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Biosynthesis of Puromycin by *Streptomyces alboniger*: Characterization of Puromycin *N*-Acetyltransferase†

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ABSTRACT: Puromycin *N*-acetyltransferase from *Streptomyces alboniger* inactivates puromycin by acetylating the amino position of its tyrosinyl moiety. This enzyme has been partially purified by column chromatography through DEAE-cellulose and Affigel Blue and characterized. It has an M_r of 23 000, as determined by gel filtration. In addition to puromycin, the enzyme N-acetylates *O*-demethylpuromycin, a toxic precursor of the antibiotic, and chryscandin, a puromycin analogue antibiotic. The K_m values for puromycin and *O*-demethylpuromycin are 1.7 and 4.6 μM, respectively. The *O*-demethylpuromycin *O*-methyltransferase from *S. alboniger*, which apparently catalyzes the last step in the biosynthesis of puromycin [Rao, M. M., Rebello, P. F., & Pogell, B. M. (1969) *J. Biol. Chem. 244*, 112–118], also O-methylates *N*-acetyl-*O*-demethylpuromycin. The K_m values of the methylating enzyme for *O*-demethylpuromycin and *N*-acetyl-*O*-demethylpuromycin are 260 and 2.3 μM, respectively. These findings suggest that *O*-demethylpuromycin, if present in *S. alboniger*, would be N-acetylated and then O-methylated to be converted into *N*-acetylpuromycin. It might even be possible that N-acetylation of the puromycin backbone takes place at an earlier precursor.

The aminoacyl nucleoside antibiotic puromycin (Figure 1), which is produced by *Streptomyces alboniger*, is an inhibitor of both 70S and 80S ribosomes [for reviews, see Vazquez (1979) and Cundliffe (1981)]. Although ribosomes from *S. alboniger* are sensitive to puromycin, this bacterium contains an acetyltransferase that appears to inactivate the drug by acetylating its amino position (Pérez-González et al., 1983, 1985). Inactivation of antibiotics by acetylation or phosphorylation is a common process in aminocyclitol-producing *Streptomyces*. This type of drug modification has been implicated in the resistance of these bacteria to their own antibiotics products (Cella & Vining, 1975; Thompson et al., 1982; Matsuhashi et al., 1985). However, little is known about the role that the inactivating enzymes might play in the modification of antibiotic precursors. It is possible that at least some precursors are lethal for the producing organisms, being inactivated by the modifying enzymes [for a review, see Davies & Yagisawa (1983)]. *O*-Demethylpuromycin (Figure 1), the last precursor of puromycin biosynthesis (Rao et al., 1969), inhibits the ribosomes from *S. alboniger*, although at a lower extent than puromycin. *O*-Demethylpuromycin is acetylated by an enzymic activity that is present in cell-free extracts from this organism, the resulting *N*-acetyl-*O*-demethylpuromycin being inactive as tested in vitro (Pérez-González et al., 1985). These findings suggest that, in *S. alboniger*, puromycin may not be directly acetylated and the acetylation occurs at an earlier step in the biosynthetic pathway. To investigate this possibility, we have carried out a biochemical study of PAC. The results suggest that puromycin biosynthesis proceeds via *N*-acetyl-*O*-demethylpuromycin and that acetylation occurs on the *O*-demethylpuromycin intermediate or on an earlier intermediate.

Materials and Methods

Streptomyces Strains, Media, and Cell Growth. Streptomyces lividans 1326, *S. alboniger* ATCC 12461, and *S. lividans* JN8 (carrying plasmid pFV8; Vara et al., 1985) 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 and 5 mM $MgCl_2$. Cultures were inoculated with spore suspensions that had been kept frozen in sterile 50% glycerol at −20 °C, according to Chater et al. (1982). Growth

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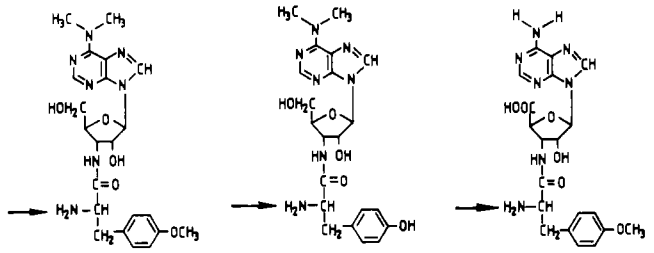

FIGURE 1: Chemical structure of puromycin, *O*-demethylpuromycin, and chryscandin. The arrows indicate the site of acetylation.

took place in an air incubator at 300 rpm.[1]

Preparation of Cell Extracts. To prepare extracts to be assayed for PAC activity, mycelia were collected from liquid cultures by centrifugation when the cultures reached an A_{660} of 0.85 (measured in a Bausch & Lomb Spectronic 20 using a 1-cm path-length tube), except when otherwise indicated. Mycelia were washed twice with TGE buffer [50 mM Tris-HCl, pH 8.5, 10% glycerol (v/v), 2 mM EDTA] by centrifugation. At this point cells can be kept at −20 °C up to 1 year without any apparent loss of PAC activity. Mycelium (16 g wet weight) and 24 g of alumina were ground in a mortar at 4 °C until most of the cells were broken, as determined by the increase in viscosity due to the release of DNA. DNase (5 μg/mL final concentration) was then added, and the paste was mixed with 20 mL of TGE buffer. The extract was centrifuged at 10000*g* at 4 °C for 10 min, and the resulting supernatant was centrifuged again at 105000*g* at 4 °C for 2.5 h. The supernatant from this last centrifugation (S-100 fraction) was taken as a source of PAC activity. If required, the S100 fraction can be stored frozen at −20 °C for 2–4 weeks without apparent loss of PAC activity.

Extracts used to determine *O*-demethylpuromycin *O*-methyltransferase activity were prepared as described above, but using 50 mM phosphate buffer, pH 7.8, and 2 mM EDTA, except when specifically indicated.

Enzyme Assays. PAC activity was determined spectrophotometrically essentially as described by Shaw (1975) for the chloramphenicol acetyltransferase enzyme. Reaction mixtures (1 mL) contained 100 mM Tris-HCl, pH 8.5, 0.04 mg/mL 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 125 μM acetyl coenzyme A, and enzyme preparation (3–25 μg). Reaction mixtures were made in a cell of 1-cm path length, which was maintained for 1–2 min at 30 °C until absorption at 412 nm was stabilized. The reaction was started by addition of puromycin or *O*-demethylpuromycin (50 μM each, except where otherwise indicated) to the sample cell and water to the reference cuvette. The increment in absorbance was related to the amount of acetyl groups transferred to puromycin by using an extinction coefficient value of 13.6 μM^{-1} cm^{-1} (Shaw, 1975). One unit of PAC activity corresponds to 1 μmol of acetyl groups transferred to puromycin or *O*-demethylpuromycin per minute under the stated conditions. As used, the assay system was not saturated by 1.5–20 milliunits of PAC activity, and the assay was linear in the absorption range used (A_{412} = 0.02–0.25).

The specific activity of *O*-demethylpuromycin *O*-methyltransferase was assayed as described elsewhere (Sankaran & Pogell, 1973) except that the reaction mixtures were extracted with a scintillation fluid containing toluene (1 L), 2,5-diphenyloxazole (4 g), and 1,4-bis(2,5-diphenyloxazolyl)benzene (50 mg). The conversion factors from cpm to enzymatic units with *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]SAM) were found to be identical with those given by Sankaran & Pogell (1973). One unit of *O*-demethylpuromycin *O*-methyltransferase activity corresponds to 1 nmol of methyl groups transferred to *O*-demethylpuromycin or *N*-acetyl-*O*-demethylpuromycin per minute. When acetyl-*O*-demethylpuromycin was used as substrate, its final concentration was 20 μM.

Protein Estimation. Protein eluting from column chromatography was determined from absorbance at 280 nm. To calculate enzyme specific activities, protein was determined as described by Bradford (1975). For very diluted enzyme preparations, a variant to this method was used (Vara & Serrano, 1981).

Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of SDS was performed as described by Laemmli (1970). Protein bands in the gels were stained as described by Fairbanks et al. (1971). Gels were photographed on Kodak Plus-X PXP-120 film and prints obtained on Kodalith Ortofilm type 3 paper. The copies were analyzed by densitometry in a Cromoscan 3 (Joyce-Loebl Ltd., Gateshead, England).

Enzymic Synthesis and Determination of the Site of Acetylation of Puromycin. The reaction mixture (10 mL), made up in 100 mM Tris-HCl, pH 8.5, 2 mM EDTA, and 10% (v/v) glycerol (TGE) buffer, contained 27.5 μmol of puromycin, 28.7 μmol of acetyl coenzyme A, and 1500 milliunits of PAC activity (from a crude S100 extract). Incubation at 30 °C for 30 min was followed by extraction of the reaction products with 50 mL of $CHCl_3$. The solvent was evaporated under vacuum at room temperature, and the desiccated material was dissolved in 0.5 mL of $CHCl_3$. The solution was applied to a preparative silica gel 60 F_{254} (Merck) TLC support, which was developped with ethyl acetate–methanol (5:1). A single band absorbing UV light and cochromatographying with chemically synthesized *N*-acetylpuromycin (R_f 0.75) was scrapped off and the powder extracted with $CHCl_3$–methanol (3:1). The extracted material was dried at 40 °C in a rotatory evaporator. The final product weighed 8 mg and was then subjected to ^{1}H NMR analysis. ^{1}H NMR spectra of both free and acetylated puromycin were obtained from 10 mM drug solution in D_2O, pH 6.2 (Stohler, 99.8%).

Chemical Synthesis of N-Acetylpuromycin. Puromycin (50 mg) was dissolved in 10 mL of H_2O and then cooled to 0 °C. Portions of acetic anhydride (up to 2 mL) were added, with occasional shaking, until they went into solution (about 30 min). The process was again repeated with another 2 mL of acetic anhydride. The solution was then lyophylized. The product was identified as *N*-acetylpuromycin by ^{1}H NMR analysis (not shown).

Chemical Synthesis of O-Demethylpuromycin and of N-Acetyl-O-demethylpuromycin. O-Demethylpuromycin (Figure 1) was synthesized as described by Pogell (1975) except that the *N*,*O*-(dicarbobenzoxy)-L-tyrosine was synthesized as described by Katchalski and Sela (1953). Its structure and purity (>97%) was determined by ^{1}H NMR analysis (not shown).

[1] Abbreviations: Ac-ODMP, acetyl-*O*-demethylpuromycin; cAMP, adenosine cyclic 3′,5′-monophosphate; DEAE, diethylaminoethyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; nm, nanometers; NMR, nuclear magnetic resonance; ODMP, *O*-demethylpuromycin; PAC, puromycin *N*-acetyltransferase; *pac*, gene for PAC; PAGE, polyacrylamide gel electrophoresis; rpm, revolutions per minute; SA, specific activity; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SDS, sodium dodecyl sulfate; TGE, 50 mM Tris-HCl, pH 8.5, 10% glycerol (v/v), and 2 mM EDTA; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; *V*, volume.

Absorption maxima and molar extinction coefficients were identical with those reported previously by Rao et al. (1969) and Pogell (1975).

N-Acetyl-*O*-demethylpuromycin was synthesized following the procedure described for the preparation of *N*-acetylpuromycin but using *O*-demethylpuromycin instead of puromycin. The product was characterized by ^{1}H NMR analysis (not shown).

Partial Purification of PAC Activity. A crude S100 extract (24 mL), obtained as described above, was applied onto a DEAE-cellulose (DE-52, Sigma) column (2.2 × 8 cm) previously equilibrated with TGE buffer. The column was washed with 63 mL of TGE, and 1.5-mL fractions were collected at a flow rate of 0.5 mL/min. None of these fractions had any PAC activity. The enzyme was then displaced with a continuous gradient of potassium acetate (0–0.5 M) made up in TGE. Fractions (numbers 50–58; Figure 2) with PAC activity were collected, pooled, and dialyzed at 4 °C overnight against 1000 volumes of TGE buffer.

A 2-mL portion of this preparation was then applied onto an Affigel Blue (1 × 5 cm) column previously equilibrated with TGE. The enzyme was eluted with TGE, 1-mL fractions being collected at a flow rate of 0.5 mL/min. Increasing the size of the column did not improve either the yield or the recovery. Indeed, recovery was drastically diminished, and the retained activity could not be eluted by high salt concentration (not shown). This protocol was repeated 6 times until all the preparation obtained from the DEAE-cellulose column passed through the Affigel Blue column.

Fractions containing PAC activity (numbers 13–25; Figure 4) were pooled and concentrated by passing the enzyme through a DEAE-cellulose column (1 × 5 cm) previously equilibrated with TGE. Enzyme activity was recovered with 6 mL of 0.5 M potassium acetate in TGE. Two 1-mL fractions containing PAC activity were collected, pooled, and dialyzed at 4 °C vs. 1000 volumes of TGE. This partially purified enzyme was immediately used for subsequent studies or stored at −20 °C until used.

Partial Purification of O-Demethylpuromycin O-Methyltransferase Activity. The procedure described by Pogell (1975) was followed to partially purify the *O*-demethylpuromycin *O*-methyltransferase activity. The crude S-100 extract contained 30 mg of protein, and the enzyme was purified 8-fold.

Determination of the Molecular Weight of PAC by Gel Filtration. A 0.8-mL aliquot of a partially purified (30-fold) PAC activity from a DEAE-cellulose column was chromatographed at 4 °C through a Sephadex G-100 column (1 × 60 cm) previously equilibrated with TGE and calibrated with blue dextran, bovine serum albumin (M_r 66 000), ovalbumin (M_r 45 000), α-chymotrypsinogen (M_r 24 000), and lysozyme (M_r 14 400). TGE was used for elution, with fractions (1 mL) being collected every 17 min. When the elution volumes were plotted against the log of the molecular weight of the standards, a straight line was obtained. From this, the molecular weight of the PAC enzyme was deduced.

Determination of Kinetic Constants. The K_m values of the PAC enzyme for puromycin, *O*-demethylpuromycin, chryscandin (Figure 1), and acetyl coenzyme A were determined with initial velocities obtained from the spectrophotometric assay (see above). The substrate concentrations varied in the range 1–10 μM for puromycin, 1–10 μM for *O*-demethylpuromycin, and 25–500 μM for chryscandin (all in the presence of 125 μM acetyl coenzyme A) and 5–50 μM for acetyl coenzyme A (in the presence of 25 μM puromycin). For each assay, 0.25–1.7 milliunits of PAC enzyme (purified 30-fold) was added. Absorption at 412 nm was continously measured for 2–5 min.

Table I: Partial Purification of PAC Enzyme

purification step	vol (mL)	tot. act. (%) [milliunits (%)]	protein (mg/mL)	sp act.	purification (*x*-fold)
S-100	24	4291 (100)	3.0	59	1
DEAE	12	3432 (82)	0.72	397	7
Affigel blue	2	1695 (39)	0.097	8741	146

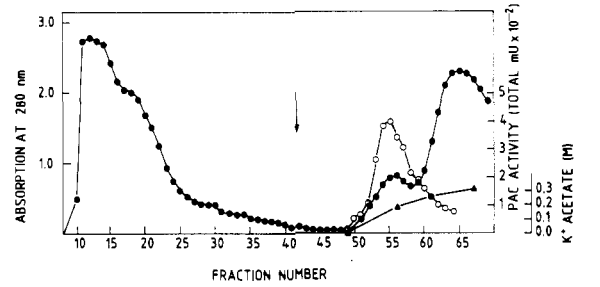

FIGURE 2: DEAE-cellulose column chromatography of PAC enzyme. See Materials and Methods for details. The arrow indicates the start of addition of potassium acetate. (●) Protein; (○) PAC activity; (▲) potassium acetate concentration.

The K_m values of the *O*-demethylpuromycin *O*-methyltransferase for both *O*-demethylpuromycin and *N*-acetyl-*O*-demethylpuromycin were determined with initial velocities obtained from the radiochemical method (see above). The substrate concentrations varied in the range 0.25–2 mM for *O*-demethylpuromycin and 2.5–20 μM for *N*-acetyl-*O*-demethylpuromycin. For each assay, 0.63 milliunit of *O*-demethylpuromycin *O*-methyltransferase (purified 8-fold) was used. Incubation of the reaction mixtures containing 50 μM [^{3}H]SAM was at 37 °C for 20–40 min.

Source of the Reagents. Reagents were obtained from Sigma (St. Louis, MO). [^{3}H]Puromycin and *S*-adenosyl-L-[*methyl*-^{3}H]methionine were obtained from Amersham (U.K.). Chryscandin was a gift from Dr. Mishio Yamashita, Fujisawa Pharmaceutical Co., Osaka, Japan.

RESULTS

Site of Acetylation of Puromycin. Our initial studies on the site of modification of puromycin by the puromycin acetyltransferase indicate that the acetylation took place on the amino position of the tyrosinyl moiety (Pérez-González et al., 1983). In order to demonstrate that this is the case, we have isolated the product of the enzymic reaction, as described under Materials and Methods, and subjected it to a ^{1}H NMR analysis. The results clearly showed that the acetylation had taken place in the tyrosinyl amino group (not shown). Thus, the *Hα* signal underwent a downfield shift of 0.69 ppm with respect to puromycin; the Hβ and Hβ′ signals shifted by less than 0.08 ppm, and all other signals in the spectrum shifted by less than 0.02 ppm. The signal of the methyl group in the acetyl residue appeared at 2.00 ppm with a relative intensity corresponding to three protons. No signals for O-acetylation appeared at all.

Partial Purification of the PAC Activity: Molecular Weight. A summary of the partial purification of PAC is given in Table I. After the DEAE-cellulose column chromatography step (Figure 2), a 7-fold purification was achieved. This preparation totally lacked any background of coenzyme A releasing activity in the absence of puromycin, indicating that it is free of acetyl coenzyme A hydrolase(s). In other preparations the purification values varied in the range 7–35-fold, depending on the combined selected fractions. Higher values

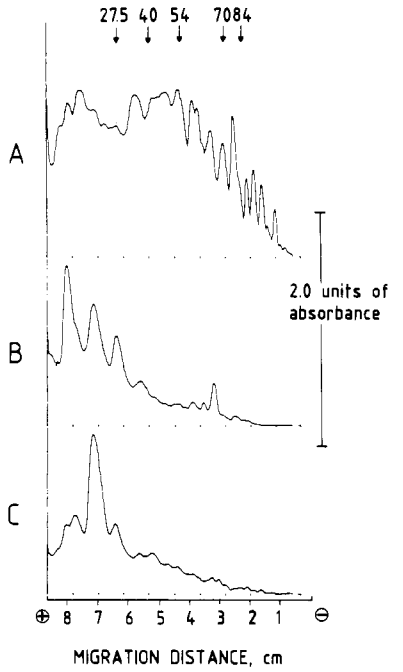

FIGURE 3: Polypeptide composition of the different fractions. See Materials and Methods for details. (A) S-100 fraction (40 μg); (B) DEAE-cellulose combined fractions (20 μg); (C) Affigel Blue combined fractions (12 μg). The arrows indicate the molecular mass in kilodaltons of the different proteins from the φ29 virus, which were used as standards (Blanco & Salas, 1984).

of purification drastically lowered the yield of PAC activity. The analysis of the polypeptide composition of the 7-fold purified preparation, by SDS–PAGE, shows an enrichment in five major peaks of protein as compared to the polypeptide pattern of the crude S100 fraction (Figure 3). Attempts to further purify the PAC enzyme by column chromatography on either CM-cellulose, hydroxylapatite, or phosphocellulose were unsuccessful since the enzyme was not retained in these columns. However, chromatography through Affigel Blue allowed further purification (Table I; Figure 4). From this column PAC activity is eluted with TGE buffer after a large peak of protein. A purification of 146-fold, with a 60% recovery of the initial activity, was achieved (Table I). SDS–PAGE of the combined active fractions shows one major peak, having a M_r of 24 000 (±500) and comprising 35% of the total analyzed protein (Figure 3C). This molecular weight is in agreement with that found for PAC by gel filtration (23 000 ± 1500) and suggests that the major peak represents the PAC enzyme. In different preparations the purification values of PAC enzyme after chromatography through Affigel Blue varied in the range 149–370-fold.

Substrate Specificity of the PAC Enzyme. S. alboniger is resistant (200 μg/mL) in vivo to the aminoacyl nucleoside antibiotics blasticidin S, sparsomycin, and gougerotin. However, *S. lividans* is sensitive to these antibiotics at concentrations of 40 μg/mL. However, none of them was acetylated by either a crude S100 fraction or a 30-fold purified preparation of PAC. Therefore, the resistance of *S. alboniger* to these drugs must be due to some other cause. The related antibiotics sinefungin, A201A, and nucleocidin, whose activity against *S. alboniger* is unknown, were neither modified. The PAC activity in the presence of puromycin was not affected by these amino nucleosides, indicating that they are not recognized by the enzyme.

The aminocyclitol antibiotics neomycin, gentamicins C_1 and C_{1a}, kanamycins A and B, sisomicin, and neamine and the antibiotic chloramphenicol can be acetylated by a variety of

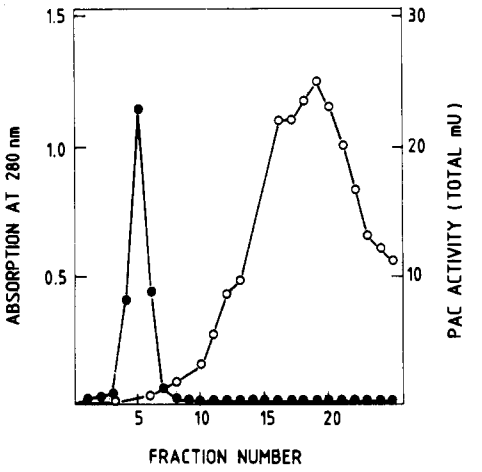

FIGURE 4: Affigel Blue column chromatography of PAC enzyme. See Materials and Methods for details. (●) Protein; (○) PAC activity.

modifying enzymes from either *Streptomyces* or clinical isolates of resistant bacteria. Again the PAC enzyme did not acetylate any of these antibiotics (not shown).

Puromycin amino nucleoside and tyrosine are the two main chemical moieties of puromycin and, therefore, were assayed as substrates for the PAC activity. Each of these compounds was not a substrate for PAC or does not compete with puromycin in the acetylation reaction (not shown).

O-Demethylpuromycin (Figure 1) is the intermediate precursor for puromycin biosynthesis (Rao et al., 1969). Chryscandin (Figure 1) is a puromycin analogue antibiotic isolated from the fungi *Chrysosporium panorum* (Yamashita et al., 1984). We have shown previously that *O*-demethylpuromycin inhibits protein synthesis, although at a lower extent than puromycin, and that it can be N-acetylated by an activity present in an S-100 fraction from *S. alboniger* (Pérez-González et al., 1985). Chryscandin also blocks protein synthesis by a mechanism similar to that of puromycin (J. A. Pérez-González, J. Vara, and A. Jiménez, unpublished results). Here we show that both *O*-demethylpuromycin and chryscandin are substrates for PAC activity, as determined by the spectrophotometric assay (Table II), and that the ratio of PAC specific activity for puromycin to that for either *O*-demethylpuromycin or chryscandin is practically identical in a crude S-100 extract and in a 35-fold purified preparation of the PAC enzyme (Table II). Moreover, this ratio was also similar in an S-100 extract and in a 7-fold purified preparation of PAC obtained from *S. lividans* JN8. This strain carries plasmid pFV8, which contains the *pac* gene in a 2.2-kb DNA fragment from *S. alboniger* (Vara et al., 1985). Therefore, the same enzyme seems to acetylate puromycin, *O*-demethylpuromycin, and chryscandin.

The possibility that the PAC activity could use acyl coenzymes A different from acetyl coenzyme A as substrates for the modification of puromycin was studied. Out of eight cofactors tested (propanoyl, butanoyl, valeryl, hexanoyl, heptanoyl, malonyl, succinyl, and glutaryl derivatives, all at 125 μM), only malonyl coenzyme A could partially (15%) replace acetyl coenzyme A. When each of these cofactors and acetyl coenzyme A were simultaneously added to the reaction mixtures, the acetylating activity was lowered by 15–35%, except for propanoyl coenzyme A, which inhibited by 85% the formation of acetylpuromycin (not shown). Thus acyl coenzymes A may compete with acetyl coenzyme A for binding to the PAC enzyme although only malonyl coenzyme A can partially replace acetyl coenzyme A as a substrate for the reaction.

Table II: Comparative Substrate Capacity of Puromycin, *O*-Demethylpuromycin, and Chryscandin for PAC Enzyme[a]

source of PAC	enzyme prepn	substrate	sp act.	ratio: SA (puromycin)/ SA (ODMP)[b]	ratio: SA (puromycin)/ SA (chryscandin)
S. alboniger	S-100	puromycin	45.0		
		ODMP[b]	45.7	0.98	
		chryscandin	11.8		3.81
	DEAE-cellulose	puromycin	1603		
		ODMP[b]	1537	1.04	
		chryscandin	396		4.05
S. lividans JN8	S-100	puromycin	3308		
		ODMP[b]	2867	1.15	
		chryscandin	901		3.67
	DEAE-cellulose	puromycin	19933		
		ODMP[b]	20268	0.98	
		chryscandin	5191		3.84

[a] The assays were performed as described under Materials and Methods. Chryscandin was used at 400 μM final concentration. [b] *O*-Demethylpuromycin.

Effect of Different Assay Conditions on the PAC Activity. The study of the effect of several pH values on the PAC activity indicated a maximum of acetylation in the range 7.8–9.5 (100 mM Tris-HCl buffers). Even at pH 9.5 the activity was 90% of that at the maximum value at pH 8.5. The effect of a variety of salts and salt concentrations on the activity of PAC enzyme was also studied. Neither the Cl^- nor acetate salts of NH_4^+, Na^+, or K^+ (each at 5 mM) showed any effect on PAC activity. However, Mg^{2+} concentrations close to 5 mM slightly stimulated the enzymic reaction.

The –SH group reagents Cu^{2+} (5 mM) and Hg^{2+} (8 μM) completely inhibited the PAC activity. This inhibition was totally reversed by 5 mM EDTA in the case of Cu^{2+} but only partially in that of Hg^{2+}, suggesting a strong binding of this cation to the –SH groups of PAC. These findings indicate that the –SH groups of PAC are required for its catalytic action.

Stability of the PAC Activity. Glycerol largely stabilized PAC activity. Thus, dialysis of a DEAE-cellulose preparation of PAC against 10 mM Tris-HCl, pH 8.5, reduced its activity by 62%. Addition of glycerol (10% v/v final concentration) after dialysis only restored 54% of the activity of the undialyzed preparation. Similarly, poly(ethylene glycol) stabilized the enzyme, although it was not as effective as glycerol; 20 mM Tris–phosphate, pH 8.5 buffer could substitute 100 mM Tris-HCl, pH 8.5 buffer with no apparent change of the acetylation values (not shown). Therefore, the effect of different salts on the stability of PAC enzyme was assayed with a preparation containing glycerol, which was incubated in the presence of different chemical agents both at 37 and at 0 °C for 1 h. When incubation took place at 37 °C, NH_4^+ (5 mM), Na^+ (5 mM), K^+ (5 mM), and Mg^{2+} (2 mM), each as Cl^- or acetate salts, inactivated by 60–85% the PAC activity. When incubation took place at 0 °C, those salts slightly inhibited (5–20%) the activity of PAC. The effect of β-mercaptoethanol or DTT was not studied since these compounds interfere with the spectrophotometric assay.

Effect of Temperature on the PAC Activity. During the course of this work *S. alboniger* was grown at 30 °C, and therefore, we have mostly used this temperature to assay the PAC enzyme. The study of the effect of other temperatures on its activity showed that the enzyme was inactivated after incubation at 55, 60, and 65 °C for 8, 5, and 2 min, respectively.

Kinetic Constants of the PAC Enzyme and the O-Demethylpuromycin O-Methyltransferase for a Variety of Substrates. The K_m values were obtained by the double-reciprocal plot (Lineweaver–Burk) as well as by the direct plot methods (Eisenthal et al., 1974; De Miguel, 1974). With both procedures the results were practically identical. The K_m value of the PAC enzyme for puromycin was 1.72 (±0.20) μM and for acetyl coenzyme A was 21.0 (±0.2) μM. Even at 1 mM final concentration of puromycin, no inhibition of PAC activity was detected. The K_m values of PAC enzyme for *O*-demethylpuromycin and chryscandin were 4.56 (±0.10) μM and 84.5 (±9.2) μM, respectively.

We have found that the *O*-demethylpuromycin *O*-methyltransferase activity from *S. alboniger* (Rao et al., 1969) also methylates *N*-acetyl-*O*-demethylpuromycin and that the reaction is linear for at least 60 min (results not shown). The K_m value of this enzyme for *O*-demethylpuromycin is 260 (±15) μM, similar to that (210 μM) found previously (Rao et al., 1969). However, the K_m value of the *O*-demethylpuromycin *O*-methyltransferase for *N*-acetyl-*O*-demethylpuromycin is 2.3 (±0.15) μM. Only a slight substrate inhibition (to 20%) of this activity was found at 2 mM final concentration of *N*-acetyl-*O*-demethylpuromycin (not shown).

It is known that puromycin inhibits the *O*-demethylpuromycin *O*-methyltransferase (Sankaran & Pogell, 1975). Therefore, we have comparatively determined the effects of either puromycin or *N*-acetylpuromycin on the methylation reaction of both substrates *O*-demethylpuromycin and *N*-acetyl-*O*-demethylpuromycin. It was found that *N*-acetylpuromycin inhibited more strongly the enzymic methylation of these two substrates than did puromycin (Figure 5).

Dependence of PAC Activity on Age of Liquid Culture. We have comparatively studied the distribution of PAC and *O*-demethylpuromycin *O*-methyltransferase activities during the growth of *S. alboniger* in liquid culture (Figure 6). Both activities were absent in the spores from which this culture was commenced or during the lag phase. Both activities appeared at the early stages of the log phase, progressively increased with the increase in the optical density readings, and reached a sharp and maximum value at the late periods of the log phase. From this point both activities sharply decreased, and while a significant amount of PAC activity remained in the advanced stationary phase, the *O*-demethylpuromycin *O*-methyltransferase activity totally disappeared at this growth stage. The distribution profile of the *O*-demethylpuromycin *O*-methyltransferase presented in Figure 6 is identical with that obtained previously for this enzyme by Sankaran & Pogell (1975), although in our study the values of the specific activities were 10 times lower than theirs. This may be attributed to differences in the composition of the culture media. Indeed, the values of the specific activities of the *O*-demethylpuromycin

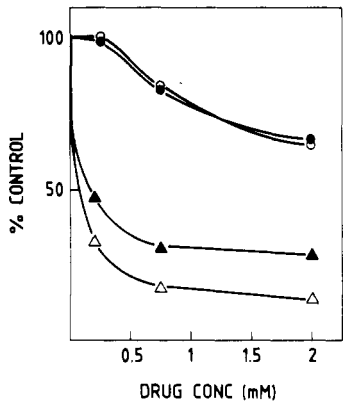

FIGURE 5: Feedback inhibition of *O*-demethylpuromycin *O*-methyltransferase activity by puromycin and *N*-acetylpuromycin. See Materials and Methods for details. In the control, 100% activity corresponds to an SA of 63 milliunits/mg of protein. (●) Substrate, *O*-demethylpuromycin; inhibitor, puromycin. (▲) Substrate, *O*-demethylpuromycin; inhibitor, *N*-acetylpuromycin. (○) Substrate, *N*-acetyl-*O*-demethylpuromycin; inhibitor, puromycin. (△) Substrate, *N*-acetyl-*O*-demethylpuromycin; inhibitor, *N*-acetylpuromycin.

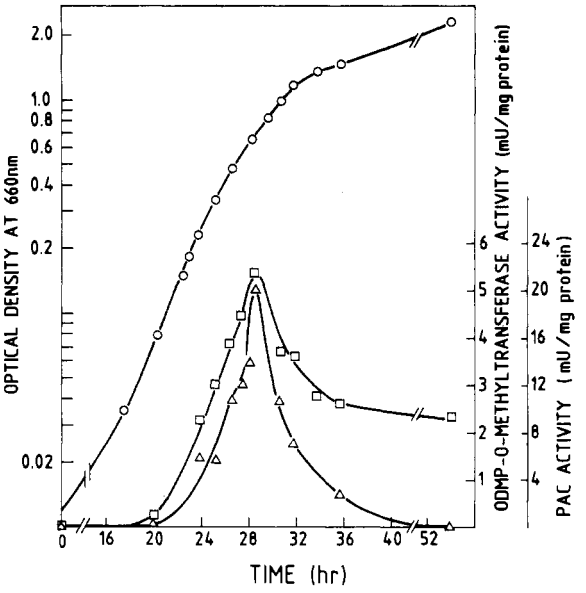

FIGURE 6: Distribution of both PAC (□) and *O*-demethylpuromycin *O*-methyltransferase (△) activities during growth of *S. alboniger*. See Materials and Methods for details. (○) Optical density.

O-methyltransferase varied widely, depending on the particular liquid medium employed (Sankaran & Pogell, 1975).

Discussion

Biochemical Properties of the PAC Enzyme. The results presented in this paper show that puromycin acetyltransferase from *S. alboniger* ATCC 12461 acetylates the primary amino group of the tyrosinyl moiety of the antibiotic, in agreement with results published previously (Perez-González et al., 1983). The enzyme has a molecular weight of approximately 23 000. No evidence for the presence of enzyme aggregates has been found. Therefore, the PAC molecule differs from both the aminocyclitol and the chloramphenicol acetyltransferases, which are usually present as tetramers (Shaw, 1975; Williams & Northrop, 1976; Coombe & George, 1982). The PAC enzyme appears to be highly specific for a small number of substrates since it only acetylates puromycin, *O*-demethylpuromycin, and the puromycin analogue antibiotic chryscandin.

A Possible Implication of PAC Enzyme in the Biosynthesis of Puromycin. Pattabiraman & Pogell (1969) found that a commercial sample of puromycin was contaminated by small amounts of N^6,N^6,O-tridemethylpuromycin, N^6,O-didemethylpuromycin, and *O*-demethylpuromycin. Therefore, they proposed that the biosynthesis of puromycin probably proceeds through these intermediates and in that order. However, *O*-demethylpuromycin, N^6,N^6-didemethylpuromycin, and the puromycin analogue antibiotics chryscandin, 3′-(homocitrullylamino)-3′-deoxyadenosine, and 3′-(lysylamino)-3′-deoxyadenosine are inhibitors of protein synthesis (Yamashita et al., 1984; Pêrez-Gonzalez et al., 1985; Lichtenthaler et al., 1979; J. A. Pêrez-Gonzâlez, J. Vara, and A. Jiménez, unpublished results); therefore, it may be assumed that the N^6,N^6,O-tridemethylpuromycin precursor will also be toxic. Ribosomes from *S. alboniger* are known to be inhibited by puromycin and *O*-demethylpuromycin (Pêrez-Gonzâlez et al., 1985). The response of these ribosomes toward other presumptive precursors has not been tested, but it seems safe to assume that they also will be sensitive. Thus, it seems likely that *S. alboniger* must present a detoxifying mechanism vs. these harmful compounds.

We have shown that PAC acetylates puromycin, *O*-demethylpuromycin, and the puromycin analogue chryscandin. This last antibiotic closely resembles the first intermediate of the biosynthetic pathway of puromycin proposed by Pattabiraman & Pogell (1969). Therefore, we propose that N-acetylation may occur on the first toxic intermediate of the pathway (N^6,N^6,O-tridemethylpuromycin), inactivating it and the following intermediates. Consequently, the biosynthetic pathway for puromycin might proceed via compounds I–VI of Figure 7. Evidently, if any of the precursors (II–V) is methylated prior to its N-acetylation, it could still be inactivated by the *N*-acetyltransferase.

Concerning even earlier steps of the pathway, it is known that [U-^{14}C]adenosine is a direct precursor for the 3′-aminodeoxyadenosine moiety of puromycin in *S. alboniger* (Suhadolnik, 1981; Goodchild, 1982). Hence, this precursor would only be required to be linked to tyrosine to be converted into the N^6,N^6,O-tridemethylpuromycin precursor. Since tyrosine is not recognized by the PAC enzyme, it would be the N^6,N^6,O-tridemethylpuromycin molecule that is the first precursor to be N-acetylated, according to our proposal. Alternatively, it is also possible that N-acetylation occurs on a putative activated form of tyrosine. This would keep all successive intermediates in an acetylated and, therefore, inactive form during puromycin biosynthesis (Figure 7).

This modified pathway is supported by the following data. First, the K_m value (2.3 μM) of the methyltransferase activity for *N*-acetyl-*O*-demethylpuromycin (compound IV; Figure 7) is 2 orders of magnitude lower than the K_m for *O*-demethylpuromycin (260 μM). This finding suggests that it is the former compound rather than the latter that is the physiological substrate for the methylase. Second, the K_m values of the PAC enzyme for puromycin (1.7 μM), *O*-demethylpuromycin (4.6 μM), and chryscandin (84 μM) are low, suggesting that in *S. alboniger* PAC would inactivate any of the intermediates of the pathway that would be present in a nonacetylated form.

The possibility of a detoxifying modification by the PAC enzyme on an early intermediate in the biosynthesis of puromycin suggests that a similar key role may be proposed for some of the antibiotic inactivating enzymes that are present in antibiotic-producing organisms, particularly when precursor molecules are toxic. Indeed, it has been suggested that streptomycin biosynthesis proceeds via phosphorylated streptidin, and it seems likely that this and all subsequent inter-

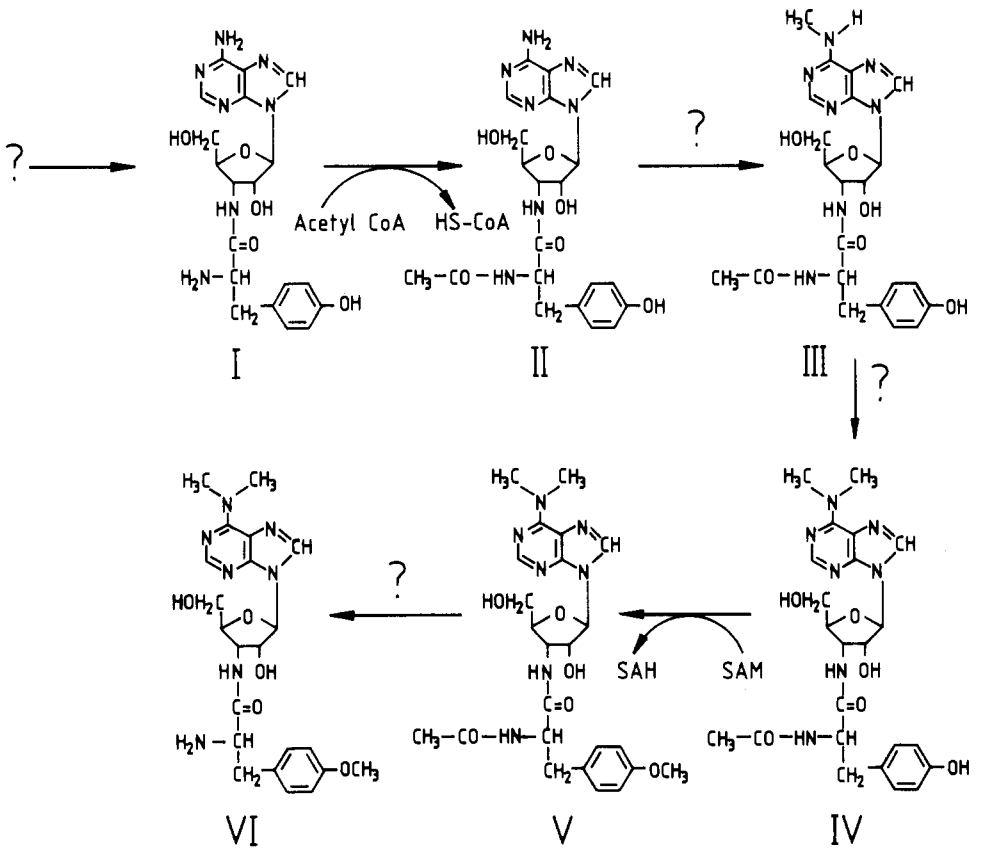

FIGURE 7: Schematic view of a possible pathway for puromycin biosynthesis [see also Pattabiraman & Pogell (1969)].

mediates in the pathway are biologically inactive [Walker & Walker, 1967; see also Cundliffe (1984)].

The synthesis of intact puromycin would require an *N*-acetylesterase activity in *S. alboniger* to deacetylate *N*-acetylpuromycin, probably at the cellular exclusion step. The presence of this enzyme has not yet been investigated in *S. alboniger*. In this respect it might be thought that the presumptive precursors found in a commercial sample of puromycin (Pattabiraman & Pogell, 1969) are derived from the relevant N-acetylated compounds. Evidence for the presence of *O*-acetylesterase has been obtained with several *Streptomyces* species, including the producer of chloramphenicol (Nakano et al., 1977).

Regulation of the Expression of PAC Enzyme. The expression of both PAC and *O*-demethylpuromycin *O*-methyltransferase enzymes appears to be regulated by a certain as yet unknown mechanism(s) (Sankaran & Pogell, 1975; this work). The finding that both PAC and *O*-demethylpuromycin *O*-methyltransferase show similar patterns of expression during growth of the *S. alboniger* cell culture (Figure 6) might suggest that they are regulated by a common mechanism. We think this is not the case. We have found that several plasmids containing the gene for puromycin *N*-acetyltransferase (Vara et al., 1985) also carry the gene for *O*-demethylpuromycin *O*-methyltransferase since they are closely linked. In *S. lividans* transformants carrying these plasmids, expression of PAC enzyme appears to be constitutive, while the expression of the *O*-methyltransferase has a profile similar to that found in *S. alboniger* (Figure 6; J. Vara and A. Jimenez, unpublished results). These results suggest that there is not a common mechanism of induction for the expression of both genes. At present, little is known on the mode of induction of these enzymes. It has been suggested that a derivative of adenine could be the specific inducer of the *O*-methyltransferase and that this derivative could also be a precursor of puromycin (Sankaran & Pogell, 1975). Moreover, the interesting possibility that the regulation of the *O*-methyltransferase could be mediated by cAMP is supported by some data (Sankaran & Pogell, 1973), although the role (if any) of the cAMP has not yet been established in *Streptomyces*.

We have shown that *N*-acetylpuromycin inhibits the *O*-methyltransferase enzyme (Figure 5), suggesting that this effect may be implicated in a regulatory function. Thus, the accumulation of *N*-acetylpuromycin may block its own biosynthesis in a feedback-type mechanism. On the other hand, we have shown that the PAC enzyme is not inhibited by either puromycin or *N*-acetylpuromycin. This finding is not surprising since this enzyme most likely protects *S. alboniger* against the toxic effects of puromycin; such inhibition could lead to suicide.

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Registry No. PAC, 87110-39-2; ODMP, 3598-42-3; NAc-ODMP, 99098-27-8; Ac-CoA, 72-89-9; *O*-demethyl-*O*-methyltransferase, 37257-04-8; puromycin, 53-79-2; chryscandin, 86936-90-5; *N*-Ac-puromycin, 22852-13-7; glycerol, 56-81-5.

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Calcium Binding to Complexes of Calmodulin and Calmodulin Binding Proteins

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ABSTRACT: The free energy of coupling for binding of Ca^{2+} and the calmodulin-sensitive phosphodiesterase to calmodulin was determined and compared to coupling energies for two other calmodulin binding proteins, troponin I and myosin light chain kinase. Free energies of coupling were determined by quantitating binding of Ca^{2+} to calmodulin complexed to calmodulin binding proteins with Quin 2 to monitor free Ca^{2+} concentrations. The geometric means of the dissociation constants ($\bar{K}_d$) for Ca^{2+} binding to calmodulin in the presence of equimolar rabbit skeletal muscle troponin I, rabbit skeletal muscle myosin light chain kinase, and bovine heart calmodulin sensitive phosphodiesterase were 2.1, 1.1, and 0.55 μM. The free-energy couplings for the binding of four Ca^{2+} and these proteins to calmodulin were −4.48, −6.00, and −7.64 kcal, respectively. The Ca^{2+}-independent K_d for binding of the phosphodiesterase to calmodulin was estimated at 80 mM, indicating that complexes beween calmodulin and this enzyme would not exist within the cell under low Ca^{2+} conditions. The large free-energy coupling values reflect the increase in Ca^{2+} affinity of calmodulin when it is complexed to calmodulin binding proteins and define the apparent positive cooperativity for Ca^{2+} binding expected for each system. These data suggest that in vitro differences in free-energy coupling for various calmodulin-regulated enzymes may lead to differing Ca^{2+} sensitivities of the enzymes.

Calmodulin (CaM)[1] mediates Ca^{2+} stimulation of a number of enzymes and is implicated in Ca^{2+} regulation of a variety of cellular functions [for reviews, see Cheung (1980), Rasmussen (1981), Klee & Vanaman (1982), and Manalon & Klee (1984)]. Recent work with intracellularly trapped fluorescent Ca^{2+} chelators (Tsien et al., 1982) indicates that free Ca^{2+} concentrations vary from 100–200 nM at rest to 500–1000 nM upon stimulation in rat myocytes (Williamson et al., 1983), PC-12 cells (Meldolesi et al., 1984), hepatocytes (Joseph et al., 1984), lymphocytes (Pozzan et al., 1982), and thymocytes and fibroblasts (Hesketh et al., 1985). However,

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[1] Abbreviations: CaM, calmodulin; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; Quin 2, 2-[[2-[bis-(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis-(carboxymethyl)amino]quinoline; Tris, tris(hydroxymethyl)aminomethane.

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