Movement of tRNA but Not the Nascent Peptide during Peptide Bond Formation on Ribosomes†

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ABSTRACT: The results from experiments involving nonradiative energy transfer indicate that a fluorescent probe on the 5′-end of $tRNA^{Phe}$ moves more than 20 Å toward probes on ribosomal protein L1 as a peptide bond is formed during the peptidyl transferase reaction on *Escherichia coli* ribosomes. The peptide itself moves no more than a few angstroms during peptide bond formation, as judged by the movement of fluorescent probes attached to the phenylalanine amino group of phenylalanyl-tRNA. Other results demonstrate that an analogue of peptidyl-tRNA, deacylated tRNA, and puromycin can be bound simultaneously to the same ribosome, indicating that there are three physically distinct sites to which tRNA is bound during the reaction steps by which peptides are elongated. The results appear to be consistent with the displacement model of peptide elongation.

Early studies of protein synthesis prompted Watson (1964) to propose the now classical two-site model to account for the fundamental requirements for peptide elongation on ribosomes. A basic feature of this model is that the nascent peptide of peptidyl-tRNA in the P (peptidyl or donor) site is transferred during the peptidyl transferase reaction to the free amino group of aminoacyl-tRNA in the A (acceptor) site. While in the A site, it cannot react with the next incoming aminoacyl-tRNA. Movement of the peptidyl-tRNA back to the P site with mRNA bound by codon–anticodon interaction was envisioned to occur in a separate reaction. However, there are many indications that this two-site model is not correct. If aminoacyl-tRNA is bound to ribosomes with EF-Tu and a nonhydrolyzable analogue of GTP such as GMPPCP[1] into what would be the A site, the peptidyl transferase reaction does not occur (Skogerson & Moldave, 1968), leading to the suggestion of an additional entry (Hardesty et al., 1969) or recognition (Lake, 1977) site. Nierhaus and co-workers have presented evidence that deacylated tRNA can bind to ribosomes in an exit site in addition to the P site (Nierhaus et al., 1986).

Our objective has been to use fluorescence techniques, especially nonradiative energy transfer between fluorophores attached to specific points on tRNA and ribosomes, to physically define the ribosomal binding sites. We reported that tRNA appeared to move into a different position or site on the ribosome during the peptidyl transferase reaction (Hardesty et al., 1986). This position was experimentally indistinguishable from that of deacylated $tRNA^{Phe}$ bound directly to the ribosomes but different from that of AcPhe-tRNA bound into what should be the ribosomal P site according to the classical model. In these studies, the tRNA fluorescence donor probes used were located near the center of the molecule according to the crystal structure of yeast $tRNA^{Phe}$ (Robertus et al., 1974; Kim et al., 1974). Energy transfer was to an acceptor attached to the single cysteine sulfhydryl residue of either ribosomal protein S21 or L11. The results were not anticipated from the classical model and prompted us to present a hypothesis we named the "displacement model" for peptide elongation (Hardesty et al., 1986). The central feature of this model is that the tRNA moves during peptide transfer whereas the nascent peptide remains in a spatially restricted site we call the peptidyl transferase center. Recently, Moazed and Noller (1989), using a completely different technique involving the shielding effect of tRNA in preventing chemical modification of specific mucleotides of the ribosomal RNA, also have concluded that deacylated tRNA and peptidyl-tRNA occupy different sites on the 50S subunit. They presented evidence that peptidyl-tRNA appears to be in about the same site on the 50S subunit whether it is in the puromycin-reactive or puromycin-nonreactive state.

Here we describe the results of a more rigorous test of the displacement hypothesis in which we measure the position and movement of an analogue of a nascent peptide and the amino acid stem of the tRNA during the peptidyl transferase reaction. Energy transfer was also measured from labeled peptidyl-tRNA to a fluorescent derivative of erythromycin.

Our results indicate that the distance from the 5′-end of tRNA to L1 decreases by greater than 20 Å upon peptidyl

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[1] Abbreviations: acp^3U_{47}, 3-(3-amino-3-carboxypropyl)uracil at position 47 of *E. coli* $tRNA^{Phe}$; AEDANS-Phe-tRNA, Phe-tRNA that was mercaptoacetylated at its α-amino group and then reacted with IAEDANS; AETUC, 3-[4-(aminoethyl)thioureidophenyl]-7-diethylamino-4-methylcoumarin; Bicine-KOH, *N,N*-bis(2-hydroxyethyl)glycine neutralized to the desired pH with KOH; CPM, 3-(4-maleimidophenyl)-7-diethylamino-4-methylcoumarin; CPM-Phe-tRNA, Phe-tRNA that was mercaptoacetylated at its α-amino group and then reacted with CPM; CITC, 3-(4-isothiocyanatophenyl)-7-diethylamino-4-methylcoumarin; DCIA, 3-[4-[(iodoacetyl)amino]phenyl]-7-diethylamino-4-methylcoumarin; dhU, dihydrouracil; EDTA, ethylenediaminetetraacetic acid; EF-G, *E. coli* elongation factor G; FITC, fluorescein 5′-isothiocyanate; FITC-erythromycin, erythromycylamine labeled at its amino group with FITC; FM, fluorescein 5′-maleimide; GMPPCP, guanosine 5′-[β,γ-methylene]triphosphate; Hepes-KOH, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid neutralized to the desired pH with KOH; IAEDANS, 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; poly(U), poly(uridylic acid); RP-HPLC, reversed-phase high-performance liquid chromatography; s^4U_8, 4-thiouracil at position 8 of *E. coli* $tRNA^{Phe}$; s^4U_8–C_{13} cross-link, the photon-catalyzed cross-link between the 4-thiouracil at position 8 and the cytosine at position 13 of *E. coli* $tRNA^{Phe}$; Tris, tris(hydroxymethyl)aminomethane.

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transfer, while little movement of the peptide relative to erythromycin or puromycin was detected. Finally, and perhaps most strikingly, we show that there is no significant difference in binding of puromycin to ribosomes carrying both deacylated tRNA and peptidyl-tRNA, the latter in the A site of the classical model, as compared to ribosomes carrying only peptidyl-tRNA in the P site. The results demonstrate unequivocally that an analogue of peptidyl-tRNA, deacylated tRNA, and puromycin can be bound simultaneously to the same ribosome. The results indicate that the binding site for the acceptor stem of aminoacyl-tRNA on the 50S subunit is open when according to the classical model it should be occupied by peptidyl-tRNA.

Materials and Methods

Materials and Chemicals. *Escherichia coli* K12, strain A19, was a kind gift from Drs. K. Nierhaus and H. G. Wittmann, Berlin, FRG. Erythromycylamine was generously provided by Dr. Robert Hamill at Eli Lilly Research Laboratories (Indianapolis, IN). The fluorescein derivative FM and coumarin derivatives CPM, CITC, and DCIA were from Molecular Probes Inc. (Junction City, OR). IAEDANS, FITC, phosphocreatine, creatine phosphokinase, purified yeast $tRNA^{Phe}$, purified *E. coli* $tRNA^{Phe}$, puromycin dihydrochloride, poly(U), ATP, GTP, GMPPCP, guanidine hydrochloride, dimethyl sulfoxide, *N*-hydroxysuccinimide, triphenylphosphine, dithiodipyridine, erythromycin, ethylenediamine (free base), and Sephadex and Sepharose products were from Sigma Chemical Co. (St. Louis, MO). *N,N'*-dicyclohexylcarbodiimide was from Schwarz-Mann (Spring Valley, NY). [^{14}C]Phenylalanine (420 Ci/mol) was from ICN Life Sciences (Irvine, CA). Trifluoroacetic acid, acetonitrile (HPLC grade), and triethanolamine were from Fisher Scientific Co. (Fair Lawn, NJ). Phenol and dimethylformamide were from J. T. Baker Chemical Co. (Phillipsburg, NJ). Phenol and dimethylformamide were redistilled before use. Nitrocellulose filters were from Millipore Corp. (Bedford, MA). All other chemicals were of reagent grade.

Solutions. Rec4 solution contained 20 mM Tris-HCl (pH 7.5), 4 mM $Mg(OAc)_2$, 400 mM NH_4Cl, 4 mM β-mercaptoethanol, and 0.2 mM EDTA. Solution A contained 30 mM Tris-HCl (pH 7.4), 20 mM $Mg(OAc)_2$, 500 mM KCl, and 1 mM dithioerythritol. TMNSH solution contained 10 mM Tris-HCl (pH 7.5), 10 mM $Mg(OAc)_2$, 100 mM NH_4Cl, and 5 mM β-mercaptoethanol.

Growth of E. coli Wild-Type and Mutant Cells. The growth of *E. coli* K12, strain A19, was carried out as previously described (Odom et al., 1980). A mutant of A19 containing altered and more readily removable ribosomal protein S21 was isolated by E. R. Dabbs (Dabbs, 1980). The mutant, designed VT442, was grown as described (Odom et al., 1984a). *E. coli* mutant strain MV17, which does not contain ribosomal protein L1 on the ribosome, was isolated by E. R. Dabbs (Dabbs et al., 1981). The mutant cells were grown in the same way as described for an L11 mutant (Deng et al., 1986).

Preparation of 70S Ribosomes and Ribosomal Subunits. 70S ribosomes and ribosomal subunits from wild-type *E. coli* were prepared as previously described (Odom et al., 1980). Preparation of S21-deficient 30S subunits from the 30S subunits derived from the *E. coli* mutant VT442 was performed as described (Odom et al., 1984a). 50S subunits lacking protein L1 (50S – L1) were prepared from the mutant cells as referenced above for wild-type *E. coli*.

Preparation and Fluorescence Labeling of Ribosomal Protein S21. Purified protein S21 was a kind gift from Dr. H. G. Wittmann (Max-Planck-Institut für Molekulare Genetik, Berlin). It has a single cysteine at position 22 (Vandekerckhove et al., 1975), which can be labeled with fluorescent probes without inactivating the protein (Odom et al., 1984a). S21 was labeled with FM by a procedure described before (Odom et al., 1984b). To ensure that no intermolecular disulfide bonds were present in S21, the sample (at 1–5 mg/mL) was first incubated at 37 °C for 30 min in a solution of 7 M guanidine hydrochloride, 10 mM Hepes-KOH (pH 8.5), and 20 mM dithioerythritol. The latter compound was then removed by passage over a Sephadex G-25 column equilibrated with 7 M guanidine hydrochloride and 10 mM Hepes-KOH (pH 7.5). To the S21 recovered from this column was added 0.01 volume of a 100 mM solution of FM in dimethylformamide. The resulting solution was incubated for 30 min at 37 °C. Excess labeling reagent was reacted with 10 mM glutathione, and the sample was passed over a second Sephadex G-25 column equilibrated with 7 M urea and 20 mM Hepes-KOH (pH 7.5) to remove guanidine hydrochloride and excess labeling reagent. FM-S21 was separated from a small amount of unlabeled S21 by HPLC over a C_{18} reversed-phase column as previously described (Odom et al., 1988). The FM-S21 sample was then evaporated to dryness, resuspended in a solution of 7 M urea, 0.1 M Tris-HCl, pH 7.5, and 0.5 M KCl, and finally dialyzed against solution A and stored at −80 °C.

Fluorescence Labeling of Ribosomal Protein L1. Purified protein L1 was kindly donated by Dr. H. G. Wittmann (Max-Planck-Institut für Molekulare Genetik, Berlin). Since L1 contains no cysteine residues, it was necessary to label the lysine ϵ-NH_2 groups. For this purpose, fluorescein isothiocyanate (FITC) was used. Labeling was in 7 M guanidine hydrochloride containing 20 mM Bicine-KOH, pH 8.5. FITC was added from a 100 mM stock solution in dimethylformamide to give a final concentration of 1 mM. Incubation was for 15 min at 37 °C, followed by passage over a Sephadex G-25 column equilibrated with 7 M urea and 20 mM Hepes-KOH (pH 7.5) to remove excess FITC and exchange the 7 M guanidine hydrochloride for 7 M urea. Finally, the labeled L1 was dialyzed against Rec4 solution and stored at −80 °C. The labeled L1 contained an average of about 1 fluorescein per molecule, but in all probability this represents an average value value of random labeling of various ϵ-NH_2 groups.

Labeled S21 was incorporated into the 30S subunit as described previously (Odom et al., 1984b), by incubation of S21-deficient 30S subunits (30 A_{260} units/mL) for 10 min at 37 °C with the stoichiometric amount of labeled S21 in a solution of 20 mM Tris-HCl, pH 7.5, 300 mM KCl, 20 mM $Mg(OAc)_2$, and 1 mM DTE, followed by centrifugation in a Beckman 50Ti rotor at 40000 rpm for 12 h and resuspension in TMNSH solution. Typically 0.60–0.65 mol of labeled S21/mol of 30S subunit was present after reconstitution. The reconstituted particles were sometimes supplemented with ribosomal protein S1, which was partially washed off during the preparation of S21-deficient 30S subunits. This was done by incubating the reconstituted particles with a stoichiometric amount of S1 in TMNSH solution for 10 min at 37 °C. This incubation with S1, however, made little difference in the energy transfer results obtained. Fluorescein-L1 was incorporated into 50S in a reaction mixture containing 400 pmol of 50S – L1, 440 pmol of fluorescein-L1, 10 mM Tris-HCl, pH 7.5, 8 mM $Mg(OAc)_2$, 150 mM NH_4Cl, and 5 mM β-mercaptoethanol in a final volume of 100 μL. Incubation was for 10 min at 35 °C. Subunits isolated by centrifugation contained at least 90% of the labeled L1, but for most ex-

periments the labeled subunits were used without isolation.

Assay for Biological Activity of Reconstituted Ribosomes. Poly(U)-directed polyphenylalanine synthesis, performed as described (Odom et al., 1980), was used to determine the activity of reconstituted ribosomal subunits. In this assay the modified subunits gave the following activities, expressed as percentages of activity with standard subunits: 30S(−S21), 41%; 30S(−S21) + FM-S21, 45%; 30S(−S21) + unlabeled S21, 48%; 30S(−S21) + unlabeled S1, 56%; 30S(−S21) + FM-S21 + unlabeled S1, 62%; 30S(−S21) + unlabeled S21 + unlabeled S1, 85%; 50S(−L1), 70%; 50S(−L1) + FITC-L1, 80%.

Labeling of E. coli tRNA^{Phe} at the s^4U_8 Position. *E. coli* tRNA^{Phe} was labeled with DCIA at the s^4U_8 position and purified by RP-HPLC as described previously (Odom & Hardesty, 1987).

Preparation of AETUC for Labeling the 5′-End of tRNA. In order to introduce a free amino group into a coumarin probe for reaction with an activated 5′-phosphate, CITC was reacted with ethylenediamine as follows. A 250-μL aliquot of a 100 mM solution of CITC in dimethylformamide was added to an equal volume of 2 M ethylenediamine in dimethylformamide. After 10 min at room temperature, the completed reaction was verified by thin-layer chromatography on silica gel (Eastman No. 6061) in ethyl acetate, on which the ethylenediamine addition product AETUC remains at the origin and the CITC migrates near the solvent front. One milliliter of 2 M NaOAc, pH 5.0, was added, and the solution was extracted twice with 0.5-mL portions of isobutyl alcohol. The isobutyl alcohol extracts were combined and extracted twice with 1-mL portions of 0.1 M K_2CO_3. The final isobutyl alcohol phase was evaporated to dryness under vacuum.

Labeling of Yeast tRNA^{Phe} at the 5′-End. Labeling of yeast tRNA^{Phe} at the 5′-phosphate group by formation of a phosphoramidate was achieved by a modification of the method of Mochalova et al. (1982). Yeast tRNA^{Phe} was suspended at 100 A_{260} units/mL in a solution of 15 mM sodium citrate, pH 7.0, 150 mM NaCl, and 10 mM EDTA. This solution was dialyzed against 1 L of 15 mM sodium citrate, pH 7.0, and 150 mM NaCl for 12 h, and then against three 4-L portions of H_2O for 4 h each. The solution was concentrated by evaporation under vacuum to 1000 A_{260} units/mL. Dry dimethyl sulfoxide (90 μL) was added to 10 μL of the tRNA solution, followed by 100 μL of 90 mM AETUC in dimethyl sulfoxide. The resulting solution was then added to a vial containing 22 mg (0.1 mmol) of dithiodipyridine and 26.2 mg (0.1 mmol) of triphenylphosphine. No attempt was made to immediately dissolve the latter reagents. Instead, they were allowed to dissolve during the course of the reaction, which consisted of shaking the mixture for 2 h at room temperature. At the end of the reaction, 2 mL of H_2O and 220 μL of 2 M NaOAc, pH 5.0, were added, and the solution was extracted with an equal volume of 70% phenol. The aqueous phase was then precipitated with 3 volumes of cold ethanol. The ethanol precipitation was repeated twice. The level of labeling was around 80%, while that of a control sample without dithiodipyridine and triphenylphosphine was near zero. The sample was further purified by RP-HPLC with a Beckman System Gold HPLC system and Beckman Ultrapore C_8 column (0.46 cm × 25 cm). The column was equilibrated and the sample loaded in a solution of 20 mM Tris-HOAc, pH 6.0, 10 mM $Mg(OAc)_2$, and 400 mM NaCl. Elution from the column was with a gradient of methanol in this same solution, achieved by admixing increasing amounts of a solution containing 60% methanol, 20 mM Tris-HOAc, pH 6.0, 10 mM $Mg(OAc)_2$, and 400 mM NaCl. Unlabeled tRNA^{Phe} eluted around 14% methanol, while most of the labeled tRNA eluted around 30% methanol. The 5′-labeled tRNA was then aminoacylated and acetylated as described below and rechromatographed on the C_8 column to remove tRNA that failed to aminoacylate. The 5′-labeled AcPhe-tRNA eluted around 41% methanol, showing excellent separation from the labeled deacylated material. The 5′-AETUC-AcPhe-tRNA had a coumarin to tRNA ratio of 1 and contained 1150 pmol of AcPhe/A_{260} unit.

Labeling of Yeast Phe-tRNA at the α-Amino Group. The phenylalanine α-amino group of yeast Phe-tRNA was prepared for labeling with sulfhydryl-specific fluorescent probes by first mercaptoacetylating it. The mercaptoacetyl group was introduced by reaction of Phe-tRNA with the succinimide ester of dithiodiglycolic acid (the disulfide of mercaptoacetic acid) followed by reduction of the disulfide bond with dithioerythritol. The succinimide ester of dithiodiglycolic acid was prepared by the *N,N′*-dicyclohexylcarbodiimide method (Anderson et al., 1967). The reaction was performed in ethyl acetate and contained 140 mM each of dithiodiglycolic acid, *N,N′*-dicyclohexylcarbodiimide, and *N*-hydroxysuccinimide. After standing overnight at room temperature, the precipitate of dicyclohexylurea was removed and the ethyl acetate was evaporated under vacuum. Since each dithiodiglycolic acid contains two carboxyl groups, presumably only half of them will be esterified and the monoester should be the predominant product. The reaction was intentionally performed in this way on the assumption that the monoester should be more soluble than the diester under the conditions used for reaction with the α-amino group of Phe-tRNA. This latter reaction was performed at 0 °C in 200 mM triethanolamine, pH 8.0, at a Phe-tRNA concentration of 10–40 A_{260} units/mL. Then 0.08 volume of a 1 M stock solution of the dithiodiglycolic monoester in dimethylformamide was added while mixing on a vortex mixer, followed quickly, also while mixing, by enough 4 M K_2CO_3 and 8 M KOH to bring the final pH to 7.9–8.0, about 0.01 volume. Incubation was for 30 min at 0 °C, after which the mixture was acidified to pH 5.0 with glacial acetic acid and precipitated with 3 volumes of cold ethanol. The ethanol precipitation was repeated twice. The disulfide bond was then reduced by incubation for 20 min at 35 °C with 100 mM dithioerythritol in 200 mM Hepes-KOH, pH 8.0, followed by acidification to pH 5.0 with glacial acetic acid and precipitation with 3 volumes of cold ethanol. The ethanol precipitation was repeated once.

The resulting mercaptoacetylated Phe-tRNA was labeled with either CPM or IAEDANS. Both reactions were performed for 30 min at 35 °C in 100 mM Hepes-KOH, pH 8.0, with 2 mM of probe but with the following differences. For reaction with CPM, the mixture contained 60% dimethylformamide and CPM was added last with mixing from a 100 mM solution in dimethylformamide to the prewarmed reaction mixture. The inclusion of the 60% dimethylformamide was necessary to maintain the CPM in solution. For reaction with IAEDANS, dimethylformamide was unnecessary and the IAEDANS was added from a 50 mM stock solution in 50% aqueous dimethylformamide. Particular care must be taken to use a freshly prepared IAEDANS solution and to exclude light. At the end of the reaction with either CPM or IAEDANS, an equal volume of H_2O was added, followed by addition of glacial acetic acid to lower the pH to 5.0 and then 0.1 volume of 2 M NaOAc, pH 5.0. The samples were extracted twice with an equal volume of 70% phenol and precipitated with 3 volumes of cold ethanol. The ethanol precipitation was repeated twice.

CPM-Phe-tRNA was then separated from unlabeled tRNA and from some material labeled elsewhere with coumarin by phenyl-Sepharose chromatography at 4 °C. The labeled tRNA was retained on phenyl-Sepharose equilibrated with a solution of 50 mM NaOAc, pH 5.0, 400 mM NaCl, and 100 mM $Mg(OAc)_2$, while the unlabeled tRNA and spuriously labeled tRNA were not bound. CPM-Phe-tRNA was then eluted with the same solution containing 20% ethanol.

AEDANS-Phe-tRNA was purified by RP-HPLC with the same C_8 column and solution as described above for the 5′-labeled $tRNA^{Phe}$. Most of the AEDANS-Phe-tRNA eluted as a single peak at 24% methanol.

Preparation of FITC-Erythromycin. FITC-erythromycin was prepared and isolated exactly as described by Vince et al. (1976), from erythromycylamine kindly provided by Dr. Robert Hamill of Eli Lilly Research Laboratories.

Aminoacylation and Acetylation of $tRNA^{Phe}$. Yeast $tRNA^{Phe}$, either unlabeled or labeled at the 5′-phosphate, was aminoacylated as previously described (Hardesty et al., 1971) except that a 0.5 M KCl salt wash of ribosomes from rabbit reticulocytes was used as the source of synthetases. The reaction mixture, in a final volume of 1 mL, contained 50 mM Tris-HCl (pH 7.5), 20 mM $Mg(OAc)_2$, 120 mM KCl, 2.5 mM dithioerythritol, 3.75 mM ATP, 60 μM [^{14}C]Phe (100 Ci/mol), 10 A_{260} units of $tRNA^{Phe}$, and 40 μg of the 0.5 M KCl ribosomal salt wash fraction prepared as described previously (Kramer et al., 1975). After incubation for 20 min at 37 °C, the tRNA was extracted twice with phenol and collected by ethanol precipitation. *E. coli* $tRNA^{Phe}$ was aminoacylated as described previously (Robbins et al., 1981).

Acetylation of Phe-tRNA was performed with the succinimide ester of acetic acid as described by Rappoport and Lapidot (1974). AcPhe-tRNA and Phe-tRNA were separated from some remaining deacylated $tRNA^{Phe}$ by C_{18} reversed-phase HPLC as described above for fluorescently labeled tRNA and previously for unlabeled tRNA (Odom et al., 1988).

Nonenzymatic Binding of tRNA to the Ribosomal P Site. Binding to the ribosomal P site of $tRNA^{Phe}$ and of AcPhe-$tRNA^{Phe}$ species labeled at different positions was carried out by a modification of the procedure of Wurmbach and Nierhaus (1979). The standard reaction mixture contained, in a total volume of 0.5 mL, 50 mM Tris-HCl (pH 7.5), 15 mM $Mg(OAc)_2$, 100 mM NH_4Cl, 5 mM β-mercaptoethanol, 60 μg of poly(U), 0.005–0.02 A_{260} unit of the respective $tRNA^{Phe}$ species, and approximately 5.6 A_{260} units of ribosomal subunits (3.6 A_{260} units of 50S subunits and 2.0 A_{260} units of 30S subunits). Ribosomes were preincubated with poly(U) for 10 min at 37 °C before addition of tRNA and then incubated an additional 20 min at 37 °C. When ribosomes with bound tRNA were isolated by gel filtration, the above procedure was modified slightly as described below.

Nonenzymatic Binding of AcPhe-tRNA to the Ribosomal A Site. Binding of AcPhe-tRNA, labeled at different positions, to the ribosomal A site was performed by a modification of the procedure previously described (Wurmbach & Nierhaus, 1979). The standard reaction mixture of 0.5 mL contained 50 mM Tris-HCl (pH 7.5), 15 mM $Mg(OAc)_2$, 100 mM NH_4Cl, 5 mM β-mercaptoethanol, 50 μg of poly(U), 5.6 A_{260} units of ribosomal subunits, and 0.15 A_{260} units of $tRNA^{Phe}$. Ribosomes were preincubated with poly(U) and deacylated tRNA for 10 min at 37 °C. Then, 0.005–0.02 A_{260} unit of AcPhe-tRNA was added and the incubation was continued for 20 min at 37 °C. The deacylated tRNA will presumably occupy the P site, thus directing the AcPhe-tRNA to the A site. When ribosomes with bound tRNA were isolated by gel filtration, the above procedure was modified slightly as described below.

Isolation of Ribosomes with Bound tRNA by Gel Filtration. Under the conditions used, binding of the various tRNA species was incomplete even if an excess of ribosomes was present in the reaction solution. This was especially true for binding to the ribosomal puromycin-nonreactive site (A site). Gel filtration chromatography was used to eliminate the small proportion of unbound tRNA. A 3.8-mL column (0.7 cm × 10 cm) of Sephacryl S-300 was equilibrated in the cold with a solution of 50 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 15 mM $Mg(OAc)_2$, and 5 mM β-mercaptoethanol. Binding of the tRNA was as described above except that the volume was reduced from 0.5 to 0.2 mL. Fractions of 0.4 mL were collected and monitored for absorbance at 260 nm and for radioactivity. Fluorescence was measured on peak fractions. Ribosomes elute in the void volume, well ahead of free tRNA.

Nonenzymatic Binding of Phe-tRNA to the Ribosomal A Site. After binding of the labeled AcPhe-tRNA species to the ribosomal P site as described above, in some cases, unlabeled Phe-tRNA was bound nonenzymatically to the A site in order to obtain acyl transfer from the P-site-bound species. This was accomplished by adding 190 pmol of nonfluorescent, nonradioactive yeast Phe-tRNA at 15 mM Mg^{2+}. Incubation was for 10 min at 20 °C. It was found to be very important to keep the temperature at or below 20 °C to prevent apparent "spontaneous translocation" of the formed peptidyl-tRNA, as judged by puromycin reactivity.

Puromycin Reactivity of AcPhe-tRNA and Phe-tRNA on the Ribosome. The puromycin reactivity of fluorescently labeled Ac[^{14}C]Phe-tRNA bound to the ribosomes was measured by a modification of the method of Wurmbach and Nierhaus (1979). A 100-μL aliquot of the sample was brought to a puromycin concentration of 1 mM and incubated for 1 h on ice. The reaction was stopped by addition of 2 M NaOAc (pH 5.0) to give a final concentration of 0.4 M. Formation of CPM-Ac[^{14}C]Phe–puromycin was measured by extraction with ethyl acetate and determining the radioactivity in an aliquot from the ethyl acetate layer in a solution containing 5 g of 2,5-diphenyloxazole/L of toluene. For AEDANS-Ac[^{14}C]Phe–puromycin, it was found that better extraction was obtained when more polar isobutyl alcohol was substituted for the ethyl acetate.

Measurement of Puromycin Binding by Fluorescence. It was found that binding of puromycin to ribosomes caused a considerable change in the fluorescence from either AEDANS-Phe-tRNA or CPM-Phe-tRNA that had been bound previously in either the puromycin-reactive or -nonreactive sites as described below. In the puromycin-reactive site at 20 °C and longer incubation times, most of the effect of puromycin on fluorescence is due to transfer of the peptidyl moiety to puromycin and subsequent release of the peptidyl puromycin from the ribosome, as evidenced by a large decrease in fluorescence anisotropy. In the puromycin-nonreactive site, on the other hand, no decrease in anisotropy was observed, but a large shift in emission maximum and, in the case of AEDANS-Phe-tRNA, in fluorescence intensity was seen, indicating binding of puromycin but no covalent reaction. Later, it was found that, with CPM-Phe-tRNA in the P site, the binding of puromycin could be largely separated from its subsequent reaction if the experiment was performed at low temperature and the fluorescence measured immediately after addition of puromycin. In this case, only a small decrease in anisotropy was observed and the emission maximum was

shifted to the blue rather than to the red, as occurred after transfer of the peptide to puromycin. Accordingly, in order to see the effect of binding and minimize the transfer of the peptide, this reaction was performed at 0 °C and the fluorescence emission spectrum taken about 1 min after addition of 1 mM puromycin. With AEDANS-Phe-tRNA in the P site, it was not possible to separate the binding of puromycin from the peptide transfer.

Preparation of Elongation Factor G. *E. coli* elongation factor G was partially purified by the method of Ravel and Shorey (1971).

Determination of Extent of Binding of Labeled tRNA from Fluorescence Anisotropy. Binding of labeled tRNA to labeled and unlabeled ribosomes was determined from measurements of fluorescence anisotropy, taking into account that the observed anisotropy (A_{obs}) is an intensity-weighted average of the anisotropies of bound tRNA (A_b) and unbound tRNA (A_f). In equation form

$$A_{obs} = \frac{(q_b/q_f)X_bA_b + (1 - X_b)A_f}{[(q_b/q_f) - 1]X_b + 1}$$

where q_b/q_f is the ratio of fluorescence intensities of bound and free tRNA, X_b is the fraction of tRNA bound, and the other terms are as described above. This equation can be rearranged to solve for X_b:

$$X_b = \frac{A_{obs} - A_f}{(q_b/q_f)(A_b - A_f) - [(q_b/q_f) - 1](A_{obs} - A_f)}$$

A_f was determined directly on free labeled tRNA without ribosomes, A_b was determined after binding of labeled tRNA to ribosomes and chromatography over Sephacryl S300 to remove any unbound tRNA as described above, and q_b/q_f was determined directly from the observed fluorescence intensities of bound and free tRNA. For tRNA labeled at the 5′-phosphate with coumarin, A_f is 0.24 and A_b is 0.35 for both AcPhe-tRNA and deacylated tRNA. The values for q_b/q_f are 1.1 for AcPhe-tRNA and 1.2 for deacylated tRNA. tRNA labeled at s^4U_8 with coumarin gave an A_f of 0.31, an A_b of 0.36, and a q_b/q_f of 1.25 for both AcPhe-tRNA and deacylated tRNA.

Fluorescence Measurements. A photon-counting spectrofluorometer, Model 8000 from SLM Instruments Inc. (Urbana, IL), was used to carry out steady-state fluorescence measurements as described before (Rychlik et al., 1983). When spectra were taken, data were accumulated at 1-nm intervals with a scanning rate of 1.0 or 2.0 s per wavelength increment. Spectra were automatically corrected for the wavelength dependence of photomultiplier sensitivity. All fluorescence measurements were made at an absorbance of less than 0.1 at the wavelength of excitation and, unless otherwise indicated, in a volume of 0.5 mL and at a temperature of 20 °C. Steady-state energy transfer measurements were performed as described before (Deng et al., 1986). Steady-state fluorescence polarization and anisotropy measurements were made with the SLM fluorometer as described (Odom et al., 1984b).

Corrections in Energy Transfer Calculations. In calculating fluorescence energy transfer, it is usually necessary to make corrections for slight concentration differences between the singly (donor only) and doubly (donor + acceptor) labeled samples, for incomplete binding of labeled tRNA to ribosomes, and for incomplete labeling of ribosomes. In the experiments described in this paper, concentration differences were corrected for by adding solid guanidine hydrochloride to both singly and doubly labeled samples after completion of the first fluorescence measurements. This causes dissociation of the ribosome–tRNA complexes, eliminating any energy transfer and making the fluorescence proportional only to probe concentration. The fluorescence of the samples is then remeasured, from which the correction factor for concentration difference is calculated. Two corrections are usually necessary when binding is incomplete. One of these corrects for *differences* in binding between singly and doubly labeled samples, and the other corrects for incomplete binding in the doubly labeled sample. The first of these corrections is only necessary if the fluorescence intensity changes upon binding, but with labeled tRNA this is usually the case. For example, if less labeled tRNA binds to labeled ribosomes than binds to unlabeled ribosomes and the fluorescence intensity increases upon binding, the labeled tRNA (which carries the fluorescence donor) would exhibit less fluorescence in the doubly labeled than in the singly labeled sample, giving a false indication of energy transfer. It is thus necessary to compute the quantity F°_{da}, the fluorescence to be expected from the donor in the doubly labeled sample in the absence of energy transfer. It can be shown that

$$F^\circ_{da} = \frac{1 - X_{b,da} + (q_b/q_f)X_{b,da}}{1 - X_{b,d} + (q_b/q_f)X_{b,d}}F_d$$

where $X_{b,da}$ is the fraction of labeled tRNA bound in the doubly labeled sample, $X_{b,d}$ is the fraction of labeled tRNA bound in the singly labeled sample, q_b/q_f is the ratio of fluorescence intensities of bound and free labeled tRNA (in the absence of energy transfer), and F_d is the observed fluorescence intensity in the singly labeled (donor only) sample. $X_{b,da}$ and $X_{b,d}$ are calculated from fluorescence anisotropies as explained above.

Energy transfer (E_{app}) is then calculated from

$$E_{app} = \frac{F^\circ_{da} - F_{da}}{F^\circ_{da}}$$

where F_{da} is the observed fluorescence intensity of the labeled tRNA in the doubly labeled sample.

If binding in the doubly labeled sample is incomplete, the above energy transfer must be corrected for this since unbound labeled tRNA will not be participating in energy transfer, causing the energy transfer calculated above to be too low. It can be shown that this correction factor is $[1 - X_{b,da}/X_{b,da}(q_b/q_f)] + 1$, so that

$$E_{corr,da} = E_{app}\left(\frac{1 - X_{b,da}}{X_{b,da}(q_b/q_f)} + 1\right)$$

Finally, a correction is made, when possible, for incomplete labeling with acceptor probe. In the present experiments, the acceptor was on the ribosome. Incomplete labeling of the ribosome with acceptor would thus cause some of the bound labeled tRNA to be unpaired with an acceptor, so that the observed energy transfer would again be too low. This correction factor is $1/X_A$, where X_A is the fractional labeling with acceptor. In the case of ribosomes labeled at protein L1, this last correction was not applied, since as stated earlier, the L1 was labeled at ϵ-NH_2 groups and, although the average extent of labeling was near unity, the fractions of unlabeled, singly labeled, and multiply labeled ribosomes were unknown.

Calculation of the Distance between Probes. The distance between two probes, R, was calculated by the equation

$$R = R_0(1/E - 1)^{1/6}$$

Table I: Energy Transfer between Probes on tRNA to Probes on S21 or L1

	5′-PO_4-coumarin			s^4U_8-coumarin		
tRNA location	E^a (%)	r^b (Å)	limits[c] (Å)	E^a (%)	r^b (Å)	limits[c] (Å)
Labeled S21, in 70S						
deacylated						
P site	<6	>87		22	64	54–77
Ac-Phe						
P site	<6	>87		10	75	63–80
+puromycin	<6	>87		21	65	55–78
+Phe-tRNA	<6	>87		20	66	55–80
Labeled L1, in 70S						
deacylated						
P site	17	72	64–82	15	69	61–78
Ac-Phe						
P site	<3	>98		11	74	65–84
+puromycin	16	73	65–83	15	69	61–78
+Phe-tRNA	14	74	65–84	12	72	64–82

[a] E is fluorescence energy transfer. [b] r is the calculated distance between the probes assuming random orientation. R_0, the distance that would give 50% energy transfer, was 55 Å for coumarin on the 5′-PO_4 to fluoresceein on either S21 or L1. The corresponding value of R_0 for coumarin on s^4U_8 was 52 Å. The lower value of R_0 for coumarin on s^4U_8 is mainly due to its lower quantum yield (0.51–0.52 vs 0.72–0.74 for 5′-PO_4-coumarin). [c] The half-height limits of distance were calculated by the method of Haas et al. (1978), with polarization values of 0.40 for fluorescein-S21·70S, 0.25 for fluorescein-L1·70S, 0.44 for 5′-PO_4-coumarin, and 0.46 for s^4U_8-coumarin.

where R_0 is the distance at which the energy transfer, E, is 0.5. Details of the calculation of R_0 have been described previously (Odom et al., 1980). The fluorescence polarization data and half-height errors were determined as described previously (Odom et al., 1980), by using the method of Haas et al. (1978).

Results

(1) Movement of the tRNA during Peptide Bond Formation. Previous distance measurements were carried out by energy transfer from probes attached to each of three points located near the center of $tRNA^{Phe}$ (dhU loop of yeast $tRNA^{Phe}$, acp^3U_{47} of *E. coli* $tRNA^{Phe}$, s^4U_8–C_{13} cross-link of *E. coli* $tRNA^{Phe}$) to acceptor probes linked to the single cysteine of protein L11 or S21 (Hardesty et al., 1986). The results showed that deacylated $tRNA^{Phe}$ and AcPhe-tRNA are bound into physically different positions on the ribosome and that the deacylated tRNA generated in the peptidyl transferase reaction moves into a conformation, site, or position that is similar to or identical with deacylated $tRNA^{Phe}$ bound directly to the ribosomes. These results were interpreted to indicate the tRNA rather than the nascent peptide chain is moved, relative to the ribosome, during the synthesis of the peptide bond.

For the experiments reported in Table I, different pairs of probes were used with the energy donor attached to other sites on $tRNA^{Phe}$ and the acceptor probe on S21 or L1. Coumarin probes were covalently attached to the 5′-terminal phosphate and to s^4U_8 of *E. coli* $tRNA^{Phe}$.

Probes to function as the acceptor in energy transfer experiments were covalently attached to ribosomal proteins S21 and L1, which were then bound to ribosomes from mutant strains of *E. coli* that lack S21 or L1. S21 was specifically labeled on the thiol group of its single cysteine residue. However, L1 contains no cysteine; thus, amino-group-reactive reagents were used to label this protein. Although the fluorophore:L1 ratio was near unity, the probe is likely to be distributed among amino groups at several positions. For this reason, the calculated absolute distance to L1 is likely to reflect a composite of energy transfer in different ribosomes. Nevertheless, differences in distance are significant and provide a sensitive measure of when and how the fluorophores move during the peptide elongation cycle of reactions.

The tRNA used in all experimental situations was nearly 100% labeled. Where necessary, this was accomplished by isolation of the specific labeled tRNA species, usually using HPLC as previously described (Odom et al., 1988). Samples used for energy transfer shown in Table I were not isolated over Sephacryl S300 to remove unbound tRNA, but values of X_b, the fraction of labeled tRNA that was bound, were calculated from fluorescence anisotropies as explained under Materials and Methods. These values were 90% or greater with FM-S21 ribosomes and unlabeled ribosomes. With FITC-L1 ribosomes, X_b was around 75% for 5′-PO_4 coumarin-labeled tRNA and around 85% for s^4U_8 coumarin-labeled tRNA. Little difference was observed between binding of AcPhe-tRNA and deacylated tRNA. Ribosomes containing no L1 also gave X_b values around 0.75, which is in contrast to the results of Sander (1983), who observed poor binding of AcPhe-tRNA to ribosomes missing L1. The reason for the difference is unclear, since polyacrylamide gel electrophoresis confirmed the absence of L1 in our 50S – L1. It should be noted, however, that we used a longer incubation time (20 min vs 30 s) and a higher ratio of ribosomes to tRNA (5 or greater vs approximately 1) than Sander.

The puromycin reactivity of the bound labeled AcPhe-tRNA was usually greater than 90% with both FM-S21 ribosomes and FITC-L1 ribosomes, indicating proper positioning of the AcPhe-tRNA on the ribosome. In addition, the *changes* in energy transfer noted upon adding puromycin or Phe-tRNA to ribosomes containing prebound labeled AcPhe-tRNA almost certainly are due to peptidyl transfer, so that any incorrectly positioned (and therefore unreactive) AcPhe-tRNA would not contribute.

The energy transfer data are summarized in Table I. The 5′-terminal probe shows a large apparent movement toward L1, from a distance of 98 Å for AcPhe-tRNA to a distance of 72 Å for the deacylated form. This is the largest change in distance that we have measured. The probe on s^4U_8 moves toward the probes on both S21 and L1 during peptide bond formation, with the larger change for S21. The results presented in Table I indicate that the 5′-end and center region of the tRNA move toward S21 and L1 as a result of peptide transfer to the incoming aminoacyl-tRNA (but see Discussion for an alternative explanation).

(2) Ribosomal Environment of the Phe-tRNA Amino Acid. How are the nascent peptide and amino acid of acyl-tRNA held in the peptidyl transferase center, and when, where, and how far do they move during the reactions of the peptide elongation cycle? To experimentally approach an answer to these questions, fluorescent probes were covalently linked to the free amino group of aminoacyl-tRNA. Either a coumarin maleimide, CPM, or an iodoacetamide derivative of 5-aminonaphthalene-1-sulfonate, IAEDANS, was covalently attached to phenylalanine in Phe-tRNA. This was accomplished by reaction with the thiol group of a mercaptoacetyl moiety that had been introduced previously onto the free α-amino group of phenylalanine in [^{14}C]Phe-tRNA (see Materials and Methods). The labeled species, CPM-Phe-tRNA or AEDANS-Phe-tRNA, was isolated by phenyl-Sepharose chromatography or by reversed-phase HPLC as described under Materials and Methods. These chromatographic procedures efficiently separate the fluorescently N-labeled Phe-tRNA from Phe-tRNA and deacylated tRNA to yield

Table II: AEDANS-tRNA and CPM-tRNA Fluorescence

conditions	AEDANS-Phe-tRNA			CPM-Phe-tRNA		
	λ_{max}[a] (nm)	I[b]	A[c]	λ_{max}[a] (nm)	I[b]	A[c]
free	502	1.00	0.022	481	1.00	0.181
bound to ribosomes[d]						
puromycin-reactive site						
no puromycin	480	0.75	0.301	471	1.52	0.379
+puromycin	496	1.41	0.045	476	1.41	0.128
puromycin-nonreactive site						
no puromycin	486	0.71	0.311	474	1.47	0.370

[a] λ_{max} is the wavelength of maximum fluorescence emission. Excitation was at 360 nm for AEDANS-labeled samples and at 390 nm for CPM-labeled samples. [b] I is relative fluorescence intensity at the emission maximum. I values in Tables II and III can be compared directly for a given type of labeled tRNA, but I values for AEDANS-Phe-tRNA are not directly comparable with those for CPM-Phe-tRNA. I for free labeled tRNA has been assigned a value of 1. [c] A is fluorescence anisotropy, measured as described under Materials and Methods. [d] After binding of AEDANS-Phe-tRNA or CPM-Phe-tRNA to ribosomes, the samples were isolated over a Sephacryl S300 column to remove any unbound tRNA.

species that are labeled with both a fluorescent probe and [^{14}C]phenylalanine. These fluorescently labeled *N*-acetyl derivatives of Phe-tRNA were then nonenzymatically bound to ribosomes in reaction mixtures containing 15 mM Mg^{2+} and at least a 5:1 molar excess of ribosomes to the tRNA under conditions in which most of the fluorescently labeled Phe-tRNA was bound to the ribosomes. Two sets of conditions were used to give what would be termed A or P site binding according to the classical model: (a) CPM-Phe-tRNA or AEDANS-Phe-tRNA was bound without deacylated tRNA directly into a puromycin-reactive site, the P site of the classical model. Separate experiments have shown that more than 90% of the bound N-labeled Phe-tRNA was reactive with puromycin, as judged by analysis of radioactivity in the resulting puromycin derivative. This level of puromycin reactivity was confirmed by calculations based on changes in fluorescence anisotropy that occur during the puromycin reaction, as indicated below. It is near the level expected for AcPhe-tRNA. (b) In another set of experiments, deacylated tRNA at a molar ratio of slightly greater than 1:1 with ribosomes was bound before CPM-Phe-tRNA or AEDANS-Phe-tRNA was added to the reaction mixture. Under these conditions, subsequent puromycin reactivity of the N-labeled Phe-tRNA species was reduced to less than 10%, indicating that most of the labeled Phe-tRNA was bound into what would be the ribosomal A site by the classical model. Samples prepared by both of the above procedures were chromatographed over Sephacryl S300 to remove any unbound tRNA, as explained under Materials and Methods.

Three types of fluorescence measurements were carried out on the samples indicated above: emission maxima, relative intensity, and fluorescence anisotropy were determined. The results are presented in Table II. The corresponding emission spectra for AEDANS-Phe-tRNA are presented in Figure 1. For AEDANS-Phe-tRNA, binding to ribosomes (no deacylated tRNA, P site) results in a shift in the emission maximum from 502 to 480 nm and corresponding changes in relative fluorescence intensity and anisotropy from 1.00 to 0.75 and 0.022 to 0.301, respectively. The change in the fluorescence emission maximum is similar to that seen between free AEDANS in aqueous solution and in a less polar environment such as ethanol (Hudson & Weber, 1973), but the decrease in intensity upon binding is in contrast to the increase in intensity seen with free AEDANS in a more nonpolar environment. This probably indicates that the quenching seen upon binding is of a more specific nature than simply a change in environment polarity. Change in anisotropy indicates that the probe is transferred from a relatively mobile state characteristic of AEDANS free in aqueous solution to a state in which its movement is relatively constrained. Addition of puromycin to the reaction mixture results in a rapid increase in relative

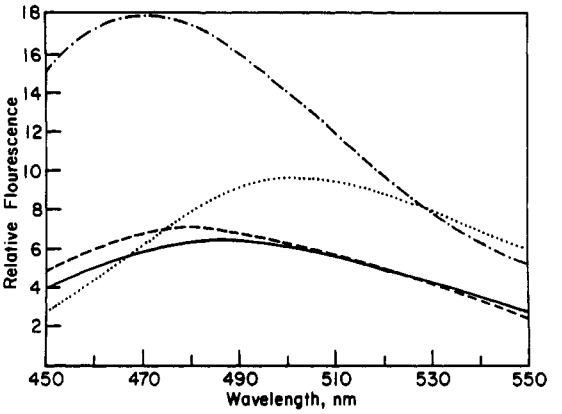

FIGURE 1: Fluorescence emission spectra of AEDANS-Phe-tRNA in various states. After the binding reaction, unbound tRNA was removed by Sephacryl S300 chromatography as described under Materials and Methods. Excitation was at 360 nm. (···), free AEDANS-Phe-tRNA; (– – –), AEDANS-Phe-tRNA in P site; (—) AEDANS-Phe-tRNA in A site; (–·–), AEDANS-Phe-tRNA in A site + 1 mM puromycin.

fluorescence intensity from 0.75 to 1.41 and a corresponding decrease in anisotropy from 0.301 to 0.045. The fluorescence anisotropy for the resulting AEDANS-Phe–puromycin is not known, but the large decrease in anisotropy indicates that nearly all of the AEDANS-Phe-tRNA in the sample has reacted with puromycin and is released from the ribosomes. Estimates of puromycin reactivity based on radioactivity indicate at least 90% or greater.

Binding of deacylated tRNA before the AEDANS-Phe-tRNA results in greatly decreased puromycin reactivity, to about 7%. The deacylated tRNA would be bound into the P site, then the AEDANS-Phe-tRNA would be bound into the ribosomal A site according to the classical model. The anisotropy in this puromycin-nonreactive site is very similar to that observed in the puromycin-reactive site. Likewise, fluorescence intensity is only slightly lower in this so-called A site, while the emission maximum undergoes a somewhat larger but still relative small shift (to the red).

Generally comparable results were obtained in experiments in which CPM-Phe-tRNA was used in place of AEDANS-Phe-tRNA. Fluorescence anisotropy for the free CPM-Phe-tRNA, 0.181, is relatively high compared to the value for CPM-cysteine in aqueous solution, 0.065. Anisotropy for CPM-Phe-tRNA bound to ribosomes approaches the theoretical limit of 0.4 and indicates that the coumarin probe is highly immobilized upon binding. Binding is associated with an increase in the relative fluorescence intensity to 1.52 and a small hypsochromic shift. As with AEDANS-Phe-tRNA, the emission maximum of CPM-Phe-tRNA before binding is near that anticipated for the free probe in aqueous solution.

Table III: Effect of Puromycin on the Fluorescence of Labeled AcPhe-tRNA in the Puromycin-Nonreactive Ribosomal Binding Site

	AEDANS-Phe-tRNA			CPM-Phe-tRNA		
additions	λ_{max}[a] (nm)	I[b]	A[c]	λ_{max}[a] (nm)	I[b]	A[c]
none[d]	486	0.71	0.311	474	1.47	0.370
puromycin[e]	472	1.95	0.308	469	1.58	0.374
puromycin + EF-G/GTP[f]	488		0.135	474		0.214

[a-c] See Table II for explanation of λ_{max}, I, and A. [d] Samples containing labeled AcPhe-tRNA in the puromycin-nonreactive site were prepared and isolated over Sephacryl S300 as described under Materials and Methods. [e] Puromycin was added directly to the fluorescence cuvette at a concentration of 1 mM, and measurements were made after a 5-min incubation at 20 °C. [f] Dithioerythritol and GTP were added to the fluorescence cuvette to give concentrations of 2 and 0.04 mM, respectively, followed by 5 μg of partially purified *E. coli* elongation factor G. Incubation was for 30 min at 20 °C.

Binding of CPM-Phe-tRNA appears to place the probe in a more hydrophobic environment, consistent with the results obtained with AEDANS-Phe-tRNA. The large value for anisotropy observed with the CPM derivative may reflect its hydrophobic character and relatively short fluorescence lifetime, near 1.7 ns for free CPM-Phe-tRNA, compared with a fluorescence lifetime near 17 ns for free AEDANS-Phe-tRNA. CPM-Phe-tRNA bound into the puromycin-nonreactive site shows fluorescence properties similar to those of CPM-Phe-tRNA in the puromycin-reactive site, the most significant difference being the small red shift in emission maximum.

In summary, results with both AEDANS-Phe-tRNA and CPM-Phe-tRNA indicate that the probes are strongly constrained and subjected to a very similar environment in the puromycin-reactive and -nonreactive ribosomal sites. Previous binding of deacylated $tRNA^{Phe}$ to give binding into the nonreactive site results in a small but experimentally significant shift in both the emission maximum and fluorescence intensity, but these shifts are small in comparison with the value for the unbound Phe-tRNA species or after reaction with puromycin. We conclude that these results are consistent with the hypothesis that the probes are in a very similar or identical position when bound in the puromycin-reactive and -nonreactive site or state.

(3) Effect of Puromycin on Probe-Phe-tRNA Fluorescence. Experiments of the type described above led us to test the effect of puromycin on the fluorescence of AEDANS-Phe-tRNA or CPM-Phe-tRNA bound to ribosomes to which deacylated tRNA had been prebound. Under these conditions, the bound probe-Phe-tRNA species is not reactive with puromycin and thus would be classified as in the A site according to the classical model. Binding of puromycin to these ribosomes is evidenced by its relatively large effect on fluorescence emission maxima and intensities (Table III). The results are particularly striking with AEDANS-Phe-tRNA, where a shift in the emission maximum from 486 to 472 nm and an increase in relative intensity from 0.71 to 1.95 are observed. This is equivalent to a 2.75-fold increase in fluorescence intensity and provides a sensitive measure of puromycin binding. The resulting fluorescence emission spectrum is included in Figure 1, in which it can be compared directly with spectra obtained in the absence of the antibiotic. Also to be noted in Table III is the fact that puromycin causes virtually no change in the anisotropy, confirming the direct assay results that no covalent reaction with puromycin occurs. As expected, puromycin causes no change in the fluorescence properties of free AEDANS-Phe-tRNA or CPM-Phe-tRNA (data not shown).

As a further test to verify that the puromycin-nonreactive labeled AcPhe-tRNA was in the classical A site and not, for example, in an inactive P site, EF-G and GTP were added after puromycin. This should translocate the labeled AcPhe-tRNA from the A site to the P site, making it puromycin-reactive. The results, shown in Table III, indicate that, with both AEDANS-Phe-tRNA and CPM-Phe-tRNA, addition of EF-G and GTP caused a large red shift in emission maximum and a decrease in anisotropy, presumably reflecting translocation, subsequent reaction with puromycin, and release of the AEDANS- or CPM-puromycin derivative from the ribosome. Reaction with puromycin was confirmed by extraction of the [^{14}C]puromycin derivatives with organic solvent and liquid scintillation counting. Sixty to seventy percent of the total bound radioactivity was extracted after EF-G, while less than 7% was extractable before EF-G.

The results unequivocally demonstrate that deacylated $tRNA^{Phe}$, AcPhe-tRNA, and puromycin can be bound simultaneously to the same ribosome. According to the classical model, puromycin should be bound only into the A site, in which it can act as the acceptor in the peptidyl transferase reaction. However, according to the classical model, the A site should be occupied by the AcPhe-tRNA derivative under the conditions used. It follows that binding of AcPhe-tRNA into the A site would be expected to prevent binding of puromycin. That the deacylated tRNA also is bound is evidenced by the failure of the AcPhe-tRNA to react covalently with puromycin and the direct effect of the deacylated tRNA on fluorescence. An estimate of the puromycin dissociation constant, K_d, was made from the changes in fluorescence intensity observed in the presence of various concentrations of puromycin. A double-reciprocal plot (not shown) constructed from these data indicated a dissociation constant in the range of 0.5 mM. This value is similar to that reported previously, 0.4 mM (Lessard & Pestka, 1972), measuring competition of puromycin with [^{14}C]chloramphenicol. The results indicate that binding of the AcPhe-tRNA in the presence of previously bound deacylated tRNA has no appreciable effect on binding of puromycin. The relatively large effect on fluorescence intensity indicates that the puromycin, probably specifically its amino group, is bound in the immediate vicinity of the AEDANS probe. It is not clear why the peptidyl transferase reaction fails when deacylated tRNA is previously bound to the ribosomes. The results with puromycin prompted the question as to whether aminoacyl-tRNA binding might also be detected by a similar procedure. No similar effect on fluorescence of AEDANS-Phe-tRNA bound to ribosomes with deacylated tRNA was observed if Phe-tRNA at a 3:1 molar ratio with ribosomes (about 1 μM) was substituted for puromycin. The latter was used at a concentration of 1 mM. The observations suggest that the presence of deacylated tRNA in what would be the P site by the classical model substantially reduces the affinity for binding of Phe-tRNA into a third position or site on the ribosome but has little or no effect on binding of puromycin, suggesting that the effect on the former may involve blockage of the codon–anticodon interaction.

The effect of puromycin on CPM-Phe-tRNA fluorescence in comparable experiments is similar to that seen with AEDANS-Phe-tRNA but smaller in magnitude (Table III). The reaction rate of puromycin with CPM-Phe-tRNA in the puromycin-reactive site is relatively slow compared with the rate with AcPhe-tRNA or AEDANS-Phe-tRNA, which appears to give the fastest reaction of the three. These striking differences in rate prompt the question of why and how the side chain of the peptidyl-tRNA analogue affects the peptidyl

Table IV: Effect of Puromycin on Fluorescence from CPM-Phe-tRNA in the Puromycin-Reactive and -Nonreactive Sites

probe-Phe-tRNA binding site	$\Delta\lambda_{max}$[a] (nm)	ΔI[a] (%)
puromycin-reactive (P site)	−7[b]	+5.1[b]
puromycin-nonreactive (A site)	−5	+7.5

[a] $\Delta\lambda_{max}$ and ΔI are the differences in emission maximum and fluorescence intensity, respectively, before and after addition of puromycin. [b] Minimum value, obtained from measurements made about 1 min after the addition of puromycin at 0 °C.

transferase reaction. In any event, the slow reaction rate with CPM-Phe-tRNA makes it possible to estimate the effect of puromycin on the fluorescence from CPM-Phe-tRNA in the puromycin-reactive site before the peptidyl transferase reaction has occurred, which is not possible with AEDANS-Phe-tRNA. The results of a very rapid determination at 0 °C are presented in Table IV. The results with CPM-Phe-tRNA bound in the puromycin-reactive ribosomal site (P site) are very similar to those for CPM-Phe-tRNA bound after deacylated tRNA into the nonreactive site (A site). The results indicate that the physical relation between the CPM probe and puromycin is very similar or identical in both situations. They suggest that the CPM-Phe-tRNA is in the same physical location on the ribosome, when it is bound into the puromycin-reactive or -nonreactive sites, the P and A sites of the classical model.

(4) Energy Transfer from Labeled AcPhe-tRNA in the Puromycin-Reactive and -Nonreactive Sites. A change in the position of the peptidyl moiety between the puromycin-reactive and -nonreactive sites should be easily detected by measuring energy transfer from a fluorescent analogue of peptidyl-tRNA to an energy acceptor probe on a ribosomal protein or RNA. A number of such experiments have been attempted, with the somewhat surprising failure to see significant energy transfer with probes attached to L1, L11, S1, and the 3′-ends of 5S and 16S RNA. Although movement of the CPM probe used in these experiments appears to be highly constrained, leading to the possibility that probe orientation might be poor for energy transfer (low value for κ^2), a more likely explanation with the number of probes tested appears to be that they all are relatively far from the peptidyl moiety. Considering the distribution of these components around the top of the 70S ribosome in the region of the central protuberance of the 50S subunit, these results may indicate that the nascent peptide is positioned between the subunits but near the bottom of the large subunit distal to its central protuberance. Such a position on the interfacing surface of the large subunit appears to be near the site of the outer surface of the 50S subunit at which the nascent peptide exits the ribosome (Bernabeu & Lake, 1980). An intriguing possibility is that the nascent peptide passes through the tunnel that has been reported to extend through the 50S subunit from the interfacing to exterior surface (Yonath et al., 1987).

Energy transfer was observed, however, from both AEDANS-Phe-tRNA and CPM-Phe-tRNA to fluorescein covalently linked to the amino group of erythromycylamine. Erythromycin appears to block the synthesis of peptides such as polylysine only after the formation of a di- or tripeptide (Otaka & Kaji, 1975). Interestingly, the antibiotic appears not to inhibit the synthesis of polyphenylalanine (Chinali et al., 1988). It has been suggested (Arevalo et al., 1988) that erythromycin binds to the 50S ribosomal subunit in a way that blocks the entrance to the tunnel considered above. Derivatives of erythromycylamine appear to bind to the same site (Vince et al., 1976; Arevalo et al., 1989), although generally less tightly than the parent compound. In any event, the fluorescein derivative of erythromycylamine appears to bind in the region of the 50S subunit near the peptidyl transferase center in a position from which efficient energy transfer can occur from the ribosome-bound derivatives of either AEDANS- or CPM-Phe-tRNA, as shown in Table V. Experimentally very similar values for energy transfer were observed with the AEDANS-Phe-tRNA derivative bound into the puromycin-reactive or -nonreactive site, about 26%; with CPM-Phe-tRNA, slightly less energy transfer was seen in the puromycin-reactive site (48.3%) than in the puromycin-nonreactive site (60.6%). This may be due to slightly different orientations of the CPM probe in the two sites, the CPM probe being more immobile apparently than the AEDANS probe, as judged by fluorescence anisotropy (Table I). These results are consistent with the conclusion that the AEDANS and CPM fluorophores are in about the same position relative to the fluorescein probe on erythromycin, whether the Phe-tRNAs to which they are attached are in the A or P site. However, the calculation of the distance between the energy donor and fluorescein probes is subject to considerable error. Unlabeled erythromycin itself has a large effect on the fluorescence quantum yield of AEDANS-Phe-tRNA or CPM-Phe-tRNA, presumably by causing changes in the local environment of the probes (manuscript in preparation). Binding of fluorescein-erythromycylamine to the ribosomes is likely to change the AEDANS or CPM fluorescence quantum yield by mechanisms that do not involve nonradiative energy transfer. We estimate the probes to be considerably closer together than calculations based on the typical R_0 values and the energy transfer data given in Table V would indicate. For these reasons, calculated distances are not given. Nevertheless, the data provide strong support for the hypothesis that the peptides or peptidyl-tRNAs bound to ribosomes in the puromycin-reactive and -nonreactive sites are in essentially the same physical position in the peptidyl transferase center of the 50S subunit.

Table V: Fluorescence Energy Transfer from AEDANS-Phe-tRNA or CPM-Phe-tRNA to Fluorescein-Labeled Erythromycin[a]

	energy transfer to FITC-erythromycin (%)	
binding site	from AEDANS-Phe-tRNA	from CPM-Phe-tRNA
puromycin-reactive (P site)	26.7	48.2
puromycin-nonreactive (A site)	25.6	60.6

[a] Samples containing ribosome-bound labeled AcPhe-tRNA were prepared and isolated over Sephacryl S300 as described under Materials and Methods. Fluorescence was measured at 460 nm, with excitation at 360 nm for the AEDANS-labeled samples and at 380 nm for the CPM-labeled samples. Unlabeled erythromycin or FITC-erythromycin was added incrementally directly to the fluorescence cuvettes after first measuring fluorescence in their absence. Concentrations of 2, 4, and 6 μM erythromycin or FITC-erythromycin were tested to verify complete binding. Controls consisting of free AEDANS-Phe-tRNA or CPM-Phe-tRNA in buffer and of unlabeled ribosomes were treated similarly.

Discussion

The results presented above indicate that a probe on the 5′-end of tRNA undergoes a relatively large movement with respect to protein L1, more than 20 Å, as it is deacylated during the formation of a peptide bond on a ribosome. This movement is larger than the change in distances from the center of the tRNA measured previously (Hardesty et al., 1986) and appears to be adequate to allow an incoming tRNA to occupy the binding site in the peptidyl transferase center. In contrast, the nascent peptide appears to move no more than a few angstroms during the peptidyl transferase reaction. These observations are inconsistent with the classical two-site

model of peptide elongation, in which the nascent peptide is envisioned to be physically moved from a tRNA in the P site to the amino acid of an incoming tRNA in the A site during the peptidyl transferase reaction.

It cannot be proven from the present energy transfer data whether it is the tRNA or L1 that moves upon peptidyl transfer. Indeed, Moazed and Noller (1989) have postulated that the 50S subunit may move with respect to the 30S subunit and tRNA during peptidyl transfer. The data in this paper and previous work (Hardesty et al., 1986) showing movement of the middle portion of tRNA with respect to protein S21, however, indicate that the relationship of tRNA to the 30S subunit is also not constant during peptidyl transfer. The apparent movement of tRNA with respect to both subunits (Hardesty et al., 1990), together with the apparent nonmovement at the anticodon binding site on the 30S subunit (Moazed & Noller, 1989), suggests some conformational change in the tRNA itself upon deacylation. We have some direct evidence for such a conformational change (Odom & Hardesty, 1987).

The apparent nonmovement of the peptide moiety between the puromycin-reactive and -nonreactive states with respect to the puromycin and erythromycin binding sites could be explained by movement in a circular path maintaining the same distance from both antibiotic binding sites, but this does not seem very probable. The very fact that puromycin and peptidyl-tRNA can be bound simultaneously in the puromycin-nonreactive state provides strong additional evidence that at least the peptidyl moiety does not shuttle between the classical P and A sites, as discussed below.

The results presented also unequivocally demonstrate that peptidyl-tRNA, deacylated tRNA, and puromycin can be bound simultaneously to the same ribosome. Binding of puromycin to the ribosomes is detected by its effect on the quantum yield of a fluorescent probe attached to the amino acid of aminoacyl-tRNA. These peptidyl-tRNA analogues react readily with puromycin unless they are bound to ribosomes to which deacylated tRNA was bound previously, in which case reaction with puromycin does not occur, indicating that deacylated tRNA must also be present on these ribosomes. The presence of either or both peptidyl-tRNA and deacylated tRNA on the ribosomes has little if any effect on their affinity for puromycin. A K_d of about 0.4–0.5 mM is observed in all cases. In addition, the effect of puromycin on the fluorescence from the peptidyl-tRNA analogue is nearly the same whether the peptidyl-tRNA is in the reactive or unreactive ribosomal site. The results indicate that puromycin binds to the same site in each case and prompts the question of why then can it not act as an acceptor in the peptidyl transferase reaction in the situation in which deacylated tRNA also is bound to the ribosome?

In contrast to the results with puromycin, the presence of both deacylated tRNA and peptidyl-tRNA on the ribosome effectively block subsequent codon-directed binding of Phe-tRNA. Of course, the latter is measured with much lower concentrations, here typically about 1 μM, rather than 1 mM used for puromycin. Puromycin acts as an analogue of the 3′-aminoacyl end of aminoacyl-tRNA and presumably interacts with the site on the 50S subunit to which this portion of the incoming aminoacyl-tRNA is bound, whereas the intact tRNA can interact at other points including its anticodon, thus leading to much tighter binding. It appears that, when deacylated tRNA is present on the ribosome, the incoming aminoacyl-tRNA is bound into what would be the A site of the classical model by interactions involving its acceptor stem and anticodon, but the latter site of interaction on the 30S subunit is blocked when both deacylated tRNA and peptidyl-tRNA are bound. It follows that there are three sites on the 50S subunit for the acceptor stem region of tRNA but perhaps only two for the anticodon region on the 30S subunit. The 50S sites would correspond to what we have called the entry site, to which incoming aminoacyl-tRNA is bound initially, the peptidyl transferase center, to which the nascent peptide and its tRNA are always bound during peptide elongation, and a site to which only deacylated tRNA is bound with high affinity, the exit site (Hardesty et al., 1986). In contrast, only two tRNAs can be bound normally by codon–anticodon interaction on the 30S subunit. During peptide elongation, one of these is always peptidyl-tRNA. The other can be either deacylated tRNA or incoming aminoacyl-tRNA but not both simultaneously. The data presented here and this interpretation appear to be consistent with the basic concepts of the displacement model (Hardesty et al., 1990). It follows that the reason the peptidyl-tRNA does not react with puromycin when deacylated tRNA is present on the ribosome is due to steric hindrance from the deacylated tRNA. The deacylated tRNA site on the 50S subunit is occupied; thus, the displacement reaction in which a second deacylated tRNA would be generated cannot take place.

The results presented here and previously appear to be at least generally consistent with those presented recently by Moazed and Noller (1989). Using an entirely different approach, they also demonstrate that the tRNA appears to move relative to the 50S subunit as the peptidyl transferase reaction takes place but find that there is little or no change in the bases that are shielded by the tRNA in the 30S subunit until the EF-G reaction takes place. The results presented here give no direct indication of when the tRNA moves, relative to the 30S subunit, except to reconfirm the old observation that aminoacyl-tRNA is not efficiently bound to ribosomes that already carry deacylated tRNA and peptidyl-tRNA. However, we find the hypothesis that the EF-G reaction breaks the anticodon–codon interaction of the deacylated tRNA to be very attractive. This might effectively open up the second site on the 30S subunit and allow for the dissociation or easy displacement of the deacylated tRNA.

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