COMPARATIVE STUDIES ON THE MECHANISM OF ACTION OF LINCOMYCIN, STREPTOMYCIN, AND ERYTHROMYCIN †

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SUMMARY. Erythromycin inhibits translocation. Lincomycin inhibits peptide bond formation. Streptomycin inhibits translocation as well as binding of aminoacyl-tRNA.

In our previous communication we described a system where one can study release of tRNA (1) and this release was found to be catalyzed by G-factor and GTP (2). Using puromycin reaction with the bound phe-tRNA one can study peptide bond formation per se without involving translocation (3). Having these systems available, we can now study various antibiotics to pinpoint the locus of their action in protein biosynthesis. It was found that LM*, like CM, inhibits peptide bond formation and EM, like FA, inhibits translocation of tRNA from one ribosomal site to the other.

MATERIALS AND METHODS. E. coli ribosomes, soluble protein (S-150) and other materials were prepared as previously described (4). T and G factors were prepared

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* Following abbreviations were used: SM: Streptomycin; LM: Lincomycin; SPM: Sparsomycin; Em: Erythromycin; FA: Fusidic acid; CM: chloramphenicol; β-ME: β-mercaptoethanol.

as described with some modifications (5). Specific radioactivity of ^{14}C-phenylalanine was 413 μc/μmole and the counting efficiency for ^{14}C radioactivity was 1.0- 1.2x10^6 cpm/μc.

Assay of peptide bond formation step. When phe-tRNA is bound only to site 2 (4), it reacts with puromycin without additional GTP or enzymes. Preferential binding at site 2 was achieved by carrying out the binding reaction at low Mg^{2+} (condition A). Phenylalanyl puromycin formation was assayed as described (6).

Combined assay of translocation and peptide bond formation. When both sites (1 and 2) were occupied by phe-tRNA, the maximum amount of puromycin derivative was formed in the presence of GTP and G-factor (7). This is because the diphenylalanyl tRNA made is located at site 1, and GTP plus G-factor translocates this diphenylalanyl-tRNA from site 1 to site 2 making it available to puromycin. Thus, the combined assay of translocation and peptide bond formation was performed by measuring formation of the puromycin derivative from the complex of the ribosomes in which both sites were occupied by phe-tRNA (condition B).

Other methods. The binding and release of tRNA from ribosomes was carried out as described previously (1).

RESULTS. Effect of various antibiotics on polyphenylalanine formation, specific binding of phe-tRNA, peptide bond formation, and translocation. Table 1 summarizes the effect of various antibiotics on steps in protein biosynthesis. It should be noted that EM had no effect on peptide bond formation (puromycin reaction under condition A), while under condition B where one measures the results of translocation and peptide bond formation, a large inhibition of formation of puromycin derivative was observed. This strongly suggests that translocation was inhibited by EM. In confirmation of this concept, release of tRNA was inhibited by this antibiotic. On the other hand, LM inhibited the puromycin reaction of the site 2-bound phe-tRNA. This shows that LM inhibits peptide bond formation per se. In accordance with this result it also inhibited the puromycin reaction under

Table 1. Effect of antibiotics on each step of polypeptide synthesis

Addition (mM)	Polyphenylalanine Formation		Phe-tRNA Binding		Puromycin Reaction		$tRNA^{Phe}$ release
	40 mM NH_4Cl	160 MM NH_4Cl	40 mM NH_4Cl	160 mM NH_4Cl	Condition A	Condition B	
	(cpm)		(cpm)		(cpm)		
None	1,085	1,636	3,453	3,528	613	1,092	2,420
SM (0.5)	69	375	1,960	2,284	503	653	1,676
LM (0.5)	886	1,292	2,854	2,914	32	356	2,302
SPM (0.01)	72	112	3,137	3,522	31	124	2,134
EM (0.5)	223	186	3,316	3,278	611	360	0

To prepare substrate for the puromycin reaction, the mixture (0.5 ml) for the binding of phe-tRNA contained 40 μmoles Tris-HCl (pH 7.1), 2.5 μmoles (Condition A) or 6.5 μmoles (Condition B) of Mg-acetate, 20 μg of NH_4Cl, 400 μg of poly U, mixture of tRNA containing 250,000 cpm of ^{14}C-phe tRNA, and 3,600 μg (Condition A) or 1,800 μg (Condition B) of ribosomes. The mixture was incubated at 22° C for 20 min and was layered on top of 4.5 ml of linear sucrose gradient (5-20%) in a buffer containing 10 mM Tris-HCl (pH 7.1), 5 mM (Condition A) or 13 mM (Condition B), Mg-acetate, 40 mM NH_4Cl, and 6 mM β-ME. After centrifugation at 380,000 rpm in a Beckman Spinco SW 50 rotor for 70 min, the complex of ^{14}C-phe tRNA, poly U, and ribosomes was isolated by taking appropriate fraction. The reaction mixture (0.25 ml) for the formation of puromycin derivative of phenylalanine and diphenylalanine contained the following in μmoles: 7.1 Tris-HCl (pH 7.1), 3.2 Mg-acetate, 40.0 NH_4Cl, and 0.9 β-ME. In addition, it contained 0.15 ml of the fraction from the sucrose gradient centrifugation containing 240 μg (Condition A) or 120 μg (Condition B) of ribosomes, and 1 mM puromycin. Under condition B 18 μg of G-factor and 0.12 μmole of GTP were added. Incubation was carried out at 22° C for 60 min. A 0.2 ml aliquot was taken, and mixed with 1.0 ml of 10 mM Tris-HCl (pH 7.8). Puromycin derivative was extracted into two 3 ml portions of ethylacetate and 5 ml of the extract were counted. If all the bound phe-tRNA had reacted with puromycin, one would expect 823 cpm and 3226 cpm in the ethylacetate fraction under conditions (A) and (B) respectively. For release of tRNA from 70 S ribosomes, the complex of $tRNA^{Phe}$, poly U and ribosomes was prepared as described previously, and the reaction mixture (0.7 ml) for release of $tRNA^{Phe}$ from ribosomes contained the following in μmoles: 98 Tris-HCl (pH 7.8), 46 KCl, 14 Mg-acetate, 4.9 β-mercaptoethanol, and 0.2 GTP. In addition it contained 24 μg of G-factor, and 139 μg of the complex. Release was performed for 10 min at 30° C. The released $tRNA^{Phe}$ was measured as described previously (1)

The values expressed as ^{14}C-phe tRNA made from the released $tRNA^{Phe}$ per the reaction mixture for release.

condition B. The inhibitory effect of SPM on the $tRNA^{phe}$ release reaction was much less than its inhibition of the puromycin reaction under both conditions A and B. Therefore, the major action of SPM is on peptide bond formation (8) and not on the release of tRNA. It has been shown that SM inhibits specific binding of phe-tRNA to ribosomes (9). Data in Table 1 not only confirms this observation, but also show that SM inhibits the translocation step. This was supported by the strong inhibition by SM of the puromycin reaction under condition B while SM does not appreciably inhibit peptide bond formation because the puromycin reaction under condition A was relatively insensitive to it. The data in the last column indicate also that SM inhibits translocation of tRNA since release of $tRNA^{phe}$ from ribosomes by G-factor and GTP can be regarded as one aspect of this reaction (2). Table 2 also shows the effect of various concentrations of EM on the puromycin reaction under condition B and on the release of $tRNA^{phe}$ from ribosomes. The effect was observed at 1×10^{-6} M EM and inhibition was virtually complete in the presence of 5×10^{-4} M EM. These results show that EM inhibits translocation of tRNA.

Table 2. Effect of Erythromycin on Translocation

Addition	Puromycin Reaction Condition B		$tRNA^{Phe}$ Release	
	cpm	% Inhibition	cpm	%Inhibition
None	798		2,244	
EM (1×10^{-6} M)	490	39	1,610	28
EM (1×10^{-5} M)	299	63	1,198	47
EM (1×10^{-4} M)	184	77	420	81
EM (5×10^{-4} M)	113	86	0	100

Puromycin reaction and $tRNA^{Phe}$ release from ribosomes were carried out as described in Table 1 except for the presence of various concentrations of EM as shown in Table.

DISCUSSION. From the studies with hybrid ribosomes between *E. coli* and *B. stearothermophilus*, it has been reported that LM inhibits binding of tRNA to 50 S subunits (10)

In contrast, Monro and Vazquez (11) concluded that LM may inhibit peptide bond formation per se from the observation that LM, like CM inhibited the formation of N-formylmethionyl puromycin. EM, like SPM, has been reported to be an inhibitor of peptide bond formation (12). In contrast to this view, Cundliffe and McQuillen (13) reported that EM is an inhibitor of translocation.

The availability of the reaction of bound phe-tRNA with puromycin under two different conditions enabled us to determine which of these two opposing views is correct. Puromycin reaction under condition A involves only peptide bond formation while puromycin reaction under condition B involves translocation and peptide bond formation. Studies on the effect of EM and LM on this system revealed that LM is an inhibitor of peptide bond formation and EM is an inhibitor of translocation. These conclusions were supported by studies on the G-factor dependent release of tRNA from ribosomes which can be regarded as a consequence of translocation of tRNA on the ribosomes.

From the fact that only FA but not EM inhibited the GTPase of the G-factor (14), and EM sensitivity was localized in a protein of 50 S subunits (15) it appears that both G-factor and ribosomal protein are involved in the translocation step. SM is known to bind 30 S subunits (16) while EM is bound to 50 S subunits. It appears therefore that these three antibiotics all act on translocation but the mode of action of these antibiotics must be different.

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