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Biochemical Basis of Resistance to Hygromycin B in *Streptomyces hygroscopicus* – the Producing Organism

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Hygromycin B, an aminocyclitol antibiotic that strongly inhibits both 70S and 80S ribosomes, is synthesized by *Streptomyces hygroscopicus*. Ribosomes from this Gram-positive mycelial bacterium are inhibited *in vitro* by the antibiotic. In contrast, the streptomycete is highly resistant to the drug *in vivo* since it possesses hygromycin B phosphotransferase activity. This enzyme has been shown by gel filtration to have a molecular weight of 42000, and to modify its antibiotic substrate to produce 7″-*O*-phosphoryl-hygromycin B which totally lacks biological activity both *in vivo* and *in vitro*.

INTRODUCTION

The aminocyclitol antibiotic hygromycin B (Fig. 1) is produced by *Streptomyces hygroscopicus* and specifically blocks the translocation step on both 70S and 80S ribosomes (Cabañas *et al.*, 1978; González *et al.*, 1978). The drug also induces misreading both *in vivo* and *in vitro* and therefore promotes phenotypic suppression (Cabañas *et al.*, 1978; Singh *et al.*, 1979). *S. hygroscopicus* contains a phosphotransferase (HPH) activity which phosphorylates hygromycin B, thus potentially providing the producing organism with autoimmunity against the toxic

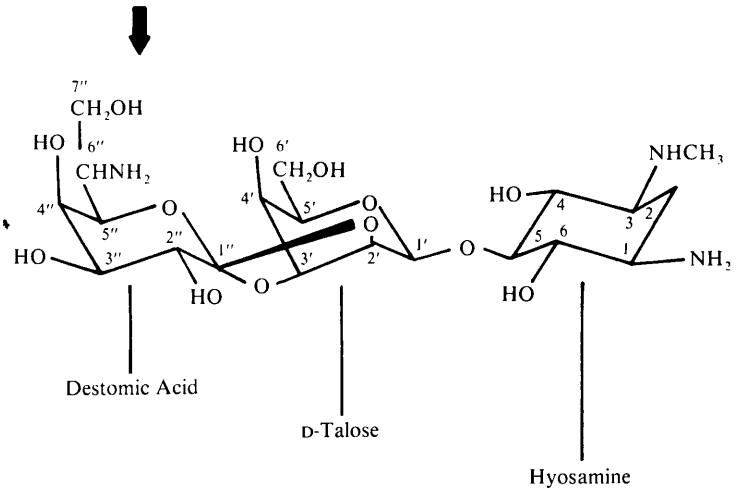

Fig. 1. Chemical structure of hygromycin B. The site of phosphorylation is indicated by the arrow.

Abbreviation: HPH, hygromycin B phosphotransferase.

effects of the drug (Leboul & Davies, 1982). This interpretation appears to be valid since the gene (*hyg*) encoding the HPH activity has now been cloned in *S. lividans* and cells containing it became resistant to hygromycin B (Malpartida *et al.*, 1983).

Such an enzymic inactivation is one of several ways by which antibiotic-producing *Streptomyces* spp. become resistant to their own secondary metabolites. Thus, neomycin, viomycin and capreomycin are inactivated by phosphorylation in *S. fradiae*, *S. vinaceus* and *S. capreolus*, respectively, and capreomycin, neomycin and puromycin are inactivated by acetylation in *S. capreolus*, *S. fradiae* and *S. alboniger*, respectively (Skinner & Cundliffe, 1980; Thompson *et al.*, 1982; Pérez-González *et al.*, 1983). Furthermore, the genes coding for the enzymes modifying neomycin (Thompson *et al.*, 1980), viomycin (Thompson *et al.*, 1982) and puromycin (Vara *et al.*, 1985) have now been cloned, thus allowing, by direct biochemical analysis, further confirmation of the important role of enzymic inactivation mechanisms in determining drug resistance in the producing *Streptomyces* spp. (Skinner & Cundliffe, 1980; Thompson *et al.*, 1982).

Resistance to hygromycin B has also been described in *Escherichia coli* where a plasmid encodes an HPH activity that modifies the antibiotic, to produce 4-phosphoryl-hygromycin B which totally lacks biological activity. The gene encoding such HPH activity has also been cloned and again confers resistance to hygromycin B (Gritz & Davies, 1983; Rao *et al.*, 1983).

In the present work we show that ribosomes from *S. hygroscopicus* are sensitive to hygromycin B *in vitro* and that the HPH enzyme from *S. hygroscopicus* is different to that of *E. coli*.

METHODS

Bacterial strains, media and cell growth. *Streptomyces hygroscopicus* NRRL 2387, *S. lividans* 1326, *Bacillus subtilis* BD54 and *Escherichia coli* MRE 600 were provided by Dr D. T. Wicklow, Professor D. A. Hopwood, John Innes Institute, Norwich, UK, Professor M. Salas, Universidad de Oviedo, Spain, and Professor J. P. G. Ballesta, Centro de Biologia Molecular, Madrid, Spain, respectively. The *Streptomyces* species were maintained on R2YE agar tubes and grown at 30 °C in liquid YEME (Chater *et al.*, 1982) supplemented with 34% (w/v) sucrose. *B. subtilis* and *E. coli* were grown at 37 °C in LB broth minus NaCl (Miller, 1972) and were maintained in this same medium supplemented with 1·5% (w/v) agar.

Preparation of cell extracts as a source of hygromycin B phosphotransferase. Cultures were grown with vigorous rotatory shaking to late exponential phase and were then collected by centrifugation. Cell pellets were washed with buffer II (10 mM-Tris/HCl, pH 7·5, 1 mM-$MgCl_2$, 0·15 mM-EDTA and 1 mM-dithiothreitol), broken with alumina, resuspended and centrifuged for 2 h at 100000***g***. The resulting supernatant (S100) was used as a crude preparation of hygromycin B phosphotransferase (HPH).

Partial purification of HPH enzyme. The S100 fraction from *S. hygroscopicus* was treated with $(NH_4)_2SO_4$ at 40% saturation and centrifuged for 15 min at 15000***g*** and the supernatant was applied to a Bio-Gel P-200 column (330 ml) previously equilibrated with buffer III [buffer II containing $(NH_4)_2SO_4$ at 10% saturation]. Elution was with buffer III and the fractions containing high HPH activity (see below) were collected, pooled and precipitated with $(NH_4)_2SO_4$ at 70% saturation. The precipitate was resuspended in 1·5 ml buffer III and was rechromatographed on Bio-Gel P200 (78 ml) as above. Fractions containing high HPH activity were pooled, concentrated to 2 ml by ultrafiltration, dialysed against 100 vols buffer II for 150 min and adsorbed on a 5 ml DEAE-Sephadex A50 column which had previously been equilibrated with buffer II. The column was washed with buffer II and the HPH enzyme was eluted with buffer II containing $(NH_4)_2SO_4$ (concentration gradient 0–500 mM). HPH activity was recovered at 240 mM-$(NH_4)_2SO_4$.

Assay of HPH enzyme activity. The method used was based on the radiochemical assay described by Haas & Dowding (1975). Reaction mixtures (50 µl) contained 10 µl buffer I (100 mM-Tris/MES, pH 7·5, 50 mM-$MgCl_2$, 500 mM-NH_4Cl, 2·5 mM-dithiothreitol), 10 µl enzyme preparation, 1 µg hygromycin B and 10 µl 1·5 mM-[γ-^{32}P]ATP [15 mCi (555 MBq) mmol^{-1}]. Assays were done for 20 min at 30 °C and the reactions were stopped by heating at 75 °C. An aliquot of the reaction mixture was applied to a strip of phosphocellulose paper (Whatman P-81) which was then washed sequentially with tap water and distilled water. Radioactivity was estimated in a liquid-scintillation spectrometer. Blanks were run in the absence of hygromycin B.

This radiochemical assay has a drawback since some nonlinearity with respect to enzyme concentration and time is observed (Goldman & Northrop, 1976). Therefore, in some experiments we included the spectrophotometric method described by Thompson *et al.* (1982).

One unit of HPH activity was defined as the amount of protein catalysing the formation of 1 pmol phosphorylated hygromycin B min^{-1} at 30 °C.

Determination of the molecular weight of the HPH enzyme by gel filtration. A postribosomal fraction from *S. hygroscopicus* was fractionated with $(NH_4)_2SO_4$ at 30% saturation and the supernatant was chromatographed on a

Bio-Gel P150 column (1·5 × 49 cm) previously equilibrated with buffer III. The HPH activity was eluted with this buffer and the active fractions were pooled and concentrated to 1 ml by ultrafiltration. This preparation was supplemented with 3 mg bovine serum albumin (mol.wt 66000), 3 mg ovalbumin (mol.wt 45000) and 3 mg lysozyme (mol.wt 14400) and then chromatographed on a column (0·9 × 48 cm) of Bio-Gel P200 previously equilibrated with the above buffer. Similar buffer was also used for elutions, with fractions being collected every 30 min. HPH activity and A_{280} were measured for each 0·75 ml fraction.

Determination of the kinetic constants of the HPH enzyme. The K_m values of the HPH enzyme for hygromycin B and ATP were determined using initial velocities obtained from both the radiochemical and the spectrophotometric assays. For the former method, phosphorylation values were obtained as described above, except that the specific activity of [γ-^{32}P]ATP was 720 mCi (26·6 GBq) mmol^{-1} and the substrate concentrations varied in the range 0·4–6 μM for hygromycin B and 10–1000 μM for [γ-^{32}P]ATP. For each assay 12–40 mU HPH activity were added. Reactions took place for 2–8 min and were stopped by heating at 90 °C.

Alternatively, and in order to have a continuous measure of the reaction velocities, the spectrophotometric method indicated above was used with 0·8 U HPH, 2·5–100 μM-hygromycin B and 30–3000 μM-ATP.

Inactivation of hygromycin B by phosphorylation. Samples of the reaction mixture from the phosphorylation assay and known amounts of hygromycin B (as positive controls) were spotted onto cellulose discs, which were then placed on LB or R2YE plates that had been seeded with either *B. subtilis* or *S. lividans*, respectively. The plates were incubated at 37 °C or 30 °C and the activities of the different samples were estimated by noting the size of the growth inhibition zone.

Dephosphorylation of phosphoryl-hygromycin B by the action of alkaline phosphatase. A sample of hygromycin B was phosphorylated with the HPH enzyme in the presence of [γ-^{32}P]ATP of a very low specific activity, and purified by chromatography on Amberlite IRC-50 (Na^+, pH 5·0) under conditions similar to those described below. Fractions containing [^{32}P]phosphoryl-hygromycin B were collected, lyophilized and resuspended in 25 mM-Tris/HCl, pH 8·0. A portion of [^{32}P]phosphoryl-hygromycin B was then treated with calf thymus alkaline phosphatase at 37 °C for 100 min. The extent of dephosphorylation (>95%) was detected using the phosphocellulose paper technique. Both modified and regenerated hygromycin B were assayed for biological activity by the paper disc method using *B. subtilis* and *S. lividans* on LB and R2YE plates, respectively. Untreated hygromycin B was used as a positive control.

Detection of [^{32}P]phosphoryl-hygromycin B by paper chromatography. Samples containing [^{32}P]phosphoryl-hygromycin B and/or hygromycin B were analysed by ascending chromatography on Whatman 3 MM paper using $MeOH/CHCl_3/NH_4OH$ (3:1:2, by vol.) as the solvent. Appropriate standards gave R_F values of 0·67 for hygromycin B and 0·33 for [^{32}P]phosphoryl-hygromycin B. Both compounds were detected by reaction with ninhydrin and in addition [^{32}P]phosphoryl-hygromycin B was detected either by exposure to X-ray films or by cutting paper strips and counting in a scintillation spectrometer.

Purification of [^{32}P]phosphoryl-hygromycin B. Hygromycin B was phosphorylated in a reaction mixture (500 ml) containing 600 μM-[γ-^{32}P]ATP [0·2 mCi (7·4 MBq) mmol^{-1}], 20 mg hygromycin B and 150 mg crude HPH in buffer I. The latter was obtained by precipitating proteins from *S. hygroscopicus* S100 with $(NH_4)_2SO_4$ at 30% saturation. The reaction took place for 16 h at 30 °C and was stopped by incubation for 15 min at 65°C. Precipitated proteins were separated by filtration through a Millipore HAWP membrane and the solution was made 50 mM with respect to sodium maleate at pH 5·0. Aliquots were assayed by the disc method which indicated greater than 95% inactivation of hygromycin B. The reaction mixture was then chromatographed through a column of Amberlite IRC-50 (Na^+ form; 1·1 × 26 cm) previously equilibrated with 50 mM-sodium maleate, pH 5·0. [^{32}P]Phosphoryl-hygromycin B was eluted 1·2 M-NH_4OH. The relevant fractions were pooled, lyophilized and dissolved in distilled water. The solution was then treated with 5 M-HCl to lower its pH to 5·0 and rechromatographed through Amberlite IRC-50 using a 0–1·2 M-NH_4OH gradient. Again, [^{32}P]phosphoryl-hygromycin B eluted in a single peak and was finally purified by chromatography through a column of Bio-Rex 70 (NH_4^+ form; 0·9 × 60 cm) which had previously been equilibrated with water. Pure [^{32}P]phosphoryl-hygromycin B was characterized by paper chromatography. Dephosphorylation with alkaline phosphatase and assay of the reaction products by the paper disc method indicated a yield of 17 mg [^{32}P]phosphoryl-hygromycin B.

Determination of the chemical structure of phosphoryl-hygromycin B. ^{1}H NMR spectra of both free and phosphorylated hygromycin B were obtained using 10 mM drug solutions in D_2O (Stohler, 99·8%; Stohler Isotope Chemicals, Waltham, Mass., USA). Spectra were taken at 360 MHz in the Fourier Transform (FT) mode on a Bruker-WM-360 instrument.

Preparation of cell-free extracts for polyphenylalanine synthesis. The procedures were essentially identical to those described by Skinner & Cundliffe (1980). However, Tris buffers were not used in cell-free protein synthesis experiments to avoid its inhibitory effect on polyphenylalanine production (E. Cundliffe, personal communication).

Mycelium was collected by centrifugation at the end of the exponential phase of growth and washed with buffer R[10 mM-HEPES/KOH, pH 7·5, 10 mM-$MgCl_2$, 50 mM-NH_4Cl, 6 mM-2-mercaptoethanol, 0·2 mM-phenylmethanesulphonyl fluoride (PMSF)]. PMSF was important in obtaining high activities for polyphenylalanine synthesis

(Mikulik *et al.*, 1983; Sugiyama *et al.*, 1980). Mycelium was broken by grinding with autoclaved alumina in a mortar at 0 °C and resuspended in buffer R (1 ml per g cells). Alumina and cell debris were removed by centrifugation for 15 min at 20000 *g* and the resulting supernatant fraction was further centrifuged for 30 min at 30000 *g*. The final supernatant (S30) was treated with DNAase (RNAase free).

Ribosomes were prepared by layering the S30 fraction over 1 vol. buffer R containing 0·5 M-NH_4Cl and 40% (w/v) sucrose followed by centrifugation at 100000 *g* for 14 h. The upper two-thirds of the supernatant fraction was collected and dialysed for 3 h against buffer R. The ribosome pellet was then resuspended in a small volume of PMSF-free buffer R. Both ribosomes and S100 fraction were stored at −80 °C. One A_{260} unit of ribosomes was equivalent to 24 pmol 70S particles.

Protein concentrations were estimated by the method described by Bradford (1976).

Synthesis of polyphenylalanine in cell-free extracts as directed by polyuridylic acid. The method used was based on that described by Skinner & Cundliffe (1980) and J. A. Salas (personal communication). Final incubation mixtures (60 µl) contained: 20 mM-HEPES/KOH buffer, pH 7·5, 50 mM-KCl, 8·5 mM-$MgCl_2$, 2·5 mM-ATP, 0·35 mM-GTP, 7·5 mM-PEP, 0·38 µM-uncharged $tRNA^{phe}$ from *Escherichia coli*, 0·5 mg polyuridylic acid ml^{-1}, 18 µg pyruvate kinase ml^{-1}, 5 µM-[^{14}C]phenylalanine [504 mCi (18·6 MBq) $mmol^{-1}$], 60 µg S100 extract and 15 pmol ribosomes. The PMSF was removed from the S100 fraction just before the assay by dialysis against PMSF-free buffer R for 1 h to avoid its inhibitory effect on polyphenylalanine synthesis (Sugiyama *et al.*, 1980). The reaction commenced after addition of ribosomes, took place for 45 min at 30 °C and was stopped by addition of 1 ml 10% (w/v) TCA. Under these conditions incorporation of [^{14}C]phenylalanine was linear for at least 60 min. Samples were processed and radioactivity determined in a liquid-scintillation spectrometer as described previously (Sánchez *et al.*, 1977). An incorporation of 1000 c.p.m. was equivalent to 1 pmol [^{14}C]phenylalanine.

Assay of aminocyclitol acetyltransferase activity. Assays of the aminocyclitol acetyltransferase activity present in S100 extracts from *S. hygroscopicus* (Leboul & Davies, 1982) were done as described by Haas & Dowding (1975).

RESULTS

Effect of hygromycin B on cell growth

S. hygroscopicus grows normally on plates containing hygromycin B (up to 1000 µg ml^{-1}) whereas *S. lividans*, *E. coli* and *B. subtilis* are sensitive to 50 µg hygromycin B ml^{-1} on R2YE and LB plates (results not shown). The possibility arises, therefore, that *S. hygroscopicus* possesses autoimmunity against the toxic effects of hygromycin B.

HPH activity from S. hygroscopicus

A supernatant extract from *S. hygroscopicus* was assayed for phosphotransferase activity with a variety of aminoglycoside antibiotics. Only hygromycin B (Fig. 1), and the closely related antibiotics 1-*N*-methyl-hygromycin B and destomycin (Table 1) were readily modified. All the other antibiotics listed in Table 1, including hygromycin B2 which contains only the hyosamine (*N*-methyl-deoxystreptamine) and D-talose moieties of hygromycin B (see Fig. 1), were unaffected by the HPH activity. These results thus confirm and extend those of Leboul & Davies (1982) who have reported that cell-free extracts from *S. hygroscopicus* contain an enzymic activity which phosphorylates hygromycin B, but lacks any significant effect on neomycin, sisomicin and streptomycin.

Some biochemical parameters of the HPH activity were determined using the crude enzyme found in supernatant fractions from *S. hygroscopicus*. For example, 400 mM-$(NH_4)_2SO_4$ protected the enzymic activity against inactivation by high temperatures – the same effect being found during attempts to purify the enzyme (results not shown). A summary of our attempts to purify HPH activity is given in Table 2. As a final result the enzyme was purified 49-fold (Table 2). Purification attempts using chromatography on CM-cellulose and Affigel-blue were unsuccessful. Furthermore, the HPH enzyme became extremely unstable after passage through DEAE-Sephadex and treatment with the potential stabilizing agents sucrose, glycerol and $(NH_4)_2SO_4$ failed to alleviate this effect. Because of this instability no further purification steps were attempted.

Molecular weight of the HPH enzyme

By applying the gel filtration technique and plotting the elution volumes against the log of the molecular weight of the standards, a straight line was obtained (not shown). From this a molecular weight of 42000 was deduced for the HPH enzyme.

Table 1. *Enzymic modifications of aminocyclitol antibiotics by S100 fraction from S. hygroscopicus*

Selected antibiotics (5 μg) were incubated with S100 fraction (13 μg protein) and either [γ-^{32}P]ATP or [^{14}C]acetyl-CoA as indicated in Methods. The extent of each reaction is expressed as pmol ^{32}P- or ^{14}C-label transferred to an antibiotic, as determined by the phosphocellulose binding technique.

Antibiotic	Phosphorylation	Acetylation	Antibiotic	Phosphorylation	Acetylation
Hygromycin B	498	–	Neamine	–	145
Destomycin	520	–	Neomycin B	–	142
1-*N*-Methyl-hygromycin B	370	–	Neomycin C	–	233
Hygromycin B2	–	ND	Paromomycin	–	ND
Gentamicin C1	–	–	Ribostamycin	–	134
Gentamicin $C1_a$	–	101	Sisomicin	–	119
G-418	–	–	Spectinomycin	–	–
Kasugamycin	–	–	Streptomycin	–	ND
Kanamycin A	–	67·5	Tobramycin	–	95·1
Kanamycin B	–	78·9	Verdamycin	–	97·2

–, No modification detected; ND, not done.

Table 2. *Partial purification of the HPH enzyme*

Stage of purification	Protein (mg)*	Total activity*†	Specific activity‡	Purification factor
S100	43·0 (100)	262 (100)	6·1	1
0–40% $(NH_4)_2SO_4$ fraction	31·2 (72·5)	279 (106)	8·9	1·45
Bio-Gel P200 (300 ml)	6·2 (14·4)	293 (112)	47·3	7·76
Bio-Gel P200 (78 ml)	2·2 (5·1)	266 (101)	121	19·8
DEAE-Sephadex	0·16 (0·4)	50·1 (19·1)	300	49·2

* Percentages are given in parentheses.
† nmol ^{32}P transferred to hygromycin B min^{-1}.
‡ nmol ^{32}P transferred to hygromycin B min^{-1} (mg protein)$^{-1}$.

Kinetic constants of the HPH enzyme

The K_m for the HPH enzyme was determined by the spectrophotometric assay described in Methods with hygromycin B as the substrate. An antibiotic concentration of 2·5 μM allowed a reproducible estimate of the reaction rate, but at hygromycin B concentrations higher than 5 μM there was substrate inhibition. Despite this limited concentration range, an approximate K_m value for hygromycin B of 2·35 μM could be deduced. Certainly the K_m value of the HPH enzyme for ATP (57 μM) could be accurately determined using this assay.

Although the radiochemical method is much more sensitive than the spectrophotometric assay, it is not linear with respect to time and enzyme concentrations (Goldman & Northrop, 1976). Indeed, these problems were increased in our work since [γ-^{32}P]ATP of high specific activity had to be used to determine initial velocities. Furthermore, since the K_m for hygromycin B is so low, the background levels of radioactivity carried over from ATP were very close to those due to phosphorylation of hygromycin B, thus increasing the inaccuracy of the method. Nevertheless, approximate K_m values of 1·5 μM and 76 μM were deduced for hygromycin B and ATP, respectively, which approached those values determined with the spectrophotometric technique.

Site of phosphorylation of hygromycin B

By determining the ^{1}H spectra of hygromycin B and its phosphorylated derivative (data not given), we have deduced that the phosphorylation site of hygromycin B is at the 7″ hydroxyl of the destomic acid ring (Fig. 1). This assignment is fully substantiated by both conventional double resonance experiments (pH 8·8) and a detailed study of shift variations between pH 2 and pH 12, for both products. Phosphorylation shifts were particularly large for $H7_a''$ and $H7_b''$,

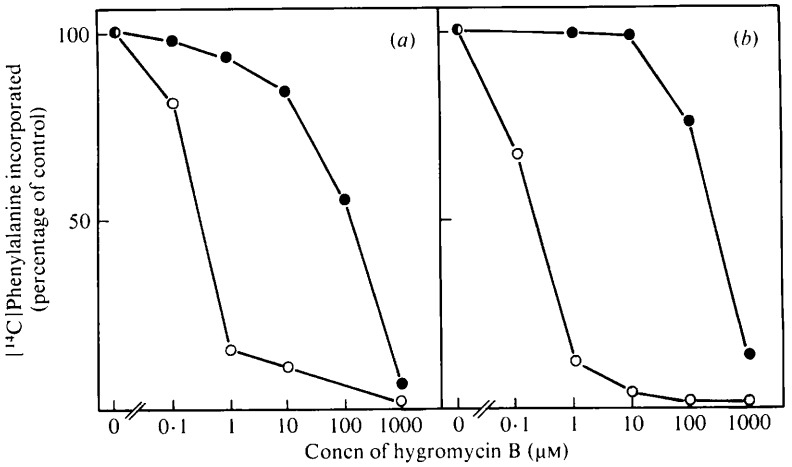

Fig. 2. Localization of hygromycin B resistance in the S100 fraction from *S. hygroscopicus*. Polyphenylalanine synthesis was performed as described in Methods. Incorporation in the controls, expressed as pmol [^{14}C]phenylalanine incorporated per pmol ribosomes, is given in parentheses. (*a*) ●, S100 fraction and ribosomes from *S. hygroscopicus* (8·8); ○, S100 fraction and ribosomes from *S. lividans* (0·66). (*b*) ●, S100 fraction from *S. hygroscopicus* and ribosomes from *S. lividans* (3·0); ○, S100 fraction from *S. lividans* and ribosomes from *S. hygroscopicus* (1·75).

H6″ and H5″ proton signals. Vicinal $^{3}J_{31p-1H}$ coupling constants (4·6 Hz) were observed for signals assigned to $H7_a''$ and $H7_b''$, which at pH 5·0 were free from other overlapping signals. Moreover, the $H7_a''$ and $H7_b''$ signals, together with some other signals of neighbouring protons, showed titration shifts (pK 5·1) due to the change of the ionization state of the phosphate group.

Effect of alkaline phosphatase on 7″-O-phosphoryl-hygromycin B

Hygromycin B was phosphorylated using the partially purified HPH enzyme and the resulting 7″-*O*-phosphoryl-hygromycin B was purified and then dephosphorylated with alkaline phosphatase. Both forms were then tested for biological activity by the paper disc method. It was clearly seen that 7″-*O*-phosphoryl-hygromycin B was totally inactive and dephosphorylation fully regenerated its growth inhibitory properties (results not shown).

Effects of hygromycin B on polyphenylalanine synthesis

Polyphenylalanine synthesis in a cell-free system from *S. lividans* was much more sensitive to hygromycin B than was a corresponding system from *S. hygroscopicus* extracts, with drug concentrations of 0·5 μM and 136 μM, respectively, causing 50% inhibition (Fig. 2). Mixing heterologous fractions indicated that the resistance resided in the S100 fraction from *S. hygroscopicus* (Fig. 2*b*). In order to correlate resistance with HPH, we measured the loss of inhibitory action on polyphenylalanine synthesis of hygromycin B which was phosphorylated by a preparation of the HPH enzyme (Fig. 3). Increasing the level of phosphorylation drastically reduced the inhibition by hygromycin B of polyphenylalanine synthesis in cell-free extracts from *S. lividans*. Thus, with 95% of the antibiotic phosphorylated, the remaining 0·5 μM-hygromycin B inhibited polyphenylalanine synthesis by approximately 50% (Fig. 3), a result in agreement with that shown in Fig. 2 where pure hygromycin B was used. Moreover, 7″-*O*-phosphoryl-hygromycin B over the concentration range 0–30 μM lacked any biological activity on cell-free protein synthesizing systems from *S. lividans* (Fig. 4*b*). Some inhibitory activity was, nevertheless, detected at higher antibiotic concentrations (Fig. 4*b*). This effect could be prevented in the presence of either S100 from *S. hygroscopicus* or extra HPH enzyme (Fig. 4*a*, *b*). Therefore, a contamination of our preparation of 7″-*O*-phosphoryl-hygromycin B with traces of native drug is implied. Indeed, when an aliquot of 7″-*O*-phosphoryl-hygromycin B was incubated with both the HPH enzyme and [γ-^{32}P]ATP of very high specific activity, 0·1%

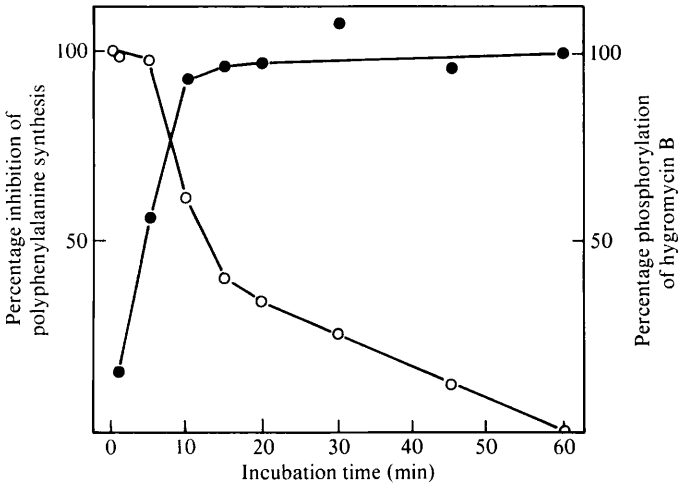

Fig. 3. Inactivation of hygromycin B by phosphorylation. A reaction mixture (600 µl) for phosphorylation of hygromycin B was prepared as described in Methods. The final concentration of hygromycin B was 50 µM. At intervals, 75 µl samples were removed, heated for 5 min at 75 °C and centrifuged. 7″-*O*-Phosphoryl-hygromycin B was estimated (from 40 µl samples) as described in Methods. Duplicate 12 µl samples were assayed for inhibition of polyphenylalanine synthesis in the *S. lividans* cell-free system. Incorporation in the control was 1·35 pmol [^{14}C]phenylalanine per pmol ribosomes. ●, Percentage of phosphorylated hygromycin B; ○, percentage of inhibition of polyphenylalanine synthesis.

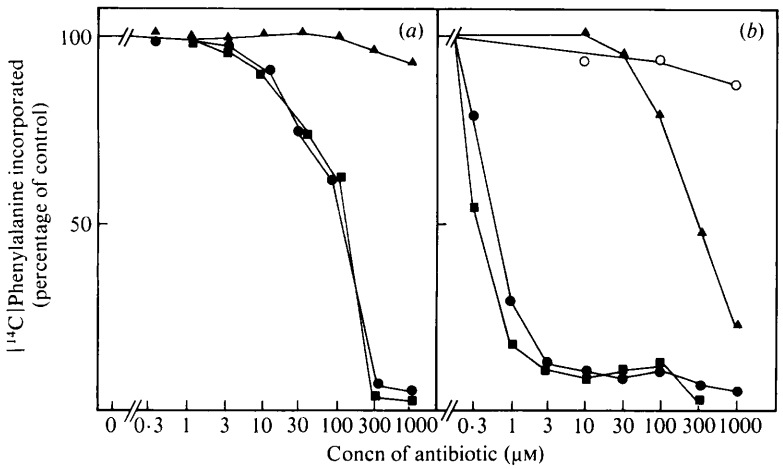

Fig. 4. Reactivation of 7″-*O*-phosphoryl-hygromycin B by treatment with alkaline phosphatase. 7″-*O*-Phosphoryl-hygromycin B was purified and then dephosphorylated as described in Methods. (*a*) S100 fraction and ribosomes from *S. hygroscopicus*; (*b*) S100 fraction and ribosomes from *S. lividans*; ●, hygromycin B; ▲, 7″-*O*-phosphoryl-hygromycin B; ■, 7″-*O*-phosphoryl-hygromycin B treated with alkaline phosphatase; ○, 7″-*O*-phosphoryl-hygromycin B and the HPH enzyme. Incorporation in the controls, expressed as pmol [^{14}C]phenylalanine incorporated per pmol ribosomes, was (*a*) 6·0 and (*b*) 1·4.

contamination with free hygromycin B was detected by phosphorylation. We conclude that 7″-*O*-phosphoryl-hygromycin B completely lacks biological activity against protein synthesis. Accordingly, alkaline phosphatase reactivated the modified drug as tested both *in vivo* (see above) and *in vitro* in a polyphenylalanine synthesizing system (Fig. 4*a*, *b*).

Aminocyclitol acetyltransferase activity from extracts of S. hygroscopicus

Leboul & Davies (1982) detected an aminoglycoside acetylation (AAC) activity in S100 extracts from *S. hygroscopicus* that modified sisomycin but had no effect on hygromycin B, and suggested that it was an AAC(3) enzyme, widely distributed within *Streptomyces* species. We have assayed for this activity with several aminocyclitol antibiotics and found that not only sisomicin, but also gentamicin $C1_a$, kanamycins A and B, neamine, neomycins B and C, ribostamycin, tobramycin and verdamycin are partially acetylated by extracts from *S. hygroscopicus* (Table 1). Hygromycin B, destomycin, 1-*N*-methyl-hygromycin B, gentamicin C1, G-418, kasugamycin and spectinomycin were not modified (Table 1). This acetyltransferase activity acts upon a range of substrates similar to that of other AAC enzymes (from *S. kanamyceticus* and several plasmid-containing strains of *E. coli*) which are known to acetylate the 6′-amino group of the aminohexose I of the modified antibiotics (Benveniste & Davies, 1973; Umezawa *et al.*, 1967). Thus, in the present work, gentamicin C1, with a substituted $-NH_2$ group in the 6′ position, was not a substrate for the enzyme, whereas gentamicin $C1_a$, with this group unsubstituted, was. Moreover, both 6′-amino groups in neomycin C appeared to be acetylated by the enzyme (Table 1). Therefore it is tempting to suggest that the *S. hygroscopicus* enzyme is a 6′-*N*-acetyltransferase. It is difficult to discover the role of this acetyltransferase since both *S. lividans*, which lacks its activity, and *S. hygroscopicus*, which possesses it, are equally sensitive to those antibiotics which can be partially acetylated by cell-free extracts from *S. hygroscopicus*.

No adenylyltransferase activity has yet been found in organisms producing aminocyclitol antibiotics. We detected no adenylylation of the aminocyclitol antibiotics tested (not shown) and Leboul & Davies (1982) did not detect adenylylation of spectinomycin, streptomycin and hygromycin B by extracts from *S. hygroscopicus*.

DISCUSSION

Leboul & Davies (1982) showed that *S. hygroscopicus*, which produces hygromycin B, contained hygromycin B phosphotransferase activity which phosphorylated this aminocyclitol antibiotic. They proposed that in *S. hygroscopicus* this enzyme could cause autoimmunity against the toxic effect of hygromycin B. We have confirmed this hypothesis by showing that ribosomes from *S. hygroscopicus* are fully sensitive to hygromycin B *in vitro* and that resistance to the antibiotic is dependent upon its enzymic modification by HPH activity. Furthermore, we have cloned the gene *hyg*, that encodes that HPH enzyme, and shown that *S. lividans* cells carrying this gene in a plasmid vector are completely resistant to hygromycin B (Malpartida *et al.*, 1983).

The modification of hygromycin B takes place on the 7″-hydroxyl group of the destomic acid moiety. In contrast, *E. coli* plasmid-determined HPH activity phosphorylates hygromycin B on the 4-hydroxyl group of the hyosamine moiety. This explains why the HPH enzyme from *S. hygroscopicus* does not modify hygromycin B2, the D-talose-hyosamine dimer, although this compound is a substrate for the *E. coli* HPH protein. However, since hygromycin B2 does not inhibit ribosomes from either *S. hygroscopicus* or *S. lividans* (data not shown), its production as a metabolite should be harmless whether or not it is an intermediate in the biosynthesis of hygromycin B. The HPH enzyme from *E. coli* has a molecular weight of 41 500 (Rao *et al.*, 1983), a figure which closely approximates to that found by us for the *S. hygroscopicus* enzyme. However, the sequence of the relevant gene from *S. hygroscopicus* is known (M. Zalacain, A. González, M. C. Guerrero & A. Jiménez, unpublished observations) and the data indicate that there are no similarities to the sequence published already for the corresponding gene in *E. coli* (Gritz & Davies, 1983; Kaster *et al.*, 1983). In contrast, the APH 3′-phosphotransferases encoded by transposons Tn*5* and Tn*601* and by DNA from *Bacillus circulans* and *S. fradiae* (Thompson & Gray, 1983; Herbert *et al.*, 1983) show striking similarities in their amino acid sequences. These results support the proposal that at least some of the genetic determinants of antibiotic-modifying enzymes in clinical isolates might have originated from antibiotic-producing organisms (Benveniste & Davies, 1972). Whether or not these are the only two types of existing hygromycin B-modifying enzymes is an open question. The same aminocyclitol

antibiotic can often be modified at different sites by specific phosphotransferases. Furthermore, hygromycin B and structurally related compounds are produced by several actinomycetes (e.g. *S. rimofaciens* and *Streptoverticillum euricidicus*) and the possibility therefore remains that other actinomycete or bacterial HPH activities might be detected in the future and be shown to phosphorylate hygromycin B on the 4- or 7″- or even on other hydroxyl groups.

S. hygroscopicus contains acetyltransferase activity which modifies several aminocyclitol antibiotics but does not affect hygromycin B (Leboul & Davies, 1982; this work). Since *S. hygroscopicus* is sensitive to all the antibiotics that are modified by its acetyltransferase, it is not clear at all why this bacterium possesses such activity. *S. hygroscopicus* apparently requires only the HPH (7″) activity to provide resistance to hygromycin B. This enzyme has a very low K_m (1–2 μM) for hygromycin B and there should be immediate inactivation of free antibiotic in the cytoplasm.

The role(s) of antibiotic inactivating enzymes in producing organisms remains to be elucidated. They may, however, have an important function in modifying precursors not only to ensure production of active antibiotic but also to render inhibitory precursors harmless to the cell (for a review see Umezawa & Kondo, 1982).

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