Enzymatic Phosphorylation of Streptomycin by Extracts of Streptomycin-producing Strains of *Streptomyces*

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Extracts of post-exponential phase mycelia of *Streptomyces bikiniensis* ATCC 11062, and other streptomycin-producers, catalyze phosphorylation of streptomycin and dihydrostreptomycin with adenosine-5'-triphosphate. The phosphate is esterified with an —OH group of the streptidine moiety. It is suggested that *O*-phosphorylstreptomycin might serve as an intracellular precursor of extracellular streptomycin or as a detoxification product of streptomycin or that it might serve an unknown physiological function in the producing organism.

Streptomycin has the structure shown in Fig. 1. The streptidine moiety is synthesized by a series of reactions from *myo*-inositol (5, 6). An *O*-phosphorylstreptidine was found to be an intermediate in the biosynthetic pathway, but it was not known at what stage in the biosynthesis of streptomycin the phosphate group was removed, i.e., before or after addition of the L-streptose and *N*-methyl-L-glucosamine moieties (4, 7, 8). In addition to the de novo biosynthetic pathway, synthesis of *O*-phosphorylstreptidine was also found to occur in intact mycelia of a number of strains of *Streptomyces* by phosphorylation of exogenous streptidine. Subsequently, it was shown that cell-free extracts from post-exponential phase mycelia of streptomycin-producing strains catalyze phosphorylation of streptidine with adenosine-5'-triphosphate (ATP; 4). The observation that phosphorylation of streptidine was strongly inhibited by streptomycin and dihydrostreptomycin suggested that perhaps the latter compounds were also phosphorylated by the same enzyme (4). In this paper, evidence is presented that enzyme preparations containing streptidine kinase activity also catalyze phosphorylation of streptomycin with ATP, and, furthermore, that the *O*-phosphoryl group appears to be located in the streptidine moiety.

MATERIALS AND METHODS

Labeled compounds. $(\beta,\gamma^{-32}P)$ATP, 77 c/mole, came from Schwarz BioResearch Inc., Orangeburg, N.Y.; L-(*guanidino*-^{14}C) arginine, 25 c/mole, came from Calbiochem, Los Angeles, Calif. Labeled aminocyclitol derivatives were prepared as described elsewhere (5).

Enzyme preparations. Lysozyme extracts of 3-day mycelia of *Streptomyces bikiniensis* ATCC 11062 were prepared as described previously (5). Nucleic acids were precipitated with $MnCl_2$, and a 0 to 70% $(NH_4)_2SO_4$ fraction was obtained; 1 ml of this fraction, containing 25 mg of protein, was applied to a Sephadex G-100 column (2.5 by 55 cm, void volume 85 ml), previously equilibrated with 0.01 M tris(hydroxymethyl)aminomethane (Tris; *p*H 7.4) and mercaptoethanol. Fractions of 2.5 ml were collected at 3 C; protein first emerged in tube 32. To aid in preserving enzyme activities during repeated freezing and thawing, 3 mg of bovine serum albumin was added to each tube.

Enzyme assays. Streptidine kinase activity was assayed radiochemically with (*N'-amidino*-^{14}C)streptidine as acceptor (4), as was done for the Sephadex fractions, or as described below for streptomycin kinase activity shown in Table 1. Streptomycin (and dihydrostreptomycin) kinase activity was assayed typically as follows: streptomycin sulfate (0.4 mg/ml), 10 µliters; 0.5 µM Tris (*p*H 8.8) containing $MgCl_2 \cdot 6H_2O$ (1 mg/ml), 5 µliters; Sephadex tube no. 48, 20 µliters; sodium $(\beta,\gamma^{-32}P)$ATP solution, 2 µliters. The mixture was incubated for 2 hr at 37 C (30 min for Fig. 2). Compounds in a 10-µliter sample were then separated by ascending paper chromatography (25 cm) with 80% phenol and 20% water in an NH_3 atmosphere (provided by adding 1 ml of concentrated NH_4OH on the inside of a jar); 1-cm horizontal strips were cut and counted in bottles by means of a liquid scintillation counter. For many studies (Tables 1 and 2; Fig. 4), the incubation volumes were scaled up 10-fold, and the mixtures subsequently were applied to small (Pasteur pipette) columns containing Dowex-1(Cl⁻) and eluted with 5 ml of water. ATP, adenosine diphosphate (ADP), adenosine monophosphate, and P_i were retained on the column; phosphorylated derivatives of streptomycin, dihy-

drostreptomycin, and streptidine were not adsorbed. Eluates were evaporated to dryness and taken up in (i) 200 μliters of water for counting (Table 1) or treatment with alkaline phosphatase, or (ii) 200 μliters of 1 N HCl for acid hydrolysis. Assay for streptidine-P in acid hydrolysates was performed as follows: hydrolysate, 10 μliters; 0.5 M Tris (pH 7.4) containing ethylenediaminetetraacetate (5 mg/ml) and mercaptoethanol (5 μliters/ml), 5 μliters; dialyzed lysozyme extract, 10 μliters; L-(guanidino-¹⁴C)arginine (33 μc/ml), 5 μliters. The mixture was incubated for

2 hr at 37 C; 10 μliters was spotted on a paper chromatogram and developed.

RESULTS

Coincidence of streptidine kinase and streptomycin kinase activities. As reported previously (4), mycelial extracts contain factors which inhibit streptidine kinase activity. Consequently, a preliminary purification was carried out before testing streptomycin as an alternate substrate; an example is shown in Fig. 2. Streptidine kinase activity can be separated more-or-less completely on a Sephadex G-100 column from a number of enzymes involved in streptidine biosynthesis, including the kinase which acts on the closely related compound, N-amidinostreptamine. Samples from the peak of streptidine kinase activity were also found to catalyze phosphorylation of streptomycin (Table 1). At lower concentrations, streptomycin is even more
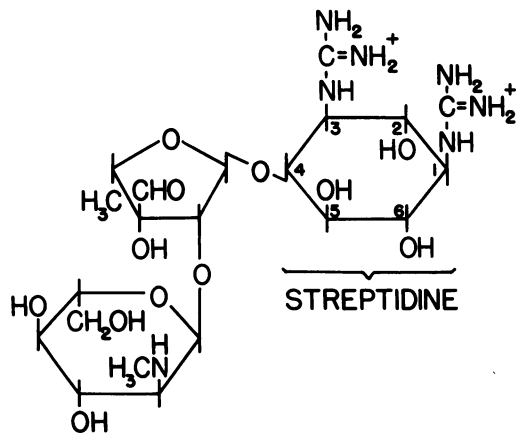

FIG. 1. Structure of streptomycin.

TABLE 1. Comparison of streptidine and streptomycin as phosphate acceptors from $(\beta, \gamma^{-32}P)ATP$ catalyzed by Sephadex G-100 fraction no. 48

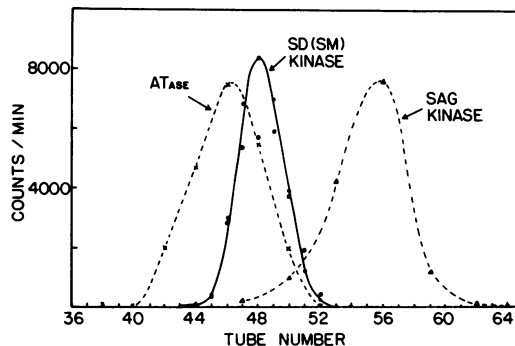| Phosphate acceptor	Phosphorylated product formed (counts/min per 5 μliters)
None	36
Streptidine, 2 mM	6,988
Streptidine, 0.4 mM	2,942
Streptomycin, 2 mM	6,903
Streptomycin, 0.4 mM	6,901

FIG. 2. Separation of streptidine and streptomycin kinase activities from other enzymes on a Sephadex G-100 column. Absolute values on the ordinate refer only to streptidine kinase activity; other activities are normalized for comparative purposes. Symbols: ●, streptidine (SD) kinase activity; ○, streptomycin (SM) kinase activity (measured with dihydrostreptomycin); ×, amidinotransferase (ATase) activity; ▲, N-amidinostreptamine (SAG) kinase activity.

TABLE 2. Localization of phosphate in streptidine moiety of streptomycin-P

Experimental conditions	Label incorporated into streptidine-P from L-(guanidino-¹⁴C) arginine (counts/min per 10 μ liters)
Streptomycin + kinase + ATP (1 N HCl, 50 C, 3 hr; neutralized, + amidinotransferase)	11,044
Streptomycin + kinase, no ATP (1 N HCl, 50 C, 3 hr; neutralized, + amidinotransferase)	32
Streptomycin + kinase + ATP (no acid hydrolysis; + NaCl + amidinotransferase)	518

active than streptidine. The profile of strepto-mycin kinase activity in the various tubes could not be distinguished from the profile of strepti-dine kinase activity (Fig. 2). Moreover, strepti-dine kinase activity partially purified on a diethylaminoethyl-cellulose column (M. S. Walker and J. B. Walker, *in press*) also catalyzed phosphorylation of streptomycin with ATP. Distribution of the two kinase activities among various strains of *Streptomyces* appears to be the same (cf. 4).

Phosphorylation of dihydrostreptomycin with ATP. The partially purified enzyme preparation from the Sephadex column also catalyzed phos-phoryl transfer from ATP to dihydrostrepto-mycin. The dihydrostreptomycin-P could be readily separated from streptidine-P, ATP, ADP, and P_i by paper chromatography with am-moniacal phenol (Fig. 3A). Results obtained with streptomycin as phosphate acceptor are shown in Fig. 3B; no activity was detected with neomycin. The phosphate group could be easily removed by hydrolysis with *Escherichia coli*
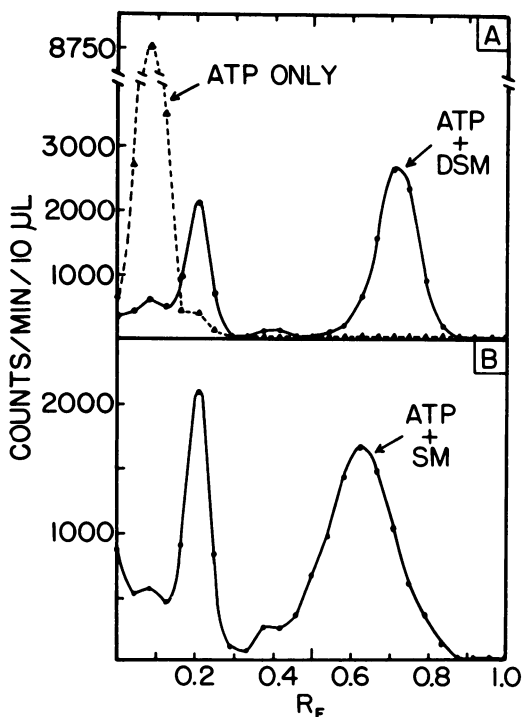

FIG. 4. *Degradation of dihydrostreptomycin-*^{32}P *(DSM-P) by means of (A) Escherichia coli alkaline phosphatase and (B) mild acid hydrolysis. Broken curves indicate DSM-P before treatment.*

FIG. 3. *Detection of phosphorylated derivatives of di-hydrostreptomycin (DSM) and streptomycin (SM) formed after incubation with (β,γ-^{32}P)ATP and Sepha-dex fraction 48. Separations were by paper chromatog-raphy. Broken curve indicates phosphate acceptor omitted.*
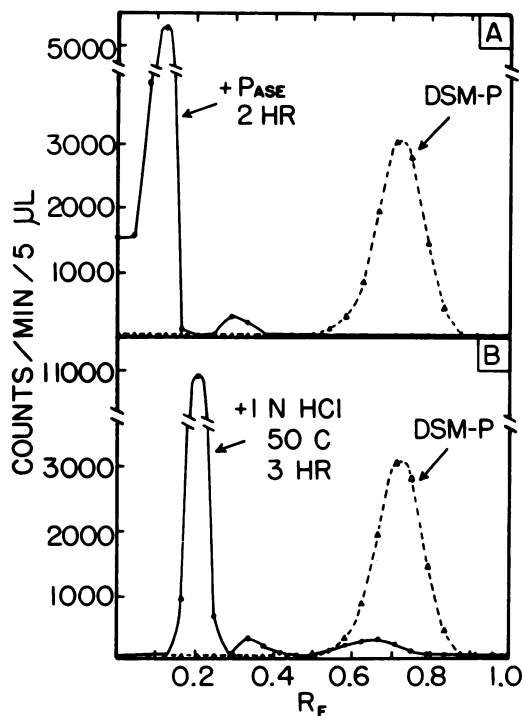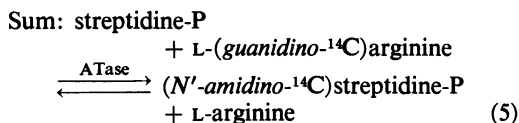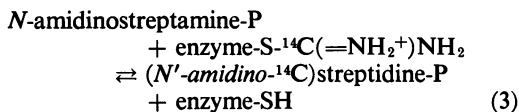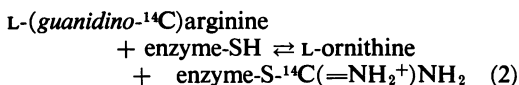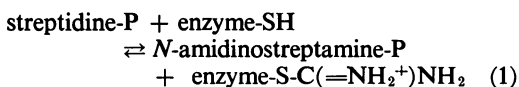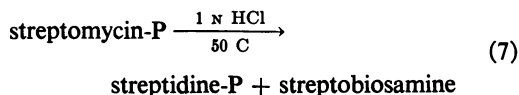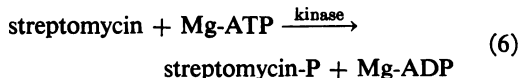
alkaline phosphatase (type III; Sigma Chemical Co., St. Louis, Mo.), as shown in Fig. 4A.

Localization of the phosphate group. If the same kinase phosphorylates both streptidine and streptomycin, the phosphate group should be located on the streptidine moiety of streptomy-cin. Mild acid hydrolysis was found to convert dihydrostreptomycin-P to a new compound with the mobility of streptidine-P (Fig. 4B). Little or no inorganic phosphate was formed, in keeping with the relative acid stability of streptidine-P. Further evidence that the product of mild acid hydrolysis was streptidine-P was provided by the experiments whose results are summarized in Table 2. A radiochemical enzymatic assay for streptidine-P, utilizing labeled arginine and amidinotransferase, was employed. This was the assay originally devised to detect accumulation of streptidine-P in mycelia of nonstreptomycin-producing strains of *Streptomyces* after strepti-dine feeding (7). The mechanism of label in-corporation is believed to be that indicated in reactions 1 to 4, with the overall reaction that of reaction 5.

streptidine-P + enzyme-SH

$$\rightleftarrows N\text{-amidinostreptamine-P} + \text{enzyme-S-C}(=NH_2{}^+)NH_2 \quad (1)$$

L-(*guanidino*-^{14}C)arginine + enzyme-SH $\rightleftarrows$ L-ornithine

$$+ \text{enzyme-S-}{}^{14}\text{C}(=NH_2{}^+)NH_2 \quad (2)$$

N-amidinostreptamine-P + enzyme-S-^{14}C($=NH_2{}^+$)NH$_2$

$$\rightleftarrows (N'\text{-}amidino\text{-}{}^{14}\text{C})\text{streptidine-P} + \text{enzyme-SH} \quad (3)$$

L-ornithine + enzyme-S-C($=NH_2{}^+$)NH$_2$

$$\rightleftarrows \text{L-arginine} + \text{enzyme-SH} \quad (4)$$

Sum: streptidine-P + L-(*guanidino*-^{14}C)arginine

$$\xrightarrow[]{\text{ATase}} (N'\text{-}amidino\text{-}{}^{14}\text{C})\text{streptidine-P} + \text{L-arginine} \quad (5)$$

Transamidination does not occur with non-phosphorylated streptidine, streptomycin, or streptomycin-P. Substantial amounts of streptidine-P, as assayed by reaction 5, are formed as a result of reactions 6 *plus* 7 (Table 2). It would appear, therefore, that the phosphate group is esterified on the streptidine moiety of streptomycin.

$$\text{streptomycin} + \text{Mg-ATP} \xrightarrow{\text{kinase}} \text{streptomycin-P} + \text{Mg-ADP} \quad (6)$$

$$\text{streptomycin-P} \xrightarrow[50\ C]{1\ N\ HCl} \text{streptidine-P} + \text{streptobiosamine} \quad (7)$$

DISCUSSION

The findings reported here are consistent with an identity of streptomycin kinase and the previously described enzyme, streptidine kinase (4). In fact, the observation that at low concentrations streptomycin is a somewhat better phosphate acceptor than streptidine suggests that the enzyme might be more accurately designated as streptomycin kinase. The precise location of the phosphate group on the streptidine moiety is not known, but it can be narrowed down to one of two positions, 5 or 6 (Fig. 1), since one of the streptidine-OH groups is in glycosidic linkage with the streptose moiety, and the -OH group located between the two guanidino groups appears not to be involved, inasmuch as 2-deoxy-streptidine can be phosphorylated by the enzyme (4).

The physiological role of streptomycin-P is not clear. Nomi and co-workers (2) have de-

tected a compound (L-component) in culture broths during streptomycin production which is converted to streptomycin on incubation with a factor (H-component) with many of the characteristics of a phosphatase, and they suggest that the L-component is a biosynthetic precursor of streptomycin. Our enzymatically synthesized streptomycin-P might well be identical with Nomi's L-component. However, the occurrence in streptomycin-producing strains of an active streptomycin kinase raises the question of whether streptomycin-P is an obligatory precursor of streptomycin or a product of enzymatic phosphorylation of preformed streptomycin. Some of the alternative possibilities are depicted in Fig. 5.

In biosynthetic pathway A, the phosphate group of streptidine-P is retained during addition of the streptose and N-methyl-L-glucosamine moieties, and streptomycin-P is the immediate precursor of streptomycin. Possible advantages of this pathway include easier retention of phosphorylated intermediates within the mycelium during biosynthesis and preservation of this potent inhibitor of protein synthesis (1) in an inactive phosphorylated form prior to secretion. A high concentration of inorganic phosphate in the culture medium might be expected to inhibit any dephosphorylation step involved in the biosynthesis of streptomycin.

In biosynthetic pathway B, intermediates following streptidine are not phosphorylated, and enzymatic phosphorylation serves as a detoxification mechanism or is in some manner related to a physiological function of streptidine-P or streptomycin-P in the producing organism. For example, streptomycin and streptomycin-P might represent active and inactive forms of a normal physiological regulator of protein synthesis (or some other process) in these organisms. It is of interest that bacteria containing certain resistance factors inactivate streptomycin not by phosphorylation but by transferring an adenyl group from ATP to the
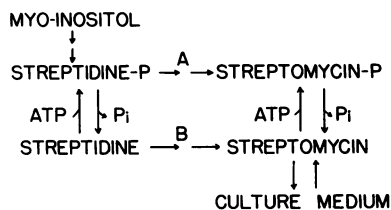

FIG. 5. *Possible physiological roles of streptomycin phosphate in Streptomyces. It is not known whether streptomycin biosynthesis proceeds by the phosphorylated pathway (A) or the nonphosphorylated pathway (B).*

3-position of the N-methyl-L-glucosamine moiety (3, 9).

Hopefully, further studies will help elucidate the function of streptomycin in these *Streptomyces*, as well as provide information of more general application to the problem of antibiotic synthesis and release into the medium.

ACKNOWLEDGMENTS

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