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MODIFIED tRNAs FOR PROBING tRNA BINDING SITES ON THE RIBOSOME*

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Unusual chemical properties of hypermodified nucleosides N^6 -(threoninocarbonyl)adenosine (t^6A) located at position 37 and 3-(3-amino-3-carboxypropyl)uridine (acp^3U) located at position 20:1 have been utilized for the introduction of photoreactive azidonitrophenyl probes to the anticodon loop and to the dihydrouridine loop of yeast $tRNA_{m}^{Met}$ and lupin $tRNA_{m}^{Met}$, respectively. The very efficient and selective modification procedures involve condensation of the carboxyl group of t^6A with ethylenediamine in the presence of a water soluble carbodiimide followed by acylation of the newly introduced amino group with the respective N -hydroxysuccinimide ester, and acylation of the primary amino group of acp^3U with the respective N -hydroxysuccinimide ester. Binding and crosslinking of the modified, uncharged tRNAs to *E. coli* ribosome have been studied in the presence and absence of poly(AUG) as a message. Both tRNAs carrying about 20 A long photoreactive probes retain their binding activity and upon irradiation with visible light crosslink to the ribosome with high yields showing their usefulness for structural studies on the tRNA-ribosome complex.

Affinity labeling technique is one of the techniques successfully applied in structural studies on the tRNA-mRNA-ribosome complex. In one approach chemical or photochemical probes are attached to tRNA by specific chemical modification of some residues such as hypermodified nucleosides or amino-acid moiety of aminoacyl(peptidyl)-tRNA. The decoding site and peptidyl transferase center are two of the sites on *E. coli* ribosome that have been well characterized by this technique. Their location delineates binding sites for the

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¹ Abbreviations: t^6A , N^6 -(threoninocarbonyl)adenosine; acp^3U , 3-(3-amino-3-carboxypropyl)uridine; NAK-Su, N -succinimidyl-6(4'-azido-2'-nitrophenylamino)hexanoate; EDA, ethylenediamine; $tRNA-EDA_{37}$ and $tRNA-EDA-NAK_{37}$, see Fig. 1; $tRNA-NAK_{20:1}$, see Fig. 3.

anticodon and aminoacid termini of both A- and P-site bound tRNA molecules [1 - 5]. The spatial arrangement of the anticodon of ribosome bound tRNAs and C-1400 of 16S rRNA has been recognized and the decoding site has been localized on 30S subunit thanks to tRNA-16S rRNA crosslinking [3, 4, 6, 7]. No proteins, however, could be identified by crosslinking *via* photoreactive probes attached to tRNA wobble base [8]. The peptidyl transferase center was analysed by crosslinking using aminoacyl-tRNAs carrying probes attached to the aminoacid residue (see [3] for review). On the other hand, very little information is yet available concerning contact sites of other parts of tRNA molecule. Protein S19 has been crosslinked to both A-site and P-site bound tRNAs *via* photoreactive probes attached to 4-thiouridine and $\text{acp}^3\text{U}_{47}$, respectively [4]. Several other proteins have been identified by a similar approach but with probes attached at unspecified location in tRNA (see [3] for review).

Identification of ribosomal components located in the decoding site and in the vicinity of the central domain of tRNA is the main goal of this project. These results taken together with information already available should greatly facilitate model building of the tRNA-mRNA-ribosome complex. In this paper preparation and analysis of modified tRNAs carrying photoreactive probes attached to the anticodon and dihydrouridine loop and preliminary ribosome binding and crosslinking results are reported. Methionine elongator tRNAs from yeast and lupin have been chosen as they contain easily modifiable nucleosides t^6A and acp^3U in the two regions of the tRNA molecule, respectively.

MATERIALS AND METHODS

tRNA_m^{Met} from lupin seeds and from baker's yeast were isolated as described before [9] by chromatography on benzoylated-DEAE-cellulose and Sepharose 4B and finally purified by h.p.l.c. on TSK-gel-DEAE-2SW (Toyo Soda) column (6.4 × 250 mm) using 300 mM NaCl, 10 mM MgCl₂, 10% methanol and 20 mM Tris/HCl (pH 7.4) as elution buffer, at room temperature and 0.3 ml/min flow rate. Yeast tRNA_m^{Met} lacked the 3'-terminal adenosine. Specific activity of both tRNAs was about 1500 pmol/A₂₆₀ unit.

Removal of the 3'-terminal adenosine: 0.2 A₂₆₀ unit of lupin tRNA_m^{Met} was incubated in 25 μl of 50 mM Tris/HCl (pH 8.0), 10 mM MgCl₂ containing 1 μg of snake venom phosphodiesterase (Boehringer, Mannheim) at 20°C for 5 min. After phenol extraction tRNA was recovered by ethanol precipitat on

tRNA 3'-end labeling: 0.1 A₂₆₀ unit of tRNA without 3'-terminal adenosine was incubated at 37°C for 1 h in 10 μl of 100 mM Tris/HCl (pH 8.0), 20 mM MgCl₂, 7 mM dithiothreitol, 70 μM CTP containing 50 - 250 μCi of [α -³²P]ATP (about 3000 Ci/mmol, Amersham) and tRNA nucleotidyltrans-

ferase (a kind gift from Dr. J. Ofengand). Labeled tRNA was purified by PAGE (15% gel, 8 M urea) and mixed with the respective unlabeled tRNA before modification.

Modification of the carboxyl group in tRNA: one A_{260} unit of yeast tRNA^{Met} was incubated at room temperature for 45 min in 0.36 ml of 200 mM ethylenediamine dihydrochloride, 40 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, 80 mM NaCl and 10 mM $MgCl_2$, at pH adjusted with HCl to 4.0 - 4.2. The reaction was quenched by addition of 1/20 vol. of 20% KOAc, pH 5.0 and tRNA was recovered by ethanol precipitation repeated 3 times to remove the reagents.

Modification of the amino group in tRNA: 1 A_{260} unit of yeast tRNA^{Met} modified with ethylenediamine or lupin tRNA^{Met} was dissolved in 60 μ l of 0.5 M sodium borate (pH 8.2) and 240 μ l of Me_2SO . NAK-Su¹ (Pierce), 3.5 mg, dissolved in 20 μ l of Me_2SO was added to the mixture in two 10 μ l portions at 45 min intervals and the reaction was carried out at room temperature for 90 min. tRNA was recovered by ethanol precipitation and the excess of the reagent was removed by 3 successive ethanol precipitations, two from 50% Me_2SO and one from water.

Analysis of tRNA modification products: 1 - 2 A_{260} units of tRNA were separated of chromatography on 1 ml benzoylated-DEAE-cellulose column equilibrated with 0.4 M NaCl (10 mM $MgCl_2$, 10 mM NaOAc, pH 4.5); unmodified tRNA was eluted with 1 M NaCl and modified tRNA was eluted with 1.5 M NaCl and 25% ethanol (both buffers contained also 10 mM $MgCl_2$, 10 mM NaOAc, pH 4.5). H.p.l.c. analysis on TSK-gel-DEAE-2SW (Toyo Soda) column (6.4 \times 250 mm) was performed using 0.27 to 0.33 mM, 4-hour gradient of NaCl in 10 mM $MgCl_2$, 10% methanol and 20 mM Tris/HCl (pH 7.4) at room temperature and a 0.4 ml/min flow rate (fractions were collected every 3 min). Labeled tRNA dissolved in 200 μ l of the starting buffer was mixed with 0.5 - 1 A_{260} unit of unmodified tRNA, incubated for 3 min at 65°C and cooled to room temperature before loading.

Ribosome binding and crosslinking: *E. coli* MRE600 ribosomes prepared as described by Noll *et al.* [10] were about 70% active in elongation factor Tu dependent binding of Phe-tRNA^{Phe} (yeast) in the presence of poly(U), at 7 mM Mg^{2+} . One A_{260} unit of ribosomes was taken as 24 pmoles. ³²P-Labeled tRNA (400 - 500 nM), ribosomes (200 - 300 nM) and poly(A,U,G) (60 μ g/ml) were incubated at 37°C for 20 min in buffer containing 50 mM Hepes/Na (pH 7.5), 50 mM NH_4Cl and 7 mM $MgCl_2$, and then chilled on ice. tRNA binding was measured by the nitrocellulose filter assay using 20 mM $MgCl_2$, 50 mM Tris/HCl (pH 7.5), 50 mM KCl as a wash. Noncovalent complexes were irradiated with visible light (150 W halogen lamp) at 0 - 4°C for 45 min. Crosslinking was measured by the nitrocellulose filtration assay using 0.1 M $MgCl_2$, 50 mM Tris/HCl (pH 7.5), 50 mM

as a wash, after diluting samples with about 100 volumes of 50 mM Tris/HCl (pH 7.5), 50 mM KCl followed by about 10 min incubation at 0°C. The measured values were corrected for non-specific binding of modified tRNAs to nitrocellulose. After irradiation ribosomes were recovered by ethanol precipitation (0.7 vol.), dissolved in 200 - 400 μ l of 20 mM Tris/HCl (pH 7.5), 0.5 mM Mg(OAc)₂, 100 mM NH₄Cl and centrifuged through 17 - 30% linear sucrose gradient in the same buffer (SW40 rotor, 31 000 r.p.m., 13 h, 2°C)

RESULTS AND DISCUSSION

Hypermodified nucleosides present in some tRNAs, located mostly at the 5'-end or next to the 3'-end of the anticodon but also in some other parts of tRNA [11], have unique chemical properties that enable introduction of a variety of labels by their specific, chemical modification (see [11] for review). Among these nucleosides t⁶A and acp³U are of special interest because of their location in some tRNAs in the anticodon loop and dihydrouridine loop, respectively.

It has been shown previously that the free carboxyl group of t⁶A can be modified very specifically and very efficiently by condensation with amines in the presence of a water soluble carbodiimide [12]. The reaction has been applied recently for attaching antigenic and photoreactive probes to the carboxyl group of uridine-5-oxyacetic acid at the wobble position of *E. coli* tRNA^{Val} [6 - 8]. The same two step procedure, including condensation with ethylenediamine and subsequent acylation of the newly introduced amino group with the respective *N*-hydroxysuccinimide ester, is now applied for yeast tRNA^{Met} containing t⁶A located next to the 3'-end of the anticodon (position 37) (Fig. 1).

The modification products were analysed by h.p.l.c. on TSK-gel-DEAE-2SW column. Both modification steps can be easily followed by this method (Fig. 2). Both of them are nearly quantitative and lead with a very high, 85 - 90%, yield to a single product carrying photoreactive probe. Yeast tRNA^{Met} treated with NAK-Su alone (without condensation with EDA) has the same retention volume as unmodified tRNA (not shown), whereas acp³U containing lupin tRNA^{Met} modified with NAK-Su separates well from its unmodified counterpart (see below). These observations confirm our earlier conclusions on the specificity of the carboxyl group condensation with amines in the presence of a water soluble carbodiimide and acylation of primary aliphatic amino groups, introduced chemically or present in some native tRNAs (see also below), by *N*-hydroxysuccinimide esters [6, 12].

The methionine specific elongator tRNA from lupin seeds contains hypermodified nucleoside acp³U located in the dihydrouridine loop (position 20). The free amino group of this nucleoside can be also very

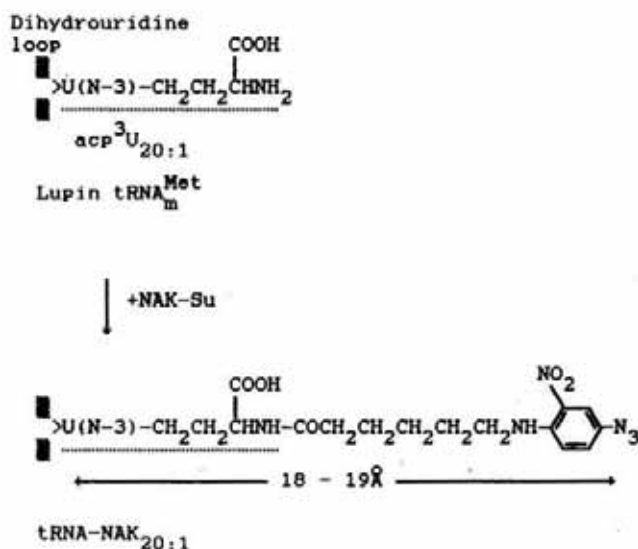


Fig. 3. acp³U modification scheme (the length calculated as the distance between N-3 atom of acp³U and the azido group in the most extended conformation of the probe).

-DEAE-cellulose (Fig. 4). Furthermore, modification of lupin tRNA_m^{Met} with radioactive reagent ([¹⁴C]NAK-Su) showed that about one molecule of the probe was attached to one tRNA molecule (not shown).

As both tRNA modification procedures were very efficient (over 80% yield), modified tRNAs were used for binding and crosslinking experiments without further purification (i.e. they contained 5 - 20% of unmodified tRNA). Both chromatographic methods described in this paper can be applied for

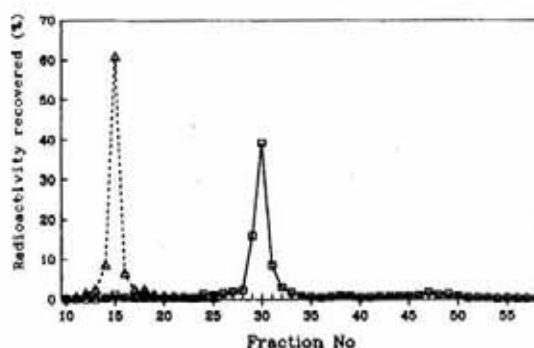


Fig. 4. H.p.l.c. analysis of lupin tRNA_m^{Met} modified with NAK-Su (tRNA_m^{Met}, Δ; tRNA-NAK_{20:1}, □). The modified tRNA was purified by chromatography on benzoylated-DEAE-cellulose. tRNAs labeled with ³²P.

product purification in the case of lower reaction yield (other tRNAs, other reagents etc.).

Binding and crosslinking properties of modified tRNAs are summarized in Table 1, and the distribution of covalently bound tRNA between ribosomal components is shown in Figs. 5 and 6. Both modified tRNAs showed increased unspecific binding to nitrocellulose. It accounted for 25 - 35% of total binding measured in the presence of ribosomes and poly(A,U,G), at high magnesium concentration (8 mM in the binding mixture and 20 mM in the wash buffer). The effect was much less pronounced at low magnesium concentration used for crosslinking yield measurements. Both binding and

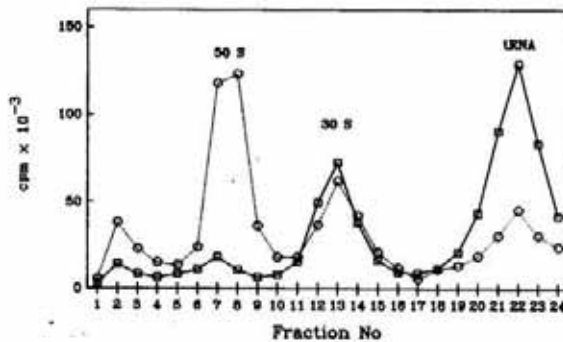


Fig. 5. Crosslinking of $[^{32}\text{P}]\text{tRNA}_{\text{Met}}^{\text{Met}}\text{-EDA-NAK}_{37}$ to *E. coli* ribosome in the presence of poly(A,U,G) (□). Analysis of the covalent complex by sucrose gradient centrifugation at 0.5 mM Mg^{2+} . Ribosomal subunits (50S and 30S) were located by A_{260} measurements (○).

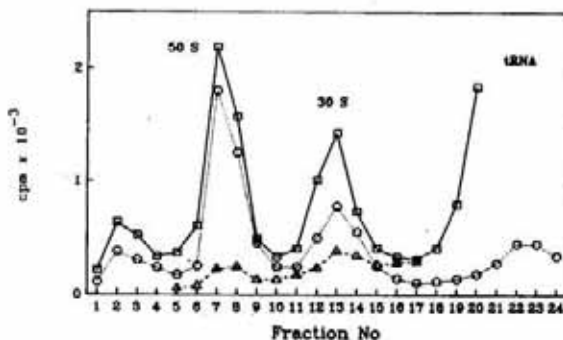


Fig. 6. Crosslinking of $[^{32}\text{P}]\text{tRNA}_{\text{Met}}^{\text{Met}}\text{-NAK}_{20:1}$ to *E. coli* ribosome in the presence of poly(A,U,G) (□). Analysis of the covalent complex by sucrose gradient centrifugation at 0.5 mM Mg^{2+} . Ribosomal subunits (50S and 30S) were located by A_{260} measurements (○). Crosslinking of pre-photolyzed $[^{32}\text{P}]\text{tRNA-NAK}_{20:1}$ is also shown (△).

crosslinking values summarized in Table 1 were corrected for this unspecific effect.

The tRNA modifications, despite their location next to the anticodon or in the dihydrouridine loop, despite the hydrophobicity of the probes, have no significant effect on binding of deacylated tRNA to the ribosome either in the presence or absence of poly(A,U,G) as a message (Table 1).

Table 1

Binding and crosslinking of yeast tRNA_m^{Met}-EDA-NAK₃₇, tRNA_m^{Met}-EDA₃₇ and lupin tRNA_m^{Met}-NAK_{20:1} and their unmodified counterparts in presence (+) or absence (-) of poly(A,U,G)

tRNA	Binding mol tRNA/mol ribos.	Crosslinking	
		mol tRNA/mol ribos. ⁻	%
poly (A,U,G)			
tRNA _m ^{Met} -EDA-NAK ₃₇			
+	1.46	0.47	32
-	0.98	0.81	82
tRNA _m ^{Met} -EDA ₃₇			
+	1.46	—	—
-	1.03	—	—
Yeast tRNA _m ^{Met}			
+	1.54	< 0.01	< 1
-	0.85	—	—
tRNA _m ^{Met} -NAK _{20:1}			
+	1.22	0.37	30
-	0.16	0.28	—
+	1.09*	0.05*	5*
Lupin tRNA _m ^{Met}			
+	1.15	< 0.01	< 1
-	0.14	< 0.01	< 1

* Modified tRNA pre-irradiated before binding

Under conditions used in this study deacylated tRNAs bind preferentially to the ribosome P-site (both in the presence and absence of the message) [13]. The results presented in Table 1 suggest that more than one molecule of deacylated tRNA_m^{Met} is bound per ribosome in the presence of poly(A,U,G) (probability of two AUG codons occurring in a row in the random 1:1:1 copolymer used in this study is less than 10%), however, detailed analysis of ribosomal sites occupied by tRNA from which crosslinking is observed requires further experiments.

Covalent crosslinks are formed with very high yields upon irradiation of non-covalent tRNA-ribosome complexes with visible light (Table 1). The high yield and stability of the photoproducts is confirmed by sucrose gradient analysis under conditions dissociating ribosomes into subunits and releasing non-covalently bound tRNA from ribosomal subunits (low Mg^{2+} concentration) (Figs. 5 and 6). On the contrary, no photocrosslinking takes place with unmodified tRNA, and for modified tRNAs pre-irradiated before ribosome binding the reaction yield is significantly diminished (6 - 8 fold) (Table 1, Fig. 6 and results not shown). The analysis shows also that tRNA is crosslinked to the small ribosomal subunit or to both ribosomal subunits for probes located in the anticodon and dihydrouridine loop, respectively.

Conclusions

High yield crosslinking of modified tRNAs described in this paper demonstrates capabilities of the approach for probing tRNA binding sites on *E. coli* ribosome and, more specifically, for probing the neighbourhood of the anticodon loop and of the central domain of the ribosome bound tRNA molecule. Identification of ribosomal components (proteins, nucleotides in rRNAs) on both subunits should yield new information on the organization of different tRNA binding sites (A, P and E) and the structure of tRNA-mRNA-ribosome complex.

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REFERENCES

1. Wittmann, H. G. (1983) Architecture of prokaryotic ribosomes. *Ann. Rev. Biochem.*, **52**, 35 - 65.
2. Lake, J. A. (1985) Evolving ribosome structure: domains in archaeobacteria, eubacteria, eocytes and eukaryotes. *Ann. Rev. Biochem.*, **54**, 507 - 530.
3. Ofengand, J., Górnicki, P., Nurse, K. & Boublik, M. (1984) On the structural organization of tRNA-ribosome complex; in *Alfred Benzon Symposium No 19: Gene expression. The translational step and its control* (Clark, B. F. C & Petersen, H., eds.) pp. 293 - 315. Munksgaard, Copenhagen.
4. Ofengand, J., Ciesiolka, J., Denman, R. & Nurse, K. (1986) Structural and functional interactions of the tRNA-ribosome complex; in *Structure, Function and Genetics of Ribosome* (Hardesty B. & Kramer, G., eds.) pp. 473 - 494. Springer-Verlag, New York and Heidelberg.
5. Stuffer, G. & Stoffer-Meilicke, M. (1986) Immuno electron microscopy on *E. coli* ribosomes; in *Structure, Function and Genetics of Ribosomes* (Hardesty, B. & Kramer, G., eds.) pp. 28 - 46. Springer - Verlag, New York and Heidelberg.

6. Górnicki, P., Nurse K., Hellman, W., Boublik, M. & Ofengand, J. (1984) High resolution localization of the tRNA anticodon interaction site on the *E. coli* 30S ribosomal subunit. *J. Biol. Chem.*, **259**, 10493 - 10498.
7. Ciesiolka, J., Górnicki, P. & Ofengand, J. (1985) Identification of the site of cross-linking in 16S rRNA of an aromatic azide photoaffinity probe attached to the 5'-anticodon base of A site bound tRNA. *Biochemistry*, **24**, 4931 - 4938.
8. Górnicki, P., Ciesiolka J. & Ofengand, J. (1985) Crosslinking of the anticodon of A and P site bound tRNAs to the ribosome via aromatic azide of variable length: involvement of 16S rRNA at the A site. *Biochemistry*, **24**, 4924 - 4930.
9. Górnicki, P., Wolański, A., Judek, M., Podkowiński, J., Gulewicz, K. & Krzyżosiak, W. J. (1986) Studies on the reaction of specific tRNAs bearing free carboxyl group with aniline in the presence of a water soluble carbodiimide. *Bull. Pol. Acad. Sci. Ser. Sci. Chem.*, **34**, 15 - 25.
10. Noll, M., Hapke, B., Schreier, M. H. & Noll, H. (1973) Structural dynamics of bacterial ribosomes. I. Characterization of vacant couples and their relation to complexed ribosomes. *J. Mol. Biol.*, **75**, 281 - 294.
11. Adamiak, R. W. & Górnicki, P. (1985) Hypermodified nucleosides of tRNA; synthesis, chemistry and structural features of biological interest. *Prog. Nucleic Acid Res. Mol. Biol.*, **32**, 27 - 74.
12. Górnicki, P., Judek, M., Wolański, A. & Krzyżosiak, W. J. (1986) Hypermodified nucleoside carboxyl group as a target site for specific tRNA modification. *Eur. J. Biochem.*, **155**, 371 - 375.
13. Nierhaus, K. H., Rheinberger, H.-J., Geigermuller, U., Gnirke, A., Saruyama, H., Schilling, S. & Wurbach, P. (1986) Three tRNA binding sites involved in the ribosomal elongation cycle; in *Structure, Function and Genetics of Ribosomes* (Hardesty, B. & Kramer, G., eds.) pp. 454 - 472. Springer-Verlag, New York and Heidelberg.