TOSYLPHENYLALANYL CHLOROMETHANE-INHIBITOR OF COMPLEX OF S_1S_3-FACTORS IN CELL-FREE PROTEIN-SYNTHETIZING SYSTEM FROM *BACILLUS STEAROTHERMOPHILUS*

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1. Introduction

The elongation of the growing peptide chain during its biosynthesis on the ribosome represents a complicated process involving the participation of several protein factors contained in the S-100 supernatant fraction. The mechanism of their action is at present intensively investigated [1, 2]. This study provides evidence showing that tosylphenylalanyl chloromethane (TPCK, 1-chloro-4-phenyl-3-tosyl-amido-2-butanone) is a selective irreversible inhibitor of the complex of S_1S_3-factors in the cell-free protein-synthetizing system from *B. stearothermophilus*. As reported elsewhere, TPCK inhibits irreversibly also the T-factor from *E. coli* [3].

2. Materials and methods

^{14}C-L-phenylalanine (103 mCi/mmole) was purchased from the Institute for Research, Production, and Use of Radioisotopes, Prague, CSSR. PolyU and TPCK were from Calbiochem, USA, GTP from Koch-Light, England, and ATP from Reanal, Hungary. Sephadex G-25 Fine was a product of Pharmacia, Uppsala, Sweden.

B. stearothermophilus, strain CCM 2184 (a generous gift of Dr. M. Kocur), was cultivated at 60° with aeration in a medium of the same composition as described for *E. coli* [4]. The cells were harvested in the late logarithmic phase of growth, washed [5], frozen and then disintegrated by 3-min treatment in a MSE Ultrasonic Generator. The washed ribosomes (20 mg/ml) were prepared according to Stenesh et al. [6]. The S-100 supernatant (10 mg of protein per ml) was prepared according to Algranati and Lengyel [5]. The S_1S_3-fraction (i.e. the mixture of the S_1 + S_3 factor [7]) and the S_2-factor were prepared from the S-100 supernatant by fractionation with $(NH_4)_2SO_4$, acetone, and chromatography on a DEAE-Sephadex column according to a modified method of Skoultchi et al. [7]. The details of the method will be described elsewhere. The S_1S_3-fraction was identified according to ^{14}C-GTP binding [8, 9]. The S_2-factor was identified on the basis of stimulation of the synthesis of polyphenylalanine from Phe-tRNA in the presence of the S_1S_3-fraction and on the basis of ribosome-dependent GTPase activity [10]. The strict dependence of PolyU directed polyphenylalanine formation upon S_1S_3-fraction and S_2 factor is demonstrated in fig. 1. The S_1S_3-fraction (0.75 mg of protein per ml) and the S_2-factor (0.3 mg of protein per ml) were stored in a buffer containing 0.01 M Tris-HCl (pH 7.4), 0.01 M β-mercaptoethanol at 4°.

The NH_4Cl washed ribosomes from *E. coli* A 19 (25 mg/ml) were prepared as reported earlier [4]. *E. coli* B tRNA was charged with ^{14}C-phenylalanine according to von Ehrenstein and Lipmann [11] and filtered through a Sephadex G-25 column.

Assay of polyphenylalanine formation. The incorporation mixture contained 0.04 M Tris-HCl (pH 7.4), 0.01 M Mg $(CH_3COO)_2$, 0.16 M NH_4Cl, 0.008 M β-mercaptoethanol, 250 μg of *E. coli* ribosomes,

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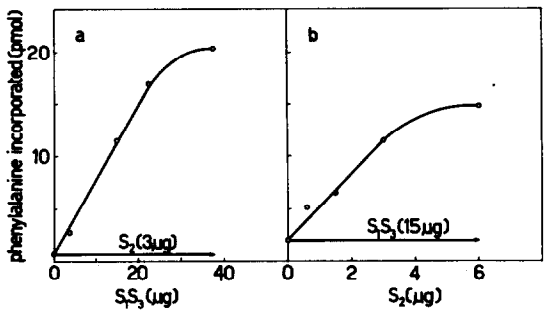

Fig. 1. Requirement for S_1S_3 and S_2 complementary fractions in polyphenylalanine formation. Effects of increasing amounts of S_1S_3 (a) and S_2 (b) on polyphenylalanine synthesis in the presence of a constant amount of the other complementary fraction as indicated. The incubation mixture contained 104 pmole of ^{14}C-Phe-tRNA and the remaining components as shown in Sect. 2. In the absence of S_1S_3 and S_2, 2.5 pmole of phenylalanine was incorporated into hot TCA-insoluble precipitate. This amount was subtracted from all experimental values.

20 µg of polyU, 2 mM GTP, 0.5 mM ATP, and ^{14}C-Phe-tRNA, the original, control or inhibited S-100 (30 µl) or S_1S_3-fraction (30 µl), and the S_2-factor (10 µl) as indicated. The total volume of the mixture was 0.1 ml. The reaction was started by adding GTP. The incubation period was 30 min at 33°. The reaction was terminated with 5% TCA solution, and the hot TCA-insoluble material was counted in a Frieseke-Hoepfner counter [4].

Inhibition by TPCK. One ml of reaction mixture contained: 0.5 mM TPCK, 1 mM GTP, 5% methanol and S-100 supernatant, or S_1S_3-fraction or S_2-factor, or ribosomes as indicated. The incubation period was 3½ hr at 4°. The control sample was incubated only with 1 mM GTP and 5% methanol. As "original" is considered the sample not preincubated with any of the reagents.

3. Results and discussion

The preincubation of the S-100 supernatant fraction from *B. stearothermophilus* with 5×10^{-4} M TPCK leads to an almost complete inhibition of its ability to stimulate polyphenylalanine synthesis in the cell-free system directed by polyU (table 1). As shown, it is not decisive whether the synthesis starts

Table 1
Effect of tosylphenylalanyl chloromethane on the activity of the S-100 supernatant fraction.

	(a) Polyphenylalanine synthesis from free phenylalanine (pmoles)		
	S-100 supernatant		
	Original	Control	Inhibited
– polyU	5.15	10.7	16.6
+ polyU	418	365	62.8
	(b) Polyphenylalanine synthesis from Phe-tRNA (pmoles)		
– polyU	0.66	0.74	0.44
+ polyU	30.7	27.1	1.5

The incubation mixture contained 300 µg of protein of the S-100 supernatant, buffer, ribosomes, polyU (where indicated), as given in sect. 2 and in addition in (a) 4 mM ATP, 0.5 mM GTP, 0.25 µC of ^{14}C-phenylalanine, and 200 µg of *E. coli* tRNA, in (b) 92.5 pmole of ^{14}C-Phe-tRNA. The incorporation of phenylalanine into hot TCA-insoluble precipitate in the absence of S-100 was in (a) 25.3 pmole in the absence of polyU and 16.6 pmole in the presence of polyU, in (b) 4.2 pmole in the absence of poly U and 4.07 pmole in the presence of polyU. These amounts were subtracted from the corresponding experimental values.

Table 2
The irreversibility of the inhibitory effect of tosylphenylalanyl chloromethane on the S-100 supernatant fraction.

	Polyphenylalanine synthesis (pmoles)		
	S-100 supernatant		
	Original	Control	Inhibited
Before Sephadex column	29.8	28.1	2.12
After Sephadex column	–	23.1	0.8

150 µl of the control or inhibited S-100 fraction was filtered through 1.2 ml of Sephadex G-25 Fine in 0.02 M Tris-HCl buffer (pH 7.8), 0.01 M Mg $(CH_3COO)_2$, 0.01 M β-mercaptoethanol, and 5% methanol. 30 µl of the peak fraction of the control or inhibited S-100 fraction was used in the experiment after the filtration. The incubation mixture contained 74 pmole of ^{14}C-Phe-tRNA and the remaining components as given in sect. 2. The incorporation of phenylalanine into hot TCA-insoluble precipitate in the absence of S-100 supernatant was 3.28 pmole. This amount was subtracted from all experimental values.

Table 3
The effect of tosylphenylalanyl chloromethane on the individual components of the protein-synthesizing system.

	Polyphenylalanine synthesis (pmoles)		
Fraction treated	Original	Control	Inhibited
(a) S_1S_3	19	14.7	1.27
(b) Ribosomes *E. coli*	22.3	20.85	20.5
(c) S_2	20	15.2	16.1
(d) Ribosomes *E. coli* + S_2	22.3	17.24	16.2
(e) Ribosomes *B. stearothermophilus*	72.5	66.3	63

The incubation mixture contained in (a) to (d) 22.5 μg of protein of the S_1S_3-fraction, 3 μg of protein of the S_2-factor, in (e) 125 μg of protein of the S-100 fraction and 200 μg of ribosomes from *B. stearothermophilus*, and further 104 pmole of ^{14}C-Phe-tRNA and the remaining components as given in sect. 2. The incorporation of phenylalanine into hot TCA-insoluble precipitate was 2.54 pmole in the absence of all factors [(a), (c)], 0.24 pmole in the absence of ribosomes [(b), (d), (e)], and these quantities were subtracted from all the corresponding experimental values.

from free phenylalanine or from phenylalanyl-tRNA. In the first case the incorporation of phenylalanine is inhibited by 83%, in the second case even by 95%. This result also indicates that the process in phenylalanine synthesis affected by TPCK is not the synthesis of Phe-tRNA but that TPCK interferes with the subsequent reactions which take place during the *de novo* synthesis after aminoacyl-tRNA had been formed.

The observed inhibitory effect of TPCK on the S-100 supernatant system is most likely irreversible as follows from the results given in table 2. The filtration of the inhibited S-100 fraction through a Sephadex G-25 column in order to remove unreacted TPCK does not change the incorporation activity of this fraction.

In other experiments we tested the effect of TPCK on the individual components of protein-synthetizing system, that is on the ribosomes from *E. coli* and *B. stearothermophilus*, on the S_1S_3-fraction, and on the S_2-factor. The results given in table 3 provide convincing evidence showing that it is the S_1S_3-fraction which is unambiguously and primarily affected by the action of TPCK. The activity of neither the S_2-factor nor of the ribosomes from *E. coli* or from *B. stearothermophilus* are influenced by this inhibitor under the conditions which lead to an inhibition of the S_1S_3fraction by TPCK by 92%.

Till now TPCK has been known as an effective inhibitor of certain proteolytic enzymes [12]. The observation of its inhibitory effect on the S_1S_3-fraction or the T-factor [3] during protein synthesis is thus surprising. A detailed elucidation of the mechanism of its effect in the process of synthesis of a new peptide on the ribosome will be the subject of our further studies.

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