The effect of GTP hydrolysis and transpeptidation on the arrangement of aminoacyl-tRNA at the A-site of *Escherichia coli* 70 S ribosomes

S.N. Vladimirov, D.M. Graifer, G.G. Karpova, Yu.P. Semenkov+, V.I. Makhno+ and S.V. Kirillov+

Institute of Bioorganic Chemistry, Siberian Division of the USSR Academy of Sciences, Novosibirsk, 630090, and *+B.P. Konstantinov Nuclear Physics Institute of the USSR Academy of Sciences, Gatchina, Leningrad District 188350, USSR*

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From the affinity labelling of 70 S ribosomes with a photoreactive derivative of Phe-tRNA^{Phe} bearing an arylazido group on guanine residues, it has been found that different sets of ribosomal proteins are labelled in the course of three successive steps of EF-Tu-dependent binding of aminoacyl-tRNA derivative at the A-site. Proteins S5, S7, S8, S16, S17, L9, L14, L15 and L24 were labelled before GTP hydrolysis; proteins S5, S7, S9, S11, S14, S18, S19, S21, L9, L21 and L29 – after GTP hydrolysis; proteins S2, S5, S7, S21, L11 and L23 – after GTP hydrolysis and transpeptidation.

Ribosome *Elongation factor Tu* *A-site* *Photoaffinity labelling* *Aminoacyl-tRNA derivative* *Ribosomal protein*

1. INTRODUCTION

The factor-dependent binding of aminoacyl-tRNA at the ribosomal A-site is a rather complicated process and includes at least two steps. Indeed, it was repeatedly noted that aminoacyl-tRNA being bound at the A-site behaved differently before and after GTP hydrolysis. If Phe-tRNA was bound in the presence of EF-Tu and GMPPCP (non-cleavable analogue of GTP), Ac-Phe-tRNA^{Phe}, located at the P-site, was able to react with puromycin. When GMPPCP was substituted for GTP, Ac-Phe-puromycin was not formed, but Phe-tRNA^{Phe} was competent in Ac-Phe-Phe-tRNA^{Phe} synthesis [1–6]. Hamburger et al. [7] compared thermostabilities of the A-site complexes of Phe-tRNA^{Phe} with ribosomes and found the complex produced with EF-Tu and GTP to be more stable than that formed in the presence of EF-Tu and GMPPCP. Yokosawa et al. [5] showed that labelled aminoacyl-tRNA bound at the A-site in the presence of EF-Tu and GMPPCP was able to exchange for the unlabelled species, whereas the exchange could not be detected if the binding was carried out in the presence of EF-Tu and GTP. In the system where the P-site was preoccupied by Ac-Phe-tRNA$^{Phe}_{ox\text{-}red}$, an analogue of peptidyl-tRNA without donor activity (i.e., the binding of aminoacyl-tRNA and peptide bond synthesis were uncoupled), the factor-dependent binding of Phe-tRNA^{Phe} was found to be much more stable than the factor-free one [8,9].

Here, we have studied the arrangement of aminoacyl-tRNA in the course of 3 well distinguishable steps of its interaction with the A-site of 70 S ribosomes:

(i) Before GTP hydrolysis and removal of EF-Tu from ribosomes (the P-site was preoccupied by Ac-Phe-tRNA^{Phe}; the binding of Phe-tRNA^{Phe} was carried out in the presence of EF-Tu and GMPPCP);

(ii) After GTP hydrolysis but before transpeptidation (Ac-Phe-tRNA$^{Phe}_{ox\text{-}red}$ at the P-site; the binding of Phe-tRNA^{Phe} – in the presence of EF-Tu and GTP);

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(iii) After both GTP hydrolysis and transpeptidation (Ac-Phe-tRNA^{Phe} at the P-site; the binding of Phe-tRNA^{Phe} – in the presence of EF-Tu and GTP).

For the modification of ribosomal components to occur, Phe-tRNA^{Phe} was substituted for its photoactivated derivative, Phe-azido-tRNA^{Phe}, bearing arylazido groups scattered statistically over tRNA guanosine residues [10–12]. The ribosomal 16 S and 23 S RNAs were not modified after UV irradiation, but the sets of ribosomal proteins cross-linked to tRNA were found to differ drastically in all 3 model complexes described above.

2. MATERIALS AND METHODS

Active 30 S and 50 S ribosomal subunits were isolated from *E. coli* MRE-600 as in [13]. Enriched preparations of [^{14}C]Phe-tRNA^{Phe} (1400 pmol/A_{260} unit), Ac-[^{14}C]Phe-tRNA^{Phe} (1400 pmol/A_{260} unit), Ac-[^{14}C]Phe-tRNA$^{Phe}_{ox\text{-}red}$ (1250 pmol/A_{260} unit) and tRNA^{Phe} (1400 pmol/A_{260} unit) were prepared as in [9,13]. ^{14}C-labelled 4-(*N*-2-chloroethyl-*N*-methylamino)benzylamine (25 mCi/mmol) was synthesized as in [14,15]. 2,4- Dinitro-5-fluorophenylazide was prepared according to [16]. Elongation factors Tu·Ts and G were isolated by the procedure of Arai et al. [17]. Free EF-Tu was obtained as in [6] and its activity was tested as in [18]. Azido-[^{14}C]tRNA^{Phe} was prepared as in [10]; the extent of modification with arylazido groups was determined to be 3 mol reagent residues/mol tRNA^{Phe}. Aminoacylation of both unmodified tRNA^{Phe} and azido-[^{14}C]tRNA^{Phe} was performed as in [12]. Ribosomal complex I, containing Ac-Phe-tRNA^{Phe} at the P-site and Phe-azido-tRNA^{Phe} at the A-site (bound in the presence of EF-Tu and GMPPCP), was formed by mixing two intermediate complexes 1 and 3 (see table 1). Complex II, where GTP hydrolysis was allowed to occur but transpeptidation was prevented by substitution of Ac-Phe-tRNA^{Phe} (at the P-site) for its analogue without donor activity (Ac-Phe- tRNA$^{Phe}_{ox\text{-}red}$), was obtained by mixing intermediate complexes 2 and 4. Complex III, where both GTP hydrolysis and transpeptidation were permitted, was obtained by mixing intermediate complexes 1 and 4. Complexes I–III were incubated 90 min at 0°C; the final concentration of Mg^{2+} was adjusted to 10 mM in all

Table 1

Composition and formation of intermediate complexes 1–4

	No.	Components	Incubation
P-site complexes	1	7.8 nmol 30 S subunits 10 nmol 50 S subunits 5 mg poly(U) 11.8 nmol Ac-[^{14}C]Phe-tRNA^{Phe}	90 min at 0°C in 3.14 ml of buffer I[a]
	2	The same, but Ac-[^{14}C]Phe-tRNA^{Phe} was substituted for Ac-[^{14}C]Phe-tRNA$^{Phe}_{ox\text{-}red}$	
Ternary complexes	3	7.8 nmol Phe-azido-[^{14}C]tRNA^{Phe} 20 nmol EF-Tu 12 μmol GMPPCP	30 min at 0°C in 1.9 ml of buffer II[b]
	4	7.8 nmol Phe-azido-[^{14}C]tRNA^{Phe} 40 nmol EF-Tu·Ts 0.45 μmol GTP	

[a] Buffer I: 20 mM Tris-HCl (pH 7.4), 15 mM $MgCl_2$, 200 mM NH_4Cl
[b] Buffer II: 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 200 mM NH_4Cl

cases. Translocation in complex III was carried out as in [6]. Inhibition of the A-site binding of aminoacyl-tRNA by tetracycline and puromycin reaction were performed according to [19]. EF-Tu-dependent binding of aminoacyl-tRNA with ribosomes was measured by centrifuging complexes through the sucrose density gradients 10–30% in buffer II. UV irradiation of the complexes ($\lambda \geqslant 350$ nm), separation of the ribosomes into subunits, isolation of ribosomal proteins and analysis of distribution of ^{14}C label among them by two-dimensional electrophoresis in polyacrylamide gel were carried out as in [20]. Before the analysis modified tRNA covalently bound to ribosomal proteins was digested by RNases T_1 and A and phosphomonoesterase. As shown earlier, the treatment produces a small oligonucleotide fragment covalently linked to ribosomal proteins and bearing charge 0 or -1. These fragments practically do not affect electrophoretic mobilities of the proteins [20]. [^{14}C]Phenylalanine (318 mCi/mmol) was from UVVVR (Czechoslovakia), poly(U) from Reanal (Hungary), 5′-guanylyl (β-γ-methylene)diphosphonate (GMPPCP) from Serva (FRG), RNases T_1 and A from Sankyo (Japan); phosphomonoesterase was purchased from Serva.

3. RESULTS

In this work we used 70 S ribosomes with fully active P- and A-sites for studying 3 model complexes (states) which imitated different steps of selection of codon-specific aminoacyl-tRNA at the A-site: before GTP hydrolysis (complex I; for details see section 2); after GTP hydrolysis but before transpeptidation (complex II); after both GTP hydrolysis and transpeptidation (complex III).

The control experiments carried out with unmodified Phe-tRNA^{Phe} showed that only in the case of EF-Tu + GMPPCP-dependent binding was peptidyl-tRNA located at the P-site able to react with puromycin (table 2). This state of aminoacyl-tRNA at the A-site (complex I), with the acceptor site of peptidyltransferase center being vacant for puromycin binding, is analogous to that observed earlier [1–4,6] and designated by Lake as a separate 'R' site [21].

To prove the peptide bond formation in the complex III carrying [^{14}C]Phe-azido-[^{12}C]tRNA it was isolated by centrifugation through 10% sucrose in buffer II. Then EF-G and GTP were added to the complex and translocation was allowed to proceed. Puromycin treatment of this post-translocation complex indicated that Ac-Phe-Phe-puromycin was synthesized in 91% complexes (not shown).

The data on EF-Tu-dependent binding of Phe-azido-[^{14}C]tRNA^{Phe} are shown in table 3. The level of this binding is 3–4-times lower than that of unmodified Phe-tRNA^{Phe}. It can be explained readily if we assume that the preparation of statistically modified tRNA^{Phe} contains some portion of molecules failing to form the ternary complex with EF-Tu and GTP (or GMPPCP). Nevertheless, the binding of a remaining active portion of Phe-

Table 2

Binding of [^{14}C]Phe-tRNA^{Phe} at the A-site of 70 S ribosome

tRNA derivative at the P-site	Components added to [^{14}C]Phe-tRNA^{Phe}				[^{14}C]Phe-tRNA^{Phe} bound, mol tRNA/mol 70 S	Ac-[^{14}C]Phe-puromycin formed, mol/mol 70 S
	EF-Tu	GTP	GMPP-CP	Tetracycline		
Ac-[^{14}C]Phe-tRNA^{Phe}	+	+	–	–	0.80	0.17
Ac-[^{14}C]Phe-tRNA^{Phe}	+	+	–	+	0.14	–
Ac-[^{14}C]Phe-tRNA^{Phe}	+	–	+	–	0.85	0.74
Ac-[^{14}C]Phe-tRNA$^{Phe}_{ox\text{-}red}$	+	+	–	–	0.81	0.00
Ac-[^{14}C]Phe-tRNA$^{Phe}_{ox\text{-}red}$	+	+	–	+	0.15	–
Ac-[^{14}C]Phe-tRNA$^{Phe}_{ox\text{-}red}$	+	–	–	–	0.19	–

Table 3

Binding and cross-linking of Phe-[^{14}C]azido-tRNA^{Phe} at the A-site of 70 S ribosome

Complex (for details see text)	Components added to Phe-[^{14}C]azido-tRNA^{Phe}				Unlabelled tRNA derivative at the P-site	Azido-tRNA bound, mol tRNA/mol 70 S	Azido-tRNA cross-linked, mol tRNA/mol subunit	
	EF-Tu	GTP	GMP-PCP	Tetracycline			30 S	50 S
I	+	–	+	–	AcPhe-tRNA^{Phe}	0.24	0.045	0.026
	+	–	–	–	AcPhe-tRNA^{Phe}	0.07		
	+	–	+	+	AcPhe-tRNA^{Phe}	0.02	0.004	0.002
	–	–	–	–	AcPhe-tRNA^{Phe}	0.01		
II	+	+	–	–	AcPhe-tRNA$^{Phe}_{ox\text{-}red}$	0.21	0.043	0.038
	+	+	–	+	AcPhe-tRNA$^{Phe}_{ox\text{-}red}$	0.03	0.005	0.003
III	+	+	–	–	AcPhe-tRNA^{Phe}	0.22	0.044	0.026
	+	+	–	+	AcPhe-tRNA^{Phe}	0.03	0.005	0.002

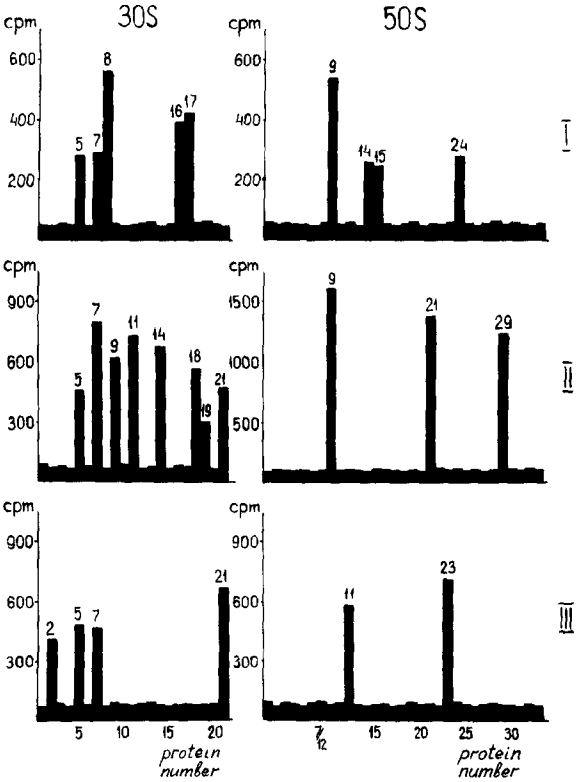

Fig.1. Distribution of the ^{14}C label among ribosomal proteins modified by Phe-azido-[^{14}C]tRNA^{Phe} at different steps of its factor-dependent binding at the A site of 70 S ribosomes: I, complex I; II, complex II; III, complex III (for details see text).

azido-[^{14}C]tRNA^{Phe} is seen to be factor-dependent and occurs at the A-site judging by the inhibitory action of tetracycline (table 3).

Complexes I, II and III carrying Phe-azido-[^{14}C]tRNA^{Phe} were irradiated with UV light ($\lambda \geqslant 350$ nm), and the distribution of ^{14}C label among ribosomal subunits was analyzed by sedimentation through a sucrose density gradient in 0.5 mM Mg^{2+}. It appeared that the amount of ^{14}C label covalently bound to the 30 S subunits did not differ in all 3 complexes. In contrast, 50 S subunits in the complex II were labeled 1.5-fold higher than those in complexes I and III (table 3). Specificity of photocross-linking of azido-tRNA to ribosomes (no reaction out of the complex) was shown earlier [12]. Sedimentation of the complexes in the presence of SDS and EDTA revealed that in all cases only ribosomal proteins rather than 16 S and 23 S RNAs were cross-linked to tRNA (5 S RNA was not tested).

The results of the analysis of the modified protein by two-dimensional electrophoresis in polyacrylamide gel (see section 2) are shown in fig.1. The sets and numbers of these proteins are seen to be different in all complexes studied. We observed only two common proteins, S5 and S7.

4. DISCUSSION

The affinity labelling of the A-site components

by Phe-azido-[^{14}C]tRNA^{Phe} was studied earlier when the P-site had been preoccupied with deacylated tRNA [12]. The set of modified proteins appeared to differ from that found in complex II in this paper. Only proteins S9, S18, S19 and L9 are common in both cases (the spot corresponding to protein L9 was erroneously identified as L8/L9 in all our previous papers [10–12,20,22] starting from [23]). One can draw the conclusion that the arrangement of aminoacyl-tRNA at the A-site depends on the ligand located at the P-site. When comparing the data on labelling of proteins at the P- and A-sites ([11,12,22] and this paper), we find 7 common proteins: S5, S7, S9, S11, S14, S19 and S21. Hence, we can conclude that these proteins are in close vicinity to both sites. Proteins S5 and S7 cross-link with aminoacyl-tRNA derivatives at all 3 states of the A-site but these proteins may be labelled by tRNA derivatives at the P-site in some cases [22]. According to Lake [21], protein S5 only participates in the formation of the R-site (this corresponds to the state, or complex I in this work). Ofengand et al. [24] observed that Phe-tRNA^{Phe} with an arylazido group attached to the s^4U_8 residue was able to cross-link at the A- rather than at the R-site, and concluded that different surroundings of the aminoacyl-tRNA exist at both sites. On the other hand, Kruse et al. [6] investigated photocross-links between Phe-tRNA^{Phe} and ribosomes at the R-site (complex I) and A-site (complex III, after GTP hydrolysis and transpeptidation). They found that cross-linking patterns for 30 S and 50 S subunits were nearly identical and concluded that both A- and R-sites are 'similar structurally'. We also observe the absence of any changes in the amounts of ^{14}C label cross-linked to both subunits in complexes I and III. But the sets of proteins labelled in A- (complex III) and R- (complex I) sites are not identical (see fig.1), indicating some differences in arrangement of the tRNA molecule in these complexes.

In the literature only few data are available on the influence of the peptidyl moiety on the arrangement of tRNA at the A-site. Lin et al. [25] reported that photocross-linking of tRNA^{Phe} derivatives with an arylazido group of the s^4U_8 residue to protein S19 was the same when the derivative of Phe-tRNA^{Phe} (situation similar to our complex II but with deacylated tRNA at the P-site) or AcPhePhe-tRNA^{Phe} (our complex III) was located at the A-site. The use of azido-tRNA^{Phe} allowed us to detect the differences in arrangement of the tRNA molecule at the A-site caused by transpeptidation.

Hence, our data directly indicate the changes in arrangement of the tRNA molecule in the course of 3 steps of enzymatic binding at the A-site: before GTP hydrolysis, after GTP hydrolysis and release of EF-Tu, and after transpeptidation.

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