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Articles

Fluorescence Characterization of the Interaction of Various Transfer RNA Species with Elongation Factor Tu·GTP: Evidence for a New Functional Role for Elongation Factor Tu in Protein Biosynthesis†

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ABSTRACT: The ubiquity of elongation factor Tu (EF-Tu)-dependent conformational changes in aminoacyl-tRNA (aa-tRNA) and the origin of the binding energy associated with aa-tRNA·EF-Tu·GTP ternary complex formation have been examined spectroscopically. Fluorescein was attached covalently to the 4-thiouridine base at position 8 (s^4U-8) in each of four elongator tRNAs (Ala, Met-m, Phe, and Val). Although the probes were chemically identical, their emission intensities in the free aa-tRNAs differed by nearly 3-fold, indicating that the dyes were in different environments and hence that the aa-tRNAs had different tertiary structures near s^4U-8. Upon association with EF-Tu·GTP, the emission intensities increased by 244%, 57%, or 15% for three aa-tRNAs due to a change in tRNA conformation; the fourth aa-tRNA exhibited no fluorescence change upon binding to EF-Tu·GTP. Despite the great differences in the emission intensities of the free aa-tRNAs and in the magnitudes of their EF-Tu-dependent intensity increases, the emission intensity per aa-tRNA molecule was nearly the same (within 9% of the average) for the four aa-tRNAs when bound to EF-Tu·GTP. Thus, the binding of EF-Tu·GTP induced or selected a tRNA conformation near s^4U-8 that was very similar, and possibly the same, for each aa-tRNA species. It therefore appears that EF-Tu functions, at least in part, by minimizing the conformational diversity in aa-tRNAs prior to their beginning the recognition and binding process at the single decoding site on the ribosome. Since an EF-Tu-dependent fluorescence change was also observed with fluorescein-labeled $tRNA^{Phe}$, the protein-dependent structural change is effected by direct interactions between EF-Tu and the tRNA and does not require the aminoacyl group. The K_d of the $tRNA^{Phe}$·EF-Tu·GTP ternary complex was determined, at equilibrium, to be 2.6 μM by the ability of the unacylated tRNA to compete with fluorescent Phe-tRNA for binding to the protein. Comparison of this K_d with that of the Phe-tRNA ternary complex showed that in this case the aminoacyl moiety contributed 4.3 kcal/mol toward ternary complex formation at 6 °C but that the bulk of the binding energy in the ternary complex was derived from direct protein–tRNA interactions. The acetylation of Phe-$tRNA^{Phe}$ had a greater effect on its ternary complex K_d than did the formylation of Met-$tRNA_f^{Met}$, which suggests that N-blocked aa-tRNA species are prevented sterically from filling the site on EF-Tu that binds the α-amino group. The affinity of EF-Tu·GTP for both elongator and initiator Met-$tRNA^{Met}$ species has also been quantified.

Protein biosynthesis in prokaryotes is catalyzed by a nonribosomal protein termed elongation factor Tu (EF-Tu)[1] (Miller & Weissbach, 1977). EF-Tu binds tightly to aminoacyl-tRNA (aa-tRNA) in the presence of GTP to form an aa-tRNA·EF-Tu·GTP ternary complex (Miller & Weissbach, 1977; Johnson et al., 1986), and it is in this form that aa-tRNA begins the recognition and binding process at the ribosome. It is therefore reasonable to assume that EF-Tu somehow

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[1] Abbreviations: aa-tRNA, aminoacyl-tRNA; EF-Tu, elongation factor Tu; s^4U, 4-thiouridine; tRNA-Fl[8], adduct between 5-(iodoacetamido)fluorescein and the s^4U base of a tRNA at position 8; PEP, phosphoenolpyruvate.

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facilitates the codon-mediated selection of aa-tRNA by the ribosomal complex, and considerable evidence to support this view has been published [e.g., see Thompson and Stone (1977) and Ruusala et al. (1982)]. However, the molecular mechanism(s) by which EF-Tu accomplishes its catalytic effect has (have) not been identified experimentally, and hence the precise role(s) of EF-Tu in protein biosynthesis is (are) still obscure.

A possible clue to EF-Tu function was provided by Adkins et al. (1983) using a fluorescent-labeled aa-tRNA that functions in protein biosynthesis almost as well as the unmodified aa-tRNA (Johnson et al., 1982, 1986). They demonstrated that the binding of EF-Tu·GTP to Phe-tRNA^{Phe}-Fl^{8}, an *Escherichia coli* Phe-tRNA with a fluorescein dye attached covalently to the 4-thiouridine base at position 8 (s^{4}U-8), caused a substantial increase in its fluorescence emission. They further showed that this spectral change resulted from a conformational change in the aa-tRNA that was induced allosterically by the protein. This last point was established by iodide ion quenching data that showed that the fluorescent dye on the Phe-tRNA was equally accessible to solvent in the presence and absence of EF-Tu·GTP, and hence that EF-Tu did not cover or interact with the dye. These observations led Adkins et al. (1983) to suggest that EF-Tu function involves, at least in part, binding to aa-tRNA molecules and inducing conformational changes in some or all of them. EF-Tu-dependent conformational changes have also been observed with four other aa-tRNAs using chemical modification (Riehl et al., 1983), nuclease digestion (Wikman et al., 1982), and spin-labeling (Kruse et al., 1978; Weygand-Durasevic et al., 1981) techniques. This suggests that EF-Tu-mediated changes are not limited to one tRNA species or to one region of the tRNA.

In order to determine whether the fluorescence-detected conformational change is unique or ubiquitous, we have examined the association of EF-Tu·GTP with four different elongator aa-tRNAs, each with a fluorescein dye covalently attached to its s^{4}U-8. This has allowed us to use the same technique to monitor the tRNA conformations and their dependence upon EF-Tu. Furthermore, in order to determine if certain structural features of the aa-tRNA are required to elicit the protein-dependent conformational change in the tRNA, we have also examined the interactions of fluorescent-labeled unacylated and initiator tRNAs with EF-Tu·GTP.

Abrahamson et al. (1985) took advantage of the EF-Tu-dependent change in Phe-tRNA^{Phe}-Fl^{8} emission intensity to examine another aspect of ternary complex formation and measure, directly and at equilibrium, the affinity of EF-Tu·GTP for aa-tRNA. This was accomplished by using fluorescence intensity to measure the extent of ternary complex formation in a sample as a function of EF-Tu concentration and thereby determine K_d. The ternary complex K_d value for an unmodified aa-tRNA was then obtained by quantifying its ability to compete with Phe-tRNA^{Phe}-Fl^{8} for binding to EF-Tu·GTP (Abrahamson et al., 1985). Previous measurements of ternary complex K_d values utilized either filter binding techniques [e.g., see Miller et al. (1973), Arai et al. (1974), and Ofengand (1974)] or protection of the aminoacyl group from either chemical deacylation [e.g., see Pingoud et al. (1977), Pingoud and Urbanke (1980), and Wagner and Sprinzl (1980)] or nuclease digestion [e.g., see Knowlton and Yarus (1980), Tanada et al. (1981, 1982), Louie et al. (1984), Louie and Jurnak (1985), Romero et al. (1985), and Seong and RajBhandary (1987)]. These nonequilibrium approaches all yielded ternary complex K_d values that were substantially higher than those that have been determined by using fluorescence (Abrahamson et al., 1985; Joshi et al., 1986; Ott et al., 1989).

The aminoacyl portion of the aa-tRNA has long been known to be essential for the tight binding of tRNA to EF-Tu (Miller & Weissbach, 1977). However, the contribution of the aminoacyl group to the high-affinity interaction has not been quantified by deacylation techniques because they require the enzymatic or chemical cleavage of a radioactive amino acid from an aminoacylated tRNA to discriminate between free and EF-Tu-bound aa-tRNAs, and cannot be used with an unacylated tRNA that lacks an amino acid. We have therefore used the fluorescence competition approach (Abrahamson et al., 1985) to measure the affinity of unacylated tRNA for EF-Tu·GTP at equilibrium, and thereby determine the fraction of the total binding energy of the ternary complex that is derived from direct tRNA–EF-Tu interactions. We have also quantified the effects of formylation and acetylation upon ternary complex binding energies. Previous such measurements utilized nuclease digestion to assess complex formation (Tanada et al., 1981, 1982; Seong & RajBhandary, 1987) but assumed that the nuclease will not cleave an aminoacyl end that is bound improperly to the protein. The spectroscopic approach is not subject to this uncertainty.

Experimental Procedures

Solvent System. All titrations were performed in a polycation-containing polymix solvent that was prepared according to Jelenc (1980), except that the buffer was titrated to pH 7.0 (instead of pH 7.5) in order to avoid precipitate formation at 6 °C (Abrahamson et al., 1985). The final incubations contained 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 8 mM putrescine, 1 mM spermidine, 5 mM NH_4Cl, 95 mM KCl, and 5 mM potassium phosphate. No reducing agent was added to any solution in this study.

EF-Tu. Crystalline EF-Tu·GDP was purified from *E. coli* B cells as described (Miller & Weissbach, 1974). EF-Tu·GDP concentrations were calculated by using $\epsilon_{280} = 41\,600\ M^{-1}\ cm^{-1}$ (Miller & Weissbach, 1977). The nucleotide binding activity of the EF-Tu was determined by using filter binding assays (Miller & Weissbach, 1974; Abrahamson et al., 1985), and the EF-Tu was completely active in these assays.

Transfer RNA. Pure *E. coli* tRNAs were purchased from Subriden RNA (Rolling Bay, WA), Plenum Scientific (Hackensack, NJ), or Boehringer (Indianapolis, IN). Fluorescent tRNAs were synthesized by reaction with 5-(iodoacetamido)fluorescein (Molecular Probes, Eugene, OR) for 17 h and purified by RPC-5 chromatography at 22 °C as described previously (Johnson et al., 1982). A purified tRNA species with the fluorescein dye attached covalently to s^{4}U-8 was designated tRNA-Fl^{8}. Complete digestion of the fluorescent tRNAs and analysis by thin-layer chromatography were also done as before (Johnson et al., 1982).

Modified and unmodified tRNA species were aminoacylated essentially as described elsewhere (Johnson et al., 1982). Incubations (3.0 mL, 37 °C, 30 min) contained 100 mM HEPES (pH 8.0), 10 mM $MgCl_2$ (20 mM for tRNA^{Phe}), 10 mM KCl (0 mM for tRNA^{Phe}), 4 mM ATP, 0.1 mM CTP, 9.7–12.2 μM radioactive amino acid, 300 μg of *E. coli* S-100 enzymes [prepared according to Johnson et al. (1976)], and 3–6 A_{260} units of pure tRNA, either modified or unmodified. Formylation of tRNA$_f^{Met}$ species was accomplished, when desired, by the inclusion of 0.1 mM folinic acid (calcium salt; Sigma) in the incubation. Uniformly labeled [^{14}C]amino acids were used undiluted; [^{3}H]amino acids (for use in competition titrations) were typically diluted with the appropriate nonra-

dioactive amino acid to a final specific activity near 15 000 dpm/pmol of amino acid. Because the advertised specific activities of some commercial radioactive amino acid preparations were incorrect, we routinely determined the actual specific activities of the commercial preparations as described previously (Abrahamson et al., 1985).

N-Acetyl-Phe-tRNA^{Phe} (AcPhe-tRNA^{Phe}) was prepared according to Rappoport and Lapidot (1974). *N*-Acetoxysuccinimide [98 mg; prepared as in Johnson et al. (1976)] in 1.0 mL of dimethyl sulfoxide was incubated (0 °C, 2 h) with 0.25 mL of 50 mM potassium acetate (pH 5.0) containing 2.4 A_{260} units of Phe-tRNA^{Phe}. Following ethanol precipitation and dialysis, the extent of acetylation was found to be greater than 98% by base hydrolysis (0.4 N KOH, 37 °C, 30 min) of an aliquot, neutralization with 4 N acetic acid, and ascending paper chromatography as described (van der Zeijst et al., 1972).

The concentration of tRNA was determined by using $\epsilon_{260} = 6.25 \times 10^5$ M^{-1} cm^{-1}. Samples were stored in 5 mM $MgCl_2$/1 mM potassium acetate (pH 5.0) in small aliquots at −75 °C.

Determination of aa-tRNA Concentration. The total amount of tRNA present in a titration sample was determined from the total radioactivity content of the sample and the previously determined specific activity (picomoles of amino acid per A_{260} unit of tRNA) of the stock aa-tRNA solution. The amount of aminoacylated tRNA was determined from aliquots that were taken directly from the cuvette at both the beginning and the end of a titration experiment, put into polypropylene microfuge tubes, and quick-frozen in liquid nitrogen. Each aliquot was later melted and chromatographed on a Sephadex G-25 gel filtration column (0.7 cm i.d. × 12.5 cm) at 4 °C. Fractions (180 μL) were collected directly into scintillation vials and counted in a Triton-based scintillation cocktail. The fraction of amino acid covalently attached to tRNA was given by the ratio of the cpm found in the void volume to the total cpm eluted from the column, after background cpm had been subtracted. The total deacylation of an aa-tRNA during a titration was usually less than 10%, and never exceeded 14%.

Deacylation Rates. Samples (225 μL) in microfuge tubes contained 10 μM GTP, 1 mM phosphoenolpyruvate (PEP), and 3–15 pmol of purified aa-tRNA in polymix. Immediately after mixing, the first aliquot (50 μL) was removed, and the sample tube was capped and placed at 6 °C. The amount of aa-tRNA present in the sample at various times was determined by gel filtration of 50-μL aliquots as described above.

Protection Assays. Samples in microfuge tubes contained 18 μg of pyruvate kinase (Sigma), 200 pmol of EF-Tu, and either 10 μM GTP and 1 mM PEP or 10 μM GDP (for the control) in polymix. After preincubation at 37 °C for 10 min to convert GDP into GTP, 25–40 pmol of [^{14}C]Met-tRNA$_m^{Met}$-Fl^8 (1240 pmol of Met/A_{260} unit of tRNA; 622 dpm/pmol of Met) was added to each sample (175-μL final volume). Immediately after mixing, duplicate aliquots (25 μL) were assayed for cold acid-insoluble radioactivity [cf. Johnson and Adkins (1984)] to determine the initial amount of aminoacylated tRNA. The samples were then placed at 37 °C, and assayed at 1 and 2 h to measure the residual cold acid-insoluble cpm. Just prior to addition of acid to an aliquot, 0.3 A_{260} unit of ribosomal RNA was added to serve as carrier.

Fluorescence Spectroscopy. Fluorescence measurements were made either in 4 mm × 4 mm quartz curvettes using an SLM 8000 or in 1 cm × 1 cm cuvettes using a Spex Fluorolog I. Each photon-counting spectrofluorimeter had two gratings in the excitation light path, utilized a 450-W xenon lamp, and was interfaced to an Apple microcomputer. Software for the Spex operation and data acquisition was written by Dr. Thomas M. Laue. Temperature was maintained at 6 °C in all experiments to minimize deacylation and was monitored by using a temperature probe that was inserted into a water-containing cuvette kept in the four place cell holder. The cell compartment was flushed with a steady stream of nitrogen gas throughout each experiment in order to prevent the condensation of water on the faces of the cuvettes. Samples were excited at 490 nm, and emission was monitored at 520 nm. The band-pass was 10 nm on the Spex and either 2 or 4 nm on the SLM. To avoid photodegradation of the samples, shutters were kept closed except during measurements.

Titrations. Elongator aa-tRNA samples (3.0 mL in large cuvettes) initially contained 10 μM GTP, 1 mM PEP, 20–40 μg/mL pyruvate kinase, and 3–30 nM aa-tRNA-Fl^8 in polymix. Samples were mixed by using a Teflon-coated magnetic stirrer. Immediately prior to a titration, the EF-Tu-containing titrant solution was incubated for 20–30 min at 37 °C with 50–67 μg/mL pyruvate kinase, 10 μM GTP, and 1 mM PEP in polymix to convert residual GDP to GTP. This titrant solution, or a dilution of it, was used to titrate the sample.

At the beginning of each titration, the fluorescence intensity of a sample was monitored until it was stable. This initial value, designated F_o, was typically reached after 2–3 h [cf. Abrahamson et al. (1985)]. However, Val-tRNA$_1^{Val}$-Fl^8 was routinely incubated in the cuvette overnight at 0 °C, since this tRNA preparation was slow to reach a stable F_o in polymix. After a stable F_o had been achieved, an aliquot was removed from the sample to determine its extent of aminoacylation by gel filtration, and then the stepwise addition of the EF-Tu-containing titrant solution was begun. After each addition of titrant, the sample was thoroughly mixed and allowed to reequilibrate at 6 °C for 8–15 min before emission intensity readings were taken. The signal of a fluorescein-free blank (a parallel titration sample that contained aa-tRNA instead of aa-tRNA-Fl^8) was subtracted from the sample signal at each point, and then the net emission was corrected for dilution due to the addition of titrant. At the end of the titration, aliquots were removed to determine the extent of aminoacylation of the sample and also the total radioactivity in the sample.

Titrations of fluorescent unacylated tRNA and initiator tRNAs were carried out as described above in a total volume of 200 μL in microcells. In order to obtain reproducible results with microcells, extra care had to be taken in mixing the samples in the cuvettes as detailed in Lu et al. (1989).

The affinities of nonfluorescent tRNA species for EF-Tu·GTP were determined by their abilities to complete with Phe-tRNA^{Phe}-Fl^8 for binding to the protein as described above and earlier (Abrahamson et al., 1985). For such titrations, the samples (3.0-mL total volume, except 0.2 mL for tRNA^{Phe} in microcells) initially contained 3–6 nM [^{14}C]Phe-tRNA^{Phe}-Fl^8 (typically 1400 pmol of Phe/A_{260} unit of tRNA; 999 dpm/pmol of Phe) and either 108 nM Ac-[^{3}H]Phe-tRNA^{Phe}, 15–23 nM [^{3}H]Met-tRNA$_m^{Met}$, 27–46 nM [^{3}H]-Met-tRNA$_f^{Met}$, 278 nM f-[^{3}H]Met-tRNA$_f^{Met}$, 5.9 μM tRNA^{Phe}, or 5.0–6.3 μM unfractionated *E. coli* tRNA.

Emission Intensities. The total fluorescence intensity (F) of any sample is given by

$$F = R_u E_u + R_f E_f + R_b E_b \quad (1)$$

where R is the concentration and E is the emission intensity per molecule of unacylated tRNA-Fl^8 (u), free aa-tRNA-Fl^8 (f), and aa-tRNA-Fl^8 bound to EF-Tu·GTP (b). The emission

intensities of $tRNA^{Ala}$-Fl[8], $tRNA^{Phe}$-Fl[8], and $tRNA_m^{Met}$-Fl[8] did not change when each was aminoacylated in a cuvette (Adkins et al., 1983; data not shown), so E_u was equal to E_f for these tRNAs. Due to a lack of material, $tRNA_1^{Val}$-Fl[8] was not tested in this way, and its E_u was assumed to be equal to its E_f.

The E values of the various elongator aa-tRNA-Fl[8] species were determined under the same solvent, temperature, and spectral conditions, and all E values in this paper are expressed relative to that of free Val-$tRNA_1^{Val}$-Fl[8], the lowest E value that we observed. The measured emission intensities were also normalized for instrumental variations over time by using the magnitude of the Raman scattering signal for water (λ_{ex} = 350 nm, λ_{em} = 399 nm) to monitor changes in lamp intensity or instrument sensitivity. Normalized emission intensities were obtained by multiplying the measured sample intensity by the ratio of the measured water Raman signals determined on an arbitrary reference day and on the day of the experiment.

The maximum fluorescence intensity of the sample, F_{max}, is observed when EF-Tu·GTP is in excess and all aa-tRNA-Fl[8] molecules are bound to the protein in the ternary complex. Then, since $E_f = E_u$

$$\frac{F_{max}}{F_o} = \frac{R_b E_b + R_u E_f}{R_t E_f} \quad (2)$$

where $R_t = R_u + R_f + R_b$. Using $R_a = R_f + R_b$, one then obtains

$$\frac{E_b}{E_f} = \frac{(F_{max}/F_o)R_t + R_a - R_t}{R_a} \quad (3)$$

The above approach could not be used to obtain an E_b value for unacylated $tRNA^{Phe}$-Fl[8] because the amount of protein required to titrate to completion (i.e., to obtain all tRNAs in a $tRNA^{Phe}$-Fl[8]·EF-Tu·GTP complex) was prohibitive. Thus, the maximum emission intensity of $tRNA^{Phe}$-Fl[8] in the EF-Tu·GTP titrations was estimated by extrapolation of a double-reciprocal plot of the data, $F_o/(F - F_o)$ vs $1/[\text{EF-Tu·GTP}]$.

Determination of K_d. K_d values for the ternary complexes of nonfluorescent tRNA species were determined by using 4.7 nM as the K_d value for the Phe-$tRNA^{Phe}$-Fl[8] complex in polymix at 6 °C (Abrahamson et al., 1985). Deacylation of aa-tRNA over the course of an experiment was taken into account at each titration point (Abrahamson et al., 1985) using the following measured deacylation constants: Phe-$tRNA^{Phe}$, 3.0×10^{-4} min^{-1}; Met-$tRNA_m^{Met}$, 1.3×10^{-3} min^{-1}; Met-$tRNA_f^{Met}$, 4.9×10^{-4} min^{-1}; fMet-$tRNA_f^{Met}$, 5.3×10^{-5} min^{-1}. Deacylation of AcPhe-$tRNA^{Phe}$ was insignificant. Since we assumed that every EF-Tu molecule was capable of binding both the nucleotide and the aa-tRNA, the K_d values reported here represent the maximum possible K_d values for the particular ternary complexes.

Results

Preparation of Fluorescent tRNAs. Five *E. coli* tRNA species, four elongator tRNAs and the initiator tRNA ($tRNA_f^{Met}$), each of which had only a single thiol base (4-thiouridine) at the same location (position 8), were reacted with 5-(iodoacetamido)fluorescein as described previously (Johnson et al., 1982). The fluorescent tRNAs were then separated from unmodified tRNAs or doubly labeled tRNAs by reversed-phase ion-exchange chromatography (Johnson et al., 1982). Analysis of each fluorescent tRNA by complete digestion and thin-layer chromatography (Johnson et al., 1982) showed that only the fluorescein adduct with s^4U was present in each case (data not shown). Thus, we conclude that the fluorescent tRNA samples are chemically homogeneous, with each tRNA having a single fluorescein dye covalently attached to its s^4U-8 base.

Table I: Emission Intensities of Fluorescent Aminoacyl-tRNA Species in the Presence and Absence of EF-Tu·GTP[a]

tRNA species	n	E_f	E_b	E_b/E_f
Val-$tRNA_1^{Val}$-Fl[8]	2	1.00 ± 0.08	2.44 ± 0.22	2.44 ± 0.03
Phe-$tRNA^{Phe}$-Fl[8]	3	1.66 ± 0.25	2.58 ± 0.22	1.57 ± 0.12
Ala-$tRNA^{Ala}$-Fl[8]	3	2.24 ± 0.10	2.57 ± 0.12	1.15 ± 0.01
Met-$tRNA_m^{Met}$-Fl[8]	2	2.85 ± 0.11	2.85 ± 0.11	1.00 ± 0.00[b]

[a] Determined in polymix at 6 °C as described under Experimental Procedures. Values given are the average values for n experiments using tRNA aminoacylated in the absence of reducing agent. E_f is the normalized emission intensity of the free fluorescent molecule, while E_b is the normalized emission intensity of the molecule when bound to EF-Tu·GTP. Standard deviations on the E_b/E_f values were obtained after averaging the ratios obtained in the n separate experiments. [b] An E_b/E_f value of 1.00 was also obtained in six other separate experiments using different spectrofluorimeters.

Emission of the Elongator tRNAs. The fluorescent signals for three elongator tRNAs, $tRNA^{Ala}$-Fl[8], $tRNA^{Phe}$-Fl[8], and $tRNA_m^{Met}$-Fl[8], were monitored as each was aminoacylated in its cuvette, and no change in emission intensity was observed (Adkins et al., 1983; data not shown). Hence, the emission properties of the probe were the same for the unacylated and aminoacylated forms of the tRNAs. It therefore appears that the environment of the dye attached to the s^4U-8 was unaffected by the aminoacylation of these tRNAs, and it follows that the aminoacylation of the tRNAs did not result in a detectable conformational change near s^4U-8. This conclusion is supported by recent time-resolved fluorescence data obtained with $tRNA^{Phe}$-Fl[8] which demonstrated that neither the lifetime nor the local and global rotational relaxation times of the tRNA-bound fluorescein were changed upon aminoacylation (Hazlett et al., 1989).

However, the individual fluorescent elongator aa-tRNAs had different emission intensities. When the observed emission intensities were normalized (see Experimental Procedures) to calculate E_f, the relative emission intensity per free aa-tRNA-Fl[8] molecule, their E_f values were found to differ by nearly 3-fold (Table I). Since the same dye is covalently attached to the same base in each tRNA, the probes in these tRNA species are chemically identical. Therefore, the difference in fluorescence intensities must arise from a difference in the environments of the fluorescein dyes in these tRNAs. This in turn indicates that the tRNAs have different conformations in solution.

During the course of this study, we observed a small variation in the E_f value obtained for different Phe-$tRNA^{Phe}$-Fl[8] preparations. This effect was ultimately traced to the presence of reducing agent in the 37 °C aminoacylation incubation, and control experiments showed that fluorescein dye was released from the tRNA in the presence of 1 mM dithiothreitol at 37 °C. A comparison of Val-$tRNA_1^{Val}$-Fl[8] samples that had been aminoacylated either in the absence or in the presence of reducing agent showed that the measured E_f and E_b values were lower (by about 10%) for the latter aa-tRNA, as would be expected if the reducing agent had caused a loss of fluorescein. As a result, dithiothreitol was omitted from all subsequent aminoacylation incubations. The data reported in this paper were all obtained using fluorescent aa-tRNAs prepared in the absence of reducing agent.

Sensitivity of Emission to Ternary Complex Formation. The emission intensity of Phe-$tRNA^{Phe}$-Fl[8] increased substantially upon association with EF-Tu·GTP, and this increase required ternary complex formation because it was not observed with an equivalent concentration of either EF-Tu·GDP

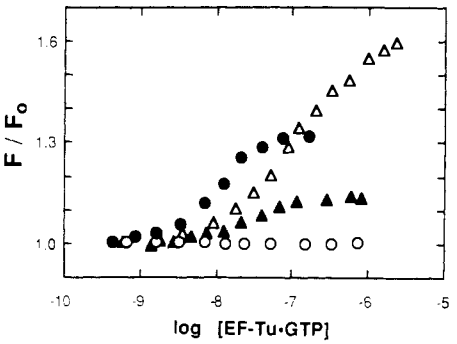

FIGURE 1: EF-Tu·GTP dependence of elongator aminoacyl-tRNA-Fl^8 fluorescence. Titrations were performed in polymix at 6 °C as described under Experimental Procedures. Initial concentrations were 6.4 nM Phe-tRNA^{Phe}-Fl^8 (●), 3.6 nM Val-tRNA$_1^{Val}$-Fl^8 (△), 11.8 nM Ala-tRNA^{Ala}-Fl^8 (▲), and 9.0 nM Met-tRNA$_m^{Met}$-Fl^8 (○).

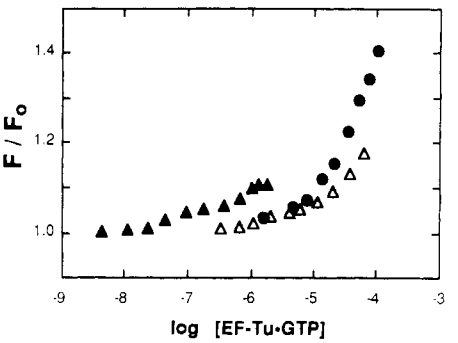

FIGURE 2: EF-Tu·GTP dependence of initiator and unacylated tRNA-Fl^8 fluorescence. Titrations were done in microcells as described under Experimental Procedures with samples that initially contained 23.6 nM Met-tRNA$_f^{Met}$-Fl^8 (▲), 39.8 nM fMet-tRNA$_f^{Met}$-Fl^8 (△), or 920 nM tRNA^{Phe}-Fl^8 (●).

or unacylated tRNA^{Phe}-Fl^8 (Adkins et al., 1983; Abrahamson et al., 1985). We therefore determined whether a fluorescence increase occurred when the other fluorescent elongator aa-tRNAs formed a ternary complex with EF-Tu·GTP. As shown in Figure 1, the emission intensities of both Val-tRNA$_1^{Val}$-Fl^8 and Ala-tRNA^{Ala}-Fl^8 also increased and reached a plateau when titrated with EF-Tu·GTP. These fluorescence changes resulted from ternary complex formation, because no intensity change was observed at these concentrations when either Val-tRNA$_1^{Val}$-Fl^8 or Ala-tRNA^{Ala}-Fl^8 was titrated with EF-Tu·GDP, or when either tRNA$_1^{Val}$-Fl^8 or tRNA^{Ala}-Fl^8 was titrated with EF-Tu·GTP (data not shown). No change in the wavelength of maximum emission was observed for either aa-tRNA upon ternary complex formation. Thus, these data indicate that each of these aa-tRNAs forms a ternary complex with EF-Tu·GTP and that the binding of the protein to the aa-tRNA induces a conformational change in the tRNA near s^{4}U-8, as was observed with Phe-tRNA^{Phe}-Fl^8.

However, the magnitude of the fluorescence change differed for these three aa-tRNAs. Whereas the emission intensity per molecule increased by 2.4-fold for Val-tRNA$_1^{Val}$-Fl^8 upon binding to EF-Tu·GTP, the increase was 57% for Phe-tRNA^{Phe}-Fl^8 and only 15% for Ala-tRNA^{Ala}-Fl^8 (Table I). Therefore the extent and/or the nature of the change in the fluorescein's environment upon ternary complex formation differed for these three aa-tRNAs.

Interaction of Met-tRNA$_m^{Met}$-Fl^8 with EF-Tu·GTP. In contrast to the other fluorescent elongator aa-tRNAs, the titration of Met-tRNA$_m^{Met}$-Fl^8 with EF-Tu·GTP did not elicit an increase in emission intensity (Figure 1). The steady-state anisotropy of the fluorescein dye was also insensitive to the protein, increasing only from 0.30 to 0.31 when excess EF-Tu·GTP was added to a sample of Met-tRNA$_m^{Met}$-Fl^8. Yet enzymatic digestion data indicated that Met-tRNA$_m^{Met}$ experiences a conformational change in at least some regions of the tRNA upon association with EF-Tu·GTP (Wikman et al., 1982). In order to ascertain whether the fluorescent aa-tRNA was in fact binding to the protein, the ability of EF-Tu·GTP to protect the aminoacyl bond of Met-tRNA$_m^{Met}$-Fl^8 from hydrolysis was examined, and EF-Tu·GTP was found to retard greatly the nonenzymatic deacylation of the fluorescent aa-tRNA (data not shown). Therefore, the EF-Tu·GTP did associate with Met-tRNA$_m^{Met}$-Fl^8 to form a ternary complex, and the absence of a fluorescence change in this case does not result from an inability of the aa-tRNA to bind to the protein. Thus, either there was no EF-Tu-dependent structural change near the s^{4}U-8 or the nature of the conformational change was such that it caused no change in the emission intensity of the fluorescein.

Emission Intensities. The data summarized in Table I show that there is a wide variance both in the emission intensity of the free elongator aa-tRNA molecules (E_f varies by almost 3-fold) and in the magnitude of the EF-Tu·GTP-dependent emission intensity changes (E_b/E_f ranges from 1.0 to 2.4). These spectral differences result from differences in the tRNAs, because the spectral parameters were kept constant throughout, the probe was the same in each case, and the measured intensities were normalized to a standard reference signal, the Raman signal of doubly distilled water, to compensate for instrumental variations over time. However, the most striking aspect of the data in Table I is the fact that the E_b values are very similar for these aa-tRNAs, despite the great differences in the E_f and E_b/E_f values. The implications of this result are discussed below.

Structural Requirements for the EF-Tu-Dependent Conformational Change. Fluorescent tRNA molecules that normally do not interact with EF-Tu·GTP were also examined in order to determine whether or not these tRNAs exhibited an EF-Tu-dependent spectral change. Met-tRNA$_f^{Met}$-Fl^8 was titrated with EF-Tu·GTP, and a small, saturable increase in fluorescein emission intensity was observed (Figure 2). This demonstrated both that EF-Tu·GTP formed a ternary complex with the nonformylated fluorescent initiator tRNA and that this association resulted in a structural change in the tRNA near s^{4}U-8. A fluorescence increase was also observed when fMet-tRNA$_f^{Met}$-Fl^8 was titrated with EF-Tu·GTP (Figure 2), though only at very high protein concentrations because the formyl group interferes with the association between the two macromolecules (see below). Saturation of the spectral change was not achieved in this titration due to the limited amount of EF-Tu available. Since formylation did not prevent the spectral change, a free α-amino group is not required for the induction of a conformational change in the tRNA.

Unacylated tRNA^{Phe}-Fl^8 was titrated with EF-Tu·GTP in order to assess directly whether an interaction between the aminoacyl moiety and the protein is required to obtain the EF-Tu-mediated change in tRNA conformation. As shown in Figure 2, an increase in emission intensity was observed at very high concentrations of EF-Tu·GTP. This spectral change was EF-Tu-dependent, because only a slight decrease in intensity was observed in control samples titrated with titrant lacking EF-Tu (data not shown). Saturation of the spectral change was not obtained because the limited quantity of available EF-Tu precluded titration to higher protein concentrations. The need for a high protein concentration is consistent with the low affinity of unacylated tRNA for EF-Tu·GTP (see below). Double-reciprocal plots of these data were linear and provided, by extrapolation, an estimate of the

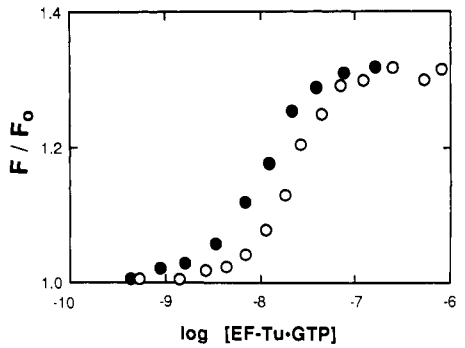

FIGURE 3: Titration of Phe-tRNA^{Phe}-Fl^8 in the presence or absence of nonfluorescent Met-tRNA$_m^{Met}$. Samples initially contained 6.4 nM Phe-tRNA^{Phe}-Fl^8 (●) or 6.4 nM Phe-tRNA^{Phe}-Fl^8 plus 19.4 nM Met-tRNA$_m^{Met}$ (○), and were titrated with EF-Tu·GTP as described under Experimental Procedures.

Table II: Affinity of EF-Tu·GTP for Various Nonfluorescent tRNA Species[a]

tRNA species	n	K_d (nM)	$\Delta G°$[b] (kcal/mol)
Phe-tRNA^{Phe}	5	1.16[c]	11.4
Met-tRNA$_m^{Met}$	3	1.8 ± 0.5	11.1
AcPhe-tRNA^{Phe}	3	380 ± 114	8.2
tRNA^{Phe}	4	2610 ± 380	7.1
tRNA, unfractionated	3	2820 ± 1360	7.1
Met-tRNA$_f^{Met}$	3	17.3 ± 1.9	9.9
fMet-tRNA$_f^{Met}$	2	136 ± 5.1	8.7

[a] The K_d values were determined in polymix at 6 °C as detailed under Experimental Procedures. Values given are the average values for n experiments. [b] These are the $\Delta G°$ values at 6 °C for the dissociation of the ternary complex, and hence are all positive. [c] From Abrahamson et al. (1985).

maximum intensity change and a value of 1.69 for E_b/E_f, close to the value found with the aminoacylated tRNA. Thus, the existence of an EF-Tu-dependent fluorescence change with tRNA^{Phe}-Fl^8 and the apparent similarity of the magnitude of the change to that obtained with Phe-tRNA^{Phe}-Fl^8 indicate that the amino acid of the elongator aa-tRNA is *not* required to obtain an EF-Tu-mediated structural change near the s^{4}U-8 of the tRNA.

Affinity of EF-Tu·GTP for Met-tRNA$_m^{Met}$. The affinity of EF-Tu·GTP for unmodified Met-tRNA$_m^{Met}$ was determined at equilibrium by its ability to compete with Phe-tRNA^{Phe}-Fl^8 for binding to the protein (Abrahamson et al., 1985). The results of such a competition experiment are shown in Figure 3, where it is clear that the addition of the nonfluorescent aa-tRNA as a competitor for binding to EF-Tu·GTP has caused the Phe-tRNA^{Phe}-Fl^8 fluorescence transition to occur at a higher protein concentration. The average K_d value measured for this ternary complex was 1.8 nM (Table II). Therefore, EF-Tu·GTP binds strongly to Met-tRNA$_m^{Met}$.

Affinity of EF-Tu·GTP for Unacylated tRNA. Competition experiments (Abrahamson et al., 1985) were employed both to demonstrate that unacylated tRNA forms a ternary complex with EF-Tu·GTP and to measure the K_d of that ternary complex. The titration experiments depicted in Figure 4 show that pure, unmodified, and unacylated tRNA^{Phe} molecules competed with Phe-tRNA^{Phe}-Fl^8 for association with the protein because the fluorescence transition occurred at a higher concentration of EF-Tu·GTP in the presence of tRNA^{Phe} than in its absence. Analysis of these equilibrium data using methods described previously (Abrahamson et al., 1985) yielded a K_d value of 2.6 μM for the tRNA^{Phe}·EF-Tu·GTP ternary complex (Table II).

The effect of the aminoacyl group on EF-Tu affinity for tRNA is clearly evident by comparing the K_d values obtained

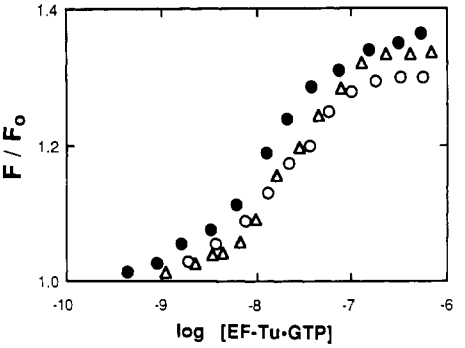

FIGURE 4: Competition between unacylated tRNA and Phe-tRNA^{Phe}-Fl^8 for binding to EF-Tu·GTP. Samples initially contained 3.4 nM Phe-tRNA^{Phe}-Fl^8 (●), 3.7 nM Phe-tRNA^{Phe}-Fl^8 plus 5.9 μM tRNA^{Phe} (○), or 3.7 nM Phe-tRNA^{Phe}-Fl^8 plus 5.9 μM tRNA (△) and were titrated with EF-Tu·GTP as in Figure 2. The final emission intensity values differ because the fraction of tRNA^{Phe}-Fl^8 that was aminoacylated at the end of these titrations was different.

with tRNA^{Phe} and Phe-tRNA^{Phe} under the same conditions and using the same approach (Table II). The K_d for the ternary complex is reduced by more than 3 orders of magnitude by the presence of the amino acid. The standard free energies associated with the various ternary complex equilibria under these conditions are also shown in Table II. This provides a direct evaluation of the contribution of the aminoacyl group to the formation of the ternary complex.

Competition experiments were also done using unfractionated *E. coli* tRNA, and the titration curve was quite similar to that obtained with tRNA^{Phe} (Figure 4). Although the measured K_d value for the unfractionated tRNA represents a weighted-average value for the various unacylated tRNA species present in the sample, this K_d value (2.8 μM; Table II) was very similar to that obtained for the purified tRNA^{Phe}.

Affinity of EF-Tu·GTP for AcPhe-tRNA^{Phe}. Although some experiments have indicated that EF-Tu·GTP and N-blocked aa-tRNAs do associate [e.g., see Campuzano and Modolell (1981)], it is well-known that peptidyl-tRNA binds poorly to EF-Tu·GTP (Miller & Weissbach, 1977). In order to quantify how poorly, we examined the ability of AcPhe-tRNA^{Phe}, an analogue of peptidyl-tRNA, to compete with Phe-tRNA^{Phe}-Fl^8 for binding to EF-Tu·GTP. The data (not shown) from competition experiments similar to those shown in Figure 3 yielded an average K_d value of 380 nM for the AcPhe-tRNA^{Phe}·EF-Tu·GTP ternary complex (Table II). Thus, this peptidyl-tRNA analogue does bind to EF-Tu·GTP and form a ternary complex. However, its K_d value is more than 2 orders of magnitude higher than that of the Phe-tRNA^{Phe}·EF-Tu·GTP ternary complex.

Affinity of EF-Tu·GTP for Initiator tRNAs. The affinities of unmodified, nonfluorescent Met-tRNA$_f^{Met}$, both formylated and nonformylated, for EF-Tu·GTP were determined by competition experiments (titration data not shown), and the K_d values for the corresponding ternary complexes are listed in Table II. These results demonstrate that both initiator tRNA species bind to the elongation factor, though not as well as the elongator aa-tRNA species we have tested (Abrahamson et al., 1985; Table II). In addition, these data show that the formylation of the Met-tRNA$_f^{Met}$ species reduced the total binding energy of its ternary complex by 1.1 kcal/mol at 6 °C.

Discussion

The various fluorescent elongator tRNA species examined in this study differ in their relative emission intensity by nearly 3-fold when free in solution (Table I). Since each aa-tRNA

has the same dye covalently attached via the same linkage to the same base in the primary structure, the fluorophore environment must differ in these aa-tRNAs.

This difference in emission intensities is, for two primary reasons, most likely caused by a difference in the tertiary structures of the tRNAs. First, other investigators using different techniques have shown that tRNA conformations do vary [e.g., see Vlassov et al. (1981), Crothers and Cole (1978), and Rigler and Wintermeyer (1983)] and that they are flexible (Crothers & Cole, 1978; Rigler & Wintermeyer, 1983). Crystallographic data have also revealed structural differences in the s^4U-8 regions of crystallized yeast $tRNA^{Phe}$ (Rich & RajBhandary, 1976) and yeast $tRNA^{Asp}$ (Moras et al., 1986) that result from a difference in the relative orientation of the two arms of the tRNA at their junction near s^4U-8. The fluorescence results of Table I are therefore consistent with, and provide independent evidence to support, the variability of tRNA structure reported by others. Second, these modified tRNAs function in aminoacylation, in EF-Tu·GTP binding, and, in the case of $tRNA^{Phe}$-Fl^8, in protein synthesis and its partial reactions as well or nearly as well as the unmodified tRNAs, despite the presence of the dye (Johnson et al., 1982, 1986; Adkins et al., 1983; Abrahamson et al., 1985; this paper). This suggests that the dye can act as a probe of structure but does not interfere with structure. Although it is possible that the presence of the dye in these tRNAs alters the tRNA conformation to some extent, such a structural alteration, if it occurs, must be minor because tRNA functionality is preserved.

We have also considered other explanations for the fluorescence differences. It is conceivable that the dyes are sensitive to primary structure differences in the free aa-tRNAs rather than tertiary structure differences, but if this is the case, one would then not expect the fluorescence intensity differences, and hence the structural differences, to disappear upon binding to EF-Tu·GTP (Table I). It is also conceivable that the tRNA conformations were originally equivalent and that the attached fluorescein moieties altered the conformations of the aa-tRNAs differently to yield different E_f values. Yet the fluorescent probe and its linkage were the same for each tRNA, and the addition of the same chemical moiety to each tRNA would be expected to elicit the same or similar changes (if any) in each. In view of the tRNA structure variability observed by others (see above), the different E_f values are more likely to result from the different intrinsic conformations of different tRNA species than from the same extrinsic probe inducing different conformations in tRNAs.

The fluorescein dye on the s^4U-8 therefore appears to constitute a sensitive spectral probe of aa-tRNA conformation at that location. The fluorescence data reported here and previously (Adkins et al., 1983; Abrahamson et al., 1985; Hazlett et al., 1989) indicate that this region of the aa-tRNA, near the elbow of the molecule, (i) is not structurally uniform for all species, (ii) is dynamic and flexible, and (iii) is allosterically (e.g., since EF-Tu·GTP does not bind directly to the dye; Adkins et al., 1983; Hazlett et al., 1989) sensitive to the interactions of the aa-tRNA with other molecules.

The different sensitivities of the probes in the fluorescent aa-tRNAs to EF-Tu·GTP are quite striking: the EF-Tu-dependent increase in fluorescein emission intensity ranged from 2.4-fold to *zero* (Table I). Therefore, in contrast to our expectation at the beginning of this study, not all aa-tRNAs experienced a conformational change near s^4U-8 upon binding to EF-Tu·GTP. Our initial surprise at the large range of observed E_b/E_f values, and the zero value in particular, was followed by the realization that the aa-tRNA species with the lowest initial intensity reading had the largest increase in intensity upon association with EF-Tu. It then became obvious that the E_b values were very similar for these aa-tRNAs (within 9% of the average E_b value; Table I), despite the great differences in their E_f and E_b/E_f values. This similarity indicates that the environments of the fluorescein probes are close to equivalent in these ternary complexes and hence that the tRNA conformations near s^4U-8 are almost the same in the ternary complexes. Thus, these aa-tRNAs have different conformations in the s^4U-8 region when free in solution but have a similar, and possibly uniform, conformation at that location when bound to EF-Tu·GTP.

The observed similarity in ternary complex emission intensity (Table I) was not due to an interaction between the EF-Tu and the dye in the ternary complex: quenching data showed that the fluorescein was equally accessible to iodide ions (and therefore solvent) in the presence and absence of EF-Tu·GTP (Adkins et al., 1983), and no change in the rotational freedom of the dye was observed when the protein bound to Phe-$tRNA^{Phe}$-Fl^8 (Hazlett et al., 1989). Also, since these aa-tRNAs have different primary structures, it appears that the tertiary structure near s^4U-8, as detected by the fluorescein emission intensity, was not sensitive to the sequence of an aa-tRNA that was bound to EF-Tu·GTP. In effect, any primary structure differences in aa-tRNAs appear to be obscured in the ternary complex.

A purpose for this ET-Tu-mediated conformational change is suggested by the following considerations. First, each of the many different aa-tRNAs in a cell must be decoded at a single recognition site on the ribosome. Second, the conformations of various tRNA species are similar, but not the same (Table I; Crothers & Cole, 1978; Vlassov et al., 1981; Rigler & Wintermeyer, 1983). It follows that the different aa-tRNAs can be evaluated *uniformly* at a *single* ribosomal site only if there is some means to minimize the conformational differences between the various aa-tRNAs before they encounter the ribosome. Minimizing such differences would both maximize the uniformity in the codon–anticodon interaction and simplify the structural requirements for the recognition site on the ribosome that has to accommodate a large number of different tRNA species. Our data indicate that EF-Tu functions, at least in part, by binding to an aa-tRNA and inducing or selecting a tRNA conformation that is the same, or nearly so, for each aa-tRNA species. In this way, EF-Tu would catalyze protein synthesis by ensuring uniformity in the conformation of different aa-tRNA species at the beginning of the recognition and binding process.

Aside from the anticodon, the structural features of the aa-tRNA that are important in proper factor-dependent binding to ribosomes have not yet been established. However, a point mutation in the dihydrouridine stem of *E. coli* $tRNA^{Trp}$ converts it to a suppressor tRNA (Hirsch, 1971; Hirsch & Gold, 1971), and the photo-cross-linking of its s^4U-8 to the cytidine at position 13 alters the coding properties of the mutant tRNA (Vacher & Buckingham, 1979). Hence, regions near the elbow of the tRNA, and far from the anticodon, are involved, directly or indirectly, in the decoding process. In this regard, it should be noted that the s^4U-8 region is exposed in the ternary complex and available for interaction with the ribosome (Adkins et al., 1983). It should also be noted that the strength of a codon–anticodon interaction depends upon the conformations of the triplets involved in the base pairing (Grosjean et al., 1978). Hence, one might expect that an EF-Tu-dependent conformational change in an aa-tRNA

would include its anticodon region so that codon–anticodon complementarity could also be examined uniformly by the ribosome at its single decoding site. Consistent with this possibility, both crystallographic and chemical modification data indicate that the anticodon loop is conformationally coupled to the region where the two arms of the tRNA meet, near s^4U-8 and the D and T loops (Moras et al., 1986). Codon-dependent conformation changes in the midsection of the tRNA have also been reported [see Schwarz et al. (1976) and Wagner and Garrett (1979) and references cited therein]. In addition, the EF-Tu-dependent structural changes in the aa-tRNA that have been identified by using a variety of techniques have been localized both in the corner of the L-shaped tRNA and in the anticodon loop (Kruse et al., 1978; Weygand-Durasevic et al., 1981; Wikman et al., 1982; Adkins et al., 1983; Riehl et al., 1983).

The structural origin of the EF-Tu-dependent fluorescence change is not certain, but it probably results from a relocation of the sugar–phosphate backbone near s^4U-8 (Adkins et al., 1983). Previous studies have shown that the fluorescence change associated with the binding of EF-Tu·GTP to Phe-$tRNA^{Phe}$-Fl^8 was accompanied by an increase in fluorescein absorbance (Adkins et al., 1983) without a change in fluorescein lifetime (and presumably quantum yield) (Hazlett et al., 1989). Since the absorbance, and therefore the emission, of fluorescein is very sensitive to pH (Mercola et al., 1972), it seems likely that the binding of EF-Tu to the tRNA caused a phosphate negative charge(s) to be repositioned further away from the fluorescein dye, thereby increasing the ionization of its ring hydroxyl by altering the local pH and/or the pK_a of the hydroxyl group. The same reasoning suggests that the different E_f values observed for the different aa-tRNA-Fl^8 species (Table I) result from variations in the disposition of phosphate charges around the fluorescein dye in the aa-tRNA species.

The fluorescence-detected structural change in the tRNA near s^4U-8 can be effected by the binding of EF-Tu·GTP to a tRNA even if the tRNA has a blocked α-amino group on its amino acid (fMet-$tRNA_f^{Met}$-Fl^8), or lacks a base pair at the 5′ end of its acceptor arm (Met-$tRNA_f^{Met}$-Fl^8), or lacks the aminoacyl moiety entirely ($tRNA^{Phe}$-Fl^8) (Figure 2). These fluorescence changes were not observed except at high concentrations of EF-Tu·GTP because the above structural elements at the aminoacyl end of the tRNA are important in dictating the affinity between the tRNA and the protein (see below), and hence the macromolecular concentrations necessary to obtain complex formation. However, the existence of these fluorescence changes indicates that the EF-Tu-dependent conformational change in the tRNA is accomplished through a direct interaction between the protein and the tRNA (presumably the acceptor arm).

The affinities of several tRNA species for EF-Tu·GTP were determined, directly and at equilibrium, by their ability to compete with Phe-$tRNA^{Phe}$-Fl^8 for binding to the protein. The K_d values and the standard free energy changes associated with the dissociation of those ternary complexes are shown in Table II. Since these K_d values were measured by using the same technique, they can be compared directly, and this allows us to make several conclusions about the importance of the aminoacyl moiety to ternary complex formation.

Several different groups have reported that unacylated tRNA binds to EF-Tu·GTP (e.g., Shulman et al., 1974; Johnson et al., 1978; Jonák et al., 1980; Österberg et al., 1981; Pingoud et al., 1982; van Noort et al., 1982; Picone & Parmeggiani, 1983; Heerschap et al., 1986; Figure 2), most likely via the acceptor arm of the tRNA [for references, see Miller and Weissbach (1977), Wikman et al. (1982), and Adkins et al. (1983)]. However, the nature and extent of direct interaction between EF-Tu and the tRNA have not been determined. We have now quantified the affinity of unacylated $tRNA^{Phe}$ for the protein (Table II). The K_d value of 2.6 μM determined here was much less than the 0.1–1.0 mM values reported earlier (Österberg et al., 1981; Heerschap et al., 1986), presumably because the sensitivity of the fluorescence technique permitted us to examine the concentration dependence of the association at the low concentrations of macromolecules needed to obtain a meaningful titration curve. Since the dissociation constants for $tRNA^{Phe}$ and for unfractionated tRNA are very similar (Table II), it would appear that most of the unacylated tRNA species have approximately equivalent affinities for EF-Tu·GTP.

The absence of the aminoacyl moiety increases the K_d of the $tRNA^{Phe}$ ternary complex by 2250-fold, an amount consistent with the importance of an aminoacyl group for ternary complex formation. However, when the equilibria are considered in terms of standard free energy changes at 6 °C, it is clear that a substantial fraction of the total free energy of binding is provided by a direct interaction between EF-Tu·GTP and the tRNA portion of the aa-tRNA. In fact, if one assumes that the $tRNA^{Phe}$ conformation is not altered upon aminoacylation and that the protein conformation is the same when bound to tRNA and to aa-tRNA, then most of the binding energy is derived from protein–tRNA interactions and/or contacts. These interactions probably include both electrostatic and nonelectrostatic components, since the binding of aa-tRNA to EF-Tu·GTP has been shown to involve a minimum of two salt bridges (Abrahamson et al., 1985) and a substantial nonelectrostatic contribution (Pingoud et al., 1977; Abrahamson et al., 1985). The interactions also extend beyond the CCA end, since tRNAs lacking their 3′-terminal CCA interact with EF-Tu (Picone & Parmeggiani, 1983). By subtraction, and making the same assumptions as above, interactions between the phenylalanyl moiety and EF-Tu·GTP provide 4.3 kcal/mol of the total ternary complex binding energy for Phe-$tRNA^{Phe}$ at 6 °C in polymix.

The acetylation of Phe-$tRNA^{Phe}$ to form AcPhe-$tRNA^{Phe}$, a peptidyl-tRNA analogue, greatly reduced its affinity for EF-Tu·GTP (Table II). Since the free energy of formation of the AcPhe-$tRNA^{Phe}$ ternary complex was reduced to a value near that of the $tRNA^{Phe}$ ternary complex, the extension of the α-nitrogen moiety by amide bond formation decreased by 74% (3.2/4.3) the contribution of the aminoacyl moiety to the binding energy between the two macromolecules. Formylation of Met-$tRNA_f^{Met}$ also reduced its affinity for EF-Tu·GTP (Table II), but in this case, the difference in $\Delta G°$ values between the ternary complexes of N-blocked and unblocked aa-tRNA species was less (1.2 kcal/mol for Met-$tRNA_f^{Met}$ vs 3.2 kcal/mol for Phe-$tRNA^{Phe}$). The significant difference in the effects of the formyl and acetyl groups on ternary complex formation suggests that the loss of the positive charge on the α-amino group, a common result of these modifications, was not primarily responsible for the loss of binding energy. Instead, since the acetyl group was more effective in reducing productive binding interactions with EF-Tu·GTP than was the formyl group, it appears that the α-amino group normally fits (perhaps in an uncharged state) into a binding site or pocket on EF-Tu·GTP and that N-blocked aminoacyl groups are excluded from the site for steric reasons. Both N-blocked species bound to the protein with about 1 kcal/mol more binding energy than the unacylated tRNA, which suggests that

some aminoacyl moieties, most likely the atoms in the aminoacyl bond itself, do interact with EF-Tu even in a peptidyl-tRNA·EF-Tu·GTP complex.

Both Met-tRNA$_f^{Met}$ and fMet-tRNA$_f^{Met}$ form ternary complexes with EF-Tu·GTP (Table II; Figures 2 and 3), as has been shown by others (Schulman et al., 1974; Tanada et al., 1981, 1982; Louie et al., 1984; Louie & Jurnak, 1985; Fischer et al., 1985; Seong & RajBhandary, 1987). In this paper, we have determined the affinities of three Met-tRNA species for EF-Tu·GTP at equilibrium, in part to address the differences in the relative K_d values reported by others. The effect of the initiator tRNA on tenary complex formation can be evaluated by comparing the K_d values of the ternary complexes containing Met-tRNA$_f^{Met}$ and Met-tRNA$_m^{Met}$. In these complexes, the aminoacyl group is the same, and only the tRNAs differ. EF-Tu interacts less well with Met-tRNA$_f^{Met}$ than with Met-tRNA$_m^{Met}$, and the difference in the binding energies of the elongator and initiator Met-tRNAs for EF-Tu·GTP is relatively small, totaling only 1.2 kcal/mol at 6 °C in polymix (Table II). Thus, neither the different base sequences of these two tRNAs nor the lack of base pairing at the 5′ terminus of tRNA$_f^{Met}$ constitutes a major impediment to Met-tRNA$_f^{Met}$ association with the elongation factor. Of course, formylation of the initiator Met-tRNA increases the ability of EF-Tu·GTP to discriminate between Met-tRNA$_m^{Met}$ and fMet-tRNA$_f^{Met}$ (Table II).

The relative affinities of the formylated and unformylated Met-tRNA$_f^{Met}$ species for EF-Tu·GTP reported in Table II are significantly closer than those reported earlier by others (Tanada et al., 1981, 1982; Seong & RajBhandary, 1987). This difference probably results from the different techniques used to monitor ternary complex formation. Nuclease digestion techniques (Tanada et al., 1981, 1982; Seong & RajBhandary, 1987) focus on the aminoacyl end of the tRNA, and cleaved fMet or Met residues are assumed to be generated only from (f)Met-tRNAs that are not bound to EF-Tu·GTP. Yet the formyl group may be sterically prevented or inhibited from binding to an EF-Tu·GTP binding pocket (see above), and hence be more exposed and subject to nuclease digestion at some rate even if the tRNA is bound via its acceptor arm to the protein. If this did occur, it would cause an underestimation of the extent of ternary complex formation with fMet-tRNA$_f^{Met}$ and an overestimation of the difference in ternary complex K_d values for fMet-tRNA$_f^{Met}$ and Met-tRNA$_f^{Met}$. The unpaired 5′-terminal base of tRNA$_f^{Met}$ may also influence nuclease accessibility to the aminoacyl moiety of tRNAs that are bound to EF-Tu, and this may contribute to the differences in the relative K_d values reported here and earlier for the various Met-tRNA species. In contrast, the fluorescence technique directly counts the number of Phe-tRNA^{Phe}-Fl^8 molecules that occupy EF-Tu·GTP binding sites at equilibrium because those molecules, and only those, experience an increase in steady-state emission intensity.

In sum, the application of the same technique to four identically labeled ternary complexes has allowed us to compare them directly at equilibrium and under nondestructive conditions. Since a single model satisfactorily and reasonably explained the fluoescence data obtained with these ternary complexes, it is now our working hypothesis that one of the functions of EF-Tu is to minimize conformational diversity in aa-tRNAs prior to their beginning the recognition stage of protein biosynthesis. Yet it is important to emphasize that the fluorescein probe monitors only a single region of the aa-tRNA structure. Confirmation of the model will require both a larger sample size of aa-tRNAs and the use of conformationally sensitive spectroscopic probes positioned at other locations in the aa-tRNA to determine whether EF-Tu standardizes the conformation along the entire tRNA molecule.

In addition, the spectroscopic determination, at equilibrium, of the affinity of EF-Tu·GTP for various tRNA species has revealed that the bulk of the free energy of association is derived from interactions between the tRNA and the protein (Table II). In the case of Phe-tRNA^{Phe}, the intact aminoacyl moiety provides less than 40% of the change in free energy associated with ternary complex formation at 6 °C in polymix. Although the identity of the tRNA affects the strength of the ternary complex (Table II; Pingoud & Urbanke, 1980; Wagner & Sprinzl, 1980; Tanada et al., 1982; Louie et al., 1984; Seong & RajBhandary, 1987), the tRNA-dependent effects are relatively small, which indicates that the protein–tRNA interaction is primarily mediated through structural elements common to all tRNAs, such as the CCA end and the ribose–phosphate backbone. A modification at the aminoacyl end of an aa-tRNA, such as the formation of a peptidyl-tRNA, reduces its affinity for the protein, but not to the level of an unacylated tRNA. The nucleotide dependence of the ternary complex and its sensitivity to aurodox (*N*-methylkirromycin) have also been examined recently using fluorescence spectroscopy (Dell et al., 1990).

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