THE EFFECT OF ERYTHROMYCIN ON PEPTIDE BOND FORMATION AND THE TERMINATION REACTION

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1. Introduction

The 50 S ribosomal subunit catalyzes the synthesis of the peptide bond (peptidyl transferase reaction) [1] and also participates in the termination reaction of protein synthesis [2, 3]. We have previously shown that a number of inhibitors of the peptidyl transferase activity of the 50 S subunit also inhibit the termination reaction in parallel [2, see also 4], raising the possibility that the same ribosomal enzymatic center catalyzes both reactions. We have now extended this study by examining the effects of the antibiotic erythromycin and wish to report (a) additional similarities between the two ribosomal activities and (b) certain observations on the action of erythromycin.

Erythromycin, an inhibitor of protein synthesis that interacts with the 50 S subunit, does not inhibit the peptidyl transferase reaction and is believed to exert its effect in a different way. It does, however, prevent or reverse the binding and effect of chloramphenicol, an antibiotic which does inhibit the peptidyl transferase reaction [reviewed in 5]. We have found that erythromycin affects the termination reaction in the same way, that is, it does not inhibit the termination reaction but it cancels the inhibition of this reaction by chloramphenicol.

We have also observed that although erythromycin does not inhibit the peptidyl transferase reaction, it does nevertheless affect the reaction, but in the opposite way, causing a marked enhancement of the reaction rate [see also 6]. This effect was seen only under certain conditions.

2. Experimental

2.1. *Materials*

Erythromycin was the gift of Dr. G.B. Whitfield, Jr., The Upjohn Co., Kalamazoo, Mich., and blasticidin S, of Prof. H. Yonehara and Dr. N. Otake, the Institute of Applied Microbiology, The University of Tokyo. Other antibiotics and materials, including 70 S ribosomes, initiation and termination factors, and f(^{14}C)Met-tRNA, were supplied or prepared as described previously [2]. Buffer 5 was 50 mM tris-HCl (pH 7.3), 100 mM NH_4Cl, 5 mM $Mg(OAc)_2$. Buffer 30 was the same, but with 30 mM $Mg(OAc)_2$.

2.2. *Binding of f(^{14}C)Met-tRNA to ribosomes*

f(^{14}C)Met-tRNA (233 mCi/mmole, 430 cpm per pmole) was bound to 70 S ribosomes in buffer 5 for 15 min at 30° in the presence of initiation factors, GTP and AUG [2]. The mixture was chilled to 0° and brought to 30 mM $Mg(OAc)_2$, preserving the original concentrations of tris and NH_4CL (buffer 30). Termination factor was added here when termination was assayed [2]. This is the 'prebound complex'. 30 µl samples corresponding to 20 µl of the original binding mixture were taken to measure binding [2] and identical samples were taken to be assayed at 0°, with or without antibiotics, for peptidyl transferase (assay (*b*), see below) or termination activity.

2.3. *Assays*

Peptidyl transferase activity was assayed in two different ways. (*a*) With the alcohol reaction [7, 8], where the ribosome-catalyzed formation of fMet-

Table 1
Peptidyl transferase reaction (alcohol reaction) in the presence of inhibitors: influence of erythromycin on reaction rate.

Experiment:		1		2		3	
Puromycin concn. (μM):		80		80		8	
Erythromycin concn. (μM):		0	8	0	24	0	24
Inhibitor	Concn. (μM)	Reaction (%)					
None	–	100	93	100	92	100	93
Chloramphenicol	40	–	–	65	88	28	89
	80	62	91	55	89	18	91
	240	37	82	40	90	7	80
Lincomycin	80	44	93	–	–	–	–
	240	22	90	–	–	–	–
Hydroxypuromycin	240	–	–	96	87	69	65
Sparsomycin	8	19	22	–	–	–	–
	80	–	–	8	8	1	1
Blasticidin	24	5	5	–	–	–	–

The reaction mixture of 125 μl contained 25 ρmoles of fMet-tRNA, 175 μg (65 ρmoles) of 70 S ribosomes, 25 μl of methanol, 1 or 10 nmoles of puromycin, and antibiotics as indicated. In experiment 1 erythromycin was added 5 min after the inhibitor and incubation was continued for 20 min before the reaction was started with the addition of puromycin and methanol. In experiments 2 and 3 all other components were mixed and the reaction was started with the addition of ribosomes and fMet-tRNA. Incubation was 15 min 0°. 100% reaction was equivalent to 4.4 (expt. 1), 5.9 (expt. 2) or 5.6 (expt. 3) ρmoles of fMet-puromycin above a background (no puromycin) of 0.05–0.2 ρmoles.

puromycin from fMet-tRNA and puromycin takes place in the presence of alcohol and the absence of messenger RNA. The reaction was stopped with KOH, the mixture was heated 10 min at 40° and neutralized with potassium phosphate (pH 7.2), and fMet-tRNA was extracted with ethyl acetate and counted [8]. (*b*) With prebound substrate, where the prebound complex is reacted with puromycin to form fMet-puromycin in the absence of alcohol [2]. The reaction was stopped, etc., as in (*a*).

The termination reaction was assayed with the prebound complex, fMet being released from ribosome-bound fMet-tRNA in response to termination factor and the termination codon UAG [2, 9]. The reaction was stopped with sodium phosphate (pH 2.0), and the released fMet extracted with ethyl acetate and counted.

All assays were run at 0° and were stopped when half or less of the substrate had reacted, so that the values obtained roughly reflected reaction rate. Details are given in the legends to the tables and in the references cited.

3. Results

Table 1 shows the effect of erythromycin on the action of five known inhibitors [10, 11] of the ribosomal peptidyl transferase as assayed with the alcohol reaction. Erythromycin had no effect on the inhibitory action of hydroxypuromycin, sparsomycin and blasticidin. It reversed the inhibitory action of chloramphenicol and lincomycin. Erythromycin itself did not affect the ribosomal peptidyl transferase activity.

Table 2 shows a similar experiment, except that a different ribosomal activity was assayed, the catalysis of the termination reaction. Of the antibiotics

Table 2
Termination reaction in the presence of inhibitors: influence of erythromycin on reaction rate.

Inhibitor	Concn. (μM)	Erythromycin (μM)	
		0	30
		Reaction (%)	
None	–	100	102
Chloramphenicol	50	38	111
	100	21	87
Hydroxypuromycin	200	36	39
Sparsomycin	5	−3	−6
	10	−4	−5
Tetracycline	50	43	33
	100	13	9

To 30 μl of prebound complex containing 135 μg of 70 S ribosomes (50 ρmoles), 25 ρmoles of fMet-tRNA of which 9.2 ρmoles were ribosome-bound, and 20 μg of termination factor, was added 15 μl of buffer 30 containing antibiotics where indicated. The reaction was started 5 min later with 0.25 A_{260} units of UAG in 5 μl of buffer 30. Incubation was for 20 min at 0°. 100% reaction was equivalent to 4.0 ρmoles of fMet above a background (without UAG) of 1.31 or 1.38 ρmoles without and with erythromycin, respectively.

Table 3
Peptidyl transferase reaction (prebound substrate) in the presence of inhibitors: influence of erythromycin on reaction rate.

Inhibitor	Concn. (μM)	Erythromycin (μM)	
		0	30
		Reaction (%)	
None	–	100	167
Chloramphenicol	10	29	163
	100	10	142
Lincomycin	10	39	172
	30	21	170
Hydroxypuromycin	300	16	37
Sparsomycin	10	2	2
Blasticidin	30	2	2

To 30 μl of prebound complex containing 175 μg of 70 S ribosomes (65 ρmoles) and 21.5 ρmoles of fMet-tRNA, of which 15 ρmoles were ribosome-bound, were added the antibiotics in a total of 15 μl of buffer 30. The inhibitor was added 5 min after the erythromycin. 20 min later the reaction was started with 5 μl of 3×10^{-4} M puromycin in buffer 30 (final concentration, 30 μM). Incubation was for 1 min at 0°. 100% reaction was equivalent to 4.0 ρmoles of fMet-puromycin above a background of 0.05 ρmoles.

tested, all known inhibitors of this reaction [2, 4], tetracycline interacts with the 30 S ribosomal subunit and the others with the 50 S subunit [5]. Erythromycin reversed the inhibitory effect of chloramphenicol but not that of the others (lincomycin was not tested). As with peptidyl transferase, erythromycin alone had no effect on termination.

In the experiment of table 3 the ribosomal activity assayed was again, as in table 1, the peptidyl transferase activity. However, a different assay was employed. In contrast to the alcohol reaction assay of table 1, the substrate, fMet-tRNA, was first bound to the ribosome before it was reacted with puromycin. When carried out in this way, the peptidyl transferase reaction was much faster than under the conditions of the alcohol reaction, and the time allowed for the reaction was accordingly reduced from 15 min to 1 min in order to ensure that the rate of the reaction was still being approximately measured. Under these conditions, in contrast to the previous results, erythromycin was seen to affect the reaction. The effect was a marked increase in rate. The extent of the reaction was not changed; when the incubation time was extended, nearly all of the prebound substrate reacted and the same amount of fMet-puromycin was formed in the presence or absence of erythromycin. When chloramphenicol or lincomycin were present, erythromycin not only abolished their inhibitory action, as before, but raised the rate to the enhanced value seen with erythromycin alone. As before, the inhibition caused by sparsomycin and blasticidin was unaffected. With hydroxypuromycin the results suggest that erythromycin did not reverse the inhibition but stimulated the remaining activity. The enhancement was not due to the changes in Mg^{2+} concentration that took place during the experiments (5 mM in the binding reaction, changed to 30 mM during the addition of erythromycin). The same enhancement was seen when the experiment was performed entirely at 5 mM Mg^{2+} or when the

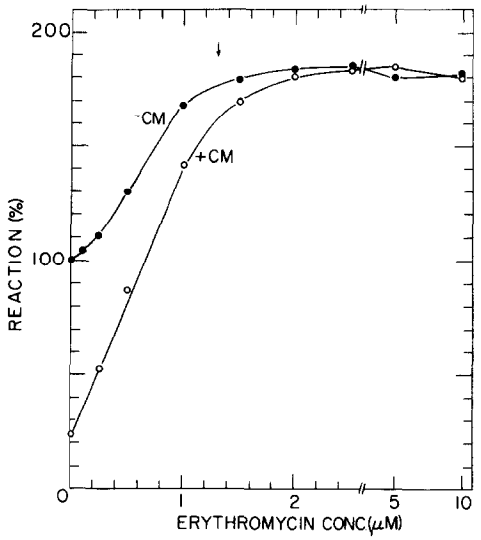

Fig. 1. The effect of erythromycin on the peptidyl transferase reaction (assayed with prebound substrate) in the presence and absence of chloramphenicol. The experiment was carried out as in table 3. The quantities of ribosomes and fMet-tRNA were the same, except that only 12 ρmoles of the latter were ribosome-bound. The concentration of chloramphenicol (CM), where present, was 30 μM. The arrow shows the erythromycin concentration at which the reaction mixture contained 1 molecule of erythromycin per ribosome. 100% reaction (no chloramphenicol or erythromycin) was equivalent to 2.6 ρmoles of fMet-puromycin above a background (no puromycin) of 0.05 ρmoles.

erythromycin was added only after the Mg^{2+} concentration had already been brought to 30 mM.

The enhancing effect of erythromycin was studied in more detail in the experiment shown in fig. 1. The assay of table 3 with prebound fMet-tRNA was used to follow the rate of the peptidyl transferase reaction as a function of erythromycin concentration. With increasing concentrations of erythromycin the reaction was accelerated both in the absence and presence of chloramphenicol, finally reaching the same maximum rate in both cases. Nearly maximal enhancement was attained when the mixture contained 1 molecule of erythromycin per ribosome, and the further addition of erythromycin had little effect. In a parallel experiment (not shown) the binding of ^{14}C-chloramphenicol to ribosomes was tested as a function of erythromycin concentration. Erythromycin reduced the amount of chloramphenicol bound but did not remove it completely, leaving a constant residual level of 50% of the original value, which was reached when about 50 ρmoles of erythromycin had been added to a mixture containing 65 ρmoles of ribosomes.

The stimulation by erythromycin of the peptidyl transferase reaction of prebound fMet-tRNA was also examined at different puromycin concentrations (results not shown). The rate of the reaction rose with rising puromycin concentration in the range studied, 3–100 μM, and erythromycin enhanced the rate at all these puromycin concentrations.

4. Discussion

Erythromycin, an antibiotic which interacts with the 50 S ribosomal subunit [12], inhibits protein synthesis but does not exert its inhibitory effect directly on the peptide-forming step (the peptidyl transferase reaction). It does, however, prevent and reverse the binding and action of two antibiotics which do inhibit this step, chloramphenicol and lincomycin [5, 13]. It has therefore been proposed that chloramphenicol and lincomycin interact with the peptidyl transferase site and that the binding site of erythromycin is not identical but is nearby, perhaps overlapping [14–16]. Our present results confirm that erythromycin reverses the effect of chloramphenicol and lincomycin on the peptidyl transferase reaction. Elsewhere, we report that when the peptidyl transferase activity of the 50 S subunit is inactivated by mild treatment [8, 17] the subunit loses the ability to bind both chloramphenicol and erythromycin [18]. This provides further support to the notion that the sites for peptidyl transferase activity, chloramphenicol binding, and erythromycin binding are interdependent. Either they are identical or overlapping, as proposed, or else they are coupled through the structure of the ribosome in such a way that an event at one site (e.g., inactivation, interaction with an antibiotic) influences other sites even if they are not identical or contiguous.

What our present findings add to the picture is that erythromycin does have a direct effect on the peptidyl transferase reaction. The effect is not inhibitory but the opposite, a marked enhancement of the reaction [see also Cerna et al., 6]. This effect was not seen when the reaction was assayed under

the conditions of the alcohol reaction, where fMet-tRNA, puromycin and ribosomes are mixed without messenger RNA and undergo interaction during the assay. Erythromycin did not affect the rate although it does interact with the ribosome under these conditions, as evidenced by its reversal of the inhibition caused by chloramphenicol and lincomycin. When fMet-tRNA was prebound to the ribosome, as in the second assay, the rate of the peptidyl transferase reaction rose markedly, and erythromycin increased it still more. It would appear that the rate-limiting step in the alcohol reaction assay is the interaction between the ribosome and fMet-tRNA and that this step is not affected by erythromycin. When this step is eliminated by prebinding the fMet-tRNA to the ribosome, the new rate-limiting step is one that is enhanced by erythromycin. This step has not been identified. Among several possibilities are (a) the interaction between the ribosome and the acceptor (puromycin) and (b) the action of the catalytic site.

We have previously shown that the ability of the ribosome to catalyze the termination reaction is affected by a number of antibiotics and by ribosomal inactivation and reactivation in the same way as the ribosomal peptidyl transferase activity, raising the possibility that both reactions share at least part of the same enzymatic apparatus [2]. The present experiments provide additional analogies between the two reactions, namely, that in neither case does erythromycin inhibit the reaction and that in both cases erythromycin reverses the inhibition by chloramphenicol. On the other hand, erythromycin did not stimulate the termination reaction, although it was carried out with prebound substrate under conditions where peptidyl transferase activity was stimulated. However, under these essentially identical conditions the termination reaction is much slower than the peptidyl transferase reaction, probably because it is more complex, requiring the ribosomal binding of a termination codon and a protein termination factor which are not required for the peptidyl transferase reaction. If one of these binding reactions is rate-limiting and is not affected by erythromycin, both of which seem likely, then the stimulating effect of erythromycin might not be expressed.

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