EVIDENCE FOR ONE FUNCTIONAL PHENYLALANYL-tRNA BINDING SITE ON THE 30S RIBOSOMAL SUBUNIT*

BY Kazuei IgarnaSHI AND Akira KAJI†

DEPARTMENT OF MICROBIOLOGY, SCHOOL OF MEDICINE, UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA

Communicated by Seymour S. Cohen, November 25, 1968

Abstract and Summary.—NH₂ terminal analysis of polyphenylalanine formed from 30S subunit-bound ¹⁴C-phenylalanyl-tRNA suggests that there is only one site in a 30S subunit for specific binding of phenylalanyl-tRNA. Assuming that no movement of the bound phenylalanyl-tRNA takes place during association of 30S subunits with 50S ribosomal subunits, the binding site of the 30S ribosomal subunit corresponds to site 2 (—NH₂ side with respect to the growing polypeptide chain) of the two binding sites of 70S ribosomes.

It has been found that the 30S ribosomal subunit binds phenylalanyl-tRNA in the presence of poly U,¹⁻⁴ and that the addition of the 50S ribosomal subunit to this complex results in approximately twofold stimulation of the binding of phenylalanyl-tRNA.⁵ ⁶ From the evidence that two phenylalanyl-tRNA molecules are bound to the complex of 70S ribosomes and poly U,⁷ ⁸ it was suggested that the 30S ribosomal subunit binds one phenylalanyl-tRNA; the addition of the 50S ribosomal subunit creates a second site for the binding of phenylalanyl-tRNA which accounts for the twofold stimulation of the binding of phenylalanyl-tRNA.⁹ In this communication we present additional evidence that the 30S ribosomal subunit binds one phenylalanyl-tRNA.

Materials and Methods.—E. coli extract and other materials: Preparation of ribosomes, tRNA from E. coli B, and aminoacyl tRNA have been described in the preceding communications.¹ ⁹ The ribosomes were washed three times and were free of the aminoacyl tRNA transfer factor¹⁰ or initiation factors.¹¹ ¹² For preparation of 30S and 50S ribosomal subunits, a linear sucrose gradient (5–25% in a buffer containing 0.01 M Tris-succinate (pH 8.0), 0.0001 M magnesium acetate) was prepared in a Beckman B IV zonal rotor. The 70S ribosomes (150 mg, prepared as above) in 10 ml of 0.01 M K-phosphate buffer (pH 7.0), 0.0001 M magnesium acetate, were applied through the center of the rotor to the gradient. The rotor was centrifuged at 40,000 rpm for 5 hr. The fractions containing 30S and 50S subunits were separately pooled, and Mg⁺⁺ concentrations were adjusted to 0.01 M. The subunits were pelleted by centrifugation at 200,000 g for 20 hr. The pellets were resuspended in 0.01 M Tris-succinate buffer (pH 8.0) containing 0.01 M magnesium acetate and were stored in liquid nitrogen until used. Preparation of the E. coli S-150, or soluble protein fraction, free of ribosomes was as described previously.¹³ Specific radioactivity of material used in this paper was ¹⁴C-phenylalanine 385 μc/μmole, and counting efficiency was 1.0–1.5 × 10⁶ cpm/μc.

Reaction mixture for the binding of ¹⁴C-phenylalanyl-tRNA to 30S ribosomal subunits: A typical reaction mixture for the formation of a complex of poly U, 30S ribosomal subunit, and phenylalanyl tRNA contained the following in μmoles/0.75 ml: 60 Tris-HCl (pH 7.1), 30 KCl, and 15.75 magnesium acetate. In addition, it contained 360 μg of poly U, 125,000 cpm of ¹⁴C-phenylalanyl tRNA, and 800 μg of 30S ribosomal subunits. The reaction mixture was incubated for 20 min at 22°C.

Formation of 70S ribosomes from the complex of 30S ribosomal subunits, poly U, and phenylalanyl-tRNA by the addition of 50S ribosomal subunits: The reaction mixture (1.5
ml) for the formation of 70S ribosomes contained the following in μmoles: 60 Tris-HCl (pH 7.1), 22.5 magnesium acetate, and 120 KCl. In addition, it contained 0.75 ml of the above solution containing the complex of poly U, 14C-phenylalanyl tRNA, and 30S ribosomal subunits, 1.66 mg of 50S ribosomal subunits, and 12C-phenylalanyl tRNA (6.76 μmoles) in 19.2 mg of mixture of tRNA's. It is important to add 14C-phenylalanyl tRNA before the addition of 50S ribosomal subunits. The mixture was incubated at 37°C for 3 or 10 min for the formation of 70S ribosomes and then chilled to 0°C. The complex of 70S ribosomes, 14C-phenylalanyl tRNA, 12C-phenylalanyl tRNA, and poly U was separated from unbound phenylalanyl tRNA by sucrose density gradient centrifugation. A 0.5-ml aliquot of the 70S ribosome complex was placed on top of a 5-20% sucrose gradient (4.5 ml) in 10 mM Tris-HCl (pH 7.1), 15 mM magnesium acetate, 80 mM KCl, and 6 mM β-mercaptoethanol. The tube was centrifuged in a Beckman Spinco SW-50.1 rotor for 70 min at 48,000 rpm. The distribution of bound 14C-phenylalanyl tRNA is shown in Figure 1. It is clear from this figure that most of the bound

![Figure 1](attachment:image.png)

**Fig. 1.**—Formation of the complex of 14C-phenylalanyl tRNA, poly U, and 70S ribosomes by association of 50S ribosomal subunits with the complex of poly U, 30S ribosomal subunits, and 14C-phenylalanyl tRNA. The reaction mixture (0.25 ml) for the binding of 14C-phenylalanyl tRNA to 30S ribosomal subunits was as described in the text, except that it contained 15,000 cpm of 14C-phenylalanyl tRNA, 400 μg of 30S ribosomal subunits, and 150 μg of poly U. The mixture was incubated at 22°C for 20 min. The reaction mixture (0.5 ml) for association of the complex with 50S ribosomal subunits was as described in the text, except that it contained 875 μg of 50S subunits and 900 μmoles of 14C-phenylalanyl tRNA in 2.56 mg of mixture of tRNA. The mixture was incubated for 3 min at 37°C and immediately subjected to sucrose density gradient centrifugation as described in the text. Three-drop fractions were collected from the bottom, and ribosome-bound 14C-phenylalanyl tRNA in each fraction was measured by the Millipore filter method.15 Absorbancy at 260 μm was measured after eightfold dilution of each fraction. ——O—O—, Absorbancy at 260 μm; ———, bound 14C-phenylalanyl tRNA per 0.15 ml of each fraction.

14C-phenylalanyl tRNA was found with 70S ribosomes. In the absence of added 50S ribosomal subunits, only 30S subunit fractions contained bound 14C-phenylalanyl tRNA (see Fig. 2). Fractions 3 through 8 were pooled and used for polyphenylalanine formation.

**Polyphenylalanine formation from the complex of 14C-phenylalanyl-tRNA, 14C-phenylalanyl-tRNA, 70S ribosomes, and poly U:** The reaction mixture for the formation of polyphenylalanine from the complex of 70S ribosomes as prepared above contained the following in μmoles per 5.0 ml: 217 Tris-HCl (pH 7.8), 82 magnesium acetate, 282 KCl, 22.2 β-mercaptoethanol, 6.0 phosphoenolpyruvate, and 0.3 GTP. In addition, it contained 3 ml of the 70S fraction isolated as described above, 84 μg of pyruvate kinase, 5.6 mg (protein) of S-150, 9.5 μmoles of 14C-phenylalanyl tRNA in 27 mg of tRNA mixture. The reaction mixture was incubated at 37°C for 15 min. To stop the reaction, 0.1 ml of 0.05 M 14C-phenylalanine and 1 ml of 50% trichloroacetic acid were added at the end of the incubation period. The precipitated polyphenylalanine was subjected to NH2-terminal analysis as described previously.7 After the hydrolysis of dinitrophenyl polyphenylalanine, dinitrophenylalanine was extracted with ether. The ether phase (NH2-terminal) and the aqueous phase (the remainder of the chain) were counted for radioactivity.
Fig. 2.—Dimerization of 30S ribosomal subunits in the presence of various concentrations of Mg$^{++}$ and K$^+$. The reaction mixture (0.5 ml) for binding of $^{14}$C-phenylalanyl tRNA to 30S ribosomal subunits was identical to that of Fig. 1, except that it contained 720 µg of 30S ribosomal subunits and various concentrations of K$^+$ and Mg$^{++}$. After incubation at 37°C for 20 min, the mixture was placed on 4.5 ml of linear sucrose gradient (5-20%) containing 10 mM Tris-HCl (pH 7.1) and corresponding concentrations of Mg$^{++}$ and K$^+$. The tube was centrifuged for 90 min at 48,000 rpm in a Spinco SW-50.1 rotor. After the centrifugation, three-drop fractions were collected from the bottom of the tubes. The following combinations of Mg$^{++}$ and K$^+$ were employed: (I) 13 mM Mg$^{++}$, 40 mM K$^+$; (II) 21 mM Mg$^{++}$, 40 mM K$^+$; (III) 13 mM Mg$^{++}$, 0 M K$^+$; and (IV) 21 mM Mg$^{++}$, 0 M K$^+$. ●—●, Bound $^{14}$C-phenylalanyl tRNA to 30S subunits per 0.15 ml of each fraction; ○—○, absorbancy at 260 mµ after 11-fold dilution.

Results.—Position in polyphenylalanine of $^{14}$C-phenylalanine derived from 30S subunit bound $^{14}$C-phenylalanyl-tRNA: In the experiment described below, the position in the polyphenylalanine chain of phenylalanine derived from 30S subunit-bound $^{14}$C-phenylalanyl tRNA was determined. This experiment was performed in three steps. The first step was to bind $^{14}$C-phenylalanyl tRNA to 30S ribosomal subunits in the presence of poly U. The second step was to add 50S ribosomal subunits to form 70S ribosomes. Since the second phenylalanyl tRNA is bound by association with 50S ribosomal subunits, it is essential to perform the association reaction in the presence of excess of $^{14}$C-phenylalanyl tRNA to avoid the binding of labeled phenylalanyl tRNA at this step. Thus, the 70S
 ribosomes formed contained $^{14}$C-phenylalanyl tRNA which was originally bound to the 30S ribosomal subunit and the $^{12}$C-phenylalanyl tRNA subsequently bound due to the formation of 70S ribosomes. The third step was to isolate this complex of 70S ribosomes by sucrose density gradient centrifugation from the unbound $^{14}$C-phenylalanyl tRNA. With the use of this complex, formation of polyphenylalanine was performed with $^{14}$C-phenylalanyl tRNA. The position of $^{14}$C-phenylalanine in the polyphenylalanine was then determined by NH$_2$-terminal analysis with dinitrofluorobenzene. If our original hypothesis that the 30S subunit binds one phenylalanyl tRNA is correct, one would expect that the phenylalanine which was bound initially to the 30S ribosomal subunit would be located at the NH$_2$-terminal end of the polyphenylalanine. Thus, we would expect 50 per cent of the radioactive phenylalanine at the NH$_2$ terminal. As shown in Table 1, most of the radioactivity was found at the NH$_2$-terminal position of the polyphenylalanine. The binding of phenylalanyl tRNA to 30S ribosomal subunits was carried out under a variety of ionic conditions because of the possibility that two phenylalanyl tRNA molecules might bind to one 30S ribosomal subunit under certain ionic conditions. The results show that the concentration of K$^+$ or Mg$^{++}$ in the binding mixture did not appreciably influence the conclusion. Similar experiments were carried out under conditions where the 30S ribosomal subunits were not saturated with $^{14}$C-phenylalanyl tRNA. In this case also the major portion of radioactivity was found at the NH$_2$ terminal.

Table 1. Position in polyphenylalanine of $^{14}$C-phenylalanine derived from bound $^{14}$C-phenylalanyl-tRNA to 30S ribosomal subunits.

<table>
<thead>
<tr>
<th>Ionic Conditions during the Binding Reaction (mM)</th>
<th>Per Cent of Radioactivity at NH$_2$ Terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Condition A</td>
</tr>
<tr>
<td>K$^+$ Mg$^{++}$</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>40</td>
<td>13</td>
</tr>
<tr>
<td>40</td>
<td>21</td>
</tr>
</tbody>
</table>

Under condition A, the reaction mixture (0.75 ml) for binding of $^{14}$C-phenylalanyl tRNA to 30S ribosomal subunits was as described in the text. For association of the complex with 50S ribosomal subunits, the reaction mixture (1.5 ml) was prepared as described in the text. Approximately 3800 cpm of $^{14}$C-phenylalanyl tRNA was bound to the reassociated 70S ribosomes. Formation of polyphenylalanine and NH$_2$-terminal analysis were performed as described in the text. Approximately 30–40% of bound radioactivity was incorporated into polyphenylalanine. Under condition B, the reaction mixture (1.5 ml) for the binding of $^{14}$C-phenylalanyl tRNA to 30S ribosomal subunits was as in Fig. 1, except that it contained 30,000 cpm of $^{14}$C-phenylalanyl tRNA, 2.4 mg of 30S ribosomal subunits, and various concentrations of K$^+$ and Mg$^{++}$ as indicated in the table. The reaction mixture (3 ml) for the association of the 30S subunits complex with 50S ribosomal subunits was identical to that described in the text, except that it contained 1.5 ml of the above solution containing complex of 30S ribosomal subunits, 360 μmoles of KCl, 5.15 mg of 50S subunits, and 3.38 mM moles of phenylalanyl tRNA in 9.6 mg of tRNA mixture. In B, 2 mM spermidine was added to facilitate subunit association, but results were essentially the same as those without spermidine. The mixture was incubated at 37°C for 10 min and was subjected to sucrose density gradient centrifugation to isolate the 70S ribosome complex. Polyphenylalanine formation and NH$_2$-terminal analysis were carried out as described in the text. Under condition B, 60–70% of bound radioactivity was incorporated into polyphenylalanine. The degree of saturation of 30S ribosomal subunits with $^{14}$C-phenylalanyl tRNA in conditions A and B was, respectively, 4 and 1 of Fig. 3.
Effect of various concentrations of $^{14}$C-phenylalanyl-tRNA on the position in polyphenylalanine: 

If two molecules of $^{14}$C-phenylalanyl tRNA are bound to a 30S ribosomal subunit, one site may have easier access to phenylalanyl tRNA than the other. In the case of the binding to 70S ribosomes, the site 1 (the $-\text{COOH}$ side of the growing polypeptide chain) had higher affinity than site 2 (the site on the side of $-\text{NH}_2$ terminal of the growing polypeptide chain). On the other hand, if only one $^{14}$C-phenylalanyl tRNA can be bound to a 30S ribosomal subunit, the amount of $^{14}$C-phenylalanyl tRNA in the binding reaction mixture should not influence the position in polyphenylalanine derived from the 30S subunit-bound phenylalanyl tRNA.

In the experiment summarized in Table 2, the ratio of $^{14}$C-phenylalanyl tRNA to 30S ribosomal subunit in the binding mixture was changed in such a way that 30S subunits obtained various degrees of saturation with $^{14}$C-phenylalanyl tRNA. It is clear from this table that even under conditions where the 30S subunits are almost saturated with $^{14}$C-phenylalanyl tRNA (condition 4), the major portion of the radioactivity was found to be at the $\text{NH}_2$ terminal of polyphenylalanine. Figure 3 illustrates the degree of saturation under which the above experiment was performed.

**TABLE 2.** Position in polyphenylalanine of $^{14}$C-phenylalanine derived from 30S subunit-bound $^{14}$C-phenylalanyl-tRNA: Effect of increasing amounts of $^{14}$C-phenylalanyl-tRNA in the binding mixture.

<table>
<thead>
<tr>
<th>Conditions of binding</th>
<th>Per cent of radioactivity at $\text{NH}_2$ terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 of Fig. 3</td>
<td>87</td>
</tr>
<tr>
<td>2 of Fig. 3</td>
<td>87</td>
</tr>
<tr>
<td>3 of Fig. 3</td>
<td>84</td>
</tr>
<tr>
<td>4 of Fig. 3</td>
<td>91</td>
</tr>
</tbody>
</table>

The reaction mixture (1.5 ml) for the binding of $^{14}$C-phenylalanyl tRNA to the 30S ribosomal subunits was as condition B of Table 1, except that it contained 31.5 µmoles of magnesium acetate and 60 µmoles of KCl; various amounts of $^{14}$C-phenylalanyl tRNA were added to obtain various degrees of saturation of 30S ribosomal subunits with $^{14}$C-phenylalanyl tRNA as shown in Fig. 3.

![Fig. 3](image-url) —Binding of $^{14}$C-phenylalanyl tRNA to 30S ribosomal subunits in the presence of various amounts of $^{14}$C-phenylalanyl tRNA. The reaction mixture (0.05 ml) for the binding of $^{14}$C-phenylalanyl tRNA was as that of Fig. 1, except that it contained various concentrations of $^{14}$C-phenylalanyl tRNA and 20 µg of poly U. Bound $^{14}$C-phenylalanyl tRNA after 20 min incubation was plotted against the amounts of $^{14}$C-phenylalanyl tRNA added to the mixture; 1 µl of $^{14}$C-phenylalanyl tRNA corresponds to 4000 cpm of radioactivity.
ments were carried out. The arrows in this figure represent the conditions where the experiments in Table 2 were carried out. In a separate experiment, the binding of phenylalanyl tRNA was carried out under the conditions where the molar ratio of phenylalanyl tRNA to 30S ribosomal subunits was 3:1. Even under such conditions, the major portion (85%) of the radioactivity was found at the NH₂ terminal of the polyphenylalanine.

Possibility that _¹⁴C-phenylalanyl-tRNA is released from the complex of _¹⁴C-phenylalanyl-tRNA and the 30S ribosomal subunit_: Although all the data presented in the preceding sections are consistent with the concept that only one phenylalanyl tRNA is bound to each 30S ribosomal subunit, the possibility exists that during the association with 50S ribosomal subunits, phenylalanyl tRNA is preferentially released from one of two binding sites on the 30S subunit, resulting in a false conclusion that one phenylalanyl tRNA is bound to 30S ribosomal subunits. The experiment charted in Figure 4 eliminated this possibility.

In this experiment, the loss of radioactivity from 30S ribosomal subunits was studied in the presence and absence of added 50S ribosomal subunits. It is clear from this figure that the presence of 50S ribosomal subunits did not facilitate the loss of radioactivity from 30S subunits. In the experiments described in the preceding section, the incubation period for association with 50S subunits was three minutes and the reaction mixture was cooled to 0°C. As shown in this figure, at most 15% of the bound radioactivity was lost during this period; and the loss was effectively stopped by cooling the mixture to 0°C. Even if one assumes that preferential loss of radioactivity took place from one of the two possible sites of the 30S ribosomal subunit, our results on the NH₂-terminal analysis of polyphenylalanine could not be explained on the basis of two sites on the 30S ribosomal subunit. It should be pointed out that once the 70S ribosomes are formed, the bound phenylalanyl tRNA is stable and is not released from the ribosomes during sucrose gradient centrifugation.

Possibility of dimerization of 30S ribosomal subunits: The 30S subunit has been reported to dimerize to form 50S material in the presence of 20 mM Mg++. It was therefore of interest to examine whether the 30S ribosomal subunits used in these experiments form the dimer during the binding of phenylalanyl tRNA. In the experiment shown in Figure 2, the mixture of 30S ribosomal subunits, poly U, and phenylalanyl tRNA was prepared in the presence of various concen-
trations of Mg++ and K+ and subjected to sucrose gradient centrifugation. As shown in this figure, a major portion of 30S subunits exists as 30S monomers in the presence of K+, whereas they exist as dimers (50S material) in the absence of K+. Since the 30S ribosomal subunit binds only one phenylalanyl tRNA regardless of the ionic conditions (Table 1), it appears that aggregation of 30S ribosomal subunits does not influence the amount of phenylalanyl tRNA binding. It should be pointed out that the bound phenylalanyl tRNA is mostly localized at the 30S position. This suggests either that the dimer of 30S cannot bind, or that the binding of phenylalanyl tRNA to the dimer is so weak that it is dissociated during the centrifugation. It should be noted that the presence of K+ inhibits the formation of 30S dimer at 20 mM Mg++. Thus, very little dimer formation was observed in the presence of 40 mM K+. The role of K+ in the dimerization of 30S subunits is currently under investigation.

Effect of Mg++ and K+ on the binding of phenylalanyl-tRNA to 30S ribosomal subunits: To explore the possibility that 30S ribosomal subunits may bind two phenylalanyl tRNA’s and that binding of one phenylalanyl tRNA is influenced by the concentration of K+ or Mg++, an experiment was performed to investigate the effect of these ions on the binding of phenylalanyl tRNA to 30S ribosomal subunits. As shown in Figure 5, over the range of concentrations measured no appreciable stimulation by increasing concentration of K+ was observed. This was true at low (5 mM) as well as at high (13, 21, or 29 mM) concentrations of Mg++. Thus, under our experimental conditions we have not been able to obtain any evidence that two phenylalanyl tRNA molecules are bound in the presence of relatively high K+ concentration.

Discussion.—From the amino terminal analysis of polyphenylalanine formed from the complex of 14C-phenylalanyl tRNA, poly U, and 70S ribosomes, it has been concluded that there are two sites for phenylalanyl tRNA binding on each 70S ribosome. These two binding sites on the 70S ribosomes have been named site 1 (—COOH side) and site 2 (—NH₂ side) with respect to the growing polypeptide chain.

The data presented in this paper indicate that the 30S subunit has one functional site for binding of 14C-phenylalanyl tRNA. The fact that 14C-phenylalanine of 30S subunit-bound 14C-phenylalanyl tRNA was located almost exclusively at the NH₂ terminal of polyphenylalanine suggests two possibilities as to the

![Fig. 5.—Effect of K+ and Mg++ on phenylalanyl tRNA binding to 30S ribosomal subunits. The reaction mixture (0.1 ml) for binding 14C-phenylalanyl tRNA to 30S ribosomal subunits contained various amounts of magnesium acetate, KCl, 8 μmoles of Tris-HCl (pH 7.1), and 20 μg of poly U, 45 μg of 30S ribosomal subunits, and 25,000 cpm of 14C-phenylalanyl tRNA. Bound 14C-phenylalanyl tRNA per reaction mixture was plotted against concentration of KCl.

- - - , 28 mM Mg++; O--O, 21 mM Mg++;
- - - , 13 mM Mg++; Δ--Δ, 5 mM Mg++.](image)
nature of the 30S ribosomal binding site for phenylalanyl tRNA. The first is that the site of the 30S subunit is site 2. The other possibility is that the site on the 30S subunit is site 1 but, during the process of association with 50S ribosomal subunits, the bound phenylalanyl tRNA moves from site 1 to site 2. In an attempt to check this possibility, the association with 50S ribosomal subunits was carried out in the presence of sparsomycin. Sparsomycin has been found to inhibit formation of peptidyl puromycin13 as well as GTP-dependent release of deacylated tRNA from ribosomes.14 It was found that even if sparsomycin was added during the formation of 70S ribosomes, the major portion of the radioactivity is found predominantly at the NH₂ terminal of polyphenylalanine. This suggests that the 30S subunit binds one molecule of phenylalanyl tRNA at the site corresponding to site 2. Nevertheless, until sparsomycin is shown to be inhibitory to the movement of tRNA, the question still remains as to whether the binding site on the 30S subunit is site 1 or site 2 of the 70S ribosome.

* Supported by grants from the National Science Foundation (NSF GB-7454), U.S. Public Health Service (PHS GM 12,053-65), and Damon Runyon Cancer Fund (DRG 799 C).
† Established Investigator of the Helen Hay Whitney Foundation.
5 Suzuka, I., H. Kaji, and A. Kaji, these PROCEEDINGS, 55, 1483 (1966).
7 Igarashi, K., and A. Kaji, these PROCEEDINGS, 58, 1971 (1967).
10 Nathans, D., and F. Lipmann, these PROCEEDINGS, 47, 497 (1961).
14 Kuriki, Y., and A. Kaji, these PROCEEDINGS, 61, 1399 (1968).