Comparison of Ribosomal Entry and Acceptor Transfer Ribonucleic Acid Binding Sites on *Escherichia coli* 70S Ribosomes. Fluorescence Energy Transfer Measurements from Phe-tRNA^{Phe} to the 3′ End of 16S Ribonucleic Acid†

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ABSTRACT: Distances were measured by nonradiative energy transfer from fluorescent probes specifically located on one of three points of yeast or *Escherichia coli* Phe-tRNA^{Phe} enzymatically bound to the entry site or to the acceptor site of *E. coli* 70S ribosomes to energy-accepting probes on the 3′ end of the 16S ribonucleic acid (RNA) of the 30S subunit. The Y base in the anticodon loop of yeast tRNA^{Phe} was replaced by proflavin. Fluorescein isothiocyanate was attached to the X base (position 47) of *E. coli* tRNA^{Phe}. *E. coli* tRNA^{Phe} which had been photochemically cross-linked between positions 8 and 13 followed by chemical reduction to form a fluorescent probe was also used. Labeled tRNAs were aminoacylated and enzymatically bound to the ribosome in the presence of elongation factor Tu and guanosine 5′-triphosphate (acceptor-site binding) or a nonhydrolyzable analogue (entry-site binding). Nonradiative energy transfer measurements were made of the distances between fluorophores located on the Phe-tRNA and the fluorophore at the 3′ end of 16S RNA. Calculations were based on comparison of the fluorescence lifetime of the energy donor, located on the Phe-tRNA, in the absence and presence of an energy acceptor on the 3′ end of the 16S RNA. Under both sets of binding conditions, the distances to the 3′ end of 16S RNA were found to be the following: cross-linked tRNA, >69 Å; Y base of tRNA, >61 Å. The distance between the 3′ end of 16S RNA and the X base of tRNA was found to be 81 Å under acceptor-site binding conditions but greater than 86 Å under entry-site binding conditions.

In prokaryotic systems, elongation factor Tu (EF-Tu)[1] promotes GTP-dependent codon-directed binding of aminoacyl-tRNA to the acceptor site of ribosomes (Ravel, 1967) with concomitant hydrolysis of GTP and peptide bond formation. Release of deacylated tRNA from the ribosome is associated with hydrolysis of a second GTP by elongation factor G. Movement of mRNA and tRNA through the ribosome is associated with these processes, but the mechanism is not understood. Watson (1964) proposed the two-site model for ribosomal binding of tRNA to account for the basic requirements of protein synthesis. Evidence interpreted to reflect a third functionally distinct site was presented by Hardesty et al. (1969). The P (peptidyl or donor) site has been defined as the tRNA binding site on the ribosome from which peptidyl-tRNA can react with puromycin (de Groot et al., 1970), while peptidyl-tRNA bound to the ribosome which is not puromycin reactive is in the A (acceptor or aminoacyl) site. If aminoacyl-tRNA is bound to the ribosome in the presence of EF-Tu and a nonhydrolyzable derivative of GTP while P site bound peptidyl-tRNA remains puromycin reactive, then the aminoacyl-tRNA is in a functionally distinct site called the E site hypothesized by Hardesty et al. (1969). When aminoacyl-tRNA is bound to the entry site, it cannot participate in peptide bond formation. The EF-Tu complexed with the analogue of GTP remains bound to the ribosome under the conditions of E-site binding (Skoultchi et al., 1970). No evidence has been presented that the E site constitutes a physically distinct location or position of tRNA on the ribosome. Lake (1980) proposed that tRNA was rotated about 180° around its short axis during the E-site (called the recognition or R site by Lake) to A-site transition.

The topography of the A site has been studied by using affinity labeling techniques [cf. Ofengand (1980)]; these experiments have been extended to include entry-site binding conditions (Johnson & Cantor, 1980; Ofengand, 1980). When Phe-tRNA^{Phe} carrying a *p*-azidophenacyl (APA) probe (approximately 9 Å long) on position 8 was enzymatically bound to the A site of 70S ribosomes, it could not be cross-linked to the 16S RNA. In other affinity labeling experiments, it was found that aminoacyl-tRNA derivatives cross-linked to the same targets when bound to the A site as when bound to the R site (Johnson & Cantor, 1980).

Here we describe fluorescence energy transfer measurements used to examine the distance between the 3′ end of 16S RNA and probes on Phe-tRNA^{Phe} and compare the results for measurements performed under A-site and entry-site binding conditions. The differences between the A- and entry-site bound tRNA in relation to the 3′ end of the 16S RNA are discussed.

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[1] Abbreviations: RNA, ribonucleic acid; tRNA, transfer RNA; tRNA^{Phe}, phenylalanine-accepting tRNA; Ac-Phe-tRNA, *N*-acetyl-Phe-tRNA; tRNA$^{Phe}_{XLR}$, tRNA^{Phe} that has been photochemically cross-linked from position 8 to position 13 followed by reduction of the cross-link with $NaBH_4$; tRNA$^{Phe}_{X\text{-}F}$, *Escherichia coli* tRNA^{Phe} that has been labeled at the X base with FITC; tRNA$^{Phe}_{Pf}$, yeast tRNA^{Phe} in which the Y base has been replaced by proflavin; FTSC, fluorescein thiosemicarbazide; FITC, fluorescein isothiocyanate; ETSC, eosin thiosemicarbazide; poly(U), poly(uridylic acid); EF-Tu, elongation factor Tu; TP30, total protein from 30S ribosomal subunits; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTP, guanosine 5′-triphosphate; GDP-NP, guanosine 5′-(β,γ-imidotriphosphate); $Mg(OAc)_2$, magnesium acetate; NaOAc, sodium acetate; BD-cellulose, benzoylated DEAE-cellulose; DEAE, diethylaminoethyl.

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Experimental Procedures

Materials

Escherichia coli K12, strain A19, fluorescein thiosemicarbazide (FTSC), eosin thiosemicarbazide (ETSC), proflavin hemisulfate, and yeast $tRNA^{Phe}$ were obtained as described (Robbins et al., 1980). Fluorescein isothiocyanate (FITC) was purchased from Molecular Probes, Inc., Junction City, OR. [^{14}C]Phe-$tRNA^{Phe}_{XLR}$ (49 Ci/mol, 1209 pmol/A_{260} unit) was a generous gift from Dr. J. Ofengand, Nutley, NJ. All other chemicals were of reagent grade.

Methods

Preparation of Ribosomal Components and $tRNA^{Phe}$ from E. coli. Isolations of *E. coli* 30S and 50S ribosomal subunits, 16S RNA, and total protein from 30S subunits were carried out as described before (Odom et al., 1980). Methods described in the same reference were used to oxidize the 3′ end of the 16S RNA, label it with FTSC or ETSC, and reconstitute labeled or unmodified 16S RNA with TP30 to form active 30S ribosomal subunits. The activity of the reconstituted particles was measured by using the poly(U)-directed poly(phenylalanine) synthesis assay described previously (Odom et al., 1980). *E. coli* $tRNA^{Phe}$ was isolated as described (Robbins et al., 1981).

Isolation of EF-Tu from Thermus thermophilus. T. thermophilus cells were grown and harvested as described (Rychlik et al., 1983). Elongation factor Tu was isolated and purified from these cells as described before (Rychlik et al., 1983).

Replacement of the Y Base. Removal of the natural Y base from yeast $tRNA^{Phe}$ and subsequent replacement with proflavin were carried out as before (Robbins et al., 1981).

Labeling of E. coli $tRNA^{Phe}$ at the X Base. E. coli $tRNA^{Phe}$ was labeled at the X base (position 47) with FITC by using the method of Plumbridge et al. (1980). Approximately 1 mL of $tRNA^{Phe}$ (130 A_{260} units) was dialyzed for 8 h against a solution of 1 M NaCl and 50 mM Hepes–KOH (pH 8.0). After dialysis of the tRNA, a dimethylformamide solution containing 100 mM FITC was added to give a final dye concentration of 10 mM. The reaction mixture was incubated for 5 h at 37 °C and brought to 100 mM KCl, and the tRNA was precipitated by addition of 2 volumes of ethanol and storage for 2 h at −20 °C. The labeled tRNA was further separated from unreacted dye by two additional ethanol precipitations. Unlabeled tRNA was removed by passing the tRNA over a BD-cellulose column (Plumbridge et al., 1980). The column was equilibrated with a solution containing 400 mM NaCl, 50 mM NaOAc (pH 5.0), and 10 mM $Mg(OAc)_2$. The labeled tRNA was loaded in a minimal volume of the same solution. A salt gradient of 0.4–2 M NaCl was applied to the column. As described before (Plumbridge et al., 1980), the unlabeled tRNA eluted first, followed by elution of $tRNA^{Phe}_{X\text{-}F}$.

Enzymatic Binding of Phe-$tRNA^{Phe}$ Species to 70S Ribosomes. Elongation factor Tu dependent binding of Phe-$tRNA^{Phe}$ to *E. coli* 70S ribosomes was performed on the basis of the method of Johnson & Cantor (1980). The maximum amount of A-site binding was empirically found to occur by using a 30S:50S molar ratio of 2:1.

A volume of 30 μL contained 6 A_{260} units of native 50S subunits and 6 A_{260} units of 30S subunits reconstituted with labeled or unmodified 16S RNA, 25 μg of poly(U), 33 μM GTP (in the case of A-site binding) or GDP-NP (in the case of E-site binding), 50 mM Tris-HCl (pH 7.5), 80 mM NH_4Cl, 70 mM KCl, 5 mM 2-mercaptoethanol, 12 mM $Mg(OAc)_2$, and 3.6 A_{260} units of unmodified deacylated *E. coli* $tRNA^{Phe}$. The mixture was incubated for 15 min at 37 °C. The volume then was increased 2-fold, keeping all salt concentrations except Mg^{2+} constant while reducing the $Mg(OAc)_2$ concentration to 6 mM, and 0.11 A_{260} unit of the appropriate Phe-$tRNA^{Phe}$ species was added with 75 μg of EF-Tu. The samples then were incubated for 20 min at 37 °C.

Following the incubation, samples were centrifuged in a Spinco Ti-50 rotor at 50000 rpm for 1 h at 4 °C. Pelleted ribosomes were taken up in 0.3 mL of a solution containing 50 mM Tris-HCl (pH 7.5), 80 mM NH_4Cl, 70 mM KCl, 7 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. Resuspended ribosomes were immediately subjected to energy transfer measurements, followed by assays for biological activity.

Puromycin Reactivity of Phe-tRNA Samples. In order to examine the amount of nonenzymatic P-site binding of Phe-tRNA in samples, the puromycin reactivity of the [^{14}C]Phe-tRNA species was measured by a modification of the method of Wurmbach & Nierhaus (1979). Following fluorescence energy transfer measurements, samples were brought to a puromycin concentration of 1 mM and incubated for 30 min on ice. The reaction was stopped by addition of an equal volume of 300 mM NaOAc (pH 5.0) saturated with $MgSO_4$. Formation of Phe-puromycin was measured by extraction with ethyl acetate and determining the radioactivity of the ethyl acetate layer in a solution containing 5% Biosolv and 5 g of 2,5-diphenyloxazole/L of toluene. The remaining aqueous layer was treated with an ice-cold solution of 5% trichloroacetic acid and filtered through glass fiber filters (Schleicher & Schuell, no. 30). The amount of [^{14}C]Phe-tRNA bound to ribosomes that was not puromycin reactive was determined by drying the filters and determining the radioactivity by liquid scintillation in a solution of 5 g of 2,5-diphenyloxazole/L of toluene.

Fluorescence Measurements. Quantum yields (Q), R_0 values (the distance at which energy transfer proceeds at half of its maximum efficiency), and bounds on measured distances due to variations in κ^2 (the dipole–dipole orientation factor in the definition of R_0) were evaluated as before (Robbins et al., 1981). The method of Haas et al. (1978) was used to calculate the bounds on measured distances based on the fluorescence polarizations of the donor and the acceptor. Table I lists the values of polarization and half-height error limits. Polarization measurements were made on an SLM PR8000 spectrofluorometer designed to correct for instrument response variations of the basic formula (Förster, 1951)

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ is the fluorescence intensity of light polarized parallel to the excitation light and $I_{\perp}$ is the intensity of light polarized perpendicular to the excitation beam.

The efficiency of energy transfer, E, between pairs of probes was calculated from the fluorescence lifetime of the energy donor fluorophore determined in the presence and absence of the energy acceptor by

$$E = 1 - \tau/\tau_0$$

where τ_0 is the fluorescence lifetime of the energy donor with no acceptor present and τ is the lifetime in the presence of acceptor. Fluorescence lifetimes were measured by using an Nd:YAG mode-locked laser (NG50, Quantel International, Sunnyvale, CA) as a source of excitation light and a Hamamatsu R306 photomultiplier tube for detection of fluorescence. The experimental setup including a description of the laser

Table I: Polarization Data of Donor–Acceptor Pairs Used and Distance Limits from Fluorophore Orientation

probe location		polarization (P)[a]		half-height limits of distance, $Q(r'/r)$[b]
tRNA^{Phe}	16S RNA	tRNA	16S RNA	
Phe-tRNA$^{Phe}_{XLR}$	eosin	0.22	0.41	0.88–1.14
	fluorescein	0.22	0.38	0.88–1.14
yeast Phe-tRNA$^{Phe}_{Pf}$	eosin	0.30	0.41	0.91–1.11
Phe-tRNA$^{Phe}_{X\text{-}F}$	eosin	0.04	0.41	0.90–1.03

[a] Polarization of fluorophores was measured as before (Robbins et al., 1981). Excitation and emission wavelengths, respectively, of energy donors were 380 and 440 nm for the cross-link moiety, 400 and 500 nm for proflavin, and 465 and 500 nm for fluorescein on the X base. Excitation and emission wavelengths, respectively, of energy acceptors were 440 and 520 nm for eosin and 440 and 500 nm for fluorescein. Values for the polarization of energy donors bound to the ribosomal A site and E site were identical within experimental error. [b] The half-height limits are determined as the full width at half-maximum height of the function $Q(r'/r)$ which is the apparent distribution of distances between donor and acceptor estimated from polarization data by the method of Haas et al. (1978). r' is the calculated distance between the donor–acceptor pair assuming $\kappa^2 = {}^2/_3$, and r is the actual distance.

Table II: Millipore Binding and Puromycin Reactivity of Phe-tRNA Species Bound to tRNA·Ribosomal Complexes[a]

amount of deacylated tRNA^{Phe} incubated with 70S ribosomes in first incubation step (A_{260} units)	amount of Phe-tRNA$^{Phe}_{XLR}$ which has puromycin reactivity (pmol)	amount bound to Millipore filters (pmol)
	6.0	14.1
0.35	2.0	13.9
0.7	1.6	11.5
1.75	0.4	11.0

[a] In duplicate samples, 1.2 A_{260} units of 30S subunits and 1.2 A_{260} units of 50S subunits were incubated with poly(U) and the indicated amount of deacylated *E. coli* tRNA^{Phe} for 10 min at 37 °C in 50 μL of 33 μM GTP, 50 mM Tris·HCl (pH 7.5), 80 mM NH_4Cl, 70 mM KCl, 5 mM β-mercaptoethanol, and 12 mM $Mg(OAc)_2$. The volume was increased 2-fold, and 0.045 A_{260} unit of Phe-tRNA$^{Phe}_{XLR}$ was added with 3 μg of EF-Tu. After incubation for a further 20 min at 37 °C, the duplicate samples were each split into two equal aliquots. Two aliquots were used to measure the amount of Phe-tRNA·70S complex which bound to Millipore filters, and the remaining two were assayed for puromycin reactivity as described under Methods. Thus, each sample should contain 0.022 A_{260} unit of Phe-tRNA and approximately 36 pmol of [^{14}C]Phe-tRNA$^{Phe}_{XLR}$ (specific activity 49 Ci/mol).

and detection system, as well as data acquisition and processing, has been described previously (Robbins et al., 1981; Foyt, 1981). The excitation light was 353 nm, and emission was measured at 440 nm (for cross-linked tRNA) or 500 nm (for fluorescein or proflavin) by using the optics described before (Robbins et al., 1981). In all cases, a one-exponential decay was convoluted with the impulse response curve to fit the observed fluorescence decay.

Results

Biological Activity of Reconstituted Subunits and Labeled tRNA^{Phe}. For the experiments described below, *E. coli* 16S ribosomal RNA was labeled with fluorescent probes as described under Methods. It has been shown (Odom et al., 1980) that 16S RNA labeled at its oxidized 3′ end with FTSC or ETSC retains its activity. It can be reconstituted into 30S ribosomal subunits as readily as unlabeled 16S RNA. Reconstituted 30S subunits were found to have between 60 and 80% of the activity of native 30S subunits when assayed for poly(U)-directed poly(phenylalanine) synthesis (Odom et al., 1980).

Labeling of the X base in tRNA^{Phe} from *E. coli* with FITC did not appreciably decrease the ability of the tRNA^{Phe} to accept phenylalanine. The *E. coli* tRNA^{Phe} was labeled on the X base with FITC as described under Methods and aminoacylated with [^{14}C]Phe to give about 1300 pmol/A_{260} unit. Yeast tRNA^{Phe} was labeled with proflavin at the Y base as described under Methods and aminoacylated to 525 pmol/A_{260} unit. Cross-linked *E. coli* tRNA^{Phe} was prepared and aminoacylated to 1209 pmol/A_{260} unit by Ofengand and coworkers (Robbins et al., 1981).

Enzymatic Binding and Puromycin Reactivity of Phe-tRNA Species. Species of Phe-tRNA^{Phe} labeled with fluorescent probes were bound to the A site or E site of *E. coli* 70S ribosomes in the presence of EF-Tu and GTP or GDP-NP, respectively, as described under Methods. Johnson & Cantor (1980) obtained optimal A-site binding at 7–8 mM $Mg(OAc)_2$. Experiments performed at 3–7 mM Mg^{2+} showed that binding of aminoacyl-tRNA was EF-Tu dependent (Shorey et al., 1971; Johnson & Cantor, 1980). At higher concentrations of Mg^{2+} (8–20 mM), Phe-tRNA can bind to the ribosome in the absence of EF-Tu and GTP (Shorey et al., 1971). EF-Tu-dependent binding of Phe-tRNA^{Phe}, unmodified or labeled with fluorescent probes, was carried out as detailed under Methods except that the $Mg(OAc)_2$ concentration was varied from 0 to 10 mM. The results obtained were similar to those reported by Johnson & Cantor (1980). Binding of Phe-tRNA^{Phe} species was performed for energy transfer experiments at 6 mM Mg^{2+}, a magnesium concentration which allows minimal nonenzymatic binding and near-maximal A- and E-site binding, according to the results of Johnson & Cantor (1980). In order to allow direct comparison of A-site and E-site measurements, the same magnesium concentration was used for both sets of binding conditions.

Preparation of samples for energy transfer experiments involved an initial incubation of 70S ribosomes with unmodified deacylated tRNA^{Phe} as detailed under Methods. The tRNA^{Phe} was nonenzymatically bound to the 70S ribosomes at 12 mM Mg^{2+} to ensure efficient P-site binding (Seeds et al., 1967). The Mg^{2+} concentration was reduced to 6 mM by doubling the reaction mixture volume with a solution containing the other salt components in their initial concentrations. Measurement of the ability of [^{14}C]Phe-tRNA$^{Phe}_{XLR}$ to bind to the resulting deacylated tRNA·ribosome complexes in the presence of EF-Tu and GTP as determined by Millipore filter binding is presented in Table II for different amounts of deacylated tRNA^{Phe} used in the initial preincubation. For each case, nonenzymatic binding of [^{14}C]Phe-tRNA$^{Phe}_{XLR}$ to the puromycin-reactive P site was assayed by its puromycin reactivity as described under Methods. The results presented in Table II show that the amount of puromycin-reactive Phe-tRNA binding can be reduced by the use of higher concentrations of deacylated tRNA^{Phe} to block the P site. When EF-Tu-dependent binding of Phe-tRNA$^{Phe}_{XLR}$ in the presence of GTP was performed as described under Methods but with no deacylated tRNA^{Phe} present in the preincubation step, it was found that a total of 0.63 pmol of Phe-tRNA$^{Phe}_{XLR}$ was bound per pmol of 70S ribosomes, as determined by the radioactivity of the Phe-tRNA·ribosome complex bound to Millipore filters (see Table II). Of this, 0.27 pmol of Phe-

Table III: Measurements from A-Site- or E-Site-Bound Phe-tRNA^{Phe} to the 3′ End of 16S RNA

Phe-tRNA	nucleotide used[a]	label on 16S RNA	Q[b]	R_0 (Å)[c]	E (%)[d]	r' (Å)[e]	r_{max} (Å)[f]	r_{min} (Å)[f]
Phe-tRNA$^{Phe}_{XLR}$	GTP	eosin	0.65	47.8	<10	>69		>60
		fluorescein	0.66	50.8	<10	>73		>63
	GDP-NP	eosin	0.65	47.7	<10	>69		>60
yeast Phe-tRNA$^{Phe}_{Pf}$	GTP	eosin	0.43	46.0	<10	>61		>56
	GDP-NP	eosin	0.43	46.0	<10	>61		>56
Phe-tRNA$^{Phe}_{X-F}$	GTP	eosin	0.63	60.4	15	81	88	77
	GDP-NP	eosin	0.60	60.0	<10	>86		>82

[a] Binding of Phe-tRNA^{Phe} to 70S ribosomes was performed in the presence of EF-Tu and GTP (A-site binding) or GDP-NP (E-site binding) as described under Methods. [b] Quantum yields, Q, for the energy donors were calculated as described under Methods. [c] R_0 is the distance in angstroms for 50% efficiency of energy transfer, assuming $\kappa^2 = 2/3$. [d] E is the percent quenching. [e] r' is the distance between the donor-acceptor pair calculated by assuming $\kappa^2 = 2/3$. [f] r is the actual distance between the donor and acceptor. The limits for r are calculated from the data of Table I.

tRNA$^{Phe}_{XLR}$ was puromycin reactive. When the amount of deacylated tRNA^{Phe} used to block the P site was increased to the level described under Methods for A- or E-site experiments, the total amount of Phe-tRNA$^{Phe}_{XLR}$ bound decreased to 0.49 pmol bound per pmol of 70S ribosomes while the puromycin reactivity decreased to approximately zero (0.02 pmol or less of Phe-tRNA$^{Phe}_{XLR}$ bound per pmol of 70S ribosomes).

EF-Tu dependence of Phe-tRNA^{Phe} binding, puromycin reactivity, and binding of the Phe-tRNA·ribosome complex to Millipore filters were performed in the presence of GDP-NP (E-site binding) as for A-site binding with comparable results (data not shown).

Distances from Phe-tRNA to the 3′ End of 16S RNA in Phe-tRNA·Ribosome Complexes. Phe-tRNA$^{Phe}_{XLR}$, Phe-tRNA$^{Phe}_{X-F}$, and Phe-tRNA$^{Phe}_{Pf}$ were prepared and bound to 70S ribosomes in the presence of EF-Tu, GTP (A-site binding), or GDP-NP (E-site binding), with deacylated tRNA^{Phe} in the P site. The 70S ribosomes were composed of native 50S subunits and 30S subunits which had been reconstituted with TP30 and unmodified 16S RNA or 16S RNA which had been oxidized and labeled with FTSC or ETSC at the 3′ end. The reconstituted 30S subunits with or without fluorescent labels were preincubated with poly(U), native 50S subunits, and unmodified deacylated tRNA^{Phe} to block the ribosomal P site. Phe-tRNA^{Phe} with the appropriate fluorescent probe was added with EF-Tu and either GTP or the nonhydrolyzable derivative GDP-NP. Ribosomal complexes were isolated by ultracentrifugation, resuspended in a binding buffer, and examined with fluorescence lifetime measurements. Each experiment consisted of two samples. One sample contained labeled Phe-tRNA^{Phe} bound to 70S ribosomes containing 30S subunits reconstituted with unlabeled 16S RNA. The second sample contained the same Phe-tRNA^{Phe} species bound to 70S ribosomes formed with 30S subunits which had been reconstituted with labeled 16S RNA.

The results, given in Table III, indicate that energy transfer was below a detectable level in measurements involving the cross-link or the anticodon loop of Phe-tRNA; these portions of the tRNA molecule, therefore, are greater than 73 Å and greater than 61 Å, respectively, from the 3′ end of the 16S RNA, when the Phe-tRNA is bound to either the A site or the E site of 70S ribosomes. Energy transfer from FITC on the X base of A-site-bound *E. coli* Phe-tRNA^{Phe} to ETSC on the 3′ end of 16S RNA was 15% efficient, corresponding to a donor–acceptor separation of 81 Å, assuming $\kappa^2 = 2/3$. Less than 10% energy transfer was observed for the same donor–acceptor pair when E-site binding conditions were used. This corresponds to a distance of greater than 86 Å. These results indicate that the 3′ end of the 16S RNA is closer to the X base of A-site-bound Phe-tRNA than to the X base of E-site-bound Phe-tRNA. Thus, there is a measurable difference in the locations of the A and E binding sites of Phe-tRNA relative to the ribosome. This difference appears to be at least 5 Å or greater.

Discussion

The 3′ region of the 16S RNA has been hypothesized to be involved in peptide initiation (Steitz, 1980) as well as peptide elongation (Ofengand, 1980; Shine & Dalgarno, 1974), suggesting that the 3′ end of the 16S RNA might be located near the messenger RNA decoding region of the 30S subunit and therefore near the anticodon loop of the P-site-bound tRNA. Although base 1400 of the 16S RNA is adjacent to the anticodon loop (Prince et al., 1982), it was found that the 3′ end of the 16S RNA (base 1541) is closer to the center of the P-site-bound tRNA (Robbins et al., 1981). It was determined that the distance between the cross-link moiety of P-site-bound Ac-Phe-tRNA$^{Phe}_{XLR}$ was approximately 57 Å from the 3′ end of the 16S RNA (Robbins et al., 1981) whereas the same cross-link bound to the A or E site is greater than 69 Å from the 3′ end of 16S RNA. The X base of *E. coli* tRNA^{Phe} was found to be 81 Å from the 3′ end of 16S RNA when measured from the A site, but no energy transfer was observed of FITC on the X base of E-site-bound Phe-tRNA. These results indicate that the tRNA molecule bound to the A site must be farther from the 3′ end of the 16S RNA than the P-site-bound tRNA, while E-site-bound tRNA is too far from the same point for energy transfer to be observed. This provides experimental evidence that the E site is a physically separate site from the A site.

Ofengand et al. (1981) have proposed four possible arrangements of the Phe-tRNA·tRNA·ribosome complex. In the most likely model (A), the anticodon of the P-site-bound tRNA is bound within the cleft of the 30S subunit in agreement with its position as determined by affinity immunoelectron microscopy (Keren-Zur et al., 1979). If it is assumed that tRNA has an L-shaped structure, the middle and aminoacyl acceptor stems are positioned around the head of the 30S subunit such that the plane of the tRNA is approximately perpendicular to the long axis of the small subunit and the acceptor stem is pointing away from the platform (Robbins et al., 1981). It has been determined that the X base of deacylated *E. coli* tRNA^{Phe} nonenzymatically bound to the P site is approximately 82 Å from the 3′ end of the 16S RNA and that the dhU bases of deacylated yeast tRNA^{Phe} bound to the P site are approximately 79 Å from the same point (Robbins, 1983). If the 3′ end of the 16S RNA is above the middle of the P-site-bound tRNA, approximately equidistant from the X base and dhU bases, and the A site bound X base is approximately 81 Å from the 16S RNA, this places the

A-site-bound tRNA close beside the P-site-bound tRNA, as shown in model A or B of Ofengand et al. (1981). It can be concluded that the same X base of E-site-bound Phe-tRNA is more distant, greater than 86 Å from the 3′ end of the 16S RNA, a minimum change of about 5 Å from the E site to the A site.

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Registry No. Proflavin, 92-62-6; FITC, 27072-45-3; ETSC, 87434-80-8.

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