MECHANISM OF PROTEIN SYNTHESIS INHIBITION BY BOTTROMYCIN A_2: STUDIES WITH PUROMYCIN

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Bottromycin A_2 inhibits polylysine synthesis with poly A at low concentrations of *E. coli* ribosomes. Peptide release by puromycin from ribosomes was significantly inhibited by blasticidin S, chloramphenicol and thiophenicol, but not by bottromycin A_2 in the absence of GTP and G factor. Puromycin-dependent release of peptide from ribosomes was stimulated by the presence of GTP and G factor; it was inhibited by bottromycin A_2. The results indicate that bottromycin A_2 inhibits translocation of peptidyl-tRNA on the ribosomes, but does not affect peptide bond synthesis.

Bottromycin A_2, a peptide antibiotic, was demonstrated to inhibit protein synthesis in the *in vivo* and *in vitro* bacterial systems[1,2]. The inhibition was highly dependent on the base composition of messenger polyribonucleotide. The inhibitory activity was observed to be significant with poly C, poly UC, or poly UG, but not with poly U nor with poly A, using a relatively high concentrations of the ribosomes. The antibiotic neither affected synthesis of aminoacyl-sRNA nor formation of aminoacyl-sRNA-messenger-ribosome complex; but it inhibited amino acid transfer from aminoacyl-sRNA to protein on the ribosomes.

The detailed site of action of bottromycin A_2 was further studied using puromycin-dependent release of peptide from the ribosomes in the absence and presence of GTP and G factor[3]. The results are presented in this publication.

Materials and Methods

Bottromycin A_2 was the same preparation as used in the previous studies[1,2]. Poly A was a product of Miles Laboratories Inc., Indiana, U.S.A.; ^{14}C-Lysine (214 mC/mM) and ^{3}H-lysine (141 mC/mM) were obtained from Daiichi Chemicals Co., Tokyo.

Preparations of ribosomes, sRNA, and G factor of *E. coli*:

The ribosomes were isolated from extracts of *E. coli* B cells in the logarithmic phase of growth as described in a previous paper[2]. The ribosomal pellets were washed 5 times in 0.005 M Tris-HCl buffer, pH 7.8, containing 0.5 M NH_4Cl, 0.012 M Mg acetate, and 0.01 M 2-mercaptoethanol. The supernatant fraction was dialyzed against 0.005 M Tris-HCl buffer, pH 7.8, containing 0.1 M NH_4Cl, 0.012 M Mg acetate, and 0.01 M 2-mercaptoethanol.

E. coli B sRNA was prepared from fully grown cells by the method of VON EHRENSTEIN and LIPMANN[5]. G factor of *E. coli* B was prepared, following the method of NISHIZUKA and LIPMANN[3].

Preparations of polylysine-labeled ribosomes:

^{14}C- or ^{3}H-Polylysine-charged ribosomes were prepared by incubating ^{14}C- or ^{3}H-lysine and ribosomes with poly A for 20 minutes at 35°C. The reaction mixture

contained, in 5 ml, 10 mg ribosomes, 0.75 mg poly A, 5 mg sRNA, 2 mg protein of the supernatant fraction, 2 μC ^{14}C- or ^{3}H-lysine, 10 μmoles ATP, 20 μmoles creatine phosphate, 0.25 mg creatine phosphokinase, 0.2 μmoles GTP, 25 μmoles Tris-HCl, pH 7.4, 60 μmoles Mg acetate, 0.5 mmoles NH_4Cl, and 50 μmoles 2-mercaptoethanol. After incubation the mixture was chilled and centrifuged at 105,000$\times g$ for 2 hours. The pellet was then washed 5~6 times in 0.005 M Tris-HCl buffer, containing 0.1 M NH_4Cl, 0.012 M Mg acetate, and 0.01 M 2-mercaptoethanol.

Polylysine synthesis:

Polylysine synthesis with poly A was assayed by measuring the incorporation of radioactive lysine into hot TCA-tungstate-insoluble material as described in previous papers[1,2].

Puromycin-dependent release of polylysine from the ribosomes:

The puromycin effect was examined by a modification of the method of RYCHLIK[6]. After treatment of ^{14}C- or ^{3}H-polylysine-charged ribosomes with puromycin, 0.1 or 0.2 mg of yeast RNA was added to 0.25 or 0.5 ml of the reaction mixture as a carrier for precipitation. After the addition of 9 volumes of cold 5 % TAC, the precipitates were collected by centrifugation at 30,000$\times g$ for 20 min., and dissolved in dilute ammonia, and dried on a planchet. The radioactivity was determined in a windowless gas flow counter. It is a measure of the ^{14}C- or ^{3}H-polylysyl-sRNA retained on the ribosomes because polylysyl-puromycin is soluble in 5 % TCA.

Results

Effects of bottromycin A_2 on polylysine synthesis with poly A in an *E. coli* ribosomal system

Bottromycin A_2 was observed to inhibit polylysine synthesis with poly A in an *E. coli* ribosomal system. The degree of inhibition was markedly influenced by the concentration of ribosomes employed. Using 10^{-4} M of bottromycin A_2, approximately 20 % inhibition was demonstrated at a ribosome concentration of 8.0 mg/ml and

Table 1. Influence of concentration of ribosomes on bottromycin A_2 inhibition of polylysine synthesis directed by poly A in an *E. coli* ribosomal system.

Concentration of ribosomes (mg/ml)	Bottromycin A_2	^{14}C-Lysine incorporated	
		cpm/0.2 mg ribesomes	% inhibition
8.0	—	5,004	
4.0	—	10,570	
2.0	—	14,480	
8.0	10^{-4} M	4,172	17
4.0	10^{-4}	5,570	48
2.0	10^{-4}	5,860	60
8.0	—	4,850	
2.0	—	9,330	
0.5	—	28,650	
8.0	10^{-4} M	3,890	20
2.0	10^{-4}	5,520	42
0.5	10^{-4}	12,500	57

The reaction mixture, in 0.2 ml, contained 0.1 to 1.6 mg ribosomes, 30 μg poly A, 80 μg protein of the supernatant fraction, 0.2 μmoles bottromycin A_2, 0.4 μmoles ATP, 1 μmole creatine phosphate, 10 μg creatine phosphokinase, 1 μmole Tris, pH 7.4, 20 μmoles NH_4Cl, 2.4 μmoles Mg acetate, 2 μmoles 2-mercaptoethanol, and 0.04 μc ^{14}C-lysine. Incubation was carried out at 35°C for 20 minutes.

about 60 % inhibition at 2.0 mg/ml of ribosomes. The degree of inhibition was significantly less than that of polypeptide synthesis with poly C, poly UC or poly UG[1,2]. The results are summarized in Tables 1 and 2. Little inhibition of polylysine synthesis with poly A by bottromycin A_2 was demonstrated in a previous paper because a higher concentration of ribosomes was employed[1].

Table 2. Effect of bottromycin A_2 on poly A-directed polylysine synthesis in an *E. coli* system.

Systems	^{14}C-lysine incorporated	
	cpm/0.2 mg ribosomes	% inhibition
Complete	5,837	
− poly A	327	
− ATP, CP*, CP kinase**	163	
− supernatant fraction	93	
+ Bottromycin A_2 1.25×10^{-4} M	3,368	43
1.25×10^{-5}	4,374	26
1.25×10^{-6}	5,311	9

* CP : Creatine phosphate
** CP kinase : Creatine phosphokinase
The same reaction mixture, containing 0.4 mg ribosomes per 0.2 ml as described in Table 1 was employed.

Influence of bottromycin A_2 on puromycin-dependent release of polylysine from the ribosomes in the absence of GTP and G factor

^{14}C-Lysine was incorporated into peptide on *E. coli* ribosomes with poly A and supernatant enzymes. The ribosomes prelabeled with ^{14}C-polylysyl-sRNA were washed, and the release of polylysine by puromycin was observed by precipitation in 5 % TCA, in which polylysyl-puromycin was soluble. Bottromycin A_2 did not significantly affect the puromycin reaction, whereas chloramphenicol, thiophenicol and blasticidin S markedly inhibited it. The results are presented in Table 3.

Table 3. Effects of bottromycin A_2 and other antibiotics on puromycin-dependent release of ^{14}C-polylysine from *E. coli* ribosomes in the absence of GTP and G factor.

System	^{14}C-Polylysine (cpm)		% inhibition of puromycin reaction
	retained on ribosomes	released from ribosomes	
Control	5,281		
+ puromycin $10^{-4.5}$ M	1,775	3,506	
// + Bottromycin A_2 10^{-4} M	2,117	3,164	10
2×10^{-5}	1,483	3,798	—
4×10^{-6}	1,259	4,022	—
// + Chloramphenicol 10^{-4} M	4,103	1,178	66
2×10^{-5}	3,499	1,782	49
4×10^{-6}	2,875	2,406	31
// + Thiophenicol 10^{-4} M	3,674	1,607	54
10^{-5}	3,043	2,238	36
10^{-6}	2,593	2,688	23
// + Blasticidin S 10^{-4} M	4,153	1,128	69
2×10^{-5}	3,134	2,147	39
4×10^{-6}	1,933	3,348	5

The reaction mixture, in 0.25 ml, contained 125 μg ribosomes charged with ^{14}C-polylysyl-sRNA (5,221 cpm), 12.5 μg poly A, 1.25 μmoles Tris-HCl, pH 7.4, 25 μmoles NH_4Cl, 3 μmoles Mg acetate, 2.5 μmoles 2-mercaptoethanol, and 5 μg puromycin (3.2×10^{-5} M). Puromycin was added at last. The mixture was incubated as 35°C for 10 minutes. The subsequent procedure is described in Materials and Methods.

Effects of GTP and G factor on puromycin-dependent release of polylysine from the ribosomes

The release of ribosome-bound polylysine by puromycin was not significantly affected by separate addition of GTP, G factor, or 105,000×g supernatant fraction. It was stimulated by simultaneous addition of GTP and G factor, or by GTP and supernatant fraction. The results are shown in Table 4.

Table 4. Effects of GTP, G factor, and the supernatant fraction on puromycin-dependent release of ribosome-bound ^{14}C-polylysyl-sRNA.

System	^{14}C-Polylysyl-sRNA (cpm)			
	retained on ribosomes		released by puromycin	puromycin reaction stimulated by GTP, G, and supernatant
	−puromycin	+puromycin		
Control	4,073	1,484	2,589	
+ G factor 5 μg	4,021	1,367	2,654	65
+ Supernatant fr. 25 μg	4,014	1,532	2,482	—
+ GTP 2.5×10^{-2} μmoles	3,975	1,668	2,307	—
+ G factor 5 μg+GTP 2.5×10^{-2} μmoles	4,220	843	3,377	788
+ Supernatant fr. 25 μg protein+GTP 2.5×10^{-2} μmoles	4,233	670	3,563	974

The control reaction mixture contained the same ingredients as those in Table 3. Prior to the treatment with puromycin, the reaction mixture was incubated at 35°C for 10 minutes.

Effects of bottromycin A_2 on puromycin-dependent release of ^{3}H-polylysine from the ribosomes in the presence of GTP and G factor

The *E. coli* ribosomes were prelabeled with ^{3}H-polylysyl-sRNA by the incorporation of ^{3}H-lysine with poly A. ^{3}H-Polylysine was released by $10^{-4.5}$ M puromycin without GTP and G factor. The release of peptide was not significantly affected by bottromycin A_2 at a concentration of 10^{-4} M or $10^{-4.5}$ M.

Table 5. Effect of bottromycin A_2 on puromycin-dependent release of ribosome-bound ^{3}H-polylysine in the presence of GTP and G factor.

System	^{3}H-polylysyl-sRNA (cpm)				
	retained on ribosomes	released by PM	(% inhibition)	PM reaction stimulated by G & GTP	(% inhibition)
Control (−PM)	3,042				
+PM $10^{-4.5}$ M	1,610	1,432			
+ BM 10^{-4} M +PM $10^{-4.5}$ M	1,745	1,297	(9)		
+ BM $10^{-4.5}$ M+PM $10^{-4.5}$ M	1,629	1,413	(1)		
+PM $10^{-4.5}$ M+G+GTP 10^{-4} M	732	2,310		878	
+PM $10^{-4.5}$ M+G	1,614	1,428		—	
+PM $10^{-4.5}$ M+GTP 10^{-4} M	1,360	1,682		250	
+ BM 10^{-4} M +PM $10^{-4.5}$ M+G+GTP 10^{-4} M	1,229	1,813		381	(57)
+ BM $10^{-4.5}$ M+PM $10^{-4.5}$ M+G+GTP 10^{-4} M	959	2,083		651	(24)

BM: Bottromycin A_2 PM: Puromycin G: G factor 10 μg/ml
GTP: Guanosine triphosphate

The control reaction mixture, in 0.5 ml, contained 400 μg ^{3}H-polylysyl-sRNA-charged ribosomes (3,000 cpm), 25 μg poly A, 2.5 μmoles Tris-HCl, pH 7.4, 50 μmoles NH_4Cl, 6 μmoles Mg acetate, and 5 μmoles 2-mercaptoethanol. The procedure was the same as described in Table 4.

More ^{3}H-polylysine was released by puromycin from the ribosomes in the presence of GTP and G factor. The puromycin reaction, stimulated by GTP and G factor, was significantly inhibited by the presence of bottromycin A_2: 57 % inhibition at a concentration of 10^{-4} M of the antibiotic, and 24 % inhibition at a concentration of $10^{-4.5}$ M. As reported in a previous paper[4], the difference in the amounts of polylysine in the presence and absence of GTP and G factor may be due to the translocation of peptidyl-sRNA from the "acceptor site" to the "donor site" on the ribosomes. The results are summarized in Table 5, and indicate that bottromycin inhibits the translocation of peptidyl-sRNA on the ribosomes.

Discussion

The puromycin reaction is regarded as an analogous reaction, by which peptide bonds are formed[7,8,9]. Puromycin releases peptidyl-sRNA bound to the "donor site" of the ribosomes, by forming peptidyl-puromycin, but does not release peptidyl-sRNA bound to the "acceptor site"[9]. The reaction neither requires GTP nor the supernatant factors[4,6,10]. GTP and G factor enhance the puromycin reaction, by stimulating translocation of peptidyl-sRNA from the "acceptor site" to the "donor site" on the ribosomes. The process of translocation of peptidyl-sRNA may be detected, by comparing puromycin reactions in the absence of GTP and G factor with those in the presence of GTP and G factor[4,9,10].

In the present experiments, puromycin-dependent release of polylysine is determined after precipitating the ribosomes in 5 % TCA, thereby taking advantage of the fact that polylysyl-puromycin is soluble in 5 % TCA, whereas lysyl- or polylysyl-sRNA bound to the ribosomes is left insoluble. The method has been originally devised by RYCHLIK, using a crude system[6]. It is successfully applied to a purified system with salt-washed ribosomes and G factor. GTP and G factor are demonstrated to be necessary for translocation of peptidyl-sRNA on the ribosomes in these experiments.

Bottromycin A_2 seems to inhibit translocation of peptidyl-sRNA on the ribosomes, because it is demonstrated in the present experiments to inhibit the puromycin reaction, stimulated by the presence of GTP and G factor. Bottromycin A_2 may not affect peptide bond synthesis because it does not significantly inhibit puromycin reaction in the absence of GTP and G factor. In this sense, the mechanism of protein synthesis inhibition by bottromycin A_2 is related to that of fusidic acid[4]. However, fusidic acid and related antibiotics inhibit ribosome-dependent GTPase activity of G factor, but bottromycin A_2 does not inhibit it (KINOSHITA & TANAKA, unpublished data). The activity of bottromycin A_2 is highly dependent on the base composition of messenger RNA[1,2]. It suggests that bottromycin A_2 may affect a process concerning the movement of messenger RNA on the ribosomes, which occurs simultaneously with the translocation of peptidyl-sRNA on the ribosomes.

It was reported in a previous paper[1] that polylysine synthesis with poly A was not inhibited by bottromycin A_2, but in the present experiments it is inhibited by the antibiotic. The discrepancy is due to much higher concentrations of ribosomes employed in the previous experiments. The activity of bottromycin on the ribosomes is markedly reversible, as shown in earlier dialysis experiments[2]. It suggests that bottromycin A_2 may affect the ribosomes and consequently inhibit translocation of peptidyl-sRNA or movement of messenger RNA on the ribosomes through a certain ribosomal mechanism.

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