MECHANISM OF PROTECTION OF PROTEIN SYNTHESIS AGAINST STREPTOMYCIN INHIBITION IN A PRODUCING STRAIN

MASANORI SUGIYAMA, HIROSHI MOCHIZUKI, OSAMU NIMI and RYOSAKU NOMI

Department of Fermentation Technology, Faculty of Engineering, Hiroshima University, Sendamachi 3, Naka-ku, Hiroshima 730, Japan

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The influence of streptomycin (SM) on protein synthesis in a SM-producing strain was investigated using polyuridylic acid-directed polyphenylalanine synthesis in cell-free extracts. Tolerance of protein synthesis to SM developed with increasing culture age of cells and could be attributed to a decrease in affinity of the ribosomes for SM and an increase in SM 6-kinase activity in the cells. SM 6-phosphate produced from SM by SM 6-kinase did not bind to ribosomes and, furthermore, ribosome-bound SM was effectively released on phosphorylation with SM 6-kinase. Also a decrease in cell permeability to SM during the production phase may contribute in protecting protein synthesis from the antibiotic.

Streptomycin(SM)-producing microorganisms must be protected from the lethal effect of their own antibiotic products. One of the possible protection mechanisms is enzymic modification of the antibiotic so that it can no longer interact with its target in the cell. Phosphorylation of SM by SM 6-kinase has been shown to inactivate the antibiotic. However, the lack of activity of SM 6-phosphate(SM 6-P) has usually been determined by bioassay with bacteria sensitive to SM; this result does not necessarily indicate a failure of SM 6-P to act on protein synthesis. Thus, there has been no confirmation that SM 6-P is inactive as a protein synthesis inhibitor although TERAOKA and TANAKA[1] showed that dihydrostreptomycin(DSM) phosphate prepared with a cell-free extract of *Streptomyces griseus* was unable to bind to *Escherichia coli* ribosomes. The effect of SM 6-P on *in vitro* protein synthesis of the SM producer has been difficult to determine due to degradation of ribosomes by protease action during isolation[2~5].

In the present study, we have isolated active ribosomes and an S-150 fraction from *S. griseus* in the absence of protease activity. We have shown that SM 6-P does not bind to the ribosomes of the SM producer, and in addition that ribosome-bound SM was effectively liberated by SM 6-kinase. The affinity of the ribosomes for SM gradually decreased during growth.

Materials and Methods

Microorganisms

Streptomyces griseus HUT 6037 produced SM about 200 μg/ml in 1% glucose - meat extract - peptone (GMP) medium[6] at 28°C with shaking. *Streptomyces griseus* KSN, a threonine auxotroph isolated from strain HUT 6037 by treatment with ultraviolet radiation produced neither SM nor SM 6-kinase, and was susceptible to SM.

Preparation of Ribosomes and S-150 Fraction

The ribosomes and S-150 fraction were prepared as described previously[5]. Since these preparations were contaminated with protease inhibitors used to prevent denaturation of the ribosomes, they were dialyzed against buffer I[5] at the final step of preparation. Elimination of the protease inhibitors

realized three times as much protein-synthesizing activity.

The amount of ribosomes was calculated by assuming that 1 A_{260} unit was equal to 70 μg or 25.7 pmole[7]. The quantity of S-150 fraction was expressed as protein determined by the method of LOWRY *et al.*[8] using bovine serum albumin as a standard.

Assay of Protein Synthesis *In Vitro*

Conditions for assay of polyphenylalanine synthesis were as described previously[9] except that ATP-Na_2 and L-[U-^{14}C]phenylalanine were present at 0.9 mM and 0.05 μCi (100 μCi/μmole), respectively, and the incubation was carried out at 28°C for 30 minutes.

Determination of SM 6-Kinase in S-150 Fraction

The SM 6-kinase activity was expressed as the amount of SM 6-P produced from SM in a reaction mixture consisting of 20 μl of 40 mM SM sulfate (pH 7.0), 20 μl of 120 mM ATP-Na_2(pH 7.0) containing 0.1 μCi adenosine 5′-[γ-^{32}P]triphosphate as a tracer, 20 μl of 250 mM glycylglycine buffer (pH 7.0) containing 25 mM magnesium sulfate, and 40 μl of the S-150 fraction. After incubation at 30°C for 1 hour, a 20 μl portion was poured onto a column of Amberlite CG-400 (Cl^- form, 0.5 cm × 3 cm). The column was washed with one ml of deionized water and the effluent containing SM 6-[^{32}P]phosphate was collected in a scintillation vial containing 15 ml of a hydrophilic scintillation cocktail which consisted of 2.6 g 2,5-diphenyloxazole, 0.08 g 1,4-bis-[2-(5-phenyloxazolyl)]benzene, 300 ml toluene and 500 ml methyl cellosolve. The amount of SM 6-P produced in the reaction mixture was calculated from the radioactivity of the effluent and the specific radioactivity of ATP in the reaction mixture.

Binding of DSM and SM 6-P to Ribosomes

The ribosomes were dialyzed overnight at 4°C against the buffer consisting of 10 mM tris-HCl (pH 7.65), 60 mM potassium chloride, 10 mM magnesium acetate and 6 mM 2-mercaptoethanol. The reaction mixture contained, in 125 μl, 50 mM tris-HCl (pH 7.65), 16 mM magnesium acetate, 140 μg of ribosomes, 605,000 c.p.m. (329 pmole) of [^{3}H]DSM and the indicated concentration of potassium chloride. After incubation at 28°C for 20 minutes, the mixture was cooled immediately in an ice bath and diluted with 5 ml of the cold buffer consisting of 10 mM tris-HCl (pH 7.65), 16 mM magnesium acetate and the same concentration of potassium chloride as in the reaction mixture. The complex of [^{3}H]DSM with ribosomes was collected on a Millipore filter (HA, 0.45 μm) prewashed with 3 ml of the cold buffer and washed 5 times with 3 ml of the same buffer. The bound [^{3}H]DSM was determined by liquid-scintillation spectrometry.

Binding of SM 6-P to ribosomes was determined using SM 6-[^{32}P]phosphate which was prepared from SM and [γ-^{32}P]ATP with the S-150 fraction from stationary phase cells and isolated by column chromatography with Amberlite CG-50[10].

Results

Influence of SM on Protein Synthesis

To determine the influence of SM on protein synthesis in the producing strain, the extent of inhibition by SM of polyuridylic acid-directed polyphenylalanine synthesis in cell-free extracts was compared between a producing strain and a non-producing one. As Fig. 1 shows, polyphenylalanine synthesis in the former strain was only weakly inhibited by 0.1 to 1 μg/ml SM, while synthesis in the non-producing strain was strongly inhibited over the same concentration range. This indicated that protein synthesis in the SM-producing strain is tolerant to SM. The influence of SM on polyphenylalanine synthesis was also determined in cell-free systems prepared from cells of the producing strain isolated from middle (16 hours) and late (22 hours) exponential phases, and stationary (46 hours) phase of growth (Fig. 2). When 5 μg/ml or more SM was used, polyphenylalanine synthesis was inhibited in all three, showing stronger inhibition at higher concentrations of SM. It was noticeable that cell-free systems from the older cells was less susceptible to inhibition by SM.

Fig. 1. Inhibition of polyphenylalanine synthesis by SM in *in vitro* systems prepared from SM producer and non-producing mutant.

Inhibition (%) by a given concentration of SM was expressed as a ratio of the reduced amount of polyphenylalanine synthesis to that synthesized in the respective SM-free reaction mixture. The ribosomes (60 μg) and S-150 fraction (240 μg) from the late exponential phase cells were used. 1; SM producer (*S. griseus* HUT 6037). 2; SM-non-producing mutant (*S. griseus* KSN).

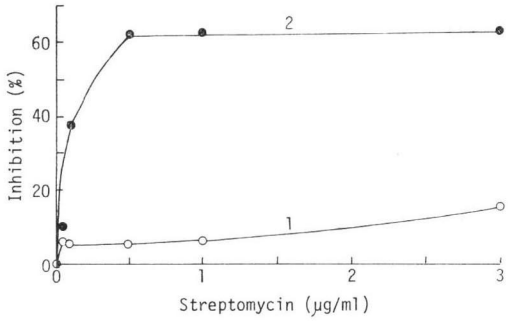
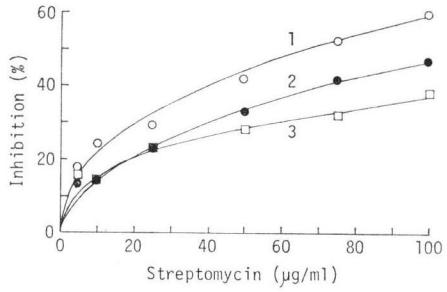


Fig. 2. Inhibition of polyphenylalanine synthesis by SM in *in vitro* systems prepared from *S. griseus* HUT 6037 at three stages of growth.

Inhibition ratio (%) by SM was expressed as in Fig. 1. Amounts of ribosomes and S-150 fraction used were 45 μg and 180 μg, respectively, in all systems. 1, 2 and 3 indicate components derived from the middle, late exponential, and the stationary phase, respectively.



In order to elucidate the causes of these variations in SM inhibition, we studied the roles of SM 6-kinase and the affinity of ribosomes for SM in protecting polyphenylalanine synthesis from the inhibitory effects of SM.

Table 1. Activity of SM 6-kinase in S-150 fractions from *S. griseus* HUT 6037 cells at three stage of growth.

Age of cells (hour)	Specific activity
16	0.29
22	0.59
46	1.58

Specific activity of enzyme was expressed as μmole of SM 6-phosphate produced from SM in one hour by one mg protein in S-150 fraction.

Content of SM 6-Kinase in S-150 Fraction

Since the SM-non-producing strain KSN produces no SM 6-kinase, the enzyme content of the SM-producing strain was determined in the three S-150 fractions used in the above experiments. As shown in Table 1, SM 6-kinase was detected in all three, however the specific activity from the stationary phase extract was about five times that of the middle exponential phase extract.

Affinity of Ribosomes for SM 6-P

The above results encouraged us to examine the binding affinity of ribosomes for SM 6-P. As Fig. 3 shows, SM 6-P did not bind to the ribosomes of *S. griseus*, suggesting that phosphorylation of SM by SM 6-kinase was an effective means of protecting the ribosomes from the antibiotic.

Binding of DSM to *S. griseus* Ribosomes

The binding affinity of the ribosomes for DSM was determined using the three ribosomal preparations described above. Since the affinity was affected by monovalent cations such as K^+ or $NH_4^{+1)}$, the binding was determined under various concentrations of potassium chloride. As Fig. 4 shows, the affinity of ribosomes for DSM decreased with increasing phase of growth. The ribosomes from stationary phase cells had 60% of the affinity for DSM compared to ribosomes from the middle exponential phase cells regardless of potassium chloride concentration, suggesting that a decrease in the affinity of the ribosomes for SM is a cause for reduction of SM susceptibility of protein synthesis during later stages of growth.

Fig. 3. Affinity of binding of SM 6-P to ribosomes.

The ribosomes from the late exponential phase cells were used. The reaction mixture was consisted of 50 mM tris-HCl (pH 7.65), 16 mM magnesium acetate, 25.6 mM potassium chloride, 104.3 pmole ribosomes, and either 400 pmole SM 6-[^{32}P]phosphate (1) or 329 pmole [^{3}H]DSM (2), in 125 μl.

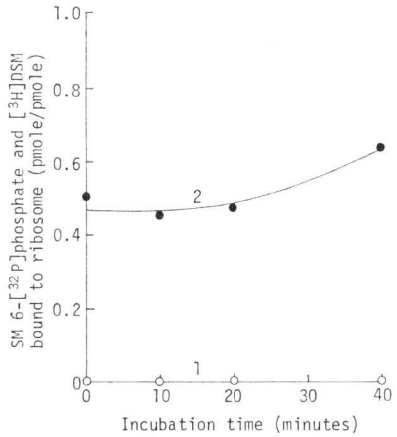


Fig. 4. Binding of [^{3}H]DSM to the ribosomes isolated from three stages of growth of *S. griseus*.

The ribosomes used were derived from the same three growth stages as described in Fig. 2. Binding ratio of antibiotic to the ribosomes was calculated on the molar basis.

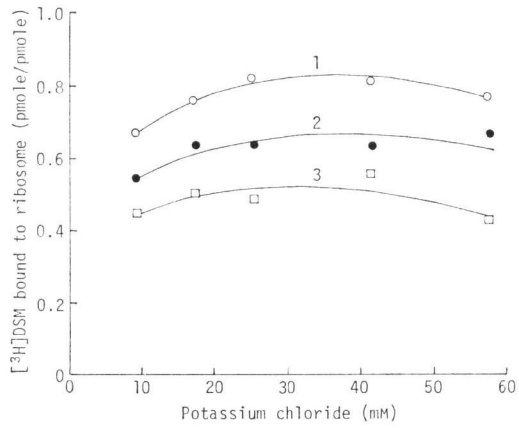


Fig. 5. Release of ribosome-bound [^{3}H]DSM by phosphorylation.

The ribosomes from middle exponential phase cells were used. Four reaction mixtures with the composition of 77.1 pmole ribosomes, 50 mM tris-HCl (pH 7.65), 16 mM magnesium acetate, 25.6 mM potassium chloride and 329 pmole [^{3}H]DSM were incubated at 28°C. After 10 minutes of incubation, one of the following solutions was added to one of the reaction mixtures, and the [^{3}H]DSM bound to the ribosomes was determined at indicated times.

The added solutions:

1; 20 μl of buffer A* and 5 μl of buffer B**.

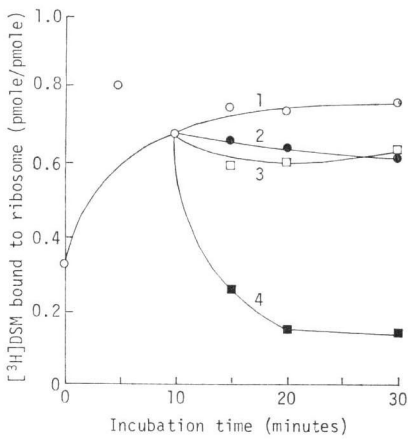
2; 20 μl of buffer A and 5 μl of 2 mM ATP-Na_2 dissolved in buffer B.

3; 20 μl of the S-150 fraction (258 μg) prepared from the stationary phase cells and 5 μl of buffer B.

4; 20 μl of the S-150 fraction and 5 μl of 2 mM ATP-Na_2.

* Buffer A: 10 mM tris-HCl (pH 7.65), 10 mM magnesium acetate, 30 mM NH_4Cl and 6 mM 2-mercaptoethanol.

** Buffer B; 10 mM tris-HCl (pH 7.65), 16 mM magnesium acetate and 25.6 mM potassium chloride.



Release of Ribosome-bound SM by Phosphorylation

As above observed, a significant amount of SM can bind to ribosomes at all stages of growth. It was of interest to know if SM bound to the ribosomes could be phosphorylated by SM 6-kinase. In experiments determining the affinity of ribosomes to DSM, an S-150 fraction and ATP were added to the reaction mixture after the ribosomes were saturated with DSM. As Fig. 5 shows, the bound [^{3}H]DSM was liberated effectively from the ribosomes, most probably as a result of phosphorylation with SM 6-

kinase. The results imply that, even if SM binds to the ribosomes in the cells, it can be released by phosphorylation.

Uptake of SM by the Producing Strain

From the above experiments, both the phosphorylation of SM and a decrease in affinity of ribosomes for SM seem to play significant roles in the protection of ribosomes from SM in *S. griseus*. It is noticeable that older cells contained greater SM 6-kinase activity and also showed a lower affinity of ribosomes for SM. Nevertheless, even in cell-free extracts of stationary phase cells, polyphenylalanine synthesis was inhibited to a significant extent by SM under the conditions used in Fig. 2. Thus the protection of ribosomes by SM 6-kinase and the reduced affinity of ribosomes for SM appears to be incomplete when a large amount of SM is added to the protein-synthesizing system. On the other hand, a SM-producing organism takes up extracellular SM[11,12] and the SM-producing strain used in the present studies produces about 200 μg/ml SM. We conclude that some mechanism other than those described above must operate in an SM producer for protection of protein synthesis against the antibiotic produced. The rate of uptake of DSM was measured using the exponential and stationary phase cells according to the method described by PIWOWARSKI and SHAW[11] except that 5.4 μmole [^{3}H]DSM (1,113 μCi/mmole) was added to 10 ml of the suspended mycelium. The initial rate of uptake was 300 c.p.m. of [^{3}H]DSM /mg dry cell weight/minute in late exponential phase cells and 50 c.p.m. in the stationary phase cells. This indicates that the permeability of *S. griseus* cells to SM is reduced during growth.

Discussion

TERAOKA and TANAKA[1] have reported that the binding of DSM to the ribosomes of *S. griseus* was very low and PIWOWARSKI and SHAW[11] showed that the ribosomes of *S. bikiniensis* (SM producer) would bind 0.3 molecules DSM per 30S subunit. CELLA and VINING[2] demonstrated that cell-free extracts from *S. griseus* contained some factors inhibitory to *in vitro* protein synthesis. These factors were shown to be proteases by VALU and SZABÓ[3,4] and SUGIYAMA *et al.*[5]. In the present paper, ribosomes of *S. griseus* were prepared under conditions that provided protection against protease activity but which were shown to bind DSM to a significant extent through all stages of growth, though the binding affinity did decrease with the growth development. Therefore, polyphenylalanine synthesis in *in vitro* protein-synthesizing systems using these ribosomes were inhibited by SM. This inhibition, however, could be prevented effectively if the SM was phosphorylated by SM 6-kinase. Even the ribosome-bound SM was liberated by phosphorylation.

Uptake of external SM by cells of SM producers decreases with growth. CELLA and VINING[12] have stated that reduced cell permeability to SM was important for protection of cells from the lethal effect of a high antibiotic concentration in the culture broth. By contrast, PIWOWARSKI and SHAW[11] showed that decreased uptake did not appear to play a significant role in protecting cells, because a SM-susceptible mutant which produced neither SM nor SM 6-kinase showed DSM-uptake equal to or less than the parent strain at the same stage in the growth cycle. The present studies using a SM-producing strain of *S. griseus* revealed that protein synthesis could be inhibited by SM even in stationary phase cells containing a considerable amount of SM 6-kinase, in the presence of sufficiently large amounts of the antibiotic. Thus our results support the suggestion of CELLA and VINING, indicating a role of reduced drug uptake in protection of a SM-producing organism from the inhibitory effects of its own antibiotic. However, the major contributor to resistance appears to be the SM 6-kinase (Biotechnol. Lett. 3: 357~362, 1981).

Acknowledgement

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