Effect of the antibiotic purpuromycin on cell-free protein-synthesizing systems

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Purpuromycin, an antibiotic isolated from the culture broth of *Actinoplanes ianthinogenes*, which is very active against Gram-positive bacteria and fungi, inhibits protein synthesis in both prokaryotic and eukaryotic cell-free systems. The ID_{50} was $9\ \mu\text{M}$ with the endogenous mRNA-directed rabbit reticulocyte lysate, $17\ \mu\text{M}$ with a poly(U)-directed system from *Escherichia coli* and $69\ \mu\text{M}$ with a poly(U)-directed system from *Artemia salina* cysts. Of the three steps of elongation, purpuromycin does not affect the peptidyl-transferase reaction, inhibits the elongation factor 1 (EF-1) dependent binding of phenylalanyl-tRNA and stimulates the GTP-dependent binding of EF-2. When protein synthesis is stopped by the addition of purpuromycin, the nascent peptide chains are found in the puromycin-reactive P site. The results suggest that the mechanism of action of purpuromycin is similar to that of fusidic acid. Both antibiotics would seem to produce a stable guanine nucleotide-ribosome-EF-2 complex which allows one round of translocation but prevents, because of a common or overlapping ribosomal binding site for the two elongation factors, the subsequent EF-1-dependent binding of aminoacyl-tRNA.

INTRODUCTION

Purpuromycin ($\text{C}_{26}\text{H}_{18}\text{O}_{13}$; molecular mass 538.4 Da) is an antibiotic isolated from the culture broth of *Actinoplanes ianthinogenes*. The antibiotic molecule contains a naphthazarin and an isocoumarin moiety. Its complete structure has been determined (Bardone *et al.*, 1974) and is reported in Fig. 1 (Coronelli *et al.*, 1974). It is very active against Gram-positive bacteria [minimal inhibitor concn. (MIC) 5–100 ng/ml] and fungi (MIC 50–1000 ng/ml), and has variable activity against Gram-negative bacteria (MIC 0.05–128 $\mu\text{g}/\text{ml}$) (Coronelli *et al.*, 1974). Little information is however available on its mechanism of action at the molecular level.

The present paper describes the effect of purpuromycin on cell-free protein-synthesizing systems of prokaryotic and eukaryotic origin.

MATERIALS AND METHODS

Purpuromycin was supplied by the Lepetit Research Center, Gerenzano, Italy. The bright purple crystals were dissolved in dimethyl sulphoxide at 1 mg/ml, and appropriate dilutions were made in water.

Fig. 1. Structure of purpuromycin

Diphtheria toxin, purified by the method of Pope & Stevens (1958), was generously supplied by the Istituto Sieroterapico e Vaccinogeno Toscano 'Sclavo', Siena, Italy; diphtheria toxin fragment A (M_r 24000) was prepared as described by Chung & Collier (1977).

[adenosine- ^{14}C]NAD $^+$ (260 mCi/mmol), L-[^{14}C]phenylalanine (513 mCi/mmol), L-[^{14}C]leucine (348 mCi/mmol) and [^3H]puromycin (5.7 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Poly(U) was from Boehringer, Mannheim, Germany; NAD $^+$, nucleotides and tRNA $^{\text{Phe}}$ were products of Sigma Chemical Co., St. Louis, MO, U.S.A.; tRNA $^{\text{Phe}}$ was charged with [^{14}C]phenylalanine by the method of Hultin & Näslund (1978).

Ribosomes and the postribosomal enzymic fractions were prepared from *Escherichia coli* as described by Wilhelm & Haselkorn (1971) and from *Artemia salina* cysts by the method of Sierra *et al.* (1974). Rabbit reticulocyte lysate was prepared as described by Allen & Schweet (1962). EF-1 and EF-2 were obtained from rat liver 'pH 5 supernatant' and resolved as described by Montanaro *et al.* (1973). Protein synthesis assays were performed using the following methods: that of Hosokawa *et al.* (1966) for poly(U)-directed polyphenylalanine synthesis by *E. coli* ribosomes; that of Sargiacomo *et al.* (1983) for endogenous mRNA-directed protein synthesis by the rabbit reticulocyte lysate; and that of Barbieri *et al.* (1980) for poly(U)-directed synthesis of polyphenylalanine by *A. salina* ribosomes. Hot-acid-insoluble radioactivity was collected as described by Montanaro *et al.* (1978). EF-1-dependent phenylalanyl-tRNA binding was carried out essentially as described by Sperti *et al.* (1976), with NAD $^+$ and diphtheria toxin fragment A present in the preincubation mixture of ribosomes in order to

Abbreviations used: MIC, minimal inhibitor concentration; EF-1, elongation factor 1; EF-2, elongation factor 2; EF-G, elongation factor G; A site, aminoacyl-tRNA site; P site, peptidyl-tRNA site; ID_{50} , concentration of inhibitor causing 50% inhibition of protein synthesis.

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inactivate possible traces of contaminating EF-2. The preparation of [^{14}C]ADP-ribosyl-EF-2 and its binding to ribosomes was essentially as described by Montanaro *et al.* (1975). The reactivity of nascent peptides with [^3H]puromycin during protein synthesis was measured following the method of Sperti *et al.* (1976) using a rabbit reticulocyte lysate system to which haemin was added to inhibit the haemin-controlled repressor (Pelham & Jackson, 1976). All experimental details are in the legends to the Figures and the Table.

Fig. 2. Inhibition by purpuromycin of prokaryotic and eukaryotic cell-free protein-synthesizing systems

The fractional activity is plotted against the log of the inhibitor concentration to calculate the ID_{50} values in the different systems. The ID_{50} is the concentration of inhibitor causing 50% inhibition of protein synthesis. The *E. coli* system (●) contained, in 100 μl , 10 mM-Tris/HCl, pH 7.8, 50 mM-KCl, 10 mM-magnesium acetate, 0.03 mM-GTP, 1 mM-ATP, 6 mM-mercaptoethanol, 1 mM-dithiothreitol, 15 mM-phosphocreatine, 6 μg of creatine phosphokinase, 0.05 mM amino acids [except tyrosine, which was 0.025 mM, and phenylalanine, present as [^{14}C]phenylalanine (0.15 μCi)], 2.5 pmol of ribosomes and 75 μg of postribosomal enzymic fraction ('s-30'). After 10 min at 37 °C, the reaction was started by the addition of 100 μg of poly(U). Incubation was for 30 min at 37 °C. The rabbit reticulocyte lysate system (○) contained, in a final volume of 62.5 μl , 10 mM-Tris/HCl, pH 7.4, 100 mM-ammonium acetate, 2 mM-magnesium acetate, 1 mM-ATP, 0.2 mM-GTP, 15 mM-phosphocreatine, 3 μg of creatine phosphokinase and 0.05 mM amino acids [except leucine, which was present as [^{14}C]leucine (0.1 μCi)]. The reaction was started by the addition of 25 μl of lysate, containing 2.5 pmol of ribosomes. Incubation was for 7 min at 28 °C. The *Artemia salina* system (Δ) contained, in 100 μl , 80 mM-Tris/HCl, pH 7.4, 120 mM-KCl, 7 mM-magnesium acetate, 2 mM-dithiothreitol, 2 mM-GTP, 80 μg of poly(U), 16 pmol of [^{14}C]phenylalanyl-tRNA and 2.5 pmol of ribosomes. The reaction was started by the addition of 100 μg of the postribosomal enzymic fraction ('s-105 supernatant'). Incubation was for 20 min at 24 °C. Data are based on four independent experiments.

RESULTS AND DISCUSSION

Fig. 2 shows that purpuromycin inhibits protein synthesis in both prokaryotic and eukaryotic systems. The ID_{50} was 9 μM with the rabbit reticulocyte lysate, 17 μM with the poly(U)-directed system from *E. coli*, and 69 μM with that from *A. salina* embryos. The *E. coli* and *A. salina* systems contained the minimal amount of supernatant enzyme fraction which gave the maximal rate of polyphenylalanine synthesis in the presence of 2.5 pmol of ribosomes, which was the amount present in the rabbit reticulocyte system.

Since undeveloped *A. salina* embryos are deficient in mRNA and in one or more initiation factors (Sierra *et al.*, 1974) and since translation of poly(U) bypasses the

Fig. 3. Effect of purpuromycin on protein synthesis and on the reactivity of ribosomal nascent peptides with puromycin

The protein-synthesizing mixture was the standard rabbit reticulocyte lysate system, supplemented with 10 μM -haemin. In a parallel mixture, [^{14}C]leucine was substituted with the non-radioactive amino acid. At zero time and after 3 min of incubation at 28 °C, 62.5 μl aliquots were withdrawn in duplicate from each mixture. The remaining volume of each mixture was divided into two samples, to which purpuromycin (final concentration 37 μM) or an equal volume of dimethyl sulphoxide were added respectively (arrow). The incubation was allowed to proceed at 28 °C and duplicate 62.5 μl aliquots were withdrawn from each sample after 10 and 20 min of total incubation. Protein synthesis (—) was measured from the incorporation of [^{14}C]leucine in the samples containing the radioactive amino acid. The reactivity of nascent peptides with puromycin (---) was measured by adding 12.8 μl of 2 M- NH_4Cl and 4.7 μl of [^3H]puromycin to the samples without the radioactive amino acid; after 20 min at 0 °C, the trichloroacetic acid-insoluble radioactivity was collected and measured. ●, Purpuromycin absent; ○, purpuromycin present. Each point is the mean of duplicate assays from two experiments; the results obtained from the two experiments were very similar.

Table 1. Effect of purpuromycin on the EF-1-dependent binding of phenylalanyl-tRNA and on the binding of ADP-ribosyl-EF-2 to *A. salina* ribosomes

Expt. 1: EF-1-dependent phenylalanyl-tRNA binding. The experiment was performed in two steps. (1) Ribosomes (15 pmol) were preincubated for 5 min at 24 °C in 15 μ l of 80 mM-Tris/HCl, pH 7.4, 80 mM-KCl, 5 mM-magnesium acetate and 2 mM-dithiothreitol containing 2 mM-NAD⁺ and 3 μ g of diphtheria toxin fragment A; purpuromycin (17 nmol) was either absent or present. (2) EF-1 (45 μ g of protein), 200 μ g of poly(U), 22 pmol of [¹⁴C]phenylalanyl-tRNA and 0.5 μ mol of GTP were added in 85 μ l of 80 mM-Tris/HCl, pH 7.4, 100 mM-KCl, 9 mM-magnesium acetate and 2 mM-dithiothreitol. Incubation was at 24 °C for 30 min. For measurement of protein synthesis, NAD⁺ and diphtheria toxin fragment A were omitted from step 1, and 0.4 pmol of EF-2 were added along with EF-1 in Step 2. Expt. 2: ADP-ribosyl-EF-2 binding. Ribosomes (27 pmol) were incubated in 150 μ l of 80 mM-Tris/HCl, pH 7.4, 120 mM-KCl, 7 mM-magnesium acetate, 2 mM-dithiothreitol, containing 1 mM-GTP and 78 pmol of [¹⁴C]ADP-ribosyl-EF-2; purpuromycin (45 nmol) was either absent or present. After 20 min at 24 °C, samples were layered on 1.8 ml of 0.25 M-sucrose in the same buffer and centrifuged for 2 h at 175000 g. Pellets were resuspended in water, the ribosome concentration was estimated from the A_{260} , and the [¹⁴C]ADP-ribosyl-EF-2 bound was measured from the acid-insoluble radioactivity collected on glass-fibre filters. Data are based on four independent experiments.

Purpuromycin	Experiment 1		Experiment 2
	Phe-tRNA bound (pmol/100 pmol of ribosomes)	Protein synthesis (pmol of phenylalanine incorporated/100 pmol of ribosomes)	ADP-ribosyl-EF-2 bound (pmol/100 pmol of ribosomes)
Absent	38.7	84.1	13.1
Present	0.9	2.1	54.8

initiation step, purpuromycin must, in this system, act on some elongation step.

The EF-1-dependent binding of aminocyl-tRNA and the GTP-dependent binding of EF-2 were studied with *A. salina* ribosomes and the elongation factors from rat liver. The EF-1-dependent binding of aminoacyl-tRNA was 50% inhibited by 94 μ M-purpuromycin (results not shown). The results reported in Table 1 confirm that the enzymic binding of phenylalanyl-tRNA was inhibited to the same extent as was protein synthesis. In contrast, the binding of ADP-ribosyl-EF-2 was increased by 4-fold.

The third step of elongation, that catalysed by ribosomal peptidyltransferase, was assayed by measuring, in the reticulocyte lysate, the reactivity of nascent peptides with [³H]puromycin. This reaction was not affected by the presence of purpuromycin (results not shown).

Since the puromycin reaction was not inhibited by purpuromycin, [³H]puromycin was used to titrate the number of nascent peptides located in the ribosomal peptidyl site (P site) when protein synthesis is stopped by the action of purpuromycin (Fig. 3). The experiment was designed to clarify whether the EF-1-catalysed step or the EF-2-catalysed step is primarily affected by purpuromycin in the complete elongation cycle.

The proportion of ribosomes bearing peptide chains in the P site, reactive with puromycin at 0 °C (Inoue-Yokosawa *et al.*, 1974), increases during protein synthesis (Sperti *et al.*, 1976). In the presence of inhibitors like ricin (Sperti *et al.*, 1976) or diphtheria toxin (Montanaro *et al.*, 1976), which allow a single round of translocation and not inhibit the EF-1-catalysed binding of aminoacyl-tRNA (Sperti & Montanaro, 1979), the arrest of protein synthesis is accompanied by a decrease in the number of ribosomes bearing nascent peptides at the P site. After the single translocation, the EF-1-catalysed binding of aminoacyl-tRNA followed by peptide-bond formation occurs normally; the nascent peptides are now in the A site and, since a second translocation is prevented, they remain at this site.

As shown in Fig. 3, this shift of the nascent peptides from the P to the A site did not occur after the addition of purpuromycin to ribosomes engaged in protein synthesis. On the contrary, the number of ribosomes bearing nascent peptides in the P site continued to increase, although at a lower rate than in the absence of purpuromycin. Thus, during the complete cycle of elongation, purpuromycin must impair the EF-1-catalysed binding of aminoacyl-tRNA; this may be a consequence of the formation of a stable guanine nucleotide-EF-2-ribosome complex (Table 1, Expt. 2) at a ribosomal site common to the two elongation factors. Translocation which is catalysed by this complex, not followed by the binding of aminoacyl-tRNA to the A site, would leave the peptide chains confined to the P site.

The mechanism by which purpuromycin inhibits protein synthesis in cell-free systems appears to be very similar to that of fusidic acid. This steroid antibiotic produced by *Fusidium coccineum* forms, in the presence of ribosomes, GTP and either EF-G or EF-2, a stable 'fusidic acid-GDP-ribosome-EF-G (or EF-2)' complex which prevents the subsequent EF-1-dependent binding of aminoacyl-tRNA (Vázquez, 1979).

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