Studies on the Formation of Transfer Ribonucleic Acid-Ribosome Complexes

VII. THE ROLE OF THE 3'-HYDROXYL-TERMINAL END OF TRANSFER RIBONUCLEIC ACID FOR INTERACTION WITH RIBOSOMES AND RIBOSOMAL SUBUNITS*

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SUMMARY

The ability of altered transfer ribonucleic acid to compete with binding of [14C]aminoacyl- and [14C]tRNA was used as an estimate of its capacity to bind to ribosomes. Transfer ribonucleic acid with the terminal 3'-hydroxyl, C, A, and C, C, A enzymatically removed could bind to 70 S ribosomes with progressively lower efficiency; in contrast, binding to 30 S subunits was unimpaired. Transfer ribonucleic acid with the entire CCA terminus removed could bind to 70 S ribosomes about half as well as intact tRNA. Periodate oxidation reduced the binding of tRNA to 70 S ribosomes by 80%. Periodate, therefore, not only oxidizes the terminal vicinal hydroxyl groups of tRNA, but also 1 internal residue of certain tRNA species.

Several steps in protein biosynthesis involve the interaction of tRNA with ribosomes (2-4). In binding to ribosomes both tRNA and aminoacyl-tRNA can specifically recognize template codons (3, 5). Since both acylated and deacylated tRNA are present in cells (6), their relative proportions may influence the rate of protein synthesis.

The translation of individual messenger RNA codons involves the interaction of a specific tRNA region, the anticodon, with the codon on the 30 S subunits of Escherichia coli ribosomes (7-10). For binding of the amino acid end of aminoacyl-tRNA to ribosomes addition of the 50 S subunit is required (11, 12) as it is for protein biosynthesis (13, 14). Previous studies (15, 16) have suggested that an intact 3'-hydroxyl end (-C, C, A) is necessary for binding of tRNA to 70 S ribosomes. On the other hand, an anticodon-containing fragment of formylmethionine tRNA can bind to ribosomes in response to a specific codon (17).

Since the binding of deacylated tRNA to ribosomes may regulate the rate of protein synthesis, the present studies were directed at elucidating some of the structural requirements of tRNA for interaction with ribosomes and ribosomal subunits. Some of these requirements are outlined in this report.

EXPERIMENTAL PROCEDURE

Ribosomes and Cell Extracts—Ribosomes and cell extracts were prepared from E. coli cells grown to mid-log phase. The procedures for the preparation of cells, cell fractions, ribosomes, and ribosomal subunits have been described previously (1, 10, 18, 19). Preparations of tRNA and [14C]aminoacyl-tRNA have been reported previously (18). Ribosomes washed in 1 M ammonium chloride were prepared from E. coli W-3100. Protein was determined by the method of Lowry et al. (20).

Chemicals, Radioisotopes, and Materials—Uniformly labeled [14C]L- and [12C]phenylalanine (333 and 1500 mCi per mmole, respectively) and [14C]lysine (221 mCi per mmole) were obtained from New England Nuclear. [14C]2-Uracil (50 mCi per mmole) was obtained from Schwarz BioResearch. Nitrocellulose membrane filters were type HA (0.45 μm pore size), 25-mm diameter Millipore filters. Poly U1 and poly A1 were obtained from Miles Laboratories (Elkhart, Indiana). Unless otherwise noted, tRNA was prepared from E. coli W-3100; E. coli B tRNA was obtained from General Biochemicals. Snake venom phosphodiesterase and E. coli alkaline phosphatase were purchased from Worthington.

Determination of [14C]Aminoacyl-tRNA Binding to Ribosomes—[14C]tRNA and [14C]aminoacyl-tRNA binding to ribosomes was determined by washing the ribosomes on nitrocellulose filters as previously reported (21).

Preparation of Periodate-oxidized tRNA—Transfer RNA was oxidized with sodium metaperiodate by the procedure of Neu and Heppel (22). After oxidation the tRNA was dialyzed to remove any salts and side products. Alkaline phosphatase was used to remove terminal phosphates from periodate-treated tRNA as previously described (22).

Preparation of [14C]tRNA—[14C]tRNA was prepared from E. coli W-3100. [14C]Uracil was added to log phase cells growing in minimal medium (23) with 1% glucose. Final uracil concen-
Fig. 1. Phenylalanine acceptance as a function of venom phosphodiesterase treatment of tRNA. Phenylalanine acceptance was determined as described under "Experimental Procedure" with purified phenylalanyl-tRNA synthetase and with unfractionated synthetases containing the enzymes which incorporate CMP and AMP into the amino acid acceptor end of tRNA. Reactions contained the following components in a volume of 0.65 ml: 0.05 m potassium cacodylate, pH 7.0; 0.03 m KCl; 0.03 m MgCl₂; 0.01 m ATP; 2.8 × 10⁻⁶ M n-phenylalanine (specific activity adjusted to 100 mCi per pmole with unlabeled l-phenylalanine); tRNA, 0.2 to 0.5 AZeO units per reaction mixture; enzyme mixture, 2 mg per ml. Incubations were performed for 20 min at 37° and percentage acylation was determined from a curve of acylation as a function of tRNA concentration for each tRNA preparation. On the graph acylation is expressed as a percentage of the value of an untreated control tRNA. The 100% value for the untreated tRNA is 40 pmoles per A₂₆₀ unit of tRNA. ●, acceptance remaining, determined with the use of the partially purified phenylalanine synthetase; ○, acceptance remaining, determined with the use of the unfractionated enzyme mixture; Δ, acceptance remaining, determined with the use of the unfractionated enzyme mixture and 0.0002 M CTP in the incubations.

As can be seen in Fig. 1, loss of the terminal adenylate residue is a function of time of incubation of the tRNA with the venom diesterase. To estimate the loss of the first terminal cytidylic acid residue, acylation was carried out in the usual reaction mixture with the unfractionated enzyme eluted from DEAE-cellulose (28). This enzyme mixture also contained the enzyme which incorporates AMP and CTP into the 3'-hydroxyl end of tRNA molecules. Thus any loss in acceptor activity can be considered a reflection of loss of a single residue or more from the 3'-hydroxyl terminus of the molecule.

Fig. 2 One AZeO unit is the amount of material which in 1.0 ml would yield a value of 1.0 fo the absorbance measured at 260 μg in a cuvette with a path length of 1.0 cm.
Table I

<table>
<thead>
<tr>
<th>Phenylalanine-accepting species</th>
<th>Phosphodiesterase</th>
<th>Acid-soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>tRNA</td>
<td>X_C_C_A</td>
<td>X_C_C_A</td>
</tr>
<tr>
<td>tRNA*</td>
<td>X_C_C_A</td>
<td>X_C_C_A</td>
</tr>
<tr>
<td>tRNA</td>
<td>X_C_C_A</td>
<td>X_C_C_A</td>
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<tr>
<td>tRNA</td>
<td>X_C_C_A</td>
<td>X_C_C_A</td>
</tr>
</tbody>
</table>

*This molecule terminates in a phosphorylated 3'-OH: tRNA = -X_C_C_P.

Table II

<table>
<thead>
<tr>
<th>tRNA fraction</th>
<th>[14C]Phe accepted pmoles/A260 unit tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>tRNA (unfractionated)</td>
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</tr>
<tr>
<td>tRNA$_{Ph}$</td>
<td>493</td>
</tr>
<tr>
<td>tRNA$_{Ala}$</td>
<td>0</td>
</tr>
<tr>
<td>tRNA$_{Met}$</td>
<td>0</td>
</tr>
<tr>
<td>tRNA$_{Ala}$</td>
<td>0</td>
</tr>
</tbody>
</table>

Table III

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Phenylalanine-accepting tRNA after various treatments</th>
</tr>
</thead>
</table>
| E. coli B tRNA was treated with venom phosphodiesterase or with sodium metaperiodate as described under "Experimental Procedure." The tRNA preparations designated with subscripts 1 to 4 were treated with snake venom phosphodiesterase for the times indicated in the table; tRNA refers to unfractionated E. coli B tRNA, not treated with the diesterase; tRNA$_{periodate}$ refers to sodium metaperiodate-oxidized tRNA; tRNA$_{periodate}$ phosphatase refers to periodate-oxidized tRNA, which was dephosphorylated with alkaline phosphatase subsequent to the periodate oxidation. The "percentage of total tRNA made acid-soluble" refers to the venom phosphodiesterase reaction only. The integrity of the amino acid acceptor end of the phenylalanine-accepting tRNA species was estimated from data presented in Fig. 1 as described under "Experimental Procedure." Molecules of tRNA which could not accept any phenylalanine are indicated in the column "Minus ≥4 Terminal Bases (or Inactive)."

Phenylalanine acceptance by various tRNA fractions

Phenylalanine acceptance by the tRNA fractions was determined as described under "Experimental Procedure" and in the legend to Fig. 1 with partially purified phenylalanyl-tRNA synthetase.
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Fig. 2. Binding of [3H]Phe-tRNA to 70 S ribosomes in the presence of intact and modified tRNA preparations. Binding of [3H]Phe-tRNA to 70 S ribosomes was determined as described under "Experimental Procedure" in 0.050 ml reaction mixtures containing the following components: 5.7 pmoles of [3H]Phe-tRNA (0.18 A260 unit); 4.0 A260 units of the various tRNA preparations described in Table I where indicated; 0.02 M magnesium acetate; 0.05 M potassium acetate; 0.05 M Tris-acetate, pH 7.2; 23 nmoles of nucleotide residues of poly U; and 2.3 A260 units of 70 S ribosomes. Incubations were performed at 24°. A solution of ribosomes and poly U was added last to start the reaction. (a), no additions; (b), unfractionated tRNA; (c), tRNA_A; (d), tRNA_B; (e), tRNA_4; (f), periodate-oxidized tRNA; (g), tRNA oxidized by periodate, followed by removal of terminal phosphates by alkaline phosphatase.

Fig. 3. Binding of [3H]Phe-tRNA to 70 S ribosomes as a function of concentration of various altered tRNA preparations. Binding of [3H]Phe-tRNA to ribosomes was determined as described under "Experimental Procedure" in 0.050-ml reaction mixtures containing the following components: 6.4 pmoles of [3H]Phe-tRNA (0.2 A260 unit); 0.26 nmole of poly U; 0.5 A260 unit of 70 S ribosomes; 0.05 M potassium acetate and 0.05 M Tris-acetate, pH 7.2; 0.02 M and 0.01 M magnesium acetate A and B, respectively. The various tRNA preparations used are described in Table I; their concentrations are indicated on the abscissa. Reactions were started by adding last a solution of ribosomes and poly U to the tubes. Incubations were performed at 24° for 20 min. (a), unfractionated tRNA from E. coli B; (b), tRNA_A; (c), tRNA_B; (d), periodate-oxidized tRNA; (e), tRNA oxidized by periodate, followed by removal of terminal phosphates by alkaline phosphatase.

Fig. 4. Binding of [3H]Phe-tRNA to 30 S subunits in the presence of intact and modified tRNA preparations. Binding of [3H]Phe-tRNA to 30 S subunits was performed as described in the legend to Fig. 2 except that 0.47 A260 unit of 30 S subunits was present in each reaction instead of 70 S ribosomes. (0), no additions; (a), unfractionated tRNA; (b), tRNA_A; (c), tRNA_B; (d), tRNA_4; (e), tRNA_5; (f), periodate-oxidized tRNA; (g), tRNA oxidized by periodate, followed by removal of terminal phosphates by alkaline phosphatase.

Nevertheless, the periodate-oxidized tRNA was able to bind to 70 S ribosomes at both 0.01 M and 0.02 M magnesium ion about one-fifth as well as intact deacylated tRNA. The data in Table I indicate that the percentage of unoxidized tRNAPhe in the periodate-treated preparation is less than 1. Therefore, the competition of periodate-oxidized tRNA with [3H]Phe-tRNA is probably due to oxidized tRNA rather than any unoxidized tRNA species in the preparation. Similarly, the data in Table I indicate that the competition of tRNA_A and tRNA_B with [3H]Phe-tRNA for ribosomal binding sites appears to be due to tRNA with essentially no terminal adenylic acid and, in the case of tRNA_4, the C,C,A terminus.

Similar experiments performed with 30 S ribosomal subunits indicate that enzymatic removal of the terminal CCA ends has little or no effect on the ability of these tRNA preparations to compete with [3H]Phe-tRNA for binding to 30 S ribosomes (Fig. 4A). Periodate-oxidized tRNA interferes with [3H]Phe-tRNA binding to 30 S ribosomes, but is only about two-thirds as effective as an equivalent amount of intact deacylated tRNA (Fig. 4B).

Analogous experiments were performed with [3H]Lys-tRNA in the presence of poly A (Fig. 5). The results indicated that periodate-oxidized and venom phosphodiesterase-treated tRNA preparations competed with [3H]Lys-tRNA binding to 70 S ribosomes in a manner similar to their competition with [3H]Phe-tRNA for binding to ribosomes. It is assumed that the results presented in Table I probably extrapolate to tRNA_A and other species which may compete with [3H]Lys-tRNA binding to ribosomes.

To determine whether these results applied only to tRNA bound to ribosomes in response to a template, analogous studies with 70 S ribosomes and 30 S subunits were performed with the use of [3H]tRNA. The results suggest that tRNA with the terminal -A removed is as effective as intact deacylated tRNA in inhibiting [3H]tRNA binding to ribosomes. Removal of the
FIG. 5. Binding of $[^{14}C]$Lys-tRNA to 70 S ribosomes in the presence of intact and modified tRNA preparations. Binding of $[^{14}C]$Lys-tRNA to 70 S ribosomes was determined as described under "Experimental Procedure" in 0.050-ml reaction mixtures containing the following components: 8.7 pmoles of $[^{14}C]$Lys-tRNA (0.27 A$_{260}$ unit); 1.0 A$_{260}$ unit of the various tRNA preparations described in Table I where indicated; 0.02 M magnesium acetate; 0.05 M potassium acetate; 0.05 M Tris-acetate, pH 7.2; 0.22 A$_{260}$ unit of poly A; and 2.3 A$_{260}$ units of 70 S ribosomes. Incubations were performed at 24°C. A solution of ribosomes and poly A was added last to start the reaction. , no additions; , unfractionated tRNA; , tRNA$_{1}$; , tRNA$_{2}$; , periodate-oxidized tRNA; , tRNA oxidized by periodate, followed by removal of terminal phosphates by alkaline phosphatase.

FIG. 6. Binding of $[^{14}C]$tRNA to 70 S ribosomes in the presence of intact and modified tRNA preparations. Binding of $[^{14}C]$tRNA to 70 S ribosomes was determined as described in the legend to Fig. 5 with the following changes in each 0.050-ml reaction mixture: 14 pmoles of $[^{14}C]$tRNA (0.028 A$_{260}$ unit); 0.10 A$_{260}$ unit of 70 S ribosomes; 0.2 A$_{260}$ unit of each unlabeled tRNA where designated. Incubations were performed at 24°C. , no additions; , 0.2 A$_{260}$ unit of unfractionated E. coli B tRNA; , 0.2 A$_{260}$ unit of tRNA$_{1}$; , 0.2 A$_{260}$ unit of tRNA$_{2}$; , 0.2 A$_{260}$ unit of periodate-oxidized tRNA; , 0.2 A$_{260}$ unit of periodate-oxidized tRNA, dephosphorylated with alkaline phosphatase.

penultimate pC and then the antepenultimate pC progressively interfered with the ability of these tRNA preparations to compete with $[^{14}C]$tRNA for binding to 70 S ribosomes (Fig. 6A). Periodate-treated tRNA (both with and without the 3'-terminal phosphate) competed with $[^{14}C]$tRNA for binding to ribosomes at about one-third the rate and extent compared to intact deacylated tRNA (Fig. 6B). Thus, these results with the use of $[^{14}C]$tRNA differ from the results in which $[^{14}C]$Lys and $[^{14}C]$-Phe-tRNA binding to ribosomes was measured (compare Figs. 2, 5, and 6B) in the presence of periodate-treated tRNA. These differences may possibly be due to the use of a template in one case and not the other or to differences in susceptibility of various tRNA species to periodate oxidation at other than terminal sites. Since tRNA$_{Phe}$ from E. coli contains a 4-thiouridylic acid residue (36), periodate oxidation may alter the molecule at this site as well as at the terminal 3' hydroxyl end.

Alternations of the 3'-hydroxyl end of the tRNAs by enzymatic treatment or periodate oxidation had very little effect on the ability of these tRNAs to compete with $[^{14}C]$tRNA for binding to 30 S ribosomes (Fig. 7). Periodate-treated tRNA (both with and without the 3'-terminal phosphate) competed with $[^{14}C]$tRNA for binding to ribosomes at about one-third the rate and extent compared to intact deacylated tRNA (Fig. 6B). Thus, these results with the use of $[^{14}C]$tRNA differ from the results in which $[^{14}C]$Lys and $[^{14}C]$-Phe-tRNA binding to ribosomes was measured (compare Figs. 2, 5, and 6B) in the presence of periodate-treated tRNA. These differences may possibly be due to the use of a template in one case and not the other or to differences in susceptibility of various tRNA species to periodate oxidation at other than terminal sites. Since tRNA$_{Phe}$ from E. coli contains a 4-thiouridylic acid residue (36), periodate oxidation may alter the molecule at this site as well as at the terminal 3' hydroxyl end.
The present results evaluate some structural requirements for tRNA binding to 30 S and 70 S ribosomal particles of *E. coli*. The ability of an altered tRNA to compete with the binding of [*4C*]labeled tRNA to ribosomes in response to poly U was examined in the presence of various purified tRNA preparations (Fig. 8). As can be seen, all of the tRNA preparations tested inhibited the binding of [*4C*]labeled tRNA to ribosomes to some extent. Of all of the fractions, tRNA*Ph* (about 40% pure) was most effective. Since the other tRNA preparations contained no detectable phenylalanine acceptor activity (Table II), their inhibition of [*3H*]Phe-tRNA binding to ribosomes was due to binding of species other than tRNA*Ph* to ribosomes.

**FIG. 8.** The binding of [*3H*]Phe-tRNA*Ph* to ribosomes as a function of the concentration of tRNA*Ph*, tRNA*Phe*, tRNA*Val*, and tRNA*Met*. Each 0.050-ml reaction mixture contained the following components: 0.05 M Tris-acetate, pH 7.2; 0.05 M potassium acetate; 0.02 M magnesium acetate; 10 pmoles of base residues of poly U; 0.14 A_{600} unit of ribosomes; 0.6 pmole of [*4C*]labeled tRNA (0.005 A_{600} unit); 0.66 unit of tRNA*Ph*, tRNA*Phe*, tRNA*Val*, and tRNA*Met* as indicated on the abscissa. Reactions were incubated at 24° for 20 min and assayed as described under "Experimental Procedure." The purified tRNA preparations are described under "Experimental Procedure." The purified tRNA preparations can compete significantly with [*4C*]labeled tRNA for binding to 30 S ribosomes in response to poly U (Figs. 4B and 8A). Periodate-oxidized tRNA competes with [*4C*]labeled tRNA for binding to both 30 S and 70 S ribosomes with specific reaction with [*4C*]labeled tRNA for binding to 30 S ribosomes (Figs. 6 and 7).

Discussions

The present results evaluate some structural requirements for tRNA binding to 30 S and 70 S ribosomal particles of *E. coli*. The ability of an altered tRNA to compete with the binding of [*4C*]labeled tRNA to ribosomes was examined in the presence of various purified tRNA preparations (Fig. 8). As can be seen, all of the tRNA preparations tested inhibited the binding of [*4C*]labeled tRNA to ribosomes to some extent. Of all of the fractions, tRNA*Ph* (about 40% pure) was most effective. Since the other tRNA preparations contained no detectable phenylalanine acceptor activity (Table II), their inhibition of [*4C*]labeled tRNA binding to ribosomes was due to binding of species other than tRNA*Ph* to ribosomes.
contained no phenylalanine acceptor activity, they contained several other contaminating tRNA species, for they were 30, 10, and 89% pure, respectively. Thus, those or other contaminating species of tRNA and tRNA\textsuperscript{32P} compete with \textsuperscript{3H}Phe-tRNA\textsuperscript{32P} for binding to ribosomes. Consistent with these results, Ile-, Val-, and Met-tRNA have been shown to bind to ribosomes in response to poly U codons\textsuperscript{3} (45).

Furthermore, in order for deacylated tRNA to compete maximally with acylated tRNA species, the CCA end must be essentially intact; however, tRNA lacking the terminal CCA end can still interact with 70 S ribosomes, albeit at a reduced rate compared to intact deacylated tRNA. The competition of deacylated tRNA for binding to ribosomes may reflect competition between several sites on the tRNA molecule other than the terminal CCA end. It is thus possible that the CCA terminus, itself, does not interact strongly with ribosomes unless an amino acid is attached.

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REFERENCES
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