Ribosomal protein L16 binds to the 3′-end of transfer RNA

Toivo Maimets, Jaanus Remme* and Richard Villems*+

Laboratory of Molecular Biology, Tartu State University and **Laboratory of Molecular Genetics, Institute of Chemical Physics and Biophysics, 14/16 Kingissepa Street, 202400 Tartu, Estonian SSR, USSR*

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Escherichia coli 50 S ribosomal subunits were reconstituted with and without protein L16 present. The latter particles, although active in puromycin reaction, were unable to use CACCA-Phe as an acceptor substrate. We also found that L16 interacts directly with this oligonucleotide and, in the complex with tRNA, protects its 3′-end from pancreatic ribonuclease digestion. We suggest that the role of L16 is in the fixation of the aminoacyl stem of tRNA to the ribosome at its A-site.

Peptidyl transferase *Protein L16* *tRNA–ribosome interaction*

1. INTRODUCTION

Protein L16 is known to cross-link to various tRNA analogues in situ (review [1,2]), as well as to restore the peptidyl transferase (PT) activity of LiCl-stripped 50 S core particles [3]. Our earlier experiments revealed two seemingly contradictory phenomena: L16 interacts with tRNA in solution [4], whereas it is not needed for the reaction carried out with puromycin as the peptide acceptor [5]. We here try to resolve this problem.

2. EXPERIMENTAL

Escherichia coli MRE600 50 S ribosomal subunits, 2 M LiCl core particles and split proteins, individual L16 and split proteins lacking L16 were obtained as in [4,5]. Reconstitution of 50 S subunits was as in [6]. CACCA-[^{14}C]Phe (18.5 GBq/mmol) was a kind gift from Dr M. Kukhanova, f[^{35}S]Met–tRNA$_f^{Met}$ was prepared [7], using commercial (Boehringer, Mannheim) tRNA$_f^{Met}$ and [^{35}S]methionine (Amersham International, 46.4 GBq/mmol).

Dipeptide formation between CACCA-Phe and fMet–tRNA$_f^{Met}$ was carried out as follows. Reconstituted 50 S subunits (0.5 mg) were dissolved in 50 μl of 20 mM Tris–HCl (pH 7.5) containing 20 mM $MgCl_2$, 250 mM NH_4Cl and 2 mM 2-mercaptoethanol. After activation (40°C, 10 min), 80 pmol of CACCA-[^{14}C]Phe and 15 pmol of f[^{35}S]Met–tRNA$_f^{Met}$ were added and the final concentrations of NH_4Cl (550 mM) and $MgCl_2$ (40 mM) were adjusted by their concentrated stock solution. An equal amount (122 μl) of methanol was added and the reaction was allowed to proceed for 90 min at 30°C. The reaction was stopped by adding 12 μl of 10 M NaOH.

The precipitate was collected by centrifugation and washed twice with 200 μl of ethanol. Combined supernatants were evaporated under vacuum to the vol. (~50 μl) and electrophoresed (Whatman no.2, 50 V/cm, 90 min) in · pyridine:acetic acid:H_2O (1:10:89). The paper strips were dried, cut into 1-cm pieces and their radioactivity was counted in a dioxane-based scintillation cocktail.

The interaction between individual protein L16 and CACCA-[^{14}C]Phe was studied using the nitrocellulose filtration technique. Similarly to [4], constant amounts of oligonucleotide were incubated with increasing amounts of L16. The radioactivity retained by nitrocellulose membrane filters (Schleicher & Schüll, BA85) was taken as the measure of the oligonucleotide in the complex.

+ To whom correspondence should be addressed

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Ribonuclease protection experiments were performed as follows. f[^{35}S]Met–tRNA$_f^{Met}$ (4×10^{-7} M) was incubated with or without 6 ng of RNase A (EC 3.1.4.22) in the presence or absence of the individual protein L16 (10^{-5} M). Incubation was carried out in 100 μl 20 mM Tris–HCl (pH 7.5), 150 mM NH_4Cl, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol and 0.05% Triton X-100 at 0°C. In these conditions almost all the tRNA is in the complex with the protein [4]. After various time intervals (legend to fig.2) samples were withdrawn and pipetted directly into 1 ml of 5% trichloroacetic acid, kept on ice for 30 min and filtered through glass-fiber filters (Schleicher & Schüll, GF92). The filters were washed with ice-cold 5% trichloroacetic acid, ethanol, dried and their radioactivity was counted in a dioxane scintillation cocktail.

3. RESULTS

That under certain ionic conditions L16 is not needed for the PT reaction as such became evident from experiments where puromycin was used as an acceptor substrate [4]. We have found, however, that by replacing puromycin with a longer 3′-end analogue of tRNA, CACCA-Phe, dipeptide formation depends upon the presence of L16. 2 M LiCl-stripped 50 S core particles, as well as those reconstituted with split proteins lacking L16 are both inactive in fMet-Phe formation, whereas subunits reconstituted from the total fraction of split proteins or from the core particles and individual L16 are active in this reaction (table 1).

In our earlier paper we demonstrated that, in solution, protein L16 interacts with tRNA with $K_d \sim 5 \times 10^{-7}$ M and suggested that L16, in particular its N-terminal region, is involved in tRNA binding to the ribosome [4]. Therefore, it was interesting to study whether puromycin and CACCA-Phe also interact with this protein.

Using several methods, among them equilibrium dialysis, we could not detect any interaction between L16 and puromycin (maximum concentration 3×10^{-5} M and 10^{-3} M, respectively). This, however, is not surprising: this antibiotic interacts very weakly even with the ribosome [8] and, according to in situ cross-linking experiments, is not in the vicinity of L16 but rather of L23 [9]. Since puromycin is an analogue of the 3′-terminal aminoacylated adenosine, one might speculate that L16 is not involved in the binding of this end of the aminoacylated tRNA to the ribosome.

Table 1

Dipeptide formation with CACCA-[^{14}C]Phe and f[^{35}S]Met–tRNA$_f^{Met}$ using different reconstituted 50 S ribosomal subunits

Particle	Dipeptide formed (%)
50 S	100
50 Sc	0
50 Sc + SP	45
50 Sc + SP$_{-L16}$	4
50 Sc + SP$_{-L16}$ + 5 × L16	43

fMet-Phe synthesis was carried out as described in section 2. Dipeptide formation by intact 50 S ribosomal subunits was taken as 100% and is equal to 8.1 pmol of f[^{35}S]Met–tRNA$_f^{Met}$ utilized. 50 Sc, 2 M LiCl-stripped 50 S subunits; SP, split proteins; SP$_{-L16}$, split proteins deprived of protein L16; 5 × L16, 5-fold molar excess of L16 over 50 Sc

In contrast, CACCA-Phe interacts with L16 (fig.1). Because of the limited amount of the fragment, we have been unable to characterize this interaction in detail; the fact that it forms a plateau at about 0.7 mol of the fragment/mol of L16 suggests that this interaction is stoichiometric.

Still, these results do not explain why the particles reconstituted without L16 were found to be

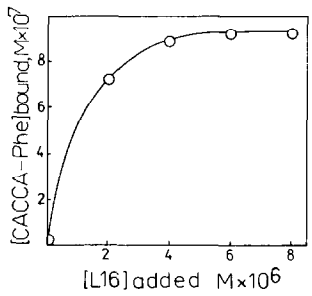

Fig.1. The binding of CACCA-[^{14}C]Phe to 50 S ribosomal subunit protein L16. A constant amount of CACCA-Phe (1.6×10^{-6} M) was incubated with increasing amounts of protein L16. The samples were passed through nitrocellulose membrane filter disks and the radioactivity retained to filters was counted. For other details see section 2.

inactive in dipeptide formation with CACCA-Phe. One of the possible reasons may be in the too low concentration of the fragment ($\sim 3 \times 10^{-7}$ M). Notably, very little, if any, peptidyl puromycin formation occurs when puromycin is used at 10^{-4} M or lower concentrations. Therefore, even if CACCA-Phe does interact with 50 S particles in the absence of L16 through its terminal adenine and aminoacyl moiety (the counterparts of puromycin), this contact might occur too rarely to allow the covalent reaction to proceed at a significant rate.

Anyhow, it can be equally well suggested that the other nucleotides of CACCA-Phe are responsible for its binding to L16. Therefore, it was interesting to know whether L16 can protect this region of the tRNA against RNase cleavage. A simple experiment was designed where we made use of the fact that only short oligonucleotides do not precipitate in 5% trichloroacetic acid. To control this assumption we first digested f[^{35}S]Met–tRNA$_f^{Met}$ with RNase A and U_2. The 3′-terminal sequence of tRNA$_f^{Met}$ is –GCAA-CCA$_{OH}$ [10]. Hence, RNase A could yield the following short oligonucleotides labelled by f[^{35}S]Met: A-fMet and CA-fMet, whereas the shortest possible fragment generated by RNase U_2 is CCA-fMet. Since trichloroacetic acid-soluble radioactive products have not been formed by RNase U_2, we conclude that CCA-fMet is already too long to be found in this sort of experiment. In contrast, RNase A digestion left all radioactivity in the acid soluble fraction, revealing that at least A-fMet does not precipitate in 5% trichloroacetic acid.

Next, we compared the amounts of acid-soluble material generated by RNase A from f[^{35}S]Met–tRNA$_f^{Met}$ with and without L16 present. As controls, we compared these results with those obtained in the presence of two other proteins, S3 and S4. Both of them give, like L16, stable complexes with tRNA with comparable K_d values (in preparation). Fig.2 shows the results of these experiments. During 30 min of incubation, about 60% of the label was found in the trichloroacetic acid supernatant (line 1). Neither S3 nor S4 had much influence on the RNase action (lines 2,3), whereas in the presence of L16 only about 10% of radioactivity was in the supernatant (line 4). The identity of rates of the spontaneous deacylation of

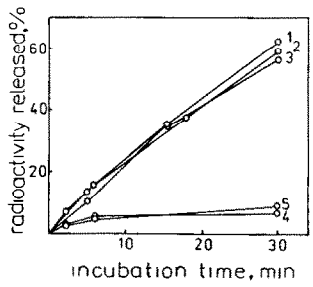

Fig.2. The hydrolysis of f[^{35}S]Met–tRNA$_f^{Met}$ and its various protein complexes with RNase A. After incubation at 0°C for a given time, cold trichloroacetic acid was added up to 5%. The samples were passed through glass-fiber filters and the trichloroacetic acid-precipitated radioactivity retained to filters was measured. fMet–tRNA$_f^{Met}$ (4×10^{-7}) was incubated with RNase A (line 1); RNase A + protein S3 or S4 (lines 2,3); RNase A + L16 (line 4); without RNase or protein present (line 5). Concentration of the proteins was 10^{-5} M. For other details see section 2.

fMet–tRNA$_f^{Met}$ in the presence and absence of protein L16 (lines 4,5) shows that this protein itself has no influence, neither stabilizing nor destabilizing, on the chemical bond linking the amino acid to the 3′-terminal ribose of tRNA$_f^{Met}$. This tRNA was chosen in this experiment precisely for its comparatively slow spontaneous deacylation compared with aminoacyl tRNAs.

Therefore, although the precise location of the RNase cleavage sites in tRNA$_f^{Met}$ in our experiments was not estimated, they are certainly located within the last few 3′-terminal nucleotides. As far as the protection by L16 is concerned, we suggest that phosphodiester bonds between C-75 and C-76 and/or C-76 and A-77 of the tRNA are shielded.

Here, the question about the possible protective role of L16 was addressed only to the 3′-end sequence of tRNA. Whether or not the presence of L16 protects some other parts of tRNA as well was not investigated.

4. DISCUSSION

It is well established that, within the ribosome, the 3′-terminal region of tRNA is strongly protected against chemical probes. Thus, according to slow tritium exchange experiments, the purines of

the terminal ACCA sequence of $tRNA^{Phe}$ have much slower exchange rates when $tRNA^{Phe}$ is bound to the ribosome [11]. The two terminal cytidine residues of the ribosome-bound $tRNA^{Phe}$ are efficiently shielded from methylation by dimethyl sulfate; the same cytidines are the only accessible cytidines of $tRNA^{Phe}$ in solution [12]. Authors in [12] argue that C-74 and C-75 of $tRNA^{Phe}$ interact with the ribosome by base-pairing: the protection from methylation occurs at N-3 of cytidines, which, in a G·C base pair is hydrogen-bonded. On the other hand, N-3 of cytidine may as well form a hydrogen bond with amino acids of a polypeptide. Therefore, the very fact of this type of protection is not necessarily an indication of the involvement of the CCA-end of tRNA in a complementary RNA–RNA interaction.

The results presented here and in [4,5] allow us to speculate that ribosomal protein L16 is involved in the fixation of the 3′-end of tRNA to the ribosome. Since L16 is not needed for the puromycin reaction [5], but the CACCA-Phe-mediated dipeptide formation depends upon its presence, we suggest that L16 may interact with the A-site bound tRNA. In particular, our experiments predict that the N-terminal residues 10–16 of L16 bind the 3′-end sequence of tRNA bound to the ribosomal A-site.

The fact that the modification of unidentified histidyl residues of 50 S ribosomal subunit proteins, possibly also the His-13 of L16, abolishes the interaction of the 3′-end of aminoacylated tRNA with the ribosomal A-site [13], lends further credence to the suggestion made here ([4] for further discussion).

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