INTERFERENCE OF SULFHYDRYL COMPOUNDS WITH THE INHIBITORY EFFECT OF N-TOSYL-L-PHENYLALANYLCHLOROMETHANE ON ELONGATION FACTOR T_u

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The rate of the inhibition of elongation factor T_u from *Bacillus stearothermophilus*, caused by N-tosyl-L-phenylalanylchloromethane, is substantially decreased in the presence of 2-mercaptoethanol or dithiothreitol. It is demonstrated that SH-compounds react with the inhibitor and reduce its concentration in the medium.

N-Tosyl-L-phenylalanylchloromethane (Tos-Phe-CH_2Cl) had first been studied as a specific active site directed irreversible inhibitor of chymotrypsin alkylating histidine in its active centre[1]. However, it was shown later that in the molecule of some other proteolytic enzymes, *e.g.* papain or ficin, it is the cystein residue which is subjected to the attack of the inhibitor resulting in enzyme inactivation[2,3]. Recently, it was demonstrated in this laboratory that Tos-Phe-CH_2Cl can also be used as an irreversible inhibitor of elongation factor T_u from *Escherichia coli* or *B. stearothermophilus*[4,5] (confer also ref.[6]) reacting with a specific SH-group in the factor molecule[7].

As it is shown in this paper, 2-mercaptoethanol or dithiothreitol can interfere with the inhibitory effect of Tos-Phe-CH_2Cl. It is demonstrated that the addition of SH-compounds results in a sharp drop of the inhibitor concentration in the medium and this could explain the protective effect of 2-mercaptoethanol or dithiothreitol on EF-T_u during the treatment with Tos-Phe-CH_2Cl. The readiness of Tos-Phe-CH_2Cl to attack 2-mercaptoetanol or dithiothreitol is also in agreement with what can be assumed about its reactivity on the basis of its chemical structure.

Abbreviations used: ATP, adenosine 5′-triphosphate; GTP, guanosine 5-triphosphate; CTP, cytidine 5-triphosphate; GDP, guanosine 5-diphosphate; Tos-Phe-CH_2Cl, N-tosyl-L-phenylalanylchloromethane, L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one; poly (U), polyuridylic acid; 2-ME, 2-mercaptoethanol; DTT, dithiothreitol.

EXPERIMENTAL

Chemicals

ATP (sodium salt) and CTP (sodium salt) were purchased from Reanal, Hungary. GTP (sodium salt) and dithiothreitol came from Koch-Light, England, GDP (lithium salt) from Boehringer, Germany. N-Tosyl-L-phenylalanylchloromethane came from Calbiochem, and 2-mercaptoethanol was from Fluka, Switzerland. L-Phenylalanine-[U-^{14}C] (103 mCi/mol) was purchased from the Institute for Research, Production and Use of Radioisotopes, Prague.

Methods

S-100 supernatants were prepared from *B. stearothermophilus* and filtered before the experiments through a 6 × 1·6 cm column of Sephadex G-25 fine either in 10 mM Tris-HCl (pH 7·8), 10 mM magnesium acetate buffer or in the same buffer supplemented with 10 mM 2-mercaptoethanol as described earlier[8]. The treatment of S-100 supernatants with the methanolic solution of Tos-Phe-CH_2Cl (final concentration of methanol 5%) and in the presence or absence of 1 mM GDP was also carried out according to previously used procedure[8]. The control samples were preincubated with 5% methanol and with or without GDP (as indicated). The assay of Tos-Phe-CH_2Cl-treated samples and of control samples for polyphenylalanine synthesis directed by polyU and the calculation of inhibition in per cents from the difference in the incorporation of phenylalanine into polyphenylalanine by control and Tos-Phe-CH_2Cl-treated samples have already been described[8].

Elongation factor T from *B. stearothermophilus* (specified as the S_1S_3-complex) and elongation factor G from *B. stearothermophilus* (specified as the S_2 factor) were prepared as described earlier[4,7].

Transfer RNA from *E. coli* B was prepared according to Littauer and coworkers[9]. Transfer RNA was charged with L-phenylalanine(^{14}C) in a reaction mixture (final volume 25 ml) containing 50 mg of transfer-RNA, 1 μmol of L-phenylalanine-(U-^{14}C), 60 μmol of ATP, 12·5 μmol of CTP, 1·25 mmol of Tris-HCl buffer (pH 7·8), 375 μmol of $MgCl_2$, 12·5 μmol of ethylenediaminotetraacetic acid (pH 7·0), 125 μmol of 2-mercaptoethanol and 14 mg of protein of S-100 supernatant from *E. coli* A_{19}. After 20 min at 35°C, 2·7 ml of cold 2M potassium acetate (pH 5·0) were added and Phe-(^{14}C)-tRNA was isolated by repeated phenol extraction at 20°C and ethanol precipitation at −20°C. The precipitate was dissolved in 4 ml of 0·1M potassium acetate (pH 5) and filtered through a column (16 × 1·6 cm) of Sephadex G-25 fine equilibrated with the same buffer. Fractions containing Phe-tRNA were pooled, precipitated with cold ethanol washed twice with 75% ethanol, dissolved in distilled water and freeze-dried.

The products of the reaction between Tos-Phe-CH_2Cl and 2-mercaptoethanol or dithiothreitol were separated (immediately after mixing) by ascending thin-layer chromatography on silica gel with an ultraviolet indicator (Silufol plates) in a system consisting of benzen–chloroform–methanol (19 : 1 : 2). The position of the individual compounds on the chromatogram was determined in ultraviolet light.

RESULTS AND DISCUSSION

The ability of 2-mercaptoethanol to decrease the rate of inactivation of elongation factor T_u by Tos-Phe-CH_2Cl in S-100 supernatant is shown in Fig. 1. In the absence of

SH-compounds, the treatment of S-100 supernatant with 0·5 mM Tos-Phe-CH_2Cl results in a loss of 50% of its polyphenylalanine synthesizing activity in about 20 s; in the presence of 10 mM 2-mercaptoethanol, the same effect was observed after about 50 min treatment.

The inhibitor reacted with the S-100 supernatant in both cases under optimal conditions, *i.e.* in the presence of GDP which was found to accelerate the rate of inhibition of EF-T_u by Tos-Phe-CH_2Cl (ref.[8]) This finding is confirmed in this work in Fig. 1 (compare curve *2* with curve *1*, *i.e.* with the time course of inhibition in the absence of GDP and SH-compounds).

A similar protective effect of 2-mercaptoethanol was also observed while determining the dependence of the rate of inhibition of EF-T_u (in S-100 supernatant) on the Tos-Phe-CH_2Cl concentration (Fig. 2). Two types of S-100 supernatants were examined – one from bacteria harvested during the log phase of growth and the

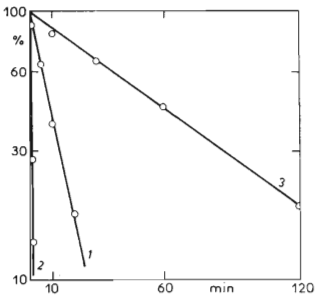

FIG. 1

The Effect of 2-Mercaptoethanol on the Time Course of the Inhibition of S-100 Supernatant by Tos-PheCH_2Cl (*1*), Tos-Phe-CH_2Cl + GDP (*2*) and Tos-Phe-CH_2Cl + GDP in the Presence of 10mM 2-Mercaptoethanol (*3*)

S-100 supernatant was preincubated with 5% methanol (control) or with a methanolic solution of 0·5 mM Tos-Phe-CH_2Cl in the presence or absence of GDP. At given time intervals, 30 µl samples (180 µg of protein) from the control as well as from the Tos-Phe-CH_2Cl-treated S-100 supernatant (prepared from bacteria harvested in the early stationary phase of growth) were assayed for phenylalanine polymerization in the incubation mixture (0·1 ml) containing 67 pmol Phe-tRNA and other components as described in Experimental part. When control samples of S-100 supernatant were tested, 30–33·5 pmol of phenylalanine were incorporated into the precipitate insoluble in hot trichloroacetic acid and blank values in the absence of S-100 supernatant were about 0·5 pmol. The abscissa shows the inhibition of polyphenylalanine synthesis in per cent.

other isolated from bacteria harvested in the early stationary phase of growth – which were found to differ[8] in their sensitivity toward the combined effect of Tos-Phe-CH_2Cl and GDP. It is quite obvious from Fig. 2 that in the presence of 10 mM 2-mercaptoethanol the Tos-Phe-CH_2Cl concentration must be approximately higher by two orders of ten to bring about the same inactivation of the factor in both S-100 supernatants tested as in the absence of 2-mercaptoethanol.

The presumption that the ability of SH-compounds to interfere with the effect of Tos-Phe-CH_2Cl on the protein synthesizing system is due to the protection of elongation factor T is confirmed by the results shown in Table I. The higher the concentration of dithiothreitol in the preparation of elongation factor T, with which Tos-Phe-CH_2Cl is preincubated, the lower the inhibition of the activity of the factor in polyphenylalanine synthesis catalysed by the treated factor.

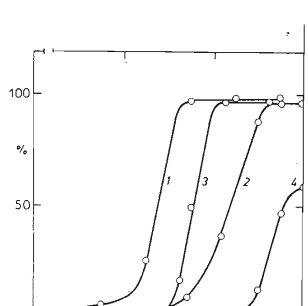

FIG. 2

Dependence of Inhibition on Tos-Phe-CH_2Cl Concentration and the Effect of 2-Mercaptoethanol

The polyphenylalanine polymerizing activity of S-100 supernatant prepared from cells harvested in the early stationary phase of growth (*1*, *2*) and of S-100 supernatant prepared from cells harvested in the exponential phase of growth (*3*, *4*) treated with an increasing concentration of Tos-Phe-CH_2Cl in the presence of 1 mM GDP for 3·5 h was examined[8]. Curves *2* and *4* show the dependence of inhibition on the Tos-Phe-CH_2Cl concentration when 10 mM 2-mercaptoethanol was present during the treatment. The procedure was carried out as described earlier[8]. Control or Tos-Phe-CH_2Cl treated samples of S-100 supernatant from the stationary phase (180 μg) or of S-100 supernatant from the exponential phase (100 μg) were assayed in the incubation mixture containing 60–64 pmol Phe-tRNA and other components as described in Experimental part. Incorporation of phenylalanine into the hot trichloroacetic acid insoluble precipitate by control samples of S-100 supernatants (in pmol): *1*, 29·6; *2*, 25·9; *3*, 25·7 and *4*, 37. Blank values in the absence of S-100 supernatants were 0·4–0·9 pmol. Abscissa, the inhibition of polyphenylalanine synthesis in per cent, *c*, Concentration of Tos-Phe-CH_2Cl.

The protection by dithiothreitol seems to be more effective than by 2-mercaptoethanol and is generally much more pronounced if the inhibition takes place in the absence of GDP. This is in agreement with the previous observation that GDP may change the sensitivity of the EF-T_u molecule so that Tos-Phe-CH_2Cl can attack the proper SH-group (s) of the factor more easily (conformation change)[8]. This is obvious also from Fig. 1, where it is shown that if Tos-Phe-CH_2Cl acts on S-100 supernatant in the presence of added GDP, the rate of inactivation is more than 10 times higher.

If the chemical constitution of Tos-Phe-CH_2Cl is taken into consideration one can expect Tos-Phe-CH_2Cl to react directly with such compounds like 2-mercaptoethanol and dithiothreitol. Results shown in Fig. 3 indicate that this indeed is so. A model experiment was carried out where Tos-Phe-CH_2Cl and 2-mercaptoethanol or dithiothreitol were mixed and products of the reaction were separated by thin-layer chromatography. The relative concentrations of the inhibitor and the SH-compounds were the same (although absolutely higher, to enable the localisation of products in ultraviolet light) as in the case when 0·5 mM Tos-Phe-CH_2Cl reacted with the S-100 supernatant or EF-T containing 10 mM 2-mercaptoethanol or 10 mM dithiothreitol.

Results of chromatography show that spots corresponding to Tos-Phe-CH_2Cl almost or entirely disappeared following the addition of 2-mercaptoethanol or dithio-

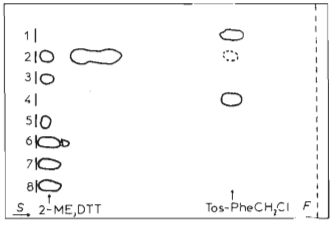

FIG. 3

Thin-layer Chromatography of Products of the Reaction between Tos-Phe-CH_2Cl and 2-Mercaptoethanol or Dithiothreitol

On start of the chromatography the following samples were applied: *1*, 5 µl of 50 mM Tos-Phe-CH_2Cl in methanol; *2*, 30 µl from the mixture of 100 µl of buffer A (10 mM Tris-HCl, pH 7·8, 10 mM magnesium acetate, 0·2M 2-mercaptoethanol, 50% methanol) + 20 µl of 50 mM Tos-Phe-CH_2Cl in methanol; *3*, 1 µl of 6M 2-mercaptoethanol; *4*, 30 µl from the mixture of 100 µl of buffer A without 2-mercaptoethanol + 20 µl of 50 mM Tos-Phe-CH_2Cl in methanol; *5*, 25 µl of buffer A; *6*, 30 µl from the mixture of 100 µl of buffer B (the same as buffer A but instead of 2-mercaptoethanol, 0·2M dithiothreitol was present) + 20 µl of 50 mM Tos-Phe-CH_2Cl in methanol; *7*, 5 µl of 1M dithiothreitol and *8*, 25 µl of buffer B.

threitol, which indicates that the actual concentration of free Tos-Phe-CH_2Cl in the medium decreases considerably due to the presence of SH-compounds. This could serve as an explanation of the remarkably slower rate of inactivation of EF-T_u by Tos-Phe-CH_2Cl observed in the presence of 2-mercaptoethanol or dithiothreitol in comparison to the rate in their absence. Tos-Phe-CH_2Cl might be converted in an alkylated 2-mercaptoethanol (see that new spots appeared on the chromatogram between the inhibitor and 2-mercaptoethanol) but no attempt yet has been made to clear the point.

The reaction between Tos-Phe-CH_2Cl and SH-compounds proceeds quickly and under the conditions described above it results in a decrease of about two orders of ten in the concentration of the inhibitor in the medium. This follows from the prolongation of the time course of inhibition (apparent velocity constant of inhibition is smaller by two orders of ten in the presence of 10 mM 2-mercaptoethanol than in its absence, Fig. 1) as well as from the dependence of inhibition on Tos-Phe-CH_2Cl concentration (Fig. 2).

TABLE I

The Protection by SH-Compounds of Elongation Factor T against N-Tosyl-L-phenylalanyl-chloromethane

EF-T factor (0·65 ml, 360 μg of protein) was filtered through a column of Sephadex G-25 fine equilibrated with buffer containing 10 mM Tris-HCl (pH 7·8), 10 mM magnesium acetate and 1 mM dithiothreitol. Peak fractions were pooled, 1M dithiothreitol or 2-mercaptoethanol were added up to the final concentrations shown and 30 μl samples with or without 1mM GDP were preincubated with 5% methanol (control) or with 0·5mM Tos-Phe-CH_2Cl + 5% methanol (Tos-Phe-CH_2Cl — treated) at 4° C for 3·5 h. The preincubated samples were then assayed for polyphenylalanine synthesis in the presence of elongation factor G (4 μg of protein), 60·7 pmol Phe-tRNA and other components[4] as described in Experimental. In the absence of EF-T and EF-G about 1·3 pmol of phenylalanine was incorporated into the precipitate insoluble in hot trichloroacetic acid.

Additions to EF-T-factor mM			Phenylalanine incorporated pmol		Inhibition %
DTT	2-ME	GDP	EF-T control	EF-T Tos-Phe-CH_2Cl treated	
1	—	—	19·6	1·9	90·1
		+	17·8	—	100.0
5	—	—	18·3	16·9	7·0
		+	16·0	0.2	99·0
10	—	—	16·3	15·4	5·5
		+	13·8	3·1	77·5
1	10	—	17·5	15·7	10·0
		+	13.7	1·1	92·5

However, all the results show that even in the presence of SH-compounds, complete although much slower inactivation of EF-T_u factor can be achieved. This is in agreement with the current view that Tos-Phe-CH_2Cl inhibits the factor molecule[4-6] irreversibly. Moreover, our findings demonstrate the high affinity existing between the inhibitor and the reactive SH-group(s) of the factor[8]. This is particularly interesting considering the fact that no components involved in protein synthesis on the ribosome, expect the stringent factor[10] and elongation factor T_u, are affected by the inhibitor[4-6,11].

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