits were prepared as described above and poly(U)-directed poly(Phe) synthesis was measured using these subunits. As Table I shows, hybrid 80S ribosomes reconstituted from control 40S and colicin E3-treated 60S ribosome subunits had a lower activity to synthesize poly(Phe). This clearly shows that colicin E3 inactivated the 60S ribosomal subunits of reticulocytes and explains why colicin E3 inhibited the formation of 80S initiation complexes in a reticulocyte cell-free system.¹⁾

The sedimentation profiles of colicin E3-treated lysates

showed relatively high absorbances at fractions of 40S and 60S ribosome regions and at fractions between these subunits as compared with control lysates. Higher amounts of the ribosomal subunits are ascribable to the accumulation of 80S ribosomes, since colicin E3 inhibits the initiation of globin synthesis.¹⁾ From the absorbance profiles in Fig. 1, we roughly estimated the amounts of 40S and 60S ribosome subunits. The ratio of the amounts of 40S to 60S ribosome subunits was found to be decreased by the colicin E3-treatment of lysates. It is therefore conceivable that colicin E3 cleaves 60S ribosome subunits to release fragments and the fragments bind with 40S ribosome subunits to cause higher absorbance at fractions between 40S and 60S ribosome subunits (Fig. 1B, D).

Though Turnowsky *et al.*⁵⁾ reported that the binding of Met-tRNA_f to 80S ribosomes was not inhibited by the colicin E3-treatment of ascites ribosomes, they reported the inactivation of ascites ribosomes with colicin E3 in experiments similar to those in Table I. Since colicin E3 inactivated 60S ribosomal subunits from reticulocytes (Table I) and ascites cells,⁵⁾ it must be general in mammalian ribosomes that colicin E3 inactivates 60S ribosomal subunits.

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