Transient Conformational States of Aminoacyl-tRNA during Ribosome Binding Catalyzed by Elongation Factor Tu†

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ABSTRACT: Conformational transitions of Phe-tRNA^{Phe} that take place during elongation factor Tu (EF-Tu)-dependent binding to the A site of *Escherichia coli* ribosomes were followed by transient fluorescence measurements. The fluorescence signal of proflavin replacing dihydrouracil at position 16 or 17 in yeast tRNA^{Phe} was utilized to monitor changes of the conformation of the D loop. The ternary complex EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) was purified by gel filtration. Upon binding of the complex to the A site of poly(U)-programmed, P-site-blocked ribosomes, the fluorescence changes in several steps. First, the rapid formation of an initial complex gives rise to a small fluorescence increase. Subsequent codon–anticodon recognition leads to a conformational rearrangement of the D loop of the tRNA that is reflected in a major fluorescence increase. Fluorescence-quenching data indicate an unfolding of the D loop in this state. The latter conformational state is short-lived, and the aminoacyl-tRNA refolds during the following rearrangement that occurs after GTP hydrolysis and accompanies the release of the aminoacyl-tRNA from EF-Tu·GDP and/or its accommodation in the A site. Further experiments show that the status of the P site influences the binding to the A site in that the two rearrangement steps are slowed down when the P site is unoccupied and even more so when it is occupied with the near-cognate tRNA^{Leu2}. In contrast, the occupancy of the E site has no influence on A-site binding, and vice versa, thus excluding any coupling between the two sites.

The elongation cycle of ribosomal protein synthesis begins with the binding of the substrate, aminoacyl-tRNA, to the A site of the ribosome. In bacterial systems, the reaction is catalyzed by elongation factor Tu (EF-Tu) and requires GTP (Miller & Weissbach, 1977; Kaziro, 1978). Binding of the complex EF-Tu·GTP·aminoacyl-tRNA to the ribosome is followed by the recognition of the codon, which provides the trigger for EF-Tu to hydrolyze GTP. The latter enables the dissociation of EF-Tu·GDP from the ribosome which, in turn, allows the aminoacyl-tRNA to accommodate in the A site so as to be positioned correctly for peptidyl transfer. There is strong evidence supporting the view that the complexity of the A-site-binding reaction, at least in part, is due to the necessity to optimize speed and accuracy of aminoacyl-tRNA selection by the inclusion of proofreading during the process (Thompson & Dix, 1982; Thompson & Karim, 1982; Ehrenberg & Kurland, 1984; Ruusala et al., 1984).

The sequence of events during A-site binding has been deduced from biochemical data primarily. Structural and kinetic data are rather scarce (Dix et al., 1990; Eccleston et al., 1985; Thompson et al., 1986; Bilgin et al., 1992). It is obvious, however, that the process of the A-site binding involves the formation of numerous intermediates that are characterized by different sets of contacts between aminoacyl-tRNA, EF-Tu, and the ribosome and the various conformations thereof. The conformational changes of the interacting species have not been characterized in detail. The transition of EF-Tu from the GTP to the GDP form, which switches the affinity of the factor for binding to the ribosome (and to aminoacyl-tRNA) from high to low, is established best. Also, a change of the ribosome from a post- to a pretranslocation state, brought about by the EF-Tu-dependent occupancy of the A site (Möller & Maassen, 1986; Hausner et al., 1987), as well as changes of the conformation of aminoacyl-tRNA, taking place upon A-site binding (Robertson & Wintermeyer, 1981; Douthwaite et al., 1983; Bertram et al., 1983), have been reported.

The conformation of aminoacyl-tRNA in the complex with EF-Tu·GTP was reported to differ from that in solution. The changes have been suggested by results obtained with various approaches, including electron spin resonance (Weygand-Durasevic et al., 1981), circular dichroism (Haruki et al., 1990), oligonucleotide binding (Kruse et al., 1980), nuclease digestion (Boutorin et al., 1981; Wikman et al., 1982), chemical modification (Riehl et al., 1983), and fluorescence (Adkins et al., 1983; Janiak et al., 1990). The affected regions of the tRNA molecule include the 3′ terminus and the T stem, i.e., two regions which directly interact with the factor (Wikman et al., 1987; Joshi et al., 1986; Boutorin et al., 1981; Picone & Parmeggiani, 1983), as well as the anticodon loop, thiouridine(8), and the D loop, i.e., regions which are not in contact with the protein (Wikman et al., 1982; W. Wintermeyer et al., unpublished data).

Binding to the A site also changes the tertiary structure of the tRNA. There is evidence suggesting that codon–anticodon interaction on the ribosome induces a conformational change of the D loop of tRNA (Robertson & Wintermeyer, 1981) in a way similar to that described for tRNA in solution (Gassen, 1980; Labuda & Pörschke, 1980). For the anticodon loop, an increase of stacking upon binding to the ribosome was reported which is not directly related to codon–anticodon pairing (Paulsen et al., 1982). Data obtained by chemical modification suggest that also the T loop and the acceptor stem are affected (Douthwaite et al., 1983; Bertram et al., 1983).

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The present contribution addresses the molecular mechanism of A-site binding with respect to conformational changes of the aminoacyl-tRNA. The experimental results have been obtained by using the fluorescence stopped-flow technique utilizing the fluorescence signal of a fluorophore on the tRNA moiety of the ternary complex EF-Tu·GTP·Phe-tRNA^{Phe}, i.e., proflavin at positions 16/17 in the D loop. The data reveal that, during the interaction of ternary complex with the A site of the ribosome, the aminoacyl-tRNA upon codon recognition in the A site transiently assumes a conformational state in which the D loop is unfolded. Following GTP hydrolysis, this state folds back upon accommodation of the aminoacyl-tRNA in the A site. The conformational changes of the tRNA are coupled to concomitant changes of the factor, as revealed by measurements with a fluorescent GTP derivative (Rodnina et al., 1993; M. V. Rodnina, unpublished results). Furthermore, data are presented that show that the tRNA in the P site has an influence on A-site binding of the ternary complex, whereas in the E site it does not.

MATERIALS AND METHODS

Buffer and Reagents. Buffer A: 25 mM Tris-HCl, pH 7.5, 50 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTE, and 0.5 mM EDTA. The experiments were performed at 20 °C if not stated otherwise. Poly(U), poly(A), and other biochemicals were purchased from Boehringer Mannheim. Radioactively labeled compounds were from Amersham or UVVVR (Czech Republic).

tRNAs and Proflavin-Labeled tRNA Derivatives. tRNA^{Phe} from brewer's yeast (charging capacity of 1.7 nmol/A_{260} unit) was purchased from Boehringer Mannheim. *E. coli* tRNA^{Phe} (1.65 nmol/A_{260} unit) and tRNA^{Lys} (1.6 nmol/A_{260} unit) were from Subriden RNA. AcPhe-tRNA^{Phe}, Ac[^{3}H]Phe-tRNA^{Phe} (2330 dpm/pmol), and [^{3}H]Phe-tRNA^{Phe} (114 dpm/pmol) were prepared and purified to homogeneity (1.7–1.75 nmol/A_{260} unit) by HPLC on C-4 as described (Rodnina & Wintermeyer, 1992). Acetylation of Phe-tRNA^{Phe} was performed according to Rappoport & Lapidot (1974); acetylation was quantitative, as verified by hydrolysis and subsequent HPLC analysis on RP-8. [^{14}C]tRNA^{Phe}, a gift of Y. Semenkov, was prepared by growing *E. coli* cells with ^{14}C-labeled uridine and isolating the tRNA according to standard procedures. The fluorescent tRNA^{Phe} derivative, tRNA^{Phe}-(Prf16/17), was prepared as described previously (Wintermeyer & Zachau, 1979) and purified by gel filtration on Superdex 75. The material from the main peak contained one dye per tRNA; it was chargeable to 1.5 nmol/A_{260} unit.

Ribosomes and Elongation Factor Tu. Tight-coupled 70S ribosomes from *E. coli* MRE 600 were prepared essentially as described (Robertson & Wintermeyer, 1981), with an additional washing step at 0.5 M NH_4Cl with subsequent purification through a 1.1 M sucrose cushion prior to the zonal centrifugation. Ribosome concentrations were calculated from absorption measurements on the basis of 23 pmol/A_{260} unit. The activity of the ribosomes was 85–90% in binding AcPhe-tRNA^{Phe} to both A and P sites and greater than 80% in peptide bond formation. Ribosome concentrations given in the text are based on tRNA binding activity.

EF-Tu was prepared from *E. coli* K12 using a procedure combined from the protocols of Lebermann et al. (1980) and Ehrenberg et al. (1990) with the following modifications. The postribosomal supernatant was purified on a DEAE-Sepharose CL-6B column using a gradient of 0–0.4 M NaCl in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTE, 100 μM PMSF, 3 mM NaN_3, and 30 μM GDP. EF-Tu was localized in the eluate by both [^{3}H]GDP binding and SDS–polyacrylamide gel electrophoresis and was precipitated by adding ammonium sulfate (0.3 g/mL). The factor was purified further by gel filtration on Sephacryl S300 in 50 mM potassium phosphate, pH 7.5, 10% glycerol, 100 μM PMSF, 30 μM GDP, 3 mM NaN_3, and 1 mM DTE. Fractions containing EF-Tu were pooled and applied to DEAE-Sepharose CL-6B; the column was developed with a gradient of potassium phosphate from 50 mM, pH 7.5, to 250 mM, pH 6.9, containing 10% glycerol, 100 μM PMSF, 30 μM GDP, 3 mM NaN_3, and 1 mM DTE. The final purification was achieved by gel filtration on a preparative Superdex 75 column (HiLoad, Pharmacia). EF-Tu was more than 95% pure according to SDS–polyacrylamide gel electrophoresis; it was devoid of any detectable contamination by elongation factor G (EF-G) on the basis of ribosome-dependent GTPase.

The concentration of EF-Tu was determined both colorimetrically (Bradford, 1976) and by absorbance at 280 nm using an extinction coefficient of 32 900 M^{-1} cm^{-1} (Block & Pingoud, 1981); both measurements gave the same values within 5%. The activity of the preparation of EF-Tu used for the present experiments was close to 100% in [^{3}H]GDP binding, measured by nitrocellulose filtration. All the factor was competent in Phe-tRNA^{Phe} binding, as shown by the absence of any unbound factor when the EF-Tu·GTP·Phe-tRNA^{Phe} complex was prepared by adding Phe-tRNA^{Phe} in excess and analyzed by analytical FPLC on Superdex 75 (HR 10/30, Pharmacia), as described below.

Preparation of EF-Tu·GTP·Phe-tRNA Complexes. About 7 nmol of EF-Tu was incubated for 10 min at 37 °C with 1 mM GTP (or GTP analog), 3 mM ATP, 6 mM phosphoenolpyruvate, and 0.1 μg/mL of pyruvate kinase in 200 μL of buffer A; then, 3 nmol of [^{14}C]Phe-tRNA^{Phe}(Prf16/17) (800 dpm/pmol) was added (final volume, 230 μL), and the incubation was continued for 5 min. The ternary complex was purified by FPLC in buffer A at 20 °C on two columns of Superdex 75 (HR 10/30, Pharmacia) operated in tandem. Fractions of 0.16 mL were collected, and 10-μL aliquots were taken for counting. EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe}(Prf16/17) was completely separated from unbound [^{14}C]Phe-tRNA^{Phe}(Prf16/17), tRNA^{Phe}(Prf16/17), and EF-Tu. The concentration of the complex was taken as equivalent to that of [^{14}C]Phe-tRNA^{Phe}(Prf16/17) in the sample, which usually was 0.7–0.9 μM. The complex was stored on ice and diluted to the usual working concentration (0.2 *M) immediately before the stopped-flow experiment. The stability of the complex after dilution to 0.2 μM was assessed by the loss of TCA-precipitable [^{14}C]Phe upon RNase A treatment (0.25 mg/mL RNase A, 37 °C, 15-s incubation). Less than 10% of [^{14}C]Phe-tRNA was released from the complex during the time required for a stopped-flow experiment (about 30 min at 20 °C). The same procedure was used to prepare the ternary complex containing [^{3}H]Phe-tRNA^{Phe}.

Preparation of Ribosome Complexes. To block the P site, the ribosomes were incubated in buffer A for 15 min at 37 °C either with a 1.1-fold excess (relative to ribosomes active in binding) of AcPhe-tRNA^{Phe} (or tRNA^{Phe}) and 2 A_{260} units/mL of poly(U) or with a 1.1-fold excess of tRNA^{Lys} and 1 A_{260} unit/mL of poly(A). The final concentration of the complexes was 0.6 μM.

Biochemical Assays. The amount of [^{14}C]Phe-tRNA^{Phe}-(Prf16/17) bound to the ribosomes was determined by the nitrocellulose filter assay by applying aliquots of the reaction mixture to the filters (Sartorius) directly and subsequently washing with binding buffer. To determine the extent of

dipeptide formation on ribosome complexes with AcPhe-tRNA^{Phe} in the P site and [^{14}C]Phe-tRNA^{Phe}(Prf16/17) in the A site, an aliquot of the binding mixture was treated with 0.5 M KOH for 1 h at 37 °C, acidified, and analyzed by HPLC on an RP-8 column using a gradient of acetonitril (0 to 40%) in 0.1% trifluoroacetic acid. ^{14}C radioactivity in the eluate was monitored by a flow-through monitor (Ramona, Raytest) using a solid scintillator. The dipeptide Ac[^{3}H]Phe-[^{3}H]Phe was determined in a similar way, except that the radioactivity in the eluate was measured by liquid scintillation counting.

Stopped-Flow Experiments. The apparatus used for the stopped-flow measurements was equipped with a 200-W Hg–Xe arc lamp (Hanovia) and a double monochromator in the excitation beam. The fluorescence of proflavin was excited at 436 nm and measured with two photomultipliers after passing KV 500 filters (Schott). Rapid mixing (dead time, 1–2 ms) was performed by the Bio-Logic stopped-flow module synchronized with the data acquisition system (Bio-Logic, France). Measurements were performed in parallel in four different time ranges of 1000 data points each, and several shots were averaged per experiment. For evaluation, the data were combined to one data set of about 3600 points. The data were evaluated by fitting to the data an expression which contained the sum of up to three exponential terms (characterized by variable time constants, k_{app}, and amplitudes, A) and another variable for the final signal by using a Marquardt nonlinear least-squares fitting procedure. The reproducibility of the rate constants given is about ±15%; that of the amplitudes, about ±20%. The curves displayed in the figures are averages of four to eight measurements. They usually represent an intermediate time range or, in some cases, an overlay of a short and an intermediate time range.

The experiments were performed by rapidly mixing equal volumes (60–80 μL each) of the ternary complex, purified by gel filtration, and the ribosome complex to give final concentrations of 0.1 and 0.3 μM, respectively. If not stated otherwise, the temperature was maintained at 20 °C. After the stopped-flow experiment, aliquots were routinely analyzed by nitrocellulose filtration for the amount of ternary complex bound to the ribosome and, when appropriate, for the amount of AcPhe-[^{14}C]Phe dipeptide. More than 90% of the ternary complex was found bound to the ribosome, and about 75% of the ribosome-bound [^{14}C]Phe-tRNA^{Phe}(Prf16/17) incorporated the amino acid into the dipeptide AcPhe-[^{14}C]Phe when AcPhe-tRNA^{Phe} was in the P site. Both binding and dipeptide formation were complete within 10 s at 20 °C.

RESULTS

Several Steps of A-Site Binding Reported by Proflavin in the D Loop. To initiate A-site binding, the purified ternary complex EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe}(Prf16/17) was rapidly mixed with an excess (3-fold or higher) of poly(U)-programmed ribosomes, which in the P site carried either AcPhe-tRNA^{Phe} or deacylated tRNA^{Phe}. When the proflavin fluorescence is monitored, two main phases of the reaction are distinguished (Figure 1): a rapid increase of the fluorescence is followed by a slower decrease. Practically the same picture is observed independent of whether AcPhe-tRNA^{Phe} or deacylated tRNA^{Phe} is in the P site; hence, the formation of the peptide bond is not reflected in the observed signal changes. For the conditions of Figure 1, the reaction is described by the sum of two exponential functions with amplitudes of opposite signs and apparent first-order rate constants, k_{app}, of 24 and 6 s^{-1}. The concentration dependence of the two steps is described below (Figure 4).

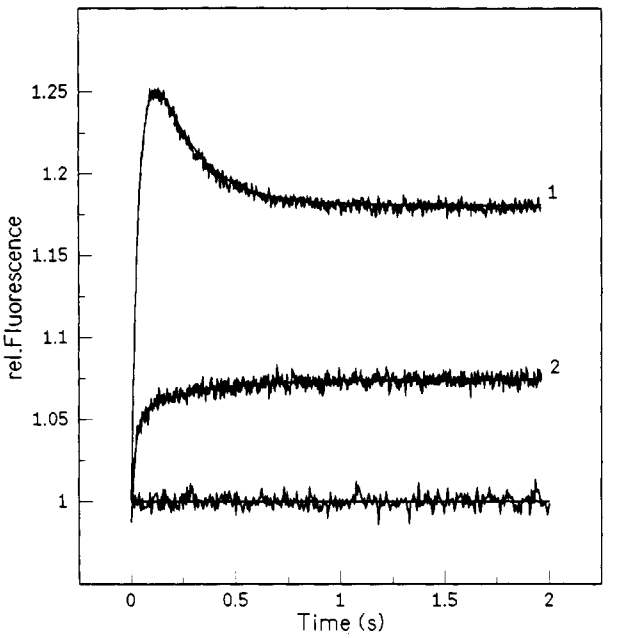

FIGURE 1: Time course of the binding of EF-Tu·GTP·Phe-tRNA^{Phe}·(Prf16/17) to the A site of 70S ribosomes monitored by proflavin fluorescence. Ribosomes programmed with poly(U) (1) or poly(A) (2) contained AcPhe-tRNA^{Phe} or tRNA^{Lys} in the P site, respectively. Fluorescence is given relative to the initial signal of the ternary complex. The displayed curves are averages of four measurements. The smooth lines represent the functions fitted with the sum of two exponential terms with the following parameters: (1) k_{app1} = 24 s^{-1}, A_1 = 35%; k_{app2} = 6 s^{-1}, A_2 = −17%; (2) k_{app1} = 43 s^{-1}, A_1 = 5%; k_{app2} = 3 s^{-1}, A_2 = 2%.

Table 1: Fluorescence and Biochemical Characterization of Various States of A-Site Binding of Phe-tRNA^{Phe}(Prf16/17)[a]

state	binding	dipeptide	F/F_0	k_{app} (s^{-1})[b]
initial complex	≈0.2[c]	0[d]	1.05	43
recognition complex				
with GTP	n.d.[e]	n.d.	1.35	24
with GTPγS	0.85	n.d.	1.12	22
with caged GTP	0.90	0.60	1.38	20
with GTP/kirromycin	0.70	0[d]	1.32	22
A site bound	0.93	0.75	1.18	6

[a] Binding was determined by nitrocellulose filtration and is given relative to the amount of [^{14}C]Phe-tRNA^{Phe}(Prf16/17) added in the complex with EF-Tu. The amount of AcPhe-[^{14}]Phe dipeptide was determined by HPLC analysis (Materials and Methods) and is also given relative to [^{14}C]Phe-tRNA^{Phe}(Prf16/17) added as EF-Tu·GTP complex. F/F_0 is the ratio of the fluorescence of the intermediate (F) and the starting fluorescence (F_0) of the EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe}(Prf16/17) complex as given by the amplitude of the respective step (see captions of figures). The same biochemical data were obtained for the EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} complexes containing unmodified and fluorescent tRNA. [b] Apparent rate constant for the formation of the respective state at the conditions of Figure 1. [c] Complex is unstable and dissociates rapidly. [d] Below detection limit of 0.02. [e] Not determined.

Initial Binding Is Codon-Independent. In order to identify the codon-recognition step, ribosomes programmed with poly(A) and containing tRNA^{Lys} in the P site were used for the binding experiments. As shown in Figure 1, there is binding also in the noncognate system. Compared to the poly(U) system, the fluorescence increase observed with poly(A) is faster and has a smaller amplitude, while the slow decrease is no longer seen; instead, there is a slow increase of the signal. The evaluation by fitting a sum of two exponential functions yields k_{app} values of 43 and 3 s^{-1} for the two steps at this particular condition. The biochemical analysis reveals that the noncognate complex is labile (Table 1). Upon rapid filtration of the sample without prior dilution, a small and

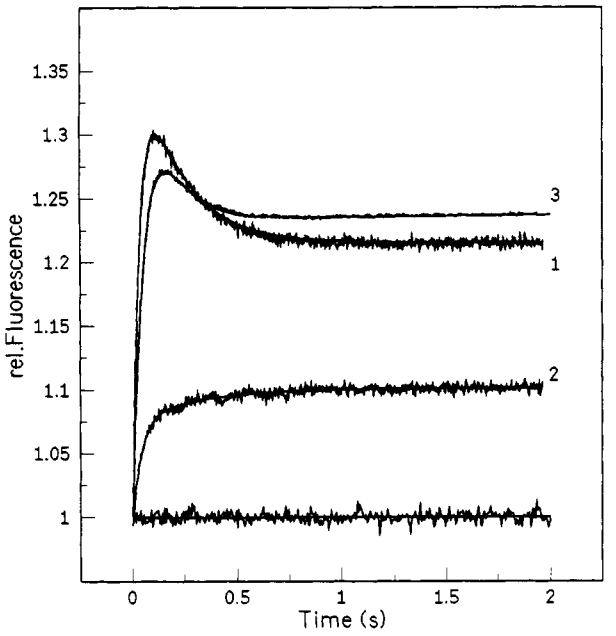

FIGURE 2: Replacement of GTP with GTP analogs during A-site binding of ternary complex. Ribosomes were programmed with poly(U) and contained $tRNA^{Phe}$ in the P site; the complex of EF-Tu with Phe-$tRNA^{Phe}$(Prf16/17) contained GTP (1), GMPPNP or GTPγS (2), or caged GTP (3). The displayed curves are averages of four (curve 3, eight) measurements. Fitting parameters: (1) $k_{appl} = 23\ s^{-1}$, $A_1 = 41\%$; $k_{app2} = 6\ s^{-1}$, $A_2 = -19\%$; (2) $k_{appl} = 22\ s^{-1}$, $A_1 = 12\%$; $k_{app2} = 2\ s^{-1}$, $A_2 = 2\%$; (3) $k_{appl} = 20\ s^{-1}$, $A_1 = 38\%$; $k_{app2} = 8\ s^{-1}$, $A_2 = -14\%$.

variable fraction (about 15–25%) of ribosomes which carry [^{14}C]Phe-$tRNA^{Phe}$ is found on the nitrocellulose filter; the complex is no longer observed when the sample is diluted shortly before filtration.

It is to be noted that the formation of the initial binding state, although it is not specific with respect to codon–anticodon interaction, involves a conformational change of the tRNA in the D loop that causes the fluorescence change observed. This indicates some specificity of the interaction and distinguishes the initial binding complex from the first encounter complex, which is not seen in our experiments. The slow fluorescence increase with $k_{app} = 3\ s^{-1}$ (Figure 1, poly(A)) does not seem to reflect any of the sequence of steps during the A-site binding, but rather represents an additional fluorescence change of the tRNA the nature of which is not known at present.

Nonhydrolyzable GTP Analogs Yield Inhomogeneous Results. To uncouple codon effects from GTP hydrolysis, we replaced GTP with the nonhydrolyzable derivatives GMPPNP, GTPγS, or caged GTP (P^3-1-(2-(nitrophenyl)ethyl)guanosine 5′-triphosphate; Schlichting et al., 1989). On the gel filtration column, the ternary complexes containing the GTP derivatives behave as the GTP-containing complex. In all three cases, the ternary complexes are bound stably to poly(U)-programmed ribosomes according to nitrocellulose filtration (90% of the ternary complex bound; no significant dissociation within 15 min following 15-fold dilution). In the stopped-flow experiment, the GTP derivatives exhibit some differences. With GTPγS and GMPPNP, a fast fluorescence increase (23 s^{-1}) followed by a slow (2 s^{-1}) further increase of very small amplitude is observed (Figure 2, curve 2), while the fluorescence decrease observed with GTP is absent; the amplitude of the fast step is only about one-quarter of that observed for the GTP-containing ternary complex. In contrast, caged GTP gives a picture similar to that of GTP (Figure 2, curve 3).

Since GMPPNP is not hydrolyzed at all and GTPγS not hydrolyzed in the time range of the stopped-flow experiments (hydrolysis rate ≈ 0.01 s^{-1}), the small amplitude observed with the two analogs may be explained by assuming that in the presence of the analogs only codon recognition takes place, while subsequent rearrangement(s) that depend upon GTP hydrolysis—and lead to the major fluorescence increase—are blocked. This model would require that GTP is hydrolyzed at least at the rate of the conformational change in question, 24 s^{-1}. However, quenched-flow experiments performed at exactly the conditions of the present stopped-flow experiments reveal an apparent rate constant of GTP hydrolysis of about 12 s^{-1} (M. V. Rodnina, unpublished). This suggests that GTP hydrolysis takes place only after the high-fluorescence tRNA intermediate has formed. Thus, the failure of GTPγS and GMPPNP to promote the formation of the transient high-fluorescence tRNA intermediate is not due to the lack of GTP hydrolysis but rather indicates that the ternary complexes containing those analogs differ from the GTP-containing complex in features which are critical for the rearrangement of the tRNA to occur.

On the other hand, caged GTP leads to fluorescence results which are the same as those obtained with GTP. In this case—in contrast to the biochemical results obtained with the other GTP analogs—the biochemical analysis reveals that, following the binding of the ternary complex containing caged GTP, the peptide bond is formed, although caged GTP is not hydrolyzed (Table 1). We conclude that EF-Tu that contains caged GTP is dissociating from the aminoacyl-tRNA and the ribosome without hydrolysis of the triphosphate, thus allowing the aminoacyl-tRNA to complete the binding process, i.e., to accommodate in the A site and take part in peptide bond formation.

Kirromycin Inhibits the Further Rearrangement of the Recognition Complex. The antibiotic kirromycin has been shown to inhibit the dissociation of EF-Tu·GDP from the ribosome, presumably by fully or partially blocking the structural transition of the factor which normally is induced by GTP hydrolysis (Parmeggiani & Swart, 1985). Thus, the antibiotic should be useful to assign the fluorescence changes that take place after GTP hydrolysis. Since kirromycin also promotes GTP hydrolysis in the absence of ribosomes, the antibiotic in the stopped-flow experiments was added only to the ribosome complexes to minimize GTP hydrolysis prior to the binding of EF-Tu·GTP·Phe-$tRNA^{Phe}$ to the ribosome. The concentration of the antibiotic (0.2 mM) was sufficient to keep the rate of binding of kirromycin to EF-Tu higher than that of EF-Tu·GTP·Phe-$tRNA^{Phe}$ association with the ribosome. In the presence of kirromycin, the binding of Phe-$tRNA^{Phe}$ to the ribosome, as measured by nitrocellulose filtration, was reduced to about 70%, while the formation of dipeptide was completely suppressed (Table 1).

When kirromycin is present during the binding of EF-Tu·GTP·Phe-$tRNA^{Phe}$(Prf16/17) to poly(U)-programmed ribosomes, the fast fluorescence increase is not affected, except for a slight reduction of the amplitude, while the slow fluorescence decrease is completely suppressed (Figure 3). The decrease of the amplitude of the fast step is in good correlation with the lower amount of the ternary complex bound to the ribosome found by filter binding and is apparently due to kirromycin-induced hydrolysis of GTP on part of the EF-Tu·GTP·Phe-$tRNA^{Phe}$ complexes. Thus, kirromycin inhibits—by blocking the structural transition of the factor—the release of the factor from the tRNA which, in turn, inhibits the corresponding conformational change of the tRNA.

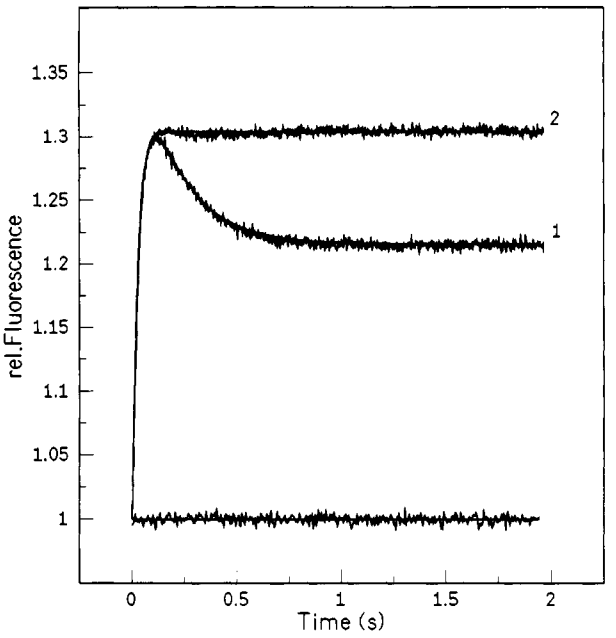

FIGURE 3: Effect of kirromycin on the A-site interaction of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17). Ribosomes were programmed with poly(U) and contained AcPhe-tRNA^{Phe} in the P site: (1) no kirromycin added, (2) 0.2 mM kirromycin added to the ribosomes before mixing. Fitting parameters: (1) $k_{appl} = 22\ s^{-1}$, $A_1 = 40\%$; $k_{app2} = 5\ s^{-1}$, $A_2 = -18\%$; (2) $k_{app\ 1} = 22\ s^{-1}$, $A_1 = 32\%$; $k_{app\ 2} = 4\ s^{-1}$, $A_2 = -1\%$.

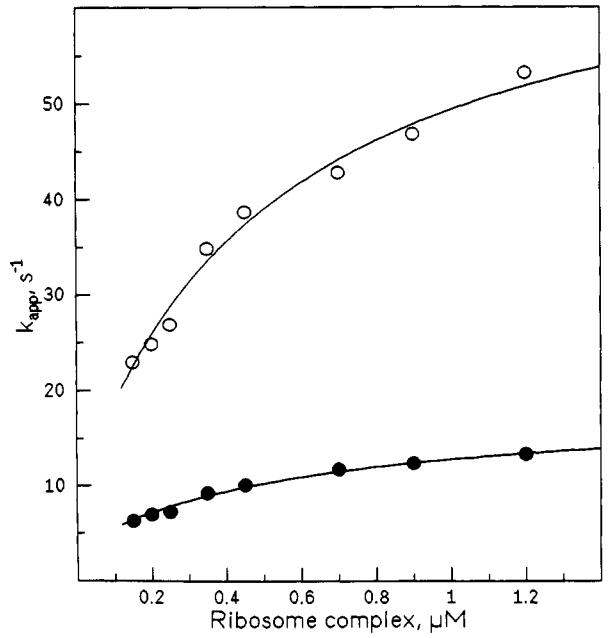

FIGURE 4: Concentration dependence of A-site binding of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17). The stopped-flow experiments were performed as in Figure 1 with increasing concentrations of poly(U)-programmed ribosomes carrying AcPhe-tRNA^{Phe} in the P site; the concentration after mixing is indicated. The concentration of ternary complex was 0.1 μM. The apparent first-order rate constants of steps 1 (○ symbols) and 2 (● symbols) were estimated as in Figure 1.

Fluorescence Changes Indicate Rearrangements of the Initial Complex. The k_{app} values of both steps observed in the poly(U) system (Figure 1) increase with increasing concentration of ribosomes and reach saturation around 60 and 15 s^{-1}, respectively, at about 1.5 μM ribosomes (Figure 4). The saturating behavior of both steps shows that they represent rearrangements of an initial complex, i.e., that they do not represent the bimolecular binding step. The formation of the initial complex is observed separately in the experiment with poly(A)-programmed ribosomes (Figure 1). Due to its small amplitude and due to the fact that its rate over the range of accessible ribosome concentrations is similar within about a factor of 2 (40–100 s^{-1}; data not shown) to the rate of the following codon-recognition step, 18–60 s^{-1}; Figure 4), which has a large amplitude, the initial binding step is difficult to separate in experiments performed in the cognate system. At the concentrations used for most of the experiments, however, the contribution of initial binding to the total fluorescence change is small and does not significantly influence the quantitative evaluation of the following steps.

Fluorescence Quenching Reveals a Conformational Change of the D Loop Due to Codon–Anticodon Interaction. Changes of the fluorescence emission intensity are due to changes of the chemical environment of the fluorophor. On the molecular level, they may have two different reasons: a change of the extent to which the factor or the ribosome protect the reporter group or else a change of the tRNA conformation that leads to a change of the interactions of the fluorophor with neighboring bases. To distinguish between the two possibilities, we studied the solvent accessibility of the fluorophor in the D loop of Phe-tRNA^{Phe} in different stages of A-site binding by fluorescence-quenching experiments both in the steady state and in transient kinetics.

In the steady state, fluorescence was measured in the presence of increasing amounts of potassium iodide, and the data were plotted according to the Stern–Volmer equation to determine the quenching constants, K_{SV}. The experiment was performed with Phe-tRNA^{Phe}(Prf16/17) free in solution, in the complex with EF-Tu·GTP, and in the complex with EF-Tu·GMPPNP bound to poly(U)- or poly(A)-programmed ribosomes (data not shown). In all cases, linear plots are obtained the slopes of which yield the same quenching constant, 7.5 M^{-1}, as previously measured for both free and A-site-bound tRNA^{Phe}(Prf16/17) (Robertson & Wintermeyer, 1981). Preliminary measurements indicate that at the same time the fluorescence lifetimes are shorter in the ribosome complex compared to free and EF-Tu-bound Phe-tRNA^{Phe}-(Prf16/17) (unpublished data). Thus, the quenching rate constant, i.e., the accessibility, of the dye is increased in the ribosome complex. Hence, the D-loop label remains at least as accessible as in the free tRNA when the aminoacyl-tRNA is in the ternary complex or the A site.

To assess the exposure to the solvent of the label in Phe-tRNA^{Phe}(Prf16/17) in the transient conformational states during A-site binding, stopped-flow experiments were performed in the presence of various concentrations of KI. If the fluorescence in all intermediates were quenched to the same extent, then the relative amplitudes of the various kinetic steps would be expected to be the same in the presence or the absence of the quencher. Alternatively, if the fluorescence of the short-lived intermediate relative to the other states were to decrease this would indicate a higher exposure of the dye in that state. Stopped-flow experiments with EF-Tu·GTP·Phe-tRNA^{Phe}-(Prf16/17) and poly(U)-programmed ribosome complexes were performed in the presence of different concentrations of KI and KCl such that the concentration of KCl + KI was 50 mM. The presence of KI affects the fluorescence amplitudes in a way that the relative contribution of the high-fluorescence intermediate is decreased while the amplitudes of the other steps are not affected (Figure 5A).

To determine the quenching constant of the affected intermediate, the traces obtained in the presence of various concentrations of KI were subtracted from the one obtained

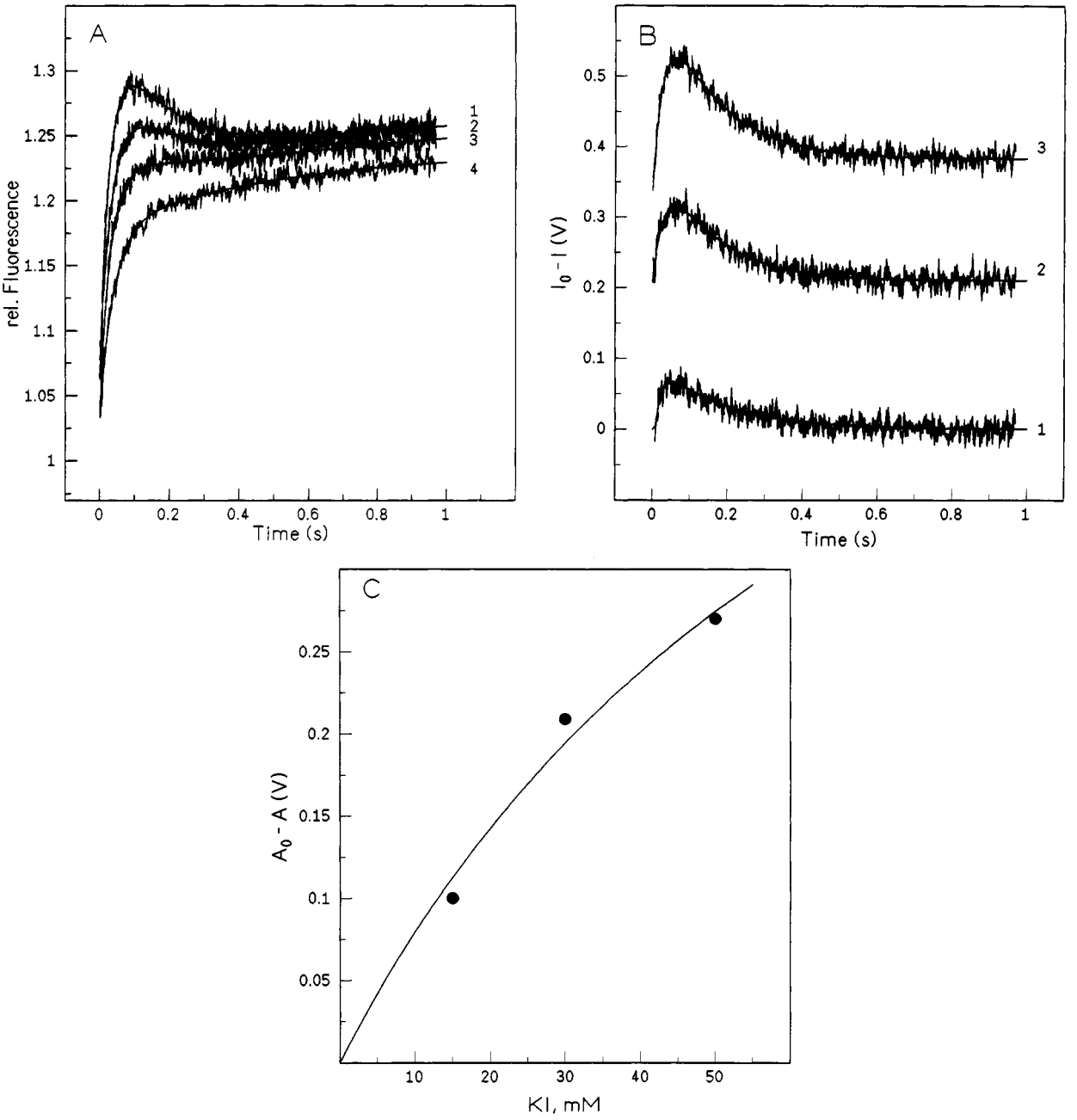

FIGURE 5: Iodide quenching of the fluorescence of Phe-tRNA^{Phe}(Prf16/17) intermediates upon binding to the A site. (A) Time course of EF-Tu·GTP·Phe-tRNA^{Phe}(Prf16/17) binding to the A site of poly(U)-programmed 70S ribosomes containing AcPhe-tRNA^{Phe} in the P site in buffer A containing, in addition, 50 mM KCl (1), 35 mM KCl and 15 mM KI (2), 20 mM KCl and 30 mM KI (3), or 50 mM KI (4). (B) Kinetic isolation of the transient intermediate state of Phe-tRNA^{Phe}(Prf16/17) at different concentrations of KI: 15 (1), 30 (2), and 50 mM (3). The differential curves were obtained by subtracting the curves obtained in the presence of KI (I) from the one in the absence of quencher (I_0). Fitting parameters: (1) $k_{app\ 1} = 31\ s^{-1}$, $A_1 = 12$ mV; $k_{app\ 2} = 6\ s^{-1}$, $A_2 = -10$ mV; (2) $k_{app\ 1} = 32\ s^{-1}$, $A_1 = 21$ mV; $k_{app\ 2} = 7\ s^{-1}$, $A_2 = -21$ mV; (3) $k_{app\ 1} = 35\ s^{-1}$, $A_1 = 30$ mV; $k_{app\ 2} = 7\ s^{-1}$, $A_2 = -27$ mV. (C) Determination of the Stern–Volmer quenching constant of the transient intermediate of Phe-tRNA^{Phe}(Prf16/17) upon binding to the A site. $A_0 - A$ is the amplitude of the fluorescence decrease determined from the differential curves presented in Figure 3B. Data were fitted according to the equation: $A_0 - A = A_0(1 - 1/(1 + K_{SV}[KI]))$ to give $K_{SV} = 11.5 \pm 1.5\ M^{-1}$.

without KI (Figure 5B). The resulting differential curves can be decomposed into two exponential terms, characterized by apparent rate constants of 30^{-1} and 6 s^{-1}, that represent the formation and the decay of the affected intermediate. To determine the quenching constant, K_{SV}, the differential amplitudes of the slow step are plotted vs. the concentration of KI (Figure 5C), and the plot is evaluated by regression according to the Stern–Volmer relationship. A quenching constant $K_{SV} = 11.5\ M^{-1}$ results for the affected intermediate, which is significantly higher than the one of either free, or EF-Tu-bound, or A-site-bound Phe-tRNA^{Phe}(Prf16/17) determined in the steady state, 7.5 M^{-1}. The differential amplitudes of the fast step yield a quenching constant around 12 M^{-1} (not shown). The observation that the quenching constant increases rather than decreases shows that the observed fluorescence increase is due to a conformational change around the dye rather than due to increased shielding against the access of solute molecules quenching the fluorescence, and indicates a higher exposure of the dye in the intermediate state.

Influence of the P Site-Bound tRNA on A-Site Binding. Stopped-flow experiments were performed with poly(U)-programmed ribosomes which had the P sites empty or occupied with cognate tRNA^{Phe} or with near-cognate [^{14}C]-

Table 2: Influence of the P-Site Ligand on A-Site Binding of the Ternary Complex[a]

P site	k_{app1} (s^{-1})	A_1 (%)	k_{app2} (s^{-1})	A_2 (%)
$tRNA^{Phe}$	23	41	6	−17
$tRNA^{Leu2}$	13	20	3	−13
free	17	49	3	−26

[a] Fluorescence stopped-flow experiments were performed with EF-Tu·GTP·[^{14}C]Phe-$tRNA^{Phe}$(Prf16/17) and poly(U)-programmed ribosomes the P site of which was occupied with *E. coli* $tRNA^{Phe}$ or $tRNA^{Leu2}$ or unoccupied. Apparent first-order rate constants, k_{app}, and amplitudes, A, of the fluorescence changes relative to the initial signal (positive, signal increase; negative, signal decrease) were determined by two-exponential fitting. Both rate constants and amplitudes are accurate to ±5% for this set of experiments. The fraction of [^{14}C]Phe-$tRNA^{Phe}$(Prf16/17) bound to ribosomes according to nitrocellulose filtration was >85% in all three cases.

$tRNA^{Leu2}$(GAG). Blocking of the P site with $tRNA^{Phe}$ was quantitative, as assessed by the indicator binding test described previously (Lill et al., 1984). Also with [^{14}C]$tRNA^{Leu2}$, the P site was occupied to more than 90%, as measured directly by nitrocellulose filtration. The extent of EF-Tu-dependent binding of [^{14}C]Phe-$tRNA^{Phe}$ to the A site, measured by nitrocellulose filtration without prior dilution, was not influenced by the ligand in the P site. More than 90% of ternary complex was bound within 30 s after adding the respective ribosome complex in excess (Table 1).

The results of the kinetic experiments are summarized in Table 2. While in the three cases that we compared the binding to the A site gives rise to qualitatively similar signal changes, the quantitative picture varies. With the cognate $tRNA^{Phe}$ in the P site, the two steps have the highest rates and amplitudes. With $tRNA^{Leu2}$ in the P site, both the apparent rate constant, k_{app1}, and the amplitude, A_1, of the first step are reduced to about one-half the values observed with $tRNA^{Phe}$; the parameters of the second step, k_{app2} and A_2, behave in about the same way. When the P site is not occupied at all, the differences are smaller, although the tendency is similar.

Occupancy of the E Site Does Not Affect A-Site Binding. It has been proposed that the exit (E) site and the A site of the ribosome are coupled in a negatively cooperative manner, i.e., the occupation of the E site reduces the affinity of the A site, and vice versa (Nierhaus, 1990). However, we have not observed any effect of A-site binding of AcPhe-$tRNA^{Phe}$ on the level of $tRNA^{Phe}$ bound to the E site (Robertson & Wintermeyer, 1987). Table 3 shows that also the level of A-site binding of ternary complex, determined by dipeptide formation with P-site-bound Ac[^{3}H]Phe-$tRNA^{Phe}$, is not significantly affected by occupancy of the E site. Rather, both A and E sites are fully occupied on these ribosomes, which are nearly 100% active in tRNA binding and peptide bond formation. To get around the problem of blocking the A site when the excess of $tRNA^{Phe}$ necessary to fill the E site is added to the ribosomes alone, $tRNA^{Phe}$ was added together with the ternary complex. Thus, the steady-state experiments performed in this system, which is highly purified, do not provide any indication for the negative cooperativity between E and A sites put forward by Nierhaus (1990).

In order to check whether there is an effect of E-site occupancy on the kinetics of A-site binding, stopped-flow experiments were performed. The ribosome complex with AcPhe-$tRNA^{Phe}$ in the P site was rapidly mixed with ternary complex either alone or together with $tRNA^{Phe}$ in sufficiently high concentration (5 μM) to ensure rapid binding of the tRNA to the E site (50 s^{-1}; unpublished data); the binding of

Table 3: A-Site Binding and E-Site Occupancy[a]

addition	ribosome binding (pmol) [^{14}C]-$tRNA^{Phe}$	ribosome binding (pmol) Ac[^{3}H]Phe-$tRNA^{Phe}$	dipeptide (pmol) Ac[^{3}H]Phe-[^{3}H]Phe
ternary complex (7.5 pmol)		13.8	7.3
ternary complex (7.5 pmol) + [^{14}C]$tRNA^{Phe}$ (100 pmol)	20.9	13.8	7.5

[a] Poly(U)-programmed ribosomes (15 pmol) carrying Ac[^{3}H]Phe-$tRNA^{Phe}$ (16.5 pmol added, 2330 dpm/pmol) in the P site were mixed with purified ternary complex EF-Tu·GTP·[^{3}H]Phe-$tRNA^{Phe}$ (7.5 pmol, 114 dpm/pmol) alone or together with [^{14}C]$tRNA^{Phe}$ (100 pmol, 600 dpm/pmol) and incubated at 20 °C for 10 s in a total volume of 50 μL. To determine the binding, the mixture was filtered through nitrocellulose filters and the ^{3}H and ^{14}C radioactivity was measured after dissolving the filters in scintillation fluid. The amount of Ac[^{3}H]Phe-[^{3}H]Phe dipeptide was determined in identical samples prepared in parallel by HPLC following alkaline hydrolysis (Materials and Methods). The binding of [^{3}H]Phe-$tRNA^{Phe}$ to the A site by nitrocellulose filtration was found to be quantitative (7.5 pmol) as estimated from the difference of the ^{3}H counts measured before (binding of Ac[^{3}H]Phe-$tRNA^{Phe}$) and after the addition of the ternary complex to the ribosomes. The binding of [^{14}C]$tRNA^{Phe}$ is mainly to the E site (15 pmol); the remainder may be in the P site or the A site. The amount of unoccupied A sites is sufficient to bind all ternary complex added.

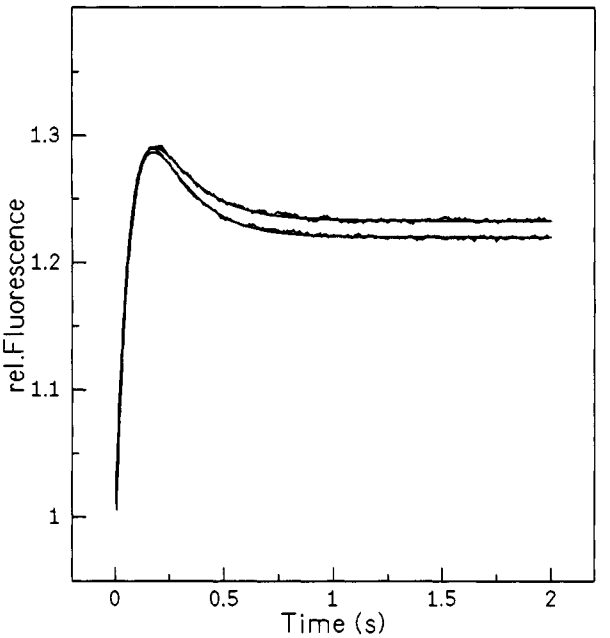

FIGURE 6: Influence of E-site occupancy on A-site binding. Poly(U)-programmed ribosomes with Ac[^{3}H]Phe-$tRNA^{Phe}$ in the P site (0.6 μM) were mixed with EF-Tu·GTP·[^{3}H]Phe-$tRNA^{Phe}$(Prf16/17) (0.2 μM) without (upper curve) or with *E. coli* [^{14}C]$tRNA^{Phe}$ (5 μM, lower curve).

the deacylated tRNA to the A site is much too slow to compete efficiently with the binding of the ternary complex. As evident from Figure 6, also the kinetics of A-site binding of the ternary complex is not affected, neither qualitatively nor quantitatively, by the occupancy of the E site.

DISCUSSION

The formation of intermediate conformational states of aminoacyl-tRNA in the sequence of A-site binding of the complex EF-Tu·GTP·Phe-$tRNA^{Phe}$ is demonstrated by the present results. The first step, reported by the fluorescent label in the D loop but not by a fluorophor in the anticodon loop (Rodnina et al., 1993), is the initial binding of EF-Tu·GTP·Phe-$tRNA^{Phe}$ to the ribosome. It does not depend on the presence of the cognate codon. Both ribosomal subunits

are involved in the initial binding, indicating that the initial binding site of the ternary complex is located at the interface side of both subunits (Rodnina et al., 1993). Interestingly, the occupancy of the A site with aminoacyl-tRNA does not inhibit the initial binding (Rodnina et al., 1993), which suggests that it takes place in another site of the ribosome. Hence, the initial binding state differs from the recognition state defined biochemically by using GTP analogs (Hardesty et al., 1969; Lake, 1977), in which the ternary complex is bound to the A site and the anticodon is in contact with the codon, but the aminoacyl end is not yet reactive in the peptidyl-transfer reaction. To indicate its intermediate character between the factor-binding site and the A site, the recognition state was called the T/A state (Moazed & Noller, 1989). Adopting this nomenclature, the initial binding state defined by the present results is the T/T state.

The second step is the codon–anticodon recognition step, which follows the initial binding and leads to the recognition state (Hardesty et al., 1969), or T/A state (Moazed & Noller, 1989). In this state, the tRNA, while it has established the contact with the codon in the A site, is still in close contact with EF-Tu·GTP. The observation that codon recognition is accompanied by fluorescence changes of both wybutine (Rodnina et al., 1993) and proflavin suggests that the rearrangement in the D loop is caused by codon–anticodon interaction via a conformational change in the anticodon loop. Results obtained with a fluorescent GTP derivative suggest that the conformational change induced by codon binding on the ribosome is transmitted from the tRNA to the effector region of the factor, thereby stimulating GTP hydrolysis (Rodnina et al., 1993, and unpublished data). The addition of kirromycin blocks the system in this state, presumably by inhibiting the factor's transition into the GDP form, which would be released from the ribosome.

In the conformational state that is induced by codon–anticodon recognition, the tRNA is altered structurally, as evident from the 35% fluorescence increase that accompanies the reaction. At the same time, the fluorescence-quenching constant observed for this state is increased. This can be due to an increase of the lifetime of the fluorophor or a change of the accessibility of the dye as a result of a conformational change of the D loop, or both. However, even if the observed fluorescence increase by 35% (Figure 1) were due exclusively to a lifetime increase, which it is not (cf. above), it would not account for the 60% increase of the quenching constant. Thus, a more plausible explanation for the observed fluorescence increase is that the conformation of the D loop changes in a way that leads to partial unstacking of the dye from the neighboring guanine, thereby releasing static quenching of fluorescence that prevails in free $tRNA^{Phe}$(Prf16/17) (Wintermeyer & Zachau, 1979). The increased quenching then indicates an enhanced exposure of the proflavin to the solvent rather than a shielding of the dye by the ribosome or EF-Tu. Thus, the fluorescence changes of proflavin in the D loop that take place during codon-dependent binding to the A site result from structural rearrangements of the tRNA molecule in the region of the D loop. On the basis of the fluorescence and quenching data, we consider likely that the conformational changes consist in an unfolding and subsequent refolding of the D loop.

The latter rearrangement, i.e., the third step distinguished by fluorescence, takes place after the hydrolysis of GTP, which itself does not cause a fluorescence change, and accompanies the dissociation of EF-Tu and/or the accommodation of the aminoacyl-tRNA in the A site. Besides the D loop of the tRNA, the conformation of the G domain of EF-Tu is also affected during this transition (Rodnina et al. 1993). When the dissociation of EF-Tu·GDP from the ribosome is suppressed by kirromycin, the final rearrangements of both tRNA and EF-Tu are blocked, suggesting that the dissociation of EF-Tu and the accommodation of the tRNA in the A site are coupled.

Taken together, the kinetic data reported in this paper and elsewhere (Rodnina et al., 1993) lead to a minimal scheme of EF-Tu-dependent A-site binding of aminoacyl-tRNA that comprises five steps: (i) initial binding, (ii) codon recognition, (iii) GTP hydrolysis, (iv) release of EF-Tu·GDP and accommodation in the A site, and (v) peptide bond formation. The steps from codon recognition to GTP hydrolysis involve tightly coupled conformational rearrangements of the tRNA and EF-Tu; i.e., the two molecules are reacting as a structural unit until they are separated in the transition (step iv) following GTP hydrolysis. Step iv probably consists of two or more reactions that cannot be described separately yet. As to the kinetic mechanism, the concentration dependencies of the apparent first-order rate constants determined up to now are consistent with a model in which the steps are following each other in a sequential fashion. The confirmation of a sequential model and the quantitative evaluation of individual rate constants is a complex problem which requires more extensive data in order to make the mathematical treatment feasible. This work is in progress right now in our laboratory.

It has been proposed that the tRNA in the P site influences the binding to the A site, either via tRNA–tRNA interactions (Labuda & Pörschke, 1980; Bossi, 1983; Carrier & Buckingham, 1984; Murgola et al., 1984; Gutman & Hatfield, 1989; Smith & Yarus, 1989) or through the conformation of the mRNA that may be different for the cognate and the noncognate codon–anticodon complex (Kato et al., 1990). The present results show that, in fact, the ligand in the P site is affecting all of the steps observed in A-site binding. When the P site is not occupied, the binding step is slightly affected while the final rearrangement of the aminoacyl-tRNA, presumably the accommodation in the A site, is slowed down significantly. The effect may indicate an interaction of the P-site-bound tRNA with the tRNA to accommodate in the A site. On the other hand, $tRNA^{Leu2}$ in the P site of poly(U)-programmed ribosomes, in addition to slowing down the final step, also slows down significantly the codon-recognition step. This is explained best by assuming an influence of codon–anticodon interaction in the P site on the structure of the contiguous codon exposed in the A site. The mismatch in the codon–anticodon complex in the P site, created by the binding of $tRNA^{Leu2}$(GAG), seems to disturb the conformation of the codon in the A site more than when the P-site codon is free. Of course, more systematic studies are necessary to substantiate this contention.

While the influence of the occupancy of the P site is clearly seen in the present experiments, the presence or absence of a tRNA ligand in the E site does not influence the binding of the ternary complex to the A site, neither the steady-state level nor the kinetics. Ribosomes programmed with a heteropolymeric mRNA yield the same results (data to be published elsewhere). These results are at variance with reports by Nierhaus and colleagues (Nierhaus, 1990) who have proposed an anticooperative coupling between A and E sites of *E. coli* ribosomes programmed with either homo- or heteropolymeric mRNA. We feel that one reason for the discrepancy may be that we are using a highly purified and highly active system. It may also be related to a somewhat anomalous behavior of the ribosomes used by Nierhaus and

colleagues in binding only one molecule of AcPhe-tRNA, while ours (Lill et al., 1984) and others' ribosomes (Kirillov & Semenkov, 1982) consistently bind two. Furthermore, the purification of the ternary complex is particularly important since deacylated tRNA contained in unpurified mixtures of aminoacyl-tRNA with EF-Tu, as used by Nierhaus and colleagues, may cause the loss of labeled tRNA from the E site by exchange. Thus, our results do not support the allosteric three-site model of the ribosome (Nierhaus, 1990). Rather, as pointed out before (Wintermeyer et al., 1990), the data suggest that the E and A sites are independent of each other with respect to tRNA binding.

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