EFFECT OF CYCLOHEXIMIDE ON THE REACTION OF PUROMYCIN WITH POLYSOME-BOUND PEPTIDYL-tRNA

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1. Introduction

Using a cell-free protein-synthesizing system prepared from rat liver, we [1, 2] obtained evidence that cycloheximide inhibits translocation of peptidyl-tRNA from the aminoacyl (A) site to the peptidyl (P) site on the ribosome, a reaction that requires transferase II (translocase) and GTP. To confirm this in a simpler system, we have exploited the reaction of ^{3}H-puromycin with peptidyl-tRNA. Formation of peptidyl-puromycin, which is catalyzed by the peptidyl-transferase activity of the ribosome, only occurs when the peptidyl-tRNA is in the P position on the ribosome [3]. Consequently, peptidyl-tRNA present at the A position has first to be translocated to the P position by transferase II before it can react with puromycin. In this way we have demonstrated that cycloheximide and fusidic acid inhibit the reaction of puromycin with peptidyl-tRNA in the A position but not in the P position. This implies that translocation is affected by both of these inhibitors. In contrast, emetine does not inhibit the puromycin reaction with either population of ribosomes. While this work was in progress, McKeehan and Hardesty [4] published results obtained with reticulocyte polysomes that agree with our identification of the site of cycloheximidine inhibition.

2. Methods

Polysomes, prepared from livers of fasting 150 g rats [5], were further purified by centrifugation through a discontinuous sucrose gradient consisting of 4 ml of 0.5 M sucrose and 4 ml of 2 M sucrose, each made up in 50 mM tris-HCl pH 7.6, KCl 25 mM, $MgCl_2$ 5 mM (TKM buffer) containing 0.5 M NH_4Cl. Transferases I and II were resolved from liver cell sap by the method of Gasior and Moldave [6]. The protein content of the polysomes and enzymes was measured by the Lowry procedure [7].

The assay for formation of peptidyl puromycin contained, in a total volume of 0.5 ml of 50 mM tris-HCl buffer pH 7.6, 5 mM $MgCl_2$, 300 mM NH_4Cl, 0.15 to 0.25 mg of purified polysomes and 0.5 mμmoles of ^{3}H-puromycin (3×10^5 cpm) purchased from New England Nuclear Corporation. After incubation, an equal volume of 10% TCA was added and the mixture was heated at 90° for 15 min. The precipitates were collected on glass fiber filters, washed with 5% TCA and then 90% ethanol, and dried. The precipitates with filters were digested in scintillation vials with 0.5 ml Nuclear Chicago Solubiliser at 55° for 30 min, and counted in a toluene-based solution in the Beckman LS-150 scintillation counter.

Incubation mixtures for sedimentation analysis of polysome profiles were first diluted with 0.7 vol. of 0.01 M tris-HCl buffer pH 7.5, then layered over a linear gradient of 10 to 50% sucrose in TKM buffer. The gradient was centrifuged at 38,000 rpm in the SW-50 rotor of Spinco Model L-2 ultracentrifuge for 70 min. The absorption profile at 260 mμ was recorded automatically with a flow cell device in a Gilford model 2000 spectrophotometer. Radioactivity on the gradient was measured on fractions of 12 drops; the protein was precipitated with carrier albumin and the hot trichloroacetic acid insoluble material was collected on a glass fiber filter and counted as described above.

3. Results and discussion

Rat liver polysomes, washed by one passage through a discontinuous 0.5 M and 2 M sucrose gradient containing 0.5 M NH_4Cl, were incubated with ^{3}H-puromycin. The puromycin continued to react with the peptidyl-tRNA on the ribosomes at a diminishing rate for at least 60 min (fig. 1), to give a final uptake of about 40 $\mu\mu$moles per mg of ribosomes. The reaction was only moderately dependent on addition of GTP to the medium. This indicates that the ribosomes still had significant amounts of adhering GTP and transferase II, since Skogerson and Moldave [7] who used rat liver ribosomes repeatedly washed in NH_4Cl found them to be considerably dependent on added GTP and transferase II for reaction with puromycin. This allowed the peptidyl-tRNA to migrate from the aminoacyl position to the peptidyl position on the ribosome where it then reacted with the puromycin.

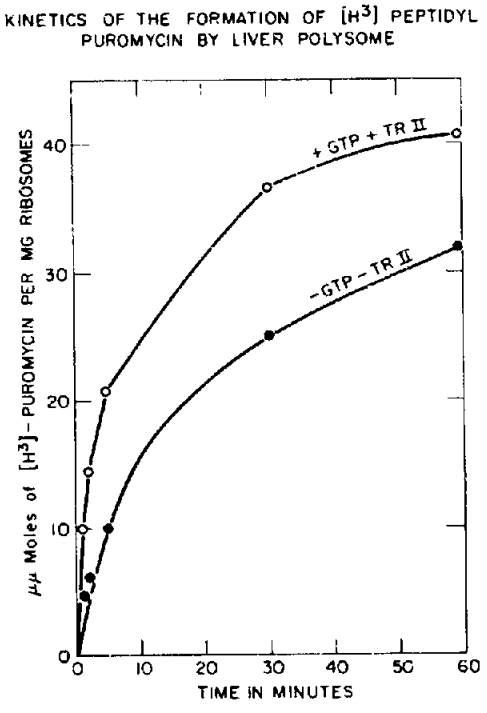

Fig. 1. Rate of synthesis of puromycin peptide by liver polysomes in the presence and absence of transferase II and GTP. The incubation mixture (0.5 ml) contained buffer, 0.6 mg polysomes and 0.5 mμmole ^{3}H-puromycin (3×10^5 cpm) and was incubated at 37° for various times.

When the reaction products were resolved on a linear sucrose gradient (fig. 2), incubation with ^{3}H-puromycin in the absence of antibiotics resulted in release of most of the ^{3}H-puromycin peptide to the top of the gradient. The reaction and particularly the release was inhibited almost completely by sparsomycin, a known antagonist of peptide bond formation by peptidyl transferase [6]. High concentrations of cycloheximide were also inhibitory, whereas

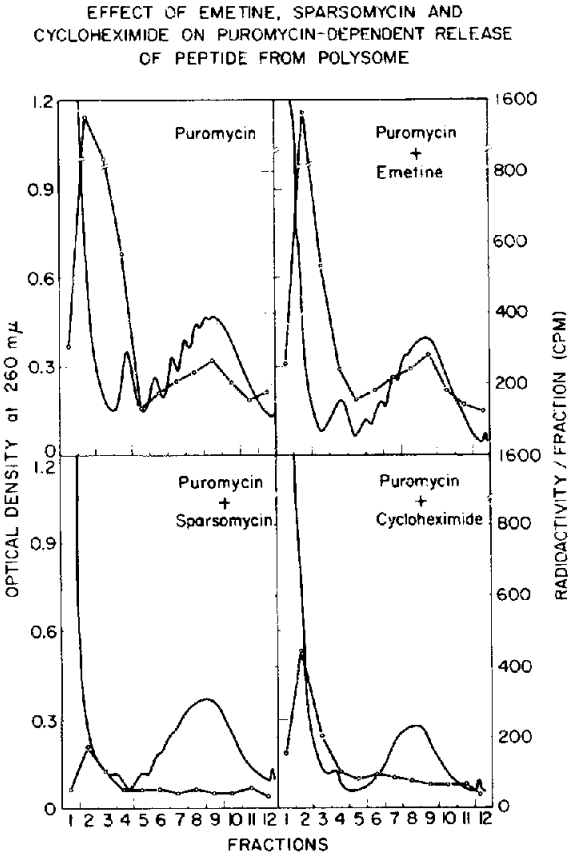

Fig. 2. Effect of cycloheximide, sparsomycin, and emetine on puromycin peptide formation. The incubation mixture (0.5 ml) containing polysomes was preincubated at 37° for 3 min with either sparsomycin (2 μg/ml), cycloheximide (1 mg/ml) or emetine (100 μg/ml). A control tube was also preincubated without any addition. After the preincubation, each tube received 0.5 mμmole of ^{3}H-puromycin (3×10^5 cpm), and was incubated for a further 5 min. The reaction products were subjected to sucrose gradient resolution. Each diagram shows the polysome profile (——) and the amount of radioactivity (o——o) along the gradient.

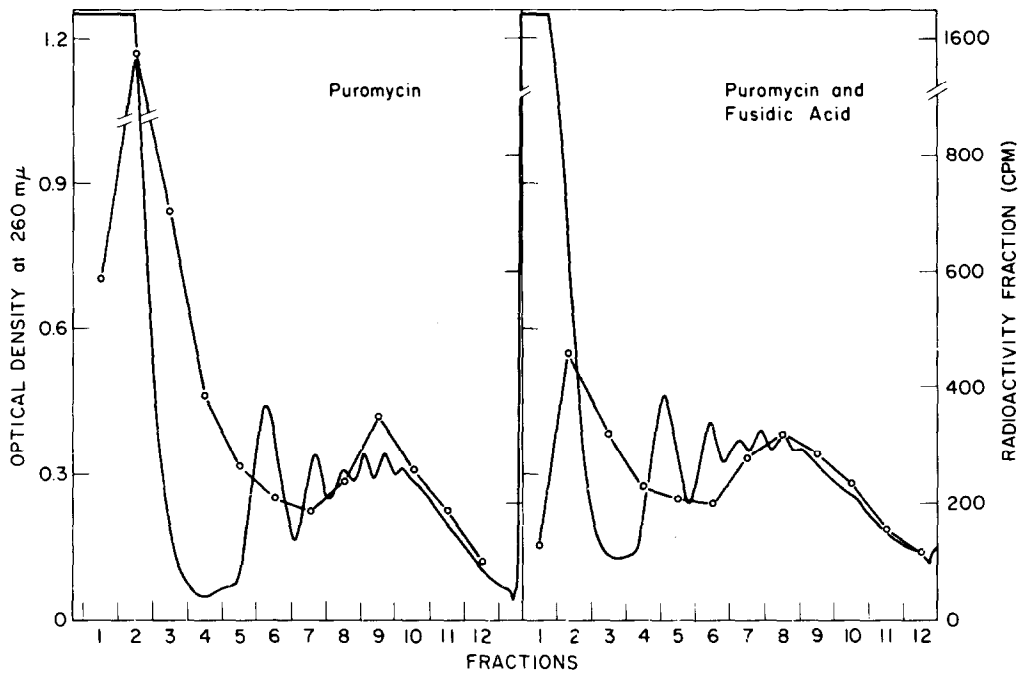

Fig. 3. Effect of fusidic acid (600 μg/ml) on puromycin peptide formation. (See legend to fig. 2 for incubation conditions).

emetine was ineffective. Finally, fusidic acid acted like cycloheximide in being partially inhibitory (fig. 3).

The freshly harvested ribosomes generally have some 20% of the peptidyl-tRNA at the peptidyl position on the ribosomes [7] which can react directly with puromycin. The remaining 80% of ribosomes have the peptidyl-tRNA at the aminoacyl position and require translocation to the peptidyl position before reaction with puromycin. If cycloheximide and fusidic acid act on the translocation step, this would explain why they exert an extensive but not complete inhibitory effect on ^{3}H-puromycin peptide formation (fig. 2). If this is correct, these inhibitors should no longer be effective when all the peptidyl-tRNA is at the peptidyl site. Accordingly, we preincubated the polysomes briefly before applying these antibiotics; as indicated above, the polysomes are contaminated with enough transferase II and GTP to allow translocation from the aminoacyl to the peptidyl site to take place (fig. 1). Addition of cycloheximide or fusidic acid *after* this preliminary incubation failed to inhibit puromycin peptide synthesis, whereas control tubes not preincubated showed inhibition (table 1). In contrast, spar-

Table 1
The effect of preincubation of polysomes on inhibition of puromycin peptide formation by cycloheximide and other related antibiotics.

Antibiotic added (amount/ml)	μμmoles puromycin peptide/mg ribosomes	
	Not preincubated	Preincubated
None	29.0	29.3
Cycloheximide (1 mg)	7.5	27.1
Fusidic acidic (600 μg)	6.7	28.3
Emetine (100 μg)	28.5	28.3
Sparsomycin (2 μg)	3.5	4.2

The reaction mixture contained, in a total volume of 0.5 ml of 50 mM tris-HCl pH 7.6, 10 mM $MgCl_2$, 300 mM NH_4Cl, together with 0.5 mμmoles ^{3}H-puromycin (3×10^5 cpm) and 0.5 mg polysomes. Incubations were carried out at 37° for 60 min. When the reaction was carried out in two steps, the polysomes and buffer were preincubated for 5 min. After the preincubation, additional buffer, ^{3}H-puromycin and the inhibitors were added and incubated for a further 50 min. The experiment was replicated twice.

somycin was inhibitory under both conditions. These results indicate that cycloheximide and fusidic acid inhibit the translocation reaction and not peptide bond formation, which is inhibited only by sparsomycin.

The inhibitory action of cycloheximide in this system can be also prevented by addition of exogenous transferase II and GTP. Thus, in one series of studies under conditions similar to table 1, the synthesis of puromycin peptide in $\mu\mu$moles per tube was 38.0 for the control incubation, 11.6 in presence of cycloheximide (1 mg/ml), 32.8 in presence of cycloheximide and GTP (0.2 mM), and 36.2 in presence of cycloheximide with GTP (0.2 mM) and transferase II fraction (100 μg protein/ml). This is further evidence that cycloheximide interacts with the translocation system.

Acknowledgements

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