Purification and Characterization of Streptomycin 6-kinase, an Enzyme Implicated in Self-protection of a Streptomycin-producing Micro-organism

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Streptomycin 6-kinase of the streptomycin-producing strain *Streptomyces griseus* HUT 6037 was purified by fractionation with $(\text{NH}_4)_2\text{SO}_4$ and chromatography on DEAE-Sephadex A-25, hydroxyapatite and Sephadex G-100. After PAGE of the final fraction, a protein band corresponding to streptomycin 6-kinase was detected, together with a less intense band having no enzyme activity. Molecular weights determined by SDS-PAGE and by Sephadex G-100 chromatography were about 36000 and 38000, respectively, suggesting that the enzyme was a monomer. The isoelectric point of the enzyme was pH 6.6. Among the nucleoside 5'-triphosphates tested, ATP was the preferred phosphoryl donor. The K_m values for streptomycin and ATP were 3.5 mM and 0.4 mM, respectively. The enzyme activity was strongly inhibited by EDTA and AgNO_3 . It was shown by using an *in vitro* protein-synthesizing system that purified streptomycin 6-kinase could protect polyphenylalanine synthesis of the streptomycin-susceptible *S. griseus* strain KSN from inhibition by streptomycin.

INTRODUCTION

Walker & Walker (1967) found an enzyme which catalysed phosphorylation of streptidine with ATP in extracts of post-exponential phase mycelia of streptomycin (SM)-producing micro-organisms. Since this enzyme phosphorylated SM and dihydrostreptomycin in preference to streptidine (Miller & Walker, 1969), it was suggested that the enzyme might be more accurately designated as streptomycin kinase. Nimi *et al.* (1971*a, b*) also found a kinase which phosphorylated the C6 hydroxyl group of the streptidine moiety of SM in the presence of ATP and Mg^{2+} , and it was very probably the same enzyme as that described by Miller & Walker (1969). This streptomycin 6-kinase (SM 6-kinase; EC 2.7.1.72, ATP:streptomycin 6-phosphotransferase) has been supposed to play a role in the phosphorylation of some intermediate on the biosynthetic pathway to SM, or to phosphorylate streptomycin in the cell as a means of self-protection from the lethal effect of the drug (Nimi *et al.*, 1971*a, b*). We demonstrated recently that while the ribosomes of SM-producing *Streptomyces griseus* bound SM (Sugiyama *et al.*, 1980, 1981*a, b*), the bound SM was released by phosphorylation with SM 6-kinase (Sugiyama *et al.*, 1981*b*). SM 6-phosphate did not inhibit protein synthesis in extracts of the SM-producer (Sugiyama *et al.*, 1982). From these results, it was concluded that although the ribosomes of the SM-producer were susceptible to the antibiotic product, protein synthesis could be protected from the action of the drug by phosphorylation of the latter with SM 6-kinase. Nimi *et al.* (1971*b*) partially purified SM 6-kinase. The present study involved further purification of the enzyme and the determination of some of its properties.

Abbreviations: PMSF, phenylmethane sulphonyl fluoride; SM, streptomycin; SM 6-kinase, streptomycin 6-kinase.

METHODS

Micro-organisms. The SM-producing *S. griseus* HUT 6037 strain and the non-producing *S. griseus* KSN strain (Sugiyama *et al.*, 1981*a*, *b*, 1982) were used. The latter strain, derived from HUT 6037, did not produce SM 6-kinase and was susceptible to SM.

Preparation of an acetone powder. *Streptomyces griseus* HUT 6037 was grown in 1% (w/v) glucose/meat extract/peptone (GMP) medium (Nimi *et al.*, 1976) at 28 °C in a 30 l jar fermenter and the mycelium was harvested at the late exponential phase of growth. The mycelium was washed twice with 0.5% (w/v) NaCl at 0 °C, then thoroughly washed with acetone and dried *in vacuo*.

Determination of protein. The protein in eluates from column chromatography was monitored by following the absorbance at 280 nm. The protein in enzyme solutions was determined by the Lowry method using BSA as a standard.

Assay of SM 6-kinase activity. SM 6-kinase activity was determined by two methods. Method A involved determination of SM 6-[³²P]phosphate produced by the enzyme from SM and adenosine 5'-[γ-³²P]triphosphate (Sugiyama *et al.*, 1981*b*). Method B involved determination by bioassay of the decreased amount of active SM after phosphorylation of SM by the enzyme (Nimi *et al.*, 1971*b*). Since the former method was inadequate when salt concentration in the enzyme solution was high, the latter method was employed to determine enzyme activities of eluates from columns of hydroxyapatite and DEAE-Sephadex A-25, as well as the specificity of phosphoryl donor for SM and the K_m value for ATP.

Purification of the enzyme. All procedures were carried out at 0 to 6 °C and, when necessary, enzyme solutions were stored at -20 °C.

(a) **Preparation of extract.** Approximately 48 g of the acetone powder of *S. griseus* HUT 6037 was suspended in 950 ml TMDP buffer [50 mM-Tris/HCl, pH 8.0, 10 mM-MgSO₄·7H₂O, 0.5 mM-dithiothreitol and 0.4 mM-phenylmethane sulphonyl fluoride and kept in an ice-bath for 1 h with gentle shaking. The supernatant fluid was obtained by centrifugation at 12000 *g* for 40 min.

(b) **(NH₄)₂SO₄ fractionation.** Solid (NH₄)₂SO₄ was added to the fluid with stirring to give 45% saturation. After centrifuging at 12000 *g* for 40 min, additional (NH₄)₂SO₄ was added to the supernatant to give 75% saturation. The precipitate was collected by centrifugation and dissolved in 182 ml TMDP buffer. The solution was dialysed against 9 l of the same buffer for 6 h.

(c) **DEAE-Sephadex A-25 chromatography I.** The dialysed solution was applied to a column (2 × 50 cm) of DEAE-Sephadex A-25 equilibrated with TMDP buffer. The column was washed with 200 ml of the same buffer and then with 300 ml TMDP buffer containing 0.2 M-NaCl. The enzyme was eluted with TMDP buffer containing 0.3 M-NaCl.

(d) **Hydroxyapatite chromatography.** The active fractions of SM 6-kinase collected from step (c) were loaded on to a column (1.6 × 22 cm) of hydroxyapatite (Seikagaku-kogyo Co.) equilibrated with TMDP buffer. The column was washed with 100 ml of the same buffer and then with 80 ml of buffer containing 2% (w/v) (NH₄)₂SO₄. Elution was carried out with TMDP buffer containing 2 to 7% (NH₄)₂SO₄ (linear gradient of concentration). The active fractions were saturated with solid (NH₄)₂SO₄ and kept in an ice-bath overnight. The resulting precipitate was collected by centrifugation at 27000 *g* for 20 min and dissolved in about 2 ml TMDP buffer.

(e) **Sephadex G-100 chromatography.** The enzyme solution obtained at step (d) was loaded on to a column of Sephadex G-100 (2 × 115 cm), previously equilibrated with TMDP buffer. Elution was with TMDP buffer.

(f) **DEAE-Sephadex A-25 chromatography II.** The enzyme fraction at the above step was applied to a column of DEAE-Sephadex A-25 (0.7 × 15 cm) previously equilibrated with TMD buffer which had been prepared by omitting PMSF from the TMDP buffer. The column was washed with 50 ml TMD buffer and then with 50 ml of the buffer containing 0.15 M-NaCl, before being eluted with TMD buffer containing 0.15 to 0.4 M-NaCl (linear gradient of concentration). The enzyme was recovered in the fractions containing about 0.2 M-NaCl. The fractions giving high enzyme activity were pooled, dialysed against TMD buffer and then used for determination of some properties of the enzyme.

In these purification steps, addition of PMSF to the buffer was necessary to prevent inactivation of SM 6-kinase by proteolytic activity. Without use of the protease inhibitor, 80% of SM 6-kinase activity in the extract of the acetone powder was lost within 2 d at 5 °C. The purified enzyme was stable at -20 °C for a few months.

Disc gel electrophoresis. Enzyme purity was determined by disc gel electrophoresis at pH 8.0 using a 7% (w/v) polyacrylamide gel (0.5 × 0.5 cm stacking gel and 0.5 × 5 cm separating gel) (Williams & Reisfeld, 1964). Coomassie brilliant blue R250 [0.2% (w/v) in 50% (v/v) methanol/7.5% (v/v) acetic acid solution] was used for staining protein bands and 20% methanol/7.5% acetic acid solution was used for de-staining.

Isoelectric focusing. The isoelectric pH of enzyme was determined by using Servalyt-Precotes no. 42967 (SERVA Feinbiochemica, Heidelberg, F.R.G.) containing carrier polyampholytes, pH 3 to 10. Cathode and anode solutions were 1 M-NaOH and 1 M-H₃PO₄, respectively. Isoelectric focusing was performed for 3 h at 1000 V. The protein band was stained with Coomassie brilliant blue after treatment of the Precotes with a fixing solution consisting of 150 ml methanol, 350 ml deionized water, 57.6 g TCA and 17.25 g sulphosalicylic acid. De-staining was done as described above.

Molecular weight determination. Molecular weight was determined by SDS-PAGE according to the method of Laemmli (1970) and by Sephadex G-100 column chromatography. Marker proteins used in the electrophoresis were phosphorylase b (94000), BSA (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100), and α -lactalbumin (14400). Marker proteins loaded on to a Sephadex G-100 column (2 $\times$ 115 cm) together with the enzyme solution were BSA, egg-white albumin (45000), chymotrypsinogen A (25000) and cytochrome *c* (12500). The column was eluted with TMD buffer at the rate of 11.4 ml h⁻¹ and 3.8 ml fractions were collected. A portion (40 μ l) of each fraction was assayed for SM 6-kinase activity by method A. Positions of marker proteins were established by their absorbance at 280 nm.

Preparation of ribosomes and S-150 fraction. Cells of *S. griseus* KSN, harvested at the late exponential phase of growth, were washed twice with buffer I (10 mM-Tris/HCl, pH 7.65, 10 mM-magnesium acetate, 1 M-KCl, 6 mM-2-mercaptoethanol and 5 mM-EDTA dipotassium magnesium salt), and then twice with buffer II (10 mM-Tris/HCl, pH 7.65, 10 mM-magnesium acetate, 30 mM-NH₄Cl, 6 mM-2-mercaptoethanol, 5 mM-EDTA dipotassium magnesium salt, 0.2 mM-diisopropyl fluorophosphate and 3.45 mM-PMSF). The washed cell pellet was ground with quartz sand and extracted with buffer II containing 2 μ g DNAase I ml⁻¹. The extract was centrifuged at 30000 *g* for 30 min. From the supernatant, the ribosomes were sedimented by centrifugation at 150000 *g* for 3 h, then washed with buffer III (20 mM-Tris/HCl, pH 7.65, 1 M-NH₄Cl, 10 mM-magnesium acetate, 6 mM-2-mercaptoethanol, 1.1 M-sucrose, 5 mM-EDTA dipotassium magnesium salt, 0.2 mM-diisopropyl fluorophosphate and 3.45 mM-PMSF) by the method of Fahnstock *et al.* (1974), and then dialysed against buffer IV (10 mM-Tris/HCl, pH 7.65, 10 mM-magnesium acetate, 30 mM-NH₄Cl and 6 mM-2-mercaptoethanol). The upper one-third portion of the supernatant obtained by centrifugation at 150000 *g* was dialysed against buffer IV and used as the S-150 fraction.

Assay of *in vitro* protein synthesis. The *in vitro* protein synthesis was performed by modifying the method of Kobayashi *et al.* (1977). The reaction mixture (125 μ l) contained 60 mM-Tris/HCl, pH 7.8, 8 mM-magnesium acetate, 80 mM-NH₄Cl, 6 mM-2-mercaptoethanol, 0.4 mM-spermidine, 0.9 mM-ATP (disodium salt), 0.2 mM-GTP (disodium salt), 4 mM-phosphoenolpyruvate, 2 μ g pyruvate kinase, 0.05 μ Ci L-[U-¹⁴C]phenylalanine (100 μ Ci μ mol⁻¹; 3.7 MBq μ mol⁻¹), 4 μ M each of 19 amino acids except phenylalanine, 25 μ g tRNA from *Escherichia coli* MRE 600 (Boehringer), 25 μ g polyuridylylate, 60 μ g ribosomes and 240 μ g S-150 fraction as protein. After incubating at 28 °C for 30 min, 10% (w/v) cold TCA (1 ml) was added prior to heating at 90 °C for 15 min. The precipitate was collected on a glass fibre disc (Whatman GF/F, 25 mm in diameter) and washed twice with 5 ml 5% TCA. The radioactivity on the filter was estimated with a liquid-scintillation spectrometer using BBOT/toluene scintillation mixture.

RESULTS AND DISCUSSION

Purity of the enzyme. The results of a typical purification are summarized in Table 1. PAGE of the enzyme from step (*f*) (see Methods) revealed a main protein band which corresponded to the single peak of SM 6-kinase activity measured in gel slices, together with a less intense protein band which had no SM 6-kinase activity.

Substrate specificity and kinetics of the enzyme. Pure SM 6-kinase was obtained by slicing the electrophoresis gels and was compared with the enzyme solution at step (*f*) of the purification with respect to the K_m value for SM. Since the K_m values of these two preparations were 4 mM and 3.5 mM, respectively, activity of SM 6-kinase was apparently hardly affected by the contaminating protein. Thereafter, the enzyme fraction from step (*f*) was employed for characterization. The highly purified enzyme catalysed the phosphorylation of SM, dihydrostreptomycin and streptidine. The phosphorylating affinity (V_{max}/K_m) of SM 6-kinase for SM was about 20-fold and 4-fold higher than that for streptidine and dihydrostreptomycin, respectively (Table 2).

Molecular weight and isoelectric pH of the enzyme. The molecular weight of SM 6-kinase was estimated to be about 36000 by SDS-PAGE, and about 38000 by chromatography on Sephadex G-100. These results suggest that the enzyme is a monomer. The isoelectric pH of SM 6-kinase was found to be 6.6.

Phosphoryl donor specificity. ATP was the preferred phosphoryl donor to SM (K_m = 0.4 mM). CTP, UTP and GTP gave hardly any activity.

Metal ion requirement. Mg²⁺ (10 mM) was required for maximal activity. Co²⁺ and Cu²⁺ (10 mM) were rather less stimulatory than Mg²⁺. Mn²⁺, Zn²⁺ and Fe²⁺ all stimulated activity slightly at 10 mM but Ca²⁺ (0.1 to 100 mM) had no significant effect on activity.

Inhibitors of the enzymic activity. The activity was strongly inhibited by AgNO₃ (0.1 mM) and EDTA (25 mM). *p*-Chloromercuribenzoate (1 mM), KCN, NaN₃ and monoiodoacetic acid (up

Table 1. *Purification of SM 6-kinase*

Purification step	Volume (ml)	Total protein (mg)	Specific activity of SM 6-kinase*	Yield (%)	Purification (fold)
(a) Extract from acetone powder	743	2191	0.49	—	1
(b) (NH ₄) ₂ SO ₄ precipitate	182	1001	0.71	66.1	1.45
(c) DEAE-Sephadex A-25	187	58	11	58.9	22.4
(d) Hydroxyapatite	110	5.2	80	39.1	163
(e) Sephadex G-100	38.5	1.92	130	23.1	265
(f) DEAE-Sephadex A-25	20.0	0.1	681	6.3	1390

* SM 6-kinase activities were determined by method B; specific activities are expressed as $\mu\text{mol SM 6-phosphate formed (mg protein)}^{-1} \text{ h}^{-1}$.

Table 2. *Substrate kinetics of SM 6-kinase*

Values were obtained using the enzyme preparation that contained a contaminant protein [step (f) of the purification].

Substrate	K_m (mM)	$V_{\max}$ [$\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$]	$V_{\max}/K_m$ ratio
SM	3.5	28.6	8.17
Dihydrostreptomycin	11	22.2	2.02
Streptidine	28	11.1	0.40

Fig. 1. Protection of polyphenylalanine synthesis from streptomycin inhibition by SM 6-kinase. Percentage inhibition is expressed as the ratio of the lowered level of polyphenylalanine synthesis in the presence of SM to that in the SM-free reaction mixture. A 12.5 μl portion of the highly purified enzyme (62.5 ng) solution from step (f) (●), or the same volume of TMD buffer (O), was added to the reaction mixture.

to 10 mM) gave little or no inhibition. Among the analogues of ATP, significant inhibitory effects were observed with adenosine, AMP, ADP and pyrophosphate, but not with adenine (even up to 10 mM). Inhibition by AMP, ADP and pyrophosphate was non-competitive and the K_i values were 6, 5 and 3 mM, respectively. However, inhibition by adenosine was competitive and the K_i value was 0.8 mM.

Protection of protein synthesis by the highly purified SM 6-kinase against inhibition by SM. *Streptomyces griseus* KSN was susceptible to SM. *In vitro* polyphenylalanine synthesis by the ribosomes and S-150 fraction from this strain was strongly inhibited by 0.1 to 0.5 $\mu\text{g SM ml}^{-1}$

(Fig. 1). However, the synthesis was protected when the highly purified enzyme was added to the reaction mixture (Fig. 1).

SM 6-kinase has been thought to play an important role in self-protection of the SM producer, because the product, SM 6-phosphate, does not inhibit protein synthesis (Sugiyama *et al.*, 1982) and this observation has now been confirmed by using the highly purified enzyme.

REFERENCES

- FAHNESTOCK, S., EDERMANN, V. & NOMURA, M. (1974). Reconstitution of 50S ribosomal subunits from *Bacillus stearothermophilus*. *Methods in Enzymology* **30**, 554–562.
- KOBAYASHI, H., KOBAYASHI, K. & KOBAYASHI, Y. (1977). Isolation and characterization of fusidic acid-resistant, sporulation-defective mutants of *Bacillus subtilis*. *Journal of Bacteriology* **132**, 262–269.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- MILLER, A. L. & WALKER, J. B. (1969). Enzymatic phosphorylation of streptomycin by extracts of streptomycin-producing strains of *Streptomyces*. *Journal of Bacteriology* **99**, 401–405.
- NIMI, O., ITO, G., SUEDA, S. & NOMI, R. (1971*a*). Phosphorylation of streptomycin at C₆-OH of streptidine moiety by an intracellular enzyme of *Streptomyces griseus*. *Agricultural and Biological Chemistry* **35**, 848–855.
- NIMI, O., ITO, G., OHATA, Y., FUNAYAMA, S. & NOMI, R. (1971*b*). Streptomycin-phosphorylating enzyme produced by *Streptomyces griseus*. *Agricultural and Biological Chemistry* **35**, 856–861.
- NIMI, O., KOKAN, A., MANABE, K., MAEHARA, K. & NOMI, R. (1976). Correlation between streptomycin formation and mucopeptide biosynthesis. *Journal of Fermentation Technology* **54**, 587–595.
- SUGIYAMA, M., KOBAYASHI, H., NIMI, O. & NOMI, R. (1980). Susceptibility of protein synthesis to streptomycin in streptomycin-producing *Streptomyces griseus*. *FEBS Letters* **110**, 250–252.
- SUGIYAMA, M., MOCHIZUKI, H., NIMI, O. & NOMI, R. (1981*a*). Roles of streptomycin 6-kinase and ribosomal affinity to streptomycin in self-protection of streptomycin producer. *Biotechnology Letters* **3**, 357–362.
- SUGIYAMA, M., MOCHIZUKI, H., NIMI, O. & NOMI, R. (1981*b*). Mechanism of protection of protein synthesis against streptomycin inhibition in a producing strain. *Journal of Antibiotics* **34**, 1183–1188.
- SUGIYAMA, M., MOCHIZUKI, H., NIMI, O. & NOMI, R. (1982). Assessment of competitive action of streptomycin 6-kinase and streptomycin 6-phosphatase in the in vitro protein synthesis of a streptomycin-producing microorganism. *FEBS Letters* **139**, 331–333.
- WALKER, J. B. & WALKER, M. S. (1967). Streptomycin biosynthesis. Enzymatic synthesis of O-phosphoryl-streptidine from streptidine and adenosine triphosphate. *Biochimica et biophysica acta* **148**, 335–341.
- WILLIAMS, D. E. & REISFELD, R. A. (1964). Disc electrophoresis in polyacrylamide gels: extension to new conditions of pH and buffer. *Annals of the New York Academy of Science* **121**, 373–381.