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Biochemical Characterization of Two Cloned Resistance Determinants Encoding a Paromomycin Acetyltransferase and a Paromomycin Phosphotransferase from *Streptomyces rimosus* forma *paromomycinus*

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The mechanism conferring resistance to paromomycin in *Streptomyces rimosus* forma *paromomycinus*, the producing organism, was studied at the level of both protein synthesis and drug-inactivating enzymes. Ribosomes prepared from this organism grown in either production or nonproduction medium were fully sensitive to paromomycin. A paromomycin acetyltransferase and a paromomycin phosphotransferase, both characteristic of the producer, were highly purified from extracts prepared from two *Streptomyces lividans* transformants harboring the relevant genes inserted in pIJ702-derived plasmids. In vitro, paromomycin was inactivated by either activity. In vivo, however, *S. lividans* clones containing the gene for either enzyme inserted in the low-copy-number plasmid pIJ41 were resistant to only low levels of paromomycin. In contrast, an *S. lividans* transformant containing both genes inserted in the same pIJ41-derived plasmid displayed high levels of resistance to paromomycin. These results indicate that both genes are required to determine the high levels of resistance to this drug in the producing organism. Paromomycin is doubly modified by the enzymes. However, whereas acetylparomomycin was a poorer substrate than paromomycin for the phosphotransferase, phosphorylparomomycin was modified more actively than was the intact drug by the acetyltransferase. These findings are discussed in terms of both a permeability barrier to paromomycin and the possible role(s) of the two enzymes in the biosynthetic pathway of this antibiotic.

The process(es) that confers resistance to aminocyclitol antibiotics in those actinomycetes that produce them appears to be complex. Mechanisms for self-protection involve either enzymatic inactivation of the antibiotics by *O*-phosphotransferases (APH) or *N*-acetyltransferases (AAC) and/or ribosomal modification (by 16S rRNA methylases) (4, 22). Study of these systems is facilitated by cloning the genes determining resistance and expressing them in a drug-sensitive organism such as *Streptomyces lividans*. Thus, *Streptomyces hygroscopicus*, the producer of hygromycin B, has been reported to contain a single gene for a hygromycin B APH (15). In contrast, *Streptomyces fradiae*, the neomycin producer, contains two genes for resistance, encoding either APH or AAC activity (24). Interestingly, *S. lividans* clones harboring either activity display only low levels of resistance to neomycin, although an *S. lividans* clone carrying both genes is resistant to high drug concentrations (24). Antibiotic-inactivating enzymes have not been found in *Streptomyces tenjimariensis*, the producer of istamycin (28, 29). Instead, the strain produces a 16S rRNA methylase which renders ribosomes resistant to this drug (22). Similar results have been obtained with *Micromonospora purpurea*, a gentamicin producer (20).

Other actinomycetes present an even more complex picture. *Streptomyces kanamyceticus* has both resistant ribosomes and AAC activity (16), and *Streptomyces tenebrarius* contains resistant ribosomes in addition to AAC and APH activities (30). The reasons for this multiplicity of mechanisms are not clear, although the development or acquisition of the genetic determinant(s) for resistance in any actinomycete may have been imposed by the chemical composition of the antibiotic, its mode and site of action, and/or its biosynthetic pathway. The expression of a gene for resistance caused by a metabolic constraint seems to occur in *S. kanamyceticus*, in which resistant ribosomes were found to be present only in cells grown in a production medium (16).

Streptomyces rimosus forma *paromomycinus* produces the aminocyclitol antibiotic paromomycin and contains both a paromomycin phosphotransferase PPH and a paromomycin AAC (18). The genes encoding these enzymes from *S. rimosus* have been cloned in *S. lividans* (14, 18). In this work, we examined the biochemical properties of the enzymes. We also considered how these genes, either individually or in concert, are implicated in determining resistance to paromomycin in *S. lividans*.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. The paromomycin producer *S. rimosus* forma *paromomycinus* NRRL 2455 was provided by D. T. Wicklow (Northern Regional Research Center, Peoria, Ill.). *S. lividans* 1326, the host for cloning *Streptomyces* plasmids, and plasmids pIJ41 (23) and pIJ702 (10) were provided by D. A. Hopwood (John Innes Institute, Norwich, England). Plasmid pMJ1.2 (a pIJ702 derivative) encoding the PPH enzyme from *S. rimosus*, was described elsewhere (18). Other plasmids were obtained as indicated in Fig. 1.

S. rimosus was grown in liquid production medium MAS, which contained (per liter): 20 g of soluble starch (E. Merck AG, Darmstadt, Federal Republic of Germany), 20 g of maltose, 10 g of $NaNO_3$, and 30 g of soybean meal. The pH was adjusted to 7.0. *S. lividans* was grown on liquid YEME

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medium (8) supplemented with 34% sucrose and 5 mM $MgCl_2$. Solid medium for *Streptomyces* spp. was R5 (2). Resistance to paromomycin was examined in minimal solid medium (7).

Cloning procedures. Plasmid DNA was prepared as described elsewhere (11). DNA recombinant techniques and transformation of *S. lividans* were also carried out as described elsewhere (23, 26).

Preparation of cell extracts. Cell fractions intended for poly(U)-directed polyphenylalanine synthesis were obtained as reported elsewhere (19). To prepare the soluble fraction (S100) for purification of enzyme activities, mycelia were collected by centrifugation and washed once with buffer B (10 mM Tris hydrochloride [pH 7.4], 10 mM $MgCl_2$, 50 mM NH_4Cl, 5 mM 2-mercaptoethanol). Mycelia were broken with alumina (1.5 to 2 g/g [wet weight] of mycelium) at 4°C, resuspended in buffer B, and centrifuged at 17,000 × *g* for 10 min. The resulting supernatant was treated with DNase (5 μg/ml) and centrifuged at 100,000 × *g* for 2 h. The supernatant (S100 fraction) was used immediately or kept at −70°C.

Enzyme assays. The methods used to assay PPH and AAC activities were based on the radiochemical assay described by Haas and Dowding (5). For PPH, reaction mixtures (50 μl) contained, except as otherwise indicated, 100 mM Tris hydrochloride (pH 7.4), 300 μM [γ-^{32}P]ATP (specific activity, 300 mCi/mmol), 1 mM $MgCl_2$, 100 μM antibiotic, and 10 ng of purified PPH. For AAC, reaction mixtures (50 μl) contained, except as otherwise indicated, 10 mM Tris hydrochloride (pH 7.4), 30 mM $MgCl_2$, 200 μM [1-^{14}C]acetyl coenzyme A (acetyl-CoA; specific activity, 57 mCi/mmol), 100 μM antibiotic, and 30 ng of purified AAC. Reactions took place at 30°C for 20 min (except as otherwise indicated). Portions (35 μl each) were then spotted on phosphocellulose (P-81; Whatman, Inc., Clifton, N.J.) strips, which were immediately immersed in tap water, washed several times with distilled water, and dried. Radioactivity was estimated in a liquid scintillation spectrometer. One unit of PPH or AAC activity was defined as the amount of protein catalyzing the modification of 1 nmol of paromomycin per min at 30°C.

Inactivation of paromomycin. Inactivation of paromomycin by either phosphorylation or acetylation was determined by the paper disk method, using *Bacillus subtilis* as the sensitive organism (17).

Purification of enzyme activities. PPH activity was purified by affinity column chromatography essentially as described earlier (31) except that a 50% ammonium sulfate cut of an S100 fraction from *S. lividans*(pMJ1.2) (see Fig. 1) was used and paromomycin (instead of hygromycin B) was coupled to epoxy-activated Sepharose 6B. The final resin was stable for several months at 4°C. PPH was eluted with a 2-mg/ml solution of paromomycin in 10 mM Tris hydrochloride buffer (pH 7.4), 10 mM $MgCl_2$, 400 mM $(NH_4)_2SO_4$, and 6 mM 2-mercaptoethanol. Paromomycin was removed from the enzyme preparation by successive dialysis and a short incubation with ATP (31). PPH was purified 42-fold.

Purification of AAC activity was also achieved by affinity column chromatography. The resin was prepared as described elsewhere (31) except that epoxy-activated Sepharose 6B (1.25 g) was treated with 40 ml of 0.1 M sodium carbonate (pH 10.85) containing 20 mg of paromomycin sulfate per ml. The final resin was stable at 4°C for only a few weeks. A 40 to 60% ammonium sulfate cut was applied to the column, and enzyme was eluted by applying a 50- to 500-mM NaCl gradient made up in buffer B. The active fractions were concentrated by chromatography through a DEAE-cellulose column, from which AAC enzyme was eluted with 250 mM NaCl in buffer B. Finally, AAC activity was purified by Bio-Gel P-200 (Bio-Rad Laboratories, Richmond, Calif.) column chromatography. AAC was purified 377-fold.

Protein concentrations were estimated as described elsewhere (3). Proteins eluted from columns were detected by continuous recording of A_{280}.

Determination of enzyme molecular weights. A sample of each of the purified PPH and AAC activities was combined with molecular weight marker proteins. The resulting mixtures were passed, independently, through a Bio-Gel P-200 column. Enzyme activities and A_{280} were measured in each fraction of the eluates.

In addition, molecular weights and purities of the enzyme preparations were estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described by Laemmli (12).

Determination of kinetic constants. The K_m values of PPH and AAC for the various substrates were determined by using initial velocities. For PPH, reaction mixtures (50 μl) were made up in 10 mM Tris hydrochloride (pH 7.5)–1 mM $MgCl_2$. The specific activity of [γ-^{32}P]ATP was 300 Ci/mol, and the substrate concentrations varied from 1 to 12 μM for paromomycin (in the presence of 300 μM ATP) and 25 to 800 μM for ATP (in the presence of 100 μM paromomycin). Reaction mixtures were prewarmed at 30°C, and the assays were started by adding 17.4 and 104.4 mU of PPH for paromomycin and ATP, respectively. Incubation was continued for 4 min.

For AAC, reaction mixtures (50 μl) were made up in 10 mM Tris hydrochloride (pH 8.5)–30 mM $MgCl_2$. The specific activity of [1-^{14}C]acetyl-CoA was 57 mCi/mmol, and the substrate concentrations were varied between 1 and 5 μM for paromomycin (in the presence of 200 μM [1-^{14}C]acetyl-CoA) and between 10 and 225 μM for [1-^{14}C]acetyl-CoA (in the presence of 100 μM paromomycin). No higher concentrations of acetyl-CoA were used because the acetylation reaction was then inhibited. Reaction mixtures were prewarmed at 30°C, and the assays were started by addition of 1.8 and 28.8 U of AAC for paromomycin and acetyl-CoA, respectively. Incubation was continued for 10 min.

Purification of phosphorylparomomycin. Paromomycin was phosphorylated with [γ-^{32}P]ATP of low specific activity and purified PPH enzyme. Phosphorylparomomycin was purified by column chromatography through Amberlite IRC-50 (Rohm & Haas Co., Philadelphia, Pa.) and preparative thin-layer chromatography on Silica Gel 60 F_{254} (Merck). A sample (100 μg) of final product had no activity on *B. subtilis* as determined by the disk method. Activity was recovered after treatment of phosphorylparomomycin with alkaline phosphatase.

RESULTS AND DISCUSSION

Effect of paromomycin on polyphenylalanine synthesis by *S. rimosus* ribosomes. Poly(U)-directed polyphenylalanine synthesis by homologous and heterologous combinations of supernatant and ribosome fractions from *S. lividans* and *S. rimosus* showed that *S. rimosus* ribosomes were sensitive to paromomycin (10-μM drug concentrations giving higher than 90% inhibition). This sensitivity was independent of the growth conditions, in either YEME or production (MAS) medium, of *S. rimosus*. In addition, supernatants from *S. rimosus* inactivated paromomycin in the presence of either ATP or acetyl-CoA as determined by the disk assay against *B. subtilis* (results not shown). These data suggest that

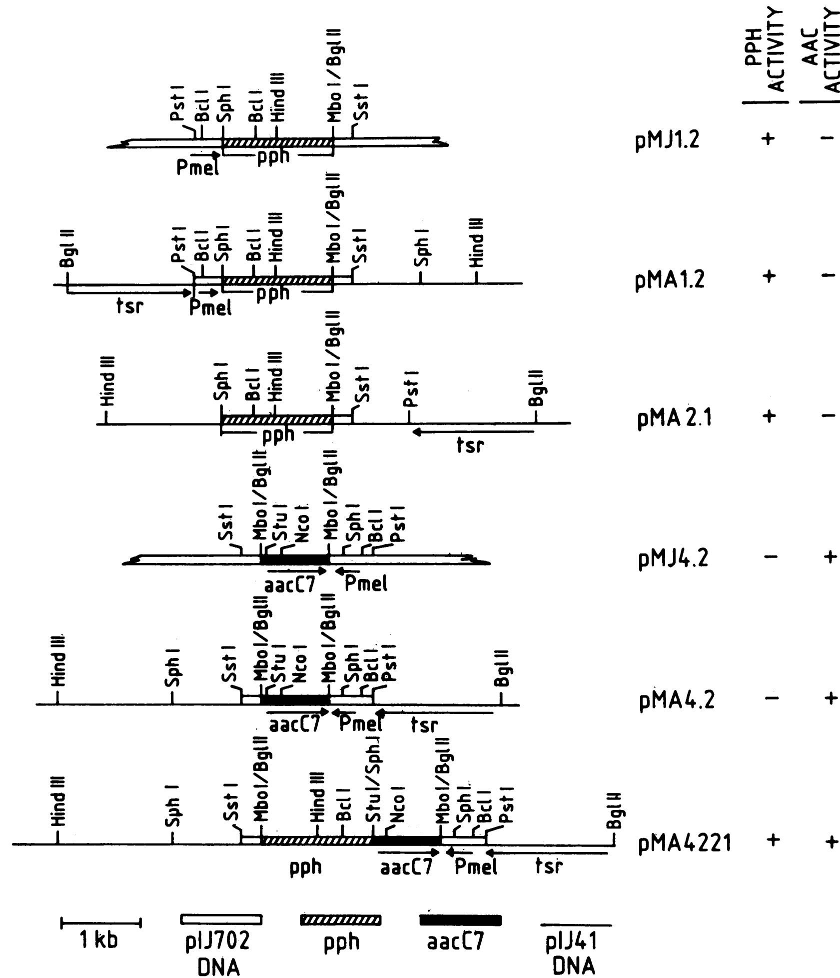

FIG. 1. Restriction maps of *Streptomyces* plasmids. The restriction map of plasmid pMJ1.2 was taken from Pérez-González and Jiménez (18). To prepare pMA1.2, the *Pst*I-*Sst*I DNA fragment, containing *pph*, from pMJ1.2 was ligated to the *Pst*I-*Sst*I replicon fragment from pIJ41 (22). To prepare pMA2.1, the *Sph*I-*Sst*I DNA fragment, containing *pph*, from pMJ1.2 was ligated to the *Sph*I-*Sst*I replicon fragment from pIJ41 (22). To obtain pMJ4.2, plasmid pMJ4 (13) was partially digested with *Mbo*I, and the resulting DNA fragments were ligated to *Bgl*II-restricted pIJ702. *S. lividans* protoplasts were then transformed with the ligation mixture, and transformants were selected in the presence of paromomycin. One of these transformants contained MJ4.2. To construct pMA4.2, the *Sst*I-*Pst*I DNA fragment, containing *aacC7*, from pMJ4.2 was ligated to the *Sst*I-*Pst*I replicon fragment from pIJ41 (22). To prepare pMA4221, the *Sph*I-*Sst*I DNA fragment from pMJ1.2 was ligated to *Sst*I-plus-*Stu*I-restricted pMJ4.2. The overhanging *Stu*I and *Sph*I sites were then filled with Klenow enzyme, followed by blunt-end ligation. Arrows indicate direction of transcription. PmeI and tsr indicate tyrosinase gene promoter and thiostrepton resistance gene, respectively.

resistance to paromomycin in *S. rimosus* may be mediated by drug inactivation, with no involvement of the ribosomes. This would contrast with the situations for *S. kanamyceticus* (16) and *S. tenebrarius* (30), in which both ribosomal resistance and drug-inactivating enzymes have been found.

Purification and biochemical characterization of PPH and AAC activities. PPH and AAC were purified from *S. lividans* clones harboring plasmid pMJ1.2 (Fig. 1) or pMJ4.2, as described in Materials and Methods. The final enzyme preparations were analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. PPH showed only one band, of M_r 33,000, and AAC had a main band of M_r 32,000 contaminated with a faint band of much higher molecular weight (results not shown). These values are identical to those obtained for PPH and AAC by gel filtration on a Bio-Gel P-200 column (results not shown). Therefore, the two enzyme activities appeared to be highly purified and accordingly were used to carry out the biochemical studies described below.

PPH activity was totally dependent on Mg^{2+}, with 1 mM being the optimum concentration. This value is rather lower than that (about 10 mM) found for other aminocyclitol APHs. The AAC enzyme was active in the absence of Mg^{2+}, although maximum activity was at a 30-mM concentration. PPH displayed maximum activity at pH 7.5 (Tris hydrochloride buffer), and AAC had two maxima at pH 6 (citrate-phosphate buffer) and 8.5 (Tris hydrochloride buffer).

The K_m values of PPH for paromomycin and ATP were 4.16 and 70 μM, respectively. These values could be accurately measured because the rate of phosphorylation of paromomycin was linear against time, in contrast to what is generally found with aminocyclitol APH. The K_m values of AAC for paromomycin and acetyl-CoA were 2.76 and 125 μM, respectively.

The substrate specificities of PPH and AAC are presented in Table 1. Paromomycin, neomycin C, ribostamycin, and, to a lesser extent, neomycin B were good substrates for PPH activity. In contrast, paromamine and neamine (two-ring

TABLE 1. Substrate specificities of PPH and AAC activities

Substrate	Sp act[a]	
	PPH	AAC
Amikacin	0.00	0.00
Butirosin	0.05	0.00
G-418	0.00	0.19
Gentamicin C_1	0.01	0.19
Gentamicin C_{1a}	0.01	0.31
Hygromycin B	0.00	0.00
Kanamycin A	0.12	0.53
Kanamycin B	0.15	0.86
Kasugamycin	0.00	0.00
Lividamine	0.08	0.06
Lividomycin A	0.01	0.61
Neamine	0.13	0.05
Neomycin B	0.40	0.57
Neomycin C	0.88	0.54
Netilmicin	0.00	0.54
Paromamine	0.09	0.07
Paromomycin	0.87	0.49
Ribostamycin	0.90	0.08
Sisomicin	0.00	0.54
Spectinomycin	0.00	0.00
Streptomycin	0.00	0.00
Tobramycin	0.01	0.75

[a] Assays were performed as indicated in Materials and Methods. Data are expressed as nanomoles of transferred ^{32}P or ^{14}C per nanomole of substrate.

derivatives), lividamine, and kanamycins A and B were poor substrates. Lividomycin A, a member of the neomycin group but lacking the 3'-hydroxyl group, and other compounds listed in Table 1 were not substrates for PPH. These results are similar to those found with APH enzymes of the 3' type (25), although, in contrast to these enzymes, PPH did not actively modify the kanamycins.

AAC clearly modified paromomycin, neomycins B and C, lividomycin A, kanamycins A and B, gentamicins C_1 and C_{1a}, tobramycin, sisomicin, netilmicin, and G-418. Paromamine, neamine, ribostamycin, and lividomycin A were only slightly acetylated. Other aminocyclitol antibiotics indicated in Table 1 were not substrates for AAC. This range of substrates is characteristic of aminocyclitol 3-*N*-acetyltransferase type III [AAC(3)-III] enzymes (25). Therefore, the activity from *S. rimosus* may be included in this group of aminocyclitol acetyltransferase activities. In agreement with this conclusion is the finding that the amino acid sequence of AAC, as deduced from the nucleotide sequence of the corresponding gene, has strong similarities to that of two other AAC(3)-III and -IV enzymes (14). Two additional AAC(3) activities have been described, one in *Micromonospora inoyoensis* (21) and the other in *S. griseus* (9). Therefore, we suggest that AAC(3)-VII provides the AAC activity of *S. rimosus*. In the presence of acetyl-CoA, paromomycin was totally inactivated by AAC(3)-VII (results not shown).

Paromomycin resistance and enzyme activities. To examine more closely the implications of the PPH and AAC(3)-VII activities in the paromomycin resistance phenotype of *S. rimosus*, we constructed a number of plasmids carrying the genes for these two enzymes. Cloning and expression of these genes in *S. lividans* have been reported elsewhere (14, 18). To diminish gene dosage interferences, the genes *pph* (encoding PPH) and *aacC7* [encoding AAC(3)-VII] were inserted in the low-copy-number plasmid pIJ41 (20) (Fig. 1). The direction of transcription of *pph* is not yet known. Therefore, to examine any possible influence from plasmid promoter(s), plasmids pMA1.2 and pMA2.1, containing the

TABLE 2. Paromomycin resistance and PPH and AAC activities of several *Streptomyces* strains

Organism	Plasmid	Sp act (mU/mg of protein)[a]		Paromomycin resistance (μg/ml)[b]
		AAC	PPH	
S. lividans 1326		0.008	0	<2
S. rimosus f. *paromomycinus*		0.200	31.00	3,000
S. lividans				
MA1.2	pMA1.2	0.010	26.95	60
MA2.1	pMA2.1	0.012	25.70	50
MA4.2	pMA4.2	2.300	0	120
MA4221	pMA4221	1.893	38.78	1,000

[a] Determined as described in Materials and Methods; 1 mU is equivalent to 1 nmol of acetylated or phosphorylated paromomycin produced per min.

[b] Determined by the ability of the clones to grow in minimal agar plates containing the indicated concentrations of paromomycin (7).

inserted DNA in the two possible orientations, were constructed (Fig. 1). *S. lividans* transformants harboring either pMA1.2 or pMA2.1 expressed similar amounts of PPH activity (Table 2), which suggests that expression of the *pph* gene in both clones is not affected by foreign sequences. The values for PPH activity in cell extracts from these transformants and from *S. rimosus* were similar. However, the paromomycin resistance levels in the transformants were about 30-fold lower than in the producing organism (Table 2). A similar situation was found in *S. lividans* MA4.2, a transformant harboring *aacC7* in a pIJ41-derived plasmid (pMA4.2) (Table 2 and Fig. 1). In this plasmid, the DNA fragment containing *aacC7*, whose direction of transcription is known (14), replaced most of the *neo* gene, including its promoter (1, 23). Although any effect of a plasmid promoter could not be discounted, such an effect would not modify the interpretation of the results described below. Thus, clone *S. lividans* MA4.2 contained about 11-fold-higher levels of AAC(3)-VII activity than did *S. rimosus*; however, its paromomycin resistance was 25-fold lower than that of the parental strain (Table 2). Therefore, it is clear that neither *pph* nor *aacC7* per se can determine the high levels of resistance found in *S. rimosus* (Table 2). When both genes were included in the same construction (plasmid pMA4221; Fig. 2), the relevant *S. lividans* transformant had a high level of paromomycin resistance, which still was threefold lower than that in *S. rimosus* (Table 2). Note that the levels of enzyme activities in the *S. lividans* clone containing both *aacC7* and *pph* (MA4221) were similar to those in the *S. lividans* clones MA2.1 and MA4.2, which harbor a single gene (Table 2). Thompson et al. (24) also reported that *S. lividans* clones harboring a single *aph* or *aac* gene had only low levels of resistance to neomycin, but clones carrying both genes displayed high resistance levels which were similar to those of *S. fradiae*, the producing organism.

A possible explanation for these findings is that either phosphorylparomomycin or acetylparomomycin is a better substrate for AAC(3)-VII or PPH, respectively, than is unmodified paromomycin. Acetylparomomycin was a poorer substrate for PPH than was the intact antibiotic (Fig. 2A), but phosphorylparomomycin was a better substrate for AAC(3)-VII than was paromomycin (Fig. 2B). As suggested by others, the doubly modified drug might block the antibiotic uptake mechanism more efficiently than does the singly modified one (24). An increased modification rate of phosphorylparomomycin by AAC(3)-VII could facilitate this protective effect against the extracellular drug.

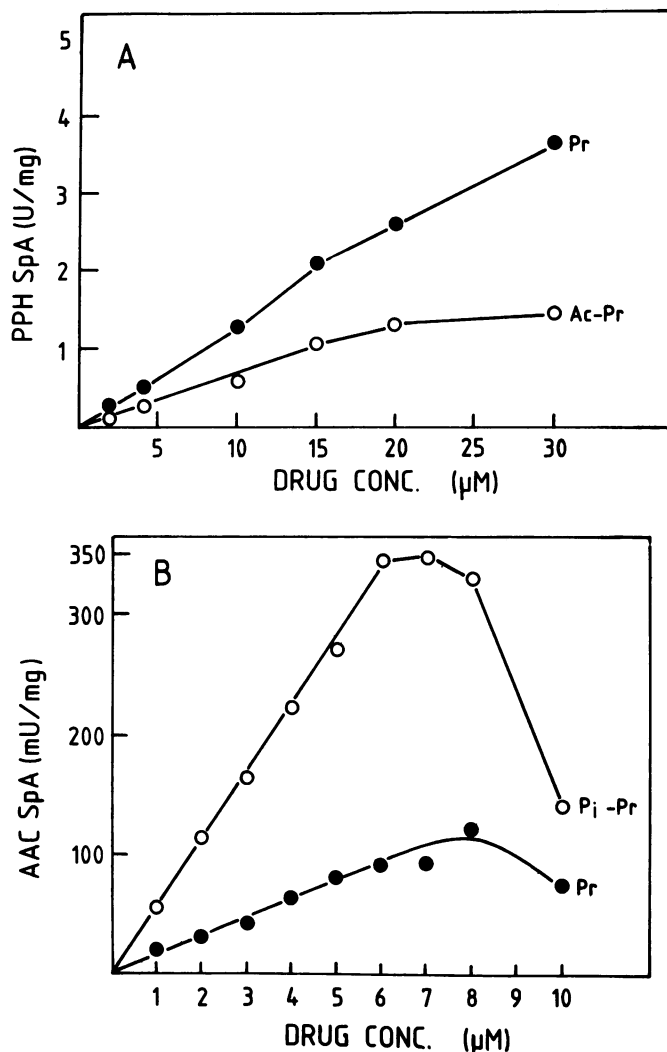

FIG. 2. Single and double modification of paromomycin. (A) Phosphorylation of paromomycin and acetylparomomycin. Paromomycin (15 nmol) was fully acetylated with an S100 fraction (150 μg of protein) from clone *S. lividans* MA4.2. Similarly, 15 nmol of paromomycin was incubated with an S100 fraction (150 μg of protein) from *S. lividans* 1326. Samples of acetylparomomycin (○) and paromomycin (●) were then used to perform the phosphorylation reaction, using an S100 fraction from *S. lividans* MA1.2 (30 μg of protein per 50 μl of reaction mixture). To measure initial rates of phosphorylation, reactions took place at 30°C for only 4 min. (B) Acetylation of paromomycin and phosphorylparomomycin. The indicated amounts of paromomycin (●) and purified phosphorylparomomycin (○) (see Materials and Methods) were acetylated in reaction mixtures (50 μl) containing an S100 fraction from *S. lividans* MA4.2 (90 μg of protein per incubation). Incubations took place at 30°C for 15 min.

The different rates of acetylation of intact paromomycin and phosphorylparomomycin allow us to speculate that if these modifications take place on intermediates of the biosynthetic pathway, the resulting molecules could be better substrates for the successive enzymatic step(s). In this respect, it has been shown that *N*-acetyl-*O*-demethylpuromycin is used more actively by the *O*-demethylpuromycin *O*-methyltransferase than is *O*-demethylpuromycin (24).

A number of aminocyclitol-producing actinomycetes contain ribosomes that are resistant to the relevant antibiotics. *S. lividans* transformants harboring the genes for ribosomal resistance display similar levels of resistance to the producing organisms, which suggests that in the latter no other form of self-protection against aminocyclitols is required (22). However, *S. kanamyceticus* and *S. tenebrarius* also contain antibiotic-inactivating enzymes (16, 27). Although the reason for the coexistence of several resistance processes is not clear, ribosomal resistance to the final antibiotic molecule may not prevent the toxicity of certain precursors; such toxic intermediates could be inactivated by the modifying enzymes.

Whether *S. rimosus* contains resistance-conferring genes other than the two described here remains an open question. The streptomycin producer *S. griseus* has recently been shown to have two different APH activities, which modify either the 3″- or 6-hydroxyl groups of streptomycin (6). Also, *Streptoverticillum eurocidicus* (M. D. Abarca and A. Jiménez, unpublished observations) and *S. tenebrarius* (E. Cundliffe, personal communication) contain the genes for two different destomycin PPHs and two different nebramycin AACs, respectively.

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