The Mechanism of Resistance to Puromycin and to the Puromycin-precursor *O*-Demethyl-puromycin in *Streptomyces alboniger*

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Ribosomes from *Streptomyces alboniger* are sensitive *in vitro* to puromycin and, to a lesser extent, to the puromycin-precursor *O*-demethyl-puromycin. The puromycin-inactivating enzyme (puromycin *N*-acetyltransferase) from *S. alboniger* also *N*-acetylates *O*-demethyl-puromycin. This finding indicates that in certain antibiotic-producing organisms the antibiotic-inactivating enzymes may play a role in self-defence against toxic precursor molecules.

INTRODUCTION

Puromycin is an aminoacyl-adenosine antibiotic which is produced by *Streptomyces alboniger*. It blocks protein synthesis by forming a peptide bond with the peptidyl moiety of peptidyl-tRNA on both 70S and 80S ribosomes and releasing the nascent peptide chain (for a review see Cundliffe, 1981). *S. alboniger* contains a puromycin acetyltransferase (PAC) which inactivates this antibiotic by *N*-acetylation (Pérez-González *et al.*, 1983). The gene *pac* encoding the puromycin *N*-acetyltransferase has been cloned and *Streptomyces lividans* carrying this gene in a plasmid vector became resistant to puromycin (Vara *et al.*, 1985). These findings suggest that the PAC enzyme provides the producer with a mechanism for puromycin resistance. Inactivation of aminocyclitol antibiotics by acetylation and/or phosphorylation has been found in the relevant *Streptomyces* producers (Cella & Vining, 1975; Skinner & Cundliffe, 1980; Thompson *et al.*, 1982; Pardo *et al.*, 1985). However, other mechanisms of resistance could coexist with antibiotic-inactivating enzymes, since *Streptomyces tenebrarius* contains both resistant ribosomes and aminocyclitol-modifying enzymes (Yamamoto *et al.*, 1982).

Little is known about detoxification of those possible harmful intermediates which are synthesized in the biochemical pathways for antibiotic biosynthesis. *O*-Demethyl-puromycin *O*-methyltransferase is present in extracts of *S. alboniger* and converts *O*-demethyl-puromycin (Fig. 1) to puromycin (Rao *et al.*, 1969). Therefore, this enzyme is thought to catalyse the last step for puromycin biosynthesis. A method for the chemical synthesis of *O*-demethyl-puromycin is readily available (Pogell, 1975). Hence, this drug can be prepared and studied as a possible inhibitor of protein synthesis and as a substrate for the PAC enzyme.

In the present work we have examined, for the first time, the response of *S. alboniger* ribosomes to puromycin and to *O*-demethyl-puromycin *in vitro*. Moreover, we have also examined the capability of the puromycin *N*-acetyltransferase to inactivate *O*-demethyl-puromycin.

METHODS

Bacterial strains, media and cell growth. *Streptomyces lividans* 1326 and *Streptomyces alboniger* ATCC 12461 were grown at 30 °C in either R2YE agar (Chater *et al.*, 1982) or liquid YEME (Chater *et al.*, 1982) supplemented with 34% (w/v) sucrose. *Staphylococcus aureus* was grown at 37 °C in LB broth (Miller, 1972) minus NaCl, supplemented with 1.5% (w/v) agar.

Abbreviation: PAC, puromycin acetyltransferase.

Fig. 1. Chemical structures of puromycin and *O*-demethyl-puromycin. The sites of acetylation are indicated by the arrows.

Preparation of cell extracts for polyphenylalanine synthesis. The procedures were essentially identical to those described previously (Skinner & Cundliffe, 1980; Pardo *et al.*, 1985). Mycelia were collected by centrifugation when the cultures reached an OD_{660} of 0.7–0.8 (measured in a Bausch & Lomb Spectronic 20 with a tube of 1 cm path length) and washed twice with buffer A [10 mM-Tris/HCl, pH 7.5; 10 mM-MgCl₂; 500 mM-NH₄Cl; 6 mM-2-mercaptoethanol; 0.2 mM-phenylmethanesulphonyl fluoride (PMSF)] and once with buffer B (buffer A with 50 mM-NH₄Cl). Mycelia were broken with alumina and resuspended in buffer B. The extracts were then centrifuged twice at 30000 *g* for 20 min to obtain the S30 fraction, which was then treated with DNAase.

Ribosomes were prepared by layering the S30 fraction over 1 vol. buffer A containing 40% (w/v) sucrose followed by centrifugation at 100000 *g* for 8 h. The upper two-thirds of the supernatant fraction was collected and dialysed against buffer B. The ribosome pellet was resuspended in a small volume of PMSF-free buffer B.

High-salt washed ribosomes from *Escherichia coli* and *Bacillus subtilis* were provided by Professor J. P. G. Ballesta, Centro de Biología Molecular, Madrid, Spain.

Assay of cell-free protein synthesis. The poly(U)-directed synthesis of polyphenylalanine was assayed as described by Pardo *et al.* (1985) but [³H]phenylalanine (specific activity 1 Ci mmol⁻¹; 37 GBq mmol⁻¹) was used instead of [¹⁴C]phenylalanine and the final concentration of uncharged tRNA^{Phe} was lowered fivefold (to 0.076 μM). This last modification was introduced since higher concentrations of tRNA^{Phe} diminished the puromycin-induced inhibition values of polyphenylalanine synthesis (not shown), an effect that was also observed with the *E. coli* cell-free system. This effect may reflect a competition of puromycin and tRNA^{Phe} for binding to the peptidyl transferase centre on the 50S ribosomal subunit, since an excess of tRNA^{Phe} could displace puromycin from its acceptor site.

Preparation of cell extracts as a source of PAC activity and assay of this enzymic activity. *S. alboniger* was grown in liquid culture as described above. Mycelia were collected by centrifugation in a bench centrifuge and washed twice with buffer TGE [10 mM-Tris/HCl, pH 8.5; 10% (v/v) glycerol; 1 mM-EDTA]. The final pellet was resuspended in cold TGE buffer (one-tenth original culture volume) and sonicated twice for 15 s at 0 °C, and cell debris was removed by a short centrifugation in the cold. DNAase was added to the supernatant, which was then centrifuged at 105000 *g* for 2.5 h. The resulting supernatant was dialysed against TGE buffer and used as a crude preparation of PAC activity (S100 fraction). PAC activity was assayed spectrophotometrically as previously described (Vara *et al.*, 1985). One unit of PAC activity transfers 1 μmol of acetyl groups to puromycin per min.

Enzymic inactivation of puromycin: effect on polyphenylalanine synthesis in vitro. Reaction mixtures (400 μl) contained 10 mM-Tris/HCl, pH 8.5, 1 mM-EDTA disodium salt, 10% (v/v) glycerol, 2.8 mM-acetyl coenzyme A and *S. alboniger* supernatant fraction (600 μg protein; 50 mU PAC activity mg⁻¹) either in the presence or in the absence of 2.25 mM-puromycin. The reaction took place at 30 °C and, at intervals, 45 μl samples were removed, heated for 2.5 min at 70 °C and centrifuged. *N*-Acetyl-puromycin was estimated by diluting 8 μl samples in a mixture (1 ml) containing 100 mM-Tris/HCl, pH 8.5 and 0.04 mg 5,5'-dithiobis(2-nitrobenzoic acid) ml⁻¹ and reading the increment in absorption at 412 nm. Samples of 8 μl with no puromycin added were used as the relevant blanks. The increment in absorbance served to estimate the amount of *N*-acetyl-puromycin synthesized, using molar absorption coefficient of 13.6 μM⁻¹ cm⁻¹. Duplicated 8 μl samples were assayed for inhibition of polyphenylalanine synthesis in the *S. lividans* cell-free system. The controls for polyphenylalanine synthesis in the absence of puromycin contained 8 μl of the sample taken from the assay done in the absence of puromycin. This was required since addition of the heat-inactivated S100 fraction from *S. alboniger*, independently of the incubation time, reduced polyphenylalanine polymerization by 80% (not shown).

Fragment reaction assay. *N*-Acetyl-[³H]leucyl-ACCAC was obtained as described elsewhere (Monro *et al.*, 1968) and used as donor substrate in the fragment reaction assay. This reaction, a model assay for peptide bond formation, was done as described by Monro *et al.* (1968) using as an acceptor substrate either puromycin or *O*-demethyl-puromycin.

Chemical synthesis. The puromycin-precursor *O*-demethyl-puromycin (Rao *et al.*, 1969) was synthesized as described elsewhere (Pogell, 1975). The chemical structure of the final product, as well as the purity (>97%), was confirmed by NMR analysis (not shown).

N-Acetyl-*O*-demethyl-puromycin was prepared similarly to *N*-acetyl-puromycin (Pérez-González *et al.*, 1983). An aqueous solution (1.2 mM) of *O*-demethyl-puromycin (900 µl) was treated consecutively with 2 × 200 µl acetic anhydride, each time at 0 °C for 30 min, before *N*-acetyl-*O*-demethyl-puromycin was finally isolated by lyophilization.

Antibacterial activity tests. Suspensions of spores or cells were spread on agar plates. Antibiotic-containing paper discs (Difco; 6 mm diam.) were placed on the plates which were incubated at the required temperature (see above).

RESULTS

Effect of puromycin and O-demethyl-puromycin on bacterial cell growth

S. alboniger was not highly resistant to puromycin since it grew poorly on agar plates containing drug concentrations greater than 300 µg ml⁻¹. *S. lividans* was sensitive to puromycin concentrations of 50 µg ml⁻¹, although some colonies appeared even at 100 µg ml⁻¹. This partial resistance may have been due to a permeability barrier, since *S. lividans* mutants sensitive to lower concentrations of puromycin were easily isolated (Vara *et al.*, 1985). In contrast, *Staphylococcus aureus* did not grow on plates containing 50 µg puromycin ml⁻¹. Interestingly, *Staphylococcus aureus* and *S. lividans* were resistant to *O*-demethyl-puromycin as assayed by the disc method (containing up to 100 µg drug). This lack of *in vivo* effect may be attributed to a permeability barrier since this drug was biologically active *in vitro* (see below).

Enzymic acetylation of O-demethyl-puromycin

An S100 fraction from *S. alboniger* was assayed for acetyltransferase activity with *O*-demethyl-puromycin as substrate and [acetyl-¹⁴C]acetyl coenzyme A as donor of [¹⁴C]acetyl groups. The incubation mixture was then extracted with chloroform and the extract was analysed by thin-layer chromatography. Since radioactivity co-migrated with chemically synthesized *N*-acetyl-*O*-demethyl-puromycin (Fig. 2), this result indicates that the *N*-acetyltransferase activity of *S. alboniger* also *N*-acetylates the *O*-demethyl-puromycin precursor. Enzymic acetylation of this precursor molecule by S100 from *S. alboniger* was also shown spectrophotometrically (results not shown).

Ability of O-demethyl-puromycin to form peptide bonds

Considering the close chemical similarities between puromycin and *O*-demethyl-puromycin and, on the other hand, the known mode of action of other nucleoside antibiotics and puromycin analogues (Lichtenthaler *et al.*, 1979), it was thought that *O*-demethyl-puromycin could block protein synthesis by forming a peptide bond with the nascent peptide, followed by a release of the *N*-peptidyl-*O*-demethyl-puromycin from the ribosome. To test this hypothesis, *O*-demethyl-puromycin was assayed as a substitute for puromycin in the 'fragment' reaction assay. In this reaction *N*-acetyl-leucyl-ACCAC (obtained as a fragment of *N*-acetyl-leucyl-tRNA) reacts with puromycin in the presence of ribosomes and ethanol to yield *N*-acetyl-leucyl-puromycin which can be extracted with ethyl acetate. *O*-Demethyl-puromycin behaved similarly to puromycin and possessed acceptor capacity for *N*-acetyl-leucyl groups in the presence of 70S ribosomes from *S. alboniger*, *S. lividans*, *E. coli* and *B. subtilis* (Table 1). These results indicate (a) that *O*-demethyl-puromycin acts in a similar, but less effective, fashion to puromycin, and (b) that ribosomes from *S. alboniger* are sensitive to both puromycin and *O*-demethyl-puromycin.

Fig. 2. Identification of *N*-acetyl-*O*-demethyl-puromycin by thin-layer chromatography. *O*-Demethyl-puromycin (25 nmol) was incubated in a reaction mixture (16 μ l) containing 10 μ l S100 fraction from *S. alboniger* (1.5 mU PAC activity) and 40 nmol [14 C]acetyl coenzyme A (specific activity 1 mCi mmol $^{-1}$; 37 GBq mmol $^{-1}$). The reaction took place at 30 $^{\circ}$ C for 30 min. Then, 80 μ l buffer containing 0.1 M-sodium borate (pH 9.0) plus 5 M-NaCl (Pogell, 1975) was added and the mixture was extracted twice with 500 μ l chloroform. Under these conditions, [14 C]acetyl coenzyme A is insoluble in chloroform. The extract was dried under vacuum, dissolved in 10 μ l chloroform and chromatographed on 13181 silica gel (Kodak) with the solvent ethyl acetate : methanol (3 : 1). Spots of the various standards were detected by UV fluorescence. Radioactivity was estimated by cutting 5 mm strips that were counted in a liquid-scintillation spectrometer. ODMP, *O*-demethyl-puromycin. The arrow indicates the position of the solvent front.

Table 1. *Capacity of puromycin and O-demethyl-puromycin to take part in peptide bond formation*

The ability of puromycin and *O*-demethyl-puromycin to accept *N*-acetyl-[3 H]leucyl residues was determined by the 'fragment reaction' assay (Monro *et al.*, 1968). When *O*-demethyl-puromycin was present in the reaction mixtures puromycin was not added. The final concentrations of antibiotic, ribosomes and 'fragment' were 10^{-3} M, 0.14×10^{-6} M and 0.07×10^{-6} M, respectively. In controls, 0.01 pmol of labelled material was extracted with ethyl acetate in the absence of ribosomes. This background was subtracted from each experimental point.

Source of ribosomes	N-Acetyl-[14 C]leucyl residues (pmol) reacted with:	
	Puromycin	<i>O</i> -Demethyl-puromycin
<i>S. alboniger</i>	0.119	0.035
<i>S. lividans</i>	0.092	0.047
<i>E. coli</i>	0.086	0.027
<i>B. subtilis</i>	0.076	0.040

Effects of the different drugs on polyphenylalanine synthesis

The action of increasing concentrations of puromycin and *O*-demethyl-puromycin on poly(U)-directed polyphenylalanine synthesis by cell-free extracts from both *S. alboniger* and *S. lividans* was studied. The results indicated that both cell-free systems were sensitive to drugs although in both cases the precursor was about 400 times less active than puromycin (Fig. 3). It is clear, therefore, that the protein synthetic machinery of *S. alboniger* is sensitive both to the precursor and to the intact antibiotic. In contrast, over the concentration range 0 – 10^{-3} M, the *N*-acetyl derivatives of puromycin and *O*-demethyl-puromycin did not affect the poly(U)-directed polyphenylalanine system of *S. lividans* (Fig. 3). Thus, it seemed possible that *S. alboniger* might use *N*-acetylation of the precursor and the final antibiotic as an autoimmunity mechanism. In this context, the importance of the *O*-methyl group for the activity of puromycin in inhibiting protein synthesis is striking. This effect may indicate that the hydrophobicity introduced into the puromycin molecule by the *O*-methyl group increases the binding affinity of the drug for the hydrophobic peptidyl transferase centre of the 50S ribosomal subunit.

Fig. 3. Effect of concentration of different drugs on polyphenylalanine synthesis. Reactions were done as described in Methods. Levels of incorporation in the controls, expressed as pmol [^3H]phenylalanine polymerized per pmol ribosomes, are given in parentheses: (a) *S. alboniger* extracts (1.18); (b) *S. lividans* extracts (0.40). ●, Puromycin; ○, *O*-demethyl-puromycin; ▲, *N*-acetyl-puromycin; □, *N*-acetyl-*O*-demethyl-puromycin.

Fig. 4

Fig. 5

Fig. 4. Reversion by acetyl coenzyme A of the puromycin-induced inhibition of polyphenylalanine synthesis by *S. alboniger* extracts. Incubation mixtures (200 μl) contained the components indicated in Methods. When required, 3×10^{-4} M-puromycin and/or 5×10^{-4} M-acetyl coenzyme A was present in the reaction mixtures. At the indicated times, 60 μl samples were taken and then processed for counting. (a) Supernatant fraction and ribosomes from *S. alboniger*; (b) supernatant fraction and ribosomes from *S. lividans*; (c) supernatant fraction from *S. alboniger* and ribosomes from *S. lividans*; (d) supernatant fraction from *S. lividans* and ribosomes from *S. alboniger*. ●, Control; △, plus puromycin; ▲, plus acetyl coenzyme A; ○, plus acetyl coenzyme A and puromycin.

Fig. 5. Enzymic inactivation of puromycin by acetylation: effect on polyphenylalanine synthesis *in vitro*. Experimental details are given in Methods. Incorporation in the control was 0.1 pmol [^3H]phenylalanine per pmol ribosomes: ●, acetylated puromycin; ○, inhibition of polyphenylalanine synthesis.

We then studied the effect of including acetyl coenzyme A in the cell-free systems on polyphenylalanine synthesis. The inhibitory action of puromycin was decreased in the *S. alboniger* cell-free system in the presence of acetyl coenzyme A while this compound had no significant effect on a similar system from *S. lividans* (Fig. 4a, b). Similar results were obtained

using *O*-demethyl-puromycin instead of puromycin (not shown). Using heterologous ribosomes and supernatant fractions from *S. alboniger* and *S. lividans*, it was shown that the resistance to puromycin resided in the supernatant fraction from *S. alboniger* (Fig. 4c, d). Similarly, resistance to *O*-demethyl-puromycin resided in the supernatant fraction from *S. alboniger* (not shown), although, in the presence of acetyl coenzyme A, the inhibitory action of neither drug was completely eliminated. This result was probably due to an incomplete acetylation under the conditions for polyphenylalanine synthesis.

In order to correlate more precisely resistance with the activity of PAC, we measured the loss of inhibitory action on polyphenylalanine synthesis when puromycin was acetylated by a preparation of the PAC enzyme (Fig. 5). Increasing the level of acetylation drastically lowered the inhibition of polyphenylalanine synthesis in a cell-free system from *S. lividans*. Thus, with 50% of the puromycin acetylated, the remaining unmodified puromycin (1.5×10^{-4} M) blocked polyphenylalanine synthesis by approximately 50% (Fig. 5), a result in agreement with that shown in Fig. 3 where pure puromycin was used.

DISCUSSION

Enzymic inactivation of antibiotics by phosphorylation and/or acetylation has been found with cell-free extracts from several antibiotic-producing *Streptomyces* species (Davies *et al.*, 1979; Skinner & Cundliffe, 1980; Thompson *et al.*, 1982; Pérez-González & Jiménez, 1984; Pardo *et al.*, 1985). The ribosomes from these bacteria are generally sensitive to their own antibiotic products and, therefore, their autoimmunity system seems to involve primarily detoxification of the drugs. A similar situation has been found for *S. alboniger* in the present work, since its ribosomes are sensitive to puromycin and resistance to the antibiotic seems to be dependent upon its enzymic inactivation by the PAC activity. Moreover, we have cloned the gene *pac* that encodes the PAC enzyme, and have shown that *S. lividans* cells carrying this gene in a plasmid vector become resistant to puromycin (Vara *et al.*, 1985).

The role of antibiotic-inactivating enzymes in the producing organisms is not yet clear. Small amounts of *N*-acetylated and *O*-phosphorylated aminocyclitols have been isolated from several producing organisms. This finding gave rise to the hypothesis that the modifying enzymes may have a function in both detoxification of drug precursors and biosynthesis of antibiotics (for a review see Davies & Yagisawa, 1983). The present results show that *O*-demethyl-puromycin, the last intermediate for puromycin biosynthesis, is an inhibitor of protein synthesis (although much less active than the final product), and inhibits ribosomes from *S. alboniger*. Since, in addition to puromycin, the precursor is also inactivated by the PAC enzyme, it appears that this enzyme might be involved in the enzymic detoxification mechanism for both the antibiotic and precursor molecules. The presence of either *N*-acetyl-puromycin or *N*-acetyl-*O*-demethyl-puromycin in extracts or culture media from *S. alboniger* has not been reported (if, indeed, they have ever been looked for); nevertheless, commercial samples of puromycin are usually contaminated with small amounts of *O*-demethyl-puromycin, *N*⁶-monodemethyl-*O*-demethyl-puromycin and *N*⁶,*N*⁶-didemethyl-*O*-demethyl-puromycin, which were considered intermediates in the biosynthetic pathway for puromycin by Pattabiraman & Pogell (1969). Whether these products are derived from the *N*-acetylated precursors or represent real precursors is not known. A better knowledge of the biochemical pathway for puromycin biosynthesis might provide valuable information on the role of the PAC enzyme in this metabolic route.

The fungi *Cordyceps militaris* (Kredish & Guarino, 1961; Guarino & Kredish, 1964) and *Chrysosporium panorum* (Yamashita *et al.*, 1984), which produce aminoacyl-adenosine antibiotics, may also inactivate their antibiotic products in much the same way as does *S. alboniger*. Indeed, we have detected a puromycin acetyltransferase activity in *C. militaris* that inactivates puromycin as tested by the disc method versus *B. subtilis* (unpublished results).

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