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Crosslinking of elongation factor Tu to $tRNA^{Phe}$ by *trans*-diamminedichloroplatinum (II)

Characterization of two crosslinking sites on EF-Tu

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In a preceding paper [(1987) Nucleic Acids Res. 15, 5787–5801], we have used *trans*-diamminedichloroplatinum (II) to induce reversible RNA-protein crosslinks within the ternary EF-Tu/GTP/Phe-$tRNA^{Phe}$ complex and have identified two crosslinking sites on the tRNA. The aim of the present paper is to determine the crosslinking sites on EF-Tu. Two tryptic peptides located in domain I could be identified, a major one (residues 45–74) and a minor one (residues 117–154). The use of *Staphylococcus aureus* V8 protease led to the isolation of two major peptides (residues 56–68 and 64–68) and one minor peptide (118–124). These results are discussed in the light of the current knowledge of the topography of the EF-Tu/tRNA complex.

Elongation factor EF-Tu; tRNA; Reversible crosslinking

1. INTRODUCTION

In the protein biosynthesis process, elongation factor EF-Tu promotes the binding of the aminoacyl-tRNAs to the ribosomal A site. Formation of the ternary EF-Tu/GTP/aminoacyl-tRNA complex is a prerequisite for this reaction. Understanding of the molecular mechanism of polypeptide elongation requires detailed knowledge of the specificity of the ternary complex formation and of the conformation of the protein and tRNA molecules within the complex. To determine how EF-Tu interacts with the tRNA, two general approaches have been employed. In the first one, amino acid residues that are in contact with the tRNA within the ternary complex are identified by selective amino acid modification: e.g. the binding of L-1-tosylamido-2-phenylethylchloromethylketone (TPCK) to cysteine 81 prevents the interaction of EF-Tu with the aminoacyl-tRNA [1]; histidines 66 and 118 become protected by the aminoacyl-tRNA from photooxidation [2,3] and lysines 2, 4, 237, 248, 263 and 282 at their ϵ-amino group from labeling with ethylacetimidate [4]. In the second approach, specific crosslinks between EF-Tu and the aminoacyl-tRNA are generated within the ternary complex. So far, only two successful crosslinking experiments were reported: one takes place through histidine 66 and the amino acid residue of N^{ϵ}-bromoacetyllysyl-tRNA [5], the other through lysines 208 and 237 and the periodate-oxidized tRNA in the presence of kirromycin [6,7].

Recently, the use of *trans*-diamminedichloroplatinum (II) (*trans*-DDP) as a reversible crosslinking agent has been developed in our laboratory and has been applied to promote RNA-protein crosslinks in the ribosome [8–10] and in aminoacyl-tRNA synthetase/tRNA complexes [8]. In previous work, Wikman et al. [11] have used *trans*-

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DDP to induce specific crosslinks within the ternary EF-Tu/GTP/Phe-tRNA^{Phe} complex at a sufficient yield to isolate the coordinated complex and to identify two tRNA regions crosslinked to EF-Tu: a major one located in the 3'-part of the T-stem and a minor one in the 3'-strand of the anticodon stem. In the present study, we focused on the characterization of the regions of EF-Tu crosslinked to Phe-tRNA^{Phe}.

2. MATERIALS AND METHODS

2.1. *Chemicals and biochemicals*

Phospho*enol*pyruvate (PEP), dithioerythritol (DTE) and *trans*-diamminedichloroplatinum(II) (*trans*-DDP) were from Sigma (USA). ATP, GTP, 1-*O*-octyl-β-D-glucopyranoside (OGP), pyruvate kinase (EC 2.7.1.40) and *Staphylococcus aureus* V8 protease (EC 3.4.21.19) were from Boehringer (France). Chymotrypsin (EC 3.4.21.1), RNase A (EC 3.1.27.5) and TPCK-treated trypsin (EC 3.4.21.4) further purified from contaminating nucleases [12] were from Worthington Biochemical Corp. (USA). AcA 44 gel was from LKB (Sweden), Fractogel TSK DEAE-650 (M) from Merck (France) and DEAE-cellulose from Whatman (England). All other reagents (analytical grade) were from Merck (France) except those employed for the sequencer and PICO·TAG systems, which were from Applied Biosystems and Waters (USA), respectively.

We have purified EF-Tu/GDP from a crude *Escherichia coli* (IBPC 5421) extract by chromatographies on DEAE-cellulose and AcA 44 as described in [13]. Purified EF-Tu exhibited a single band on SDS-polyacrylamide gel electrophoresis (results not shown). A specific activity of 20000 U/mg was determined by the GDP-exchange assay [14]. Brewer's yeast tRNA^{Phe} was purified by counter current distribution and chromatography on BD-cellulose [15]. Yeast phenylalanyl-tRNA synthetase was purified as in [16].

2.2. *Ternary complex formation and crosslinking*

To convert EF-Tu/GDP in EF-Tu/GTP, 12 nmol of EF-Tu/GDP was incubated in 50 μl of buffer A (50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 150 mM NH_4Cl; 0.02 mM 2-mercaptoethanol; 0.005% OGP) with 30 μg pyruvate kinase, 1 mM GTP and 40 mM PEP, for 20 min at 37°C. Aminoacylation of 150 μg (6 nmol) tRNA^{Phe} was done in 100 μl of buffer A containing 160 μM [^{14}C]phenylalanine, 2 mM ATP and 10 μg phenylalanyl-tRNA synthetase for 6 min at 37°C. The ternary EF-Tu/GTP/[^{14}C]Phe-tRNA^{Phe} complex was achieved by incubating EF-Tu/GTP and Phe-tRNA^{Phe}, for 2 min at 37°C, then 20 min on ice. The ternary complex was isolated by HPLC with a Waters chromatograph (ALC/GPC-204) using a TSK G2000 SW (7.5 × 600 mm) column (LKB-Produkter, Sweden). Elution was with buffer B (12.5 mM potassium phosphate, pH 7.2; 0.005% OGP) at a flow rate of 0.4 ml/min. 1 min fractions were collected and those containing the ternary complex were pooled.

Crosslinking was performed by incubating the ternary complex with a freshly prepared *trans*-DDP (0.35 mM final concentration) for 1 h at room temperature in the dark. A final concentration of 0.05% OGP and 0.15 M NaCl was then added. The crosslinked complexes were fractionated from the non-crosslinked species by chromatography on a Fractogel TSK DEAE-650 (M) column (300 μl-packed in a Pasteur pipette). Elution was at a flow rate of 0.4 ml/min with 12.5 mM potassium phosphate, pH 7.2, 0.05% OGP, in the presence of increasing concentrations of NaCl as described in [11]. 1 min fractions were collected.

2.3. *Analysis of the crosslinked peptides*

In order to remove NaCl from the sample, the crosslinked complex was rechromatographed on a TSK G2000 SW column and eluted with 12.5 mM potassium phosphate, pH 7.2, 0.05% OGP. Digestion with RNase-free trypsin or *Staphylococcus aureus* V8 protease was achieved in the dark at 20°C (enzyme:substrate ratio of 1:20 by weight) for 6 h. The digest was chromatographed on a TSK G2000 SW column under the same conditions as just described. The peak coeluting with the tRNA was pooled and the crosslinks were reversed by a 1 h treatment with 20 mM DTE at room temperature. Liberated peptides were fractionated on a TSK G2000 SW column as indicated above. The tryptic peptides were then dried, dissolved in 10% acetic acid for 2 h at room temperature and evaporated again to dryness. Chymotryptic digestion of the tryptic peptides was performed in 12.5 mM potassium phosphate, pH 7.2, 0.05% OGP for 16 h at 37°C (enzyme:substrate ratio of 1:20 by weight).

Amino acid analysis of the released peptides was done with the socalled PICO·TAG method. Acid hydrolysis was accomplished in vapors of 6 M HCl at 110°C for 18 h and phenylisothiocyanate (PITC) precolumn derivatization procedure was performed according to Waters recipe. The phenylthiocarbamyl-amino acids (PTC) were analyzed by chromatography on a PICO·TAG column (3.9 × 150 mm).

Peptides were subjected to automated N-terminal degradation with PITC on an Applied Biosystems 470 A sequencer. Samples were applied to a polybrene-treated and precyclated glass-fiber filtre. Amino acid phenylthiohydantoins (PTH) were identified by chromatography on a C18 column Brownlee (2.1 × 200 mm) coupled with the 120 A PTH-analyzer, according to Applied Biosystems chromatographic parameters.

3. RESULTS AND DISCUSSION

trans-DDP has a square planar geometry where the two chlorines span a 7 Å long distance. As described in detail by Tukalo et al. [8], these chlorines can easily be substituted by stronger nucleophilic groups. Binding positions are specific: on RNA, platinum mainly binds by coordination to position N7 of guanines and to a smaller extent to N1 of adenines and N3 of cytosines. On the protein side, at neutral pH, *trans*-DDP coordinates to the unprotonated nitrogen atoms of the imidazole ring of histidines and to the sulphur atom of cysteines and methionines. These crosslinks can either be kept stable or can easily be reversed by the addi-

tion of stronger nucleophilic groups such as DTE.

Crosslinks induced by *trans*-DDP within the ternary EF-Tu/GTP/Phe-tRNA^{Phe} were achieved under the conditions described in a previous work [11]. Under these conditions, an average percentage of 35 to 40% of the complex could be converted into crosslinked species. The specificity of the crosslinking reaction was also tested as described in detail in the above mentioned work: after the platination reaction, the non-crosslinked complex was dissociated in the presence of 0.5 M NaCl prior to the addition of a large excess of [^{14}C]Phe-tRNA^{Phe}; elimination of the salt and appropriate incubation led to the recovery of a 1:1 EF-Tu/tRNA complex, indicating that the tRNA was crosslinked at its specific site.

In order to identify the regions of the protein involved in the crosslinking reaction, the ternary complex was formed as described in the experimental section prior to the platination experiment. The non-crosslinked complex was then dissociated in the presence of NaCl and the crosslinked EF-Tu/tRNA complex was fractionated (see section 2). The crosslinked complex was then subjected to tryptic digestion and the resulting crosslinked tRNA-peptide complexes were separated from unbound peptides by chromatography on a TSK G2000 SW column (fig.1). Two peaks could be obtained: peak 1 containing the crosslinked complex as shown by the measure of the tRNA radioactivity, and peak 2 containing the unbound peptides. A *Staphylococcus aureus* V8 protease digestion yielded an identical profile (result not shown). In order to insure that no contaminating peptidic material was coeluted with the tRNA, a control experiment was performed in which an EF-Tu digest was cochromatographed with tRNA under the same conditions. In that experiment, no peptides could be isolated from the tRNA peak, showing that the peptides present in the tRNA peak after platination only correspond to the crosslinked peptides.

The crosslinks were then reversed by treatment with DTE and the resulting peptides rechromatographed under the same conditions as above. Again, two peaks were isolated, one containing the radioactive tRNA, the other one containing the released peptides. The tRNA fractions were subjected to an amino acid analysis as described in section 2 in order to insure that the

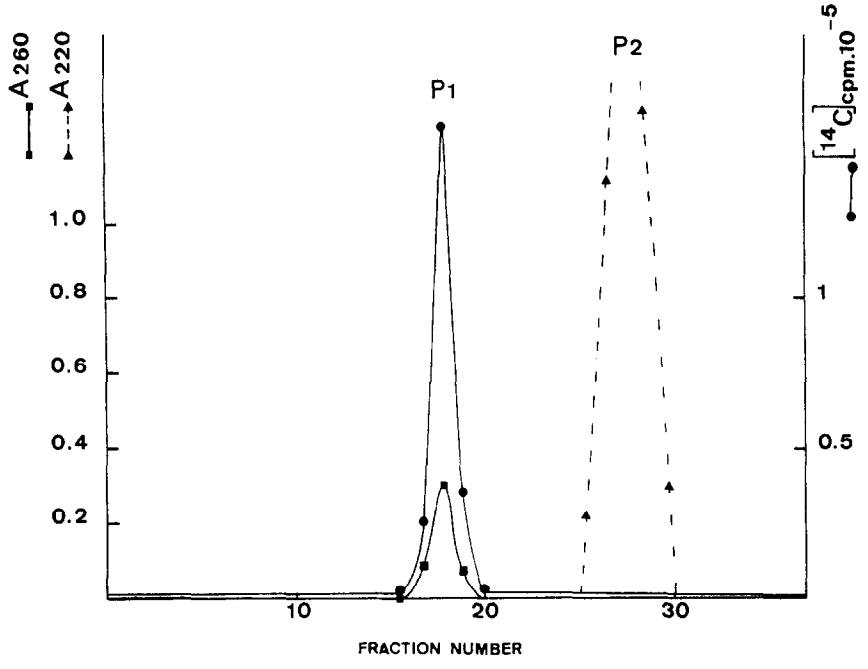

Fig.1. Fractionation of the crosslinked tRNA-peptides complexes by chromatography on a TSK G2000 SW column.

crosslinks were completely reversed. From the knowledge of the complete amino acid sequence of EF-Tu [17], the determination of the amino acid composition of the released peptides and automated Edman degradation strongly suggested that two tryptic peptides could be characterized, a major one Ta (78%) encompassing residues 45–74, and a minor one Tb (22%) containing residues 117–154 (fig.2). However, the quantitative recovery of PTH-amino acids was very low indicating that Edman degradation did not proceed easily. Indeed, only residues 45–53 of peptide Ta could be sequenced unambiguously, whereas Edman degradation did not go over the N-terminal Glu 117 of peptide Tb. This difficulty in sequence analysis, often due to the configuration of the peptides themselves, could be overcome by dissolving the above tryptic peptides in 10% acetic acid, drying and further subjecting them to a chymotryptic digestion in the appropriate buffer. Unambiguous sequencing of the resultant smaller chymotryptic peptides indicates that they belong to the two tryptic fragments Ta (45–74) and Tb (117–154) (see fig.2). Furthermore, four peptide bonds including a basic amino acid were resistant to tryptic cleavage in the crosslinked complex at positions K56–A57, R58–G59, R123–Q124, K136–C137 (fig.2). It has been reported that the peptidic chain at arginines 44 and 58 is easily cleaved in the free EF-Tu by trypsin even under limited digestion conditions, with a concomitant loss of aminoacyl-tRNA binding activity [18,19]. The high resistance to the tryptic cleavage at Arg 58 within the complex suggests that this area is protected by the close neighbourhood of the tRNA. Indeed, after reversion of the crosslink this bond became cleavable even by chymotrypsin, as this enzyme itself has also some tryptic activity.

Identification of the above tryptic peptides is confirmed by sequencing the peptides generated by cleaving the crosslinked EF-Tu/tRNA complex with *Staphylococcus aureus* V8 protease. Three sequences could be identified (see fig.2): two major peptides S1a (56–68) and S2a (64–68), and a minor one Sb (118–124). S2a is a subfragment of S1a which is a part of peptide Ta, whereas Sb is located at the N-terminus of peptide Tb. This strongly suggests that the major crosslinked residue(s) is located in fragment 64–68, and the minor one in fragment 118–124. Both sequences contain a histidine residue (His 66 and His 118) which are potential targets for the platination reaction. Comparison of the primary structure of EF-Tu from different sources (fig.3) shows that both crosslinked fragments Ta and Tb exhibit a very high degree of homology (70%). It is remarkable to point out that both protected histidines 66 and 118 found in the ternary complex by the tRNA in other experiments (see section 1) are conserved in all species studied so far.

The crosslinked peptides are represented on the three-dimensional structure model of EF-Tu proposed by Nyborg and La Cour [20] (fig.4). Summarizing all the biochemical data dealing with the

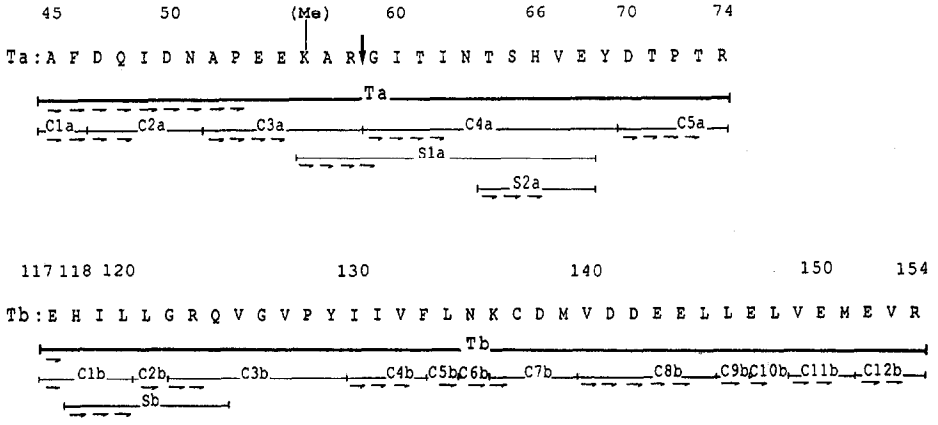

Fig.2. Amino acid sequences of the EF-Tu regions crosslinked to the RNA. (→) Amino acid determined by automated Edman degradation. Residues are noted by the one-letter abbreviation system. T, tryptic peptides; C, chymotryptic peptides; S, *S. aureus* V8 protease peptides. (↓) Cleavage by limited tryptic digestion.

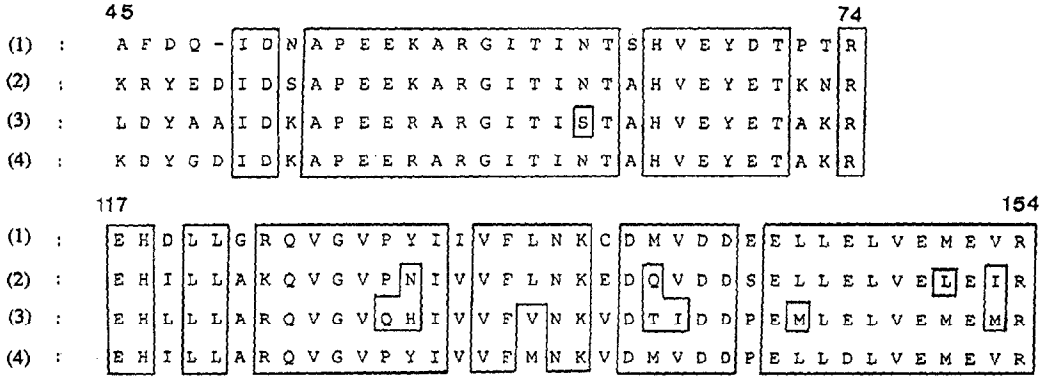

Fig.3. Sequence comparison of the two tryptic peptides 45–74 and 117–154 from *E. coli* (1) EF-Tu crosslinked to the tRNA with EF-Tu sequences from *Euglena gracilis* chloroplast (2) [28], *Saccharomyces cerevisiae* mitochondria (3) [29] and *Thermus thermophilus* (4) [30]. Homologous areas are boxed.

protection of the amino acid residues by the tRNA (see section 1) and with the crosslinking data, it is striking that all these residues are exclusively located in domains I and II (His 66 and 118, Cys 81, Lys 2, 4, 208, 237, 248, 263, 282).

In a previous paper [11], we have identified two

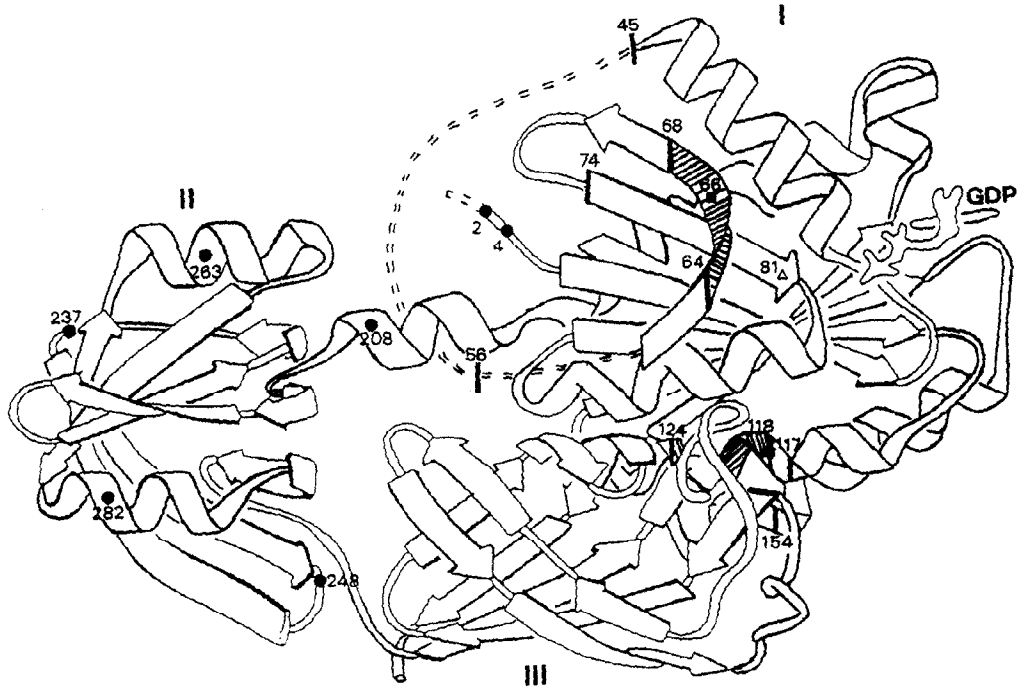

Fig.4. Location on the three-dimensional structure model of EF-Tu proposed by Nyborg and La Cour [20] of the shortest crosslinked peptides 64–68 and 118–124 (dashed boxes). (▮) The different tryptic and *S. aureus* V8 protease cleavages.

T1 oligonucleotides crosslinked to EF-Tu in the ternary complex by *trans*-DDP, a major one located in the 3'-strand of the T-stem in which G65 would be the residue involved in the crosslinking reaction, and a minor one encompassing the anticodon region in which the crosslinking presumably occurred at G43 or G45 at the 3'-end of the anticodon stem. A stereoscopic view of $tRNA^{Phe}$ showed that the 3'-part of both crosslinked regions is located on the same side of the tRNA [11]. This suggests a model of interaction in which the L-shaped tRNA lies parallel onto the protein as already proposed by Kabsch et al. [21]. This model provides large surface contacts as supported by the high-binding constants [22,23] and by the protection of tRNA against nuclease digestion in the complex (for a review see [24]). If we assume that the major crosslinked peptide (residues 56–68 or even 64–68) is linked to the major crosslinked T1 fragment (nucleotides 58–65), one can postulate that the potential crosslinking residue in this peptide is His 66 which would be separated by a 7 Å distance (length of *trans*-DDP) from the postulated G65. His 66 was already found to be protected by aminoacyl-tRNA and crosslinked to the chemically modified amino acid of the aminoacyl-tRNA [5]. This would also fit with the location of the 3'-end of the tRNA near the GDP-binding site [24]. In this arrangement, the 3'-strand of the amino acid- and T-stems are in contact with EF-Tu but not the T-loop. This orientation is also consistent with the footprinting experiments [24]. However, this localization of the 3'-end of the tRNA is not in agreement with the crosslinking of the 3'-oxidized tRNA to lysines 208 and 237 in the presence of kirromycin [6,7]. As for the second minor crosslinking site (peptide 118–124), the candidate for the platination is His 118. Indeed His 118 was also found to be protected by the tRNA [4]. The crosslinking of His 118 with G43 or G45 would also fit with the crosslinking data of Kao et al. [25] who crosslinked nucleotide 47 in the extra arm of *E. coli* $Phe\text{-}tRNA^{Phe}$ with a 20 Å bridging reagent to EF-Tu. However, multiple orientations of the anticodon arm with respect to EF-Tu remain possible, especially if one would conciliate these results with the protection of the lysines in domain II mentioned in section 1, the footprinting data, small X-ray and small-angle scattering studies [26,27] which suggested that the anticodon loop would be protruding from the protein. Experiments are presently in progress to isolate the crosslinked oligonucleotide-peptide fragments in order to identify those residues that are involved in the crosslinking reaction. This would greatly help in the correct positioning of the tRNA on EF-Tu within the ternary complex.

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