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Modification of *Bacillus subtilis* elongation factor Tu by N-tosyl-L-phenylalanyl chloromethane abolishes its ability to interact with the 3′-terminal polynucleotide structure but not with the acyl bond in aminoacyl-tRNA

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Modification of *B. subtilis* EF-Tu by *N*-tosyl-L-phenylalanyl chloromethane destroyed its ability to promote protein synthesis and resulted in selective dissociation of the two binding activities of the protein for aminoacyl-tRNA. The modified EF-Tu was completely ineffective in the protection of the 3′-terminal CCA structure of tRNA against pancreatic ribonuclease, while remaining almost fully active in the protection of the ester bond between the 3′-terminal adenosine and the amino acid residue in aminoacyl-tRNA.

Elongation factor Tu; Tosyl-L-phenylalanyl chloromethane, *N*-; EF-Tu-aminoacyl-tRNA interaction; Aminoacyl-tRNA-binding site; (*Bacillus subtilis*)

1. INTRODUCTION

The formation of a ternary complex between a nonribosomal protein designated elongation factor Tu, aminoacyl-tRNA, and GTP is an obligatory step in the elongation cycle of protein synthesis. To date, about five different approaches have been used to estimate the affinity of EF-Tu·GTP for aminoacyl-tRNA: protection of the ester bond between amino acid and tRNA by EF-Tu·GTP from nonenzymatic deacylation [1] – the hydrolysis protection assay [2]; EF-Tu·GTP protection of the aminoacyl end of aminoacyl-tRNA from ribonuclease digestion – the RNase-resistance assay [3–5]; protection of EF-Tu by aminoacyl-tRNA from labelling by *N*-tosyl-L-phenylalanyl chloromethane – the TPCK assay [6–8]; monitoring the fluorescence change that accompanies the association of EF-Tu·GTP and fluorescent aminoacyl-tRNA [9]; reduction by aminoacyl-tRNA of the amount of EF-Tu bound to nitrocellulose filters [10,11].

The first four assays are based on the binding properties of the acceptor stem of aminoacyl-tRNA. The mechanism of the filter assay is less obvious. Whereas the hydrolysis protection assay as well as the RNase-resistance assay approach the formation of the complex via the integrity of the aminoacyl-tRNA molecule, the specificity of the TPCK assay rests on the monitoring protein EF-Tu integrity. The rate of modification of an essential cysteine residue of prokaryotic EF-Tus in the Asp_{80}-Cys-Pro-Gly_{83} (*E. coli*) consensus sequence is a measure of the affinity of individual ligands for the aminoacyl-tRNA-binding site of EF-Tu [6,7,12].

In our previous experiments we found that modification of EF-Tu from *E. coli* or *B.*

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Abbreviations: TPCK, *N*-tosyl-L-phenylalanyl chloromethane or L-1-tosylamido-2-phenylethylchloromethyl ketone; EF-Tu, elongation factor Tu

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stearothermophilus by TPCK resulted in a loss of its ability to catalyse protein synthesis because the aptitude to interact with aminoacyl-tRNA was abolished [13,14]. In the present study, we analysed the behaviour of TPCK-modified EF-Tu from another bacterium, *B. subtilis*. To our surprise, we found that although the factor was completely unable to promote protein synthesis, the results of the hydrolysis protection assay did not indicate that it had lost its capacity to interact with aminoacyl-tRNA.

2. MATERIALS AND METHODS

The preparation of EF-Tu·GDP, EF-G, and 1.5 M NH_4Cl-washed ribosomes from *B. subtilis* by modified classical procedures will be described elsewhere. EF-Tu, at least 95% pure, was 64% active in GTP-binding based on a standard nitrocellulose binding assay [10]. The procedure for the preparation of *E. coli* [^{14}C]Phe-tRNA (713 cpm/pmol) has been described [15]. Pancreatic ribonuclease (EC 3.1.27.5) was purchased from Boehringer, nitrocellulose filters (HA type) from Millipore Corporation. All other chemicals were as reported in [16].

2.1. *Assay of EF-Tu activities*

The ability of control or TPCK-treated EF-Tu from *B. subtilis* to interact with aminoacyl-tRNA was measured either by the hydrolysis protection assay [2] as described in [16] or by the ribonuclease-resistance assay [3] as described below. 10 μM EF-Tu was first preincubated with 0.8 mM GTP, 6 mM phospho*enol*pyruvate, and 87 μg/ml pyruvate kinase in a buffer containing 20 mM Tris-HCl (pH 8.1), 12 mM magnesium acetate, 100 mM NH_4Cl, and 10 mM 2-mercaptoethanol for 10 min at 35°C to form EF-Tu·GTP from EF-Tu·GDP. Then 500 pmol EF-Tu·GTP was incubated with 90 pmol [^{14}C]Phe-tRNA in 413 μl of reaction buffer containing 10 mM Tris-HCl (pH 7.6), 12 mM magnesium acetate, 40 mM NH_4Cl, 10 mM 2-mercaptoethanol, 3 mM phospho*enol*pyruvate, 17.4 μg pyruvate kinase, and 0.05 mM GTP at 35°C for 3 min to form a ternary complex. The mixture was chilled to 4°C and a 100 μl aliquot was withdrawn and precipitated by 3 ml of cold 10% trichloroacetic acid to measure the initial radioactivity. To the rest of the reaction mixture 10 μl of pancreatic ribonuclease (0.1 mg/ml) was added and 100 μl aliquots were withdrawn at 15, 40 and 90 s and precipitated by 3 ml of cold 10% trichloroacetic acid to stop the digestion. The precipitates were collected on Millipore filters, rinsed thrice with trichloroacetic acid, dried, and counted. Blank samples, containing the same components but without EF-Tu, were treated in the same way as the complete assay system and run simultaneously. The values obtained were subtracted from the values obtained with EF-Tu. The phenylalanine polymerization activity of EF-Tu was assayed in a buffer containing 10 mM Tris-HCl (pH 7.6), 12 mM magnesium acetate, 40 mM NH_4Cl, and 10 mM 2-mercaptoethanol, essentially as described in [15] except that EF-G and ribosomes from *B. subtilis* were used.

2.2. *Inhibition of EF-Tu from* B. subtilis *by* N-*tosyl-L-phenylalanyl chloromethane*

A preparation of 7.9 μM EF-Tu·GDP in buffer containing 20 mM Tris-HCl (pH 8.1), 10 mM magnesium acetate, 100 mM NH_4Cl, 0.5% (v/v) glycerol, 0.5 μM GDP, and 5% (v/v) methanol was incubated with 0.5 mM *N*-tosyl-L-phenylalanyl chloromethane at 4°C for 4–5 h. Control EF-Tu was incubated under the same conditions but without the reagent. Then aliquots of control and TPCK-treated EF-Tu were tested in all three assays described above.

3. RESULTS

Treatment of EF-Tu from *B. subtilis* with *N*-tosyl-L-phenylalanyl chloromethane destroyed the ability of the factor to promote protein synthesis (fig.1). For *E. coli* and *B. stearothermophilus* elongation factors studied before, it had been established that TPCK-modified EF-Tus failed to catalyse polymerization because their interaction with aminoacyl-tRNA had been abolished [13,14]. Therefore, *B. subtilis* EF-Tu modified by TPCK was also tested for this partial activity in one of the widely used assay systems – the hydrolysis protection assay [2]. To our surprise, however, the factor, fully inactive in polymerization, was only very slightly affected in its ability to protect from nonenzymatic deacylation (fig.2). The results of this assay thus indicated that, in contrast to *E. coli* or *B. stearothermophilus* EF-Tus, the ability of TPCK-modified EF-Tu from *B. subtilis* to interact with aminoacyl-tRNA was not affected. The modified protein was then screened for other functions to explain the inhibitory effect of TPCK. It

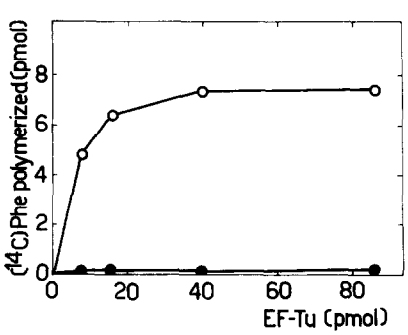

Fig.1. Effect of TPCK on EF-Tu activity. Control (○) and TPCK-treated (●) EF-Tu were assayed for activity promoting the poly(U)-directed phenylalanine polymerization as described in section 2.

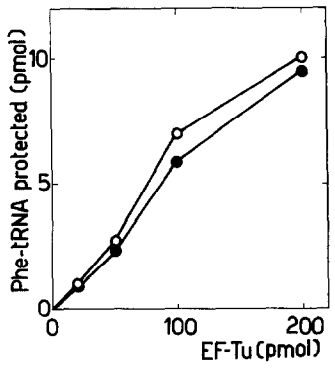

Fig.2. Lack of inhibition of protection by TPCK-treated EF-Tu of the acyl linkage in phenylalanyl-tRNA against nonenzymatic hydrolysis. (○) Control EF-Tu; (●) TPCK-treated EF-Tu.

was found that only the activity promoting the transfer of aminoacyl-tRNA onto the ribosome and the GTPase activity of the modified elongation factor were partly inhibited, though not to the same extent as its polymerization activity (data not shown). This strongly suggested that despite the negative results of the hydrolysis protection assay the inactivation of EF-Tu must occur at the level of the formation of the EF-Tu-tRNA complex. Therefore, this ability of the TPCK-modified EF-Tu was tested again using a different assay, the RNase-resistance assay [3,5]. This time, as can be seen in fig.3, the protecting activity of the elongation factor against ribonuclease was found almost completely abolished by the TPCK treatment. The rate of digestion of phenylalanyl-tRNA by

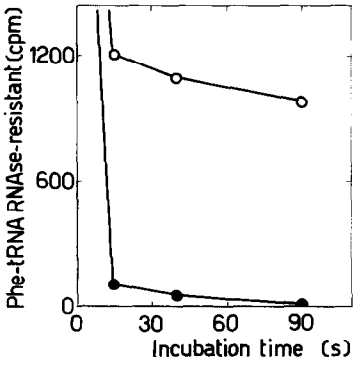

Fig.3. Effect of TPCK on protection by EF-Tu of aminoacyl-tRNA against digestion by pancreatic ribonuclease. (○) Control EF-Tu; (●) TPCK-treated EF-Tu.

ribonuclease was almost the same irrespective of the presence or absence of the modified EF-Tu.

4. DISCUSSION

The treatment with TPCK made it possible for the first time to dissociate two activities of EF-Tu involved in binding with aminoacyl-tRNA: interaction with the acyl linkage between the 3′-terminal adenosine and the phenylalanine residue of phenylalanyl-tRNA from the interaction with the 3′-terminal CC polynucleotide structure of the aminoacyl-tRNA. This implies that in *B. subtilis* EF-Tu, the binding site for the 3′-terminus of aminoacyl-tRNA involves at least two rather independent regions: one for the polynucleotide structure (the target site for the ribonuclease) and the other for the ester bond of the 3′-terminal aminoacyl-adenosine (susceptible to nonenzymatic deacylation). The former region can be selectively disabled by modification with TPCK, either directly or through a conformational change(s), while the latter region is only slightly affected. This suggests that the 3′-end polynucleotide structure of aminoacyl-tRNA represents an essential region for EF-Tu binding, because lack of interaction at this region completely abolished the proteosynthetic activity of EF-Tu even though the interaction at the ester bond region was retained.

The unexpected finding of discrepancy between the results obtained by the hydrolysis protection assay and by the RNase-resistance assay has also some methodological significance for determination of equilibrium dissociation constants between EF-Tu and aminoacyl-tRNAs. Any assay based on a partial function of a protein only, need not necessarily detect all irregularities that can occur even within the same binding site of the protein. This may be still more relevant to heterologous systems consisting of components isolated from different species (e.g. as above: EF-Tu from *B. subtilis*, Phe-tRNA from *E. coli*).

We observed no such dissociation of partial functions in TPCK-labelled EF-Tu from *E. coli* (unpublished results). This is interesting, because the target structure for TPCK, a tryptic peptide 15 amino acid residues long, has the same sequence both in *E. coli* [12] and *B. subtilis* EF-Tus (unpublished results). To understand these variations,

more detailed structural work on the elongation factors will be required.

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