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Biochemistry

Release of (oligo)peptidyl-tRNA from ribosomes by erythromycin A

(antibiotics/protein synthesis/donor site of ribosomes)

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ABSTRACT **Erythromycin A released peptidyl-tRNA in the *in vitro* polypeptide synthesis system with bacterial components programmed by synthetic polynucleotide. This is consistent with our hypothesis that erythromycin A inhibits translocation by preventing proper situation of oligopeptidyl-tRNA in the donor (D) site on ribosomes.**

Erythromycin A has been shown to inhibit the translocation step (1–3) as well as the peptidyl transferase step (4) of polypeptide formation. In an attempt to correlate these observations, a unitary hypothesis was proposed in which erythromycin inhibits translocation by preventing polypeptidyl-tRNA from situating itself properly at the donor (D or P) site while it would still allow the movement of the polypeptidyl-tRNA to the vicinity of the D-site (1).

One logical extension of the above hypothesis is that peptidyl-tRNA which was hindered by erythromycin from situating itself correctly at the D site might well be unstable and would, therefore, be released from ribosomes. This communication presents evidence that this is indeed the case. Erythromycin A releases oligopeptidyl-tRNA from ribosomes in an *in vitro* protein synthesizing system. This finding correlates with the accumulation of oligopeptide in the presence of erythromycin A (5, 6), as well as instability of ribosomal complexes blocked by erythromycin (20).

MATERIALS AND METHODS

***Escherichia coli* Extract and Other Materials.** *E. coli* Q13 (middle of the logarithmic phase of growth) was purchased from General Biochemicals Co. Preparations of soluble enzymes, S-150 (8), and ribosomes (9) were described previously. Soluble enzymes free of peptidyl-tRNA hydrolase were prepared as described elsewhere‡. The sources of the antibiotics were described previously (1, 8, 10, 11). Details of each experiment can be found in the legends.

RESULTS

Releasing action of erythromycin A on peptidyl-tRNA in an *in vitro* protein synthesizing system with synthetic polynucleotide

In the experiment shown in Table 1, [^{14}C]polyphenylalanine was formed in the presence of poly(U), and the ribosome-bound and unbound [^{14}C]polyphenylalanine were measured in the presence and absence of erythromycin A. It is clear from this table that in the presence of 0.1 mM erythromycin A, the ratio of released polyphenylalanine to that bound to ribosomes increased at least 4-fold, indicating that release of polyphenylalanine takes place in the presence of this antibiotic. In confirmation of previous results (1), erythromycin had only a slight effect on the overall polyphenylalanine synthesis.

Since it is known that oligolysine accumulates in the presence of erythromycin A (5), the possibility that oligolysyl-tRNA is released from ribosomes in the presence of erythromycin A was also examined. In the experiment shown in Table 2, erythromycin A was added to a polylysine synthesis system and the released materials were analyzed by paper chromatography to separate oligolysine from mono- and polylysine (12). It is clear from this table that the amounts of released dilysyl- and trilysyl-tRNA were markedly increased in the presence of erythromycin A, while in the presence of micrococcin, which is known to fix aminoacyl-tRNA to ribosomes (11), no appreciable release of oligolysyl-tRNA was observed.

In order to establish the nature of the material which is released from ribosomes, the radioactive material in the soluble fraction was subjected to sucrose gradient centrifugation. As shown in Fig. 1, the peak of the radioactivity was found at the position where [^{14}C]lysyl-tRNA was sedimented, indicating that the radioactivity released from ribosomes was still bound to tRNA. To further establish the fact that the radioactive material is bound to tRNA, the peak fractions were treated with alkali or RNase A. Almost all radioactivity became acid soluble (data not shown). The analysis of the radioactivity released by the alkaline treatment indicated that 31.4% and 32.2% were tri- and dilysine, respectively.

It has been reported that in the presence of erythromycin A oligolysine rather than oligolysyl-tRNA accumulates (5). In the present polylysine-forming system, we have used a soluble fraction free of peptidyl-tRNA hydrolase (13–17), thereby avoiding the complication of formation of oligolysine. As shown in Fig. 2, the material released by erythromycin in the polylysine synthesis system is susceptible to this enzyme, which was in the crude soluble fraction, supporting further the notion that released oligolysine is covalently linked to tRNA. The data in this figure also show that our partially purified soluble fraction is free of peptidyl-tRNA hydrolase and peptidyl-tRNA remains intact.

The release of formyl-[^{14}C]methionyl-peptidyl-tRNA from ribosomes

If the notion that erythromycin A releases oligopeptidyl-tRNA is correct, one would expect formyl-[^{14}C]methionyl-peptidyl-tRNA to be released in the presence of erythromycin A in the natural polypeptide synthesis system. In the experiment shown in Table 3, a complex of ribosome, formyl-

Abbreviations: D and A, donor and acceptor sites, respectively, on ribosomes.

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‡ K. Ogawa and A. Kaji, in preparation.

Table 1. The release of [^{14}C]polyphenylalanine from the ribosomes in the presence of erythromycin A

Antibiotics	Ribosome-bound poly(Phe) (cpm) (A)	Ribosome-unbound poly(Phe) (cpm) (B)	(B)/(A) x 100
None	33,736	1,157	3.4
Erythromycin A (0.1 mM)	29,384	4,479	15.3

The mixture (0.35 ml) for the formation of polyphenylalanine contained 50 mM Tris·HCl (pH 7.8), 60 mM NH_4Cl, 13 mM Mg acetate, 1 mM dithiothreitol, 0.2 mM GTP, 7 mM phosphoenolpyruvate, 38 μg of pyruvate kinase, 642 μg of ribosomes, 178 μg of S-150, 60 μg of poly(U), and 235 μg of tRNA mixture containing 1.26×10^5 cpm of [^{14}C]Phe-tRNA. Where indicated, erythromycin A (0.1 mM) was added. After incubating for 20 min at 36°, 0.3 ml of the mixture was placed on 4.8 ml of a sucrose gradient (5–20% in 50 mM Tris·HCl (pH 7.4), 50 mM NH_4Cl, 13 mM Mg acetate, and 1 mM dithiothreitol). The tubes were centrifuged for 60 min at 48,000 rpm with a SW 50.1 rotor. The drops were collected from the bottom of the tube and the radioactivity insoluble in hot trichloroacetic acid in each fraction was measured by the filter disc method (24).

[^{14}C]methionyl-tRNA and phage MS2 RNA was prepared and the polymerization of amino acids was allowed to proceed in the presence or absence of erythromycin A. The presence of radioactivity of formyl-[^{14}C]methionyl group (mostly as formyl-[^{14}C]methionyl-peptidyl-tRNA) was assayed on the ribosomes. As shown in this table, the number of formyl-[^{14}C]methionyl groups remaining on the ribosomes decreased more than half in the presence of erythromycin A under the conditions where the incorporation of [^{3}H]valine was almost completely inhibited. It should be pointed out that erythromycin does not release the bound f-Met-tRNA from the ribosomes. On the other hand, under a similar inhibition by micrococcin, the [^{14}C]methionine groups remain bound to the ribosomes. These results are consistent with the notion that erythromycin A releases peptidyl-tRNA from ribosomes.

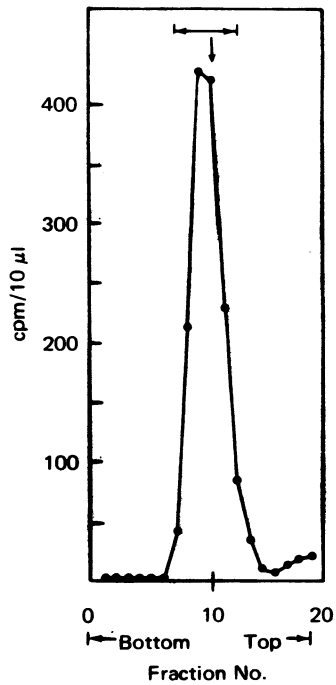

FIG. 1. The sedimentation behavior of the materials released by erythromycin A. The reaction mixture for polylysine synthesis and the preparation of the released oligolysine were described in Table 2. The alcohol-precipitable materials were isolated by adding two volumes of ethanol and the precipitate was dissolved in water. After removing the residual ethanol with ether, the solution (0.19 ml) was placed on 4.8 ml of a sucrose gradient [5–20% in 10 mM Tris·HCl (pH 7.2), 10 mM NH_4Cl, and 13 mM Mg acetate] and the tubes were centrifuged for 17 hr at 45,000 rpm with a SW 50.1 rotor. The drops were collected from the bottom of the tube and the radioactivity (in 10 μl) insoluble in cold trichloroacetic acid was measured by the filter disc method. Peaks of the radioactivity (fractions 7–12) were pooled and were used in the experiment described in Fig. 2. The arrow indicates the position where a known sample of [^{14}C]Lys-tRNA sedimented in a separate gradient.

Table 2. Release of di- and trilysyl-tRNA from the ribosomes by erythromycin A

Antibiotics added	Polylysine formed (cpm)	Released: Dilysyl-tRNA (cpm*)	Released: Trilysyl-tRNA (cpm*)
—	90108	4301	5102
Erythromycin A (10 μM)	22680	18502	28194
Micrococcin (1.4 μM)	23450	0	0

The mixture (0.35 ml) for the formation of polylysine was essentially the same as that for polyphenylalanine formation except that it contained 18 mM Mg acetate, 480 μg of peptidyl-tRNA-hydrolase-free soluble enzymes, 30 μg of poly(A) and 756 μg of tRNA containing 1.3×10^5 cpm of [^{14}C]Lys-tRNA. Where indicated, erythromycin A (10 μM) or micrococcin (1.4 μM) was added. The conditions for incubation and sucrose gradient analysis were essentially the same as that for the poly(U) system. For analysis of the released materials, ribosome-free fractions were pooled and the pooled fraction was treated with 0.3 N KOH for 30 min at 36°. The oligolysine thus released from tRNA was analyzed by the paper chromotography method (13).

* This represents the amounts obtained from the alkaline-sensitive radioactivity.

DISCUSSION

The mode of action of erythromycin A has been a somewhat controversial matter. It was originally proposed that erythromycin A may be a specific inhibitor of translocation *in vivo* (3) as well as *in vitro* (2). On the basis of the observation that the release of tRNA accompanying the translocation process was strongly inhibited by erythromycin A, it was concluded that the translocation step was inhibited *in vitro*. Supporting evidence that erythromycin A may inhibit in a similar fashion *in vivo* has been obtained from the observation that *in vivo* treatment with erythromycin A resulted in the accumulation of peptidyl-tRNA at the acceptor (A) site (3). On the other hand, erythromycin A has been shown to be an effective inhibitor of peptide bond formation depending on the type of peptidyl-tRNA. Thus, in the case of diphenylalanyl-tRNA, *N*-acetyldiphenylalanyl-tRNA, prolyl-glycyl-tRNA, phenylalanyl-leucyl-tRNA, and phenylalanyl-glycyl-tRNA the puromycin reaction with these oligopeptidyl-tRNAs is clearly inhibited (4). Since the inhibition of peptidyl-puromycin formation occurred under conditions not requiring an enzymatic translocation step (18), this suggested that erythromycin is a peptidyl transferase inhibitor.

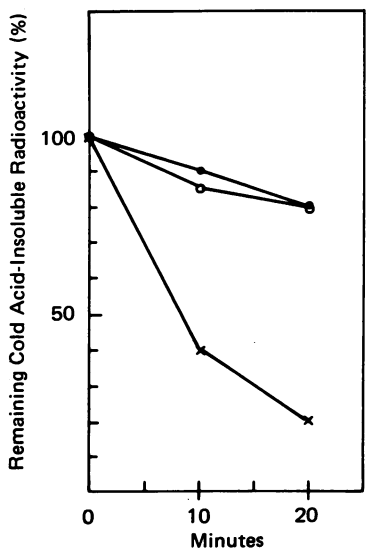

FIG. 2. The sensitivity of the materials released by the action of erythromycin A in the polylysine synthesis system to peptidyl-tRNA hydrolase. For the hydrolysis of the released materials by peptidyl-tRNA hydrolase, the mixture (0.35 ml) contained 50 mM Tris·HCl (pH 7.8), 1 mM dithiothreitol, 60 mM NH_4Cl, 18 mM Mg acetate, and 2800 cpm of the pooled fractions described in Fig. 1. Where indicated, S-150 or peptidyl-tRNA-hydrolase-free soluble enzymes were added. The incubation was carried out at 36° and 100 μl of the mixture were taken at the indicated times. The radioactivity insoluble in cold trichloroacetic acid was measured by the Millipore filter method. (●), no addition; (○), 480 μg of peptidyl-tRNA-hydrolase-free soluble enzymes; and (×), 510 μg of S-150. The values are expressed as the time course for the disappearance of cold trichloroacetic-acid-insoluble radioactivity.

In an attempt to unify these contradictory observations, a hypothesis was proposed which suggested that erythromycin A allows translocation of peptidyl-tRNA to a point very close to the D site, but not to the extent that this peptidyl-tRNA will push out the unesterified tRNA which is occupying the D site (donor site, or often called P site). This hypothesis was supported by the observation that (*a*) evacuation of the A site during the translocation reaction was not inhibited by erythromycin A, (*b*) depending on the type of peptidyl-tRNA, the inhibitory effect of erythromycin on the peptide bond formation with puromycin varied, indicating that erythromycin A is not a universal inhibitor of peptide bond formation, and (*c*) the release of tRNA which accompanies translocation was inhibited by erythromycin A (1). This hypothesis, however, did not explain the observation that while polylysine formation was markedly inhibited in the presence of erythromycin A, oligolysine accumulates. To accommodate this observation, we extended our previous hypothesis by postulating that the peptidyl-tRNA which was moved into the vicinity of the D site but not to the D site itself is unstable, so that it will often be released from ribosomes. As presented in this communication, in the system with a synthetic polynucleotide such as poly(A) peptidyl-tRNA is released in the presence of erythromycin A.

It should be pointed out that erythromycin A did *not* release peptidyl-RNA in the absence of translocation (unpublished observation). We can, therefore, summarize the action of erythomycin A as follows: erythromycin A inhibits translocation in such a manner that peptidyl-tRNA cannot completely situate itself at the D site. When the peptidyl group of that particular tRNA does not have a strong affinity for the D site or peptidyltransferase, eventual release from ribosome will often occur. This can explain the accumulation of oligolysine in the presence of erythromycin A. Thus, in the presence of erythromycin A, di- or trilysyl-tRNA can be formed. When this is translocated, it will not be situated exactly at the D site, by virtue of erythromycin A action. The oligolysyl-tRNA is then released from the ribosomes and ordinarily it would then be subjected to peptidyl-tRNA hydrolase. In our system we have removed this enzyme, and thus were able to observe the release of peptidyl-tRNA as such and not the release of the peptide group. The recent observation (19) that erythromycin increased the amount of peptidyl-tRNA *in vivo* in a mutant with a temperature-sensitive hydrolase correlates very well with the present hypothesis. The efficiency of inhibition and release by erythromycin is dependent on the nature of peptide group. Thus, erythromycin inhibits polylysine synthesis more than polyphenylalanine synthesis (1).

The observation that oligopeptidyl-tRNA is released from ribosomes is consistent with the recent report (20) that erythromycin blocks the ribosomes shortly after initiation and the blocked complexes are unstable. It appears that erythromycin cannot exert its action on the ribosomes with long polypeptidyl-tRNA because of its inability to bind to such ribosomes (20–23), while single free ribosomes or initiating ribosomes are susceptible. The unstable nature of the ribosome–peptidyl-tRNA complex with erythromycin is reminiscent of

Table 3. Release of initiator methionine during protein synthesis directed by RNA of phage MS2 in the presence of erythromycin A

Antibiotic added	[^{3}H]Valine incorporated (pmol)	[^{14}C]Methionine remaining on ribosomes (pmol)
—	2.00	0.52
Erythromycin A (0.1 mM)	0.18	0.20
Micrococcin (2 μM)	0.15	0.44

The mixture (0.5 ml) for the formation of MS2 RNA, f[^{14}C]Met-tRNA, and ribosomal complex contained 50 mM Tris·HCl (pH 7.2), 100 mM NH_4Cl, 10 mM Mg acetate, 1 mM dithiothreitol, 0.2 mM GTP, 800 μg of crude initiation factors, 1 mg of MS2 RNA, 5.6 mg of ribosomes, and 835 μg of tRNA containing 3×10^5 cpm of f[^{14}C]Met-tRNA (200 Ci/mol). After incubating for 20 min at 37°, the mixture was placed on 4.8 ml of a sucrose gradient (5–20% in the same buffer as that for the reaction) and the tubes were centrifuged for 65 min at 48,000 rpm with SW 50.1 rotor. The drops were collected from the bottom of the tube and the initiation complex was pooled. The mixture (0.15 ml) for the assay of the release of f[^{14}C]Met-peptidyl-tRNA contained 50 mM Tris·HCl (pH 7.8), 1 mM dithiothreitol, 60 mM NH_4Cl, 10 mM Mg acetate, 0.2 mM GTP, 7 mM phosphoenolpyruvate, 6 μg of pyruvate kinase, 6.5 nmol each of 19 amino acids, 5 μCi of [^{3}H]valine (specific activity 2.53 Ci/mmol), 1 mM ATP, 128 μg of tRNA mixture, 168 μg of S-150, and 1.7 A_{260} unit of the initiation complex containing 4.47 pmol of formyl-[^{14}C]methionyl-tRNA. Not all of the formyl-[^{14}C]methionyl-tRNA remained on the ribosomes due to handling of the complex. Where indicated, erythromycin or micrococcin was added. After incubating for 15 min at 30°, the mixture was diluted with 3 ml of a buffer containing 50 mM Tris·HCl (pH 7.2), 60 mM NH_4Cl, and 15 mM Mg acetate. The diluted solution was poured through a Millipore filter and the filter was washed with 9 ml of the buffer. The filter was dried and the radioactivity was measured. Under the experimental conditions, oligopeptide and most of the polypeptide formed would pass through the filter while the polypeptidyl-tRNA and aminoacyl-tRNA bound to ribosomes are retained on the filter because the ribosomes are trapped by the filter.

the similar action of streptomycin (7), which has an inhibitory action on translocation (2).

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