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Crosslinking of elongation factor Tu to $tRNA^{Phe}$ by trans-diamminedichloroplatinum (II). Characterization of two crosslinking sites in the tRNA

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SUMMARY

Trans-diamminedichloroplatinum (II) was used to induce reversible crosslinks between EF-Tu and Phe-$tRNA^{Phe}$ within the ternary EF-Tu/GTP/Phe-$tRNA^{Phe}$ complex. Up to 40% of the complex was specifically converted into crosslinked species. Two crosslinking sites have been unambiguously identified. The major one encompassing nucleotides 58 to 65 is located in the 3'-part of the T-stem, and the minor one encompassing nucleotides 31 to 42 includes the anticodon loop and part of the of 3'-strand of the anticodon stem.

INTRODUCTION

Elongation factor Tu (EF-Tu) promotes the codon-dependent binding of the aminoacyl-tRNA to the A site of the ribosome. For this purpose, the GTP induced conformation of EF-Tu has to bind each of the elongator aminoacyl-tRNAs utilized in protein biosynthesis. Understanding of the molecular mechanism of polypeptide elongation requires knowledge of the specificity of the ternary EF-Tu/GTP/aminoacyl-tRNA complex formation and of the conformations of the protein and tRNA molecules within the complex. The formation of the ternary complex has been extensively studied by kinetic studies (1-3). It has also been suggested that its formation is tRNA sequence dependent as demonstrated by the comparative study of the initiator and elongator Met-tRNA binding (4). The conformation of the ternary complex has also been approached by small angle X-ray (5) and neutron scattering (6,7) and by NMR studies (8). Numerous investigations have been attempted to determine the regions of the tRNA molecule in close contact with the protein factor within the ternary complex, and to study the conformational changes of the tRNA induced by the factor interaction. These experiments were essentially based on footprinting experiments, either by chemical probing (9-11) or by nuclease accessibilities (12-16), and on crosslinking experiment (17).

Despite some discrepancies between the published results, the involvement of the tRNA aminoacid arm and the T-arm in the interaction with EF-Tu are strongly suggested.

In the present study we have investigated a crosslinking approach to determine the RNA regions in the close neighbourhood of EF-Tu within the ternary EF-Tu/GTP/Phe-tRNA^{Phe} complex. This RNA-protein crosslinking is based on the use of a novel reversible crosslinking agent : the trans-diamminedichloroplatinum (II). This reagent has been successfully used in our laboratory to promote RNA-protein crosslinks between tRNA and aminoacyl-tRNA synthetase, between RNA and proteins within the ribosome particle (18), and to determine the 16S rRNA region crosslinked to the initiation factor 3 within the specific IF3-30S complex (19). As described in details in ref. 18, trans-DDP has a square planar geometry where the two chlorines span a 7 Å long distance. These chlorines can easily be substituted by stronger nucleophilic groups. Binding positions are specific : on the RNA, platinum mainly coordinates to N7 of guanine and at a less extent to N1 of adenine and N3 of cytosine residues. On proteins, at neutral pH, it binds by coordination to sulfur atoms of cysteines and methionines, and to the nitrogen atoms of the imidazole ring of histidines (18). Because of the nature of the coordination bonds between platinum and the acceptors, these crosslinks can either be kept stable under certain solvent conditions or can be reversed by the addition of stronger nucleophilic groups.

The specific crosslinks induced by trans-DDP within the ternary EF-Tu/GTP/Phe-tRNA^{Phe} complex are of sufficient yield to make possible the isolation of the coordinated complex and the identification of the tRNA regions crosslinked to EF-Tu.

MATERIALS AND METHODS

1- Materials

2 [N-Morpholino]ethanesulfonic acid (MES), phosphoenolpyruvate (PEP) and trans-diamminedichloroplatinum II (trans-DDP) were from Sigma (USA). [^{3}H]phenylalanine (49 Ci/mmol), [α^{32}P]ATP (410 Ci/mmol) and [γ^{32}P]ATP (3200 Ci/mmol) were from Amersham (France). Unlabelled ATP and GTP, 1-O-octyl-ß-D-glucopyranoside (OGP), and pyruvate kinase were from Boehringer (FRG). RNase T1 was from Sankyo (Japan), T4 polynucleotide kinase and RNase kits for oligonucleotide sequencing were from PL Biochemicals (FRG). AFFI-SEP DEAE was from Senetek PLC (Denmark) and

Ultrogel AcA 44 and 54 from LKB (Sweden). Sephadex G-100 was purchased from Pharmacia (Sweden).

2- Biochemical material

Brewer's yeast $tRNA^{Phe}$ was purified by counter current distribution (20) followed by conventional chromatography on BD-cellulose. Phenylalanyl-tRNA synthetase from yeast was purified as described by Kern et al. (21, 22). Buffer A contained 10 mM MES pH 7.2, 7 mM $MgSO_4$, 12.5 mM $(NH_4)_2SO_4$, 0.02 mM 2-mercaptoethanol and 0.005% OGP. Buffer B contained 12.5 mM potassium phosphate pH 7.2, 0.05% OGP and different concentrations of NaCl as described.

3- Ternary complex formation and isolation

Standard conditions used here to form the ternary complex are the following. EF-Tu/GTP complex was formed by incubating 500 µg (12 nmol) EF-Tu in 50 µl of Buffer A containing 30 µg pyruvate kinase, 1 mM GTP and 40 mM PEP, for 20 min at 37°C. After incubation, the mixture was kept on ice for 30 min. 3'-labelled $[^{32}P]tRNA^{Phe}$ was achieved as described elsewhere (23). The 3'-CCA triplet was previously removed and restored in the presence of $[\alpha^{32}P]ATP$, CTP and tRNA nucleotidyl transferase. Aminoacylation of 150 µg (6 nmol) of unlabelled $tRNA^{Phe}$ (with $[3'^{32}P]tRNA^{Phe}$ as a marker) was done in 100 µl of Buffer A containing 160 µM $[^3H]$phenylalanine, 2 mM ATP and 10 µg phenylalanyl-tRNA synthetase, for 6 min at 37°C. Ternary complex was then achieved by adding the EF-Tu/GTP complex prepared as above. Incubation was continued for 2 min at 37°C followed by placing on ice for 20 min.

The ternary complex was isolated by chromatography on a AcA 44 column (44 x 1 cm) previously equilibrated with EF-Tu / GTP in Buffer A. Elution was with Buffer A. Fractions containing the complex were pooled.

4- Formation and isolation of the crosslinked EF-Tu/tRNA complex

Crosslinking was performed by adding trans-DDP at a final concentration of 0.35 mM. Aqueous solution of trans-DDP (2 mM) was prepared prior use. Incubation was for 1h at room temperature in the dark.

After incubation the reaction mixture was made 0.05% in OGP and 0.15M in NaCl. The mixture was then applied to a AFFI-SEP DEAE column (300 µl packed in a Pasteur pipette and equilibrated with Buffer B). The column was carefully washed with Buffer B to remove excess of trans-DDP, then with Buffer B-0.2 M NaCl to remove non crosslinked EF-Tu. The crosslinked complex was eluted with Buffer B-0.45 M NaCl. The non-crosslinked tRNA was eluted with 1 M NaCl.

5- Isolation of the crosslinked EF-Tu/oligonucleotide complexes

Fractions containing the crosslinked EF-Tu/tRNA complex were collected and diluted with Buffer B to 0.15 M NaCl. RNase T1 (0.15 U / µg tRNA) was added for a 30 min incubation at room temperature. The resulting crosslinked EF-Tu/oligonucleotide complexes were isolated by two different methods. The first one was chromatography on an AcA 54 column (22 x 1cm) with Buffer B-0.15 M NaCl as elution buffer. The exclusion peak containing the crosslinked EF-Tu/oligonucleotide complexes was located by U.V. spectroscopy. The peak fractions were pooled and dialyzed 2 x 30 min against 0.005% OGP and finally against H_2O before lyophilisation. In the second method, the crosslinked EF-Tu/T1 oligonucleotide complexes were fractionated from free T1 oligonucleotides by nitrocellulose membrane filtration. The hydrolysis mixture was made 0.45 M NaCl and filtered on nitrocellulose filter (Millipore type HA, 45 µm pore size, 25 mm diameter), previously soaked for 30 min in the buffer. The complex retained on the filter was washed with 20 ml of the same buffer containing 0.45 M NaCl and 5 mM EDTA (19).

6- Reversion of the crosslinks and labelling

The crosslinked EF-Tu/oligonucleotide complexes isolated by AcA 54 chromatography were lyophilized and resuspended in the appropriate buffer. The oligonucleotides were labelled at their 5'-end within the complex in the presence of T4 polynucleotide kinase and 200 µCi [γ^{32}P]ATP, according to a procedure derived from that published in ref. 24. Thiourea (1 M final concentration) was added to the labelling mixture after 15 min of incubation in order to reverse the crosslinks and incubation was continued for 15 min at 37°C. The liberated oligonucleotides were precipitated twice with ethanol, in the presence of 10 µg unlabelled pCp as carrier. When the crosslinked complexes were isolated by filtration, the nitrocellulose filters were soaked in 450 µl 1 M thiourea. The supernatants were removed and the filter washed with 350 µl 1 M thiourea. The liberated tRNA fragments were precipitated and labelled as above.

The 5'-labelled RNA fragments were fractionated by electrophoresis on a 15% polyacrylamide/8 M urea slab gel. After autoradiography, bands were excised, eluted according to Maxam and Gilbert (25), and the products repurified by a second electrophoresis on a 18% polyacrylamide/8 M urea gel. The fragments were eluted, precipitated with ethanol in the presence of 10 µg of tRNA as carrier, dissolved in 10 µl bidistilled water and sequenced by rapid gel electrophoresis methods using RNase statistical

hydrolysis of the oligonucleotides. Digestion was with RNase T1 (0.005 U/µg tRNA), RNases U2, PhyM and from **B. cereus** (0.1-0.5 U/µg tRNA), respectively. Incubation was at 55°C for 15 min in a citrate buffer 0.02 M pH 7,5 containing 1 mM EDTA, in the presence of 8M urea for RNases T1, U2, PhyM, and in the absence of urea for RNase from **B. cereus.** The ladder was formed in a 50 mM Na carbonate buffer pH 9, containing 1 mM EDTA, for 20-30 min at 90°C. Analysis of the digests was carried out by electrophoresis on a 15-25% polyacrylamide/8 M urea slab gel.

The 5'-terminal nucleotide was identified by total hydrolysis (12h at 37°C) of the 5' labelled-RNA fragments with RNase P1 (0,5 µg) in the presence of 5 µg tRNA as carrier. The liberated nucleotides were separated by chromatography on thin-layer cellulose plate in the presence of HCl/2-propanol/H_2O (17.6/68/14,4) as solvent.

RESULTS

1- Formation and isolation of the crosslinked complex

Standard conditions used here to obtain aminoacylated $tRNA^{Phe}$, conversion of EF-Tu/GDP in EF-Tu/GTP, formation and isolation of the ternary EF-Tu/GTP/Phe-$tRNA^{Phe}$ complex were derived from those described by Wikman et al. (15). Trans-DDP crosslinking of the ternary complex and isolation of the crosslinked EF-Tu/tRNA complex was as described in Materials and Methods. Figure 1 shows a typical elution profile of the platinated complex. In order to identify the regions of the tRNA molecule

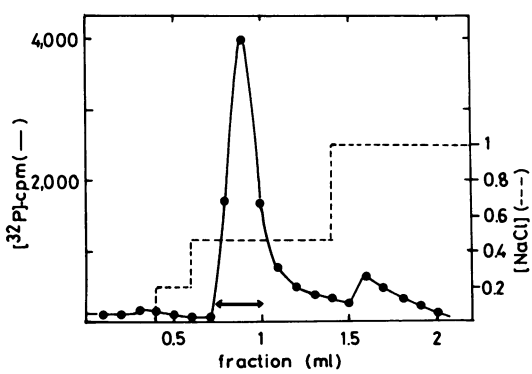

Figure 1: Isolation of the crosslinked EF-Tu/Phe-$tRNA^{Phe}$ complex by chromatography on a AFF1-SEP DEAE column equilibrated with a 12.5 mM potassium phosphate buffer pH 7,2, 0.05% OGP (buffer B). Elution was with different concentrations of NaCl.

TABLE I : YIELD AND SPECIFITY OF THE CROSSLINKING REACTION

(1) Ternary complex content (pmol)	(2) Content after crosslinking (pmol)	(3) Ternary complex (2) +200 pmol Phe-tRNA (pmol)
EF-Tu : 100	EF-Tu : 100	EF-Tu : 100
Phe-tRNA : 100	Phe-tRNA : 36	Phe-tRNA : 98

involved in the crosslinking reaction, the crosslinked EF-Tu/tRNA complex was subjected to a limited RNase T1 digestion. The resulting crosslinked EF-Tu/oligonucleotide complexes were separated from unbound tRNA fragments either by a AcA 54 chromatography or by nitrocellulose filtration in the presence of high salt concentration (see the experimental procedure).

2- Yield and specificity of the crosslinking reaction

The yield and the specificity of the crosslinking reaction was studied separately as follows. Unlabelled $tRNA^{Phe}$ was aminoacylated with [^{3}H]phenylalanine and isolated as described earlier (10). The ternary EF-Tu/GTP/[^{3}H]Phe-tRNA complex was formed in the presence of an excess of EF-Tu and isolated as described in Materials and Methods. The concentration of EF-Tu was precisely calculated by the determination of its aminoacid composition with a Durrum D500 analyser (the sample was previously hydrolysed with HCl 6 M in the presence of 0.02% 2-mercaptoethanol and 0.1% (v:v) saturated phenol, for 18 h at 110°C under nitrogen). The concentration of [^{3}H]Phe-tRNA bound to EF-Tu was evaluated by the nitrocellulose filtration procedure described by Louie and Jurnak (3). This test takes into advantage the protection of the aminoacylated 3'-end of the tRNA by EF-Tu in the ternary complex against limited RNase A digestion.

The ternary complex (100 pmoles) was treated with trans-DDP. Non crosslinked complexes were dissociated in 0.2 M NaCl and the crosslinked tRNA was separated from the free tRNA on a Sephadex G100 column (40 x 0.6 cm). Elution was with Buffer B in the presence of 0.15 M NaCl. Fractions containing the crosslinked complexes and unbound EF-Tu were pooled. The

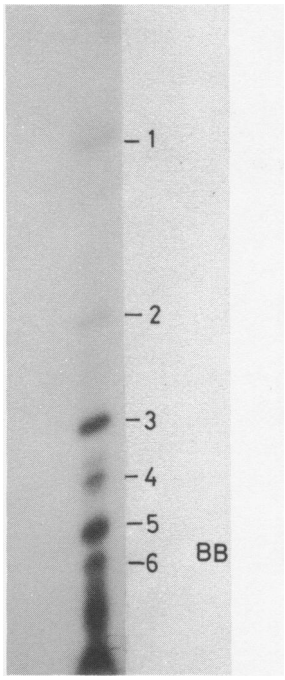

Figure 2: Polyacrylamide/urea gel electrophoresis of the crosslinked oligonucleotides. The crosslinked EF-Tu/T1 oligonucleotides complexes were subjected to 5'end labelling and to reversion of the crosslinks before fractionation.

EF-Tu content and the crosslinked [^{3}H]Phe-tRNA were measured as described above. An average percentage of crosslinking of 35 to 40% was found. Controls were performed in the absence of the crosslinking reagent, indicating that no non specific binding occurred during the isolation procedure.

To test the specificity of the crosslinking reaction, an experiment was conducted in which 36% of crosslinking occurred. The fractions containing the crosslinked complex and unbound EF-Tu were pooled and dialysed against Buffer A. In order to insure that all non crosslinked EF-Tu is complexed with GTP, the sample was submitted to the conversion procedure of GDP into GTP, as described in Materials and Methods, before addition of a large excess of [^{3}H]Phe-tRNA for the ternary complex formation. Results of such a typical experiment are summarized in table I. Recovery of a 1:1 EF-Tu/GTP/Phe-tRNA complex strongly suggests that the tRNA was crosslinked at its specific site.

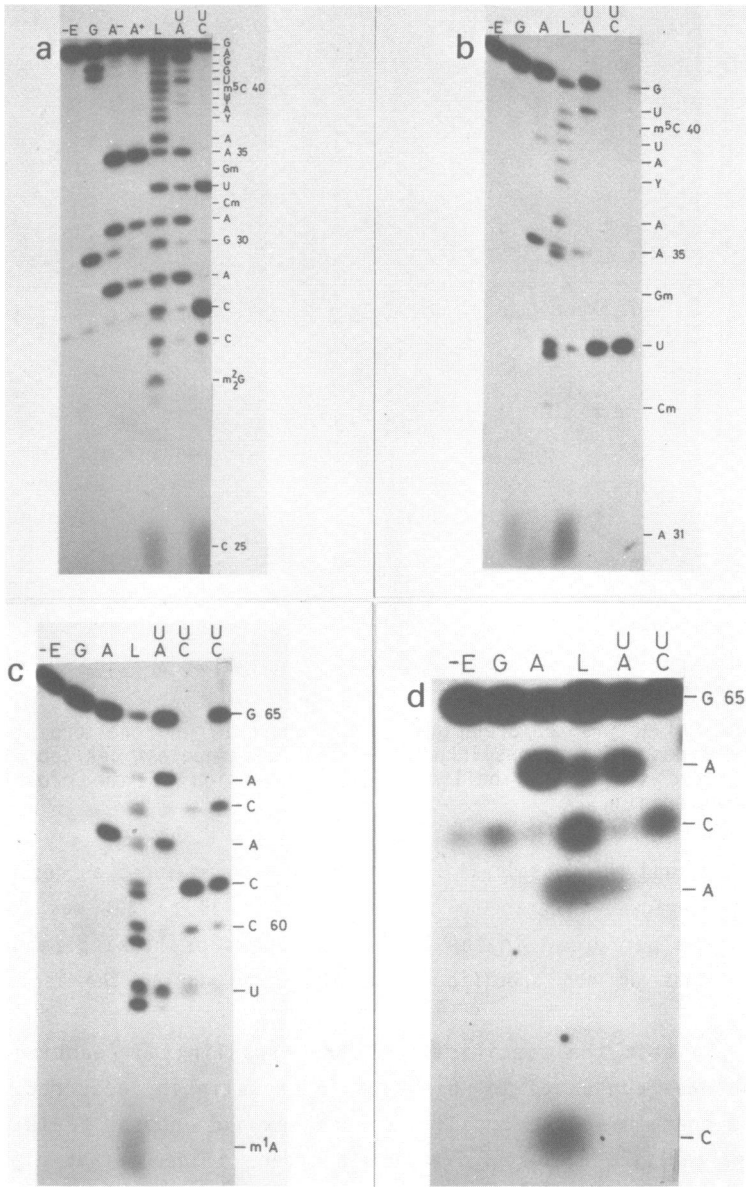

Figure 3: Sequence analysis of some of the crosslinked tRNA fragments isolated on Figure 2. Autoradiographies a, b, c, d correspond to fragments 1, 2, 3 and 4, respectively. Lane (-E) : control in the absence of enzyme. Lanes (G), (A), (AU), (CU) : accessibility to RNases T1, U2, Phy M, **B. cereus**, respectively. Conditions of digestions are detailed in Materials and Methods.

3- Sequence analysis of the crosslinked oligonucleotides

The RNA fragments in the crosslinked EF-Tu/T1 oligonucleotide complexes previously separated by AcA 54 gel filtration were labelled at their 5'-ends. The crosslinks were reversed by thiourea treatment and the fragments were isolated by electrophoresis on polyacrylamide/urea gel (Fig. 2). In order to insure that no contaminating RNA fragments comigrate with the crosslinked complex, $tRNA^{Phe}$ was digested in the presence of non platinated EF-Tu and chromatographed in the same conditions as above. Fractions containing EF-Tu were pooled and submitted to the 5'-labelling procedure. The sample was then fractionated by gel electrophoresis : no significant amount of material could be detected (result not shown).

When the crosslinked EF-Tu/T1 oligonucleotide complexes were retained on nitrocellulose filters, 5'-labelling was made after reversion of the crosslinks (see Materials and Methods). Gel electrophoresis of the labelled material showed that the same fractionation pattern could be obtained as in Figure 2 although with a greater loss of material. The same controls as above were performed, showing that only the crosslinked fragments were retained on the nitrocellulose filters by the protein.

All crosslinked fragments were repurified by a second electrophoretic migration on polyacrylamide/urea gel. The RNA fragments were excized from the gel, eluted and sequenced using RNase statistical hydrolysis followed by polyacrylamide/urea gel electrophoresis. Several examples of sequence gels are presented in Figure 3. The 5'-labelled nucleotide of the different fragments was identified separately (by total RNase P1 digestion and chromatography). Results are summarized in Figure 4. From a total of four experiments, it became evident that a RNase T1 octamer (nucleotides 58-65) containing a part of the T-loop and the 3'-strand of the corresponding helix was found crosslinked at a high and stable extent. Two subfragments encompassing nucleotides 58m_1A-U-C-C-A-C63 and 61C-A-C-A-G65 resulting from a non enzymatic phosphodiester cleavage between C63 and A64, C60 and C61, respectively, were also found. These types of cleavages occurring essentially in pyrimidine-adenine phosphodiester bonds have already been described and reflect the intrinsic fragility of the RNA chain (26). It is noteworthy that the octamer 58-65 migrates under two different forms (fragments 3 and 4 in Figure 2). A likely explanation should be that the platinum adduct is not

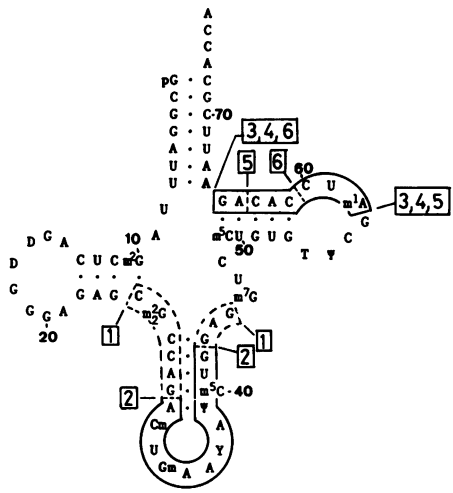

Figure 4: Cloverleaf structure of $tRNA^{Phe}$. Regions reproducibly crosslinked are boxed and regions crosslinked at a variable extent are delimited by a broken line. The numbers in boxes indicate the extremities of the crosslinked fragments. Fragments are numbered as in Figures 2 and 3.

totally removed. When present, it perturbs the migration of the RNA fragment.

A second tRNA region was found crosslinked to EF-Tu at a weaker and more variable extent than the octamer 58-65. This region encompassing nucleotides 25 to 45 contains the anticodon stem and loop (Fig. 4). A subfragment encompassing nucleotides 31 to 42 containing the anticodon loop and the 3'-strand of the corresponding helix, was also found crosslinked. It has to be mentioned that sequencing of the anticodon region of $tRNA^{Phe}$ by the statistical enzymatic cleavage method presents some difficulties since several residues could not be detected by enzymatic accessibilities. Due to the mechanism of splitting of RNases and of alkaline digestion, nucleotides methylated at the ribose (Cm32, Cm34) could not be detected. With the methodology used here to detect the crosslinked fragments, the relative amount of crosslinking cannot be accurately estimated since the intensity of labelling does not necessarily reflect the amount of RNA because phosphorylation is sequence and structure dependent.

DISCUSSION AND CONCLUSION

Crosslinking approaches have been widely used to identify neighbourhood or contact areas between RNA and proteins within ribonucleoprotein complexes. However, several limitations have been pointed out, especially linked to the low yields of crosslinking, to the steric hindrance of the probes when bifunctional reagents are used, and to the irreversibility of the crosslinks.

In the present work, we have used trans-DDP as a reversible crosslinking reagent to study the tRNA regions in close contact with EF-Tu within the ternary EF-Tu/GTP/Phe-tRNA^{Phe} complex. Results reported here show that an average yield of 40% of tRNA could be crosslinked to the elongation factor within the ternary complex by coordination through trans-DDP. This high yield of crosslinking facilitated the isolation and the identification of the crosslinked tRNA regions. Two regions were found crosslinked to EF-Tu. The major one, encompassing nucleotides 58 to 65 contains the 3'part of the T-loop and stem. This finding confirms footprinting experiments which strongly suggest the involvement of the T-arm in the interaction with EF-Tu (9-16). Two subfragments of the T1 octamer 58-65 were also identified: 58m_1A-U-A-C-C63 and 61C-A-C-A-G65 resulting from unspecific phosphodiester cleavages. The second crosslinked region was unexpected since it encompasses the anticodon stem and loop. Although this region was found at a weak and variable extent, it was identified in all our experiments.

It was deduced from footprinting experiments (15) that the anticodon arm is not in direct contact with EF-Tu, although weak protection against single stranded nuclease could also be observed in the anticodon loop (at positions 33, 34, 36, 37) of Phe-tRNA^{Phe} in the ternary complex (15). These authors focussed their attention on the RNase cuts which were found increased in the anticodon arm in the presence of the protein, suggesting a conformational change of this region (15). This conformational rearrangement was also detected by the study of a tRNA spin labelled in the anticodon loop during its participation in ternary complex formation (14). Strikingly, no crosslinks were found in the aminoacid acceptor stem although this region was defined as involved in the binding with EF-Tu.

Since the crosslinks were reversed before sequencing of the coordinated oligonucleotides, the platinated residues could not be

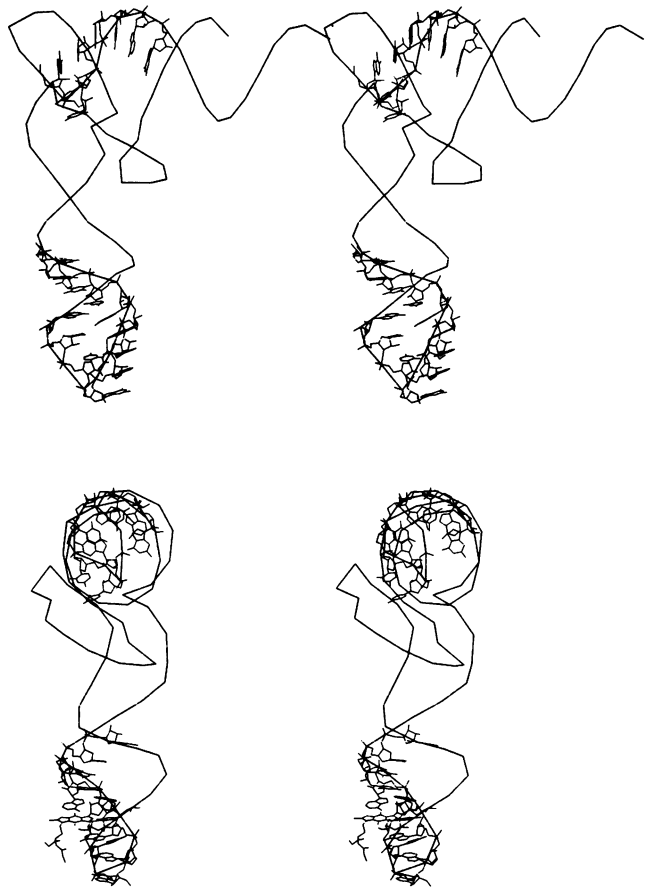

Figure 5: Stereoscopic view of the backbone structure of yeast $tRNA^{Phe}$ in two different orientations. For clarity, only the nucleotides corresponding to the crosslinked regions are represented. Coordinates of yeast $tRNA^{Phe}$ are from Quigley et al. (32). The display pictures were taken on an Evans and Sutherland MPS colour display using the program FRODO developed by Jones (33) and adapted for the PS 300 by Pflugrath and Saper (34).

identified. However, it should be mentioned that previous work on $tRNA^{Phe}$ crystals soaked with trans-DDP (27, 28) showed coordination of the platinum adduct at position N7 of G18, Gm34, G43 and at position N1 of A73. If this holds true in our experiments, crosslinking of regions 31 to 42, 43 or 45 can easily be explained by a 7 Å neighbourhood of position N7 of residues Gm34 and/or G43 to EF-Tu. An additional residue is obviously

platinated in our experiments located in region 58-65 which could be G65. But the potential platination of residues G18 in the D-loop and A73 in the aminoacid acceptor arm does not lead to crosslinks with the protein. However, in our experiment trans-DDP treatment was performed in solution on the ternary complex and the resulting platination pattern is not necessarily the same as in the tRNA crystal. Also conformational changes in the tRNA molecule induced by the interaction with EF-Tu could also happen as well as some protection of potentially reactive residues by the protein (e.g. in the aminoacid acceptor stem), changing the accessibility of the platinum derivative.

The stereoscopic view of $tRNA^{Phe}$ (Fig. 5) shows that the 3'-part of both crosslinked regions are located on the same side of the tRNA. This suggests a model of interaction in which the L-shaped tRNA is lying parallel onto the protein as already proposed by Kabsch et al. (29). Such a model is compatible with the shape and dimension of both molecules and agrees with protection experiments of ε-amino groups of lysines of the protein in the ternary complex against ethyl acetimidate (30). This model provides large surface contacts as supported by the high binding constants (2, 3) and by the protection of the tRNA against RNase digestion in the complex (15). Our results are in agreement with the proposed model in which the aminoacid acceptor- and T-helices lie on the head of the protein (in domain I) near the GDP binding site (31). Spatial localization of G65 (Fig. 5) suggests that this base could be the crosslinked residue. The anticodon arm is located at the other end of the protein (at the bottom of domain III). This should agree with the possible crosslinking of Gm34 and G42, 43 or 45 and also with the concept that a part of the anticodon is protruding from the protein as mentioned by small angle X-ray scattering studies of the complex formation (5). Experiments are now in progress to characterize the nucleotide residue and the protein regions involved in the binding with both crosslinked regions of the tRNA.

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