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# Transient Kinetics of Transfer Ribonucleic Acid Binding to the Ribosomal A and P Sites: Observation of a Common Intermediate Complex†

Wolfgang Wintermeyer* and James M. Robertson

ABSTRACT: The mechanism of tRNA–ribosome complex formation has been studied in fluorescence stopped-flow as well as slow kinetic experiments. As a fluorescent probe, proflavin was inserted into the anticodon loop of yeast $tRNA^{Phe}$ ($tRNA^{Phe}_{Prf37}$). Complex formation of $tRNA^{Phe}_{Prf37}$ or *N*-Ac-Phe-$tRNA^{Phe}_{Prf37}$ with poly(U)-programmed, vacant *Escherichia coli* ribosomes results in a biphasic change of both intensity and polarization of fluorescence characterized by relaxation times in the 100-ms ($\tau_{fast}$) and 1-s ($\tau_{slow}$) time range. The data are consistent with a sequential two-step mechanism of tRNA–ribosome complex formation, in which a rapid association–dissociation equilibrium is followed by a rearrangement step. From the dependence of the inverse relaxation time upon ribosome concentration, the following parameters are obtained (20 °C): $k_{12} = (1.5 \pm 0.2) \times 10^7$ $M^{-1}$ $s^{-1}$, $k_{21} = 0.5 \pm 0.2$ $s^{-1}$, $k_{23} = 0.3 \pm 0.2$ $s^{-1}$, and $k_{32} \leq 0.1$ $s^{-1}$. Neither the amplitude of the fluorescence change nor the rate constants of the fast second-order step are affected by the presence or absence of poly(U) or by preoccupancy of the P site with nonfluorescent tRNA. This suggests that the fast step leads to an intermediate complex in which the tRNA is bound to a site other than the P site in a codon-independent manner. An activation energy of 9 ± 2 kcal/mol indicates that the apparent second-order step in fact represents a fast rearrangement that is coupled to the probably diffusion-limited formation of a weak encounter complex. The first-order rearrangement ($k_{23}$) is related to the binding of the tRNA into the P site. It is completely depressed by blocking the P site with nonfluorescent tRNA. Furthermore, codon–anticodon interaction takes place during this step. Provided the P site is occupied, the intermediate tRNA–ribosome complex very slowly (minute time range) rearranges to the A-site complex. The presence of poly(U) is required for this rearrangement to occur. Experiments with Phe-$tRNA^{Phe}_{Prf37}$ show that elongation factor Tu and GTP strongly accelerate the rearrangement but not the formation of the intermediate complex.

Protein synthesis in *Escherichia coli* cells proceeds at a rate of 15 amino acids incorporated into protein $s^{-1}$ ribosome$^{-1}$, implying elementary steps of the elongation cycle in the time range of milliseconds (Gouy & Grantham, 1980). Although the steady-state rates of protein synthesis in the current in vitro systems are much slower—for instance, with *E. coli* ribosomes and factors more than 100 times—these figures nevertheless indicate that fast kinetic techniques have to be applied in order to determine the rates of individual steps and to isolate short-lived intermediates of ribosomal protein synthesis. In the past, kinetic techniques like stopped flow, pressure jump, or temperature jump have been utilized only scarcely for studies in the ribosome system, the well-documented examples being limited to studies on the association–dissociation equi-

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librium or ribosomal subunits (Wishnia et al., 1975; Schulz et al., 1976; Görisch et al., 1977; Chaires et al., 1979). The broader application of these techniques was hampered by the lack of observables suitable to selectively monitor a single component of the system with high time resolution. A promising way out of this difficulty is the use of fluorescent markers. Recently, fluorescent derivatives of initiation factor 3 from *E. coli* have been utilized to study the complex formation with 30S ribosomal subunits by the stopped-flow technique (Goss et al., 1980; Woolley & Box, 1979).

In a similar fashion, in order to study the kinetics of tRNA ribosome complex formation, we have utilized a fluorescent derivative of yeast $tRNA^{Phe}$, in which the base naturally occurring next to the 3′ side of the anticodon had been replaced with proflavin ($tRNA^{Phe}_{Prf37}$)[1] (Wintermeyer & Zachau, 1979). While the insertion of the fluorescent group was shown previously not to appreciably impair the activities of $tRNA^{Phe}$ in ribosome binding and polyphenylalanine synthesis (Wintermeyer & Zachau, 1975a,b), this report shows that the $tRNA^{Phe}$ proflavin derivative is useful for kinetic studies of tRNA binding to either the P or the A site of *E. coli* ribosomes. Experiments on the influence of elongation factor Tu on the kinetics of A-site binding are also reported. Preliminary results from this work have been reported briefly before (Wintermeyer et al., 1979); some of the previous observations were proven artifactual and are corrected here.

## Materials and Methods

*Biochemicals.* $tRNA^{Phe}$ from brewer's yeast, $tRNA^{Phe}_{Prf37}$, and 70S ribosomes ("tight couples") from *E. coli* MRE 600 were prepared as described elsewhere (Robertson & Wintermeyer, 1981). Preparative aminoacylations of $tRNA^{Phe}_{Prf37}$ with L-[$^{14}$C]phenylalanine (513 Ci/mol) (1.3 nmol of [$^{14}$C]-Phe/$A_{260}$ unit) and the preparation of *N*-Ac[$^{14}$C]Phe-$tRNA^{Phe}$ (1.6 nmol of *N*-Ac[$^{14}$C]Phe/$A_{260}$ unit) and of *N*-Ac[$^{14}$C]-Phe-$tRNA^{Phe}_{Prf37}$ (1.5 nmol of *N*-Ac[$^{14}$C]Phe/$A_{260}$ unit) were performed as described elsewhere (Robertson & Wintermeyer, 1981). EF-Tu·Ts and poly(U) were purchased from Boehringer Mannheim. The concentrations of $tRNA^{Phe}$, $tRNA^{Phe}_{Prf37}$, and ribosomes were determined spectrophotometrically with extinction coefficients ($\mu M^{-1}$ cm$^{-1}$) at 260 nm of 0.571, 0.595, and 43.5, respectively.

*Biochemical Assays.* The aminoacylation, Millipore filtration, and puromycin assays have been described elsewhere (Robertson & Wintermeyer, 1981).

*Kinetic Measurements.* (*1*) *Apparatus.* The stopped-flow experiments were performed in a fluorometer that allowed the measurement of both fluorescence intensity and polarization (Robertson et al., 1977). Proflavin fluorescence was excited at 436 nm (light from 200-W Hg–Xe lamp was passed through a grating monochromator and an interference filter, 436 ± 0.5 nm) and measured after passing cutoff filters (KV 500, Schott). Due to the light sensitivity of proflavin, the exciting light had to be attenuated considerably in order to obtain signals that were stable with time.

The stopped-flow cell was adapted from the gross design of a published construction (Rigler et al., 1974); a four-jet mixing chamber (5-$\mu$L volume) was used. The solutions were driven by nitrogen at a pressure of about 5 atm, resulting in a dead time below 2 ms. The flow was controlled by an electromagnetically actuated pneumatic valve at the outlet of the cuvette (Union Giken Ltd., Tokyo).

The data were stored in a transient recorder (Model 805, Biomation, Palo Alto, CA; 2048 data points of 8 bits each) from where they were displayed on an oscilloscope and transferred to a computer (Tektronix 4052) for evaluation (see next paragraph).

For slow kinetic measurements, a Schoeffel RRS 1000 spectrofluorometer equipped with a double monochromator for excitation was used. Proflavin fluorescence was excited at 436 nm (1-nm bandwidth) and measured at 500 nm (3-nm bandwidth).

(*2*) *Sample Preparation and Conditions.* The experiments were performed by mixing equal volumes (50 $\mu$L each) of the tRNA solution and the solution containing ribosomes and, when required, poly(U). The tRNA solution had been preincubated for at least 30 min at the temperature chosen for the experiment (20 °C; except in experiments in which the temperature was varied) and the ribosome solution with or without poly(U) for 20 min at 37 °C, both in the buffer used for the respective experiment. For experiments in which A-site binding was to be studied, the ribosomes, following preincubation with poly(U), were incubated with a 1.1-fold molar excess of $tRNA^{Phe}$ for an additional 15 min at 20 °C in order to block the P site (see Figure 1). The following buffer was used for all experiments: 50 mM Tris-HCl, pH 7.6 (20 °C), 25 mM KCl, 70 mM ammonium chloride, and 3 mM 2-mercaptoethanol, containing 18 mM magnesium acetate, if not stated otherwise. The concentration of poly(U) was 3 $A_{260}$/mL, if present.

(*3*) *Data Evaluation.* The kinetic curves were evaluated by fitting the function

$$I(t) = A_0 + \sum_i A_i \exp(-t/\tau_i)$$

with one or two exponential terms. Routinely, electronically generated exponential functions were visually fitted to the measured curves on an oscilloscope screen. In addition, selected representative experiments were numerically fitted with a computer program that by iterative parameter variation searched for the minimum of the sum of the squared residuals. As starting values of the parameters, the values obtained by visual fitting were used. In all cases where comparisons have been made, the two fitting procedures yielded the same parameters within the limits of error. The quality of the computer fits was judged from the random distribution of residuals and standard deviations of the measured points, ranging from about ±2% (slow measurements) to ±4% (fast measurements) of the respective amplitudes. The error of the relaxation times (±10–20% depending on the amplitude of the signal change) was estimated from the variation of several determinations.

## Results

*Specificity of tRNA–Ribosome Complex Formation.* The aim of these experiments, to measure the kinetics of tRNA binding to the ribosomal A and P sites separately, requires that the two sites can be discriminated with sufficient specificity. As shown by the high puromycin reactivity (Table I), *N*-AcPhe-$tRNA^{Phe}_{Prf37}$ binds to the P site of poly(U)-programmed vacant ribosomes with high preference over the A site, provided the ribosomes are present in at least an equimolar amount. Nearly stoichiometric binding is achieved under the present experimental conditions. After the P site has been occupied, for instance, with deacylated $tRNA^{Phe}$, *N*-AcPhe-$tRNA^{Phe}$ binds to the A site, as defined by the puromycin reactivity before and after incubation with elongation factor G and GTP (Figure 1). *N*-AcPhe-$tRNA^{Phe}_{Prf37}$ behaves very similarly

[1] Abbreviations: $tRNA^{Phe}_{Prf37}$, yeast $tRNA^{Phe}$ in which wybutine at position 37 next to the anticodon is replaced with proflavin; EF, elongation factor; Tris, tris(hydroxymethyl)aminomethane.

Table I: Ribosome Binding of *N*-AcPhe-tRNA$^{Phe}_{Prf37}$ and Phe-tRNA$^{Phe}_{Prf37}$ [a]

| compd | P site preoccupied with tRNA$^{Phe}$ | $Mg^{2+}$ (mM) | charged tRNA$^{Phe}_{Prf37}$ bound (% of input) | puromycin react. (%) [b] −EF-G | puromycin react. (%) [b] +EF-G |
|---|---|---|---|---|---|
| *N*-AcPhe-tRNA$^{Phe}_{Prf37}$ | − | 18 | 97 | 100 | 100 |
| | + | 18 | 75 | 20 | 100 |
| Phe-tRNA$^{Phe}_{Prf37}$ | − | 18 | 93 | 65 | nd |
| | + | 18 | 94 | 18 | nd |
| | + | 6 [c] | 93 | 10 | nd |

[a] The experiments were performed as described under Materials and Methods with 30 pmol of ribosomes and 10 pmol of charged tRNA$^{Phe}_{Prf37}$ per 100-μL assay. Translocation was accomplished by incubating the A-site complexes with 0.3 μM EF-G and GTP (0.5 mM) for 10 min at 25 °C. [b] Percentage of bound charged tRNA$^{Phe}_{Prf37}$. nd, not determined. [c] 0.25 μM EF-Tu·Ts and 0.25 mM GTP added.

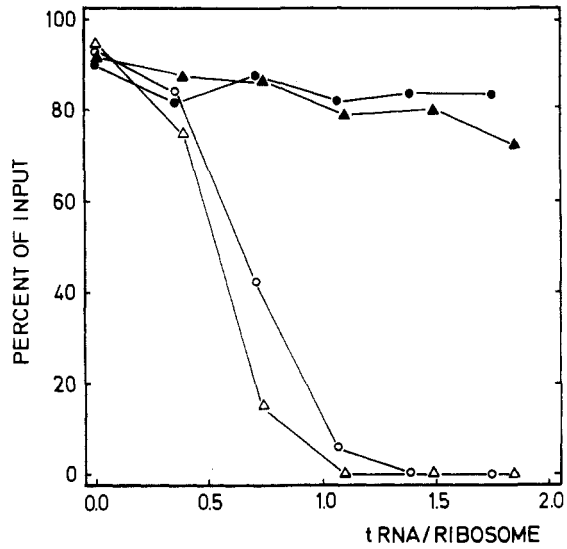


FIGURE 1: Specificity of P-site binding of tRNA$^{Phe}$ (triangles) or tRNA$^{Phe}_{Prf37}$ (circles). Poly(U)-programmed ribosomes (0.3 μM) were incubated with increasing amounts of tRNA$^{Phe}$ (tRNA$^{Phe}_{Prf37}$) for 15 min at 20 °C; then *N*-Ac[$^{14}$C]Phe-tRNA$^{Phe}$ was added (0.1 μM) and the incubation continued for another 30 min. The amount of *N*-Ac[$^{14}$C]Phe-tRNA$^{Phe}$ bound (closed symbols) and reactive with puromycin (open symbols) was determined as described previously (Robertson & Wintermeyer, 1981).

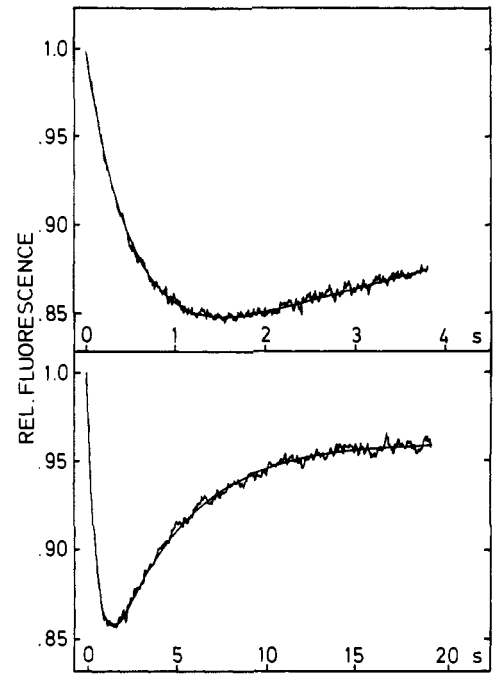


FIGURE 2: Fluorescence changes in stopped-flow experiments on binding of tRNA$^{Phe}_{Prf37}$ to poly(U)-programmed ribosomes. The experiments were performed as described under Materials and Methods at a concentration of 6 nM tRNA$^{Phe}_{Prf37}$ and of 85 nM ribosomes. Total signal was about 2 V; rise time filter was 10 ms. The solid lines represent the functions calculated from the parameters obtained by two-exponential fitting, as described under Materials and Methods.

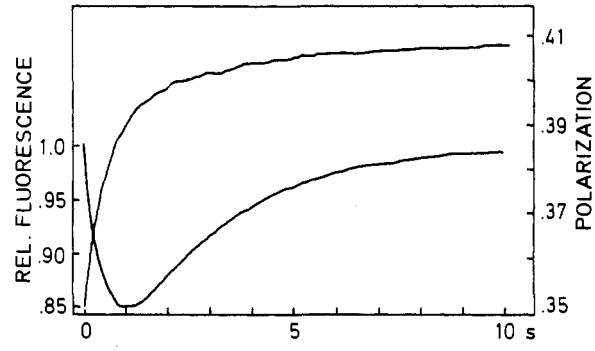


FIGURE 3: Correlation of fluorescence intensity (lower final level) and polarization (upper final level) changes occurring during complex formation of tRNA$^{Phe}_{Prf37}$ with poly(U)-programmed ribosomes. Concentrations were 3 times higher than those in Figure 2.

(Table I). The same is true for Phe-tRNA$^{Phe}_{Prf37}$, as verified by assaying both nonenzymatic and enzymatic binding (Table I).

With the ribosomes used for this work, the formation of the complexes was completely dependent on poly(U) at $Mg^{2+}$ concentrations up to 10 mM. At higher $Mg^{2+}$ concentrations, poly(U)-independent binding to the P site became increasingly apparent, reaching about 50% efficiency at 18 mM $Mg^{2+}$. The binding experiments reported below have been performed at this latter $Mg^{2+}$ concentration, if not stated otherwise.

*Two Resolved Kinetic Steps in P-Site Binding of tRNA$^{Phe}_{Prf37}$.* The stopped-flow experiment shows that the total fluorescence change observed upon P-site binding of tRNA$^{Phe}_{Prf37}$ (Robertson & Wintermeyer, 1981) takes place in two steps (Figure 2). A fluorescence decrease taking about 2 s (relaxation time $\tau_{fast}$ = 0.4 s but see below) is followed by a slow increase of similar amplitude taking about 15 s ($\tau_{slow}$ = 4 s), after which time the signal does not increase anymore. The experiments were performed under pseudo-first-order conditions with at least a 5-fold excess of ribosomes over tRNA. Thus, chemical relaxation times are obtained by straightforward two-exponential evaluation of the reaction progress curves (Bernasconi, 1976; Hiromi, 1979). Within the error limits, additional relaxation effects can be excluded from the fits obtained (Figure 2). In a preliminary report on P-site binding of fluorescent tRNA$^{Phe}$ derivatives, we described two slow relaxations in addition to the two faster ones reported here (Wintermeyer et al., 1979). As is evident from these data, those slow fluorescence changes, the amplitudes of which were rather small, were artifactual. They are probably related to slow structural alterations of the tRNA$^{Phe}$ derivatives, which require a long preincubation period before adding the ribosomes (Robertson & Wintermeyer, 1981).

The two steps observed by fluorescence intensity changes are correlated to a biphasic increase of the polarization of fluorescence (Figure 3). The major effect is associated with the fast reaction, indicating that this step represents or is strongly coupled to the binding step. Very similar biphasic changes of fluorescence and polarization are observed in analogous experiments with *N*-AcPhe-tRNA$^{Phe}_{Prf37}$ (data not shown).

Table II: Kinetic Parameters of Complex Formation of $tRNA^{Phe}_{Prf37}$ with Poly(U)-Programmed Ribosomes[a]

| | $k_{12}$ [$(\mu M \cdot s)^{-1}$] | $k_{21}$ ($s^{-1}$) | $k_{23}$ ($s^{-1}$) | $k_{32}$ ($s^{-1}$) | $E^*_{12}$ (kcal/mol) | $E^*_{23}$ (kcal/mol) |
|---|---|---|---|---|---|---|
| P site free | 15 ± 2 | 0.5 ± 0.2 | 0.3 ± 0.2 | <0.1 | 9 ± 2 | 10 ± 2 |
| P site blocked | 14 ± 2 | 0.3 ± 0.2 | | | nd | |

[a] The data of Figure 5 have been evaluated according to eq 1 as described in the text. The activation energies have been obtained from Arrhenius plots (see text). nd, not determined.

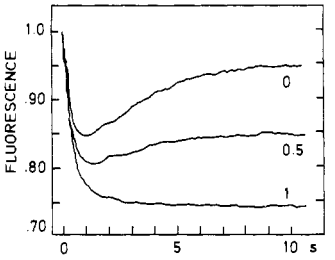


FIGURE 4: Effect of blocking the P site with $tRNA^{Phe}$ on the kinetics of complex formation of $tRNA^{Phe}_{Prf37}$ with poly(U)-programmed ribosomes. Experimental conditions were as in Figure 2 except that the ribosomes were incubated with $tRNA^{Phe}$ at the indicated tRNA/ribosome ratios prior to the measurement.

In order to prove that the observed relaxation effects are due to tRNA ribosome interactions, we performed several controls. Experiments in which $tRNA^{Phe}_{Prf37}$ was mixed with poly(U) containing buffer showed no relaxation of either intensity or polarization of fluorescence. Furthermore, the scattering signal did not change with time when poly(U)-programmed ribosomes were mixed with buffer. A shift in the poly(U)–ribosome equilibrium due to anchoring of the poly(U) by the tRNA binding can be excluded from results of experiments utilizing a 3′ proflavin labeled poly(U) (chain length of about 30 nucleotides). While mixing the fluorescent poly(U) derivative with vacant ribosomes resulted in a considerable increase of the polarization of proflavin fluorescence, addition of $tRNA^{Phe}$ to the preformed complex of fluorescent poly(U) with ribosomes caused no further polarization increase (data to be presented elsewhere). This result suggests that the poly(U)–ribosome equilibrium does not change significantly upon mixing with $tRNA^{Phe}$ at the conditions of these kinetic experiments.

The amplitude of the slow fluorescence change decreases when the P site is partially preoccupied with nonfluorescent $tRNA^{Phe}$ and is completely depressed at a stoichiometric $tRNA^{Phe}$ to ribosome ratio (Figure 4).[2] This observation shows that the slow signal change is related to the binding of the tRNA into the P site or a site overlapping with the P site. In fact, data obtained by the filtration assay indicate that the P-site complex is formed within about 20 s, the time span of the stopped-flow experiments. However, the correlation of fluorescence change and P-site complex formation will have to be confirmed by time-resolved experiments employing both the filtration and the puromycin assays.

*Evaluation of a Kinetic Scheme for tRNA–Ribosome Complex Formation.* The results of Figures 2 and 4 already show that the formation of the P-site complex takes place in at least two steps. In order to evaluate a binding mechanism, we determined the dependence of the inverse relaxation times on the ribosome concentration (Figure 5). The linear concentration dependence of $1/\tau_{fast}$ and the saturating behavior

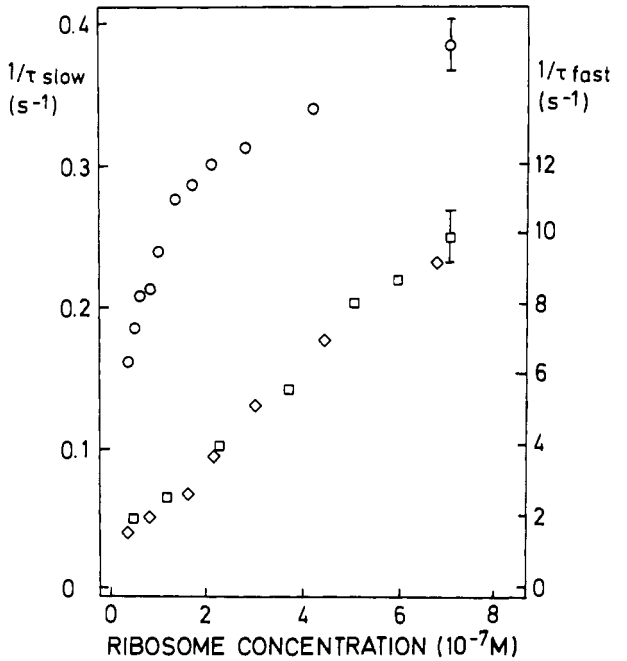


FIGURE 5: Dependence of the reciprocal relaxation times $1/\tau_{fast}$ (□; ◇) and $1/\tau_{slow}$ (○) upon ribosome concentration. The experiments were performed as in Figure 2 except that the concentration of vacant (□) or P-site blocked (◇) poly(U)-programmed ribosomes was varied, as indicated. Averaged values from at least three independent measurements are plotted, the bars indicating the maximal variation.

of $1/\tau_{slow}$ suggest a two-step binding mechanism, as depicted in eq 1 (R denotes poly(U)-programmed, vacant ribosomes;

$$R + T \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} R{\cdot}T' \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} R{\cdot}T^P \qquad (1)$$

T denotes the fluorescent $tRNA^{Phe}$). The kinetic parameters of the model have been evaluated by plotting both the sum and the product of the two inverse relaxation times vs. ribosome concentration (Bernasconi, 1976); both plots were perfectly linear (not shown) and yielded the rate constants summarized in Table II. Given the precision of these data, the intercept of the product plot was not distinguishable from zero, indicating that $k_{32}$ is smaller than 0.1 $s^{-1}$.

The concentration dependence of the fast step has also been measured with P site blocked ribosomes. The slope and intercept of the plot of the inverse relaxation time vs. ribosome concentration (Figure 5) yield the rate constants $k_{12}$ and $k_{21}$, respectively, given in Table II. It should be noted that blocking of the P site has no influence on the fast step.

In order to determine the activation energies of the two steps observed during P-site binding, we measured the kinetics at six temperatures between 10 and 37 °C. By plotting the inverse relaxation times vs. the inverse absolute temperature, we obtained linear Arrhenius plots (not shown) yielding the activation energies given in Table II.

*Influence of the mRNA on P-Site Complex Formation.* Steady-state data show that the tRNA–ribosome complexes formed in the absence of mRNA are only slightly less stable than the ones containing poly(U), at least under these ex-

[2] In fact, a slow fluorescence increase following the fast relaxation is observed in the minute range. It accompanies the formation of the A-site complex, considered below. For the evaluation of the stopped-flow data, A-site binding can be disregarded.

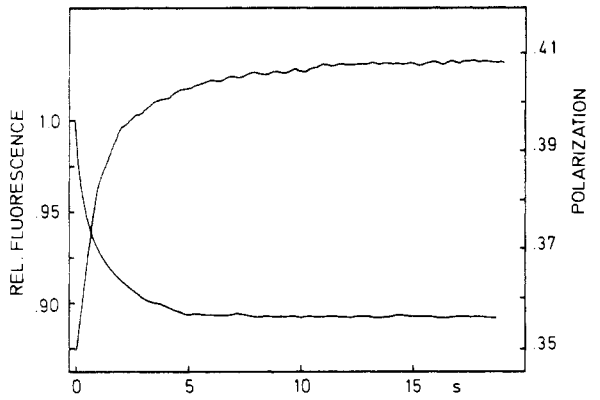


FIGURE 6: Binding of $tRNA^{Phe}_{Prf37}$ to nonprogrammed ribosomes. Polarization (upper final level) and intensity (lower final level) of fluorescence were measured as in Figure 3 except for the omission of poly(U).

perimental conditions (18 mM $Mg^{2+}$). Furthermore, fluorescence polarization and quenching data indicate a rather similar binding mode of the anticodon loop of $tRNA^{Phe}_{Prf37}$; in both types of complexes, the label is highly immobilized and shielded against solvent access, the only difference being a poly(U)-dependent enhancement of fluorescence by about 15% (Robertson & Wintermeyer, 1981).

Stopped-flow experiments with $tRNA^{Phe}_{Prf37}$ and nonprogrammed ribosomes (Figure 6) or ribosomes programmed with poly(A) reveal that this difference is connected to the slow rearrangement step ($\tau_{slow}$). The fluorescence increase in the second range observed in the presence of poly(U) is now no longer seen. Nevertheless, the complex is formed in a biphasic manner as demonstrated by the polarization measurement (Figure 6), which clearly shows two steps similar to those of Figure 3. Thus, the slow transition to the P-site complex occurs also in the absence of correct codon–anticodon interaction, albeit without a fluorescence change.

In the absence of poly(U), the dependence on ribosome concentration could only be measured for the fast relaxation effect with sufficient precision. The inverse relaxation time linearly increases with increasing ribosome concentration (measured up to 0.5 $\mu$M). The rate constants evaluated from the plot, $k_{12} = (1.4 \pm 0.2) \times 10^7\ M^{-1}\ s^{-1}$ and $k_{21} = 0.2 \pm 0.1\ s^{-1}$, within the error limits are the same as the ones obtained in the presence of poly(U) (Table II). These results clearly show that the second-order step of eq 1 is not influenced by the presence or absence of mRNA and suggest that it is the rearrangement step where codon–anticodon interaction takes place.

*Influence of the Ionic Conditions on the Kinetics of P-Site Complex Formation.* As shown in Figure 7, the two relaxation times are moderately accelerated with increasing ionic strength at the higher $Mg^{2+}$ concentration (18 mM) but are only slightly influenced at the lower $Mg^{2+}$ concentration (10 mM). The major effect of ionic strength (and $Mg^{2+}$ concentration) variation is seen in the amplitudes of both relaxations, which increase slightly at low ionic strength and decrease at high ionic strength. The pronounced shift of the maximal amplitudes toward lower ionic strength, observed upon lowering the $Mg^{2+}$ concentration, suggests that competition of ammonium ions for $Mg^{2+}$-binding sites destabilizes the tRNA–poly(U)–ribosome complex. On the other hand, the slight increase of both amplitudes and inverse relaxation times in the early part of the titration at 18 mM $Mg^{2+}$ probably reflects a stabilization of the complex caused by screening of repulsive charges.

*Kinetics of A-Site Complex Formation.* Nonenzymatic binding of the Phe-$tRNA^{Phe}$ or *N*-AcPhe-$tRNA^{Phe}$ to the A

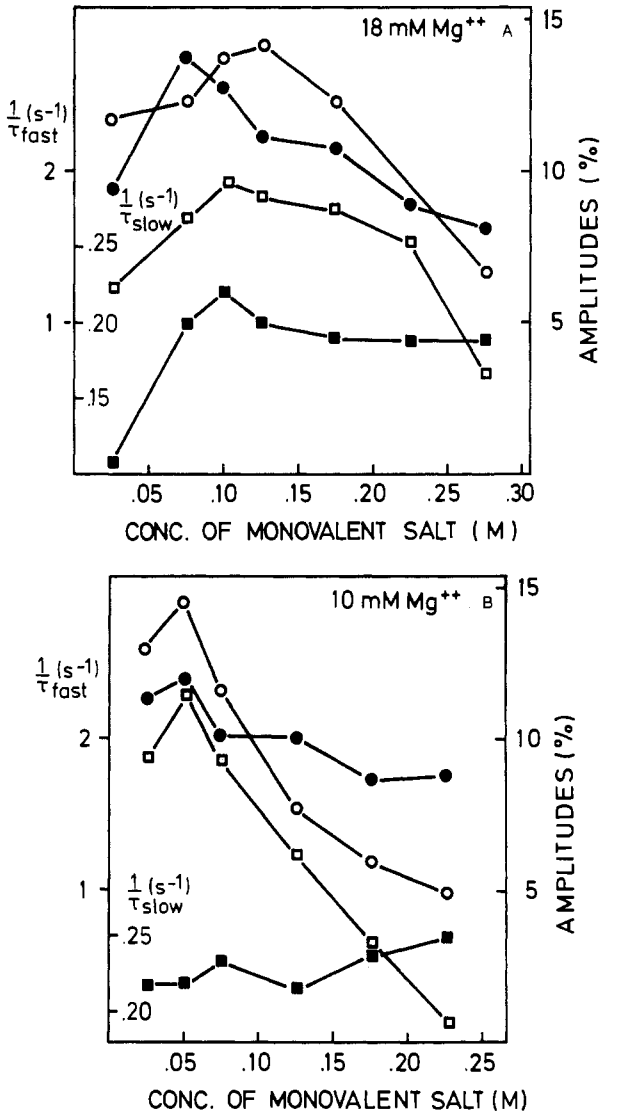


FIGURE 7: Ionic strength dependence of the kinetics of complex formation at 18 mM $Mg^{2+}$ (A) and 10 mM $Mg^{2+}$ (B). The binding of $tRNA^{Phe}_{Prf37}$ to poly(U)-programmed ribosomes (concentrations as in Figure 2) was measured in 25 mM Tris-HCl, pH 7.6, 18 (10) mM $Mg^{2+}$, and 3 mM 2-mercaptoethanol at varying concentrations of ammonium chloride. The inverse relaxation times (closed symbols) and amplitudes (open symbols) of the fast (circles) and the slow (squares) relaxation of Figure 2 have been obtained by the fitting procedure described under Materials and Methods.

site of P site blocked poly(U)-programmed ribosomes is rather slow, taking several minutes in the filtration assay. The proflavin-substituted $tRNA^{Phe}$ derivatives behave similarly. The formation of the A-site complex is accompanied by a small increase of proflavin fluorescence (not shown). As shown above (Figure 4), the slow step is preceded by a fluorescence decrease taking place in the 100-ms range. This effect both qualitatively (Figure 4) and quantitatively (Figure 5, Table II) is identical with the effect corresponding to step 1 (eq 1) in P-site binding. We conclude that the formation of the intermediate complex R·T′ is common to the pathways leading to the binding of the tRNA into the P site or, if the P site is already occupied, into the A site.

*Kinetic Experiments in the Presence of Tetracycline.* The apparently identical second-order step observed during binding of $tRNA^{Phe}_{Prf37}$ or *N*-AcPhe-$tRNA^{Phe}_{Prf37}$ to both P and A sites raises the question, as mentioned earlier (Wintermeyer et al., 1979), whether binding to the P site may proceed through transient binding to the A site. Since tetracycline is known

Table III: Effect of Tetracycline on Ribosome Binding of *N*-AcPhe-tRNA$^{Phe}_{Prf37}$ [a]

| tetracycline added | P site preoccupied with tRNA$^{Phe}$ | *N*-AcPhe-tRNA$^{Phe}_{Prf37}$ bound (% of input) | A-site occupancy (%) [b] |
|---|---|---|---|
| − | − | 96 | 2 |
| + | − | 95 | 2 |
| − | + | 75 | 80 |
| + | + | 25 | 30 |

[a] Experiments were performed as described under Materials and Methods and in Table I. The concentration of tetracycline was 50 μM. [b] Fraction of ribosome-bound *N*-AcPhe-tRNA$^{Phe}_{Prf37}$ that did not react with puromycin.

to specifically inhibit the binding of aminoacyl-tRNA to the A site (Zagorska et al., 1971) without affecting P-site binding, the antibiotic may affect such a step. However, the presence of tetracycline at a concentration (50 μM) sufficient to inhibit the formation of the stable A-site complex of *N*-AcPhe-tRNA$^{Phe}_{Prf37}$ by more than 50% (Table III) did not change either relaxation times or, more important, amplitudes of the two steps observed in P-site binding of *N*-AcPhe-tRNA$^{Phe}_{Prf37}$ or of tRNA$^{Phe}_{Prf37}$ (data not shown). The fast step observed with P site blocked ribosomes was not affected either. Thus, the inhibitory effect of tetracycline appears to be confined to the slow rearrangement leading to the stable A-site complex.

The lack of any effect of tetracycline on the fast second-order step excludes the possibility that the tRNA during that step is bound to the A site in the functional sense. On the other hand, since topology and molecular mechanism of the tetracycline inhibition are not known, the negative result does not exclude transient binding of the tRNA molecule to the A site in the physical sense.

*Influence of EF-Tu on the Kinetics of A-Site Complex Formation.* Under physiological conditions, the binding of aminoacyl-tRNA to the A site requires EF-Tu and GTP and is accompanied by GTP hydrolysis (Miller & Weissbach, 1977). In order to determine the kinetic step(s) on which EF Tu has an influence, we carried out experiments with Phe-tRNA$^{Phe}_{Prf37}$ and EF-Tu·Ts at 6 mM $Mg^{2+}$. It was found that the addition of increasing amounts of EF-Tu·Ts in the presence of GTP considerably enhances the rate of the fluorescence increase accompanying A-site binding (Figure 8). The amplitudes of the fluorescence changes are correlated to the yield of complex, as determined by membrane filtration of the samples after 40 min. An 8-fold increase of the ribosome concentration (from 0.1 to 0.8 μM) does not increase the rate, indicating that the step that is accelerated by EF-Tu is a first-order rearrangement of an intermediate complex, possibly equivalent to R·T′ in eq 1. Preliminary stopped-flow data show that the formation of the intermediate complex itself is not appreciably affected by the addition of EF-Tu·GTP, $\tau_{fast}$ being shorter by only a factor of 1.5 at the highest concentration of EF-Tu used in the experiments of Figure 8.

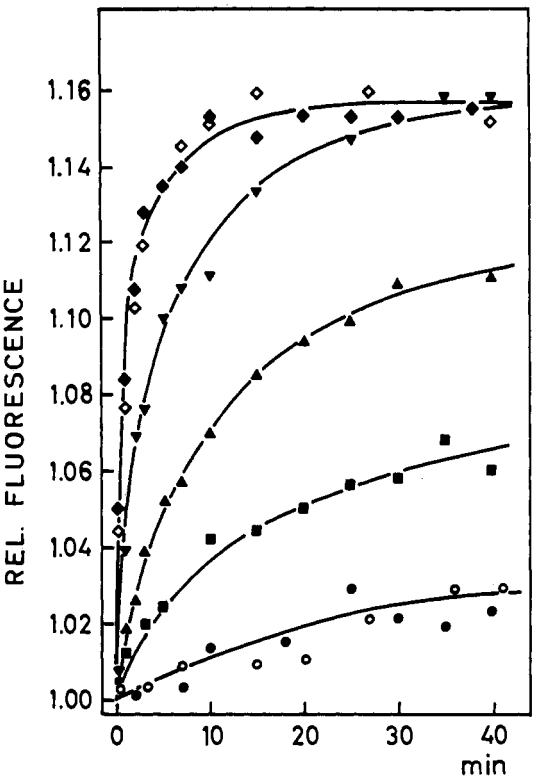


FIGURE 8: Kinetics of EF-Tu-dependent binding of Phe-tRNA$^{Phe}_{Prf37}$ to poly(U)-programmed, P site blocked ribosomes. Fluorescence at 500 nm was measured at varying times after adding ribosomes (final concentration 0.1 μM), which had been preincubated with poly(U) and tRNA$^{Phe}$ (0.12 μM), to Phe-tRNA$^{Phe}_{Prf37}$ (13 nM) in the presence of 0.25 mM GTP and varying concentrations of EF-Tu·Ts (μM): (●) no factor added; (■) 0.03; (▲) 0.08; (▼) 0.17; (♦; ◇; ○) 0.25. In one experiment ribosomes were added at a higher concentration [0.8 μM (◇)]. As a control, GTP was replaced with GDP at the highest concentration of EF-Tu·Ts (○). Experimental conditions were as described under Materials and Methods except that $Mg^{2+}$ was present at a concentration of 6 mM. As determined by the filtration assay, greater than 90% of the input of Phe-tRNA$^{Phe}_{Prf37}$ was bound in the experiment with the highest concentration of EF-Tu·Ts.

## Discussion

The fluorescent probe in the anticodon loop of tRNA$^{Phe}_{Prf37}$ in stopped-flow experiments reports two distinct steps during complex formation with poly(U)-programmed vacant ribosomes. Under the experimental conditions applied, all the tRNA is eventually found in the P site. According to the detailed kinetic analysis, the first step appears to be a second-order recombination step that leads to a short-lived intermediate tRNA–ribosome complex, designated R·T′ in eq 1 (above). In a subsequent slow reaction, the P-site complex or a complex closely related to it (R·T$^P$) is formed.

It should be mentioned that a slow change of the fluorescence polarization ($k$ = 0.015 $s^{-1}$) has been observed recently during binding of N-dansylated aminoacyl-tRNAs to non-programmed ribosomes at 40 mM $Mg^{2+}$ (Goss et al., 1980). The effect has been interpreted as a first-order transformation between two tRNA–ribosome complexes. Although this observation qualitatively resembles our results, it is difficult to correlate the relatively slow effect to any of the steps reported here, particularly because the binding site of the tRNA in the complex has not been determined.

As to a molecular interpretation of the fast second-order step, the activation energy of 9 kcal/mol strongly disfavors it being a simple bimolecular encounter step, which is expected to exhibit an activation energy not greater than about 3 kcal/mol (Hiromi, 1979). Thus, the fast relaxation is likely to represent a rearrangement of a rather weak first-encounter complex, the formation of which cannot be observed directly under these experimental conditions. Interestingly, the presumed rearrangement does not depend on the presence of poly(U).

The formation of R·T′ is observed regardless of whether the P site is vacant or fully occupied by nonfluorescent tRNA$^{Phe}$. Thus, the binding site of the fluorescent tRNA in the intermediate complex does not overlap with the P site. As to a possible overlap with the A site, the kinetic data available are not conclusive yet, mainly because it is difficult to completely

block the A site with nonfluorescent tRNA. There are, however, data from equilibrium titration experiments that exclude the existence of a third independent tRNA-binding site being stronger than about $5 \times 10^5$ $M^{-1}$ (Lill, 1981). For the intermediate complex R·T′, a binding constant of $(3 \pm 2) \times 10^7$ $M^{-1}$ is calculated from the rate constants $k_{12}$ and $k_{21}$ (Table II). These figures suggest that in the intermediate complex the tRNA is transiently bound to a site that, at least partially, overlaps with the A site. On the other hand, because of its rather rapid dissociation, the intermediate complex is not detectable by the filtration assay.

Provided the P site is already occupied, the intermediate complex rearranges to the A-site complex. The rearrangement requires proper codon–anticodon interaction and is very slow in the absence of EF-Tu·GTP. The A-site complex, owing to its very slow dissociation, is readily isolated by filtration on nitrocellulose filters. It should be noted that the equilibrium binding constant does not change by the rearrangement, the affinity of the tRNA for the A site being close to the one obtained for the intermediate complex (Lill, 1981). The strong poly(U) dependence of A-site complex formation, therefore, cannot be explained on the basis of a large contribution of codon–anticodon interaction to the binding energy, as has been proposed previously (Odinzov & Kirillov, 1978). It appears more likely that the transition of the tRNA from the intermediate state to the state in which it is locked in the A site is brought about by a codon-induced conformational change of the tRNA molecule. Such a change, resulting in an exposure of the TψCG sequence, has been reported (Möller et al., 1979).

The pathway of EF-Tu·GTP-dependent binding of aminoacyl-tRNA to the A site comprises additional steps that have not yet been resolved completely. GTP hydrolysis and, probably, release of EF-Tu·GDP have to take place before a peptide bond can be formed (Miller & Weissbach, 1977). Recent rapid-mixing experiments show that the rate of peptide bond formation following enzymatic binding is limited by a step taking place after GTP hydrolysis (Thompson et al., 1980). It has been postulated that in the intermediate complex the tRNA is bound to a site overlapping with the A site and that the A-site complex is formed by a codon-dependent switching of the tRNA into the A site (Lake, 1977). The use of the fluorescence technique allowed us to directly monitor both the rapid formation of the intermediate complex and the slow rearrangement to the A-site complex. Interestingly, the strong accelerating effect of EF-Tu·GTP is confined to the rearrangement step, the binding step being essentially unaffected by the factor. It will be interesting now to relate codon–anticodon interaction, as monitored by fluorescence, to GTP hydrolysis and release of EF-Tu·GDP and to see whether the possibility of conformational changes of the tRNA during the rearrangement step can be substantiated.

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