PEPTIDYL TRANSFERASE INHIBITORS ALTER THE COVALENT REACTION OF BrAcPhe-tRNA WITH THE *E. COLI* RIBOSOME

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1. Introduction

BrAcPhe-tRNA [1–4] and other reactive peptidyl-tRNA analogues (5–8) have been used to identify 50 S proteins located in or near the donor and acceptor sites of the peptidyl transferase center of the 50 S *E. coli* ribosome. Many antibiotic inhibitors of peptidyl transferase have been shown to interfere with the ribosome binding of the 3′-end of peptidyl- or aminoacyl-tRNA [9–11]. We have examined the effect of some of these antibiotics on the pattern of reaction of BrAcPhe-tRNA with 50 S proteins. This provides more detailed information about the mechanism of these antibiotics than is available from simple binding measurements. In the absence of antibiotics, P site-bound BrAcPhe-tRNA reacts chiefly with the 50S proteins L2 and L26-27 [2,3]. The peptidyl transferase inhibitors chloramphenicol, sparsomycin, lincomycin, celesticetin, and streptogramin A preferentially inhibit reaction with L2. Sparsomycin and streptogramin A actually enhance the reaction with L26-27. Streptomycin and tetracycline, two antibiotics which are not considered peptidyl transferase inhibitors, do not show differential effects on the reaction with L2 and L26-27.

2. Materials and methods

2.1. *Materials*

E. coli ribosomes and BrAc[^{3}H] Phe-tRNA were prepared as described elsewhere [1,3]. Streptomycin and chloramphenicol were obtained from Sigma Chemical Co. Lincomycin and celesticetin were the generous gift of the Upjohn Co., Kalamazoo, Mich. Sparsomycin was the gift of Drug Research and Development, Chemotherapy, National Cancer Institute. Streptogramin A was purified from crude streptogramin (gift of Merck, Sharpe and Dohme, Rahway, New Jersey) by fractional crystallization from ethyl acetate [12].

2.2. *Ribosome labeling reactions*

Ribosome labeling reaction mixtures contained 400 A_{260} units of ribosomes, 20 A_{260} units of poly U and 1.1 μCi BrAc[^{3}H] Phe-tRNA in 2 ml buffer A (0.01 M Tris–HCl, pH 7.5, 0.02 M NH_4Cl, 0.01 M $MgCl_2$). Where indicated, antibiotics were added to the ribosomes prior to the addition of BrAcPhe-tRNA. The reaction mixtures were incubated at 37°C for 30 min. Following this incubation the labeled ribosomes were dissociated into subunits and the 50 S proteins were isolated and analyzed by gel electrophoresis as described elsewhere [3].

2.3. *Assays*

Binding of BrAc[^{3}H] Phe-tRNA to ribosomes was determined according to the method of Nirenberg and Leder [13] using aliquots from the labeling reaction mixtures containng 2 A_{260} units of ribosomes. Puromycin reaction was determined by a modification of the method of Leder and Burtzyn [14]. 0.05 ml aliquots were taken from the labeling reaction mixture and made 4 mM in puromycin. The samples were then incubated for 5 min at 37°C, and the reaction was stopped by dilution with 1.0 ml cold buffer A. 3.0 ml ethyl acetate were then added. After vigorous shaking, 1 1/2 ml of the ethyl acetate layer was withdrawn and the ^{3}H determined in a dioxane-based scintillation cocktail.

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3. Results

3.1. *Binding of BrAcPhe-tRNA to ribosomes*

Total binding of BrAc[^{3}H] Phe-tRNA to 70 S ribosomes was measured after incubation under conditions where the binding has been shown to be mostly to the P site [3,4]. As shown in table 1, the effect of five peptidyl transferase inhibitors is very small. Sparsomycin produces a slight stimulation of the binding and streptogramin A an even smaller one. Chloramphemicol, lincomycin and celesticetin inhibit the binding by very small, but reproducible amounts. Two other antibiotics, streptomycin and tetracycline produce a larger inhibition of the binding than any of the peptidyl transferase inhibitors studied here. Both of these antibiotics are thought to act chiefly on the 30 S subunit [15].

3.2. *Puromycin reaction of BrAcPhe-tRNA*

In contrast to their small effect on total binding, the five peptidyl transferase inhibitors produced a marked inhibition of the reaction of BrAcPhe-tRNA with puromycin. The puromycin reaction was measured under the same conditions used to measure the binding, by following the formation of BrAcPhe-puromycin. These results are also shown in table 1. In the presence of chloramphenicol, sparsomycin and streptogramin A, the inhibition of the puromycin reaction was nearly complete. Lincomycin and celesticetin were less effective inhibitors of this reaction, giving 68% and 60% inhibition respectively. They gave somewhat better inhibition, however, if the 37°C incubation of the samples prior to addition of the puromycin was omitted (see table 1). Perhaps these two antibiotics can be competed away from the puromycin-reactive site by the CCA end of BrAcPhe-tRNA during the 30 min incubation.

Tetracycline and streptomycin show very little inhibition of the puromycin reaction. This is consistent with the results of other authors [16–18] and with the notion that they act chiefly on the 30S subunit. The small inhibition of the puromycin reaction that is seen in their presence is similar to the amount of inhibition of the binding of BrAcPhe-tRNA.

3.3. *Covalent reaction of BrAcPhe-tRNA with the ribosome*

The effect of peptidyl transferase inhibitors on the covalent reaction of BrAcPhe-tRNA with 50S proteins is shown in fig. 1. This figure shows the results of one-

Table 1
Binding to 70S ribosomes and puromycin reaction of BrAc[^{3}H]Phe-tRNA in the presence of antibiotics

Additions	Binding	Puromycin reaction
After 37°C 30 min incubation		
None	100%	100%
1 mM Chloramphenicol	98%	4%
1 mM Lincomycin	98%	32%
1 mM Celesticetin	98%	40%
0.1 mM Sparsomycin	110%	4%
0.1 mM Streptogramin A	107%	2%
0.1 mM Streptomycin	76%	73%
0.5 mM Tetracycline	87%	82%
No preincubation		
None		100%
1 mM Lincomycin		11%
1 mM Celesticetin		32%

BrAcPhe-tRNA labeling reactions were performed with antibiotics added at the indicated concentrations prior to the addition of BrAcPhe-tRNA.

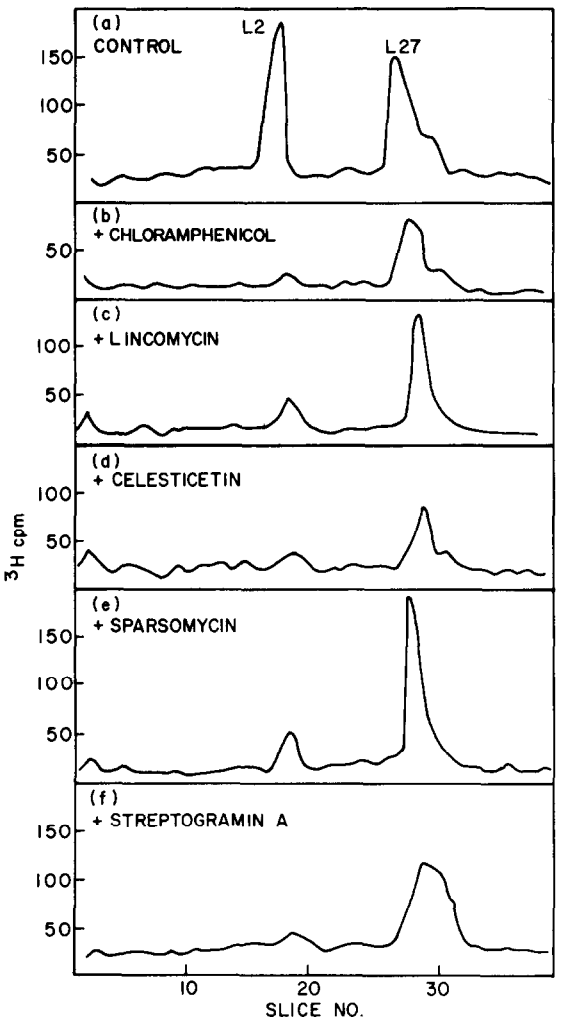

Fig. 1. Effect of Peptidyl Transferase Inhibitors on the 50 S Labeling Pattern of BrAcPhe-tRNA. Labeling reactions and one-dimensional gel analysis of labeled 50 S proteins were performed as described in Materials and methods. Where indicated, antibiotics were added to the labeling mixtures prior to the addition of BrAcPhe-tRNA at the following to the concentrations; 1 mM chloramphenicol; 1 mM lincomycin; 1 mM celesticetin; 0.1 mM sparsomycin; 0.1 mM streptogramin A. The amounts of protein applied to each gel were: a) 0.50 mg b) 0.41 mg c) 0.48 mg d) 0.53 mg e) 0.53 mg f) 0.48 mg.

dimensional acrylamide gel analysis of 50 S proteins isolated from 70 S ribosomes which have been incubated with BrAc[^{3}H] Phe-tRNA. These are two main peaks of radioactivity corresponding to proteins L2 and L26-27. The identification of these two proteins has been confirmed by two-dimensional gel analysis for the control [2,3] and the lincomycin and sparsomycin containing samples [12]. In the absence of antibiotics, the relative extent to which these proteins react with BrAcPhe-tRNA is somewhat variable depending on the preparation of ribosomes used. When parallel labeling reactions are done with ribosomes from the same antibiotic-free preparation, however, the ratio of labeling of the two proteins is constant [4]. Thus all BrAcPhe-tRNA labeling reactions in the presence of antibiotics were performed in parallel with a control sample. All five peptidyl transferase inhibitors dramatically supress the reaction of BrAc Phe-tRNA with L2. No new 50S proteins are labeled. The peaks of radioactivity always correspond to the positions on the gel of L2 and L26-27 as determined by comparison with the control gel. Table 2 shows quantitative results from two different experiments. The [^{3}H] Phe incorporated into L2 and L26-27 was determined as the area under the peaks in the one-dimensional gel analysis normalized by the amount of total 50S protein actually applied to each gel. Chloramphenicol inhibits the labeling of L2 to the greatest extent, but all the antibiotics tested inhibit this labeling by at least 73% in experiment 1 and 48% in experiment 2. Chloramphenicol, lincomycin, and celesticetin also inhibit the labeling of L26-27, but to a lesser extent than that of L2. Sparsomycin and streptogramin A, on the other hand, stimulate the labeling

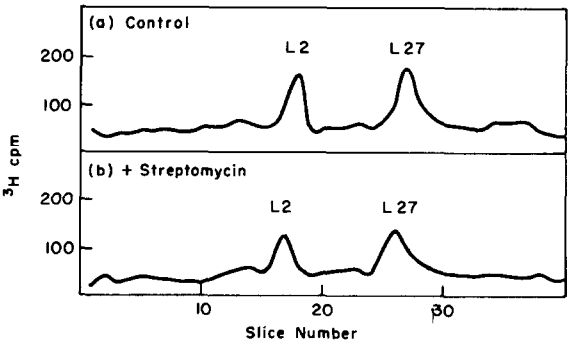

Fig. 2. Effect of Streptomycin on the 50 S Labeling Pattern of BrAcPhe-tRNA. Labeling reaction and one-dimensional gel analysis of labeled 50S proteins were performed as described in Materials and methods. Where indicated 0.1 mM streptomycin was added to the labeling mixture prior to the addition of BrAcPhe-tRNA. The amounts of proteins applied to the gels were: a) 0.42 mg and b) 0.52 mg.

Table 2
Covalent labeling of proteins L2 and L26-27 by BrAc[^{3}H]Phe-tRNA in the presence of antibiotics

	cpm/ .5 mg TP50			
	Experiment 1		Experiment 2	
	L2	L27	L2	L27
None	246	240	123	282
1 mM Chloramphenicol	15	183	34	123
1 mM Lincomycin	63	194	64	182
1 mM Celesticetin	32	109	–	–
0.1 mM Sparsomycin	67	305	61	358
1 mM Streptogramin A	35	363	59	306

70 S ribosomes were labeled in BrAcPhe-tRNA labeling reactions in the presence of antibiotics as indicated. The 50 S proteins were then isolated and analyzed by one-dimensional gel electrophoresis. All the values were then normalized to account for the amount of protein applied to the gels.

of L26-27. Thus the effect of all the peptidyl transferase inhibitors studied, apart from any effect on the total amount of covalent labeling, is to change the ratio of labeling of L2 and L26-27 to favor labeling of L26-27.

In contrast, streptomycin inhibits the total covalent labeling of BrAcPhe-tRNA somewhat but does not change the ratio of labeling of L2 and L26-27. This is shown in fig. 2. The amount of inhibition of covalent labeling is roughly the same as the inhibition of the binding of BrAcPhe-tRNA by streptomycin. Tetracycline shows equivalent effects [3]. Therefore the preferential inhibition of L2 labeling shown by all the antibiotics listed in table 2 seems related to their ability to inhibit the puromycin reaction. There is no quantitative correlation between the ability of an antibiotic to inhibit the puromycin reaction and its inhibition of L2 labeling. This may indicate that these antibiotics inhibit peptide bond formation by somewhat different mechanisms.

4. Discussion

Peptidyl transferase inhibitors produce a dramatic decrease in the covalent labeling of L2 with only a small effect on the total binding of BrAcPhe-tRNA. They appear to interfere specifically with the binding of the CCA end of the affinity label without disrupting other ribosome–tRNA interactions. The inhibition of covalent labeling by streptomycin and tetracycline is probably simply the result of their inhibition of affinity label binding.

Peptidyl transferase inhibitors could alter the covalent BrAcPhe-tRNA reaction by direct steric blocking, or by induced conformational changes. Most evidence indicates that chloramphenicol binds to the A site of the peptidyl transferase center [10,11]. The results presented here, however, indicate that chloramphenicol severely distorts the interaction of peptidyl-tRNA with the P site. This could result from a conformation change in the ribosome produced by A site-bound chloramphenicol. However, direct steric effects are also possible since it has been reported [11] that there are two chloramphenicol binding sites on the ribosome.

Sparsomycin stimulates the enzymatic binding of AcPhe-tRNA approximately 3-fold [19]. Monro et al. [20] observed a sparsomycin-induced stimulation of CACCA-Leu-Ac binding to 50 S subunits. These results were interpreted in terms of a very stable, inert complex between the CCA end of peptidyl-tRNA and the 50 S subunit in the presence of this antiobiotic. Our results show that sparsomycin inhibits the labeling of L2 while stimulating that of L26-27. This suggests that in the sparsomycin-ribosome-BrAcPhe-tRNA complex the CCA end of BrAcPhe-tRNA is shifted toward the reactive group of L26-27 and away from that of L2. It implies that L26-27 is further from the puromycin-reactive binding site than L2. This is consistent

with results reported elsewhere [21] obtained with longer peptidyl-tRNA affinity analogues.

Celma et al. have reported that chloramphenicol and celesticetin slightly stimulate the binding of CACCA–Leu–Ac [9]. Lincomycin which is structurally similar to celesticetin inhibits the fragment binding by 64% [9]. All these antibiotics inhibit the covalent reaction of BrAcPhe-tRNA with both L2 and L26-27. Streptogramin A which inhibits the binding of CACCA-Leu-Ac very effectively [9] (by 92%) inhibits the labeling of L2 but not of L26-27. Sparsomycin stimulates the binding of CACCA–Leu–Ac [9] and also stimulates the reaction of L26-27, but inhibits the labeling of L2.

It is not certain that the binding is the same under our conditions and those of Celma et al. which included 50% ethanol [9]. If it is the same, our results indicate that chloramphenicol, lincomycin and celesticetin are not simply preventing the binding of the 3′ end of BrAcPhe-tRNA. Rather they are perturbing the ribosome-tRNA interaction in such a way as to make the reactive groups of L2 and L26-27 less available for reaction. In contrast, sparsomycin and streptogramin A are producing a pertubation which makes L26-27 more available and L2 less available for reaction with the affinity label. Thus the notion that many of the peptidyl transferase inhibitors act by competing for substrate binding at the donor or acceptor site of peptidyl transferase may be an oversimplified view.

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