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Three tRNA binding sites on *Escherichia coli* ribosomes

(aminoacyl-tRNA site/peptidyl-tRNA site/chloramphenicol)

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ABSTRACT The binding of *N*-acetyl-Phe-tRNA^{Phe} (an analogue of peptidyl-tRNA), Phe-tRNA^{Phe}, and deacylated tRNA^{Phe} to poly(U)-programmed tightly coupled 70S ribosomes was studied. The *N*-acetyl-Phe-tRNA^{Phe} binding is governed by an exclusion principle: not more than one *N*-acetyl-Phe-tRNA^{Phe} can be bound per ribosome, although this peptidyl-tRNA analogue can be present either at the aminoacyl-tRNA (A) site or the peptidyl-tRNA (P) site. Two Phe-tRNA^{Phe} molecules are accepted by one ribosome in the presence of poly(U). This aminoacyl-tRNA binds enzymatically (in the presence of elongation factor Tu and GTP) and nonenzymatically to the A site and is then transferred to the P site, if that site is free. If this elongation factor G-independent movement is hampered, either by using an incubation temperature of 0°C or by the addition of the translocation inhibitor viomycin, only one Phe-tRNA^{Phe} per ribosome can be bound. The effect of the peptidyltransferase inhibitor chloramphenicol on the binding is similar to that of viomycin. In the absence of poly(U), Phe-tRNA^{Phe} cannot bind to the ribosome. Deacylated [^{14}C]tRNA^{Phe} can bind in three copies to one ribosome. The new third tRNA binding site is called the "E" site. The sequence of filling the sites is P, E, and A. The apparent binding constants for the P and the E sites are both $\approx 9 \times 10^6$ M^{-1} and that for the A site is 1.3×10^6 M^{-1}. In the absence of poly(U), only one deacylated tRNA^{Phe} can be bound per ribosome. This tRNA^{Phe} most likely occupies the P site.

The ribosomal model comprising two tRNA binding sites (the A site for aminoacyl-tRNA and the P site for peptidyl-tRNA) is generally accepted for both pro- and eukaryotic ribosomes. This model is related to, and mainly caused by, the method usually chosen for the localization of aminoacyl-tRNA—i.e., the puromycin reaction (1). A positive reaction is supposed to indicate location of an aminoacyl-tRNA at the P site, and a negative reaction indicates location at the A site.

Experimentally, we have only scant knowledge of the properties of ribosomal tRNA binding sites, and controversial data have been reported concerning even the number of these binding sites. *In vivo* labeling of tRNA and subsequent analysis of polysomes derived from rabbit reticulocytes has demonstrated that eukaryotic ribosomes (80S) can carry two tRNA molecules per ribosome (2). However, *in vitro* binding experiments with rat liver polysomes indicate that two or three tRNA molecules can bind simultaneously to an 80S ribosome (3), and binding experiments with 80S ribosomes were interpreted to reflect two binding sites for aminoacyl-tRNA (entry and A site, respectively) next to the P site (4). With prokaryotic ribosomes, even less is known about the number of binding sites. Evidence for one firm binding site (5) and for up to four binding sites (6) has been reported.

In this paper, we confirm and extend our recent finding that the *Escherichia coli* ribosome can bind three tRNA molecules simultaneously (7). In addition to the established A and P sites, a third binding site exists for the codon-dependent binding of exclusively deacylated tRNA. Furthermore, we characterize the three binding sites with respect to poly(U)-dependent and independent binding of deacylated tRNA^{Phe}, Phe-tRNA^{Phe}, and *N*-acetyl-Phe-tRNA^{Phe}.

MATERIALS AND METHODS

Tightly coupled 70S ribosomes were isolated from *E. coli* K-12 cells (strain D10) as described (7). We estimated that 1 A_{260} unit of 70S ribosomes is equivalent to 24 pmol. [^{3}H]Phenylalanine (specific activity 2959 GBq/mmol $\approx$ 80 Ci/mmol), [^{14}C]phenylalanine (specific activity 18.3 GBq/mmol $\approx$ 495 mCi/mmol), [^{14}C]ATP (18.9 GBq/mmol $\approx$ 510 mCi/mmol), and [^{14}C]CTP (17 GBq/mmol $\approx$ 460 mCi/mmol) were obtained from Amersham. *E. coli* tRNA^{Phe} was purchased from Boehringer Mannheim. The preparation of Phe-tRNA^{Phe} and *N*-acetyl-Phe-tRNA^{Phe} and the isolation of elongation factor(s) (EF)-G and -Tu followed the procedure of ref. 8. ATP (CTP):tRNA nucleotidyltransferase from baker's yeast (specific activity, 56,000 units/mg) was purified as described (9).

tRNA Labeling. To exchange the unlabeled C-C-A terminus of tRNA for a labeled one, 200 A_{260} units of *E. coli* tRNA^{Phe} was incubated in 2.5 ml of 10 mM ammonium citrate, pH 7.5/100 mM KCl/10 mM $MgSO_4$/1 mM dithioerythritol/1.0 mM [^{14}C]ATP (specific activity 38 mCi/mmol)/1.0 mM [^{14}C]CTP (17 mCi/mmol)/2.5 mM inorganic pyrophosphate containing 1200 units of ATP (CTP):tRNA nucleotidyltransferase for 3 hr at 32°C. tRNA-[^{14}C]C-[^{14}C]A was recovered by chromatography on Sephadex A-25, followed by desalting on a Bio-Gel P-2 column as described (9). The [^{3}H]phenylalanine acceptance of the labeled [^{14}C]tRNA^{Phe} was determined in a ligase assay (ref. 10; 1500 pmol/A_{260} unit) and was at least as good as that of the unlabeled tRNA^{Phe}. The total efficiences, comprising sample oxidizer and scintillation counter, were 65–70% for ^{14}C and $\approx$20% for ^{3}H and were determined in each experiment. One picomole of [^{14}C]tRNA^{Phe} was equivalent to 80 cpm. This value was confirmed by binding of Ac-[^{3}H]Phe-[^{14}C]tRNA^{Phe} to the A site (7). In this assay, the binding could be monitored either by using [^{3}H]phenylalanine of known specific activity (68 GBq/mmol) as an internal standard or by using [^{14}C]tRNA of 80 cpm/pmol. In both cases, the result was 0.75 molecules Ac-Phe-tRNA bound per 70S ribosome, indicating that 80 cpm/pmol is in fact the specific activity of the actually bound [^{14}C]tRNA.

Binding Assay and Puromycin Reactions. These were performed as described (11), except that the aliquots were one-

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Abbreviations: A site, aminoacyl-tRNA site; P site, peptidyl-tRNA site; Ac-Phe-tRNA^{Phe}, *N*-acetyl-Phe-tRNA^{Phe}; EF, elongation factor.

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fourth (25 μl) the usual size and the amount of 70S ribosomes was reduced to 4.5 A_{260} units per starting reaction volume, corresponding to 18 pmol per assay. The amounts of the various tRNAs added were as indicated. The nitrocellulose filters containing both ^{14}C- and ^{3}H-labeled compounds were assayed in a sample oxidizer, and the isotopes were collected separately. All experiments were performed in duplicate; the error of the mean values was <5%.

The binding data represent extent and not rate of reaction. Control experiments have shown that the binding data do not change significantly if the filters are washed three times instead of twice. At least 80%, usually 90–100%, of our tightly coupled 70S ribosomes were active, as indicated in binding assays under tRNA-saturating conditions. In the binding calculations, we assumed 100% active 70S ribosomes. Therefore, our binding constants may be slightly low, which however, does not affect our conclusions.

In all binding assays and at all tRNA concentrations, a blank (lacking 70S ribosomes) was determined and subtracted before calculating the binding data.

Determination of Phenylalanine and Phe_2. After binding [^{14}C]Phe-tRNA^{Phe}to the ribosomes, 100 μl of the reaction mixture was mixed with 12 μl of 1 M acetic acid to prevent further binding and peptidyltransferase activity, the ester bond was cleaved by addition of 25 μl of 1 M NaOH and incubation at 37°C for 30 min, and the sample was acidified by the addition of 25 μl of 1 M HCl. A total of 21 μl (seven 3-μl portions; ≈4000 cpm) of the final mixture was spotted on a cellulose sheet [20 × 20 cm, Polygram CEL 300, Machery and Nagel (Düren, Federal Republic of Germany)] together with 9 μl each of 1.5 mM phenylalanine and saturated Phe_2 (≤2 mM; Sigma). After 8 hr (400 V in 2% pyridine acetate, pH 4.4, containing 15% acetone), phenylalanine and Phe_2 were completely separated; Phe_2 and phenylalanine migrated ≈10 and 7.5 cm, respectively, toward the cathode and were visualized with the ninhydrin reaction. The entire lane including the origin was cut in 1-cm-long pieces, and the radioactivity was determined.

RESULTS

Binding of Ac-Phe-tRNA^{Phe} to Ribosomes. In the experiment described below, an Ac-[^{14}C]Phe-tRNA^{Phe} preparation was used that contained ≈30% tRNA^{Phe}. Adding increasing amounts of this Ac-Phe-tRNA^{Phe} to poly(U)-programmed ribosomes led to a saturation value of 1 Ac-Phe-tRNA^{Phe} molecule per 70S ribosome, when the molar excess of Ac-Phe-tRNA^{Phe} over ribosomes was at least 2 (Fig. 1). As shown by the puromycin reaction, the Ac-Phe-tRNA^{Phe} was, surprisingly, not located in one ribosomal site. At low concentrations (<1 Ac-Phe-tRNA^{Phe} molecule per ribosome), the Ac-Phe-tRNA^{Phe} was located predominantly at the P site. However, at higher concentrations, increasing amounts of Ac-Phe-tRNA^{Phe} were found at the A site, giving an equal distribution between the A and the P sites at ≈7 Ac-Phe-tRNA^{Phe} molecules per ribosome. The A-site binding of Ac-Phe-tRNA^{Phe} is obviously due to the deacylated tRNA^{Phe} moiety present in this preparation; Ac-Phe-tRNA^{Phe} can be bound to the A site only when the P site is already occupied with a cognate deacylated tRNA (11, 12, 13).

We are therefore faced with the unexpected result that, at various A site/P site distributions of Ac-Phe-tRNA^{Phe}, the total number of these molecules bound per ribosome remains constantly 1. The explanation is that, whenever a ribosome has bound an Ac-Phe-tRNA^{Phe} molecule to either the A or the P site, a second Ac-Phe-tRNA^{Phe} molecule cannot bind to the ribosome. Thus, the binding of one Ac-Phe-tRNA^{Phe} molecule excludes the binding of a second one (exclusion principle for the binding of Ac-Phe-tRNA^{Phe}).

Without poly(U), Ac-Phe-tRNA^{Phe} and deacylated tRNA^{Phe} compete for the same binding site, which is obviously the ribosomal P site. This has been demonstrated by using an Ac-[^{3}H]Phe-[^{14}C]tRNA^{Phe} plus [^{14}C]tRNA^{Phe} mixture (7), in agreement with evidence reported previously (11).

Binding of Phe-tRNA^{Phe} to Ribosomes. [^{14}C]Phe-tRNA^{Phe} was bound nonenzymatically to poly(U)-programmed ribosomes in the presence of 15 mM Mg^{2+}. At 0°C, up to 0.9 Phe-tRNA^{Phe} molecules per ribosome could be bound (Fig. 2). These Phe-tRNA^{Phe} molecules were predominantly present at the ribosomal A site (85%), as indicated by the puromycin reaction (Table 1, experiment 1). At 37°C, the number of Phe-tRNA^{Phe} molecules bound approached 1.4. The puromycin reaction showed that significant amounts of bound Phe-tRNA^{Phe} were present at the P site. In the best case, about two-thirds of the bound Phe-tRNA^{Phe} react with puromycin (Table 1, experiment

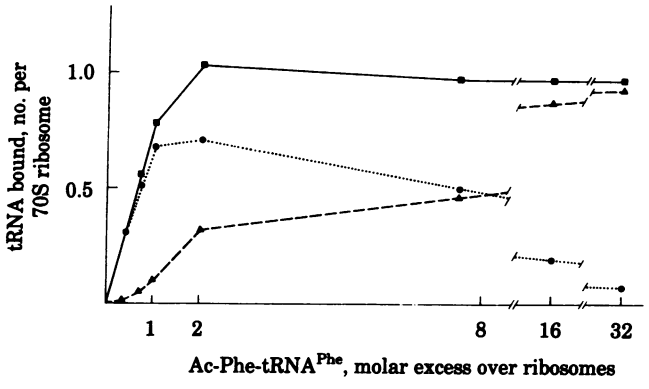

FIG. 1. Ac-[^{14}C]Phe-tRNA^{Phe} binding to poly(U)-programmed 70S ribosomes at 15 mM Mg^{2+} (■). The A and P site locations were tested by the puromycin reaction [reaction in the absence of EF-G indicates P site (●), reaction in the presence of EF-G indicates A site plus P site, and the difference is the A site (▲)]. The Ac-Phe-tRNA^{Phe} preparation contained ≈30% deacylated tRNA^{Phe}. Equivalent binding was obtained at 10 mM Mg^{2+}. With an Ac-Phe-tRNA^{Phe} preparation containing <10% deacylated tRNA^{Phe}, essentially the same binding curve was obtained, whereas the % A-site occupation was reduced.

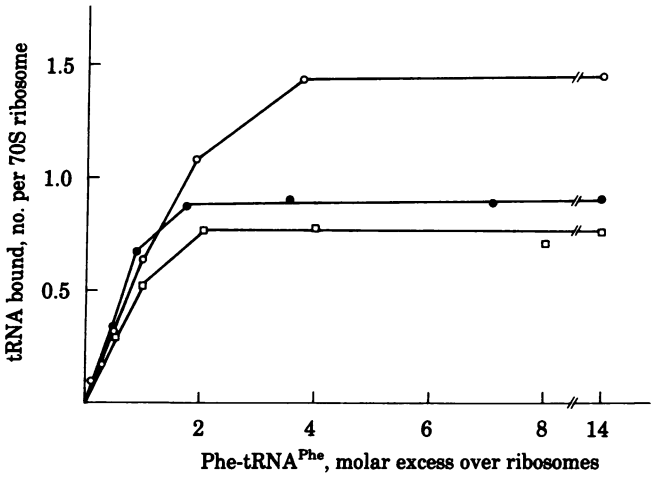

FIG. 2. Nonenzymatic [^{14}C]Phe-tRNA^{Phe} binding to poly(U)-programmed 70S ribosomes at 15 mM Mg^{2+}. Samples were incubated for 30 min at 0°C (●) or 37°C (○) in the absence of chloramphenicol and at 37°C in the presence of 0.5 mM chloramphenicol (□). Results equivalent to those in the presence of chloramphenicol were obtained in the presence of 80 μM viomycin at 37°C (data not shown; see Table 1, experiment 1).

Table 1. [^{14}C]Phe-tRNA^{Phe} binding to 70S ribosomes

	Binding conditions					Puromycin reaction, cpm		Location, %	
Exp.	Mg^{2+}, mM	Poly(U)	Temp,°C	Additions	Binding, cpm	−Ef-G	+EF-G	P site	A site
1	15	+	0	None	6,753	247	1638	15	85
			37	None	6,682	1360	2204	62	38
			37	VM	5,820	415	533	22	78
2	15	—	37	None	140				
	8	—	37	EF-Tu, GTP	190				
Control	15	+	37	None	8,740				
	8	+	37	EF-Tu, GTP	13,220				

Background values (minus 70S ribosomes for the binding measurements and minus puromycin for the puromycin reactions) have been subtracted. Experiment 1: 8.8 pmol of [^{14}C]Phe-tRNA^{Phe} was added to 20 pmol of 70S ribosomes. VM, 80 μM viomycin. The site location in the presence of viomycin was calculated by assuming the same reactivity of Phe-tRNA toward puromycin as in the absence of viomycin (415:2.204, corrected for the somehow lower binding). As, for technical reasons (11), the cpm output of the puromycin reaction is ≈50% of that of the binding assay, it is evident that after binding at 37°C in absence of viomycin, about two-thirds of the bound Phe-tRNA reacted with puromycin (2 × 2.204 cpm vs. 6.682 cpm). After the binding reaction had been performed (experiment 1), an aliquot was analyzed for oligo(Phe) formation in thin-layer electrophoresis. Labeled material was found to significant extent exclusively at the phenylalanine and Phe_2 positions (e.g., at the origin, <1% of the total radioactivity was found). The total yield of radioactivity per lane was ≈60% of the input amount; identical values were found with a control containing a defined amount of [^{14}C]phenylalanine (19,400 cpm). A further aliquot (25 μl) of the tRNA-binding reaction mixture subjected to a standard hot trichloroacetic acid precipitation yielded background values (30 cpm) only. These results demonstrate that no oligo(Phe) except Phe_2 was formed during the binding reaction. In all three cases of experiment 1, 92 ± 1% of the bound Phe-tRNA was found as phenylalanine and 8 ± 1% was found as Phe_2, taking into account that 75% of the added Phe-tRNA bound to ribosomes. In experiment 2, 14.4 pmol of [^{14}C]Phe-tRNA was added to 18 pmol of 70S ribosomes.

1, and legend), whereas with Ac-Phe-tRNA^{Phe}, a practically quantitative reaction with puromycin can be obtained (11). Thus, in our hands, ribosomal-bound Phe-tRNA^{Phe} reacted satisfactorily with puromycin, in contrast to a report stating that <5% of the bound Phe-tRNA could undergo the puromycin reaction, even in presence of EF-G and GTP (14). Our finding is consistent with the generally accepted view that two ribosomal sites, the A and the P sites, are available for aminoacyl-tRNA. Furthermore, due to the presence of ≈30% of deacylated tRNA^{Phe} in the Phe-tRNA^{Phe} preparation used in this experiment, a number significantly >1.4 Phe-tRNA^{Phe} molecules per ribosomes would not be expected, if two Phe-tRNA^{Phe} molecules can be bound per ribosome and tRNA^{Phe} competes with the Phe-tRNA^{Phe} binding. In the presence of either viomycin or chloramphenicol, ≈0.8 Phe-tRNA^{Phe} molecules per ribosome can be bound at 37°C (Fig. 2). The presence of viomycin traps the Phe-tRNA^{Phe} at the A site (78% at the A site; Table 1, experiment 1). Whether chloramphenicol exerts a similar effect cannot be tested by the puromycin reaction, as chloramphenicol inhibits peptidyltransferase activity. However, the similarities of the binding curves and the saturation levels at 0°C in the absence and at 37°C in the presence of chloramphenicol, respectively, indicate that chloramphenicol also inhibits the transfer of Phe-tRNA^{Phe} from A site to P site. Concerning nonenzymatic Phe-tRNA^{Phe} binding, we conclude that two Phe-tRNA^{Phe} molecules can be bound per ribosome and that Phe-tRNA^{Phe} (at least predominantly) first binds to the A site (entry site for aminoacyl-tRNA) and then moves, even in absence of EF-G, to the P site at 37°C, if this site is free. The EF-G-independent movement can be inhibited by viomycin or chloramphenicol.

In the absence of poly(U), Phe-tRNA^{Phe} cannot bind to 70S ribosomes, either nonenzymatically in the presence of 15 mM Mg^{2+} or enzymatically in the presence of 8 mM Mg^{2+}, EF-Tu, and GTP (Table 1, experiment 2).

Binding of Deacylated [^{14}C]tRNA^{Phe} to Ribosomes. Various amounts of deacylated tRNA^{Phe} were added to poly(U)-programmed tightly coupled 70S ribosomes. Saturation was observed at three tRNA^{Phe} molecules per ribosome in the presence of either 10 or 15 mM Mg^{2+} (Fig. 3A). The location of the deacylated tRNA^{Phe} was determined by adding a small amount of Ac-[^{3}H]Phe-tRNA^{Phe} (0.18 pmol per assay) and using the puromycin reaction. If the deacylated tRNA^{Phe} was not at the P site, the Ac-Phe-tRNA^{Phe} should bind exclusively to this site. However, if deacylated tRNA^{Phe} had already filled the P site, then Ac-Phe-tRNA^{Phe} should bind to the A site. Fig. 3A shows that one deacylated tRNA^{Phe} molecule bound per ribosome closed the P site and directed the Ac-Phe-tRNA^{Phe} to the A site. Thereafter, a second deacylated tRNA^{Phe} could be bound without affecting the A site location of Ac-Phe-tRNA^{Phe}. Only when the binding number exceeds two and approaches three is there a parallel decrease in A site-bound Ac-Phe-tRNA^{Phe}.

The fact that ≈20% of the Ac-Phe-tRNA^{Phe} remains bound to the A site (Fig. 3A) when three deacylated tRNA^{Phe} molecules have been bound can be explained by a higher affinity of Ac-Phe-tRNA^{Phe} for the A site as compared with that of tRNA^{Phe} (the respective binding constants are 2.6×10^6 M^{-1} and 1.3×10^6 M^{-1}). Therefore, Ac-Phe-tRNA^{Phe} might have chased a small amount of deacylated tRNA^{Phe} out of the A site.

It is obvious that, after filling the P site and before binding to the A site, deacylated tRNA^{Phe} occupies a third site, which we have arbitrarily designated as the "E" (for exit) site. From the data shown in Fig. 3A, we can calculate the filling sequence of the three sites (Fig. 3B). Thus, deacylated tRNA^{Phe} successively fills first the P site, then the E site, and finally the A site. These data explain our previously reported finding that, after binding of two [^{14}C]tRNA^{Phe} molecules per ribosome, one Ac-[^{3}H]Phe-[^{14}C]tRNA^{Phe} molecule could be bound to the A site, yielding a total of three tRNA molecules per ribosome (two deacylated tRNA^{Phe} molecules and one Ac-Phe-tRNA^{Phe} molecule; see ref. 7).

The binding data of deacylated [^{14}C]tRNA^{Phe} at 10 mM Mg^{2+} were plotted according to Scatchard (ref. 15; Fig. 4). The P and E sites bind deacylated tRNA^{Phe} with similar affinities (apparent $K_a = 9 \times 10^6$ M^{-1} in either case), whereas the A site has a significantly lower affinity for deacylated tRNA^{Phe} (apparent $K_a = 1.3 \times 10^6$ M^{-1}).

In the absence of mRNA, we find one deacylated tRNA^{Phe} molecule per ribosome (Fig. 3C) in accordance with ref. 16. Ac-Phe-tRNA^{Phe} binds exclusively to the P site of nonprogrammed

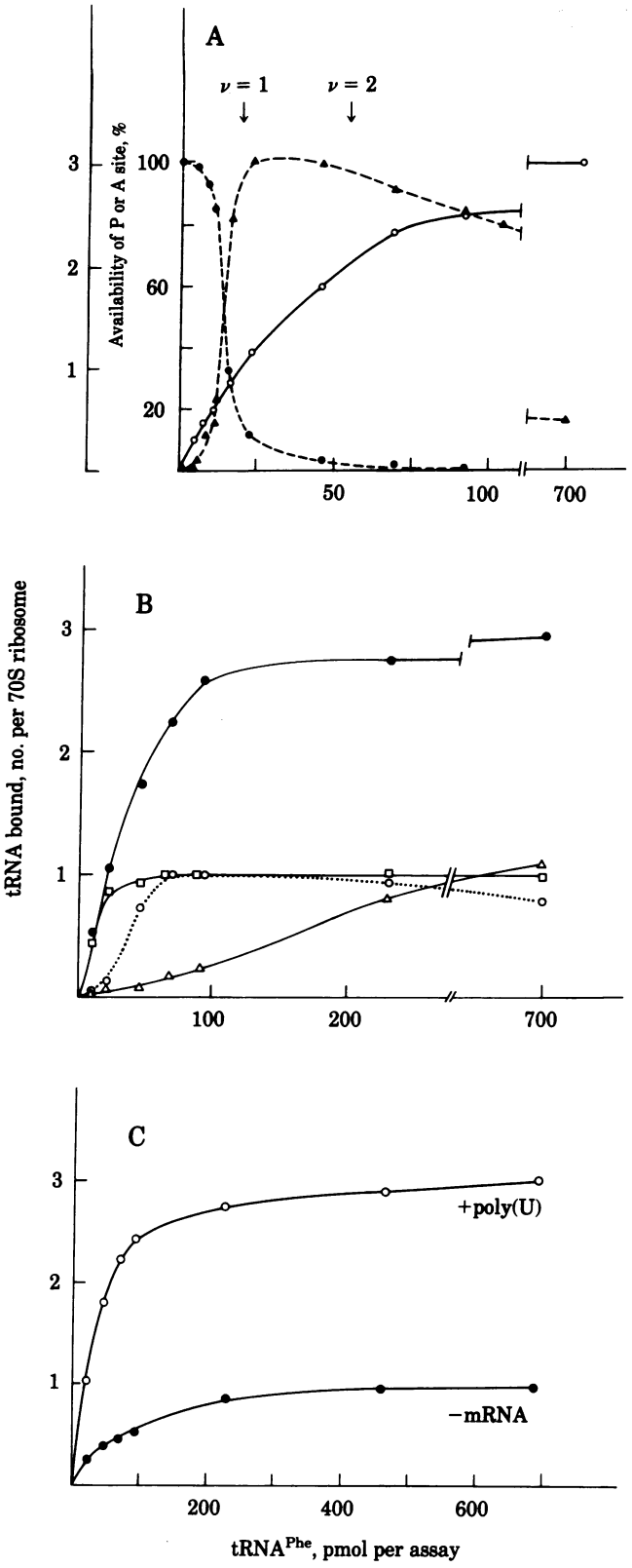

FIG. 3. Binding of deacylated [^{14}C]tRNA^{Phe} to tightly coupled ribosomes. (*A*) Poly(U)-dependent binding (○). The availability of P (●) and A (▲) sites was tested by the addition of a small amount of Ac-[^{3}H]Phe-tRNA^{Phe} (0.18 pmol per assay) followed by a puromycin assay (see legend to Fig. 1). ν, tRNA bound per 70S ribosome. (*B*) Data shown in *A* were used to calculate the filling sequence of the P (□), E (○), and A (△) sites. ●, Total binding. (*C*) Binding of [^{14}C]tRNA^{Phe} to nonprogrammed ribosomes (●). For comparison, the binding curve obtained in the presence of poly(U) (○) is included.

ribosomes (7, 11). As this Ac-Phe-tRNA^{Phe} binding to the P site could be completely blocked by deacylated tRNA^{Phe} (11), we conclude that the only binding site available for deacylated tRNA^{Phe} on nonprogrammed ribosomes is the P site.

DISCUSSION

The finding that a poly(U)-programmed 70S ribosome can bind only one Ac-Phe-tRNA^{Phe} molecule, although two binding sites (A and P) are available (Fig. 1), is the basis for the "exclusion principle" concerning peptidyl-tRNA binding (Ac-Phe-tRNA^{Phe} can be regarded as a simple analogue of peptidyl-tRNA): i.e., when a 70S ribosome has a codon-dependently bound peptidyl-tRNA in either the A or the P site, a second peptidyl-tRNA cannot bind to this ribosome. This finding may reflect the existence of one excretion channel for the peptidyl residue so that this channel holds the peptidyl residue regardless of whether the corresponding tRNA moiety is present at the A or the P site. Furthermore, this ribosomal feature could help to prevent fMet-tRNA from binding to an AUG-programmed A site (in addition to the specific properties of this tRNA species; see ref. 17) if the P site is already occupied by a peptidyl-tRNA. Such a binding of fMet-tRNA to the A site would immediately block protein synthesis.

Fig. 1 also shows that increasing amounts of Ac-Phe-tRNA^{Phe} (≈30% deacylated tRNA^{Phe}) led to an increased binding of Ac-Phe-tRNA^{Phe} to the A site. One possible explanation of this finding is that, under our conditions, tRNA^{Phe} binds with a higher affinity to the P site than Ac-Phe-tRNA^{Phe}; the respective bind-

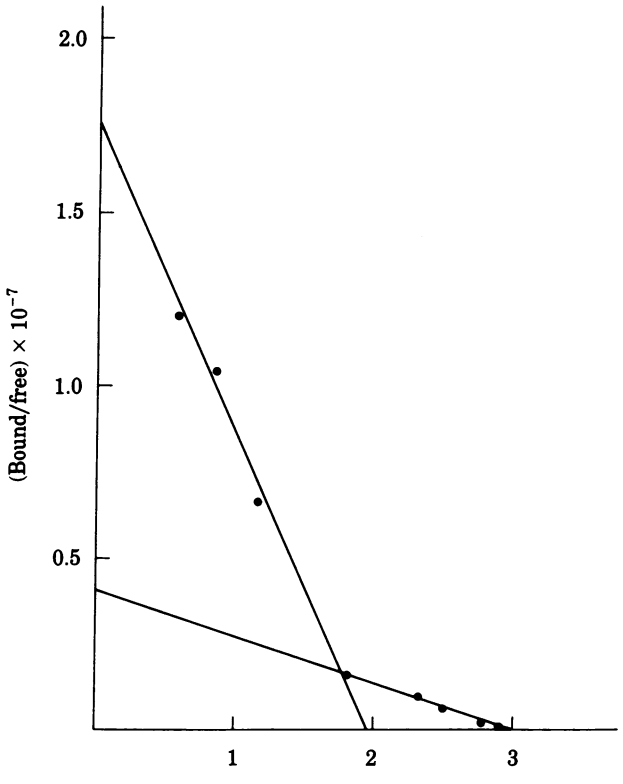

FIG. 4. Scatchard plot of binding of deacylated [^{14}C]tRNA^{Phe} to poly(U)-programmed ribosomes (see Fig. 3*A*). The steeper regression line intercepts the abscissa at $n \approx 2$, indicating that P and E sites bind deacylated tRNA with a similar affinity (apparent $K_a \approx 9 \times 10^6\ M^{-1}$). The other regression line marks the A-site binding (apparent $K_a = 1.3 \times 10^6\ M^{-1}$).

Table 2. tRNA binding to *E. coli* ribosomes

mRNA	tRNA species	Binding sites: No.	Binding sites: Location	Binding properties: One entry site*	Binding properties: Filling sequence
Poly(U)	$tRNA^{Phe}$	3	P, E, and A	No	P site first, then E site, then A site
	Phe-$tRNA^{Phe}$	2	P and A	Yes (A)	P site via transient A-site binding; also, A site without occupied P site
	Ac-Phe-$tRNA^{Phe}$	1	P or A†	No	P site; A site only after deacylated tRNA has occupied P site
None	$tRNA^{Phe}$	1	P		
	Phe-$tRNA^{Phe}$‡	0	—		
	Ac-Phe-$tRNA^{Phe}$	1	P§		

Except when otherwise indicated, binding studies were performed nonenzymatically at 10 and 15 mM Mg^{2+} concentrations with equivalent results.

* These statements are the most probable at present but await definite experimental proof.

† Exclusion principle.

‡ 8 mM Mg^{2+} plus EF-Tu GTP.

§ See ref. 7.

ing constants are $9 \times 10^6\ M^{-1}$ and $4.6 \times 10^6\ M^{-1}$. An alternative explanation postulates that deacylated $tRNA^{Phe}$ binds directly to the P site whereas Ac-Phe-$tRNA^{Phe}$ reaches the P site exclusively via a transient A-site binding. With increasing amounts of Ac-Phe-$tRNA^{Phe}$ (plus $tRNA^{Phe}$), more and more P sites would be occupied by $tRNA^{Phe}$ and the Ac-Phe-$tRNA^{Phe}$ would accordingly accumulate at the A site. However, evidence has been provided that Ac-Phe-$tRNA^{Phe}$ binds directly to the P site without transient A-site binding (11). Further support for direct P-site binding is derived from the binding capacity of nonprogrammed ribosomes, which bind $tRNA^{Phe}$ and Ac-Phe-$tRNA^{Phe}$ to the P site (7, 11), whereas Phe-$tRNA^{Phe}$ does not bind at all, either enzymatically or nonenzymatically (Table 1, experiment 2). Therefore, we currently favor the first mechanism—i.e., that the higher affinity of $tRNA^{Phe}$ to the P site explains the accumulation of Ac-Phe-$tRNA^{Phe}$ at the A site (Fig. 1).

The binding of up to two Phe-$tRNA^{Phe}$ molecules per ribosome in the presence of poly(U) (Fig. 2) is in accordance with the A site/P site model of the ribosome and documented by a wealth of data (for review, see ref. 18). Interestingly, the A site is also the entry site for nonenzymatic Phe-$tRNA^{Phe}$ binding (0°C binding curve in Fig. 2). This curve also shows that Phe-$tRNA^{Phe}$ binds to the A site in the absence of a prefilled P site, in contrast to Ac-Phe-$tRNA^{Phe}$, which only binds to the A site when the P site is occupied by deacylated $tRNA^{Phe}$ (11, 13, 14). In the presence of chloramphenicol, the binding curve obtained at 37°C approaches 0.75 Phe-$tRNA^{Phe}$ molecules per ribosome and is similar to that obtained at 0°C in the absence of the drug (Fig. 2). We conclude that, in the presence of chloramphenicol, the movement of Phe-$tRNA^{Phe}$ is inhibited, so that the Phe-$tRNA^{Phe}$ binding is restricted to the A site. Thus, chloramphenicol, which is a well-known inhibitor of peptidyl transferase activity (for review, see ref. 19), also blocks the EF-G-independent sliding of Phe-$tRNA^{Phe}$ from the A site to the P site in a manner similar to that of viomycin, which blocks translocation. Whether chloramphenicol also blocks translocation movement of peptidyl-tRNA from the A to the P site is not yet known.

As mentioned above, nonprogrammed ribosomes do not bind Phe-tRNA at all, either nonenzymatically or enzymatically (Table 1, experiment 2). This finding is in disagreement with an earlier report (16), in which equal binding of deacylated and acylated tRNA was reported. However, those authors did not document proper controls as they compared the binding of [^{14}C]aminoacyl-[^{14}C]tRNA with that of [^{14}C]tRNA. Thus, the reported aminoacyl-tRNA binding could be mimicked by the deacylated tRNA portion present in their preparation.

For deacylated $tRNA^{Phe}$, three binding sites exist on poly(U)-programmed ribosomes. In addition to the established A and P sites, a third binding site was found (Fig. 3), which was designated the E site. The filling sequence of these three sites is P, E, and, finally, A. The E site binds deacylated $tRNA^{Phe}$ in a codon-dependent manner; without poly(U), only one binding site is available for $tRNA^{Phe}$ on 70S ribosomes. This site is most probably the P site (7).

A summary of the relationships among the various tRNAs and the three binding sites is presented in Table 2; the present state of knowledge on the binding sites is as follows. The E site binds cognate deacylated tRNA in the presence of mRNA; without mRNA, there is no tRNA binding. The P site binds cognate deacylated tRNA, aminoacyl-tRNA, and peptidyl-tRNA in the presence of mRNA; without mRNA, there is binding of deacylated tRNA and of Ac-aminoacyl-tRNA. The A site binds cognate deacylated tRNA, aminoacyl-tRNA, and peptidyl-tRNA in the presence of mRNA; without mRNA, there is no tRNA binding.

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