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Ribosomal proteins S7 and L1 are located close to the decoding site of *E.coli* ribosome — affinity labeling studies with modified tRNAs carrying photoreactive probes attached adjacent to the 3′-end of the anticodon

Jan Podkowiński and Piotr Górnicki*

Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61704 Poznań, Poland

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ABSTRACT

Two photoreactive azidonitrophenyl probes have been attached to Yeast methionine elongator tRNA by chemical modification of the N6-(threoninocarbonyl)adenosine located next to the 3'-end of the anticodon. The maximum distance between the purine ring and the azido group estimated for the two probes is 16-17 and 23-24Å, respectively. Binding and cross-linking of the uncharged, modified tRNAs to *E.coli* ribosomes have been studied with and without poly(A,U,G) as a message, under conditions directing uncharged tRNAs preferentially to the P-site. The modified tRNAs retain their binding activity and upon irradiation bind covalently to the ribosome with very high yields. Protein S7 is the major cross-linking target for both modified tRNAs, in the presence or absence of poly(A,U,G). Protein L1 and to a lesser extent proteins L33 and L27 have been found to be cross-linked with the short probe. Cross-linking to 16S rRNA reaches significant levels only in the absence of the message.

INTRODUCTION

The decoding site of the *E.coli* ribosome has been studied extensively over the last decade. Its localization deep in the cleft between the head and the lateral protrusion of the small ribosomal subunit is well established [1], and the suggested multiple involvement of the 3'-end minor domain of 16S rRNA in mRNA binding and decoding [2] is now supported by many exprimental results, including cross-linking of the tRNA wobble base to C_{1400} [3-5], cross-linking of poly(A) to the same rRNA region [6], protection of several nucleotides against chemical probes induced by tRNA binding [7,8], functional analysis of mutant ribosomes [9-12] and interaction with aminoglycoside antibiotics known to interfere with the decoding process [13-15]. A detailed protein map [16,17] and a three dimensional model of the arrangement of the 16S rRNA within the small ribosomal subunit [18-20 suggest several proteins and rRNA

regions which should be located in this defined decoding site. Ribosomal proteins S7, S18 and S21 [18,21], and initiation factor 3 [22] have been cross-linked to the 3'-end of 16S rRNA. Other ribosomal proteins (S1, S3, S4, S5, S9, S12, S18, S21) have been suggested to lie near the decoding site [see 23 for review]. No ribosomal proteins could be identified in cross-linking experiments using tRNAs carrying a photoreactive probe at the 5'-end base of the anticodon [4,5].

Our interest is to determine the precise orientation of tRNA molecules in different sites on the *E.coli* ribosome with the help of a series of affinity probes. In this paper, we report the results of affinity labeling experiments with modified tRNAs carrying photoreactive probes attached adjacent to the 3'-end of the anticodon, leading to identification of ribosomal proteins located in the neighbourhood of the decoding site.

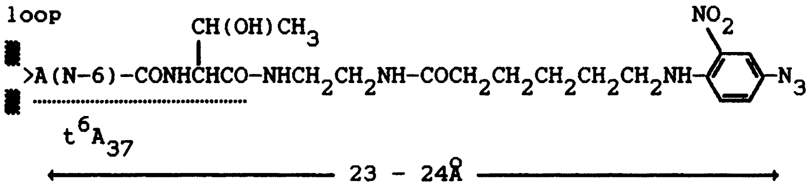
MATERIALS AND METHODS

$tRNA_m^{Met}$ from Baker yeast isolated and finally purified by HPLC on TSK-DEAE-2SW (Toyo Soda) as described before [24] lacked the 3'-terminal adenosine and had a UV-absorbtion corresponding to about 1500pmole/A_{260} unit. *E.coli* MRE600 ribosomes (tight couples) prepared as described previously [25] were 70-75% active in the EF-Tu dependent binding of Phe-$tRNA^{Phe}$ (Yeast) in the presence of poly(U), at 7mM Mg^{2+}. 1 A_{260} unit of ribosomes was taken as 24 pmoles. tRNA 3'-end labeling : 1 A_{260} unit of Yeast $tRNA_m^{Met}$ was incubated at 37°C for 1h in 40μl of 100mM Tris-HCl (pH 8.0), 20mM $MgCl_2$ 7mM DTT and 70μM CTP containing 50-100μCi of [α-^{32}P]-ATP (ca. 3000Ci/mmole, Amersham) or [^{3}H]-ATP (ca. 30Ci/mmole, Amersham), and saturating amounts of partially purified tRNA nucleotidyltransferase ([EC 2.7.7.25.], a kind gift from Dr. J.Ofengand). The mixture was then incubated for 1h at 37°C after adding ATP to a final concentration of 170μM. Labeled tRNA was purified on a 0.5ml DEAE-cellulose column. Analysis by polyacrylamide gel electrophoresis showed complete (>95%) addition of the 3'-terminal adenosine. Modification of the carboxyl group in tRNA : 1 A_{260} unit of labeled tRNA was incubated at room temperature for 45min in 0.36ml of 200mM ethylenediamine dihydrochloride, 40mM 1-ethyl-

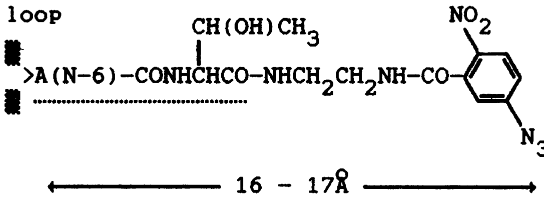
3-(3-dimethylaminopropyl)carbodiimide hydrochloride, 80mM NaCl and 10mM $MgCl_2$, the pH being adjusted to 4.0-4.2 with HCl. The reaction was quenched by addition of 1/20 vol of 20% KOAc, pH 5.0, and the tRNA was recovered by ethanol precipitation which was repeated 3 times to remove the reagents. Modification of the amino group in tRNA : 1 A_{260} unit of tRNA modified with ethylenediamine was dissolved in 60μl of 0.5M sodium borate (pH 8.2) and 240μl of DMSO. 3.5mg of N-5-azido-2-nitrobenzoyl-oxysuccinimide or N-succinimidyl-6(4'-azido-2'-nitrophenyl-amino)hexanoate (Pierce) dissolved in 20μl of DMSO was added to the mixture in two 10μl portions at 45min intervals and the reaction was allowed to proceed out at room temperature for 90min. tRNA was recovered by ethanol precipitation, and the excess of the reagent was removed by 3 succesive ethanol precipitations , two from 50% DMSO and one from water. The overall modification yield (tested by HPLC as described in Ref. 26) was greater than 85%. Ribosome binding and cross-linking : [^{32}P] labeled tRNA (160nM), ribosomes (80nM) and poly(A,U,G) (100μg/ml) were incubated at 37°C for 15min in buffer containing 50mM HEPES-Na (pH 7.5), 50mM NH_4Cl and 8mM $MgCl_2$, and then chilled on ice. tRNA binding was measured by a nitrocellulose filter assay (aliquots of the binding mixture were spotted onto wet filters) using 20mM $MgCl_2$, 50mM Tris-HCl (pH 7.5), 50mM KCl as the wash buffer. The noncovalent complexes were irradiated with >290nm UV light (HBO 300 lamp) or with visible light (150W halogen lamp) at 0°C for 30-45min. Cross-linking (covalent binding) was measured by a nitrocellulose filtration assay using 0.1mM $MgCl_2$, 50mM Tris-HCl (pH 7.50), 50mM KCl as the wash buffer, after diluting the samples with ca. 100 volumes of 50mM Tris-HCl (pH 7.5), 50mM KCl followed by incubation at 0°C for about 10min. Measured values were corrected for non-specific binding of tRNA to nitrocellulose (determined from a control without ribosomes). Separation of ribosomal subunits : after irradiation the ribosomes were recovered by ethanol precipitation (0.7 vol), dissolved in 200-400μl of 20mM Tris-HCl (pH 7.5), 0.5mM $Mg(OAc)_2$, 100mM NH_4Cl, and were centrifuged through a 17-30% linear sucrose gradient in the same buffer (SW40 rotor, 31,000rpm, 13h, 2°C). Separation of rRNAs and

r-proteins : ribosomal subunits were recovered from the sucrose gradient fractions by ethanol precipitation (2.5 vol), and were incubated in 100μl of 2% SDS, 100mM LiCl, 10 mM EDTA, 20mM Tris-HCl (pH 7.5), 0.5 mM $Mg(OAc)_2$, 100mM NH_4Cl for 5min at

tRNA-24Å/37



tRNA-17Å/37



37°C. The samples were then diluted with 3 volumes of 20mM Tris-HCl (pH 7.5), 0.5 mM $Mg(OAc)_2$, 100mM NH_4Cl and were centrifuged through a 17-30% linear sucrose gradient in the same buffer containing 0.1% SDS, 100mM LiCl and 10mM EDTA (SW40 rotor, 30,000rpm, 16h, 16°C). Radioactivity measurements: dry filters were counted with 5ml of PPO/POPOP/toluene scintillation cocktail. Sucrose gradient fraction aliquots were diluted with water to 0.5-1.0ml and counted with 5ml of Unisolve 1 (Koch-Light). Identification of ribosomal proteins cross-linked to tRNA was performed with the agarose method of Ref. 27, using the protein-tRNA cross-linked complexes from the SDS-containing sucrose gradients (above), after recovery by ethanol precipitation.

RESULTS AND DISCUSSION

The free carboxyl group of hypermodified nucleosides present in some tRNAs have unique chemical properties which enable their selective modification by condensation with amines in the presence of a water soluble carbodiimide [28]. A primary aliphatic amino group can be introduced in this way into tRNA by using ethylenediamine in the reaction. The amino group can be further modified with appropriate N-hydroxysuccinimide esters. Both reactions are very efficient and selective [1,4,24,26,28]. The modification procedure can be applied to any tRNA containing a hypermodified nucleoside with a free carboxyl group, and therefore offers a convenient method for attaching different probes at a single precisely defined site on the tRNA molecule. In this study the procedure is applied to Yeast $tRNA_m^{Met}$ which contains N^6-(threoninocarbonyl)adenosine (t^6A) located next to the 3'-end of the anticodon (position 37). The structure of the

Fig.1. Structure and approximate location of photoreactive probes in tRNA. The natural N6-(threoninocarbonyl)adenosine side chain is marked with a dotted line. In the tRNA structure, the carbon atom of the carboxyl group is marked by a full circle (4Å diameter) as the probe attachment site. It has been located in the crystal structure of Yeast phenylalanine tRNA [29] (atomic coordinates from Brookhaven Protein Data Bank) by aligning the N-1, C-6 and C-5 atoms of Wyosine 37 and N6-(threoninocarbonyl)-adenosine in its crystal structure [30]. The aminoacid arm of the tRNA molecule shown on the right points away from the viewer. The side chain of t6A points in a direction opposite to the direction of the aminoacid arm of tRNA.

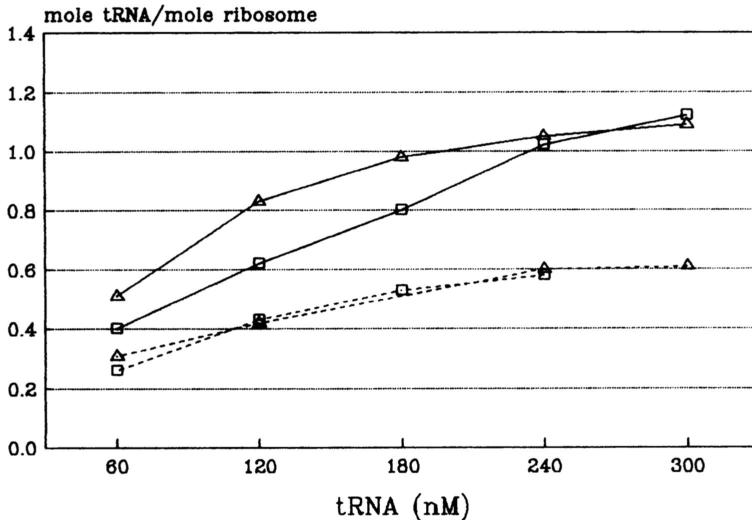


Fig.2. Binding of unmodified $tRNA_m^{Met}$ (triangles) and tRNA-17Å/37 (squares) to *E.coli* ribosomes (80nM) in the presence (solid lines) and in the absence (broken lines) of poly(A,U,G).

photoreactive probes employed in this study and the approximate location of their attachment site in the tRNA is shown in Fig.1. The distance between the N-6 atom of t^6A and the azido group was taken as the maximum extended length of the probe, and was estimated to be 16-17 and 23-24Å for the short and long probe, respectively. The 2-nitro-5-azidophenyl group of the short probe can be photoactivated by UV light (320-350nm) whereas the 2-nitro-4-azidophenyl group of the long probe is photoactivated by visible light. In the following discussion the two modified tRNA's are referred to as "tRNA-17Å/37" and "tRNA-24Å/37" for the short and long probe, respectively.

The modification, despite its location next to the anticodon, and despite the size and hydrophobicity of the probe, has only a limited effect on the binding of deacylated tRNA to the ribosome. Decreased binding was observed only in the presence of poly(A,U,G) at lower tRNA:ribosome ratios - Fig.2. The results presented in Fig.2 suggest that up to about 0.6 and 1.0 molecules of deacylated, modified or unmodified $tRNA_m^{Met}$ can bind *per* 70S ribosome in the absence and in the presence of poly(A,U,G), respectively. These values agree well with the

Table 1. Binding and cross-linking of Yeast tRNA-17Å/37 and tRNA-24Å/37.

poly(A,U,G)	Binding	Cross-linking			
		70S	50S	30S [16SrRNA rP]	70S (%)
tRNA-17Å/37					
+	.75	.13 (.10)	.025	.105 [.005 .100]	17
-	.46	.19 (.18)	.020	.170 [.025 .145]	41
tRNA-24Å/37					
+	.85	.29 (.27)	.035	.255 [.020 .235]	30
-	.51	.44 (.42)	.025	.415 [.060 .355]	71
$tRNA^{Met}_{m}$ (unmodified)					
+	1.04	<.01	-	-	<1
-	.53	<.01	-	-	<1

Binding and cross-linking yield are given in moles tRNA per mole ribosome. Cross-linking yields were measured from the sucrose gradients and also (in the case of the initial cross-linking to the 70S ribosome) by a nitrocellulose filter assay (values in brackets). In the case of cross-linking to the 30S subunit, the distribution of cross-linking between ribosomal protein and RNA is shown in square brackets. The last column shows the yield of cross-linking to the 70S ribosome, expressed as a percentage of the bound tRNA. The accuracy of the measurements is better than 10%, it depends primarily on the accuracy of the tRNA extinction coefficient estimation.

conclusions of a recent systematic survey of tRNA binding to *E.coli* ribosome [31], and suggest strongly that under the conditions used in this study uncharged tRNAs should also bind predominantly, if not exclusively, to the ribosomal P-site.

The binding and cross-linking properties of the modified tRNAs are summarized in Table 1. Non-covalent tRNA-ribosome complexes form cross-links with very high yields upon irradiation with light of an appriopriate wavelength. In contrast, no reaction takes place with unmodified tRNA (Table 1), and

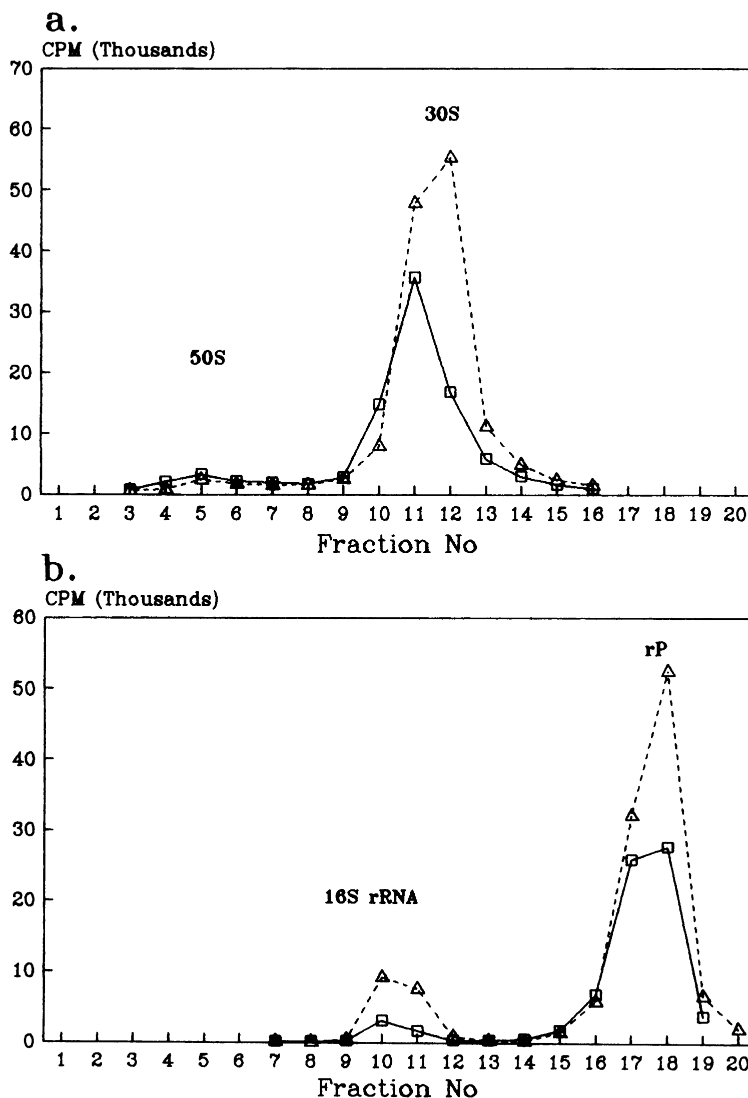


Fig.3. Sucrose gradient analysis of the distribution of cross-linked tRNA-24Å/37 between ribosomal subunits (a) and between 16S rRNA and ribosomal proteins (b), in the presence (squares) and in the absence (triangles) of poly(A,U,G). Ribosomal subunits and 16S rRNA were located by A260 measurements (not shown). Cpm values for the modified tRNA shown in (a) were corrected for ribosome recovery and by subtracting the respective bacground values measured in a parallel experiment with the unmodified tRNA (in the later case no radioactivity specifically associated with ribosomal subunits was found, proving that unmodified tRNA was not cross-linked, data not shown).

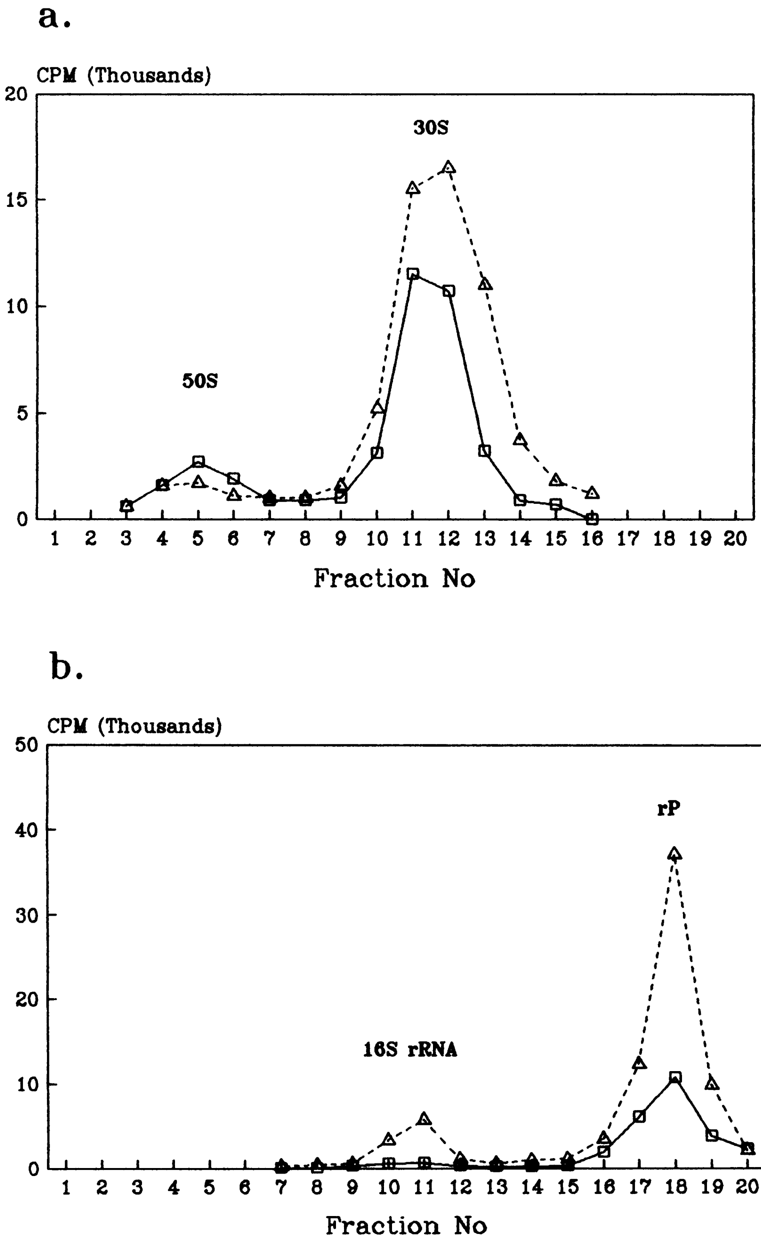


Fig.4. Sucrose gradient analysis of the distribution of cross-linked tRNA-17Å/37 between ribosomal subunits (a) and between 16S rRNA and ribosomal proteins (b), in the presence (squares) and in the absence (triangles) of poly(A,U,G). See legend to Fig.3 for further details.

pre-irradiation of modified tRNAs before binding to the ribosome decreases the cross-linking yield by more than 8-fold ([26] and data not shown). The cross-link formation is especially efficient in the absence of poly(A,U,G), and a more than two-fold increase in the cross-linking yield (% cross-linking) was found for both probes when the message was omitted from the binding mixture.

Analyses by sucrose gradient centrifugation of the distribution of the cross-linked tRNA between the ribosomal components (RNA and protein) are shown in Figs.3 and 4, and the results are summarized in Table 1. The small ribosomal subunit is the major cross-linking target, but, some cross-linking to the 50S subunit also takes place. 50S subunit cross-linking accounts for 15-25% of the total cross-linking observed in the case of the short probe (Table 1 and data not shown). Ribosomal proteins become cross-linked in both the 30S (Figs.3 and 4) and the 50S subunit (data not shown). However, 5S rRNA involvement, as well as any instability of the covalent product(s) formed upon irradiation, cannot be excluded on the basis of the sucrose gradient analysis. Furthermore, substantial cross-linking to 16S rRNA was observed in the absence of poly(A,U,G).

Although tRNA binding is stimulated almost two-fold by poly(A,U,G), the cross-linking yield is decreased in the presence of the message. The overall effect is due to a greatly reduced labeling of the 30S subunit accompanied by an almost unchanged level of 50S subunit labeling. Within the 30S subunit cross-linking to 16S rRNA and to ribosomal proteins is decreased 3-5 and 1.5 fold, respectively, in the presence of the message. The effect is essentially independent of the probe length. On the other hand, an almost two-fold increase of the cross-linking yield is achieved by increasing the length of the probe by about 7Å, and this is due to more efficient labeling of the 30S subunit protein(s) (most probably protein S7, see below).

Ribosomal proteins cross-linked to tRNA were identified by binding to goat antibodies specific for individual ribosomal proteins immobilized on Agarose-anti-goat IgG conjugate [27]. Results of the antibody assays are presented in Figs.5-7. The efficiency of the assay varied from 20 to 40% depending on the

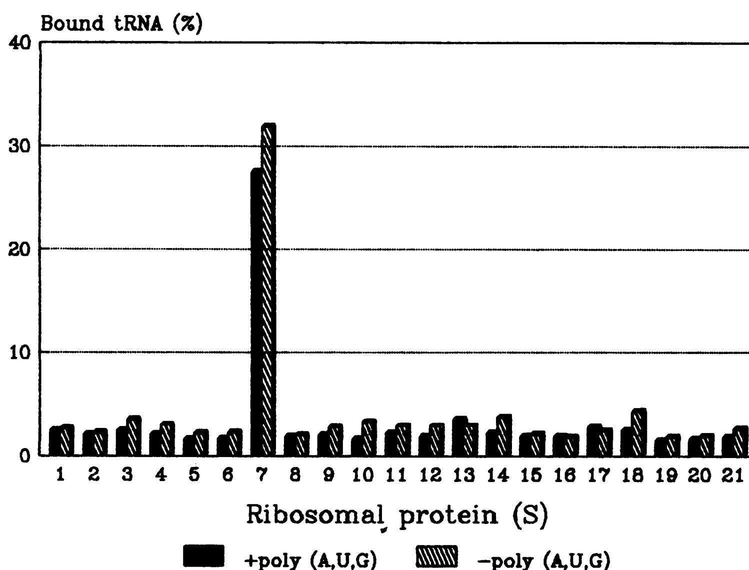


Fig.5. Identification of small subunit proteins cross-linked to tRNA-24Å/37. The bars indicate the percentage of radioactivity (label in tRNA) bound to the antibody in each test performed for indicated ribosomal protein. Values from at least two independent measurements were averaged.

product analysed, and was highest for protein S7-tRNA covalent complexes from the 30S subunit (Figs.5-6). This is the only major observed product of cross-linking to the 30S subunit, and it was identified with both probes, with and without poly(A,U,G). In addition a low level cross-linking to S1 and S18 was found for tRNA-17Å/37 in the absence of the message - Fig.6. The antibody assay itself is not quantitative, and therefore it is very likely that the tRNA-S7 adduct is the only product which contributes significantly to the high overall cross-linking yield. It could be, however, that the lower efficiency for the other products (S1, S18) in the antibody assay is due to decreased protein-antibody affinity caused by cross-link formation and/or instability of the product. For the same reasons some cross-links may have escaped detection entirely.

The cross-linked proteins found agree very well with the localization of the decoding site on the small ribosomal subunit [1] (the major cross-linking target) and with the suggestion

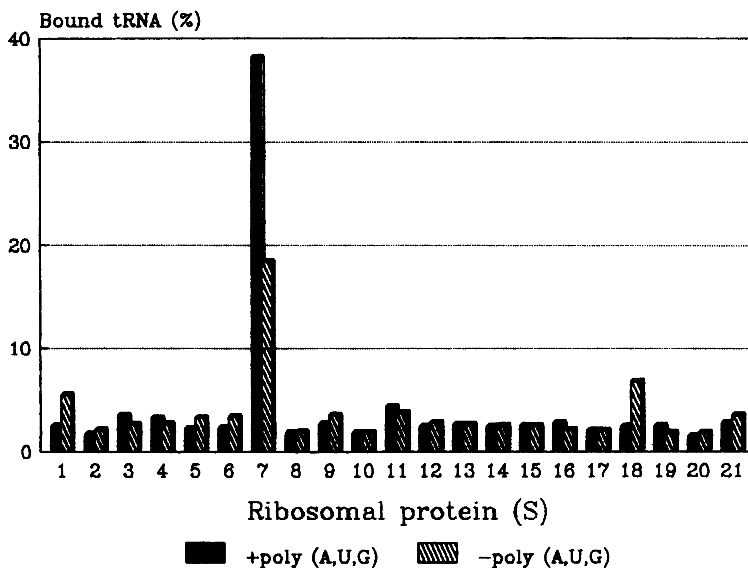


Fig.6. Identification of small subunit proteins cross-linked to tRNA-17Å/37. (Cf. Fig.5)

that codon-anticodon interaction takes place in a pocket well shielded from the solvent [4] (thus allowing high cross-linking yields). In addition, codon-anticodon interaction affects cross-linking yield and to some extent its protein specificity (as evidenced by the poly(A,U,G) dependence, Figs.5-7.). This may be due to a different orientation and/or higher flexibility of the photoreactive probe located next to the anticodon in the absence of the message, which is not allowed when the anticodon loop conformation is fixed by codon-anticodon interaction. Occupation of a different tRNA binding site, although less likely, can not be ruled out.

The very efficient cross-linking to protein S7 is of special interest. This protein has been located on the side of the head of the 30S subunit facing the protrusion [16-19], just above the cleft and the decoding site [1]. S7 can also be cross-linked, among other sites on 16S rRNA and ribosomal proteins, to the 3'-end of 16S rRNA, located on the protrusion [18,19]. It is thus feasible for the probes attached in the middle of the anticodon loop and facing in a direction opposite to the

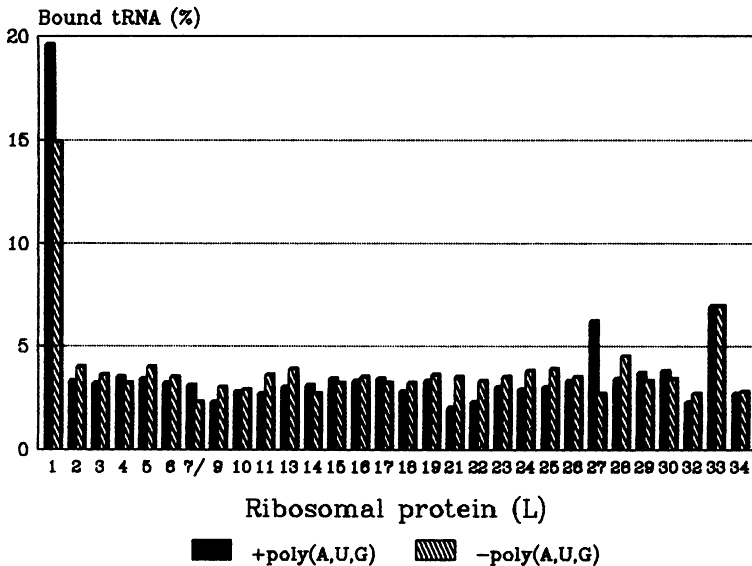


Fig.7. Identification of large subunit proteins cross-linked to tRNA-17Å/37. L7/ denotes proteins L7 and L12. (Cf. Fig.5).

direction of the aminoacid arm of tRNA (Fig.1) to reach protein S7, allowing the photoreaction to take place without too much quenching by the solvent. It seems likely that protein S7 is located along the anticodon arm and extends from the anticodon loop towards the "elbow" of the molecule. This conclusion is supported by a similarly efficient cross-linking to S7 of probes attached at position 20:1 in the dihydrouridine loop of Lupin $tRNA_m^{Met}$ bound to *E.coli* ribosomes under conditions identical to those used in this study (P.Górnicki, unpublished results), and by direct UV cross-linking of tRNA to S7 in a complex with the 30S subunit [32].

Proteins S18 and S1, which are cross-linked only in the absence of poly(A,U,G), have also been localized near the decoding site [16-19]. The distance between the mass centers of S7 and S18 in the 30S subunit model is 87Å [16], but cross-linking experiments indicate that at least some parts of the proteins are located much closer to one another (an average radius of ribosomal proteins is about 15Å assuming their spherical shape) [18]. It seems reasonable to assume that both

proteins are within reach of the 17Å, probe if different spatial orientations of the probe are involved (see discussion above).

Identification of the large subunit proteins cross-linked to the anticodon loop *via* the short probe (Fig.7) shows a similar pattern. Only protein L1 is significantly cross-linked, with traces of L27 and L33. This indicates that the decoding site located in the cleft of the 30S subunit faces the L1 protuberance, probably near its base where proteins L27 and L33 are located [33]. This orientation is fully compatible with the localization of the peptidyl transferase center on the L1 side of the central protuberance and with the relative orientation of ribosomal subunits in the 70S ribosome [3,23,34]. Cross-linking of proteins S7 and L1 to the same probe indicates that in the 70S ribosome they are close neighbours lying less than 34Å apart i.e. within reach of the 17Å probe. Our cross-linking results locate the tRNA binding site in the 70S ribosome on the L1 side of the 50S subunit (far from the L7/L12 stalk), at least as far as the decoding site is concerned.

The structural and functional implications of the results presented in this paper will be discussed in more detail when identification of the corresponding cross-link sites on 16S rRNA is completed, and when proteins cross-linked to tRNAs carrying probes at different locations and bound at different ribosomal sites have been studied.

*To whom correspondence should be addressed

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