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Identification of the *N*-tosyl-L-phenylalanyl chloromethylketone modification site in *Thermus thermophilus* elongation factor Tu

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EF-Tu from *Thermus thermophilus* was first labelled with *N*-[^{14}C]tosyl-L-phenylalaninechloromethylketone and then cleaved by the combined action of CNBr and trypsin. The resulting peptides were separated by reversed-phase HPLC. Analysis of the isolated, labelled peptide led to the identification of a sequence which was identical to residues 76–88 in *T. thermophilus* EF-Tu. The TPCK reactive site is at Cys-82. Kinetic measurements of the incorporation of TPCK into native EF-Tu and EF-Tu nicked at position Arg-59 were performed. The results provide evidence that the cleavage of the peptide bond between Arg-59 and Gly-60 does not lead to a dramatic conformational change of EF-Tu at the aa-tRNA binding site.

GTP-binding protein; Protein biosynthesis; Elongation factor Tu; *N*-Tosyl-L-phenylalanyl chloromethylketone; (*Thermus thermophilus*)

1. INTRODUCTION

The elongation factor (EF) Tu is one of the most abundant proteins in both prokaryotic and eukaryotic cells. During the elongation cycle this protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes. One of the interesting properties of the protein is its ability to interact with numerous ligands. The protein binds, in addition to aa-tRNA, GDP, GTP, ppGpp, EF-Ts, the antibiotics kirromycin and pulvomycin and certain ribosomal components (for review see [1]).

N-Tosyl-L-phenylalanyl chloromethylketone (TPCK) was used as a specific irreversible inhibitor of EF-Tus from *E. coli, Bacillus stearothermophilus* and *Bacillus subtilis* [2–5]. This compound blocks the aa-tRNA binding site of the factors by labelling Cys-81 in *E. coli* and the homologous cysteine residue in *B. stearothermophilus*.

The formation of a specifically nicked EF-Tu, which often occurs during preparation, has been reported for *T. thermophilus* [6,7] and for *T. aquaticus* [8] proteins. We previously found that the nicked EF-Tu from *T. thermophilus* is fully active in nucleotide binding and ternary complex formation but inactive in promoting a poly U-directed poly Phe synthesis [7]. The interaction with EF-Ts was also altered to some extent. We have now tested whether the conformational transition of EF-Tu upon introduction of the specific nickage alters its reactivity towards TPCK-modification.

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Abbreviations: EF-Tu and EF-Ts, elongation factors Tu and Ts; aa-tRNA, aminoacyl-tRNA; TPCK, *N*-tosyl-L-phenylalanyl chloromethylketone; PTH, phenylthiohydantoin

2. MATERIALS AND METHODS

The preparation of ^{14}C-labelled TPCK has been described elsewhere [9]. TPCK-treated trypsin was obtained from Worthington (NJ, USA). Acrylamide and *N,N'*-methylene bis(acrylamide) were from BRL (Eggenstein, FRG). Trifluoroacetic acid (sequencing grade) was from Pierce (Rodgau, FRG) and 2-propanol (LiChrosolv) was purchased from Merck. EF-Tu·GDP was isolated from *T. thermophilus* cells, strain HB8, harvested at the late log-phase, as described by Leberman et al. [10].

Limited digestion of EF-Tu was done as follows: 20 μg EF-Tu in 20 μl 100 mM *N*-methylmorpholine acetate, pH 8.1, were treated with 1 μg trypsin at 37°C. To stop the digestion, mixtures were frozen using dry-ice in 2-propanol and immediately lyophilized. Subsequent cleavage of EF-Tu with CNBr and analysis of the resulting fragments by SDS-urea-PAGE was done as described previously [11].

For total tryptic digestion, peptides (1 mg/ml) were incubated with 2% (w/w) trypsin for 4 h at 37°C in the above buffer.

Peptide analysis by HPLC was performed using a reversed-phase system with 0.12% aqueous trifluoroacetic acid as solvent A and 0.1% trifluoroacetic acid in 70% 2-propanol as solvent B and a flow rate of 0.4 ml/min through a self-packed Vydac C_4 column (4.6 × 250 mm). Fractions of 0.4 ml were collected. The Vydac material was from 'The Separation Group' (Hesperia, CA, USA).

Labelling of EF-Tu with TPCK was performed in the following manner: 50 μM EF-Tu from *T. thermophilus* was incubated with 500 μM [^{14}C]TPCK (4.16 Ci/mol) in 20 mM Tris-HCl, pH 8.1, 10 mM $MgCl_2$ and 100 mM NH_4Cl for 12 h at 4°C, then dialyzed twice against 5 mM β-mercaptoethanol in H_2O (adjusted to pH 7.0 with NH_4OH) at 4°C. The final solution was lyophilized.

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Amino acid analysis on a Durrum D-500 Amino Acid Analyzer was done as described elsewhere [12]. Sequencing of the ^{14}C-labelled peptide was carried out in an Applied Biosystems 470A gas-phase sequencer. Purified polybrene was used for the fixation of the peptide. PTH-amino acids were identified by HPLC on a column (250 × 4.6 mm) of ODS (Ultrasphere, Beckman) by isocratic elution with acetonitrile in an acetate buffer.

3. RESULTS AND DISCUSSION

Only one cysteine residue has been identified in *T. thermophilus* EF-Tu by sequencing of the gene [11,13]. This cysteine is in position 82 of the polypeptide chain and corresponds to an invariable residue present in most bacterial EF-Tus sequenced to date. It is believed to be involved in aminoacyl-tRNA binding and can be specifically modified with TPCK, as shown for EF-Tu from *E. coli* and *B. stearothermophilus* [12,14]. We have tested this reaction with the *T. thermophilus* protein using ^{14}C-labelled reagent. After incubation of EF-Tu·GDP with TPCK for 12 h the reagent was found to be crosslinked to EF-Tu in a stoichiometric amount (0.97 nmol TPCK/nmol EF-Tu·GDP). The modified protein was first treated with trypsin under conditions which lead to the specific hydrolysis of the Arg-59/Gly-60 peptide bond and subsequently cleaved with cyanogen bromide (fig.1). The resulting fragments (mixture I, fig.1) were separated by reversed-phase HPLC. The modified peptide was further digested with trypsin and the resulting peptides (mixture II, fig.1) were again separated by a reversed-phase HPLC. Two tryptic peptides had a molecular weight high enough to be retained on the Vydac-material. Only the second peptide, however, carried radioactivity (fig.2). This labelled peptide was subjected to Edman degradation yielding the sequence His-Tyr-Ser-His-Val-Asp-Xaa-Pro-Gly-His-Ala-/-Tyr. This is in complete agreement with the EF-Tu sequence His(76)-Tyr-Ser-His-Val-Asp-Cys-Pro-Gly-His-Ala-Val-Tyr(88). Instead of cysteine in the seventh position, a PTH-derivative of a modified amino acid was found in a yield of only 1/6 of the PTH amino acids from other degradation cycles. Amino acid analysis of TPCK modified EF-Tu additionally confirmed that the only modified residue is a derivative of cysteine (not shown). This result, together with the achieved TPCK labelling yield of EF-Tu of nearly 100%, allows us to conclude that only Cys-82 in *T. thermophilus* EF-Tu becomes specifically modified by TPCK.

Analysis of the protein modified by [^{14}C]iodoacetamide or [^{14}C]TPCK confirms that there is only one cysteine residue in *T. thermophilus* EF-Tu. This is in agreement with the results of the gene sequencing [11,13] and disproves the results of Nakamura et al. [15], which imply a disulfide bridge in *T. thermophilus* EF-Tu. The reason for this misinterpretation could lie in the observation that Cys-82 in the thermophilic protein could only be modified with iodoacetamide using denaturing conditions (unpublished result) whereas the corresponding cysteine (residue 81) in *E. coli* EF-Tu is reactive under native conditions [16]. As we have now

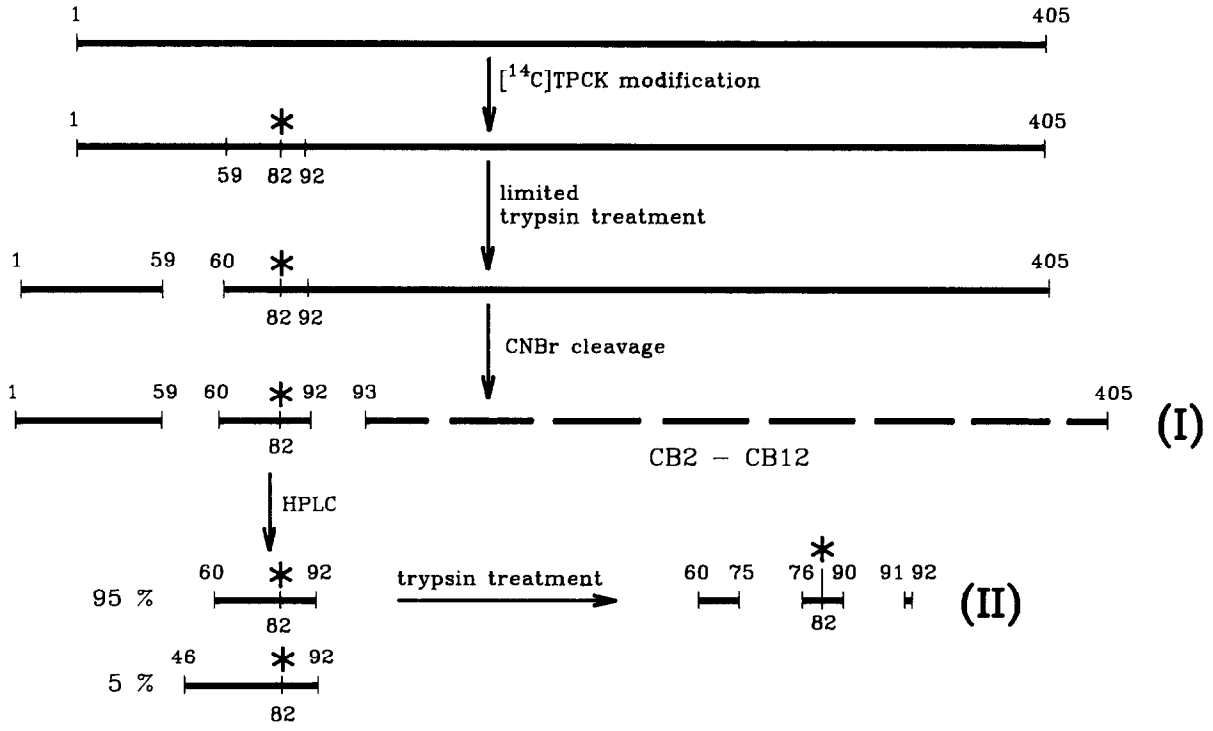

Fig.1. Scheme of the isolation of the [^{14}C]TPCK-labelled peptide His-76–Arg-90. The modified amino acid residue Cys-82 is marked by an asterisk. Mixture II was used for the HPLC separation shown in fig.2.

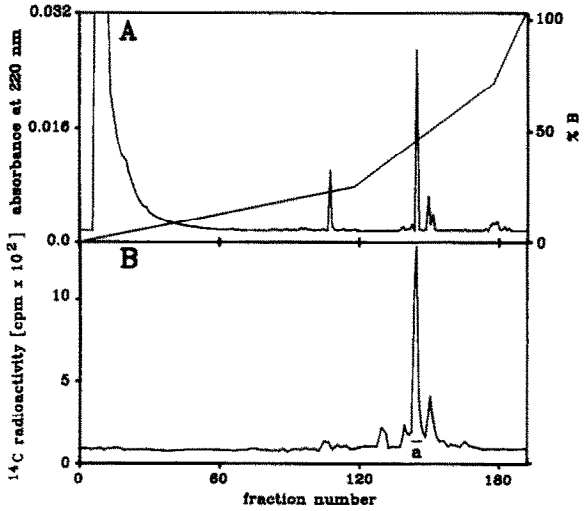

Fig.2. Isolation of the [^{14}C]TPCK-labelled peptide 76–90. A radioactive peptide spanning residues 60–92 (16 nCi) derived from a preceding HPLC isolation step was treated with trypsin and applied on a Vydac C_4 column. For conditions see section 2. (A) Absorbance. (B) Radioactivity profile. The radioactive peak 'a' was pooled and subjected to Edman degradation on a gas-phase sequencer.

shown the denaturation is necessary in the thermophilic protein in order to increase the accessibility of Cys-82 but not for a cleavage of a disulfide bond.

A lower cysteine content of extreme thermophilic proteins compared to their mesophilic variants has been predicted [17]. This can now be supported by analysis of the sequence of *T. thermophilus* EF-Tu and the recently published EF-Tu gene sequence of the extreme thermophilic eubacterium *Thermotoga maritima* [18]. The EF-Tus of these organisms contain one and two cysteine residues, respectively, compared to 3 cysteines in the mesophilic *E. coli* EF-Tu.

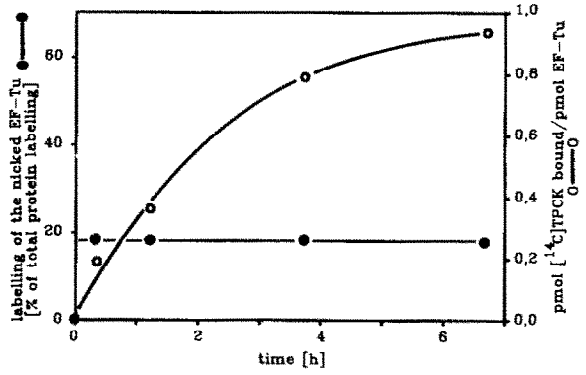

Fig.3. Kinetics of TPCK-labelling of native and nicked EF-Tu. 50 μM EF-Tu·GDP containing 20% of nicked EF-Tu was incubated with 500 μM [^{14}C]TPCK at 4°C as described in section 2. At the indicated times, aliquots of 10 μg EF-Tu were each withdrawn and analyzed by 10% SDS-PAGE and subsequent autoradiography. The exposed X-ray film was scanned at 550 nm along each lane using a DU-8 spectrophotometer (Beckman) equipped with a film scanning device. Areas above blanks were integrated.

In contrast to the iodoacetamide modification, labelling of *T. thermophilus* EF-Tu with TPCK was achieved under native conditions. This fact underlines the proposed mechanism of the TPCK reaction; namely, that TPCK mimics the binding of the 3'-end of aa-tRNA prior to the crosslinking event. The specific and quantitative modification of Cys-82 by [^{14}C]TPCK is in agreement with the results obtained with studies on EF-Tu from *E. coli* [14,19] and *B. stearothermophilus* [12].

We have previously noticed that EF-Tu cleaved at Arg-59 is not able to promote a poly U-directed poly Phe synthesis [7]. This could be due to reduced ability to bind aa-tRNA. Since the TPCK-modifiable cysteine residue is believed to be involved in the binding of the 3'-end of aa-tRNA, we compared the kinetics of TPCK binding to native EF-Tu and EF-Tu nicked at Arg-59 (during the preparation of the protein). An EF-Tu mixture containing 20% nicked protein was incubated with a 10-fold excess of [^{14}C]TPCK (fig.3). The extent of modification of native EF-Tu and EF-Tu fragment (60–405) were determined by autoradiography after separation by SDS-PAGE (fig.3). The ratio of modified native and nicked EF-Tu is the same at each time tested. The $t_{1/2}$ of TPCK modification for both EF-Tu species was 1 h 50 min. If the protein were to undergo a conformational change in the vicinity of the cysteine residue at the aa-tRNA binding site, then the accessibility of this cysteine residue would probably be reduced. The result of identical rates of TPCK modification for both EF-Tu forms implies that this is not the case. This is completely different to the situation upon conversion of EF-Tu·GDP into EF-Tu·GTP. It was shown for EF-Tu from *E. coli* and *B. stearothermophilus* that the TPCK modification rates depend directly on the conformation [12,20]. Although it had been suggested that the corresponding cysteine residue is in the vicinity of the 3'-end of the bound aa-tRNA, this residue might not be essential for aa-tRNA binding since the sequenced EF-Tu from *Micrococcus luteus* has at this position an alanine residue [21].

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