

SUSCEPTIBILITY OF PROTEIN SYNTHESIS TO STREPTOMYCIN IN STREPTOMYCIN-PRODUCING *STREPTOMYCES GRISEUS*

M. SUGIYAMA, H. KOBAYASHI*, O. NIMI and R. NOMI

Dept. of Fermentation Technology, Faculty of Engineering, Hiroshima University, Sendamachi 3, Hiroshima 730 and *Institute of Medical Science, The University of Tokyo, Shiroganedai 4, Minato-ku, Tokyo 108, Japan

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1. Introduction

Streptomycin inhibits protein synthesis in the streptomycin-sensitive bacteria by binding to their ribosomes. Since a streptomycin producer must be resistant to streptomycin, it is of interest to know if the protein synthesis of the streptomycin producer is inhibited by its own product. Cella and Vining [1] could not demonstrate the effect of streptomycin on protein synthesis in an in vitro system prepared from *Streptomyces griseus*, because the cell extract contained some factor inhibitory to polyuridylylate-dependent polyphenylalanine synthesis. Teraoka and Tanaka [2] reported that the binding of dihydrostreptomycin to ribosomes of *S. griseus* was very low. On the other hand, Piwowarski and Shaw [3] showed that the crude ribosomes of *S. bikiniensis* (streptomycin producer) bound 0.3 dihydrostreptomycin molecules/30 S ribosomal subunit.

This paper describes the establishment of an in vitro polyphenylalanine-synthesizing system in *S. griseus*. Using this system, investigations were carried out on the inhibitory effect of streptomycin on polyphenylalanine synthesis as well as on the binding affinity of streptomycin to the ribosomal subunits.

2. Materials and methods

Streptomyces griseus HUT 6037 (streptomycin producer) was grown in 1% glucose–meat extract–peptone medium [4] at 28°C. *Escherichia coli* Q13 which was sensitive to streptomycin was employed for comparison with *S. griseus* and was grown in nutrient broth at 37°C. The cells, harvested at a given

time, were washed twice with buffer I [10 mM Tris–HCl (pH 7.65), 30 mM NH₄Cl, 10 mM Mg-acetate and 6 mM β-mercaptoethanol], then ground with quartz sand and extracted with the same buffer containing 2 μg/ml of deoxyribonuclease I. The extract was centrifuged at 30 000 × g for 30 min (S30 fraction). The ribosomes were sedimented from the S30 fraction by centrifugation at 150 000 × g for 3 h, then washed with buffer II [20 mM Tris–HCl (pH 7.65), 1 M NH₄Cl, 10 mM Mg-acetate, 6 mM β-mercaptoethanol and 1.1 M sucrose] by the methods in [5]. The 150 000 × g supernatant fluid was dialyzed against buffer I and used as the S150 fraction. The reaction mixture and conditions for assay of in vitro polyphenylalanine synthesis were the same as in [6], but spermidine was 0.4 mM and t-RNA was derived from *E. coli* MRE 600 (Boehringer product). Binding of [³H]dihydrostreptomycin to ribosomes was determined as in [2].

3. Results and discussion

Using the ribosomes and the S150 fraction prepared from the logarithmic phase cells of the two microorganisms, measurements were performed on the in vitro polyphenylalanine-synthesizing capacity and the binding affinity of [³H]dihydrostreptomycin to the ribosomes. Neither polyphenylalanine synthesis nor binding was observed in the case of *S. griseus*, though synthesis and binding were evident in the case of *E. coli* (data not shown). These observations were essentially the same as those reported in [1,2].

According to [7–9], protease interfered with the protein synthesis in in vitro system of *Bacillus subtilis*. Elimination of protease activity from the in

vitro protein-synthesizing system of *S. griseus* was attempted. After the cells of *S. griseus* were harvested, they were washed twice with a buffer which contained 1 M KCl and 0.5 mM Mg-Titriplex in place of 30 mM NH_4Cl in buffer I, then twice more with the buffer which contained 5 mM Mg-Titriplex, 3.45 mM phenyl-methylsulfonylfluoride and 0.2 mM diisopropylfluorophosphate as the protease inhibitors, in addition to the components of buffer I (buffer III). The latter buffer was used also for extraction of ground cells. To wash the ribosomes, buffer II containing the 3 protease inhibitors was used. The ribosomes and S150 fraction thus prepared from logarithmic phase cells were used for the in vitro polyphenylalanine-synthesizing system. As shown in fig.1, the polyphenylalanine synthesis was clearly observed, though the ability of *S. griseus* system was a little lower than that of *E. coli*. It is obvious that protease inhibits the in vitro protein-synthesizing system of *S. griseus*. Then the effect of streptomycin was investigated on polyphenylalanine synthesis in the in vitro system prepared from logarithmic phase cells, using protease inhibitors. As shown in fig.2, streptomycin inhibited polyphenylalanine synthesis. The ribosomes, prepared by applying protease inhibitors, were examined for

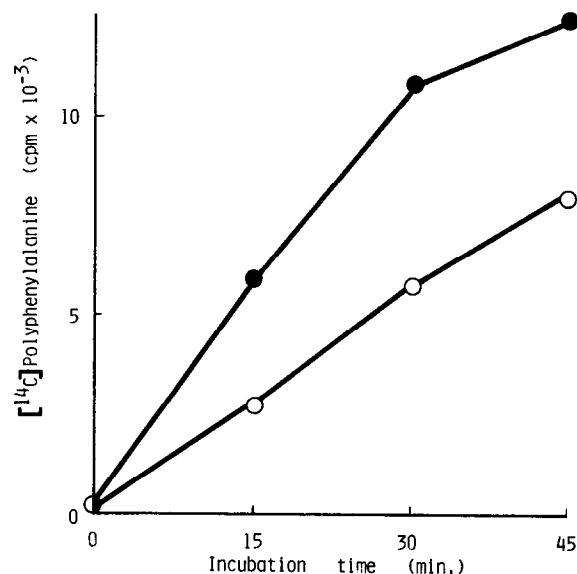


Fig.1. Polyphenylalanine synthesis by in vitro systems prepared from logarithmic phase cells of (○) *S. griseus* and (●) *E. coli*. The ribosomes and the S150 fraction from *S. griseus* were prepared in the presence of protease inhibitors. Amounts of the ribosomes and the S150 fraction employed were 52 μg and 258 μg (as protein), respectively, in both microorganisms.

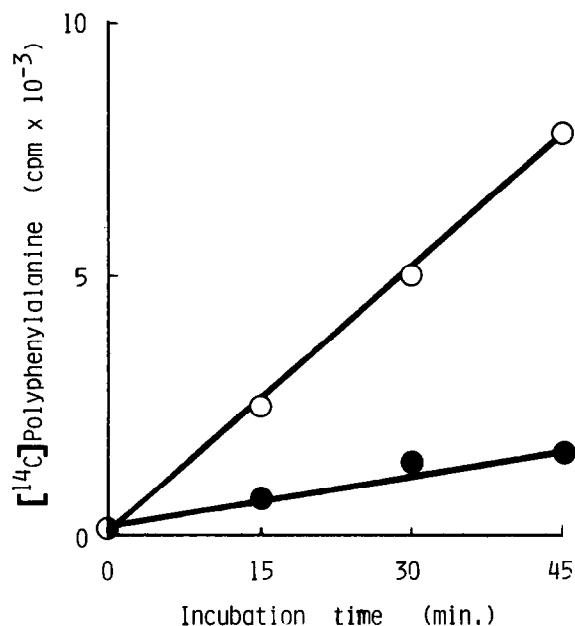


Fig.2. Effect of streptomycin on polyphenylalanine synthesis by in vitro system prepared from logarithmic phase cells of *S. griseus*. (○) Without addition of streptomycin; (●) 100 $\mu\text{g/ml}$ streptomycin was added to reaction mixture. Amounts of the ribosomes and the S150 fraction employed were 60 μg and 258 μg (as protein), respectively.

the streptomycin-binding affinity; they were dissociated by dialysis against the buffer which contained a decreased amount of Mg-acetate (0.3 mM) in buffer III. The dissociated ribosomes were incubated with $[^3\text{H}]$ dihydrostreptomycin and the mixture was fractionated by sucrose density gradient centrifugation. As shown in fig.3, the peak of $[^3\text{H}]$ dihydrostreptomycin and that of ribosomal small subunits appeared on the same fractions, indicating that the ribosomes which possessed protein-synthesizing activity bound $[^3\text{H}]$ dihydrostreptomycin on 30 S ribosomal subunits.

Since streptomycin is mainly produced in the late stages of culture, the susceptibility of protein synthesis to streptomycin in the stationary phase cells was also investigated. As shown in fig.4, polyphenylalanine synthesis was susceptible to streptomycin. From these results, it can be concluded that the ribosomes of *S. griseus* are significantly susceptible to streptomycin through all stages of growth as long as they actively synthesize proteins. Accordingly, *S. griseus* must have streptomycin-resistant mechanisms other than ribosomal resistance.

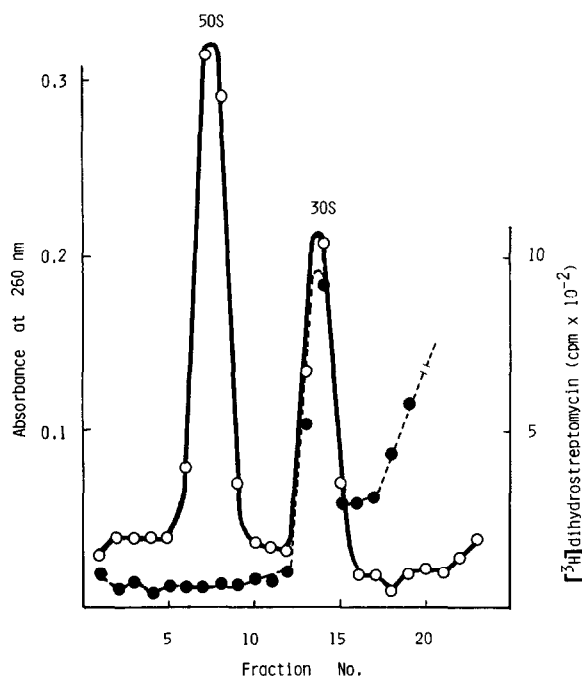


Fig. 3. Binding of [³H]dihydrostreptomycin to ribosomal subunits from *S. griseus*. The dissociated ribosomal preparation (3.7 A_{260} , 259 μ g) was incubated with 417 pmol [³H]-dihydrostreptomycin at 37°C for 15 min. The incubation mixture was layered on to a density gradient of 10–30% (w/v) sucrose in the buffer which contained a decreased amount of Mg-acetate (0.3 mM) in buffer I. The centrifugation was carried out at 70 000 $\times g$ for 17 h, then fractionation was performed. (○) A_{260} ; (●) radioactivity of [³H]dihydrostreptomycin.

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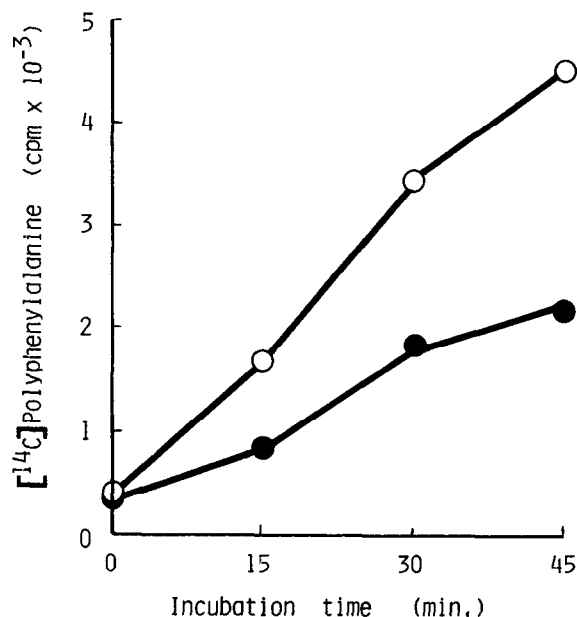


Fig. 4. Effect of streptomycin on polyphenylalanine synthesis by in vitro system prepared from stationary phase cells of *S. griseus*. (○) Without addition of streptomycin; (●) 100 μ g/ml streptomycin was added to the reaction mixture. Amounts of the ribosomes and the S150 fraction employed were 60 μ g and 258 μ g (as protein), respectively.

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