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Position of Transfer Ribonucleic Acid on *Escherichia coli* Ribosomes. Distance from the 3′ End of 16S Ribonucleic Acid to Three Points on Phenylalanine-Accepting Transfer Ribonucleic Acid in the Donor Site of 70S Ribosomes†

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ABSTRACT: *Escherichia coli* 16S RNA from 30S ribosomal subunits was isolated, oxidized at the 3′ end, and labeled with the thiosemicarbazide derivatives of fluorescein or eosin. Labeled 16S RNA was reconstituted into 30S subunits. They were almost fully active compared to 30S subunits reconstituted from unlabeled 16S RNA by using a poly(uridylic acid)-directed polyphenylalanine synthesis assay. Fluorophores were placed at three different positions of $tRNA^{Phe}$. *E. coli* and yeast $tRNA^{Phe}$ were oxidized at the 3′ end and labeled with the thiosemicarbazide derivative of fluorescein or with the hydrazide of *N*-methylanthranilic acid. The Y base in the anticodon loop of yeast $tRNA^{Phe}$ was replaced by proflavin or 1-aminoanthracene. Also, *E. coli* $tRNA^{Phe}$ was photochemically cross-linked between 4-thiouridine at position 8 and cytidine at position 13. After reduction, this site was used as a fluorescent probe. The labeled tRNAs were bound into the peptidyl site of 70S ribosomes, and then the distances from the fluorophore in the modified tRNA to the fluorophore at the 3′ end of 16S RNA were measured by nonradiative energy transfer. Calculations were based on measurements of fluorescence lifetimes. The distances to the 3′ end of 16S RNA were found to be as follows: 3′ end of tRNA, 67–74 Å; cross-linked tRNA, 53–60 Å; anticodon loop of tRNA, >61 Å.

Very little is known about the relative location of tRNA[1] bound to the peptidyl site of 70S ribosomes and the 3′ end of 16S RNA in the small ribosomal subunit. The 3′ end of 16S RNA has been found to be particularly sensitive to nucleases, chemical modification, and antibodies (Zimmermann, 1980) and has been implicated in the process of subunit association. When in 30S subunits, 16S RNA was found to react at 22 sites with the guanine-specific reagent kethoxal, most of them in the 3′ half of the molecule (Noller, 1974). Reactive sites in the 5′-end 600 nucleotides are equally reactive in either 30S subunits or 70S ribosomes (Chapman & Noller, 1977), while the middle and 3′ regions were protected in 70S ribosomes, leading to the conclusion that these portions of the 16S RNA are near the 30S–50S interface.

The 3′ region of 16S RNA also has been implicated in the recognition of a complementary nucleotide sequence in messenger RNA (Shine & Dalgarno, 1974; Steitz & Jakes, 1975;

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[1] Abbreviations used: RNA, ribonucleic acid; tRNA, transfer RNA; $tRNA^{Phe}$, phenylalanine-accepting tRNA; Ac-Phe-tRNA, *N*-acetyl-Phe-tRNA; $tRNA^{Phe}_{XL}$, $tRNA^{Phe}$ that has been photochemically cross-linked from 4-thiouridine in position 8 to cytidine in position 13; $tRNA^{Phe}_{XLR}$, $tRNA^{Phe}_{XL}$ where cross-links have been reduced with $NaBH_4$; Srd_8, 4-thiouridine at position 8 in *E. coli* $tRNA^{Phe}$; Cyd_{13}, cytidine at position 13 in *E. coli* $tRNA^{Phe}$; $tRNA^{Phe}_{Pf}$, $tRNA^{Phe}$ in which the Y base has been replaced by proflavin; DNP, dinitrophenyl moiety; FTSC, fluorescein thiosemicarbazide; ETSC, eosin thiosemicarbazide; NMA, *N*-methylanthranilic moiety; DEAE, diethylaminoethyl; BD-cellulose, benzoylated DEAE-cellulose; poly(U), poly(uridylic acid); Me_2SO, dimethyl sulfoxide; EF-G, elongation factor G; EFTu, elongation factor Tu; Tris, tris(hydroxymethyl)aminomethane; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; CP, creatine phosphate; CPK, creatine phosphokinase; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate.

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Dunn et al., 1978) and thus may function in positioning mRNA in the ribosome. Further evidence for the involvement of the 3′ end of 16S RNA in initiation includes its ability to be cross-linked to initiation factors and ribosomal proteins known to be involved in the initiation process (Brimacombe et al., 1978) and the ability of oligonucleotides complementary to the 3′ end of 16S RNA to inhibit the formation of initiation complexes (Taniguchi & Weissmann, 1978; Eckhardt & Lührmann, 1979).

The approximate position of the 3′ end of 16S RNA in 30S subunits has been deduced from studies using immunoelectron microscopy (Olson & Glitz, 1979; Shatsky et al., 1979; Lührmann et al., 1980). Antibodies were raised against an antigenic group covalently linked to the 3′ end of the 16S RNA. The antibody attachment sites were visualized at the tip of the inner surface of the large lobe of the 30S subunit (Lührmann et al., 1980), in the groove between the side "ledge" and "head" of the subunit (Shatsky et al., 1979), or on the upper portion of the subunit platform (Olson & Glitz, 1979). Despite the different descriptive terms used, the location determined by all three groups is essentially the same and is near the tip of the large projection of the 30S ribosomal subunit.

An estimate of the location of the anticodon of P site bound tRNA has been obtained by combining affinity labeling with the technique of immunoelectron microscopy. Ofengand and co-workers (Ofengand et al., 1979; Zimmermann et al., 1979; Ofengand, 1980) photochemically cross-linked the 5′-anticodon base of several *Escherichia coli* tRNAs including $tRNA_1^{Val}$ to an as yet unidentified base in the 3′ third of the 16S RNA, in a P site specific reaction. In order to visualize this attachment site in the electron microscope (Keren-Zur et al., 1979), Val-$tRNA_1^{Val}$ was labeled with DNP at the α-amino position of the valine before P site binding and subsequent cross-linking. Although correlation of the sites of anti-DNP antibody attachment with the site of cross-linking was complicated by the fact that the antigenic site was some 80 Å from the cross-linking position, extrapolating this distance from the several sites detected led to a common intersection region which appeared to be located deep in the cleft between the large projection and the body of the 30S subunit. Thus the anticodon of tRNA appears to bind in the region of the 3′ end of 16S RNA.

In this paper, we use the technique of nonradiative energy transfer to study the distances of fluorophores attached to the 3′ end of oxidized 16S RNA to fluorophores attached to the 3′ end of *E. coli* $tRNA^{Phe}$ and yeast $tRNA^{Phe}$, fluorophores replacing the Y base (position 37) of yeast $tRNA^{Phe}$, or photochemically induced fluorescent cross-linked $tRNA^{Phe}$ of *E. coli*, placed in the P site of *E. coli* 70S ribosomes.

Experimental Procedures

Materials

E. coli K12, strain A19, fluorescein 5-thiosemicarbazide (FTSC), and eosin thiosemicarbazide (ETSC) were obtained as described (Odom et al., 1980). *N*-Methylisatoic anhydride (Aldrich Chemical Co., Milwaukee, WI) was reacted with hydrazine to yield NMA hydrazide. Proflavin hemisulfate was from Sigma Chemical Co. (St. Louis, MO), and 1-aminoanthracene was obtained from ICN-K&K Laboratories (Plainview, NY). DNP-hydrazine was from ICN Pharmaceuticals (Cleveland, OH). Unfractionated brewer's yeast tRNA, from which $tRNA^{Phe}$ was fractionated according to the procedure of Wimmer et al. (1968), was purchased from Boehringer, Mannheim (West Germany).

Solutions. Solution A was 0.4 M NaCl, 50 mM NaOAc (pH 5.0), and 10 mM $Mg(OAc)_2$; solution B was 1 M NaCl, 50 mM NaOAc (pH 5.0), and 10 mM $Mg(OAc)_2$; solution C was 20 mM Tris-HCl (pH 7.6), 10 mM $Mg(OAc)_2$, 160 mM NH_4Cl, and 4 mM β-mercaptoethanol. All solutions were prepared with glass-distilled deionized water. Contact with plasticware was strictly avoided to minimize contamination by fluorescent materials. Detergent-washed glassware was washed with ethanol before use for the same reason.

Methods

Preparation of Ribosomal Subunits and Components Derived from Them. The growth of *E. coli*, subsequent isolation of ribosomal subunits, 16S RNA, and ribosomal proteins from 30S subunits, periodate oxidation, and labeling of isolated 16S RNA with fluorescein or eosin, followed by reconstitution into 30S subunits, were carried out as described previously (Odom et al., 1980).

Preparation of Fractions from the E. coli Postribosomal Supernatant. E. coli extract from 300 g of cells was clarified by two low-speed centrifugations, followed by centrifugation for 4 h at 35 000 rpm in a Ti-45 rotor (Spinco, Beckman Instruments, Palo Alto, CA). The details of this procedure have been described previously (Odom et al., 1980). The upper $^2/_3$ of the resulting supernatant, called S-150, was used for the isolation of tRNA or aminoacyl-tRNA synthetases.

For tRNA, the S-150 fraction was extracted twice with 90% phenol, and then KCl was added to the combined aqueous layers to give a final concentration of 0.1 M. RNA was precipitated by the addition of 2 volumes of ethanol. After 4 h at −20 °C the precipitate was collected by centrifugation. The resulting RNA pellet containing tRNA was resuspended in 20 mM Tris-HCl (pH 7.5) to a total volume of 30 mL.

Aminoacyl-tRNA synthetases were partially purified from the S-150 fraction by using a modification of the method reported by Tanaka et al. (1976). A volume of 100 mL of the S-150 fraction was adjusted to pH 6.5 with 1 M acetic acid. Solid ammonium sulfate was added to give 70% saturation (40.8 g/100 mL). The resulting precipitate was collected by centrifugation, resuspended in 20 mM Tris-HCl (pH 7.5), 5 mM β-mercaptoethanol, 1 mM dithioerythritol, and 200 mM KCl, dialyzed against the same solution for ~24 h, and applied to a 2.0 × 34 cm column containing DEAE-cellulose (DE-52, Whatman, Maidstone, England) equilibrated in the same solution. Aminoacyl-tRNA synthetases are not retained on the column under these conditions. Fractions containing the aminoacyl-tRNA synthetases were pooled and concentrated by ultrafiltration using an Amicon system equipped with a PM-10 membrane (Amicon Corp., Lexington, MA), dialyzed against the same solution but with only 50 mM KCl, and stored in small aliquots at −80 °C.

Isolation of E. coli $tRNA^{Phe}$. The procedure used was developed independently but is similar to that described by Roy et al. (1971). *E. coli* tRNA prepared as described above was first acylated in a reaction mixture containing, in a final volume of 150 mL, 40 mM Tris-HCl (pH 7.5), 20 mM β-mercaptoethanol, 10 mM $MgCl_2$, 12 mM KCl, 2.67 mM ATP, 8×10^{-5} M [^{14}C]Phe (1 Ci/mol), and 0.75 mL of the aminoacyl-tRNA synthetases fraction described above. Then, the tRNA was extracted with phenol and fractionated by chromatography on BD-cellulose equilibrated with solution A. All tRNAs except $tRNA^{Ser}$, $tRNA^{Tyr}$, $tRNA^{Trp}$, and Phe-$tRNA^{Phe}$ were desorbed with solution B. These four tRNAs were eluted with solution B containing 15% ethanol, concentrated by ethanol precipitation, resuspended in 50 mM Tris-HCl (pH 8.9), and incubated for 60 min at 37 °C to deacylate Phe-

$tRNA^{Phe}$. After dialysis against solution A, the sample was loaded on a BD-cellulose column equilibrated in solution A. Solution B was used to elute $tRNA^{Phe}$ from this column. The other tRNAs, $tRNA^{Ser}$, $tRNA^{Tyr}$, and $tRNA^{Trp}$, were eluted subsequently with solution B plus 15% ethanol. Deacylated $tRNA^{Phe}$ was precipitated with ethanol, resuspended in distilled H_2O, and stored at −80 °C.

Preparation of $tRNA^{Phe}_{XLR}$, Phe-$tRNA^{Phe}_{XLR}$, and Ac-Phe-$tRNA^{Phe}_{XLR}$. Photochemical cross-linking of the Srd_8 and Cyd_{13} of *E. coli* $tRNA^{Phe}$ (Plenum Scientific Research, Hackensack, NJ, sp act. 1260 pmol of Phe/A_{260} unit) was induced by irradiation of the $tRNA^{Phe}$ at 335 nm, followed by reduction with $NaBH_4$, to produce fluorescent $tRNA^{Phe}_{XLR}$ (Favre & Yaniv, 1971). The procedure was a modification of that previously described (Ofengand et al., 1974). A solution of $tRNA^{Phe}$ at 21 A_{260} units/mL in 20 mM Bicine, pH 7.5, and 10 mM $Mg(OAc)_2$ was irradiated with stirring for 30 min at 0 °C in a Rayonet reactor with 350-nm lamps plus a plastic filter (Dupont Mylar S, 92 gauge) and a solution filter of 1 cm of 1.2 M lead nitrate (Ofengand et al., 1979). Portions of 7 mL in a single 16 × 150 mm Pyrex tube were used. Under these conditions, first-order kinetics were obtained with a $\tau_{1/2}$ of 3.4 min. No decrease in cross-linking was found when irradiation was continued to 40 min. Reduction of the $tRNA^{Phe}_{XL}$ formed was performed at a tRNA concentration of 2 A_{260} units/mL, in 100 mM borate–KOH, pH 9.5, 18 mM ethylenediaminetetraacetic acid, and 2 mg/mL $NaBH_4$ for 40 min at 23 °C in the dark. Aliquots incubated for 60 and 80 min showed no further increase in fluorescence. The $tRNA^{Phe}_{XLR}$ was precipitated by adjustment of the solution to 2% KOAc, pH 5, and 72% EtOH and incubation at 0 °C for 20 min. The sample was finally passed over Sephadex G-50 in water to remove residual salts. Overall yield of $tRNA^{Phe}_{XLR}$ was 83%.

Phe-$tRNA^{Phe}_{XLR}$ was aminoacylated by using high Mg^{2+}–Me_2SO conditions, since cross-linking, or any other modification of Srd_8 in *E. coli* $tRNA^{Phe}$, interferes with the acylation activity of *E. coli* synthetase (Ofengand & Liou, 1978). The conditions were 25 mM Tris, pH 8.5, 0.5 mM ATP, 40 mM Mg^{2+}, 20% Me_2SO, 1 mM dithiothreitol, 20 μM phenylalanine, and 2 A_{260} units/mL $tRNA^{Phe}_{XLR}$ and partially-purified synthetase at 30 °C. The time and synthetase concentration were adjusted to give maximum acylation. Phe-tRNA was isolated by phenol extraction at pH 5, ethanol precipitation, and Sephadex G-50 gel filtration. The final specific activity was 1168 pmol/A_{260} unit.

Ac-Phe-$tRNA^{Phe}_{XLR}$ was prepared by acetylation of Phe-tRNA with acetic anhydride (Haenni & Chapeville, 1966) as described previously (Schwartz & Ofengand, 1978). The extent of acetylation as monitored by Cu^{2+}–Tris incubation (Schwartz & Ofengand, 1978) was 100%. The final specific activity was 1120 pmol/A_{260} unit.

Ac-Phe-$tRNA_{XLR}$ was as active as control Ac-Phe-tRNA for P site binding to tight couple ribosomes at 5 mM Mg^{2+}, under conditions of both limiting tRNA (82% of the added tRNA was bound) and tRNA excess (50% of the ribosomes were functional). Phe-$tRNA_{XLR}$ was also as active as control Phe-tRNA in EFTu-dependent A site binding at 5 mM Mg^{2+}, under both tRNA-limiting (82% bound) and tRNA-excess (63% active ribosomes) conditions.

Oxidation and Labeling of tRNA. One milliliter of solution containing 90 A_{260} units of yeast or *E. coli* $tRNA^{Phe}$, 0.1 M $NaIO_4$, and 50 mM NaOAc (pH 5.0) was incubated for 90 min at room temperature in the dark. After incubation, KCl was added to a final concentration of 0.2 M. The resulting KIO_4 precipitate was removed by centrifugation for 10 min at 10000*g*. The supernatant was dialyzed twice for 2 h each against 50 mM NaOAc (pH 5.0).

After dialysis, the solution was brought to 0.2 M NaOAc and either 0.1 M NMA hydrazide or 0.1 M FTSC in dimethylformamide was added to a final concentration of 10 mM. The reaction mixture was incubated for 4 h at 37 °C, then KCl was added to 0.1 M, and tRNA was precipitated with 2 volumes of ethanol at −20 °C. Ethanol precipitations were repeated until the supernatant remained clear of fluorescent material. Removal of dye that is not covalently linked to tRNA was more efficient when reprecipitation was carried out from 1 mL of a 1:1 (v:v) water/dimethylformamide mixture.

Oxidation and labeling of deacylated $tRNA^{Phe}_{XLR}$ with ETSC or with DNP-hydrazine were performed as described above. However, the periodate treatment reoxidizes the cross-link in addition to opening the *cis*-diol, rendering the tRNA nonfluorescent. Fluorescence could be restored by $NaBH_4$ reduction as described for the preparation of $tRNA^{Phe}_{XLR}$. After this second reduction reaction, the fluorescence from $tRNA^{Phe}_{XLR}$ was indistinguishable from that of the original preparation.

Replacement of the Y Base. Removal of the Y base of yeast $tRNA^{Phe}$ and subsequent purification of $tRNA^{Phe}_{Y}$ were achieved by using the method of Thiebe & Zachau (1968). The proflavin and 1-aminoanthracene derivatives were then prepared as before (Odom et al., 1978) by using the procedure of Wintermeyer & Zachau (1971).

Poly(U)-Dependent Polyphenylalanine Synthesis. This assay was carried out exactly as reported previously (Odom et al., 1980). Generally, 0.5 A_{260} unit of 30S subunits and 0.8 A_{260} unit of 50S subunits were used with [^{14}C]Phe (5 Ci/mol) at 5 × 10^{-5} M.

Binding of $tRNA^{Phe}$ to 70S Ribosomes. Binding of $tRNA^{Phe}$, labeled in different positions, and of Ac-Phe-$tRNA^{Phe}$ species used in the fluorescence experiments described below was carried out by a modified form of the procedure of Wurmbach & Nierhaus (1979). The standard P site binding assay contained, in a total volume of 0.36 mL, 0.15 A_{260} unit of labeled $tRNA^{Phe}$, 75 μg of poly(U), 10 mM $Mg(OAc)_2$, 40 mM Tris-HCl, pH 7.8, 0.16 M NH_4Cl, 4 mM β-mercaptoethanol, and ~12 A_{260} units of 70S ribosomes. It was found that a molar ratio of 50S/30S subunits of 1:1.4 produced the optimal amount of binding of Ac-[^{14}C]Phe-$tRNA^{Phe}$; thus 8 A_{260} units of 50S subunits and 5.6 A_{260} units of 30S subunits (labeled or unlabeled) were used. Samples were then incubated for 15 min at 37 °C.

In all cases, after incubation samples were centrifuged in a Ti-50 rotor at 50000 rpm for ~2 h, and the ribosomal pellet was taken up in 0.5 mL of solution C and immediately subjected to fluorescence measurements. Binding and puromycin reactivity of radioactively labeled tRNA species were measured in aliquots of the resuspended samples. Generally, 20 μL in duplicate was withdrawn and filtered through Millipore filters, type HA (Millipore Corp., Bedford, MA), and then the filters were washed with ~3 mL of solution C. The filters were dried, and their radioactivity was determined by scintillation counting in 10 mL of a solution containing 5 g of 2,5-diphenyloxazole/L of toluene.

Puromycin reactivity of the Ac-[^{14}C]Phe-tRNA bound to 70S ribosomes was measured by following the procedure described by Wurmbach & Nierhaus (1979). An aliquot of the resuspended ribosomes (usually 30 μL) in duplicate was incubated in the presence or absence of elongation factor G

(EF-G) for 10 min at 37 °C with GTP, CP, and CPK added under the conditions described (Wurmbach & Nierhaus, 1979). Then puromycin was added to give 1 mM final concentration, and the incubation was continued for 30 min on ice. Formation of Ac-Phe-puromycin was determined after extraction with ethyl acetate.

After all steady-state and lifetime data were taken, samples were frozen at −80 °C and assayed the following day by using a poly(U)-dependent polyphenylalanine synthesis assay as described previously (Odom et al., 1980). When samples were analyzed on sucrose gradients, they were used immediately without freezing.

Sucrose Gradient Analysis. Aliquots of 60 μL from samples to be used for fluorescence measurements were layered on a 10–30% (w/v) sucrose gradient in solution C and centrifuged in a Beckman SW60 rotor for 12 h at 20000 rpm. The gradients were fractionated with automatic scanning of the absorbance at 254 nm. Then the fluorescence of each fraction was determined. With $tRNA^{Phe}_{XLR}$ bound to 70S ribosomes, the resulting fractions were excited at 390 nm, and emission was monitored at 450 nm.

Measurement of Fluorescence Lifetimes. A Nd-YAG mode-locked laser (NG50, Quantel International, Sunnyvale, CA) was used as the excitation source for energy transfer determinations; detection, processing, and deconvolution of fluorescence signals were carried out as before (Odom et al., 1980). Excitation light was 353 nm, and emission was measured at 440 nm for NMA and $tRNA^{Phe}_{XLR}$ or 500 nm for fluorescein and proflavin by using the optics detailed previously (Odom et al., 1980) with the addition of the 440-nm band-pass filter (10-nm half-height band-pass filter with 50% transmission at 440 nm and 10^{-4}% transmission on either side of the peak, Ditric Optics, Inc., Marlboro, MA).

Deconvolution of the fluorescence decay from the observed signal was carried out by means of a least-squares program similar to that described by Knight & Selinger (1971). A one- or two-exponential function is convoluted with the impulse response curve in order to generate a model for the observed signal. The parameters of the trial function are then varied so as to minimize the standard deviation of the model from the observed signal.

The fits obtained with one-exponential functions for typical fluorescence decay curves are shown in Figure 1, which is from photographs of actual computer graphic plots from an experiment the results of which are presented in Table V. A fluorescence decay curve for Ac-Phe-$tRNA^{Phe}_{XLR}$ in the donor site and the single-exponential computer-generated curve fit to these data points are shown in Figure 1A. The corresponding decay profile of quenched fluorescence obtained in the presence of eosin on the 3′ end of the 16S RNA is shown in Figure 1B. The quenched and unquenched fluorescence lifetimes from these curves were 2.6 and 3.2 ns, corresponding to an energy transfer of 19%.

Calculation of the Distance between Probes. The distance, r, between probes was calculated relative to R_0, the distance at which energy transfer proceeds at half of its maximum rate, by

$$r = R_0(E^{-1} - 1)^{1/6} \quad (1)$$

Efficiency of Energy Transfer. E is the efficiency of energy transfer between the probes and was determined by measuring the fluorescence lifetime of the donor both in the presence and in the absence of the acceptor. This efficiency is given by

$$E = 1 - \tau/\tau_0 \quad (2)$$

where τ_0 is the fluorescence lifetime of the donor without the acceptor and τ is the lifetime in the presence of the acceptor.

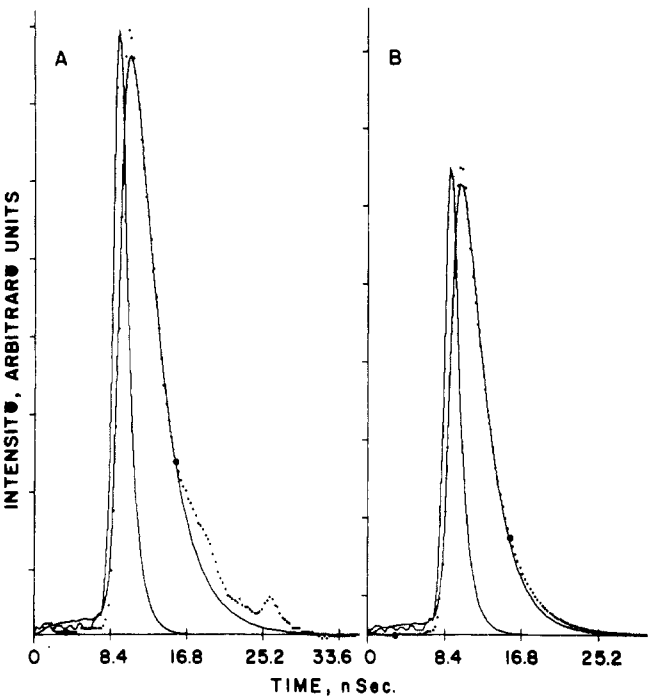

FIGURE 1: Observed and fitted curves for the decay of fluorescence from Ac-Phe-$tRNA^{Phe}_{XLR}$ in the donor ribosomal site. The figure is derived from photographs of computer graphic plots of observed and computer-fitted curves from which energy transfer was calculated for Table V. The narrow, leading pulse at ~8.4 ns in (A) and (B) is due to the pulse of excitation light and is used for the deconvolution of the fluorescence signal. (A) Unquenched fluorescence from Ac-Phe-$tRNA^{Phe}_{XLR}$ in the donor ribosomal site fitted with a single-exponential curve corresponding to a fluorescence lifetime of 3.2 ns. (B) The corresponding quenched decay curve with eosin on the 3′ end of 16S RNA as an energy acceptor. The curve is fitted with a one-exponential function corresponding to a fluorescent lifetime at 2.6 ns. (+) Data points; (—) fitted curve; (⊕) limits of data used for curve fitting.

R_0 and κ^2. R_0 was calculated by eq 3 (Förster, 1959). The

$$R_0 = (9.79 \times 10^3)(\Phi n^{-4} \kappa^2 J)^{1/6} \quad (3)$$

quantum yield of the energy donor, Φ, was determined as described below. The refractive index of the medium, n, was determined for samples by using an Abbe refractometer as detailed by Pasto & Johnson (1969).

J is the spectral overlap integral of the two fluorophores as given by Förster (1966)

$$J = \frac{\int_0^\infty F(\lambda)\epsilon(\lambda)\lambda^4 \, d\lambda}{\int_0^\infty F(\lambda) \, d\lambda} \quad (4)$$

where $F(\lambda)$ is the relative fluorescence intensity of the donor at wavelength λ and $\epsilon(\lambda)$ is the molar extinction coefficient of the acceptor at this wavelength. Absorption spectra were taken on a Cary 15 recording spectrophotometer.

κ^2 is the orientation factor for dipole–dipole transfer and may vary between 0 and 4, but it has a value of $^2/_3$ if the orientations of the donor and acceptor are random with respect to each other. In most situations the value of κ^2 cannot be determined. However, limits for κ^2 and therefore bounds on the donor–acceptor distance can be estimated from the depolarization of fluorescence from the donor and the acceptor. The method of Haas et al. (1978) was used to calculate the bounds on the donor–acceptor distances given in Tables IV–VI.

The fluorescence polarization data and half-height error limits are shown in Table IV. Fluorescence polarization was

determined as described previously (Odom et al., 1980) by evaluating

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (5)$$

where $I_{\parallel}$ is the intensity of fluorescence polarized parallel to the excitation light and $I_{\perp}$ is the intensity polarized perpendicular to the excitation (Förster, 1951). The polarization of the donor was measured at 353 nm, the excitation wavelength of the laser, while the acceptor was measured at a wavelength in the absorption band responsible for absorption of donor energy; for fluorescein-labeled samples this was 480 nm, and for eosin-labeled samples this was 490 nm. Steady-state spectra, including those for the determination of polarization of fluorophores, were taken on a steady-state photon-counting fluorometer described by Odom et al. (1978) or on an SLM PR8002 programmed to correct fluorescence spectra to allow for variations of photomultiplier tube response with wavelength.

When fluorescein is the donor, excitation at 353 nm does not excite the main absorption band; following Kasha's rule (Cantor & Tao, 1971; Rohatgi-Mukherjee, 1978), excited fluorescein will rapidly decay (nonradiatively) to the first singlet excited state, from which it will fluoresce. The resulting fluorescence is almost completely depolarized [see Scott et al. (1970)].

Quantum Yields. The quantum yields, Φ, of the fluorescent species listed in Table V were calculated with eq 6 (Berlman,

$$\tau_0 = \tau/\Phi \quad (6)$$

1971) from the theoretical natural fluorescence lifetime, τ_0, and the measured fluorescence lifetime, τ, determined under the experimental conditions with which energy transfer was measured. The natural lifetime is the inverse rate constant for fluorescence decay when radiation is the only process by which excitation energy may be lost. The natural lifetimes of fluorescein, fluorescent $tRNA^{Phe}_{XLR}$, and NMA were calculated to be 4.7, 5.7, and 23.7 ns, respectively, by using the formula (Strickler & Berg, 1962; Turro, 1965)

$$\tau_0 = 3.472 \times 10^{-4} \frac{\int_0^{\infty} I(\lambda)\lambda \, d\lambda}{n^2 \int_0^{\infty} \frac{\epsilon(\lambda)}{\lambda} d\lambda \int_0^{\infty} I(\lambda)\lambda^{-2} \, d\lambda} \quad (7)$$

where τ_0 is in units of nanoseconds, $I(\lambda)$ is the relative fluorescent intensity at wavelength λ, $\epsilon(\lambda)$ is the molar extinction coefficient at wavelength λ, and n is the refractive index of the solution.

A calculated τ_0 and measured τ have been used before for estimating the quantum yield of the naturally occurring fluorescent Y base of yeast $tRNA^{Phe}$ (Beardsley & Cantor, 1969); our calculated lifetime for fluorescein agrees with that of Strickler & Berg (1962). The quantum yield of $tRNA^{Phe}_{XLR}$ bound to ribosomes in individual experiments also was calculated from the fluorescence lifetimes determined in those experiments. The average of these values was 0.523 as given in Table V. A τ_0 value of 5.7 ns determined as indicated above was used for these calculations. The quantum yield of $tRNA^{Phe}_{XLR}$ in solution C was determined to be 0.57 by comparison with quinine sulfate, taking the quantum yield of the latter to be 0.7 (Scott et al., 1970). We have also determined the fluorescence lifetime of $tRNA^{Phe}_{XLR}$ in solution C to be 3.7 ns, corresponding to a calculated quantum yield of 0.65. Favre has determined the quantum yield of $tRNA^{Val}_{XLR}$ to be 0.42 by comparison with quinine sulfate in 0.1 N H_2SO_4 by assuming the latter to be 0.7 (personal communication). We have determined the quantum yield of $tRNA^{Val}_{XLR}$ in solution C to be 0.51 by comparison with quinine sulfate. Fluorescence from both fluorescein and $tRNA^{Phe}_{XLR}$ is somewhat environmentally sensitive. Fluorescein fluorescence is particularly sensitive to pH in the range of pH 7.5 (Mercola et al., 1972). Calculated quantum yields have the potential advantage of representing the true quantum yield under the conditions of the individual experiment.

The alternative method for determination of quantum yield is to compare the amount of fluorescence arising from a known amount of sample when compared to that of a standard of known quantum yield. This comparison method is the most common and was the one used previously (Odom et al., 1980); both methods gave comparable results for the parent compounds of fluorescein and NMA under standard conditions (data not shown). The lifetime method was used here rather than the comparison method because the energy transfer experiments are performed with 353-nm excitation light. At this wavelength the absorbance of the energy donor probes is low relative to the background absorbance of the sample. Thus, direct measurement of absorbance is subject to considerable error.

Table I: Polyphenylalanine Synthesis with Ribosomes Having 30S Subunits Reconstituted with 3′-Labeled 16 S, before and after Measurement of Energy Transfer[a]

30S subunits used	[^{14}C]Phe incorporated (pmol/A_{260} unit of 70 S) before	after
not reconstituted	1073	935
reconstituted, unlabeled	840	747
fluorescein-labeled 16 S	933	1120
eosin-labeled 16 S	793	794

[a] 30S ribosomal subunits were reconstituted from a fraction containing the total protein extracted from 30S ribosomal subunits, TP30, and the indicated form of 16S RNA. The activity of the ribosomes for poly(U)-directed polyphenylalanine synthesis was determined before and after measurement of fluorescence, as described under Methods.

Results

Activity of Reconstituted Subunits. Reconstitution of 30S subunits with labeled or unlabeled 16S RNA was carried out on a preparative scale. The subunits were collected by centrifugation and stored at −80 °C until needed. As described previously (Odom et al., 1980), subunit activity was measured by using poly(U)-directed synthesis of polyphenylalanine shortly before binding of tRNA to ribosomes for energy transfer experiments and then again after all energy transfer measurements had been completed. The results, shown in Table I, demonstrate that attachment of fluorescein or eosin to the 3′ end does not destroy the activity of the 16S RNA. Small subunit particles reconstituted from labeled 16S RNA are active in peptide synthesis. The excitation light or other conditions of the procedure by which energy transfer is measured do not appreciably reduce the capacity of the subunits to support polyphenylalanine synthesis.

The effect of the experimental procedure on components of the system also was checked by measuring the amount of Phe-tRNA bound to the ribosomes before and after the fluorescence measurements were made. Representative results are shown in Table II. For this experiment, Ac-[^{14}C]Phe-$tRNA^{Phe}_{XLR}$ was nonenzymatically bound to reconstituted 30S subunits plus native 50S subunits by the procedure used to prepare samples for fluorescence measurements, as described

Table II: Binding of Ac-Phe-tRNA$^{Phe}_{XLR}$ to 70S Ribosomes before and after Energy Transfer Experiments[a]

30S subunits used	Ac-Phe-tRNA$^{Phe}_{XLR}$ bound (mol/mol of 70 S) before	after
not reconstituted	0.45	0.42
reconstituted, unlabeled	0.22	0.21
fluorescein-labeled 16 S	0.20	0.22
eosin-labeled 16 S	0.18	0.18

[a] 30S ribosomal subunits were reconstituted with the indicated form of 16S RNA as described previously (Odom et al., 1980). Samples for energy transfer experiments contained 5.6 A_{260} units of 30S subunits and 8 A_{260} units of 50S subunits (a 1.4:1 molar excess of 30 S to 50 S). Ac-Phe-tRNA$^{Phe}_{XLR}$ was bound to the ribosomes as described under Methods. Ac-[^{14}C]Phe-tRNA$^{Phe}_{XLR}$ bound to the ribosomes was determined in 50-μL aliquots removed from the sample before and after fluorescence measurements were made.

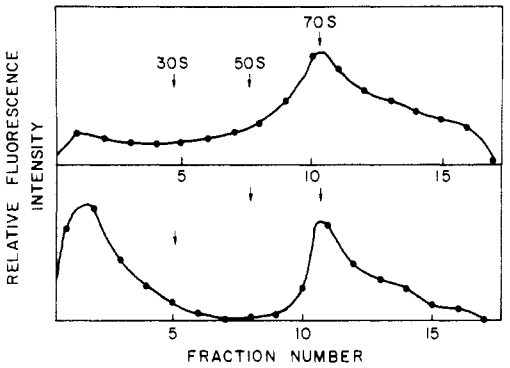

FIGURE 2: Sucrose gradient centrifugation of tRNA$^{Phe}_{XLR}$ bound to poly(U)-programmed ribosomes. Gradient analysis was carried out as described under Methods. (Top) Native 30S subunits. (Bottom) Reconstituted 30S subunits.

under Methods. Aliquots were removed from the samples before and after fluorescence measurements were carried out. The results show that 30S subunits reconstituted with unlabeled 16S RNA or 16S RNA labeled at its 3′ end with fluorescein or eosin have similar activity for tRNA binding. Also, they show that the tRNA is not lost from the ribosomes during the course of the experiment.

Typically, the molar ratio of the Phe-tRNA bound per reconstituted 30S ribosomal subunit is ~0.2. Thus, many of the subunits do not bind tRNA. A technical advantage is gained by having the energy donor on the tRNA. This arrangement provides a relatively high proportion of energy donor fluorophores paired with an energy acceptor. Thus the relatively low ratio of tRNA bound per ribosome does not compromise the results obtained. It should be noted that the calculation of energy transfer from a change in the fluorescence lifetime is independent of concentration, as briefly described below.

Samples similar to those used for measurement of fluorescence were analyzed routinely on sucrose gradients. Figure 2 shows the results from such an analysis of a sample containing deacylated tRNA$^{Phe}_{XLR}$ with reconstituted or unreconstituted 30S subunits plus native 50S subunits. Fluorescence from tRNA$^{Phe}_{XLR}$ is shown. The tRNA$^{Phe}_{XLR}$ is located within the 70S peak in both samples containing either reconstituted or unreconstituted 30S subunits. Poly(U) tends to broaden the 70S peak, presumably by addition of polymers of variable size and structure to the 70S particles.

A substantial amount of tRNA$^{Phe}_{XLR}$ is distributed through the top of the gradient of the sample containing reconstituted

Table III: Puromycin Reactivity of Ac-Phe-tRNA^{Phe} Bound to 70S Ribosomes[a]

probe location 16S RNA	tRNA	Ac-Phe-tRNA^{Phe} bound to ribosomes (pmol)	puromycin reactive (pmol) −EF-G	+EF-G
(A) unlabeled	Ac-Phe-tRNA$^{Phe}_{XLR}$	140	108	105
fluorescein	Ac-Phe-tRNA$^{Phe}_{XLR}$	83	68	66
eosin	Ac-Phe-tRNA$^{Phe}_{XLR}$	69	47	42
(B) unlabeled	Ac-Phe-tRNA$^{Phe}_{Pf}$	98	60	59
eosin	Ac-Phe-tRNA$^{Phe}_{Pf}$	73	50	45

[a] 30S ribosomal subunits were reconstituted either with unlabeled 16S RNA or 16S RNA labeled at its 3′ end with fluorescein or eosin and then reisolated by centrifugation. These reconstituted 30S subunits were incubated with 50S subunits in the amounts and under the conditions given under Methods with either 239 pmol of Ac-[^{14}C]Phe-tRNA$^{Phe}_{XLR}$ (49 Ci/mol) (A) or 204 pmol of Ac-[^{14}C]Phe-tRNA$^{Phe}_{Pf}$ (100 Ci/mol) (B). After the binding reaction, ribosomes were reisolated by centrifugation. Ac-Phe-tRNA binding was determined with one aliquot, while other aliquots were incubated in the presence or absence of EF-G, then puromycin was added, and the Ac-Phe-puromycin formed was determined as described under Methods.

30S subunits. It is likely that the tRNA$^{Phe}_{XLR}$ is not as strongly bound to the reconstituted 30S subunits, hinting at subtle differences in the interactions of the tRNA^{Phe} with native and reconstituted 30S subunits in the tRNA–70S complexes. In the experiments described below, reconstituted 30S subunits (either with unlabeled or labeled 16S RNA) were used.

P Site Binding. For interpretation of the experiments described below, it is important to know the proportion of the total bound tRNA that is in the ribosomal P site. Conditions were chosen which favor P site binding, e.g., an excess of ribosomes over tRNA and use of Ac-Phe-tRNA^{Phe} where possible. Deacylated tRNA has been shown to bind primarily into the ribosomal P site (Seeds et al., 1967). P site binding was determined directly by reaction of bound Ac-Phe-tRNA$^{Phe}_{XLR}$ with puromycin. Also, the procedure described by Wurmbach & Nierhaus (1979) was used to estimate the proportion of A site binding. In this assay system EF-G with GTP is used to translocate into the P site Ac-Phe-tRNA that may have been bound originally into the A site, thereby increasing the amount of *N*-acetyl-Phe-puromycin that might be formed in a subsequent reaction. The results are presented in Table III. These experiments were carried out with 30S subunits that were reconstituted with unlabeled, eosin-labeled, or fluorescein-labeled 16S RNA as indicated. About 60–80% of the total bound Ac-Phe-tRNA^{Phe} was recovered as the puromycin derivative. The ethyl acetate extraction procedure used does not give quantitative recovery (Leder & Bursztyn, 1966); thus, a high proportion of the Ac-Phe-tRNA must have reacted with puromycin. Furthermore, EF-G did not cause an increase in puromycin reactivity under any of the conditions tested. These data indicate that Ac-Phe-tRNA$^{Phe}_{XLR}$ is bound primarily into the P ribosomal site under the conditions used with little or no A site binding.

Distance from tRNA^{Phe} to the 3′ End of 16S RNA. Experiments in which energy transfer was measured were performed with the energy donating probe attached at one of three specific points on either yeast or *E. coli* tRNA^{Phe}. The energy-accepting probe was on the 3′ end of 16S RNA reconstituted into 30S ribosomal subunits. Native unlabeled 50S subunits were used to form 70S ribosomes. The tRNA was bound to the 70S ribosomes under conditions that give binding

Table IV: Polarization and Distance Limits from Fluorophore Orientation

probe location		polarization (P)[a]		half-height limits of distance,
$tRNA^{Phe}$	16S RNA	tRNA	16S RNA	$Q(r'/r)$[b]
(A) Ac-Phe-$tRNA^{Phe}_{XLR}$	eosin	0.22	0.41	0.88–1.14
	fluorescein	0.22	0.38	0.88–1.14
(B) 3′-labeled $tRNA^{Phe}$				
(1) *E. coli*				
fluorescein	eosin	0	0.41	0.93–1.03
NMA	fluorescein	0.17	0.38	0.88–1.14
(2) yeast				
fluorescein	eosin	0	0.41	0.93–1.03
(C) anticodon loop of yeast $tRNA^{Phe}$				
1-aminoanthracene	fluorescein	0.19	0.25	0.91–1.11
proflavin	eosin	0.30	0.41	0.91–1.11

[a] Polarization of fluorophores was measured as before (Odom et al., 1980). [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 distance between the donor–acceptor pair assuming $\kappa^2 = 2/3$, and r is the actual distance.

Table V: Distance from Three Sites on $tRNA^{Phe}$ to the 3′ End of 16S RNA

tRNA	16S RNA	Φ[a]	R_0[b] (Å)	E[c] (%)	r'[d] (Å)	r_{max}[e] (Å)	r_{min}[e] (Å)
(A) Ac-Phe-$tRNA^{Phe}_{XLR}$[f]	fluorescein	0.529	48.9	22	60	68	52
	eosin	0.529	46.1	27	54	62	47
	fluorescein	0.542	49.1	23	60	68	52
	eosin	0.529	46.1	22	57	64	50
	eosin	0.552	46.4	19	59	68	52
	eosin	0.470	45.2	23	55	63	49
	eosin	0.500	45.7	30	53	60	46
	av:	0.523			57	65	50
(B) 3′-labeled $tRNA^{Phe}$							
(1) *E. coli*							
fluorescein	eosin	0.763	61.0	27	72	74	67
NMA	fluorescein	0.089	31.0	no quenching seen			
(2) yeast							
fluorescein	eosin	0.860	64.0	30	74	76	69
(C) anticodon loop of yeast $tRNA^{Phe}$							
1-aminoanthracene	fluorescein	0.250	42.0	<15	>56		>51
proflavin	eosin	0.440	46.0	<15	>61		>56

[a] Quantum yields, Φ, for the energy donor were calculated as described under Methods. [b] R_0 is the distance in angstroms for 50% efficiency of energy transfer assuming $\kappa^2 = 2/3$. [c] E is the percent quenching. [d] r' is the distance between the donor–acceptor pair calculated by assuming $\kappa^2 = 2/3$. [e] r is the actual distance between the donor–acceptor pair. The limits for r are calculated from the data of Table IV. [f] In all cases the energy donor is located on tRNA and the acceptor is on 16S RNA.

into the P site as described above. Two otherwise identical samples were run for each experiment. One sample contained only the energy donor, and the other contained the donor–acceptor pair. These samples were used to measure the quenched and unquenched fluorescence lifetimes of the energy donor as described above. Experimentally identical samples were used to measure fluorescence polarization from the donor and acceptor probes. These results with limits for the probable distribution of the distances between the two probes estimated from the polarization data by the method of Haas et al. (1978) are presented in Table IV.

The results from a series of experiments in which energy transfer was measured between probes on the tRNA and the 3′ end of the 16S RNA are given in Table V. Numbers for individual experiments involving the cross-link in Ac-Phe-$tRNA^{Phe}_{XLR}$ to fluorescein or eosin on the 16S RNA are shown in part A of the table. The data given in parts B and C represent the average of at least two separate experiments. Quenching of the fluorescence donor was seen from the cross-linked tRNA and from fluorescein attached to the 3′ end of the $tRNA^{Phe}$. The distances from the cross-link and from the 3′ end of the *E. coli* $tRNA^{Phe}$ to the 3′ end of the 16S RNA were calculated to be about 57 and 72 Å, respectively. The latter distance was calculated to be 74 Å for yeast $tRNA^{Phe}$. The difference between the values for *E. coli* tRNA and yeast tRNA is within the error of the experimental procedure. A decrease in the fluorescence lifetime ranging from 0 to 9% was seen for energy transfer from proflavin in the anticodon loop to eosin on the 3′ end of 16S RNA in a series of five separate determinations. This level of apparent energy transfer is too low to be measured reliably; thus, a value of <15% energy transfer is given for this pair in Table V. Steady-state fluorescence measurements and energy transfer from 1-aminoanthracene in the anticodon loop to fluorescein at the 3′ end of 16S RNA were used to confirm the conclusion that these data do not nearly reflect total energy transfer. A value of <15% energy transfer for the proflavin to eosin pair corresponds to a distance of >61 Å. Polarization data for these probes indicate that this lower limit value should not be in error by more than ~6 Å due to the assumption of $^2/_3$ for κ^2.

Distance between the Cross-Link and the 3′ End of $tRNA^{Phe}$. Energy transfer was measured between the Srd_8–Cyd_{13} cross-link to either DNP or eosin attached to the 3′ end of $tRNA^{Phe}$ bound to ribosomes. For this experiment, a sample of $tRNA^{Phe}_{XLR}$ was oxidized with periodate. Portions of the sample were either reduced immediately or labeled with either DNP-hydrazide or ETSC and then reduced with sodium borohydride. The capacity of the cross-link to fluoresce is lost during the oxidation step but is regenerated during reduction. No differences could be detected between the spectral char-

Table VI: Intramolecular Energy Transfer from the Cross-Link to a Probe at the 3′ End of $tRNA^{Phe}$ in the Ribosomal P Site[a]

3′ acceptor	R_0 (Å)	% quenching	r (Å)	r_{max} (Å)	r_{min} (Å)
eosin	46.6	78	38	41	34
DNP	33.4	32	38		

[a] The terms, general experimental conditions, and calculations are as described for Tables IV and V. The average quantum yield for cross-link determined in this series of experiments was 0.529. Polarization of P site $tRNA^{Phe}_{XLR}$ was 0.22. The average polarization for 3′-eosin on P site $tRNA^{Phe}_{XLR}$ was 0.25. DNP is not fluorescent.

acteristics of cross-link fluorescence from $tRNA_{XLR}$ that had been oxidized and then reduced and unreacted tRNA (data not shown). The tRNA containing only the cross-link or the cross-link plus an energy acceptor at the 3′ end was bound to the ribosomal P site under the standard conditions described under Methods. The results of energy transfer experiments and the distance calculations are presented in Table VI. The average distance between the cross-link and eosin at the 3′ end was 38 Å with maximum and minimum values of 41 and 34 Å, respectively, as calculated from fluorescence polarization data by the method of Haas et al. (1978). A relatively high degree of quenching, 78%, was observed with the cross-link to eosin pair. Much lower quenching, 32%, was observed when DNP was used as an energy acceptor. The average distance calculated for the cross-link to DNP pair was 38 Å. Since DNP is not fluorescent, limits for the distance could not be calculated from fluorescence polarization data.

Discussion

As judged from the crystal structure of $tRNA^{Phe}$, probes at the Y base position in the anticodon loop, at the 3′ end, and at the Srd_8–Cyd_{13} cross-link give three well-separated reference points for determining the position of ribosomal-bound tRNA relative to specific sites in the ribosome. The anticodon and 3′-end probes define the two ends of the L-shaped structure while the cross-link probe is located near the center of the molecule. The data presented here indicate that the 3′ end of 16S RNA is relatively close to the cross-link near the middle of $tRNA^{Phe}$ when it is bound into the P ribosomal site. This distance is 53–60 Å, neglecting error due to the size of the fluorophores used and the side chains by which they are attached to the tRNA. The corresponding distances to the 3′ end of the tRNA and to the Y base position were 72–74 and more than 61 Å, respectively. Energy transfer was too small to be measured reliably with the proflavin to eosin pair used for the latter determination.

These results are somewhat surprising since the evidence for the involvement of the 3′-terminal region of the 16S RNA in peptide initiation (Steitz, 1980) and in peptide elongation (Ofengand et al., 1980) suggests that the 3′ terminus of the 16S RNA might be near the decoding region of the small ribosomal subunit. However, the energy transfer data indicate clearly that the 3′ rRNA terminus is actually located closer to the center of the tRNA than to the Y base position in the anticodon loop. A way to rationalize these results is suggested by the arrangement of the P site tRNA on the 30S subunit which has recently been proposed by Ofengand et al. (1981). In the most likely model (A) of the several that were proposed, the anticodon of the P site tRNA is bound deep in the cleft of the 30S subunit in agreement with its estimated position by affinity immunoelectron microscopy (Keren-Zur et al., 1979). The remainder of the L-shaped structure is arranged around the neck of the 30S subunit such that the plane of the tRNA is approximately perpendicular to the long axis of the subunit and the amino acid arm is pointing away from the large projection. The dimensions measured in this paper appear to be reasonably consistent with such an orientation, particularly since the Y base position would then be on the side of the anticodon loop *away* from the tip of the large projection.

A second surprising distance was that from the cross-link to the 3′ end of the tRNA. The measured value, 34–41 Å, is significantly less than the crystal structure distance of ~45 Å (Robertus et al., 1974). In the crystal structure the bases of the potentially flexible ACCA end of the amino acid arm are stacked and thus form an extension of the double-helical region of this arm. The data presented here suggest that the crystal structure conformation of this portion of the molecule is not retained when $tRNA^{Phe}$ is bound into the ribosomal P site. The most likely explanation is that the bases of the ACCA end are not stacked but are turned toward the cross-link near the center of the bound $tRNA^{Phe}$. This distance of 34–41 Å to the cross-link is consistent with both the 72–74-Å value to the 3′ end of 16S RNA and the 53–60-Å distance from the cross-link to the 3′ end, since in the orientation suggested above, these three points are arranged approximately as a right triangle.

Whether one model or another ultimately proves to be correct, the distance measurements provided in this paper remain as experimental facts that will need to be taken into account. Measurements from additional positions both in tRNA and in the ribosome are continuing. We believe that fluorescence energy transfer, as well as affinity labeling (Ofengand, 1980), will prove to be the most useful tools for determining the topography of the tRNA–ribosome complex.

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