Mechanism of Self-protection in a Puromycin-producing Micro-organism

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Puromycin is a potent inhibitor of bacterial protein synthesis, but puromycin-producing *Streptomyces alboniger* KCC S-0309 is tolerant to the antibiotic *in vivo*. Puromycin bound to both 30S and 50S ribosomal subunits from *S. alboniger* and inhibited polyuridylylate-directed polyphenylalanine synthesis by the ribosomes. However, the organism possessed a novel puromycin-inactivating enzyme which acetylated the antibiotic at the 2'-NH₂ group of the *O*-methyltyrosine moiety.

INTRODUCTION

The micro-organisms which produce antibiotic inhibitors of protein synthesis must be protected from the lethal effect of their own antibiotics. The producers of thiostrepton (Cundliffe, 1978; Cundliffe & Thompson, 1979; Thompson & Cundliffe, 1981), erythromycin (Teraoka & Tanaka, 1974; Skinner & Cundliffe, 1982), noshiheptide (Cundliffe & Thompson, 1981), istamycin (Yamamoto *et al.*, 1981) and berninamycin (Thompson *et al.*, 1982) all possess drug-resistant ribosomes. Enzymic inactivation of streptomycin (Sugiyama *et al.*, 1980, 1981*a,b*, 1982, 1983), neomycin (Thompson *et al.*, 1980; Hotta *et al.*, 1981), kanamycin (Satoh *et al.*, 1975; Hotta *et al.*, 1981), streptothricin (Keeratipibul *et al.*, 1983), viomycin and capreomycin (Skinner & Cundliffe, 1980) by their producers has also been demonstrated. Furthermore, in the production of streptomycin (Sugiyama *et al.*, 1981*b*; Nimi *et al.*, 1981) and chloramphenicol (Malik & Vining, 1972), it has been suggested that a decrease in cell permeability to the antibiotic at the idiophase of culture plays an important role in self-protection of the producer.

Puromycin, a functional analogue of aminoacyl tRNA, inhibits protein synthesis by substituting for the incoming coded aminoacyl tRNA, and serving as an acceptor for the nascent peptide chain of ribosome-bound peptidyl tRNA (Nathans & Lipman, 1961). *Streptomyces alboniger*, a puromycin producer, is tolerant to its own antibiotic product. The organism has been reported to express an enzyme activity which acetylates puromycin by using acetyl coenzyme A (Pérez-González *et al.*, 1983). The acetylated puromycin showed no antibiotic activity against *Bacillus subtilis*. It was suggested that puromycin was converted into *N*-acetyl-puromycin. The acetylation of puromycin and the self-protection of the producer were proposed by rather indirect procedures.

We also showed acetylation of puromycin by the producer, and have purified the acetyltransferase. In the present paper, we show that puromycin inhibits protein synthesis of the producer by binding to both 30S and 50S subunits of ribosomes. Protection of protein synthesis by puromycin-acetyltransferase was ascertained by using an *in vitro* protein-synthesizing system. The site of acetylation of puromycin was determined physico-chemically. The substrate specificity of the puromycin acetyltransferase was also defined.

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METHODS

Organism. Puromycin-producing *Streptomyces alboniger* KCC S-0309 was used.

Chemicals. Puromycin dihydrochloride was purchased from Sigma, and [8(n)-³H]puromycin dihydrochloride from Amersham. Neomycin sulphate, kanamycin sulphate and chloramphenicol were purchased from Nippon Kayaku, Meiji Seika Kaisha and Boehringer, respectively.

Preparation of ribosomes and S-150 fraction. This was done according to the methods of Sugiyama *et al.* (1983), using mycelia grown in GMP medium [1% (w/v) glucose, 0.5% (w/v) NaCl, 0.4% (w/v) peptone, 0.2% (w/v) yeast extract, 0.2% (w/v) meat extract, 0.025% (w/v) MgSO₄ · 7H₂O, pH 7.0] to an OD of approximately 250 Klett units.

Assay of protein synthesis in vitro. Polyuridylylate-directed polyphenylalanine synthesis was assayed as described previously (Sugiyama *et al.*, 1983).

Puromycin tolerance of the producer in vivo. One loopful of spore mass was inoculated into 10 ml GMP medium and cultivated at 28 °C for 48 h under shaking. A sample (0.1 ml) of this culture broth was transferred to 10 ml of the same medium containing a given concentration of puromycin. Growth was measured after incubation at 28 °C for 48 h under shaking.

Purification of acetylated puromycin. A solution (approximately 5 ml) containing 20 mg (37 µmol) puromycin dihydrochloride, 30.6 mg (38 µmol) acetyl coenzyme A and S-150 fraction (16 mg protein) from *S. alboniger* was incubated for 1 h at 28 °C, and was extracted with 10 ml ethyl acetate. The organic phase was washed twice with water by mixing vigorously and adjusting the pH value to 4, so that any trace of puromycin dissolved in the ethyl acetate would be extracted into the water phase. The organic phase was dehydrated with anhydrous sodium sulphate and was evaporated to obtain acetylpuromycin. The purity of acetylpuromycin was confirmed by TLC on a silica gel sheet (Merck Art. 5553) using a solvent system of *n*-butanol/acetic acid/water (4:1:2, by vol.). Since acetylpuromycin gave a negative ninhydrin reaction, it was detected by spraying with 10% (v/v) sulphuric acid.

RESULTS

Susceptibility of the ribosomes of S. alboniger to puromycin

When *S. alboniger* KCC S-0309 was cultivated in GMP medium with and without puromycin, as described in Methods, it was tolerant of puromycin up to 100 µg ml⁻¹ with no change in the extent of growth. However, protein synthesis in this strain was strongly inhibited by puromycin when it was examined in an *in vitro* system constructed with the ribosomes and S-150 fraction (data not shown).

Binding of puromycin to the ribosomes was examined using dissociated ribosomes which were incubated with [³H]puromycin and fractionated by sucrose density gradient centrifugation. The peaks of [³H]puromycin radioactivity appeared in the same fractions as the ribosomal 30S and 50S subunits (Fig. 1). From these results, it can be concluded that the ribosomes of strain KCC S-0309 are susceptible to puromycin, suggesting that the organism must have a puromycin resistance mechanism other than ribosomal resistance.

Inactivation of puromycin by the S-150 fraction from S. alboniger

The antibiotic activity of puromycin disappeared during inactivation with acetyl coenzyme A and the S-150 fraction from strain KCC S-0309 (Fig. 2). Substitution of ATP for acetyl coenzyme A was ineffective. When a polyphenylalanine-synthesizing reaction mixture to which puromycin had been added was also supplemented with acetyl coenzyme A, the inhibitory effect of puromycin was completely suppressed. These results indicate that the puromycin-acetylating enzyme can protect protein synthesis in extracts of KCC S-0309 against the endogenous antibiotic product.

Substrate specificity of the puromycin-acetylating enzyme

Kanamycin, neomycin, streptothricin and chloramphenicol are known to be inactivated by acetylation (Benveniste & Davies, 1973; Satoh *et al.*, 1975; Shaw & Hopwood, 1976; Thompson *et al.*, 1980; Keeratipibul *et al.*, 1983). We tested the puromycin acetyltransferase from strain KCC S-0309 to see whether it could acetylate these antibiotics. The S-150 fraction from our strain inactivated streptothricin as well as puromycin (Fig. 2). However, it should be pointed out that streptothricin was also inactivated by the S-150 fraction in the absence of added acetyl coenzyme A. The S-150 fraction appears to have some streptothricin-inactivating activity other than acetylation.

Fig. 1. Binding of [^3H]puromycin to ribosomal subunits from *S. alboniger*. Ribosomes were dissociated by dialysis against buffer consisting of 10 mM-Tris/HCl (pH 7.65), 30 mM- NH_4Cl , 0.3 mM-magnesium acetate and 6 mM-2-mercaptoethanol. The dissociated preparation (355 pmol ribosomes) was incubated with 625 pmol [^3H]puromycin at 28 °C for 1 h and then layered onto density gradients of 10 to 30% (w/v) sucrose in the same buffer. The samples were then centrifuged at 70000 *g* for 17 h. O, A_{260} ; ●, radioactivity of [^3H]puromycin.

Fig. 2. Substrate specificity of the puromycin-acetylating enzyme. Reaction mixtures (100 μl in total) contained 80 mM-Tris/HCl (pH 7.65), 10 mM-magnesium acetate, 6 mM-2-mercaptoethanol and antibiotic substrate (100 $\mu\text{g ml}^{-1}$) together with the following: (a) S-150 fraction (216 μg protein), (b) S-150 fraction and ATP (2 mM), (c) ATP, (d) S-150 fraction and acetyl coenzyme A (2 mM), or (e) acetyl coenzyme A. Substrate antibiotics used were puromycin dihydrochloride (P), neomycin sulphate (N), kanamycin sulphate (K), chloramphenicol (C) and streptothricin (S). The reaction mixtures were incubated at 28 °C for 30 min and then subjected to antibiotic assay by an agar diffusion method using *Bacillus subtilis* IFO 3134 as a test organism.

Fig 3. ^1H NMR spectra of puromycin and the acetylated product. The solvent systems of D_2O and $\text{D}_2\text{O} + \text{DCl}$ were used for (a) puromycin and (b) the acetylated product, respectively.

Table 1. Chemical shift of ^{13}C NMR

Atom*	δ (p.p.m.)		Atom*	δ (p.p.m.)	
	Puromycin	Acetylpuromycin		Puromycin	Acetylpuromycin
2	146.0	146.5	2''	55.4	56.3
4	148.4	148.9	3''	36.9	37.4
5	120.3	120.3	4''	127.2	129.4
6	149.5	148.5	5''	115.3	115.0
8	141.3	141.5	6''	131.5	131.2
6-N-CH ₃	41.3	41.8	7''	159.3	158.7
1'	90.8	91.0	8''	131.5	131.2
2'	74.1	74.5	9''	115.3	115.0
3'	56.3	56.3	7''-O-CH ₃	51.4	51.1
4'	83.2	83.3	$\begin{smallmatrix} \text{O} \\ \parallel \\ \text{C}-\text{CH}_3 \end{smallmatrix}$	-	174.8
5'	61.0	61.0	$\begin{smallmatrix} \text{O} \\ \parallel \\ \text{C}-\text{CH}_3 \end{smallmatrix}$	-	22.5
1''	170.1	174.2			

* The numbering system identifying the carbon atoms is shown in Fig. 5. The relevant carbon atom in the acetyl group is shown in **bold** type.

Fig. 4. FAB mass spectra of (a) puromycin and (b) the acetylated product.

Fig. 5. The chemical structure of puromycin ($R = H$) and the acetylated product ($R = COCH_3$).

Site of acetylation of puromycin

Acetylated puromycin, prepared enzymically, was purified and compared with authentic puromycin by TLC as described in Methods. The acetylated puromycin gave a single spot and was less polar than puromycin (data not shown). The acetylated product was analysed by 1H NMR. The spectrum was similar to that of puromycin except that the acetylated puromycin gave a distinctive singlet at 1.8 p.p.m. representing a CH_3CO- residue (Fig. 3). This residue was confirmed by analysis by ^{13}C NMR. The acetylated puromycin had two specific signals at 22.5 and 174.8 p.p.m., which were assigned to a primary and a quaternary carbon respectively, in addition to the same signals as puromycin (Table 1). In order to estimate the number of acetyl residues, the molecular weight of the modified drug was determined from the FAB mass spectrum. The protonated molecular ions (MH^+) of puromycin and the acetylated product gave m/z of 472 and 514, respectively (Fig. 4). The latter value is consistent with monoacetyl puromycin. A fragment ion at m/z 192 corresponding to an *N*-acetyl-*O*-methyltyrosine residue appeared in the mass spectrum of acetylated puromycin, showing that the acetyl group was introduced into the amino group of the *O*-methyltyrosine moiety in puromycin. In the infra red spectrum, no absorbance assignable to the ester of a carboxyl group was observed in acetylated puromycin (data not shown). These results indicated that puromycin was inactivated by *N*-acetylation and not by *O*-acetylation. Thus the chemical structure of the acetylated puromycin was determined to be that shown in Fig. 5.

DISCUSSION

Acetylpuromycin was produced from puromycin using the S-150 fraction of *S. alboniger* plus acetyl coenzyme A, and was then purified. The acetylated site was physico-chemically identified as the 2'-NH₂ group of the *O*-methyltyrosine moiety. Puromycin 2''-*N*-acetyltransferase was able to protect the protein synthetic apparatus in extracts of the puromycin producer.

It was also shown in the present study that the ribosomes of the puromycin producer were sensitive to the endogenous antibiotic. Puromycin bound to both the 30S and 50S subunits. According to Olson *et al.* (1982) and Grant *et al.* (1983), binding of puromycin to ribosomes of *Escherichia coli* also took place on both the 30S and 50S subunits, and each subunit had two binding regions. Many antibiotics which inhibit bacterial protein synthesis have been reported to bind to either one of the two ribosomal subunits. Puromycin is unusual in binding to both of the subunits; the functional differences arising from this remain to be determined.

Several antibiotic-producing micro-organisms have been reported to produce enzymes that inactivate their own products. But there is no definitive evidence that any producer is protected by its inactivating enzyme alone. We have already reported that the ribosomes of streptomycin-producing *Streptomyces griseus* were sensitive to streptomycin, but that they were protected by streptomycin 6-phosphotransferase produced by the organism (Sugiyama *et al.*, 1980, 1981*a*, *b*, 1982, 1983). While the production of this inactivating enzyme increased with the age of mycelium, sensitivity of ribosomes and also the ability of mycelium to take up extracellular streptomycin decreased at the idiophase of culture (Sugiyama *et al.*, 1981*b*). Owing to these three protecting measures *S. griseus* appeared to be able to produce a large amount of streptomycin. We are now studying the puromycin producer to see whether it shows a similar change in ribosomal sensitivity and uptake of puromycin during cultivation.

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