N-Tosyl-L-phenylalanylchloromethane reacts with cysteine 81 in the molecule of elongation factor Tu from *Escherichia coli*

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Elongation factor EF-Tu from *Escherichia coli* was labelled with *N*-[^{14}C]tosyl-L-phenylalanylchloromethane, digested with trypsin and the peptides obtained separated by HPLC. The only radioactive peak recovered corresponded to tryptic peptide containing residues 75–98. Sequencing of the peptide by automated Edman degradation identified cysteine 81 as the site of *N*-tosyl-L-phenylalanylchloromethane modification. These results confirm the importance of this residue for the interaction with aminoacyl-tRNAs.

Elongation factor EF-Tu *Cysteine 81* Escherichia coli *Modification by TPCK* *HPLC chromatography* *Tryptic peptide*

1. INTRODUCTION

N-Tosyl-L-phenylalanylchloromethane (TPCK) can be used as a specific irreversible inhibitor of elongation factor EF-Tu from *Escherichia coli* and *Bacillus stearothermophilus* [1–3]. This compound destroyed the ability of the factor to bind aminoacyl-tRNA [4,5], presumably by affecting the cysteine residue important for the interaction with aminoacyl-tRNAs [3,6,7]. ^{14}C-Labelled TPCK was shown to form a covalent equimolar complex with EF-Tu [7], and this fact established the inhibitor as a specific reagent to assay its interactions with aminoacyl-tRNAs and their analogues [8,9].

Here we report that TPCK modifies specifically cysteine residue 81 [10,11] in EF-Tu from *E. coli* B.

Abbreviations: TPCK, *N*-tosyl-L-phenylalanylchloromethane or L-1-tosylamido-2-phenylethyl chloromethyl ketone; EF-Tu, elongation factor Tu; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoine

2. MATERIALS AND METHODS

The preparation of crystalline EF-Tu·GDP, ^{14}C-labelled TPCK (4.2 Ci/mol), tRNA from *E. coli* B and [^{14}C]Phe-tRNA (270 Ci/mol) has been described in [3,8]. GDP was from Merck (FRG), polybrene was from Sigma Chemical Co. (USA), trifluoroacetic acid from Rathburn (Scotland). Nucleosil ODS 5 μm was from Macherey-Nagel (FRG) and TPCK-trypsin was the product of Worthington (USA).

Labelling of EF-Tu·GDP with [^{14}C]TPCK: 238 nmol crystalline EF-Tu·GDP were washed with 5 ml solution containing 42% saturated ammonium sulphate, 20 mM Tris–HCl (pH 7.6), 10 mM magnesium acetate, 5 mM 2-mercaptoethanol and 10 μM GDP. The sediment of crystals was dissolved in 5 ml buffer containing 20 mM Tris–HCl (pH 8.1), 10 mM magnesium acetate, 100 mM ammonium chloride and 60 μM GDP; 100 μl solution was removed as a control, and methanol was added to 4% final conc. To the rest of the solution, 2 μmol [^{14}C]TPCK in methanol were added (final conc. of methanol was 4%) and

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incubation took place for 5 h at 4°C. Both samples were dialysed against a series of buffers containing 1 mM 2-mercaptoethanol and decreasing concentration of Tris–HCl and magnesium acetate (pH 7.9), tested for their activity in the phenylalanine polymerization assay and freeze-dried. The procedure for reduction, carboxymethylation with iodoacetic acid and tryptic digestion (enzyme/substrate ratio 1:100, w/w) of EF-Tu will be described elsewhere.

Amino acid analysis on Beckman 121-M Amino Acid Analyzer and sequencing of peptides in the presence of polybrene in a Beckman 890C sequencer were as in [12]. Peptides from the lyophilized tryptic digest were separated on a column (0.8 × 25 cm) of Nucleosil ODS 5 μm particles using 0.1% trifluoroacetic acid and a gradient of ethanol at 50°C [13]. Identification of PTH derivatives of amino acids was performed by HPLC as in [14]. All separations by HPLC were performed on a Hewlett-Packard HP 1084B instrument.

3. RESULTS AND DISCUSSION

Treatment of EF-Tu with ^{14}C-labelled TPCK resulted in the almost complete loss of its ability to promote poly(U)-dependent polyphenylalanine formation (fig. 1). In the same sample, the incorporation of ~0.75 TPCK molecule/factor molecule was detected as determined by the standard TPCK-assay procedure [8,9].

To determine the amino acid residue modified by TPCK, the labelled protein, extensively dialyzed to remove unreacted TPCK was lyophilized, reduced, carboxymethylated with iodoacetic acid, subjected to tryptic digestion and the digest separated by HPLC chromatography. A typical chromatogram of ~2 nmol digest is shown in fig. 2. The chromatographic profile of the digest of TPCK-treated EF-Tu (fig. 2b) revealed the presence of a new distinct peak (indicated by arrow) which could not be detected in the hydrolyzate of control untreated protein (fig. 2a). Furthermore, measurement of the radioactivity of all the individual frac-

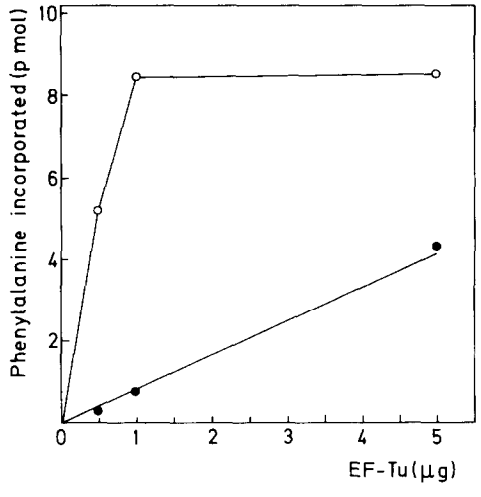

Fig. 1. Effect of TPCK on EF-Tu activity. Control (○—○) or [^{14}C]TPCK-treated (●—●) EF-Tu were assayed for the activity to promote the poly(U)-directed phenylalanine polymerization as in [3] except that the reaction mixture (80 μl) contained 10 pmol [^{14}C]Phe-tRNA (5400 cpm) and incubation took place for 7 min at 35°C.

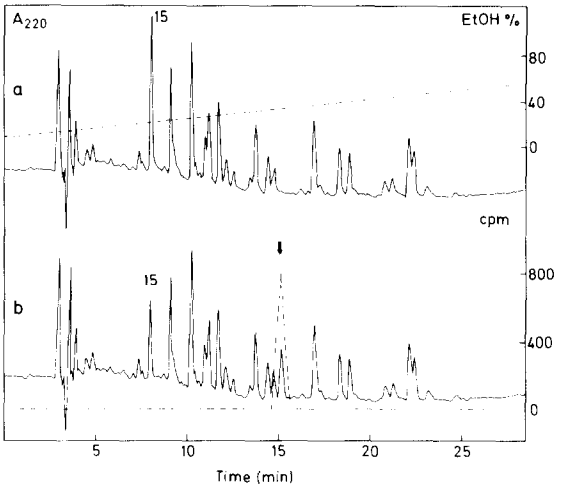

Fig. 2. HPLC of the tryptic digest of elongation factor EF-Tu from *E. coli* B: 2 nmol tryptic digest of EF-Tu were dissolved in 90 μl 0.1% TFA, mixed with the same volume of 96% ethanol, the mixture was centrifuged and the supernatant applied on the column. No radioactivity was detected in a tiny sediment. Absorbance at 220 nm was recorded and radioactivity in 100 μl aliquots from each fraction was measured: (a) control EF-Tu; (b) [^{14}C]TPCK-labelled EF-Tu.

tions obtained from the chromatography showed that the new peak was the only one containing radioactivity. However, we observed a substantial decrease in the amount of peak no. 15 in the hydrolyzate of TPCK-treated EF-Tu as compared to the same peak in the control sample. No other differences between the two chromatograms were detected.

The amino acid analysis of the radioactively labelled fraction gave the following composition (residues/molecule): Asx 1.9, Pro 0.95, Gly 1.2, Ala 2.0, Val 1.6, Tyr 1.6, Lys 1.0 and His 2.7. By dansylation of ~1 nmol peptide, the histidine residue was determined as the N-terminal amino acid. These analyses identified tryptic peptide T13 containing residues 75–89 (see the nomenclature in [11]) as the only peptide present in the radioactive peak. The failure to detect the cysteine residue which is also present in the native peptide already suggested that this amino acid could be the target of TPCK modification. This agrees with the finding that there is a loss of 0.5–0.6 cysteine residue (determined as cysteic acid) in the amino acid composition of TPCK-treated EF-Tu (J.J., unpublished).

The isolated radioactive peptide was further sequenced by automated Edman degradation. In each successive step, the radioactivity was measured, and the phenylthiohydantoine derivatives of amino acids obtained were identified by HPLC [14] (fig. 3). Almost no radioactivity was detected in the first 6 degradation steps comprising residues 75–80. This excludes His-75 and His-78 as the potential targets of TPCK reagent [15]. The analysis of PTH-derivatives of degraded amino acids in individual steps also confirmed the established sequence of this part of T13 peptide [10,11]. In step 7, the peak of radioactivity was recovered, clearly indicating that [^{14}C]TPCK modifies residue 7 of the tryptic peptide T13, which corresponds to cysteine-81. TPCK has also been reported to modify the cysteine residue in the active site of papain [16].

However, the degradation of TPCK-modified cysteine-81 by automated Edman procedure presented technical problems. This is indicated by the presence of a continuous tail of radioactivity in all the steps following step 7 as well as by the lower yield (~6-times) of PTH amino acids in these steps in comparison to steps 1–6 (not shown).

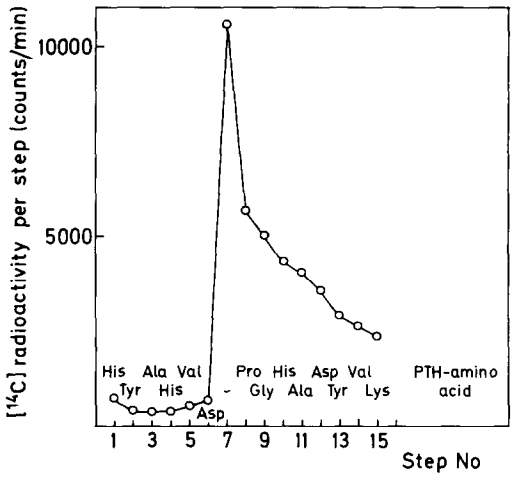

Fig. 3. Edman degradation of [^{14}C]TPCK-labelled tryptic peptide T13: ~50 nmol [^{14}C]TPCK-labelled peptide T13 (180 000 cpm) were subjected to automated Edman degradation procedure. PTH amino acids were identified by HPLC as in section 2.

Nevertheless, the identity of each of them was established with certainty, thus confirming the known sequence of this part of the molecule. That His-84 is not modified by TPCK is suggested by the lack of increase of radioactivity in step 10 and by the recovery of PTH histidine residue in this step. However, in assaying step 7 by the HPLC chromatography system currently used for the identification of PTH amino acids [14] no amino acid residue was recovered. TPCK-modified PTH-cysteine represents thus an intractable compound which disturbs the usual Edman degradation procedure and binds strongly to the HPLC column. A thiazine derivative of the cysteine, similar to that identified in the study on *N*-tosylglycylchloromethane-modified cysteine [17], may be formed, but ours is certainly more hydrophobic. This may also explain the distinct shift in the elution time of tryptic peptide T13 following TPCK-modification from its standard position in peak 15 on the chromatogram to a completely new location (fig. 2). The lack of complete disappearance of peak 15 after TPCK-treatment is due to the presence of another tryptic peptide eluting at this position, namely T39 and of a small fraction of unmodified peptide T13 (J.J., T.E.P., unpublished).

These results show that TPCK reacts specifically with cysteine 81 in EF-Tu from *E. coli* B. This finding confirms the assumptions [3,6–9,18] concerning the importance of this residue of the factor for its interaction with aminoacyl-tRNAs. This view is further supported by indications that TPCK modification of cysteine-81 is blocked by 3′-terminal analogues of tRNAs [8,9].

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