ENZYMIC SYNTHESIS OF STREPTOMYCIN. TRANSFER OF L-DIHYDROSTREPTOSE FROM dTDP-L-DIHYDROSTREPTOSE TO STREPTIDINE-6-PHOSPHATE

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1. Introduction

Our previous work on the biosynthesis of streptomycin led us to conclude that dihydrostreptomycin is very likely the primary product in streptomycin biosynthesis. This assumption was based on the fact that dTDP-L-dihydrostreptose and not dTDP-L-streptose is formed from dTDP-D-glucose by the action of 3 enzymes from *Streptomyces griseus* [1] and that only dihydrostreptomycin was found inside the mycelium at all stages of fermentation [2]. An as yet unsolved question is in which sequence the three components of dihydrostreptomycin (*N*-methyl-α-L-glucosamine (1→2) α-L-dihydrostreptose(1→4)streptidine) are linked together. Walker et al. have shown that the streptidine moiety is synthesized from glucose-6-phosphate [3,4] and have postulated that L-streptose and *N*-methyl-L-glucosamine are transferred from the corresponding nucleotide diphosphate sugars to streptidine-6-phosphate and *O*-α-L-streptose (1→4)-streptidine-6-phosphate, respectively, to form streptomycin-6-phosphate [5].

We now wish to report the enzymatic transfer of the dihydrostreptose moiety from dTDP-dihydrostreptose to streptidine-6-phosphate to form *O*-α-L-dihydrostreptose (1→4)-streptidine-6-phosphate, a likely intermediate in the biosynthesis of dihydrostreptomycin.

2. Materials and methods

2.1. *Materials and synthesis of substrates*

dTDP-D-[U-^{14}C]glucose, 50 Ci/mol, was purchased from ICN (Irvine, California). Streptidine was obtained from streptomycin (Merck AG, Darmstadt) by acid hydrolysis [6]. Phosphorylated streptidine was obtained by a published method [7] and was purified by ion exchange chromatography on Dowex 50 [8]. The amount of streptidine phosphate which was then free of streptidine was determined by the Sakaguchi reaction [9]. The streptidine phosphate was active in the amidinotransferase test [10]. For storage a 2 mM solution in 0.2 N HCl was kept frozen. Streptomycin 6-kinase and streptomycin-6-phosphate phosphatase were obtained according to a published procedure [11]

2.2. *Analytical procedures*

Descending paper chromatography was carried out on Whatman 1 and Whatman 3 MM with the following solvent systems: (1) phenol/water (8:2 w/w) in an ammonia atmosphere; (2) methylethylketone/acetic acid/saturated aqueous boric acid (8:1:1 v/v/v); (3) the system of Heding with amyl alcohol containing di(ethylhexyl)phosphate [12]. For paper electrophoresis (50 V/cm) on Macherey and Nagel MN 214 buffer 4, acetic acid/pyridine/water (10:3:487 v/v/v), pH 3.9, was used. Streptidine derivatives were detected with the following reagent: 1 part 0.1% diacetyl in water + 1 part 20% KOH + 1 part 2.5% methanolic solution of α-naphtol [13]. Dihydrostreptose was detected with aniline phthalate.

2.3. *Cultivation of* S. griseus

S. griseus strain N 2-3-11 from Kaken Chem. Co., Tokyo, was grown in a complex medium [14]. The cells were harvested after 38 h.

2.4. *Preparation of cell-free extract*

The cell-free extracts from *S. griseus* and

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S. aureofaciens were obtained as described previously [14] with the modification that 50 mM K-phosphate buffer, pH 7.5, containing 5 mM mercaptoethanol, 1 mM EDTA and 30% (v/v) glycerol was used for sonification of *S. griseus*. For removal of metal ions 3 ml of the *S. griseus* extract was dialyzed for 6 h at 4°C against 3 litres of 5 mM K-phosphate buffer, pH 7.5, containing 200 mg EDTA and 200 µl mercaptoethanol.

2.5. *Enzyme incubations*

(a) Formation of dTDP-6-deoxy-D-*xylo*-4-hexosulose [14]. Two nmol (0.1 µCi) of dTDP-[U-^{14}C] glucose was incubated for 60 min at 30°C with 15 µl of a cell-free extract from *S. aureofaciens* and 15 µl of 50 mM K-phosphate buffer, pH 7.5, containing 5 mM mercaptoethanol, 1 mM EDTA and 30% (v/v) glycerol.

(b) Formation of dTDP-L-dihydrostreptose [14]. To 40 µl of the above incubation mixture was added 10 µl (0.1 mg) NADPH, 70 µl of the cell-free extract from *S. griseus* and 14 µl of 1 M glycine-NaOH buffer, pH 9.0. The mixture was incubated for 20 min at 30°C.

(c) Transfer of dihydrostreptose to streptidine phosphate. The acceptor solution contained: 0.35 mM streptidine phosphate, 10.9 mM EDTA, 43.6 mM $MgSO_4$, 21.8 mM Tris-HCl and 25 mM NaCl. The solution was adjusted to pH 8.0. To 100 µl of the pre-incubation mixture containing dTDP-L-dihydrostreptose was added 100 µl of the cell-free extract of *S. griseus* and 50 µl of the acceptor solution. After 20 min incubation at 30°C the reaction was terminated by cooling the solution to 0°C and adding 20 µl 2 N HCl. The supernatant was then applied as a 4 cm broad band to paper and electrophoresed for 50 min in the pyridine/acetate buffer. The radioactive compounds were detected with a chromatogram scanner and eluted with water.

3. Results and discussion

Since it had been shown that the biosynthesis of streptomycin very probably proceeds via phosphorylated intermediates [5,15], streptidine phosphate prepared by chemical phosphorylation of streptidine [7,8] was used as the potential acceptor for dihydrostreptose. Streptidine phosphate was incubated with dTDP-L-[U-^{14}C]dihydrostreptose, obtained in situ from dTDP-D-[U-^{14}C] glucose [1,14] and with a dialyzed cell-free extract from *S. griseus*. Paper electrophoretic separation of the incubation mixture with buffer 4 gave four radioactive compounds. Two compounds migrated toward the cathode with migration distances of + 1 and + 9.5 cm relative to malachite green (+ 6 cm), one compound migrated to the anode (– 15 cm), and one compound remained at the origin. Since a disaccharide of dihydrostreptose and streptidine or streptidine phosphate must have a positive change at pH 3.9, the two cathodic compounds were eluted from the paper with water and the eluates hydrolysed in 4% trifluoroacetic acid for 20 min at 95°C. Co-chromatography of the hydrolysate with L-dihydrostreptose on paper with solvent system 2 proved that the total radioactivity in the two cathodic compounds was located in dihydrostreptose. After treatment of the cathodic compound which had migrated the smaller distance with streptomycin-6-phosphate phosphatase [11], this compound became more positively charged and migrated the same distance in the electrophoresis as the second compound mentioned above.

The two positively charged compounds were not formed in control experiments in which a denatured extract was used, streptidine phosphate was absent, or streptidine was substituted for streptidine phosphate. We concluded from these results that the cathodic compound with the smaller migration distance is a disaccharide of dihydrostreptose with streptidine phosphate and the compound with the higher migration distance the corresponding disaccharide with streptidine.

Further identification of this presumed disaccharide was facilitated by the fact that we received an authentic sample of *O*-α-L-dihydrostreptose (1→4) streptidine (fig.1, III R = H) from H. Paulsen, which had been synthesized in his laboratory and had been characterized by high resolution n.m.r. [16]. This substance proved to be identical with our non-phosphorylated enzymatic product in electrophoretic mobility and by co-chromatography on paper with solvent system 3, in which non-phosphorylated streptidine derivatives are well separated [12]. Further proof for the identity of one of the enzymatic products with synthetic III was obtained in the following way. Synthetic III (R = H) was phosphorylated with

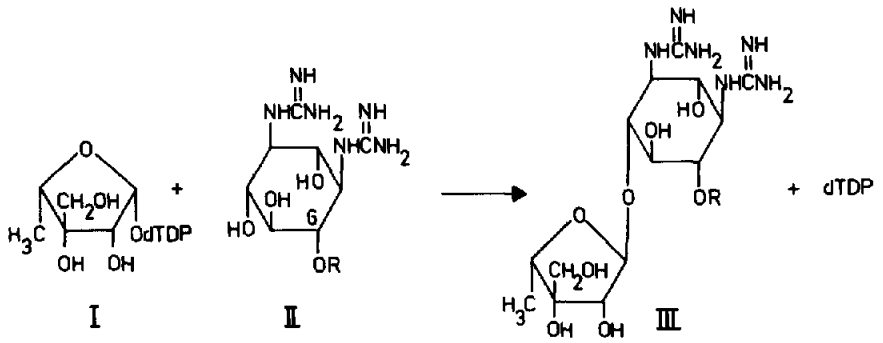

Fig.1. Formation of *O*-α-L-dihydrostreptose(1→4) streptidine-6-phosphate (III, R = phosphate) from dTDP-L-dihydrostreptose (I) and streptidine-6-phosphate (II, R = phosphate).

[γ-^{32}P]ATP and streptomycin 6-kinase [11] to the [6-^{32}P]phosphate derivative. After chromatography on paper of this derivative with the ^{14}C-labelled enzymatic product with the lower electrophoretic mobility in solvent system 1 the ^{32}P and ^{14}C activities coincided exactly (peak at R_f = 0.31).

These results prove the formation of *O*-α-L-dihydrostreptose (1→4) streptidine-6-phosphate (III, R = phosphate) from dTDP-L-dihydrostreptose and streptidine-6-phosphate with a cell-free extract from a streptomycin producing strain of *S. griseus*. III is very probably the first intermediate in the assembly of the three components of streptomycin. The formation of the non-phosphorylated product in the incubations can be explained by the presence of streptomycin-6-phosphate phosphatase in the crude extract. The fact that no transfer product was observed with streptidine again points to the importance of phosphorylated intermediates in streptomycin biosynthesis [5,15].

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