PREVENTION OF RIBOSOMAL DONOR SITE FROM OCCUPATION BY AMINOACYL tRNA DURING POLYPEPTIDE SYNTHESIS

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1. Introduction

Polypeptide chain elongation consists of three separate steps, namely, binding of aminoacyl tRNA to the acceptor site (A-site), peptide bond formation, and translocation of the peptidyl tRNA from the A-site to the donor site (D-site) [1–3]. The substrate for the translocase is a complex of the ribosome with peptidyl tRNA on the A-site and unesterified tRNA on the D-site. In the process of translocation of peptidyl tRNA from the A-site to the D-site, the unesterified tRNA is released from the D-site [4, 5]. It has been found that the presence of aminoacyl tRNA on the donor site prevents translocation because of the inability of the G factor (translocase) to release aminoacyl tRNA from the D-site. Consequently, aminoacylation of unesterified tRNA bound to the D-site would stop chain elongation. It is therefore conceivable that some mechanism exists to prevent such aminoacylation of ribosome-bound tRNA. In this paper we present evidence that such a mechanism indeed exists.

2. Results and discussion

In the experiment described in table 1, a complex consisting of ribosomes, tRNA specific for phenylalanine ($tRNA^{Phe}$), and poly U was isolated, and the phenylalanine acceptor capacity of the bound $tRNA^{Phe}$ was compared with that of unbound $tRNA^{Phe}$. As shown in this table only 5% of the bound $tRNA^{Phe}$ was aminoacylated under the condition where unbound tRNA was esterified 97%. In this experiment, the ribosomal complex was prepared in

Table 1
Inability of ribosome-bound tRNA to accept amino acid.

Substrate	Phenylalanyl tRNA formed (% of total)
A) Ribosomal complex of $tRNA^{Phe}$	5.1
B) Ribosomal complex of $tRNA^{Phe}$ prepared at 6 mM Mg^{2+}	2.8
C) Unbound $tRNA^{Phe}$	97.3

The reaction mixture (1.0 ml) for the formation of the complex of $tRNA^{Phe}$, ribosomes and poly U contained 100 μmoles of Tris-HCl, pH 7.8, 50 μmoles of KCl, 6 or 20 μmoles of magnesium acetate, 6 μmoles of 2-mercaptoethanol, 15.5 mg of NH_4Cl washed ribosomes, 8 mg of an unfractionated tRNA mixture and 0.8 mg of poly U. After incubation for 30 min at 30° the complex of $tRNA^{Phe}$, ribosomes and poly U was isolated by sucrose density gradient centrifugation. The reaction mixture (4.8 ml) for aminoacylation of bound and unbound tRNA contained the following: 100 mM Tris-HCl, pH 7.8, 50 mM KCl, 20 mM magnesium acetate, 3 mM dithiothreitol, 3 mM 2-mercaptoethanol, and 1.5 mM ATP. In addition it contained either ribosomal complex (24.4 A_{260} units in A, 24.0 A_{260} units in B) or a mixture of tRNA (175 μg), 0.9 μCi of ^{14}C-phenylalanine (385 μCi/μmole, counting efficiency 1.0×10^6 cpm/μCi), and 0.1 mg of purified *E.coli* B phenylalanyl tRNA synthetase [9]. The reaction mixture was incubated for 2.5 min and 0.6 ml of the reaction mixture was poured through a Millipore filter. Transfer RNA aminoacylated on the ribosome during this procedure was measured by counting radioactivity on the ribosomes which is trapped by the Millipore filter [12]. The amounts of total $tRNA^{Phe}$ bound to ribosomes were 6067 cpm and 7104 cpm in (A) and (B) respectively, which were measured by amino acid acceptor capacity after release of all $tRNA^{Phe}$ from ribosomes by washing buffer (100 mM Tris-HCl, pH 7.2, 50 mM KCl, 0.1 mM magnesium acetate and 6 mM 2-mercaptoethanol) [10]. Aminoacylation of the unbound tRNA was measured by counting radioactivity insoluble in cold trichloracetic acid. The total amount of unbound $tRNA^{Phe}$ was 10,475 cpm.

Table 2
Effect of G factor on aminoacylation of tRNA bound to ribosomes.

Addition	Phenylalanyl tRNA formed (% of total)
Ribosomal complex with $tRNA^{Phe}$	1.7
+GTP	1.4
+ G factor	2.5
+GTP + G factor	37.7
+GTP + G factor + fusidic acid	17.9

Experimental conditions for aminoacylation of tRNA were identical to table 1. Where indicated, 7.5 μg of G factor, 0.4 mM GTP and 1 mM fusidic acid were added. The reaction mixture (1.2 ml) was incubated for 3 min and the tRNA aminoacylated was measured by counting the radioactivity of cold TCA insoluble material. The total amount of tRNA bound to ribosome was equivalent to 6126 cpm of ^{14}C-phenylalanyl tRNA.

the presence of 6 mM or 20 mM Mg^{2+}. In the former condition the $tRNA^{Phe}$ was bound predominantly to the D-site while in the latter condition both D- and A-sites were occupied [6]. It is clear from this table that tRNA bound to the ribosome could not be aminoacylated regardless of its ribosomal site for binding.

Although data are not shown here, in the presence of low Mg^{2+} or after prolonged incubation, a considerable aminoacylation occurred due partly to release of tRNA and subsequent aminoacylation of the released tRNA. Since G factor is known to release tRNA from the ribosomal complex containing tRNA on both sites, the effect of G factor on the aminoacylation of bound tRNA was examined. As can be seen in table 2, the addition of G factor resulted in aminoacylation of $tRNA^{Phe}$ due to its action of releasing $tRNA^{Phe}$ from the ribosomes. This activity of G factor was dependent on the addition of GTP and inhibited by fusidic acid [7]. These data indicate that translocation of tRNA can be assayed conveniently by following aminoacylation of tRNA which is released from the ribosomal complex by G factor.

Another possible way to bind aminoacyl tRNA to the D-site would be to exchange the D-site bound

Table 3
Selective exchange of A-site bound $tRNA^{Phe}$ with free *N*-acetylphenylalanyl tRNA

Ribosomal complex	Incubation time (min)	*N*-acetyl ^{14}C-phenyl-alanyl tRNA bound (cpm)	*N*-acetyl ^{14}C-phenyl-alanyl puromy-cin formed (cpm)
1) ribosomes with $tRNA^{Phe}$ and poly U	30	1889	7
	60	2433	199
2) ribosomes with NAc-^{14}C-phe tRNA and poly U	30	4044	1233
	60	4112	1512

In exp.1 the reaction mixture (0.45 ml) for exchange of ribosome-bound $tRNA^{Phe}$ with *N*-acetyl-^{14}C-phenylalanyl tRNA contained the following: 100 mM Tris-HCl, pH 7.2, 80 mM KCl, 20 mM magnesium acetate and 6 mM 2-mercaptoethanol. In addition, it contained 110 μg of *N*-acetyl-^{14}C-phenylalanyl tRNA (55,000 cpm) and 2.42 A_{260} units of ribosomal complex with $tRNA^{Phe}$ prepared as described in table 1. In exp.2 ribosomal complex with *N*-acetyl-^{14}C-phenylalanyl tRNA was prepared as described in table 1 except for replacement of tRNA with *N*-acetyl-^{14}C-phenylalanyl tRNA. *N*-acetyl-^{14}C-phenylalanyl tRNA was further added to this complex under identical conditions as in exp.2. In both experiments the reaction mixture was incubated for 30 min or 60 min at 30° and 0.2 ml of the mixture was poured through a Millipore filter. *N*-acetyl-^{14}C-phenylalanyl tRNA bound to ribosome was measured by counting radioactivity on the Millipore filter. The reaction mixture for the assay of puromycin reaction with bound *N*-acetyl-^{14}C-phenylalanyl tRNA was identical to that for the exchange reaction except that it contained 0.55 mM puromycin. *N*-acetyl phenylalanyl puromycin was measured as described [11]. The total amount of bound $tRNA^{Phe}$ and *N*-acetyl ^{14}C-phenylalanyl tRNA measured by aminoacylation was 3641 cpm.

tRNA with aminoacyl tRNA. Such an exchange would stop chain elongation because of the inability of G factor to release aminoacyl tRNA from the D-site. As shown in table 3 ribosomes apparently have a built-in mechanism to prevent exchange of unesterified tRNA with aminoacyl tRNA at the D-site. In this experiment the ribosomal complex with $tRNA^{Phe}$ was prepared in 20 mM Mg^{2+}. To this complex *N*-acetyl-^{14}C-phenylalanyl tRNA was added,

and the bound N-acetyl-^{14}C-phenylalanyl tRNA and its ability to react with puromycin were measured. Since the ribosomes were already occupied with $tRNA^{Phe}$ the binding of *N*-acetyl-^{14}C-phenylalanyl tRNA must be mostly due to exchange of bound $tRNA^{Phe}$ with *N*-acetyl-^{14}C-phenylalanyl tRNA. Of the bound *N*-acetyl-^{14}C-phenylalanyl tRNA only 10% reacted with puromycin, indicating that most of the exchange took place at the A-site but not at the D-site where the puromycin reaction was expected to occur [8]. Further evidence that the exchange took place mostly at the A-site was obtained by a separate experiment. The addition of G factor and GTP caused 66% of the exchanged *N*-acetyl-^{14}C-phenylalanyl tRNA to react with puromycin at 30° for 30 min (data not shown here). As a control, a ribosomal complex was prepared with *N*-acetyl-^{14}C-phenylalanyl tRNA in the absence of $tRNA^{Phe}$ (table 3, exp.2). Under this condition a large portion of the bound *N*-acetyl-^{14}C-phenylalanyl tRNA reacted with puromycin, indicating that *N*-acetyl phenylalanyl tRNA can indeed bind to the D-site provided the D-site is not occupied with unesterified tRNA. Since the donor site will never become empty during polypeptide synthesis this situation does not occur physiologically.

In summary, the ribosome structure somehow prevents the D-site from being occupied with aminoacyl tRNA during polypeptide synthesis through the prevention of aminoacylation and the exchange reaction of the D-site bound tRNA.

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