ASSESSMENT OF COMPETITIVE ACTION OF STREPTOMYCIN 6-KINASE AND STREPTOMYCIN 6-PHOSPHATASE IN THE IN VITRO PROTEIN SYNTHESIS OF A STREPTOMYCIN-PRODUCING MICROORGANISM

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1. Introduction

While ribosomes of streptomycin (SM)-producing *Streptomyces griseus* HUT 6037 bound SM [1,2], bound SM was released by phosphorylation with SM 6-kinase in the cells [3]. The phosphate ester of SM (SM 6-P) no longer bound to the ribosomes [3]. Thus, in vitro protein synthesis of this organism was little inhibited by low [SM] [3]. However, the synthesis was inhibited by ~60% in the presence of 100 μg SM/ml [1,3], though most SM was thought to be phosphorylated by SM 6-kinase. This inhibition was attributed to the high [SM 6-P] derived from 100 μg SM/ml or SM regenerated from SM 6-P by an alkaline phosphatase which is periplasmic enzyme and inevitably contained in the cell-free extract.

According to [4], in vitro protein synthesis in *S. griseus* ISP 5236 was weakly inhibited even in the presence of 200 μg SM/ml. This result was significantly different in the inhibitory ratio from our result. They discussed that, since the cell-free extract of our strain might contain alkaline phosphatase, the effect of SM 6-kinase was possibly reduced so that the cell-free protein synthesis appeared sensitive to SM. However, the cell-free extract of strain ISP 5236 might also contain alkaline phosphatase. It would be therefore necessary to elucidate the cause of inconsistency in the inhibitory ratio between the above two strains.

The influences of high [SM 6-P] on protein synthesis and of alkaline phosphatase on regeneration of SM from SM 6-P in the cell-free protein-synthesizing system of *S. griseus* were determined. SM 6-P did not inhibit protein synthesis in the cell-free system even at high concentrations. Alkaline phosphatase in the cell-free extract could regenerate SM from SM 6-P, but the action in the in vitro protein-synthesizing system was not so strong as to reduce extensively SM 6-P produced by SM 6-kinase. Most of the SM added to the reaction mixture was transformed to SM 6-P. The significant variation in inhibition of cell-free protein synthesis as was observed between the above two strains was most possibly brought by a slight difference in concentration of free SM in the reaction mixture.

2. Materials and methods

The materials and methods used here were as in [3] except: SM 6-phosphatase (SM 6-phosphate phosphohydrolase) activity in S-150 fraction was expressed as the amount of [^{32}P]phosphate released by the enzyme from SM 6-[^{32}P]phosphate in a reaction mixture consisting of 10 μl 500 mM Tris/HCl (pH 8.5), 10 μl 100 mM $MgSO_4$, 40 μl 0.50 mM SM 6-[^{32}P]-phosphate (4.12 μCi/μmol) and 40 μl S-150 fraction. After incubation at 37°C for 2 h, the reaction mixture was applied onto a column of Amberlite CG-50 (NH_4^+ form, 0.5 × 3 cm). The column was washed with 1.5 ml deionized water and the effluent containing [^{32}P]phosphate was collected in a scintillation vial containing 15 ml hydrophilic scintillation cocktail [3] and the radioactivity was measured by liquid scintillation spectrometry. The amount of [^{32}P]phosphate produced in the reaction mixture was calculated from the radioactivity of the effluent and the specific activity of SM 6-[^{32}P]phosphate in the reaction mixture.

Para-nitrophenylphosphatase (*p*-nitrophenylphosphate phosphohydrolase) activity was estimated as follows. The reaction mixture consisted of 120 μl 500 mM Tris/HCl (pH 8.5), 120 μl 100 mM $MgSO_4$,

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600 μl 1.2 mM *p*-nitrophenylphosphate and 600 μl S-150 fraction. After incubation at 37°C for 1 h, absorbancy at 420 nm given by *p*-nitrophenol was determined. The amount of hydrolyzed *p*-nitrophenylphosphate was calculated from calibration curves with *p*-nitrophenol and the dephosphorylating activity was expressed as the amount of substrate hydrolyzed under the above condition by 1 mg protein in the S-150 fraction.

3. Results and discussion

Cell-free protein synthesis of the SM producer (*S. griseus* HUT 6037) was shown to be sensitive to SM [1–3], though a significant amount of SM added to the cell-free system was inactivated by phosphorylation with SM 6-kinase in S-150 fraction. In this case, if SM 6-P is dephosphorylated by SM 6-phosphatase, the resulting SM can inhibit protein synthesis, reducing the protecting activity of SM 6-kinase. This would lead to a wrong interpretation of self-protection by SM 6-kinase in the cells. It is important to determine the influence of SM 6-phosphatase in the cell-free protein-synthesizing system. The content of SM 6-phosphatase in the S-150 fraction was determined using the cells from the middle (16 h) and late (22 h) exponential phases and the stationary (46 h) phase of growth. As shown in table 1, SM 6-phosphatase was detected in all three S-150 fractions.

To estimate the influence of this enzyme, SM 6-P was added to the reaction mixture of poly(U)-directed poly(Phe) synthesis and the inhibitory effect was compared with that by SM. Two kinds of reaction mixtures were employed. One contained the ribosomes and S-150 fraction derived from *S. griseus* HUT 6037 (HUT 6037 system), and the other contained the ribosomes from *S. griseus* HUT 6037 and the S-150 fraction from *S. griseus* KSN (hybrid system). *S. griseus* KSN was isolated from HUT 6037 and produced neither SM nor SM 6-kinase [2,3], but produced only a very small amount of SM 6-phosphatase (table 1), though it produced *p*-nitrophenylphosphatase to the same extent as the parent strain (table 2). The results given by HUT 6037 and hybrid systems are shown in fig.1. SM 6-P (75 μg/ml) inhibited poly(Phe) synthesis

Table 1
Activity of SM 6-phosphatase in S-150 fraction from the cells at different stages of growth

Strains	Stages of growth	Spec. act.
HUT 6037	Middle exponential	0.72
	Late exponential	1.31
	Stationary	0.85
KSN	Late exponential	0.04

Specific activity of enzyme was expressed as nmol phosphate released from SM 6-[^{32}P]phosphate in 1 h by 1 mg protein in S-150 fraction

Table 2
Activity of *p*-nitrophenylphosphatase in S-150 fraction prepared from late exponential phase cells

Strains	Spec. act.
HUT 6037	0.304
KSN	0.300

Specific activity of enzyme was expressed as μmol *p*-nitrophenol transformed from *p*-nitrophenylphosphate in 1 h by 1 mg protein in S-150 fraction

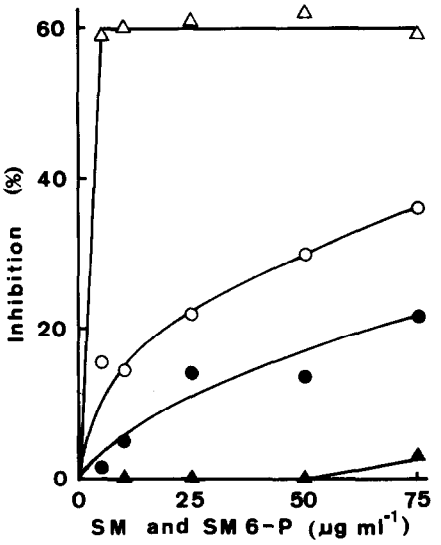

Fig.1. Inhibition of in vitro polyphenylalanine synthesis by SM and SM 6-P in HUT 6037 and hybrid systems. The HUT 6037 system was constructed with S-150 fraction and ribosomes from *S. griseus* HUT 6037. The hybrid system was constructed with S-150 fraction from *S. griseus* KSN and ribosomes from *S. griseus* HUT 6037. Inhibition (%) by a given concentration of SM and SM 6-P was expressed as a ratio of the reduced amount of poly(Phe) synthesis to that synthesized in the SM- and SM 6-P-free reaction mixture, respectively. Ribosomes (60 μg) and S-150 fraction (240 μg) from the late exponential phase cells were used. Inhibition by SM: HUT 6037 (○) and hybrid (△) systems. Inhibition by SM 6-P: HUT 6037 (●) and hybrid (▲) systems.

Table 3
Inhibition of in vitro poly(Phe) synthesis by SM in the hybrid system constructed with S-150 fraction from *S. griseus* KSN and ribosomes from *S. griseus* HUT 6037

SM (μg/ml)	Inhibition ratio (%)
0	0
0.05	15
0.1	17
0.15	25
0.2	30
0.5	32
1.0	47

Inhibition ratio is expressed as in fig.1

of the HUT 6037 system by 22%, though only ~3% inhibition was produced in the hybrid system at the same concentration of SM 6-P. From the influence of SM on the hybrid system (fig.1, table 3), ~0.12 μg SM/ml was required to produce 22% inhibition of poly(Phe) synthesis. Thus the SM converted from SM 6-P (75 μg/ml) in the HUT 6037 system in fig.1 should also be ~0.12 μg/ml. This means that, even if 75 μg SM/ml in the reaction mixture of the HUT 6037 system is completely phosphorylated by SM 6-kinase, only 0.12 μg SM/ml was regenerated by phosphatase from SM 6-P.

Also in the hybrid system, a slight inhibition of poly(Phe) synthesis by SM 6-P is attributed to a very small amount of SM which was produced from SM 6-P by a low SM 6-phosphatase activity. It is strongly suggested that SM 6-P does not inhibit protein synthesis in an SM producer.

However, inhibition of poly(Phe) synthesis by 75 μg SM/ml in the HUT 6037 system was ~36% here. This inhibition must be caused by both the residual SM which was not phosphorylated and the SM regenerated from SM 6-P. The total amount of these two kinds of SM will be only ~0.6 μg/ml on the basis of table 3 and fig.1.

Most SM in the reaction mixture of in vitro protein-synthesizing systems must be converted to inactive SM 6-P and only a small amount of SM which was not phosphorylated or/and was regenerated from SM 6-P gives a high ratio of inhibition.

In [5] protein synthesis of the SM producer in the idiophase was not inhibited by SM added extracellularly. We concluded in [3] that decrease in uptake of SM by cells in the idiophase must be indispensable for self-protection of the producer. These results support that conclusion. An SM producer takes up extracellular SM into the cells [3,5–7]. This SM is mostly inactivated by SM 6-kinase in the cells. In this case, reactivation of SM 6-P will not occur in the cells, because SM 6-phosphatase is not contained in the cytoplasm. However, the inactivation of SM in the cells is not complete at high SM concentration, because SM always gives more inhibition than SM 6-P at the same concentration (fig.1). The residual SM will inhibit protein synthesis. To keep the concentration of residual SM in the cells sufficiently low to allow protein synthesis, decrease in uptake of SM, especially in the idiophase, will be required.

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