# The effect of aminoacyl- or peptidyl-tRNA at the A-site on the arrangement of deacylated tRNA at the ribosomal P-site

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Photoreactive derivatives of *E. coli* $tRNA^{Phe}$ bearing arylazido groups on guanine residues (azido-tRNA) were used for affinity labelling of *E. coli* ribosomes in the region of the P-site when the A-site was either free or occupied by aminoacyl- or peptidyl-tRNA. Corresponding complexes of azido-tRNA with ribosomes and poly(U) were obtained both nonenzymatically and with the use of elongation factors. UV-irradiation of the complexes resulted in labelling of ribosomal proteins (preferentially of 30 S subunit). Proteins S9 and S21 were labelled only when the A-site was free; S14 – only when it was occupied; S11, S13, S19 – in both cases; S5, S7, S12, S20 – in some states.

*Photoreactive tRNA derivative* *Affinity labelling* *Ribosome* *tRNA-binding site* *Ribosomal protein*

## 1. INTRODUCTION

To understand the molecular mechanisms of elongation it is essential to know how tRNA interacts with the ribosome during each elongation step. One of the most useful tools to study tRNA–ribosome interactions is photoaffinity labelling of ribosomes by photoreactive derivatives of *E. coli* $tRNA^{Phe}$ bearing arylazido groups on guanine residues (azido-tRNA) [1–4]. Earlier we used azido-tRNA for identification of ribosomal proteins interacting with deacylated tRNA bound at the P-site nonenzymatically in the presence of poly(U) at 20 mM $Mg^{2+}$ [1] and 10 mM $Mg^{2+}$ [2] and without poly(U) at 20 mM $Mg^{2+}$ [3] as well as with Phe-azido-tRNA located at the A-site by EF-Tu and GTP (the P-site was blocked with total deacylated tRNA) [2,4]. These model states of tRNA are widely used to study tRNA–ribosome interactions but none of them is realized in a translating ribosome. During the elongation cycle, deacylated tRNA occupies the P-site only when peptidyl-tRNA is located at the A-site (pretranslocation state). When the A-site is free the P-site may be occupied only by peptidyl-tRNA (post-translocation state).

We have here studied affinity labelling of *E. coli* ribosomes with the derivatives of $tRNA^{Phe}$ bound at the P-site either nonenzymatically or with the use of elongation factors when the A-site was free or occupied with aminoacyl- or peptidyl-tRNA. From comparison of the sets of proteins labelled within corresponding complexes it was concluded that tRNA located at the A-site affects the arrangement of deacylated tRNA at the P-site, codon–anticodon interaction at the P-site being probably unbroken.

## 2. MATERIALS AND METHODS

Ribosomes were isolated from *E. coli* MRE-600 as in [5], and then purified by gel filtration on Sephadex G-200 in buffer [1.5 M $NH_4Cl$, 10 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.4), 1 mM DTT] to remove completely translation factors. Fifty % of the ribosomes were active in poly(U)-dependent binding of $Phe\text{-}tRNA^{Phe}$. Elongation factor Tu·Ts (3000 pmol/mg) and total elongation factor (13 mg/ml) were isolated as in [6]. 4-(*N*-2-Chloroethyl-*N*-methylamino)-[$^{14}$C]benzylamine (25 mCi/mmol) was synthesized as in [7,8]. 2,4-Dinitro-5-fluorophenylazide was prepared as in [9]. Amino-



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acylation of $tRNA^{Phe}$ and azido-tRNA was performed as in [2,4]. Poly(U) and [$^{14}$C]phenylalanine (360 mCi/mmol) were from Reanal (Hungary). $tRNA^{Phe}$ was purchased from Boehringer Mannheim, RNases A and $T_1$ were from Sankyo (Japan).

Azido-tRNA I and azido-tRNA II (extent of modification 2 mol reagent residues per mol $tRNA^{Phe}$ in each case) were prepared as in [10]. In azido-tRNA I arylazido groups were scattered statistically over N7 atoms of guanine residues whereas in azido-tRNA II N7 atoms of guanines participating in tertiary bond formation remained unaffected [10,11]. Nonenzymatic binding of azido-tRNA at the P-site and EF-Tu-dependent binding of Phe-$tRNA^{Phe}$ at the A-site were performed as in [2]. Peptidyl-azido-tRNA was directed to the P-site at the end of the synthesis of poly(Phe) in the reaction mixture containing 6 nmol of 70 S ribosomes, 19 nmol Phe-azido-tRNA, 3.5 mg poly(U) and 15 mg total elongation factor in 2.5 ml buffer A [0.05 M Tris–HCl (pH 7.5), 0.1 mM $NH_4Cl$, 10 mM $MgCl_2$, 5 mM DTT, 0.3 mM GTP] at 37°C for 1 h. The complex obtained (I) was isolated from nonbound azido-tRNA and the elongation factors by centrifugation through 10% sucrose in buffer A without GTP (105000 × *g*, 4°C, 4 h). In some experiments complex I was treated with puromycin as in [4]. Pretranslocation complex 70 S ribosome poly(U) $(Phe)_n$-$tRNA^{Phe}$ (A-site) azido-tRNA (P-site) was obtained by incubation of complex I with 8 nmol Phe-$tRNA^{Phe}$ and 40 nmol EF-Tu in buffer A at 0°C for 15 min and isolated by centrifugation as above.

Corresponding complexes were irradiated with UV light ($\lambda \geqslant 350$ nm) and labelled ribosomal proteins were isolated and analysed by two-dimensional gel electrophoresis as in [4]. In different experiments varying amounts of labelled material were subjected to electrophoresis so absolute values of $^{14}$C counts corresponding to protein spots are not strictly comparable for different states and subunits (fig.1).

## 3. RESULTS

To investigate the labelling of ribosomes with azido-tRNA bound at the P-site nonenzymatically two types of complexes were used: (I) azido-tRNA II·70 S ribosome·poly(U) (A-site free) and (II) azido-tRNA-II·70 S ribosome·poly(U)·Phe-$tRNA^{Phe}$ (A-site). Complex I was obtained using a 3-fold excess of ribosomes over azido-tRNA. Complex II was obtained by incubation of complex I with [$^{12}$C]Phe-$tRNA^{Phe}$, EF-Tu and GTP. To fill the A-sites completely Phe-$tRNA^{Phe}$ was taken in 1.5-fold excess over ribosomes. Occupancy of the A-site was tested in separate experiments using $2 \times 10^{-5}$ M tetracycline and [$^{14}$C]Phe-$tRNA^{Phe}$. The level of binding of azido-tRNA at the P-site did not depend on the presence of Phe-$tRNA^{Phe}$ at the A-site, being 0.26 mol azido-tRNA per mol ribosomes in both cases. The specificity of nonenzymatic binding of azido-tRNA at the P-site has been demonstrated [1–3].

tRNA–ribosome interaction in elongation factor-dependent system was studied with the use of azido-[$^{14}$C]tRNA I in 3 different states: (III) $(Phe)_n$-azido-tRNA·ribosome·poly(U) (A-site free); (IV) azido-tRNA·ribosome·poly(U) (A-site free); (V) azido-tRNA·ribosome·poly(U)·$(Phe)_{n+1}$-$tRNA^{Phe}$ (at the A-site). Complex III (posttranslocation state) was obtained in the system containing 70 S ribosomes, poly(U), azido-tRNA, elongation factors and GTP. At the end of the synthesis of poly(Phe) all azido-tRNA bound with ribosome was located at the P-site as $(Phe)_n$-azido-tRNA as revealed from puromycin reactivity (see table 1). Complex IV was obtained by treatment of complex III with puromycin. The reaction proceeded completely as tested with the use of [$^{14}$C]Phe-azido-[$^{12}$C]tRNA in separate experiments. Complex V (pretranslocation state) was obtained after EF-Tu-directed binding of Phe-$tRNA^{Phe}$ to complex III with subsequent transpeptidation. Location of $(Phe)_{n+1}$-$tRNA^{Phe}$ at the A-site of complex V was confirmed by the absence of puromycin reactivity (see table 2).

Complexes I–V were irradiated with UV light ($\lambda \geqslant 350$ nm). Distribution of the label between the ribosomal subunits was analysed by centrifugation in a sucrose density gradient under dissociating conditions (0.5 mM $Mg^{2+}$). As shown in table 2 in all cases both subunits are labelled (30 S preferentially). Cross-linking of azido-tRNA to the P-site was shown earlier to be specific (no reaction out of the complex) from the experiments on inhibition of the photoreaction in the presence of unmodified $tRNA^{Phe}$ [1–3]. Correct location of tRNA

Table 1

Binding of tRNA derivatives with ribosome after poly(U)-directed synthesis of poly(Phe) in the presence of elongation factors and GTP

| $tRNA^{Phe}$ derivative at the P-site | Component added | | Binding (mol tRNA per mol 70 S ribosome) | | Amount of [$^{14}$C]Phe residues bound with ribosomes (pmol) | |
|---|---|---|---|---|---|---|
| | Phe-$tRNA^{Phe}$ at the A-site | Puromycin | Before irradiation | After irradiation | Before irradiation | After irradiation |
| [$^{14}$C](Phe)$_n$-azido-[$^{12}$C]tRNA I | – | – | | | 46 | 42 |
| [$^{14}$C](Phe)$_n$-azido-[$^{12}$C]tRNA I | – | + | | | 2.4 | 2.0 |
| [$^{12}$C](Phe)$_n$-azido-[$^{14}$C]tRNA I | – | – | 0.320 | 0.285 | | |
| [$^{12}$C](Phe)$_n$-azido-[$^{14}$C]tRNA I | – | + | 0.290 | 0.260 | | |
| [$^{14}$C](Phe)$_n$-azido-[$^{12}$C]tRNA I | + | – | | | 42.0 | 38.0 |
| [$^{14}$C](Phe)$_n$-azido-[$^{12}$C]tRNA I | + | + | | | 40.5 | 36.0 |
| [$^{12}$C](Phe)$_n$-azido-[$^{14}$C]tRNA I | + | – | 0.300 | 0.270 | | |

Each reaction mixture contained 14 pmol of 70 S ribosomes

Table 2

Distribution of label between ribosomal subunits after irradiation of complexes of azido-[$^{14}$C]tRNA with ribosomes and poly(U)

| Complex (see text) | Mol covalently bound azido-tRNA per mol ribosomal subunit | |
|---|---|---|
| | 50 S | 30 S |
| I | 0.014 | 0.029 |
| Ia [2] | 0.018 | 0.035 |
| II | 0.012 | 0.022 |
| III | 0.03 | 0.12 |
| IV | 0.02 | 0.09 |
| V | 0.05 | 0.12 |

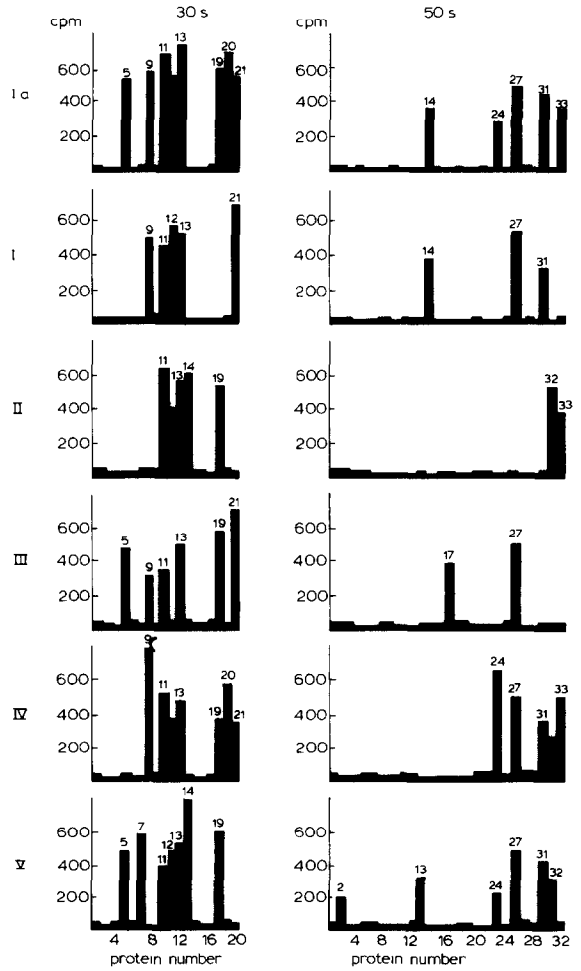



Fig.1. Distribution of the radioactive label among ribosomal proteins labelled with azido-tRNA in different states.

| State | tRNA derivative in P-site | A-site |
|---|---|---|
| Ia [2] | azido-tRNA I | – |
| I | azido-tRNA II | – |
| II | azido-tRNA II | Phe-$tRNA^{Phe}$ |
| III | (Phe)$_n$-azido-tRNA I | – |
| IV | azido-tRNA I | – |
| V | azido-tRNA I | (Phe)$_{n+1}$-$tRNA^{Phe}$ |

States Ia, I and II were obtained using nonenzymatic binding of azido-tRNA at the P-site; states III–V were obtained with the use of elongation factors.

derivatives on the ribosome as well as the conservation of functional activity of the ribosomes after irradiation of isolated complexes III–V were confirmed by:

(i) the level of the puromycin reaction in complex III did not change after irradiation;
(ii) irradiated complexes III and IV were able to bind Phe-tRNA$^{Phe}$ in the presence of EF-Tu and GTP;
(iii) the lack of puromycin reactivity in the pretranslocation complex V before and after irradiation which indicated that no translocation proceeded during the experiment (see table 1).

In all states only ribosomal proteins were labelled as analysed by centrifugation in a sucrose density gradient in the presence of SDS and EDTA (5 S rRNA not tested). Proteins were isolated from the modified subunits and analysed as in [4]. Results of the analysis are given in fig.1.

## 4. DISCUSSION

The 30 S subunit was labelled with azido-tRNA at the P-site preferentially in all states (see table 2), hence this site is formed mainly by surface of the small subunit. Using azido-tRNA I bound at the P-site nonenzymatically at 10 mM $Mg^{2+}$ we have identified proteins in the region of the P-site [2] (state Ia in fig.1). Comparing the sets of proteins labelled in states I and Ia one can see that proteins S5, S19, S20 and L24, L33 are modified only with azido-tRNA I, not azido-tRNA II. In azido-tRNA I arylazido groups are scattered statistically over guanine residues with the exception of $G_{22}$, $G_{27-30}$, $G_{69-71}$ [10,11]. In azido-tRNA II these residues are also unaffected as well as $G_{15}$, $G_{53}$, $G_{57}$, $G_{63}$, $G_{65}$, important for tertiary folding of tRNA$^{Phe}$ [10,11]. Therefore proteins S5, S19, S20 and L24, L33 interact with arylazido groups attached to $G_{15}$, $G_{53}$, $G_{57}$, $G_{63}$, $G_{65}$ or to some of them. Complexes I and Ia were obtained under exactly the same conditions so difference in the sets of labelled proteins is due only to the difference between tRNA derivatives used.

The arrangement of deacylated tRNA at the P-site depends slightly on the manner of complex formation. Modification patterns for identical tRNA derivatives within the same complexes, Ia and IV, are identical despite the fact that in one case azido-tRNA was bound at the P-site nonenzymatically (Ia) while in another – after EF-dependent synthesis of poly(Phe) with subsequent removal of the peptidyl moiety (IV). The small difference (in state IV proteins S5 and L14 are not labelled) takes place likely due to the more stringent selection of functionally active modified tRNA molecules in the course of aminoacylation and the elongation cycle in state IV as compared to state I.

Interaction of the peptidyl moiety with peptidyltransferase center probably leads to more strict fixation of the CCA-end and acceptor stem in the 50 S subunit. This may explain the reduction in contact area of peptidyl-tRNA with the 50 S subunit as compared with deacylated one (states III and IV, fig.1). Contacts of tRNA with the 30 S subunit are also altered to some extent by the peptidyl moiety.

The presence of aminoacyl or peptidyl-tRNA at the A-site affects the modification patterns for deacylated tRNA in both nonenzymatic (states I,II) and elongation factor-dependent systems (states IV,V). tRNA at the A-site may protect some proteins against modification by azido-tRNA; on the other hand it may affect the arrangement of deacylated azido-tRNA at the P-site. Therefore proteins S7, S14, L2, L13 are labelled only when the A-site is occupied (S7, L2, L13 – only in factor-dependent system); S9 and S21 – only when it is free; S11, S13 and S19 – in all cases. The latter seem to be the 'structural foundation' of the P-site. Besides those mentioned, proteins S5, S7, S12, S20 and several 50 S ones were labelled in some states. The modification pattern for state II (azido-tRNA II used) is smaller than that for the similar state IV (azido-tRNA I used) as described above.

From comparison of sets of proteins labelled by azido-tRNA located at the P-site and for oligouridylate derivatives bearing an alkylating group on the 5′-end (mRNA analogs) some conclusions concerning decoding region of the P-site may be made. Proteins S11, S13, S19 are labelled with the derivatives of tetra- and pentanucleotides (codon–anticodon interaction only at the P-site) [12,13] and with azido-tRNA located at the P-site when the A-site is free. These proteins seem to be the components of the decoding area of the P-site when the A-site is free. By analogy proteins S5, S11, S12, S13, S19 which are labelled with the

derivatives of hexa- and heptauridylates (codon–anticodon interaction at both A- and P-sites) [12–14] and with azido-tRNA located at the P-site when the A-site is occupied, are assigned to decoding region of the P-site when the A-site is occupied. Probably, the presence of either aminoacyl- or peptidyl-tRNA at the A-site affects codon–anticodon interaction of deacylated tRNA at the P-site but does not break it.

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