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Studies on the Fluorescence of the Y Base of Yeast Phenylalanine Transfer Ribonucleic Acid. Effect of pH, Aminoacylation, and Interaction with Elongation Factor Tu†

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ABSTRACT: The fluorescence spectrum and peak emission intensity of the Y base in yeast $tRNA^{Phe}$ is unchanged upon aminoacylation of $tRNA^{Phe}$. In both $tRNA^{Phe}$ and phenylalanyl-$tRNA^{Phe}$ the Y base fluorescence intensity at the emission maximum depends on the presence of Mg^{2+} and varies with pH. These results indicate that aminoacylation does not lead to changes of the tertiary structure or environment at the anticodon region of $tRNA^{Phe}$ which affect the fluorescence properties of the Y base.

The rate of deacylation of phenylalanyl-$tRNA^{Phe}$ is significantly retarded when the molecule interacts with EF-Tu and GTP to form a ternary complex. On the other hand, the fluorescence properties of the Y base in the ternary complex are not significantly different from those in uncomplexed phenylalanyl-$tRNA^{Phe}$. These observations suggest that the EF-Tu may bind to the phenylalanyl-$tRNA^{Phe}$ at its 3′ terminus. This conclusion is consistent with the results of others on the structural requirements in phenylalanyl-$tRNA^{Phe}$ and other aminoacylated-tRNAs for EF-Tu and GTP recognition.

Yeast $tRNA^{Phe}$ contains a highly fluorescent modified guanine nucleotide, commonly referred to as the Y base, located adjacent to the 3′ end of the anticodon (RajBhandary *et al.*, 1967). Even before the structure of this intrinsic chromophore had been elucidated (Nakanishi *et al.*, 1970), a number of laboratories had made extensive use of its unusual optical properties to study the conformation of yeast $tRNA^{Phe}$. These studies suggested that the fluorescence of the Y base is a very sensitive indicator of the local conformation at the anticodon region of $tRNA^{Phe}$ and is strongly dependent on external conditions, especially Mg^{2+} ion concentration (Eisinger *et al.*, 1970; Beardsley *et al.*, 1970). Robison and Zimmerman (1971) demonstrated that the increase in the Mg^{2+}-induced fluorescence of yeast $tRNA^{Phe}$ is almost completely reversed at temperatures which do not alter the secondary structure, but influence the tertiary structure of the tRNA. Such studies led to the conclusion that the highly fluorescent, Mg^{2+} stabilized, conformation is the biologically active state.

Utilizing a variety of evidence, Levitt (1969) and Cramer *et al.* (1969) proposed tentative tertiary structures for $tRNA^{Phe}$ in which, within the constraints of the basic cloverleaf arrangement, the 3′ end of the molecule and the anticodon-containing loop are maximally separated. This aspect of the proposed model was confirmed by singlet–singlet energy transfer experiments which utilized the Y base as the energy donor and a number of acceptor chromophores attached to the 3′ end of $tRNA^{Phe}$ (Beardsley and Cantor, 1970). The most recent model of the tertiary structure of $tRNA^{Phe}$ which is based on X-ray crystallographic studies at 4-Å resolution (Kim *et al.*, 1973) also shows a large separation, 82 Å, between the anticodon and amino acid acceptor end of the molecule.

In this study our objective was to utilize the fluorescence of the Y base to follow the conformational state of the anticodon-containing region of $tRNA^{Phe}$ as the molecule proceeds through certain steps of the protein-synthesizing cycle. More specifically, we have investigated some of the fluorescence properties of the Y base in $tRNA^{Phe}$ after aminoacylation with phenylalanine and after ternary complex formation of Phe-$tRNA^{Phe}$ with the protein elongation factor Tu[1] and GTP (for a review of the function of EF-Tu, see Lucas-Lenard and Lipmann, 1971). Our results indicate that the fluorescence properties of the Y base are the same in $tRNA^{Phe}$ and Phe-$tRNA^{Phe}$. These results are discussed in terms of the pro-

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[1] Abbreviation used is: EF-Tu, elongation factor Tu.

posed tertiary structure of $tRNA^{Phe}$ and in terms of other reports concerning structural changes in tRNA upon aminoacylation. We also find that the fluorescence of the Y base in Phe-$tRNA^{Phe}$ is not significantly affected by the formation of the Phe-$tRNA^{Phe}$·EF-Tu·GTP complex, but that the rate of deacylation of the Phe-$tRNA^{Phe}$ within the complex is significantly retarded. These observations are discussed in relation to previous studies on the structural requirements in aminoacyl-tRNAs for EF-Tu·GTP binding.

Materials and Methods

Yeast $tRNA^{Phe}$ (lots 7192210 and 7252408) was purchased from Boehringer–Mannheim and was used without further purification. The phenylalanine acceptor activity according to our assays was approximately 1200 pmol of phenylalanine/A_{260} unit $tRNA^{Phe}$ which corresponds to 60% purity assuming 1 mg of $tRNA^{Phe}$ equals 21 A_{260} units. *E. coli* A19 cells and pyruvate kinase were obtained from General Biochemicals; phosphoenolpyruvate was obtained from Calbiochem. The pyruvate kinase was dialyzed against 10 mM sodium acetate buffer (pH 6.3) containing 0.1 M NH_4Cl and stored in liquid nitrogen. [^{14}C]Phenylalanine (492 Ci/mol), [^{3}H]phenylalanine (3.48 Ci/mmol), [^{3}H]GTP (12 Ci/mmol), and [^{3}H]GDP (7 Ci/mmol) were purchased from Amersham Searle. Phenylalanyl-tRNA synthetase from *E. coli* was partially purified by DEAE-cellulose chromatography of ammonium sulfate concentrated soluble fractions of the cell.

All radioactivity measurements were carried out using a Nuclear-Chicago Isocap/300 multichannel liquid scintillation counter. The efficiencies of counting ^{14}C and ^{3}H were 75 and 30%, respectively.

Preparation of Yeast Phe-$tRNA^{Phe}$. Yeast $tRNA^{Phe}$ was charged with [^{14}C]phenylalanine using crude *E. coli* Phe-tRNA synthetase after pilot experiments were carried out to determine the optimum conditions for complete charging (Conway, 1964). The complete reaction mixture (5 ml) was then loaded on a 1.5 × 18 cm benzoylated DEAE-cellulose column and eluted using a combined salt and ethanol gradient as described by Litt (1968). The column fractions containing [^{14}C]Phe-$tRNA^{Phe}$ were dialyzed against H_2O or 10 mM sodium acetate buffer (pH 5.6) containing 5 mM magnesium acetate and stored at either −20 or −76°. Less than 10% deacylation occurred during the course of these experiments as a result of storage, and the purity of the various samples as judged by trichloroacetic acid insoluble [^{14}C]phenylalanine/A_{260} unit ranged between 60 and 90%. All Phe-$tRNA^{Phe}$ fractions used for fluorescence measurements were at least 80% pure. The deacylation of [^{14}C]Phe-$tRNA^{Phe}$ under various conditions was followed by placing 10-μl samples at indicated times into 5% Cl_3CCOOH, filtering on Millipore, dissolving the wet filters in Bray's solution, and counting.

Purification of EF-Tu from E. coli. Elongation factor Tu was isolated and purified as the EF-Tu·GDP complex essentially according to the procedure of Miller and Weissbach (1970), except that the final ammonium sulfate extraction step was omitted. Protein determinations were carried out as described by Lowry *et al.* (1951), and based on the GDP binding assay the purity of the various fractions was 40–60%. EF-Tu·GDP was stored in liquid nitrogen in 10 mM Tris-HCl buffer (pH 7.8) containing 10 mM magnesium acetate, 1 mM dithiothreitol, and 10^{-5} M GDP. Under these conditions no detectable loss of GDP binding activity occurred as a result of storage over a 4-month period.

Fluorescence Measurements. All fluorescence measurements were carried out at room temperature using a Perkin-Elmer Model 204 fluorescence spectrophotometer standardized with quinine trihydrate in 0.1 N H_2SO_4. The fluorescence intensity at 440 nm of $tRNA^{Phe}$ in 10 mM Tris-HCl (pH 7.0) containing 5 mM magnesium acetate relative to this standard agreed with the values published by Robison and Zimmerman (1971) under nearly identical conditions. All of the spectra were corrected for background fluorescence using appropriate blanks. The fluorescence spectrum of Phe-$tRNA^{Phe}$ in the presence of EF-Tu and GTP was measured under conditions in which essentially all of the tRNA is involved in ternary complex formation. These conditions were determined by carrying out a number of small-scale experiments in which ternary complex formation is assayed for by the reduction in the amount of EF-Tu·[^{3}H]GTP bound to nitrocellulose filters (Weissbach *et al.*, 1971). The complete reaction mixture used for fluorescence measurements of the ternary complex contained the following in a volume of 3.0 ml: 945 pmol of EF-Tu·GDP, 733 pmol of [^{14}C]Phe-$tRNA^{Phe}$, 0.3 mg of pyruvate kinase, 7.5 μmol of phosphoenolpyruvate, 120 nmol of GTP, 3 μmol of dithiothreitol, 90 μmol of Tris-HCl (pH 7.4), 30 μmol of magnesium acetate, and 60 μmol of NH_4Cl. The blank solution used to correct the fluorescence spectrum contained all of the above components except [^{14}C]Phe-$tRNA^{Phe}$.

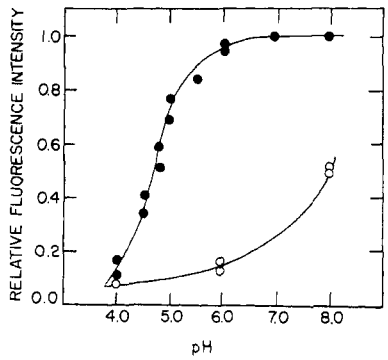

FIGURE 1: Effect of pH on the fluorescence of the Y base of $tRNA^{Phe}$. The solutions were buffered with either 10 mM sodium acetate or 10 mM Tris-HCl in the presence (●) or absence (○) of 5 mM magnesium acetate. The fluorescence at 430 nm is shown normalized to the fluorescence at pH 8.0 in the presence of 5 mM Mg^{2+}. The excitation wavelength was 318 nm, and the final $tRNA^{Phe}$ concentration was 4.5×10^{-7} M.

Results

pH Dependence of $tRNA^{Phe}$ Fluorescence. During the course of our experiments we noticed that the Mg^{2+} enhanced fluorescence of $tRNA^{Phe}$ is strongly dependent on pH. A typical fluorescence titration curve is shown in Figure 1. This dependence was completely reversible in the pH range studied. It was not accompanied by any measurable hypo- or hyperchromicity at 260 nm, suggesting that the secondary structure of $tRNA^{Phe}$ is not perturbed appreciably by the indicated changes in pH. The apparent pK_{diss} of the ionizing group(s) responsible for this change in Y base fluorescence may be estimated to be 4.7 ± 0.2. The identity of the ionizing groups is not known, but the pH dependence of the fluorescence may be due to the protonation of the adenosine and methylated cytidine residues at or near the anticodon loop. It is not clear if the effect of pH on the fluorescence reflects changes in the binding of Mg^{2+} to the molecule. The Mg^{2+} dependent fluorescence enhancement at pH 7.2 is nearly fourfold (Figure 1),

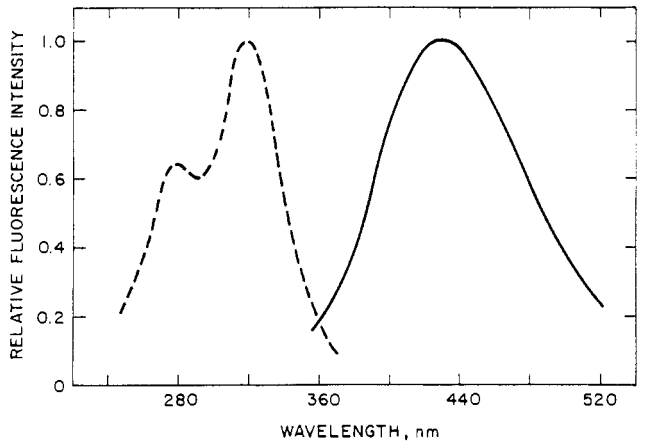

FIGURE 2: The fluorescence excitation and emission spectrum of [^{14}C]Phe-tRNA^{Phe}. The buffer consisted of 10 mM sodium acetate (pH 5.6) and 5 mM magnesium acetate. The excitation spectrum (- - -) was determined by measuring emission at 430 nm, and the emission spectrum (—) was determined by excitation at 318 nm. The Phe-tRNA^{Phe} concentration was 6.2×10^{-7} M, and the fluorescence intensity shown has been normalized to the fluorescence at 430 nm.

which is in good agreement with the results reported by Robison and Zimmerman (1971) at this pH.

Fluorescence Spectra of Phe-tRNA^{Phe}. The fluorescence emission and excitation spectrum of Phe-tRNA^{Phe} at pH 5.6 is shown in Figure 2. The overall shape of the spectra, as well as the exact position of the excitation and emission maximum, 318 and 430 nm, respectively, is identical with the spectra for uncharged tRNA^{Phe} under the same conditions (data not shown). Furthermore, it corresponds nearly exactly with the spectra for tRNA^{Phe} reported by Beardsley *et al.* (1970) and Robison and Zimmerman (1971) under slightly different conditions.

In order to compare quantitatively the fluorescence intensity of the Y base in charged and uncharged tRNA^{Phe} under identical conditions, the fluorescence intensity of [^{14}C]Phe-tRNA^{Phe} was monitored at 430 nm under conditions in which the molecule is slowly deacylated. As can be seen in Figure 3, no change takes place in the 430-nm emission intensity after nearly complete deacylation. Similar results were obtained at other wavelengths, and the fluorescence of Phe-tRNA^{Phe} was found to vary with pH in a manner similar to tRNA^{Phe} (data not shown). The half-life of the deacylation reaction under these conditions is 32 min (Figure 3).

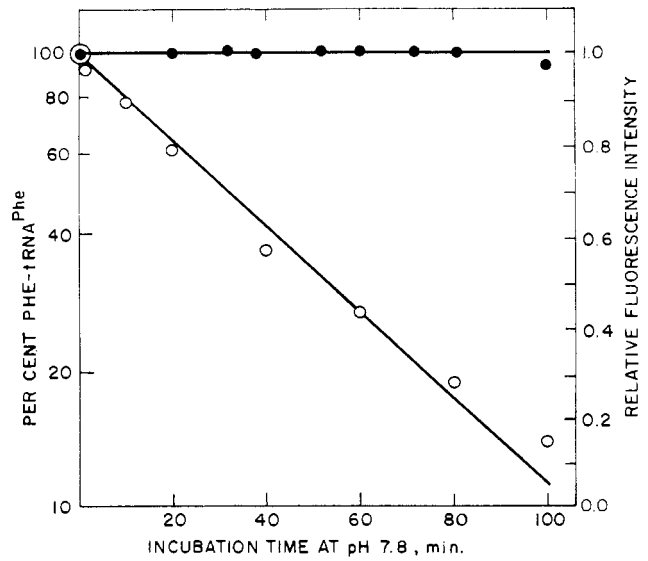

FIGURE 3: The fluorescence intensity of the Y base in [^{14}C]Phe-tRNA^{Phe} as the molecule undergoes deacylation. The fluorescence at 430 nm in 8 mM sodium acetate and 0.16 M Tris-HCl (pH 7.8) containing 4 mM magnesium acetate is shown as a function of the time of incubation (●) along with the decrease in Phe-tRNA^{Phe} concentration (○). The excitation wavelength was 318 nm and the initial Phe-tRNA^{Phe} concentration 5.3×10^{-7} M.

TABLE I: Deacylation Kinetics of [^{14}C]Phe-tRNA^{Phe}. Effect of Ternary Complex Formation.[a]

Incubn	EF-Tu Added (pmol)	Reaction Half-Life[b] (min)
1[c]	63	52
2	0	56
3	3	56
4	16	65
5	32	158
6	63	500
7	135	800

[a] The complete system for ternary complex formation described under Materials and Methods was scaled down 15-fold except for EF-Tu·GDP, which was varied as indicated. The incubation was at 30°, and each reaction mixture contained 48 pmol of [^{14}C]Phe-tRNA^{Phe}. [b] The time for 50% deacylation was estimated from the type of data presented in Figure 4. [c] Complete system without GTP, phosphoenolpyruvate and pyruvate kinase.

Effect of Ternary Complex Formation on the Rate of Deacylation of Phe-tRNA^{Phe}. Before determining whether the formation of the ternary complex with EF-Tu and GTP would affect the fluorescence of the Y base of Phe-tRNA^{Phe}, we investigated the stability of the Phe-tRNA^{Phe} under the conditions of the fluorescence experiments. This was necessary because deacylated tRNA does not interact with EF-Tu and GTP (Gordon, 1967; Ravel *et al.*, 1967). Our results indicate that interaction with EF-Tu and GTP greatly stabilizes Phe-tRNA^{Phe} against base-catalyzed deacylation (Table I and Figure 4), the rate of the reaction at 30° in the complex being approximately an order of magnitude slower than for uncomplexed Phe-tRNA^{Phe}. The results in Table I and Figure 4 also show that all of the components required for ternary complex formation are necessary for this stabilization of Phe-tRNA^{Phe}; EF-Tu alone, or the complete system without EF-Tu, does not protect Phe-tRNA^{Phe} against deacylation. These observations were confirmed during the course of the fluorescence measurements on the ternary complex which were carried out at room temperature. The decrease in deacylation rate due to the presence of EF-Tu·GTP was used, in addition to the assay described in Materials and Methods, to check the extent of ternary complex formation under the conditions of the fluorescence measurements. Control experiments were carried out to test whether the slow rate of deacylation of Phe-tRNA^{Phe} in the complete reaction mixture might be caused by the possible contamination of the EF-Tu preparation with Phe-tRNA synthetase, or of the phosphorylating system with ATP. These gave negative results.

Fluorescence of the Y Base in the Phe-tRNA^{Phe}·EF-Tu·GTP Complex. Measurements of the fluorescence spectrum of Phe-tRNA^{Phe} in the ternary complex were complicated by significant fluorescence of EF-Tu and pyruvate kinase. Typical fluo-

TABLE II: Fluorescence Properties of the Y Base in Phe-tRNA^{Phe}. Effect of Ternary Complex Formation.[a]

Excitation Wavelength (nm)	Emission Wavelength (nm)	H_2O	Fluorescence Intensity					
			Control			Complete System		
			1[b]	2[c]	Δ[d]	3[e]	4[f]	Δ[g]
280	430	0	11	26	15	17	33	16
290	430	1	20	36	16	32	47	15
310	430	2	21	57	36	45	77	32
320	430	3	20	60	40	46	81	35
340	430	3	22	41	19	31	50	19
330	410	6	25	52	27	66	90	24
330	430	3	19	48	29	39	71	32
330	450	1	13	39	26	22	48	26
330	470	0	9	29	20	13	32	19
318	430	3	22	61	39	47	91	44
318	460	1	12	43	31	20	53	33
318	480	0	8	29	21	9	32	23

[a] The complete system is described under Materials and Methods. Fluorescence intensities are expressed in arbitrary units. [b] Complete system without EF-Tu·GDP and [^{14}C]Phe-tRNA^{Phe}. [c] Complete system without EF-Tu·GDP. [d] Difference between 2 and 1. [e] Complete system without [^{14}C]Phe-tRNA^{Phe}. [f] Complete system. [g] Difference between 3 and 4.

rescence data obtained on these systems, including blanks, are presented in Table II. Although the net fluorescence of the complete system, as well as the control (with EF-Tu·GTP omitted) is obtained by subtracting rather large background values, these "corrected" numbers could be reproduced to within 15% in different experiments, thus making possible at least qualitative comparisons. The fluorescence spectra thus obtained for the Y base in the ternary complex exhibited emission and excitation maxima of 430 and 318 nm, respectively, as well as the pronounced shoulder at 280 nm in the excitation spectrum. Within the error limit mentioned above, the spectra were superimposable with those for uncomplexed Phe-tRNA^{Phe} (complete system without EF-Tu·GTP) under the same conditions. It appears then that the interaction of Phe-tRNA^{Phe} with EF-Tu and GTP does not lead to such perturbations of the Y base fluorescence which have been observed for different pH and Mg^{2+} conditions (Figure 1).

Discussion

The interpretation of the findings presented here depends on the validity of the contention that the Y base fluorescence in tRNA^{Phe} is sensitive to the environment and conformational state of the anticodon region of the molecule. As noted above, this assumption has been substantiated in various laboratories (Beardsley and Cantor, 1970; Eisinger *et al.*, 1970; Beardsley *et al.*, 1970; Robison and Zimmerman, 1971). Our observation on the pH dependence of tRNA^{Phe} fluorescence extends previous reports on the sensitivity of the Y base fluorescence to environmental conditions. It should be noted, however, that the following discussion necessarily excludes possible conformational changes in tRNA^{Phe} which do not affect the fluorescence of the Y base.

A number of laboratories have investigated possible conformational changes in various tRNAs as a result of aminoacylation in order to examine structural contributions to the strikingly different functional properties of charged and uncharged tRNA. No significant differences were found in the circular dichroism spectra of *E. coli* tRNA^{fMet}, methionyl-tRNA^{fMet}, and formylmethionyl-tRNA^{fMet}, indicating little change in their respective secondary structures (Adler and Fasman, 1970; Wickstrom, 1971). On the other hand, evidence has been presented for differences in the local tertiary conformation around the 4-thiouracil base in tRNA^{fMet} and formylmethionyl-tRNA^{fMet} (Watanabe and Imahori, 1971). This base lies between the 5′ end of tRNA and the dihydrouracil arm of most *E. coli* tRNA molecules. In addition, differences in the behavior of various uncharged, aminoacylated, and N-blocked aminoacyl-tRNA molecules during separation on methylated albumin–silicic acid columns have also been interpreted to be due to conformational variations of an undefined nature (Stern *et al.*, 1969). Similar conclusions have been reached using other methods (Chatterjee and Kaji, 1970; Gantt *et al.*, 1969). Our study on the fluorescence of the Y

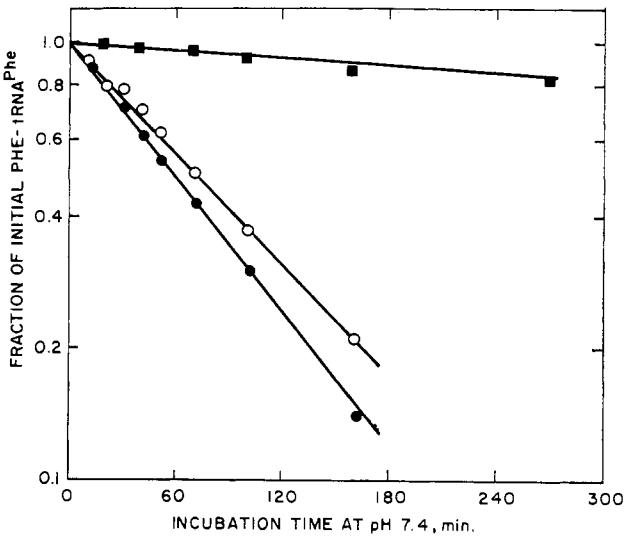

FIGURE 4: Effect of the ternary complex formation on the deacylation kinetics of [^{14}C]Phe-tRNA^{Phe}. The reaction mixture for ternary complex formation described under Materials and Methods was scaled down tenfold: (■) complete reaction mixture; (○) complete reaction mixture with Tu-GDP omitted; (●) complete mixture without GTP, phosphoenolpyruvate, and pyruvate kinase. The incubation was at 30°.

base in $tRNA^{Phe}$ and Phe-$tRNA^{Phe}$ suggests that in this case the local environment and conformation of the molecule near the anticodon is unaffected by the covalent linkage of the amino acid at the 3′ end. This conclusion is consistent with the observation reported by Hänggi and Zachau (1971) that aminoacylation of $tRNA^{Phe}$ does not change the accessibility of the anticodon region to cleavage by T2 RNase. It also agrees with the proposed models of $tRNA^{Phe}$ which describe nearly maximal separation between the 3′ terminus and anticodon loop of the molecule (Cramer *et al.*, 1969; Levitt, 1969; Beardsley and Cantor, 1970; Kim *et al.*, 1973). Our study further suggests that aminoacylation does not indirectly induce tertiary structural changes at the anticodon-containing region of $tRNA^{Phe}$. A similar conclusion has been reached concerning the local tertiary structure of the 4-thiouridine-containing region of *E. coli* $tRNA_1^{Val}$ (Krauskopf *et al.*, 1972).

Our result concerning the effect of ternary complex formation on the stability of the aminoacyl bond in Phe-$tRNA^{Phe}$ and the indication from the fluorescence study that the fluorescence of the Y base at the anticodon region is unaffected by interaction with EF-Tu·GTP complement each other. These observations confirm and extend previous reports and conclusions concerning the structural requirements in aminoacyl-tRNA for ternary complex formation. This problem is of general interest because almost all unmodified aminoacyl-tRNAs interact with EF-Tu apparently through a common mechanism. Various modifications of tRNA structure at the anticodon loop such as cleavage of the phosphodiester bond in the anticodon of *E. coli* valyl-$tRNA_1^{Val}$ (Krauskopf *et al.*, 1972), the excision of the Y base of $tRNA^{Phe}$ (Ghosh and Ghosh, 1970), or cyanoethylation of the inosine base adjacent to the 5′ end of the anticodon in *E. coli* $tRNA^{Arg}$ (Chen *et al.*, 1970) have been found not to affect EF-Tu·GTP recognition. On the other hand, modifications in the amino acid accepting region of tRNA, such as reduction of the 2′,3′ carbon–carbon bond at the 3′-terminal ribose of yeast $tRNA^{Phe}$ (Chen and Ofengand, 1970) or removal or direct modification of the amino acid itself has been shown to inhibit ternary complex formation (Ravel *et al.*, 1967; Gordon, 1967, 1968). The lengthening of the 3′ end in yeast $tRNA^{Phe}$ by an additional cytidine residue to yield a terminal pCpCpCpA sequence has been recently shown to significantly reduce the efficiency of ternary complex formation (Thang *et al.*, 1972). These findings suggest that the EF-Tu·GTP recognition site on aminoacyl-tRNA in the native state is localized near the 3′ end of the molecule, and that the amino acid itself is also involved in the interactions. Our observations that ternary complex formation stabilizes the aminoacyl bond in Phe-$tRNA^{Phe}$ against base-catalyzed hydrolysis, but does not significantly affect the fluorescence of the Y base, which is at a far removed part of the molecule, are consistent with this hypothesis.

At low Mg^{2+} concentration (5–10 mM), EF-Tu and GTP stimulate the binding of aminoacyl-tRNA to ribosomes two- to fivefold (Shorey *et al.*, 1971). Since our study suggests that interaction with EF-Tu does not affect the conformation of tRNA about the anticodon, the site of interaction with the ribosome-bound mRNA, ternary complex formation then apparently serves to stabilize the interaction between the amino acid carrying portion of the molecule and the ribosomal binding site.

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