MODE OF ACTION OF BOTTROMYCIN A_2: EFFECT ON PEPTIDE BOND FORMATION

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1. Introduction

In [1] we reported that bottromycin A_2 has releasing action of peptidyl- or aminoacyl-tRNA from the acceptor site (A site) of ribosomes, but other reports on the action of bottromycin A_2 on the peptidyl transferase have been conflicting. It has been shown that bottromycin A_2 does not interfere with the formation of polylysyl–puromycin [2], or with the puromycin reaction involving formylmethionyl-tRNA bound at the initiation site of ribosomes [3]. In contrast, it has been shown that *N*-acetyl phenylalanyl puromycin formation catalyzed by the peptidyltransferase was clearly inhibited by bottromycin A_2 [4]. In view of these contradictory reports, we have attempted to relate the inhibitory effect of bottromycin A_2 on peptide bond formation to its action on peptidyl-tRNA at the A site of the ribosomes. It was found that the inhibitory effect of bottromycin A_2 on the puromycin reaction can be understood on the basis of a change in the affinity of puromycin for the A site in the presence of bottromycin A_2. Thus one can propose a hypothesis whereby bottromycin A_2 acts by weakening the affinity of peptidyl or aminoacyl-tRNA and puromycin (analog of aminoacyl-tRNA) for the A site. This action results in the release of peptidyl-tRNA from the A site or in the inhibition of the puromycin reaction.

2. Materials and methods

2.1. *Preparation of cell extracts from* E. coli

Escherichia coli Q13 (middle log) was purchased from General Biochemical Co., ribosomes [5], run-off ribosomes [6], S-150 [6], EF G (elongation factor G) [5] and termination factor R_1 [7] were prepared as described. Bottromycin A_2 was a generous gift from Dr N. Tanaka of Tokyo University in Japan.

2.2. *Assay of polylysyl–puromycin formation*

For the formation of the ribosomal complex with poly[^{14}C]lysyl-tRNA, the mixture (0.48 ml) contained 50 mM Tris–HCl (pH 7.8), 60 mM NH_4Cl, 18 mM Mg-acetate, 6 mM β-mercaptoethanol, 0.2 mM GTP, 1 mM ATP, 7 mM phosphenolpyruvate, 60 μg pyruvate kinase, 105 μg tRNA mixture, 32 μg poly(A), 4 mg ribosomes, 240 μg S-150 and 0.4 μCi [^{14}C]lysine. After incubating for 30 min at 37°C, the complex formed was isolated by sucrose density gradient centrifugation. The specific activity of the complex was 1.2×10^3 cpm/A_{260}.

The mixture (0.2 ml) for the formation of poly-[^{14}C]lysyl–puromycin contained 50 mM Tris–HCl (pH 7.2), 100 mM NH_4Cl, 13 mM Mg-acetate, 1 mM DTT (dithiothreitol), 0.5 mM puromycin and 1.65 A_{260} units of the complex obtained above. Where indicated, 10^{-4} M bottromycin A_2, 0.2 mM GTP and 4.4 μg elongation factor G were added. After incubating for 15 min at 37°C, poly[^{14}C]lysyl–puromycin formed was measured as in [8] by following the decrease of the hot trichloroacetic acid-insoluble radioactivity.

2.3. *Preparation of the various ribosomal complexes with* N-*acetyl-[*^{14}*C]Phe-tRNA*

The ribosomal complex [1] having $tRNA^{Phe}$ and *N*-acetyl-[^{14}C]Phe-tRNA at the donor site (D or P site) and the A site, respectively, was prepared as in [9]. The ribosomal complex [11] having *N*-acetyl-[^{14}C]-Phe-tRNA at the D site was also prepared as in [9].

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2.4. *Assay of the termination reaction*

This was done essentially as in [7]. The reaction mixture (0.14 ml) contained 90 μl ribosomal complex of f-[^{14}C]Met-tRNA and AUG containing 3.2×10^3 cpm of f-[^{14}C]Met-tRNA, 0.2 A_{260} unit of UAG and various volumes of termination factor R_1 (127 μg/ml). Where indicated, 10^{-4} M bottromycin A_2 was added. Incubation was carried out for 15 min at 25°C.

3. Results

3.1. *Inhibitory effect of bottromycin A_2 on peptide bond formation*

The experiments indicated in table 1 show the effect of bottromycin A_2 on puromycin reaction involving ribosome-bound polylysyl-tRNA. In this experiment, the complex of polylysyl-tRNA, poly(A) and ribosomes was prepared in the reaction mixture for poly(A)-dependent polylysine formation. The complex was incubated with puromycin in the presence or absence of bottromycin A_2. As can be seen from the upper half of this table, a significant inhibition of polylysyl–puromycin formation was observed in the presence of bottromycin A_2. Some of the polylysyl-tRNA was apparently bound to the A site, because the addition of EFG and GTP increased the formation of polylysyl–puromycin. Even in the presence of EFG and GTP, bottromycin A_2 had a similar strong inhibitory effect. It should be pointed out that these experiments were performed under the identical conditions as the experiment in which no effect was observed [2]. It has been found that peptidyl- or aminoacyl-tRNA bound at the D site in the absence of EFG behaves differently from those peptidyl- or aminoacyl-tRNAs bound through the action of EFG [9]. It was therefore of interest to establish that bottromycin A_2 can exert its effect on peptidyltransferase activity with other aminoacyl- or peptidyl-tRNA which have been placed at D site under physiological conditions through the action of EFG. In the experiment indicated in table 2, the complex of *N*-acetyl-[^{14}C]Phe-tRNA placed at the D site was prepared with EFG. Thus, the A site bound *N*-acetyl-[^{14}C]-Phe-tRNA was treated with EFG and GTP so that is was physiologically placed at the D site. With this complex, the puromycin reaction with the ribosomal bound *N*-acetyl-[^{14}C]Phe-tRNA was studied in the presence and absence of bottromycin A_2. It is clear from this table that the strong inhibitory effect of bottromycin A_2 was observed with this complex also, indicating that bottromycin A_2 can exert its inhibitory effect on peptidyltransferase activity with puromycin as an acceptor substrate. These results are consistent with [4] and establish that peptidyltransferase activity involving *N*-acetyl-[^{14}C]Phe-tRNA and puromycin is indeed inhibited by bottromycin A_2.

It has been reported that bottromycin A_2 could not exert its inhibitory effect on the puromycin reaction with the nascent polypeptidyl-tRNA on polysomes isolated from growing *E. coli* [10]. It was therefore possible that inability of bottromycin A_2 to inhibit the puromycin reaction with polysomes may be due to the possible presence of a factor(s) in these polysomes which has not gone through an extensive

Table 1
Inhibitory effect of bottromycin A_2 on polylysyl–puromycin formation

Bottromycin A_2 (10^{-4} M)	EFG	Polylysyl–puromycin formed (cpm)
–	–	998
+	–	308
–	+	1206
+	+	326

The experimental conditions were described in the text

Table 2
Inhibitory effect of bottromycin A_2 on puromycin reaction with the substrate formed by the action of EFG

Bottromycin A_2 (10^{-4} M)	*N*-Acetyl-[^{14}C]Phenylalanyl-puromycin formed (cpm)	
	5 min	10 min
–	614	782
+	180	195

The mixture (A) (0.6 ml) for the translocation reaction contained 50 mM Tris–HCl (pH 7.2), 50 mM NH_4Cl, 13 mM Mg-acetate, 1 mM DTT, 4.65 A_{260} units of the ribosomal complex [1] containing 1.22×10^4 cpm of *N*-acetyl-[^{14}C]-Phe-tRNA, 0.2 mM GTP and 17 μg EFG. It was incubated for 30 min at 37°C to complete the translocation reaction. The mixture (0.2 ml) for the puromycin reaction contained 0.5 mM puromycin and 175 μl of the incubated mixture. Where indicated, 10^{-4} M bottromycin A_2 was added. After incubating at 37°C, 80 μl reaction mixture were mixed with 0.8 ml 10 mM Tris–HCl (pH 7.8) and 2.5 ml ethylacetate, and after shaking vigorously, 2 ml ethylacetate layer were counted

Table 3
Inhibitory effect of bottromycin A_2 on the puromycin reaction with the run-off ribosomes

Bottromycin A_2 (10^{-4} M)	*N*-Acetyl-[^{14}C]Phenylalanyl–puromycin formed (cpm)
–	352
+	67

The mixture (0.2 ml) for the puromycin reaction contained 50 mM Tris–HCl (pH 7.2) 50 mM NH_4Cl, 6 mM Mg-acetate, 1 mM DTT, 0.5 mM puromycin and 1 A_{260} unit of the ribosomal complex [11] containing 700 cpm of *N*-acetyl-[^{14}C]-Phe-tRNA. Where indicated, bottromycin A_2 was added to 10^{-4} M. After incubating for 10 min at 37°C, the *N*-acetyl-[^{14}C]Phenylalanyl–puromycin formed was measured with the ethylacetate extraction technique

Table 4
Effect of increased amounts of puromycin on the inhibitory effect of bottromycin A_2 on peptidyltransferase activity

Bottromycin A_2 (10^{-4} M)	Preincubation	Puromycin (M)	*N*-Acetyl-[^{14}C]-phenylalanyl–puromycin formed (cpm)
–	–	5×10^{-4}	344
+	–	5×10^{-4}	86
–	–	3×10^{-3}	320
+	–	3×10^{-3}	240
–	+	5×10^{-4}	342
+	+	5×10^{-4}	8
–	+	3×10^{-3}	360
+	+	3×10^{-3}	44

The mixture (0.2 ml) for the puromycin reaction was essentially the same as that of table 3 except that it contained 1.1 A_{260} unit of the ribosomal complex [11] containing 400 cpm *N*-acetyl-[^{14}C]Phe-tRNA. Where indicated, the ribosomal complex was preincubated with 10^{-4} M bottromycin A_2 for 2 min at 37°C. After incubating for 10 min at 37°C *N*-acetyl-[^{14}C]phenylalanyl–puromycin formed was measured by the ethylacetate extraction method

washing procedure. It was therefore of interest to see if a similar peptidyltransferase reaction involving puromycin and *N*-acetyl-[^{14}C]Phe-tRNA is inhibited by bottromycin A_2 if one uses unwashed ribosomes isolated from naturally occurring polysomes.

As shown in table 3, bottromycin A_2 can exert its strong inhibitory effect on peptidyltransferase activity with these unwashed ribosomes, suggesting that inability of bottromycin A_2 to inhibit peptidyltransferase activity involving polysomes is not due to the possible factor(s) associated with unwashed ribosomes. The ribosomes used in these experiments are run-off ribosomes obtained from naturally occurring polysomes [14].

3.2. *Effect of various concentrations of puromycin on the inhibitory action of bottromycin A_2 on peptidyltransferase activity*

In the experiment indicated in table 4, various concentrations of puromycin were used in the peptidyltransferase reaction assay involving the D-site-bound *N*-acetyl-[^{14}C]Phe-tRNA. It is clear from this table that if puromycin concentration was increased, the inhibitory effect of bottromycin A_2 was remarkably reduced. However the inhibitory effect was almost complete regardless of the concentration of puromycin if the ribosomal complex was preincubated with bottromycin A_2 prior to the addition of puromycin.

3.3. *Inhibitory effect of bottromycin A_2 on the termination reaction*

It has been proposed that the termination step of polypeptide formation involves peptidyltransferase [12]. One can visualize the termination reaction as the reaction of peptidyl-tRNA with water catalyzed by termination factor R_1, R_2 or R_3. Thus, the role of puromycin is played by water in this case. As shown in table 5, the termination reaction measured by the model complex having f-Met-tRNA at the D site and UAG at the A site was studied in the presence of various concentrations of R_1. As can be seen in table 5, bottromycin A_2 appears to exert a strong inhibitory effect on the termination reaction and this inhibitory

Table 5
Inhibitory effect of bottromycin A_2 on the termination reaction

Bottromycin A_2 (10^{-4}) M	Amounts of R_1 used (μl)	f-[^{14}C]Methionine released (cpm)
–	5	708
+	5	261
–	10	1068
+	10	485
–	20	1402
+	20	616
–	40	1462
+	40	805

The experimental conditions were described in the text

effect cannot be appreciably overcome by the increased concentration of the termination factor, R_1. This suggests that R_1 may bind to a part of the A site which is different from the site wherein puromycin or H_2O interacts.

4. Discussion

The results reported in this letter establish that puromycin reaction can be inhibited by bottromycin A_2. The notion that bottromycin A_2 uncouples the release of tRNA from the movement of peptidyl-tRNA from the acceptor site to the donor site [3] therefore is no longer valid because this notion was based on the premise that bottromycin A_2 does not inhibit the puromycin reaction with polylysyl-tRNA [3]. The notion that EFG can release tRNA from the D site without movement of peptidyl-tRNA from the A site [3] is consequently not valid because this was based on the claim that bottromycin A_2 uncouples the release of tRNA from the movement of peptidyl-tRNA. Our original proposal [13] that release of tRNA from the donor site during the translocation is a result of 'push out' by the movement of peptidyl-tRNA from the acceptor site to the donor site is therefore still valid. EFG does not release tRNA at the donor site if another aminoacyl- or peptidyl-tRNA is not at the A site [13].

Having established that bottromycin A_2 can inhibit the puromycin reaction with peptidyl-tRNA, this action of bottromycin A_2 must somehow be correlated with the other action of this antibiotic, namely, release of peptidyl-tRNA from the ribosomes. We propose the following hypothesis:

> Bottromycin A_2 binds to the ribosome at or near the A site and weakens the affinity of the A site for peptidyl-tRNA, aminoacyl-tRNA and puromycin. The decreased affinity for the puromycin would result in decreased reaction with puromycin as described here. The influence of puromycin concentration on the bottromycin A_2 effect suggests the binding site for this antibiotic is either at or very close to the A site.

Thus, bottromycin represents another antibiotic, like tetracyline [15] which has a relatively specific action on the acceptor site of ribosomes.

The lack of bottromycin A_2 effect on diphenylalanine formation [11] also is consistent with the notion that the primary action of bottromycin is not to inhibit peptidyltransferase as such. If the Phe-tRNAs are situated on the D site and the A site of the ribosome, peptide bond formation can take place in the presence of bottromycin A_2 before Phe-tRNA is released from ribosomes by bottromycin. Thus, di-Phe-tRNA is made and is situated at the A site. This di-Phe-tRNA may in turn be released by bottromycin A_2 from the ribosomes.

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