Crosslinking transfer RNA and messenger RNA at the ribosomal decoding region: identification of the site of reaction on the messenger RNA

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Received 24 September 1984; Accepted 16 October 1984

ABSTRACT

Wybutine (Ywye), situated next to the 3'-side of the anticodon of tRNA\(^{\text{Phe}}\) from Saccharomyces cerevisiae, can be photo-crosslinked to mRNA when bound to Escherichia coli ribosomes. Crosslinking can be obtained with poly(U) as well as with oligonucleotides such as pAUGUUU or p(U). In order to identify the site of reaction on the mRNA, 5'\(^{32}\)P-labelled pAUGUUU was crosslinked by irradiation at 320 nm with Phe-tRNA\(^{\text{Phe}}\) from yeast bound to the acceptor-site. The photoprodct was subsequently digested with P1-nuclease and analyzed by electrophoresis followed by homochromatography in the second dimension. As a result of the photoreaction the wybutine was found to be crosslinked to the U at the 5'-position of the corresponding UUU-anticodon.

INTRODUCTION

tRNAs usually contain a hypermodified nucleotide next to the 3'-side of the anticodon. In tRNA\(^{\text{Phe}}\) from yeast this site is occupied by wybutine (Ywye). Upon irradiation at 320 nm Ywye undergoes a photoreaction (1). This photoreactivity has been utilized in several photocrosslinking studies (2,3). Using ribosomal complexes Phe-tRNA from yeast was found to be photocrosslinked with poly(U) at the Ywye residue (3,4). Irradiation at 320 nm does not affect ribosomal activity. The photoreaction of Ywye can therefore serve as a highly specific probe for the study of events taking place at the ribosomal decoding site.

A prerequisite for photocrosslinking is the close proximity of the residues involved in the reaction. In the crystal structure of tRNA the anticodon-loop is found in the 3'-stacked conformation (5). In this form the hypermodified base is stacked on to the anticodon. Models of codon-anticodon interaction show the Ywye to be juxtaposed to the mRNA when the anticodon-loop is
in the 3'-stacked form (6,7). Photocrosslinking between Ywye and mRNA on ribosomes was found to occur with the same kinetics and efficiency both from the acceptor-site (A-site) and the peptidyl-site (P-site). Furthermore, the Phe-tRNA-poly(U) complex photocrosslinked at the A-site was shown to be translocated to the P-site upon addition of elongation factor-G (EF-G) and GTP. It was therefore postulated that the anticodon-loop of the aminoacyl-tRNA in the ribosomal A- and P-site is in the 3'-stacked form and that aminoacyl-tRNA and mRNA are transported together during translocation (4). Since these previous studies were performed with poly(U), the site of photocrosslinking on the mRNA could not be determined. For that reason the photocrosslinking experiments have now been repeated using short oligonucleotides as mRNAs. In this paper we show that photocrosslinking of Phe-tRNA\textsuperscript{Phe} from yeast occurs with p(U)\textsubscript{6} as well as with pAUGUUU. Using the latter oligonucleotide it is demonstrated that the photoactivated Ywye reacts with the 5'-uridyl-residue of the UUU-codon.

**MATERIALS AND METHODS**

**Materials**

70S ribosomes from *E. coli* strain MRE 600 were prepared according to the procedures of Noll (8) and Jelenc (9). [\textsuperscript{3}H]Phe (84 Ci/mmol) and [\textsuperscript{32}P] (carrier free) phosphate were from Amersham. EF-Tu/GDP was a gift from Dr. A. Pingoud, Hannover, FRG. Glyceraldehyde-3-phosphate-dehydrogenase, 3-phosphoglyceric phosphokinase, tRNA\textsuperscript{Phe} from yeast, tRNA\textsuperscript{Phe} from *E. coli*, tRNA\textsuperscript{Met} from *E. coli*, (U)\textsubscript{6} were from Boehringer-Mannheim. Polynucleotide kinase was either from Bethesda Research Laboratories or from New England Biolabs. Nuclease P1 was from Calbiochem. AUGUUU was prepared as described (10). Cellulose-acetate strips for high voltage electrophoresis (CA 250/0) were from Schleicher & Schüll. Polygram Cel 300 DEAE/HR-2115 thin layer sheets for homochromatography were from Macherey - Nagel (Düren). Yeast RNA (crude extract) for the homochromatography mixture was from Sigma.

**5'-Labelling of the Hexanucleotides**

[\textsuperscript{\gamma\textsuperscript{-32}P}]ATP was prepared according to Maxam and Gilbert (11), 5'-labelling of the hexanucleotides was performed as described.
by Donis-Keller (12); the labelled oligonucleotides were purified by ion exchange chromatography on a QAE-Sephadex A-25 column (2 x 0.4 cm). The column was washed with 0.15 M triethylammonium carbonate buffer, pH 7.8, and the labelled oligonucleotide was eluted with 2M triethylammonium carbonate. The sample was lyophilized repeatedly and dissolved in sterilized water containing 5% ethanol. Specific activities were between 10 and 100 Ci/mmol.

Binding of $[5'-%^{32}\text{P}]\text{pAUGUUU}$ and Phe-tRNA to ribosomes.

500 pmol ribosomes (9) were preincubated with 1 nmol tRNA$^{\text{Met}}$ and 15 nmol $[5'-%^{32}\text{P}]\text{pAUGUUU}$ at 37°C for 3 min in buffer A (95 mM KCl, 5 mM NH$_4$Cl, 0.5 mM CaCl$_2$, 5 mM MgCl$_2$, 4 mM putrescine, 0.5 mM spermidine, 5 mM phosphate, 1 mM dithioerythritol, pH 7.5). The temperature was subsequently lowered to 20°C, 500 pmol $[^3\text{H}]{\text{Phe-tRNA}}^{\text{Phe}}$ from yeast, 0.1 mg elongation factor-Tu (EF-Tu) and 0.2 mM GTP were added and the incubation (total volume 1 ml) was continued for 10 min.

Binding of $[5'-%^{32}\text{P}]\text{p(U)}$ and Phe-tRNA to ribosomes.

500 pmol ribosomes (8) were incubated for 10 min at 20°C in buffer B (50 mM NH$_4$Cl, 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate-sodium salt (HEPES), 10 mM MgCl$_2$, 1 mM dithioerythritol, pH 7.4) containing 0.2 mM GTP, 15 nmol $[5'-%^{32}\text{P}]\text{p(U)}$, 1 nmol uncharged tRNA$^{\text{Phe}}$ from E.coli, 500 pmol $[^3\text{H}]{\text{Phe-tRNA}}^{\text{Phe}}$ from yeast and 0.1 mg EF-Tu in a final volume of 1 ml.

Irradiation and Isolation of Crosslinked Products.

Irradiation was performed for 15 min as described (3,4). After irradiation the incubation mixture was layered on a Sepharose 6B column (10 x 0.4 cm) and eluted with buffer B in order to separate the ribosomes from unbound $[32\text{P}]$-oligonucleotides. Fractions containing the ribosomes were collected, extracted twice with phenol, once with chloroform and the RNA chromatographed on a Sephadex G-50 column (40 x 1.5 cm). The peak of tRNA containing the crosslinked oligonucleotides was collected and precipitated with ethanol.

Digestion with RNase P1 and Fractionation by Two-Dimensional Chromatography.

Partial digestion with P1-nuclease was carried out as described (13). For fractionation of the digestion products the
two-dimensional "fingerprinting procedure" (14) was applied using high voltage electrophoresis at pH 3.5 on cellulose acetate strips (16 min at 5000 V) for the first dimension and homochromatography on DEAE-cellulose thin layer sheets (20 x 20 cm) at 65° C for the second dimension.

RESULTS AND DISCUSSION
Photocrosslinking between Phe-tRNA from Yeast and 5'-[32P]labelled Hexanucleotides.

EF-Tu mediated binding of aminoacyl-tRNA to the A-site requires a minimal length of six nucleotides as mRNA (10). In addition a tRNA corresponding to the codon in the P-site has to be present in the incubation mixture. When comparing different hexanucleotides the highest degree of enzymatic Phe-tRNA binding was obtained with AUGUUU (10). For that reason photocrosslinking experiments were initially performed using ribosomal complexes containing 5'-[32P]-labelled pAUGUUU, tRNA\textsuperscript{Met} and [3H]Phe-tRNA from yeast bound in the presence of EF-Tu and GTP. Following incubation and irradiation the ribosomal complexes were purified by chromatography on Sepharose 6B in order to remove unbound [5'-32P] pAUGUUU. RNA was prepared by phenol extraction and was fractionated on a Sephadex G-50 column. The results are presented in Fig. 1. In the irradiated sample a peak of [5'-32P] pAUGUUU is found to comigrate with the peak of [3H]Phe-tRNA. From the specific activities the yield of the photoreaction was calculated to be approximately 20 %. This value is comparable to that obtained with poly(U) (3,4). As expected from previous binding studies (10), omission of tRNA\textsuperscript{Met} from the incubation mixture resulted in a substantial decrease in photocrosslinking (Fig. 1). No significant amount of [5'-32P]-pAUGUUU was found associated with [3H]Phe-tRNA in the non-irradiated control sample (Fig. 1). In a similar experiment the dependence on EF-Tu was tested. As seen in Tab. 1, omission of EF-Tu resulted in a substantial reduction of the photoreaction. The strong stimulatory effect of EF-Tu indicates that [3H]Phe-tRNA binding occurs at the A-site. The position of [5'-32P]-pAUGUUU with respect to the ribosome is therefore determined by tRNA\textsuperscript{Met} occupying the P-site and Phe-tRNA at the A-site. Al-
Fig. 1: Isolation of \([5'-\text{^{32}P}]\text{pAUGUUU}\) photocrosslinked to \([\text{^{3}H}]\text{Phe-tRNA}\) from yeast by chromatography on Sephadex G-50. 300 pmol ribosomes were incubated with \([5'-\text{^{32}P}]\text{pAUGUUU}\) in buffer A in the presence of tRNA\(_f\), \([\text{^{3}H}]\text{Phe-tRNA}\) from yeast and EF-Tu/GTP, irradiated and chromatographed on Sepharose 6B as described in Materials and Methods. Isolated RNA was subsequently fractionated on Sephadex G-50. \([\text{^{32}P}]\)-profiles of samples obtained from the complete incubation mixture (•—•), from the non-irradiated control (□—□) and upon omission of tRNA\(_f\) (Δ—Δ); profile of \([\text{^{3}H}]\text{Phe-tRNA}\) (○—○) from the complete incubation mixture.

Table 1

<table>
<thead>
<tr>
<th>Incubation</th>
<th>([5'-\text{^{32}P}]\text{pAUGUUU}) in Phe-tRNA peak fraction (cpm)</th>
<th>Stimulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+EF-Tu</td>
<td>79 900</td>
<td>100</td>
</tr>
<tr>
<td>-EF-Tu</td>
<td>6 500</td>
<td>8</td>
</tr>
</tbody>
</table>

100 pmol ribosomes were used in each incubation. RNA was prepared from purified ribosomal complexes and the photocrosslinked \([5'-\text{^{32}P}]\text{pAUGUUU} - \text{Phe-tRNA}\) complexes were isolated as described in Fig. 1. Radioactivity obtained from the non-irradiated control sample is subtracted. Specific activity of \([5'-\text{^{32}P}]\text{pAUGUUU}\) was 14 Ci/mmol.
Table 2
Photocrosslinking experiments with $[5'-\text{32P}]pU_6$.

<table>
<thead>
<tr>
<th>Phe-tRNA species</th>
<th>Irradiation</th>
<th>EF-Tu in incubation mixture</th>
<th>$[5'-\text{32P}]pU_6$ in Phe-tRNA peak (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>yeast</td>
<td>+</td>
<td>+</td>
<td>7 950</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>680</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>805</td>
</tr>
<tr>
<td>E. coli</td>
<td>+</td>
<td>+</td>
<td>710</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>740</td>
</tr>
</tbody>
</table>

Experimental conditions were as in Tab. 1. Incubations were carried out either with Phe-tRNA from yeast (upper panel) or Phe-tRNA from E. coli (lower panel) as described in Materials and Methods. Photocrosslinked $[5'-\text{32P}]pU_6$ - Phe-tRNA complexes were isolated as described in Fig. 1. Specific activity of $[5'-\text{32P}]pU_6$ was 20 Ci/mmol.

Though the stimulatory effect of EF-Tu is comparable to that of tRNA$^{\text{Met}}_E$, the molecular basis for the stimulation is different. Whereas EF-Tu directs A-site binding of $[^3H]Phe$-tRNA, the stimulatory effect of tRNA$^{\text{Met}}_E$ is primarily due to an increase in binding of pAUGUUU to ribosomes (data not shown).

For comparison photocrosslinking between $[^3H]Phe$-tRNA$^{\text{Phe}}_E$ from yeast and 5'-[$\text{32P}$]-labelled p(U)$_6$ has also been tested. The same assay system as in the previous experiment was used. The results are presented in Tab. 2. Again photocrosslinking at the A-site is demonstrated by the dependence on EF-Tu and GTP. No attachment of $[5'-\text{32P}]p(U)_6$ to the tRNA$^{\text{Phe}}_E$ is observed in the non-irradiated control sample or when Phe-tRNA$^{\text{Phe}}_E$ from E. coli is used instead of Phe-tRNA$^{\text{Phe}}_E$ from yeast. Similar results have been obtained with p(U)$_8$ (data not shown). The total amount of $[5'-\text{32P}]p(U)_6$ photocrosslinked to Phe-tRNA$^{\text{Phe}}_E$ from yeast (Tab. 2) is much lower than that of $[5'-\text{32P}]pAUGUUU$ found in the previous experiment (Tab. 1). This difference corresponds to that observed in earlier studies when the two hexanucleotides were compared in filter binding experiments: AUGUUU was shown
to be about ten times more efficient in stimulating Phe-tRNA binding to ribosomes than (U)_6 (10).

Identification of the Site of the Photocrosslink on pAUGUUU.

The experiments described so far demonstrate that the photocrosslink of Ywye of Phe-tRNA^Phe from yeast occurs within six nucleotides of the coding-region of the mRNA. In order to localize the site of the crosslink more precisely, 5'-[\textsuperscript{32}P]-labelled pAUGUUU was employed. Because of the strong dependence of photocrosslinking on the presence of tRNA^Met and EF-Tu it can be assumed that [5'-'\textsuperscript{32}P] pAUGUUU is positioned precisely on the ribosome with the AUG-codon occupying the P-site and the UUU-codon filling the A-site. Ribosomal complexes were isolated after irradiation and the RNA was fractionated by column chromatography as described. The peak of [\textsuperscript{3}H]Phe-tRNA containing the photocrosslinked [5'-'\textsuperscript{32}P]pAUGUUU was collected and the RNA was precipitated with ethanol. The RNA was subsequently incubated with nuclease P1 and the products of the partial digest were separated in a two-dimensional system employing high-voltage electrophoresis and homochromatography. The results are shown in Fig. 2. Fig. 2A presents the pattern of the P1-nuclease partial digest of [5'-'\textsuperscript{32}P] pAUGUUU, which serves as a control. The spots of the individual oligonucleotides are clearly identifiable on the chromatogram, as indicated. Fig. 2B shows the results of the P1-digestion of [5'-'\textsuperscript{32}P]pAUGUUU photocrosslinked to the tRNA^Phe from yeast. Both digestions were carried out in parallel. The pattern in Fig. 2B clearly shows the spots corresponding to [5'-'\textsuperscript{32}P]pA, [5'-'\textsuperscript{32}P]pAU and [5'-'\textsuperscript{32}P] pAUG just as in Fig. 2A. However, no spot corresponding to [5'-'\textsuperscript{32}P]pAUG is visible in the digest of the photocrosslinked sample (Fig. 2B). The pattern of the larger oligonucleotides is changed dramatically: In Fig. 2B a series of predominantly long oligonucleotides is seen which apparently originates from tRNA-fragments attached covalently to [5'-'\textsuperscript{32}P]pAUGUUU. Since the pattern up to the third nucleotide is the same as in the control, the site of the photocrosslink must be the fourth nucleotide, i.e. the 5'-U of the UUU-codon. There might still be trace amounts of [5'-'\textsuperscript{32}P]pAUGUU and [5'-'\textsuperscript{32}P]pAUGUUU present in the digest shown in Fig. 2B. These may be due to an incomplete re-
Fig. 2: Autoradiography of partial P1-nuclease digests of [5'-32P]pAUGUUU (2A) and of [5'-32P]pAUGUUU photocrosslinked on ribosomal complexes with [3H]Phe-tRNA from yeast (2B). RNA was partially digested with nuclease P1. The products were separated on a two dimensional system employing high voltage electrophoresis on cellulose acetate strips at pH 3.5 in the first dimension and homochromatography in the second dimension.

The photochemistry of the isolated Ywye has been studied in detail (1). However, nothing is known about the chemistry of the photoreaction of Ywye in tRNA with the U-residue in mRNA. From the study of pAUGUUU alone it is difficult to decide whether the photoreaction with U in position 4 and the lack of reaction with G in position 3 is due to the particular sterical arrangement or to the higher reactivity of the U-residue, or both. In order to discriminate between these possibilities, further studies have to be performed.

The data on the Ywye photocrosslinking (3,4) as well as fluorescence (15,16) and chemical modification studies (17,18) are consistent with the notion that the anticodon-loop of tRNA on the ribosome occurs in the 3'-stacked conformation both in the P- and in the A-site. Nevertheless, some differences in the arrangements of the nucleotides of the anticodon-loop might exist between P-site and A-site bound tRNA. Structural differences in the anticodon-loop of initiator-tRNA as compared to elon-
Fig. 3: Schematic representation of the photocrosslink between pAUGUUU and tRNA$^{\text{Phe}}$ from S. cerevisiae. The P-site is shown to be occupied by tRNA$^{\text{Met}}$ from E. coli and the A-site by tRNA$^{\text{Phe}}$ from yeast. Both tRNAs are assumed to interact simultaneously with their respective codons. The covalent bond between Ywye and U is symbolized by dotted lines. Gm is 2'-O-methyl guanosine.

gator-tRNAs have been observed in X-ray studies of crystals and upon nuclease digestion of tRNAs in solution (19 - 21). In tRNA$^{\text{Met}}_f$ the U$_{33}$ residue on the 5'-side of the anticodon is oriented outwards from the loop leading to a distortion of the anticodon-loop as compared to that of Phe-tRNA from yeast. Nevertheless, the 3'-stack of the anticodon-loop is maintained. Similar or related structural transitions might occur during translocation (22). Whatever the nature of such a structural change may be, it is apparently to subtle to be seen in the Ywye photocrosslinking experiments.

The result of the photocrosslink is presented schematically in Fig. 3. Codon-anticodon interaction is assumed to take place simultaneously both in P-site and in A-site, as has been suggested by several authors (4,7,23 - 25). In order to bring the Ywye into close proximity to the U-residue in position 4 of the pAUGUUU, the mRNA is drawn as being kinked. Arguments in favour of a kink in the mRNA have been put forward in several review articles (7,25,26), but experimental evidence for such a structure on the ribosome is still lacking. In the absence of any detailed information on the chemistry of the photoreaction and on the structure of the photoprodut it is difficult to make any predictions as to the exact arrangement of the individual nucleotides during tRNA-mRNA interaction. Yet it is tempting to speculate that the close proximity necessary for photocrosslinking between the Ywye in tRNA and the U-residue in mRNA.
might be brought about by a partial unstacking of the mRNA between the 3'-nucleotide of the codon in the P-site and the 5'-nucleotide of the codon in the A-site. Hypotheses as to the extent of this unstacking clearly have to await further experimentation.

In summary the data on the photocrosslink show that Ywye of tRNA$^{\text{Phe}}$ from yeast forms, upon irradiation, a covalent bond with the 5'-U of the complementary UUU-codon. The results obtained confirm and extend our previous hypothesis that the anticodon-loop of tRNA on the ribosome is in the 3'-stacked conformation.

ACKNOWLEDGEMENTS

We wish to thank A. Barta and C. Cantor for discussions, W. Sommergruber for advice on the homochromatography system, A. Gupta for technical assistance, D. Thompson for critical reading and B. Gamperl for typing the manuscript. This work was supported by a grant from the Austrian Fonds zur Förderung der wissenschaftlichen Forschung.

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