INHIBITION OF MAGIC SPOT FORMATION BY L-1-TOSYLAMIDO-2-PHENYLETHYL CHLOROMETHYL KETONE

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1. Introduction

TPCK* was recently found to interfere with protein chain elongation. The drug prevented polyphenylalanine formation by bacterial cell extracts programmed with poly U, due to the inactivation of the elongation factor EF-Tu, which normally functions in carrying aminoacyl-tRNA to ribosomes [1–3]. Ribosomes, aminoacyl-tRNA synthetases or elongation factor EF-G were not affected.

The *rel*$^+$ bacterial strains contain the stringent factor (SF) which is involved in the MS formation. In this paper we report that the *in vitro* formation of MSI and MSII* [4–6] is also inhibited if the ribosomal salt wash proteins from *rel*$^+$ strain ribosomes are first preincubated with TPCK.

2. Materials and methods

The 0.5 M NH_4Cl-washed ribosomes from *E. coli* MRE 600 were prepared essentially as described by Haseltine et al. [4]. The low Mg^{2+} treated ribosomes (73 mg/ml) were prepared according to the procedure of Pedersen et al. [5] and were stored at 4°C. The crude *rel*$^+$ factor (SF) was prepared as a 0.5 M NH_4Cl ribosomal wash as described by Haseltine et al. [4] except that the second centrifugation of the wash at 45 000 rpm was performed overnight. The partially purified SF was precipitated from the second supernatant by 35% saturation with $(NH_4)_2SO_4$ (20.9 g of solid $(NH_4)_2SO_4$ for 100 ml of the solution). The precipitate was dissolved in buffer containing 0.01 M Tris–acetate pH 7.8, 0.06 M potassium acetate, 0.01 M magnesium acetate, 1 mM dithiothreitol and 0.2 M NH_4Cl and passed through a column of DEAE-Sephadex (1.5 × 14 cm) equilibrated with the same buffer [5]. The SF emerged in the void volume from the column, was divided into aliquots and stored at a protein concentration of 250 μg/ml under liquid nitrogen.

The assay for MS synthesis used a 20 μl incubation volume as described by Pedersen et al. [5] except that 0.025 μCi of [^{14}C]GTP per reaction mixture was used instead of [α-^{32}P]GTP, and the concentration of dithiothreitol varied from 0.05–0.1 mM. Low Mg^{2+} ribosomes were present at a final concn. of 2.65 mg/ml, the SF being at concentrations indicated. After an incubation for 60 min at 37°C, the reaction mixtures were precipitated with 3 M formic acid and centrifuged, then 14 μl samples of supernatant were spotted on polyethyleneimine thin layer plates for the chromatographic procedure and autoradiography described by Pedersen et al. [5]. The spots corresponding to (MSI + MSII) and (GTP + GDP) were excised and then counted in a Packard-Tricarb scintillation spectrometer with an efficiency of about 65% using a toluene based scintillation liquid.

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* *Abbreviations:*
TPCK = L-1-Tosylamido-2-phenylethyl chloromethyl ketone; MS = magic spots, MSI + MSII; MSI is ppGpp, guanosine tetraphosphate; MSII is pppGpp, guanosine pentaphosphate.

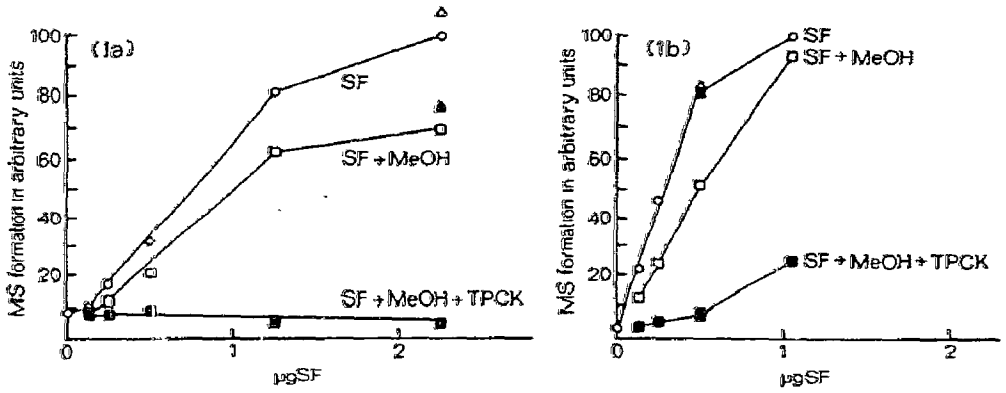

Fig. 1. Effect of TPCK on SF activity in MS synthesis. Each reaction mixture contained per 20 μl volume increasing amounts of original (o—o—o), control with MeOH (□—□—□) or TPCK-treated SF (■—■—■) as shown and the remaining components as described in Materials and methods. In addition, in fig. 1b each reaction mixture contained poly U and $tRNA^{Phe}$ (in final concn. 0.25 mg/ml and 0.6 mg/ml, respectively). 100 arbitrary units in MS formation correspond to 31% conversion of added GTP into MSI and MSII in fig. 1a and to 98% conversion of added GTP into MSI and MSII in fig. 1b. △ — Reaction mixture contained original SF and 1.25% methanol in addition to other components listed above in fig. 1a, and in fig. 1b, 0.25% methanol. ▲ — Reaction mixture contained original SF and 1.25% methanol + 2.5×10^{-4} M TPCK, in addition to other components listed above, in fig. 1a and in fig. 1b 0.25% methanol + 5×10^{-5} M TPCK.

Inhibition by TPCK: the SF or low Mg^{2+} ribosomes (as indicated) were preincubated in the presence of 2.5% methanol and 0.5 mM TPCK for 5 hr at 4°C before assembling the reaction mixtures called SF or ribosomes, TPCK-treated (see fig. 1 and table 1). Control samples were preincubated with 2.5% methanol only. Original samples were not preincubated with any of the reagents.

[^{14}C]GTP, 500 μCi/μmol was purchased from The Radiochemical Centre, England; $tRNA^{Phe}$ from *E. coli* was prepared by Mr. R. Coulson from starting material provided by the Microbiological Research Establishment, Porton, Wilts, England; poly U was the product of Miles Laboratories USA; TPCK and GTP, Li salt, came from the Sigma Chemical Company, USA; ATP, Na salt came from Koch-Light, England.

3. Results and discussion

Pedersen et al. [5] showed that further purification of the ribosomal system developed by Haseltine et al. [4] for the *in vitro* synthesis of MS resulted in a requirement for the presence of uncharged tRNA and corresponding messenger RNA in the reaction mixture. We have confirmed these results. Fig. 1 shows that, for example, in the presence of 0.5 μg SF the addition of $tRNA^{Phe}$ and poly U brought about an approx. 8-fold increase in the conversion of added GTP into MS (fig. 1b) in comparison with samples containing ribosomes and SF only (fig. 1a). (Notice that in fig. 1a and 1b, 100 units of MS formation corresponds to 31% and 98% conversion of GTP, respectively). In contrast we have found that with higher SF concentration some significant MS production took place in this system without $tRNA^{Phe}$ and poly U (fig. 1a).

The preincubation of SF with 5×10^{-4} M TPCK results in almost complete inhibition of its ability to stimulate MS production in both systems tested (fig. 1). In the system containing ribosomes and SF only, even the addition of the highest amount of SF preincubated with TPCK did not stimulate the conversion of GTP into MSI and MSII, suggesting that the SF activity is completely inhibited (fig. 1a). In the system stimulated by $tRNA^{Phe}$ and poly U, at low SF concentrations the production of MS was also almost entirely abolished after the preincubation with TPCK, whereas at the highest tested concentration of TPCK-treated SF some MS formation occurred. This suggests that a small fraction of SF, probably not affected during the preincubation with TPCK, could be detected in this system because of its greater effectiveness in comparison with the system lacking poly U and $tRNA^{Phe}$.

Table 1
The inhibitory effect of TPCK on MS formation.

a)

SF	Ribosomes	Conversion of GTP into MS (arbitrary units)
Original	Original	100
Control	Original	76
TPCK-treated	Original	13.6
–	Original	5
Original	–	4.9
Original	Control	105
Original	TPCK-treated	86
Control	Control	66
TPCK-treated	Control	17.0
Control	TPCK-treated	50
TPCK-treated	TPCK-treated	4.9

b)

SF	Ribosomes	Conversion of GTP into MS (arbitrary units)
Original	Original	100
Control	Original	84
TPCK-treated	Original	36
–	Original	2.8
Original	Original, without $tRNA^{Phe}$ and poly U	33
Original	Control	101
Original	TPCK-treated	91
Control	Control	38
TPCK-treated	Control	31
Control	TPCK-treated	70
TPCK-treated	TPCK-treated	17

Each reaction mixture contained in a) 100 µg/ml (final concn.) of original (i.e. untreated), control (i.e. + MeOH) or TPCK-treated SF (as indicated), in b) 25 µg/ml (final concn.) of original, control or TPCK-treated SF, poly U and $tRNA^{Phe}$ (in final concn. 0.25 mg/ml and 0.6 mg/ml, respectively) and the remaining components as described in Materials and methods. 100 arbitrary units correspond in a) to 51% and in b) to 98% conversion of GTP into MSI + MSII.

The triangles in fig. 1a and 1b show the production of MS with the original untreated SF preparation if methanol (MeOH) or TPCK + MeOH (in final concentrations equal to those which resulted in incubation mixtures containing TPCK-treated SF samples) was added directly to the incubation mixture. This was done to characterise the effect of TPCK on the system producing MS during the incubation period at 37°C, because in our experiments TPCK was not removed from inhibited samples before testing their activity. The results show that 2.5×10^{-4} M present in the incubation mixture slightly affects the MS production (fig. 1a), whereas the presence of 5×10^{-5} M TPCK in the incubation mixture had no effect (fig. 1b).

Methanol alone inhibits the activity of SF slightly as can be seen from the fig. 1 (control samples + MeOH), if preincubation takes place for a longer time. In other experiments (not shown) it was found that when a higher methanol concentration was used in preincubation, a lower SF activity was retained.

Both ribosomes and SF are necessary for MS formation [4] *in vitro*. Therefore, we tested the combined effect of TPCK preincubation, both with SF and ribosomes, on MS production. Results in table 1 show that the component primarily affected by TPCK is the SF preparation, whereas the preincubation of ribosomes alone with TPCK under the same conditions which resulted in substantial SF inhibition (86% in the experiment in the absence of $tRNA^{Phe}$ and poly U (table 1a) and 64% in the experiment with $tRNA^{Phe}$ and poly U (table 1b)), brought about only approx. 10% loss in activity of the system. On the other hand, if both ribosomes and SF preparation were first pre-incubated with TPCK, the residual activity of both systems further decreased. It may suggest that ribosomes could be also partly affected by TPCK action. However a more probable explanation is that addition of ribosomes preincubated with TPCK results in a higher final concentration of the inhibitor in the incubation mixture.

At present it is difficult to explain how TPCK inhibits MS formation because the overall mechanism of this reaction is not fully understood. Also the purest SF preparations presently available are not fully characterised. Future work is needed to show whether the inhibitory effect reported here is related to the previously reported inhibitory effect of TPCK on the function of EF-Tu [3, 7]. Another possibility is an uncharacterised protein concerned in the production of MS is inhibited by TPCK.

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