Streptomycin-phosphorylating Enzyme Produced by *Streptomyces griseus*

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Streptomycin-phosphorylating enzyme was reported previously to be produced in mycelium of *Streptomyces griseus* HUT 6037 at late stage of growth. In the present investigation, this enzyme was purified 200 times as high in specific activity as cell-free extract by means of salting out, chromatography on DEAE-Sephadex A-25 and gel filtration with Sephadex G-100. This enzyme was most stable at pH 8.0 and required 10^{-2} M Mg^{2+} in the reaction mixture for the highest activity. It lost the activity by heat treatment at 40°C for 15 min in absence of the substrate.

Mutant cultures were prepared on productivity of or tolerance to streptomycin, and their capacity to produce streptomycin-phosphorylating enzyme was examined. The cultures which had low to no capacity to produce streptomycin produced a small amount to none of the enzyme, suggesting that production of the streptomycin-phosphorylating enzyme had some correlation with streptomycin productivity of the culture. But no definite correlation was observed between productivity of the enzyme and the capacity to tolerate streptomycin.

In the previous paper,¹⁾ a streptomycin-phosphorylating enzyme was shown to be produced in the mycelium of *Streptomyces griseus* HUT 6037 in the stationary to autolyzing phase. This enzyme phosphorylated C₆-OH of streptidine moiety of streptomycin in presence of ATP and Mg^{2+} .

In the present paper, further purification and characterization of the enzyme were carried out, and, in the various mutants from the same culture as above, the correlation was investigated between the capacity to produce the enzyme and that to produce or tolerate streptomycin.

MATERIALS AND METHODS

The same materials and methods as those in the pre-

vious paper¹⁾ were employed in the present investigation in respect to strain, inoculum, culture medium, preparation of cell-free extract, assay of antibiotic potency and estimation of phosphorylated streptomycin.

Estimation of streptomycin-phosphorylating activity in enzyme preparation. The reaction mixture for phosphorylation of streptomycin consisted of 0.4 ml of 5 mM streptomycin sulfate, 0.4 ml of 15 mM ATP (disodium salt), 0.2 ml of 0.1 M magnesium sulfate, 0.2 ml of 1 M Tris-maleate buffer (pH 7.0), 0.8 ml of enzyme solution and a few drops of toluene. It was incubated at 30°C for 1 hr. For control, a reaction mixture was prepared substituting 0.5% aqueous sodium chloride solution for enzyme. As the phosphorylated streptomycin is biologically inactive, the quantity produced by the enzyme was shown with the difference of residual streptomycin in the above two reaction mixtures after incubation. The enzyme activity was expressed with the amount of streptomycin which was phosphorylated at 30°C for one hr

1) O. Nimi, G. Ito, S. Sueda and R. Nomi, *Agr. Biol. Chem.*, **35**, 848 (1971).

by 1 ml of enzyme solution in the reaction mixture.

Estimation of protein. Protein was estimated by the procedure described by Lowry *et al.*²⁾ and expressed as albumin.

Dry weight of mycelium. Two ml of mycelium suspension or sonicated mycelium was dried in a small dish made of aluminum foil at 60°C for 24 hr. After cooling in a desiccator, it was weighed.

Preparation of streptomycin-resistant mutants. Resistant cultures were obtained from *Streptomyces griseus* HUT 6037 by serial transfer on Bennett agar slants which were enriched with streptomycin in gradually increased amount in each transfer.

RESULTS

Partial purification of enzyme

If the streptomycin-phosphorylating enzyme is localized near the surface of the mycelium, namely external to the protoplasmic membrane, it could be liberated from the myceli-

um by osmotic shock method described by Nossal *et al.*,³⁾ giving an advantageous material for enzyme purification. But this was not the case when the same method was applied to 72 and 96 hr mycelia of *Streptomyces griseus* HUT 6037.

When ammonium sulfate was added giving 0.6 saturation to the cell-free extract of the organism grown for 96 hr, about 90% enzyme activity in the cell-free extract was salted out. The precipitate was dialyzed against 0.03 M Tris-HCl buffer (pH 8.0) for 15 hr. The dialyzate was added onto a column of DEAE-Sephadex A-25 equilibrated with the same buffer. After washing with the buffer, the column was eluted in gradient way with 0 to 5% NaCl dissolved in the buffer (Fig. 1). The enzyme was eluted in about 2% NaCl fractions. Those fractions were pooled and concentrated by freeze-drying. It was then subjected to gel filtration through a column of Sephadex G-100, eluting with the same buffer as above (Fig. 2). In Table I, purity

FIG. 1. Column Chromatography of Streptomycin-phosphorylating Enzyme on DEAE-Sephadex A-25.

Column size: 3×30 cm. Eluting solution: 0 to 5% sodium chloride solution in 0.03 M Tris-HCl buffer (pH 8.0). Fraction: 15 ml. : Enzyme activity. ○—○: Protein. —: Concentration of sodium chloride.

Enzyme activity was expressed with the amount of streptomycin (μM) which was phosphorylated by one ml of the fraction for one hr at 30°C.

2) O. H. Lowry, N. J. Rosenbraugh, A. L. Farr and R. T. Rondall, *J. Biol. Chem.*, **193**, 265 (1951).

3) N. G. Nossal and L. A. Heppel, *J. Biol. Chem.*, **241**, 3055 (1966).

FIG. 2. Gel Filtration of Streptomycin-phosphorylating Enzyme with Sephadex G-100.

Column size: 1×83 cm. Eluting solution: 0.03 M Tris-HCl buffer (pH 8.0). Fraction: 3 ml. $\square$: Enzyme activity. $\circ$ — $\circ$: Protein. $\circ$: Void volume.

Enzyme activity was expressed as in Fig. 1.

FIG. 3. Effect of pH on Enzyme Stability and Phosphorylating Reaction.

$\circ$ — $\circ$: Effect of pH on enzyme stability. $\triangle$ — $\triangle$ and $\blacktriangle$ — $\blacktriangle$: Effect of pH on phosphorylating reaction ($\blacktriangle$ — $\blacktriangle$, 0.1 M Tris-maleate buffer; $\triangle$ — $\triangle$, 0.1 M citric acid-sodium citrate buffer).

TABLE I. PURIFICATION AND RECOVERY OF STREPTOMYCIN-PHOSPHORYLATING ENZYME

Enzyme preparation	Enzyme activity* (SM: μ M/ml/hr)	Specific activity* (SM: μ M/mg/hr)	Recovery
Cell-free extract	2.38	0.42	100
After salting out and dialysis	2.42	0.86	88
Elate from DEAE-Sephadex A-25	2.0	14.28	59
Elate from Sephadex G-100	1.68	84.0	28

SM: Streptomycin

* Enzyme activity and specific activity were expressed with the amount of streptomycin (μ M) phosphorylated by one ml of the enzyme solution and one mg protein in the enzyme solution, respectively, for one hr at 30°C.

and yield of enzyme at different stages of purification are presented. The enzyme in the eluate from Sephadex G-100 was purified 200 times as high in specific activity as the cell-free extract, giving 28% yield. This eluate was used for examination of the following enzyme properties.

Influence of pH on enzyme stability

The enzyme solutions were adjusted to different pH values as is shown in Fig. 3 with HCl or NaOH solution and kept at room temperature for 15 min. After readjustment of pH to 8.0, they were made up to a fixed volume and the phosphorylating enzyme ac-

tivity was determined. The results are given in Fig. 3. This enzyme was most stable at pH 8.0, but rapidly inactivated on either side of the pH.

On the other hand, the influence of the substrate on stability of the enzyme was also examined at various pH. The reaction mixtures without buffer were adjusted to different pH (5 to 8) as are shown in Fig. 3. After addition of buffer in respective pH, the mixtures were incubated at 30°C for 1 hr. Citric acid-sodium citrate buffer (0.1 M) and Tris-maleate buffer (0.1 M) were employed for pH 5 to 6.5 and for pH 6.5 to 8, respectively. As is shown in Fig. 3, the pH range of maxi-

imum activity extended in comparison with that in absence of the substrate. This indicated that the enzyme was much stabilized in presence of the substrate. The effective pH ranged from 6 to 8 in the latter experiment and most stable pH was 8.0 in the former experiment. But pH 7.0 was adopted as the reaction pH, because phosphorylated streptomycin formed could easily be dephosphorylated at alkaline pH if the alkaline phosphatase (possibly H enzyme) contaminated the enzyme preparation.

Influence of heat on enzyme activity

The enzyme solutions which were adjusted to pH 8.0 were kept at different temperatures for 15 min, then rapidly cooled. The phosphorylating activity retained by the treated enzyme solution was determined by the procedure already indicated. The results are presented in Fig. 4. The enzyme was completely inactivated by the treatment at 40°C. However, it is noticeable that, when the enzyme was incubated with the substrate at 40°C, considerable activity was retained after 1 hr reaction, giving 2.25 $\mu\text{M}/\text{ml}/\text{hr}$ phosphorylated streptomycin, while little activity was

FIG. 4. Influence of Heat on Enzyme Activity.

retained at 50°C. This means that enzyme is stabilized in presence of the substrate.

Influence of magnesium concentration on enzyme activity

In order to determine the opt. concentration of Mg^{2+} in the reaction mixture, the influence

FIG. 5. Influence of Magnesium Concentration on Enzyme Activity.

TABLE II. COMPARISON IN STREPTOMYCIN-PHOSPHORYLATING ACTIVITY OF FOUR CULTURES WITH DIFFERENT PRODUCTIVITY OF STREPTOMYCIN

Strain No.	Streptomycin productivity ($\mu\text{g}/\text{ml}$)	Streptomycin tolerance ^{a)} ($\mu\text{g}/\text{ml}$)	Inactivated ratio ^{b)} (%)
HUT 6037 (parent)	252	500	79
4A-125	25	100	22
A-12	trace	200	0
N-1	0	< 50	0

^{a)} Cultures was inoculated on Bennett agar slants which contained different amount of streptomycin. The largest amount of streptomycin per ml in agar slant which allowed growth of the culture was described as streptomycin tolerance.

^{b)} Inactivated ratio was expressed as a percentage ratio of phosphorylated streptomycin to substrate streptomycin after one hr reaction. Cell-free extract of 96 hr mycelium in each strain was used as enzyme solution in the reaction mixture.

of magnesium sulfate on the enzyme activity was examined at the concentrations of 0, 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} M. The results are given in Fig. 5. The highest activity was obtained at 10^{-2} M of Mg^{2+} , while no activity was done in absence of the ion. Also cobaltous ion gave 80% effect of magnesium ion.

Production of phosphorylating enzyme by streptomycin-non-producing or low-producing cultures

In order to gain a clue to the physiological role of streptomycin-phosphorylating enzyme, the correlation was investigated between the productivity of streptomycin and that of phosphorylating enzyme. Streptomycin-non-producing and low-producing cultures were prepared from *Streptomyces griseus* HUT 6037 with N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich Chemical Co. Inc.). Three mutant cultures were compared with the parent one

in respect of streptomycin productivity and activity of phosphorylating enzyme in 96 hr mycelium. The results are presented in Table II. Less streptomycin-producing cultures produced less amount of phosphorylating enzyme. The streptomycin-non-producing culture gave no enzyme activity even by prolonged incubation for 20 hr.

Productivity of phosphorylating enzyme in streptomycin-resistant cultures

In the above experiments, more streptomycin-producing cultures produced more streptomycin-phosphorylating enzyme. Waksman *et al.*⁴⁾ suggested that more streptomycin-producing culture had more tolerance to streptomycin. Three cultures which were able to grow on media containing 1000, 2000 and 2500 μ g per ml of streptomycin, respectively, were prepared by serial transfers of

FIG. 6. Production of Streptomycin-phosphorylating Enzyme by Streptomycin-resistant Strains.

I shows the parent strain. II, III and IV show 1000, 2000 and 2500 μ g/ml streptomycin-tolerant strains, respectively. O—O: Specific enzyme activity. Δ --- Δ : Streptomycin in culture broth. x---x: pH.

Specific enzyme activity was expressed with the amount of streptomycin (μ M) which was phosphorylated by one mg of mycelium (dry weight) for one hr at 30°C. Cell-free extract of mycelium in each hour was used as enzyme solution.

4) S. A. Waksman, H. C. Reilly and D. B. Johnstone, *J. Bacteriol.*, **52**, 393 (1946).

the same parent culture (HUT 6037) as above on streptomycin-enriched agar slants. These resistant cultures were grown in glucose-meat extract-peptone medium and examined in each 24 hr after inoculation on production of streptomycin and the phosphorylating enzyme. As is shown in Fig. 6, two resistant cultures produced both streptomycin-phosphorylating enzyme and streptomycin, but one did only a very small amount of the enzyme and streptomycin. It seemed likely that the productivity of the enzyme had no correlation with resistant capacity of the culture. But here again the productivity of the enzyme looked to have strong correlation to that of streptomycin, though quantitative parallelism was not always observed between the two.

It is also noticeable that, in streptomycin-producing cultures among the resistant ones, the peak of production of the enzyme preceded streptomycin excretion. This is sometimes observed also in the parent culture.

DISCUSSION

As was described above, productivity of streptomycin-phosphorylating enzyme looked to have no correlation with streptomycin tolerance of the culture, but rather to associate with streptomycin productivity. From this fact, the enzyme is supposed to play a role either in direct phosphorylation of streptomycin already produced or in phosphorylation of some intermediate on the biosynthetic pathway. Also the possibility is not excluded for the enzyme to be produced by induction with streptomycin which has been produced by the organism itself.

On direct phosphorylation of the already

produced streptomycin just before excretion from mycelium, the possibility was discussed in the previous paper, denying the mechanism.

On the other hand, the authors already reported that young mycelium of *Streptomyces griseus* HUT 6037 which should have few streptomycin-phosphorylating enzyme excreted phosphorylated streptomycin (referred to as L) when it was suspended in aqueous glucose and sodium chloride solution.⁵⁻⁷⁾ Also in streptomycin-producing culture broth, L appeared outside the cell prior to streptomycin accumulation, i.e. in young culture broth where the pH is below neutral. When the pH value of culture broth was controlled to be at slightly acid side through every stage of culture, all antibiotic accumulated in the phosphorylated form (L)⁸⁾. Furthermore, few phosphorylated streptomycin are detected in free form in cell-free extract of the mycelium through all stages of growth. From these facts, it looks to be reasonable to suppose that phosphorylated streptomycin in mycelium exists in a bound form to some cell component. If the phosphorylating enzyme intrinsically participates in formation of this bound phosphorylated streptomycin, it still remains to be solved when and how the enzyme plays the role on the biosynthetic pathway.

The third possibility for induction of the enzyme formation by streptomycin is now under investigation.

5) R. Nomi, O. Nimi and T. Miyazaki, *Agr. Biol. Chem.*, **30**, 296 (1966).

6) R. Nomi, O. Nimi, T. Miyazaki, A. Matsuo and H. Kiyohara, *ibid.*, **30**, 1269 (1966).

7) *idem*, *ibid.*, **31**, 973 (1967).

8) R. Nomi, O. Nimi and T. Kado, *ibid.*, **32**, 1256 (1968).