Direct Determination of the Association Constant between Elongation Factor Tu·GTP and Aminoacyl-tRNA Using Fluorescence†

Julie K. Abrahamson,‡ Thomas M. Laue,‡ David L. Miller,§ and Arthur E. Johnson*,‡

Department of Chemistry, University of Oklahoma, Norman, Oklahoma 73019, and New York State Institute for Basic Research, Staten Island, New York 10314
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ABSTRACT: We have investigated the formation of the aa-tRNA·EF-Tu·GTP ternary complex spectroscopically by monitoring a fluorescence change that accompanies the association of EF-Tu·GTP with Phe-tRNA^{Phe}–F^8, a functionally active analogue of Phe-tRNA^{Phe} with a fluorescein moiety covalently attached to the s^{4}U-8 base. With this approach, the protein–nucleic acid interaction could be examined by direct means and at equilibrium. The fluorescence emission intensity of each Phe-tRNA^{Phe}–F^8 increased by 36–55% upon association with EF-Tu·GTP, depending on the solvent conditions. Thus, when Phe-tRNA^{Phe}–F^8 was titrated with EF-Tu·GTP, the extent of ternary complex formation was determined from the increase in emission intensity. A nonlinear least-squares analysis of the titration data yielded a dissociation constant of 0.85 nM for the ternary complex in 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (pH 7.6), 10 mM $MgCl_2$, and 50 mM NH_4Cl, at 6 °C. The $\Delta H°$ of this interaction, determined by the temperature dependence of K_d, was −16 kcal/mol; the $\Delta S°$ was therefore −16 cal mol^{-1} deg^{-1} at 6 °C in this buffer. In a more physiological polycation-containing solvent ("polymix"), the K_d was 4.7 nM. The ionic strength dependence of ternary complex formation showed that a minimum of two salt bridges and a substantial nonelectrostatic contribution are involved in the binding of aa-tRNA to EF-Tu. The affinities of unmodified aa-tRNAs for EF-Tu·GTP were determined by their abilities to compete with the fluorescent aa-tRNA for binding to the protein. As expected, titrations using two different fluorescent aa-tRNAs, Phe-tRNA^{Phe}–F^8 and Val-tRNA$_1^{Val}$–F^8, gave similar values for the K_d of the *Escherichia coli* Phe-tRNA^{Phe} ternary complex. In polymix, the K_d values for yeast Phe-tRNA^{Phe}, *E. coli* Phe-tRNA^{Phe}, and *E. coli* Lys-tRNA^{Lys} are 0.72, 1.16, and 7.7 nM, respectively. The affinity of EF-Tu·GTP for aa-tRNA is much higher than necessary to obtain ternary complex formation in the cell, and this suggests that the very tight association between the protein and the aa-tRNA is required to take full advantage of the discriminatory mechanisms of the ribosomal complex, thereby reducing translational errors.

During protein biosynthesis, aminoacyl-tRNA (aa-tRNA)[1] normally binds to the ribosomal complex as a part of a ternary complex containing GTP and a nonribosomal protein (Miller & Weissbach, 1977; Grunberg-Manago et al., 1978). This protein, designated elongation factor Tu or EF-Tu in *Escherichia coli*, catalyzes protein biosynthesis in vitro, but the mechanism(s) by which it facilitates polypeptide chain elongation is (are) not known. Since the formation of a ternary complex between EF-Tu, aa-tRNA, and GTP is an obligatory step in the elongation cycle, it is important to characterize this complex in order to clarify the nature of its critical role in the aa-tRNA recognition and binding process.

We recently investigated ternary complex conformation and topology using a fluorescent-labeled aa-tRNA (Adkins et al., 1983). Among other things, we found that the fluorescence emission intensity of Phe-tRNA^{Phe}–F^8, a functional analogue of Phe-tRNA^{Phe} with a fluorescein dye covalently attached to the s^{4}U-8 base (Johnson et al., 1982), increased by about 30% when the modified aa-tRNA bound to EF-Tu·GTP at pH 8.0 (Adkins et al., 1983). This large spectral change was EF-Tu·GTP dependent and resulted from an EF-Tu·GTP-induced conformational change in the aa-tRNA near s^{4}U-8 (Adkins et al., 1983).

Phe-tRNA^{Phe}–F^8 therefore provides a unique means of monitoring ternary complex formation and, in particular, of measuring the strength of the association between aa-tRNA and EF-Tu·GTP. Because of the large fluorescence change that accompanies Phe-tRNA^{Phe}–F^8 binding to EF-Tu·GTP, the extent of ternary complex formation in a sample can be determined directly from the magnitude of its emission intensity. From this, the equilibrium constant for ternary complex dissociation can be calculated. The primary advantage of this spectroscopic approach to measuring K_d is that the method is nondestructive and determines K_d in a sample that is actually at equilibrium throughout the time of the measurement.

To date, three different approaches have been used to estimate the affinity of EF-Tu·GTP for various aa-tRNAs: EF-Tu·GTP protection of the aminoacyl bond from nonenzymatic deacylation (Pingoud et al., 1977; Pingoud & Urbanke, 1980; Wagner & Sprinzl, 1980); EF-Tu·GTP protection of the aminoacyl end of the aa-tRNA from nuclease digestion (Knowlton & Yarus, 1980; Tanada et al., 1981, 1982; Louie et al., 1984); the reduction by aa-tRNA of the amount of EF-Tu bound to nitrocellulose filters (Miller et al., 1973;

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‡ University of Oklahoma.
§ New York State Institute for Basic Research.

[1] Abbreviations: EF-Tu, elongation factor Tu; aa-tRNA, aminoacyl-tRNA; PEP, phosphoenolpyruvate; IAAF, 5-(iodoacetamido)-fluorescein; s^{4}U, 4-thiouridine; tRNA^{Phe}–F^8, adduct between IAAF and the s^{4}U-8 base of tRNA^{Phe}; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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Arai et al., 1974; Ofengand, 1974). The dissociation constants reported in these studies have varied considerably, ranging from less than 10 nM to 1 μM for the aa-tRNAs tested.

In this paper, we report the spectroscopically determined K_d values for the dissociation of several different ternary complexes containing modified or unmodified aa-tRNAs. These values are 1–2 orders of magnitude lower than those estimated by less direct methods.

Experimental Procedures

Solvent Systems. Titrations were carried out in one of two solvent systems. Buffer A contained 50 mM Hepes (pH 7.6), 10 mM $MgCl_2$, 50 mM NH_4Cl, and 0.1 mM dithiothreitol, unless specified otherwise. Buffer B, the polycation-containing solvent ("polymix"), was prepared with 10× polymix (Jelenc, 1980) and contained 5 mM Mg^{2+}, 0.5 mM Ca^{2+}, 8 mM putrescine, 1 mM spermidine, 5 mM NH_4^+, 95 mM K^+, 0.1 or 1 mM dithiothreitol, and 5 mM potassium phosphate (pH 7.0). Because we observed a salt precipitate at pH 7.5 in the Jelenc polymix buffer at 0 °C, our buffer B solutions were titrated to pH 7.0 and 0 °C.

Transfer RNA. Escherichia coli $tRNA^{Phe}$ (Plenum Scientific, Hackensack, NJ) and $tRNA_1^{Val}$ (Boehringer, Indianapolis, IN) were reacted with IAAF (Molecular Probes, Junction City, OR) as described previously (Johnson et al., 1982), except that the time of reaction was increased to 17 h. The tRNA species with the fluorescein dye covalently attached to s^4U-8, $tRNA^{Phe}$–F^8 and $tRNA_1^{Val}$–F^8, were purified by RPC-5 chromatography (Johnson et al., 1982).

Modified and unmodified $tRNA^{Phe}$ and yeast $tRNA^{Phe}$ (Boehringer) were aminoacylated as described earlier (Johnson et al., 1982) by using *E. coli* S-100 enzymes prepared according to Johnson et al. (1976) and radioactive phenylalanine purchased from ICN (Irvine, CA). *E. coli* MRE-600 $tRNA^{Lys}$ (Boehringer) was aminoacylated with [^{3}H]lysine (ICN) as described elsewhere (Johnson & Slobin, 1980). The same procedures were used to aminoacylate $tRNA_1^{Val}$–F^8 with [^{14}C]valine (ICN), except that the dithiothreitol concentration was 1 mM instead of 5 mM.

We have found that the advertised specific activities of ICN, New England Nuclear, and Amersham amino acid preparations are occasionally incorrect and are frequently incorrect for [^{3}H]phenylalanine. Consequently, we have routinely determined the actual specific activity of radioactive amino acid preparations using parallel accepting activity assays (Johnson et al., 1982) that differ only in the ratio of nonradioactive to radioactive amino acid in each incubation.

Transfer RNA concentration was determined with $\epsilon_{260} = 6.25 \times 10^5$ M^{-1} cm^{-1}. The amount of aminoacylated tRNA was determined from the amount of radioactivity precipitated in cold trichloroacetic acid according to standard techniques. The fraction of acid-insoluble radioactivity in a sample of aa-tRNA was found to be the same as the fraction of radioactivity that eluted in the void volume following chromatography on Sephadex G-25, at 4 °C in 1 mM potassium acetate (pH 5.0) and 5 mM $MgCl_2$.

EF-Tu. Crystalline EF-Tu·GTP was purified from *E. coli* B cells as described elsewhere (Miller & Weissbach, 1974). EF-Tu·GDP concentrations were determined with $\epsilon_{280} = 41\,600$ M^{-1} cm^{-1}. (A 1.0 mg/mL solution of EF-Tu·GDP had an absorbance at 280 nm of 0.96.) GDP-binding assays showed that the EF-Tu was completely active.

The nucleotide binding activity of the EF-Tu was routinely measured by using [^{3}H]GDP and the nitrocellulose filter binding assays described by Miller & Weissbach (1974), except that no EF-Ts was present. The specific activity of the [^{3}H]GDP was periodically redetermined by using thin-layer chromatography to determine the mole fraction of GDP (Jelenc & Kurland, 1979) and evaporation to determine the amount of ^{3}H exchange into H_2O.

The GDP-binding activity of an EF-Tu sample was also estimated by using gel filtration following an 8 min, 0 °C incubation in the presence of 10 μM [^{3}H]GDP (167–236 dpm/pmol) and 0.55 mg/mL bovine serum albumin. In this case, the minimum amount of active EF-Tu was calculated from the total radioactivity eluted in the void volume from the Sephadex G-25 column (0.6 cm i.d. × 19 cm) at 4 °C.

Protection and Deacylation Assays. Samples (160 μL) were prepared in 1.5-mL capped polypropylene tubes and contained 10 μM GTP, 8 μg of pyruvate kinase, 1 mM PEP, 0.1 mM dithiothreitol, and 1.77 μg of EF-Tu·GDP, either in buffer A, in buffer B, in buffer A at pH 7.0, or in buffer A with 50, 300, or 1000 mM KCl instead of 50 mM NH_4Cl. Deacylation assays differed from the protection assays only in that EF-Tu was omitted from the samples. Following an initial 20-min incubation at 37 °C to convert all GDP to GTP, 9.6–11.7 pmol of aa-tRNA was added to each sample. Immediately after mixing, aliquots were removed and assayed for cold acid insoluble radioactivity as described by Johnson & Adkins (1984). The sample tubes were then capped and placed at 6 °C. Duplicate aliquots were assayed two more times between 2 and 8 h.

Fluorescence Measurements. All fluorescence measurements were made on a Spex Fluorolog spectrofluorometer interfaced to a Tektronix 31 programmable calculator (Adkins et al., 1983). The band-pass was 10 nm on both excitation and emission for all experiments. Slits were kept closed except during scans to avoid photodegradation of the sample. Temperature control was maintained with a Lauda K2R circulating bath attached to the four-position cell holder. Temperatures in the sample cells, determined by using a temperature probe (Fisher Scientific) in a cell containing only solvent, were between 5 and 7 °C during all experiments, unless specified otherwise. The cell holder compartment was flushed throughout each experiment with a steady stream of N_2 in order to prevent condensation on the faces of the cuvettes.

All samples were excited at 480 nm. The absorbance of the samples at 480 nm never exceeded 0.001. Emission intensity was quantified by integration of the uncorrected fluorescence signal at 1-nm intervals from 500 to 600 nm. A minimum of two emission scans were done at each titration point, and fewer than 5% of the duplicate emission intensities differed from their average by more than 0.5%. In all experiments, the background signal due to solvent was subtracted, and all data were corrected for dilution due to the addition of titrant.

In order to monitor the stability of the instrument, the emission intensity of a 8 nM solution of disodium fluorescein in 0.1 N KOH was measured prior to each set of sample scans during a titration. In 52 separate experiments of 3–9-h duration, individual measurements did not differ more than 1.2% from the mean for that experiment, and most were within 0.6% of the mean value. Hence, the lamp intensity was essentially constant during our experiments.

Titration Procedures. Prior to a titration, EF-Tu was incubated with pyruvate kinase and PEP in buffer A or B at 37 °C for 20 min to convert all of the GDP to GTP and thereby ensure that all of the EF-Tu was bound to GTP. This EF-Tu·GTP stock solution was then kept on ice during the course of the titration.

Samples were prepared without EF-Tu directly in 1 × 1 cm quartz fluorescence cuvettes. All samples (3.0 mL) contained

10 μM GTP, 135–168 μg of pyruvate kinase (Sigma), 1 mM PEP, and usually 0.01 A_{260} unit of [^{14}C]Phe-tRNA^{Phe}–F^8 (900–1080 pmol of Phe/A_{260} unit of tRNA; 405–450 Ci/mol of Phe) in buffer A or B. Thus the concentration of Phe-tRNA^{Phe}–F^8 in the samples typically ranged from 2.2 to 4.6 nM. A Teflon-coated magnetic stirrer was added to each cuvette to permit mixing without removal of the cuvettes from the cell holder; this prevented any spectral fluctuations due to temperature changes in the sample. The initial fluorescence emission intensity of each sample, F_0, was determined by averaging a minimum of four separate scans. The initial scans sometimes showed a monotonic increase in emission intensity. In those cases, scans were repeated until a constant emission was observed over six scans. Typically, a stable F_0 was established within 2 h. Robertson & Wintermeyer (1981) also observed that a preincubation of fluorescent tRNA samples was necessary in their experiments.

Following the determination of F_0, two 250- or 300-μL aliquots from each sample were assayed for acid-insoluble radioactivity to establish the initial concentration of aa-tRNA, and GDP binding to the EF-Tu in the stock solution was measured by using filter assays.

Titrations were accomplished by the sequential addition of the EF-Tu·GTP stock solution (in volumes ranging from 1 to 200 μL) to the samples in the cuvettes. After each addition, the solution was gently stirred for 1 min. Emission scans were not initiated until 7 min later in order to ensure complete equilibration and to permit the evaporation of any condensation on the cuvettes. After the final addition of EF-Tu·GTP, three to five sets of duplicate scans were taken at 8-min intervals in order to ensure attainment of equilibrium.

At the end of each experiment, three or four 400-μL aliquots of each sample were assayed for cold acid insoluble radioactivity. The GDP-binding activity of the EF-Tu in the stock solution was also measured by filter binding assay. The total volume of titrant added to a sample during a titration did not exceed 12% of the original volume (2.4 or 2.5 mL), except in the experiments at higher temperatures or high ionic strength, where a greater [EF-Tu] was required for saturation of binding.

Titrations of Val-tRNA$_1^{Val}$–F^8 were carried out as above, except that they initially contained from 6.1 to 7.4 nM [^{14}C]Val-tRNA$_1^{Val}$–F^8 (790 pmol of Val/A_{260} unit of tRNA; 240 Ci/mol of Val) in buffer A.

Ionic Strength Dependence of K_d. The dependence of K_d on the ionic strength was determined as above by using [^{14}C]Phe-tRNA^{Phe}–F^8 in buffer A containing 50, 120, 300, 550, or 1000 mM KCl instead of 50 mM NH_4Cl.

Determination of K_d. In any sample, the total observed fluorescence emission intensity (F) 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 fluorescence intensity per molecule of unacylated tRNA^{Phe}–F^8 (u), free Phe-tRNA^{Phe}–F^8 (f), and Phe-tRNA^{Phe}–F^8 bound to EF-Tu·GTP (b). Since the aminoacylation of tRNA^{Phe}–F^8 does not change its emission intensity (Adkins et al., 1983), $E_u = E_f$. In the absence of EF-Tu·GTP, the fluorescence intensity, F_0, equals $R_t E_f$, where R_t is the total concentration of tRNA^{Phe}–F^8 ($R_u + R_f + R_b$). When EF-Tu·GTP is in excess, so that all Phe-tRNA^{Phe}–F^8 is in a ternary complex, the observed emission intensity is at a maximum and the relative fluorescence emission intensity (F/F_0) is given by

$$(F/F_0)_{max} = (R_b E_b + R_u E_f)/R_t E_f \quad (2)$$

Rearrangement of eq 2 yields

$$E_b/E_f = [(F/F_0)_{max} + y - 1]/y \quad (3)$$

where $y = R_a/R_t$ and $R_a = R_f + R_b$. E_b/E_f was determined for each experiment according to eq 3. The same equations were used to analyze the Val-tRNA$_1^{Val}$–F^8 titrations.

In the presence of a large excess of GTP, the dissociation of the ternary complex is given by

$$\text{aa-tRNA·EF-Tu·GTP} \rightleftarrows \text{aa-tRNA} + \text{EF-Tu·GTP} \quad (4)$$

and this equilibrium is described by the equation

$$K_d = \frac{[\text{aa-tRNA}][\text{EF-Tu·GTP}]}{[\text{aa-tRNA·EF-Tu·GTP}]} = \frac{R_f(T_t - R_b)}{R_b} \quad (5)$$

where the concentration of the free ET-Tu·GTP (T_f) is given by the difference between the total EF-Tu·GTP concentration (T_t) and the concentration of EF-Tu·GTP bound in the ternary complex ($T_b = R_b$). Combining eq 1 and 5 yields an expression for the relative fluorescence intensity in terms of the experimentally determined parameters (R_t, R_a, T_t, E_b/E_f) and the unknown, K_d:

$$F/F_0 = 1 + \frac{(E_b/E_f) - 1}{2R_t}\{(R_a + T_t + K_d) - [(R_a + T_t + K_d)^2 - 4R_a T_t]^{1/2}\} \quad (6)$$

Values of K_d were obtained by performing a nonlinear least-squares fit of the data from individual fluorescence experiments to eq 6 with the NLIN procedure of SAS (Statistical Analysis System, SAS Institute, Inc., Cary, NC). For this analysis, the intercept of 1 (eq 6) was considered a variable since the initial fluorescence measurement does have uncertainty associated with it. The best-fit value of this initial fluorescence measurement ranged from 0.993 to 1.002 for the four sets of data in Table I. The initial estimates for K_d were calculated from the individual data points for each experiment, and the intercept was initially set to 1. Each K_d value reported in Tables I and II was obtained from a nonlinear least-squares analysis of a data set that included all of the data points collected in titrations of a particular type.

The best-fit K_d values returned by NLIN were reported with symmetric, asymptotic 95% confidence intervals. As pointed out by Johnson & Frasier (1984), such confidence limits can be seriously misleading. Therefore, the 67% confidence intervals were obtained by searching the parameter space for the variance ratios predicted by the F statistic (Johnson et al., 1981; Johnson, 1983; Johnson & Frasier, 1984). These 67% confidence intervals were frequently asymmetric and larger than the 95% confidence intervals returned by NLIN. Tests for goodness of fit were done as described by Johnson et al. (1981). The residuals of the nonlinear fit, examined both as a function of F/F_0 and as a function of T_t, were scrutinized for systematic deviations graphically and by fitting to polynomials to the third degree.

We found that neglecting the deacylation of the aa-tRNA during the experiment resulted both in significant systematic deviations in the residuals of the fit and in broader confidence intervals for K_d. Hence, the effect of deacylation on the total concentration of aa-tRNA (R_a) was taken into account at each titration point i according to the equation

$$R_{a,i} = R_{b,i-1} + (R_{a,i-1} - R_{b,i-1})e^{-k\Delta t} \quad (7)$$

where Δt is the time between titration points $i - 1$ and i. In this model, only the free aa-tRNA deacylates. R_b was calculated by using the best estimate of K_d, and the rate constant k was determined from deacylation assays (see above). All K_d values at 6 °C reported in this paper were obtained by using

$k = 1.1 \times 10^{-3}$ min^{-1} for the Phe-tRNA species in buffer A, $k = 3.0 \times 10^{-4}$ for the Phe-tRNA and Lys-tRNA species in buffer B, and $k = 2.25 \times 10^{-4}$ min^{-1} for Val-tRNA$_1^{Val}$–F^8.

Competition Experiments. Samples initially contained 2.8–4.0 nM [^{14}C]Phe-tRNA^{Phe}–F^8 in buffer A or B and either [^{3}H]Phe-tRNA^{Phe}, [^{3}H]Lys-tRNA^{Lys}, or yeast [^{3}H]Phe-tRNA^{Phe} at concentrations between 3.9 and 14.8 nM. Those experiments using Val-tRNA$_1^{Val}$–F^8 initially contained 5.5–5.6 nM [^{14}C]Val-tRNA$_1^{Val}$–F^8 and 4.4–4.8 nM [^{3}H]Phe-tRNA^{Phe} in buffer A. Titrations with EF-Tu·GTP were carried out as described above.

Determination of K_d Values for Unmodified Nonfluorescent aa-tRNAs. Since fluorescence changes are associated only with Phe-tRNA^{Phe}–F^8 binding to EF-Tu·GTP, the expression relating the relative emission intensity to the concentration of Phe-tRNA^{Phe}–F^8 in the ternary complex is the same whether or not nonfluorescent aa-tRNA is also present in the sample. From eq 1–3, this expression is

$$F/F_0 = 1 + [(E_b/E_f) - 1](R_b/R_t) \quad (8)$$

When a sample contains two different aa-tRNA species, R_b at a particular T_t value will depend upon the concentration of the competing aa-tRNA and its affinity for EF-Tu·GTP, since the following two equilibria must hold simultaneously:

$$K_{dR} = R_f T_f / R_b \qquad K_{dS} = S_f T_f / S_b \quad (9)$$

where R refers to the fluorescent aa-tRNA and S to the nonfluorescent aa-tRNA. Since K_{dR} is determined in the single-component titration experiments, and since T_f is given by $T_t - S_b - R_b$, one can derive an equation that relates the amount of bound fluorescent aa-tRNA to the experimentally determined quantities (T_t, R_t, R_a, S_t, S_a, and K_{dR}) and the single unknown, K_{dS}:

$$(K_{dR} - K_{dS})R_b^3 + (T_tK_{dS} + 2R_aK_{dS} + K_{dR}K_{dS} - R_aK_{dR} + S_aK_{dR} - T_tK_{dR} - K_{dR}^2)R_b^2 + (R_aT_tK_{dR} - R_aS_aK_{dR} - 2R_aT_tK_{dS} - R_a^2K_{dS} - R_aK_{dR}K_{dS})R_b + K_{dS}R_a^2T_t = 0 \quad (10)$$

The solutions to this equation are three real, unequal roots, and an algorithm was used to select the correct root. Nonlinear least-squares analyses were then performed to fit eq 8 with convergence on the values of K_{dS} and the intercept as described above. In these experiments, the best-fit intercepts ranged from 1.02 to 1.07. The deacylation of each aa-tRNA was included in our analysis, as detailed earlier. The confidence intervals and the goodness of fit were also determined as above.

Results

Fluorescence-Detected Ternary Complex Formation. The addition of EF-Tu·GTP to a solution of Phe-tRNA^{Phe}–F^8 caused a substantial increase in its fluorescence emission intensity (Figure 1). This enhancement resulted from the association of EF-Tu·GTP with Phe-tRNA^{Phe}–F^8 and a subsequent EF-Tu·GTP-induced conformational change in the aa-tRNA near the s^{4}U-8 base (Adkins et al., 1983).

The extent of this emission intensity increase was solvent dependent. The largest fluorescence change was observed in buffer B, where it averaged 55%. The fluorescence change was 36% in buffer A and 28% in the Hepes-containing buffer (pH 8.0) reported by Adkins et al. (1983). To identify the source of these differences, the EF-Tu·GTP-dependent fluorescence change of Phe-tRNA^{Phe}–F^8 was examined in a buffer A solution that had been adjusted to pH 7.0. The size of this fluorescence increase was found to be the same as in buffer B (see Table I). These results suggest that the magnitude of the observed fluorescence change is primarily pH,

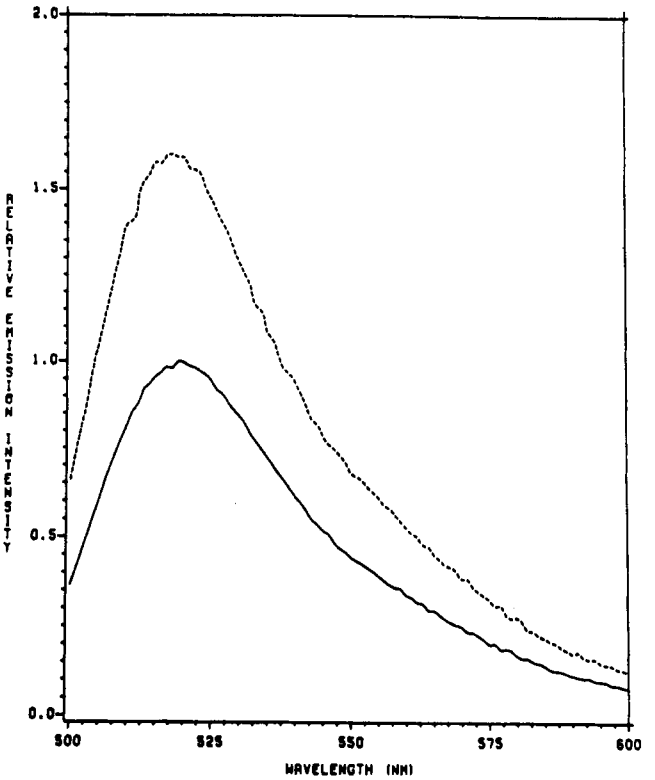

FIGURE 1: Fluorescence change upon ternary complex formation: uncorrected emission spectra for Phe-tRNA^{Phe}–F^8 in buffer B (polymix) before (—) and after (---) the addition of excess EF-Tu·GTP. The emission intensity of the Phe-tRNA^{Phe}–F^8 in the sample prior to EF-Tu addition was arbitrarily defined to be 1.0. Since the addition of EF-Tu·GTP does not alter the emission of tRNA^{Phe}–F^8 (see Figure 2), its contribution to the fluorescence signal has been subtracted.

rather than buffer, dependent and decreases with increasing pH.

The EF-Tu·GTP-induced increase in fluorescence emission intensity for Val-tRNA$_1^{Val}$–F^8 was larger than that observed for Phe-tRNA^{Phe}–F^8 in the same buffer (A). This suggests that the conformation of the Val-tRNA$_1^{Val}$–F^8 differs from that of the Phe-tRNA^{Phe}–F^8. Also, it is interesting to note that the fluorescence emission intensity per mole of free Val-tRNA$_1^{Val}$–F^8 was less than that for free Phe-tRNA^{Phe}–F^8, both in the presence and in the absence of EF-Tu·GTP. No changes in spectral shape upon ternary complex formation were observed in either buffer A or buffer B.

Titration of Fluorescent-Labeled aa-tRNA with EF-Tu·GTP. In order to determine the affinity of EF-Tu·GTP for Phe-tRNA^{Phe}–F^8, we determined the concentration dependence of the association of EF-Tu·GTP with Phe-tRNA^{Phe}–F^8. The formation of the ternary complex was monitored by the change in fluorescence, and as expected, the emission intensity reached a maximum when sufficient EF-Tu·GTP had been added (Figure 2). The fluorescence change occurred over a two-log change in EF-Tu·GTP concentration (Figure 3), which indicates that the fluorescence change monitors a single binding event (Klotz, 1982). This conclusion was further supported by the fact that our data were fit successfully to a model that includes only a single binding event. The fluorescence change was reversible upon the addition of an excess of aminoacyl-tRNA.

The formation of EF-Tu·GTP was ensured by including pyruvate kinase, PEP, and 10^{-5} M GTP in both the aa-tRNA- and the EF-Tu-containing solutions. Experiments were done at 6 °C to reduce the rate of hydrolysis of the aa-tRNA. Although experiments lasted as long as 9 h, the total deac-

Table I: Association of Fluorescent-Labeled aa-tRNA with EF-Tu·GTP[a]

aa-tRNA	buffer	expts	E_b/E_f	K_d (nM)
Phe-tRNA^{Phe}–F^8	A	4	1.36[b] (1.29–1.42)	0.85[c] (0.31–0.97)
Phe-tRNA^{Phe}–F^8	A (pH 7.0)	2	1.55	1.2 (0.77–1.8)
Phe-tRNA^{Phe}–F^8	B	13	1.55 (1.47–1.62)	4.7 (3.6–6.2)
Val-tRNA$_1^{Val}$–F^8	A	4	1.69 (1.63–1.79)	4.3 (2.8–6.5)

[a] Experimental details are given under Experimental Procedures. [b] This represents the average value of E_b/E_f, while the range of values obtained is shown in parentheses. [c] This represents the best-fit K_d returned by the nonlinear least-squares analysis of all of the titration data. The values in parentheses indicate the 67% confidence interval (see Experimental Procedures).

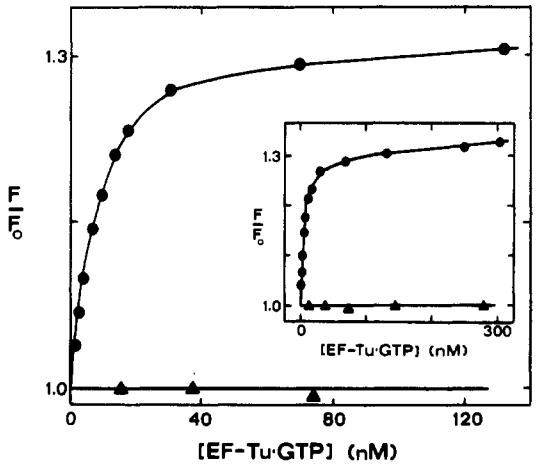

FIGURE 2: EF-Tu·GTP dependence of Phe-tRNA^{Phe}–F^8 fluorescence intensity. The titration initially contained 3.16 nM Phe-tRNA^{Phe}–F^8 (●) in buffer B at 6 °C as described under Experimental Procedures. No fluorescence change was observed when 5.4 nM tRNA^{Phe}–F^8 was titrated with EF-Tu·GTP (▲). The data in the inset are from the same experiments but are plotted on a reduced scale in order to demonstrate the saturation of the fluorescence change.

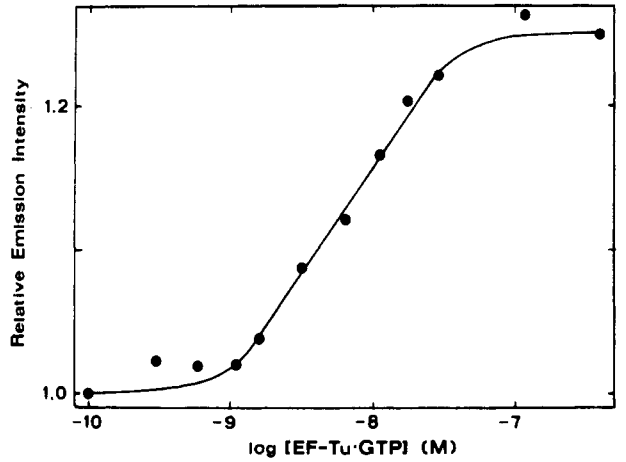

FIGURE 3: Change in fluorescence for Phe-tRNA^{Phe}–F^8, occurring within a two-log change in EF-Tu·GTP concentration. The sample initially contained 2.40 nM Phe-tRNA^{Phe}–F^8 in buffer B and was titrated as described under Experimental Procedures. For this experiment, a portion of the EF-Tu·GTP stock solution was diluted 16-fold in buffer B after the 37 °C incubation and was used for the initial titration points.

ylation never exceeded 13% at 6 °C and in nearly all cases was less than 10%. In the experiments at 15 and 25 °C, as well as in the titrations at high ionic strength, the total deacylation was greater. But in all cases, the deacylation was taken into account in our analysis procedures.

No fluorescence change was observed when Phe-tRNA^{Phe}–F^8 was titrated with EF-Tu·GDP, or when either tRNA^{Phe}–F^8 or tRNA$_1^{Val}$–F^8 was titrated with EF-Tu·GTP, within the concentration ranges used in the ternary complex titrations (see Figure 2). These results are consistent with those reported by Adkins et al. (1983) and support the conclusion that the change in fluorescence emission intensity is due to the binding of Phe-tRNA^{Phe}–F^8 to EF-Tu·GTP in a ternary complex.

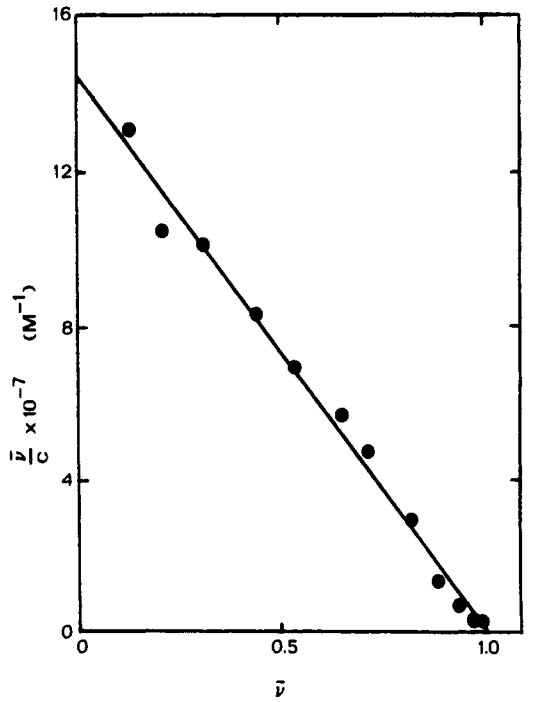

FIGURE 4: Scatchard analysis of the binding data. The data shown in Figure 2 are replotted here according to Scatchard (1949). The straight line drawn was generated by linear least-squares regression analysis of the data.

Calculation of K_d. The K_d values reported in Table I were obtained by nonlinear least-squares analysis of the combined data from all of the titrations of a particular type, as detailed under Experimental Procedures. The 67% confidence intervals for these best-fit K_d values are also shown in Table I.

The values in Table I were obtained with two different preparations of Phe-tRNA^{Phe}–F^8. K_d's calculated from experiments using a higher concentration of Phe-tRNA^{Phe}–F^8 (10.6–13.4 nM) were not significantly different from those for experiments using lower concentrations of Phe-tRNA^{Phe}–F^8 (2.2–4.6 nM) and are also included in the average K_d (for buffer B) listed in Table I. These data indicate that the calculated K_d was independent of both the concentration and preparation of the Phe-tRNA^{Phe}–F^8. Similarly, the same K_d was obtained with two different preparations of EF-Tu.

A Scatchard plot of the binding data of Figure 2 is shown in Figure 4. Linear least-squares regression analysis of the data from this particular experiment gave $n = 1.0$ and $K_d = 7.0 \times 10^{-9}$ M.

These results demonstrate that EF-Tu·GTP has a very high affinity for aa-tRNA. As noted under Experimental Procedures, the EF-Tu concentration in our experiments was determined by absorbance. Hence, the K_d values reported in this paper actually constitute the *maximum* values for K_d, since we cannot rule out the possibility that a fraction of the EF-Tu

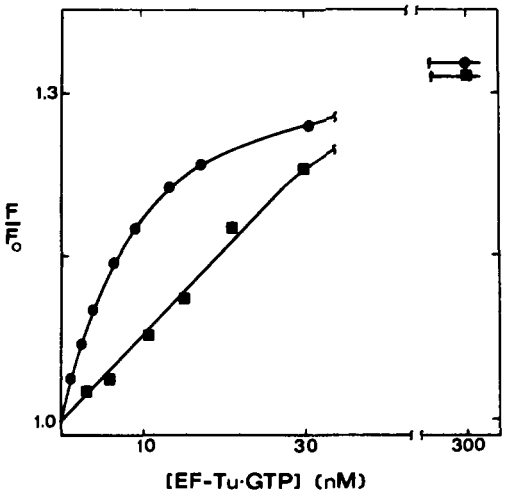

FIGURE 5: Titration of Phe-tRNA^{Phe}–F^8 with EF-Tu·GTP in the presence or absence of competing Phe-tRNA^{Phe}. One sample (●) initially contained Phe-tRNA^{Phe}–F^8 (3.2 nM), while the second sample (■) contained both Phe-tRNA^{Phe}–F^8 (3.2 nM) and Phe-tRNA^{Phe} (14.8 nM). Titrations were performed as described under Experimental Procedures.

Table II: Association of Unmodified aa-tRNA with EF-Tu·GTP[a]

unmodified aa-tRNA	aa-tRNA–F	buffer	expts	K_{dS}[b] (nM)
E. coli Phe-tRNA^{Phe}	Phe-tRNA^{Phe}–F^8	B	5	1.16 (0.55–2.3)
yeast Phe-tRNA^{Phe}	Phe-tRNA^{Phe}–F^8	B	3	0.72 (0.27–1.9)
E. coli Lys-tRNA^{Lys}	Phe-tRNA^{Phe}–F^8	B	7	7.7 (3.6–14.4)
E. coli Phe-tRNA^{Phe}	Phe-tRNA^{Phe}–F^8	A	4	0.10 (0.02–0.26)
E. coli Phe-tRNA^{Phe}	Val-tRNA$_1^{Val}$–F^8	A	5	0.28 (0.03–2.75)

[a] Experimental details are described under Experimental Procedures. [b] The best-fit K_d values for the unmodified aa-tRNAs (=K_{dS}) were determined as detailed under Experimental Procedures by using the K_d values for the fluorescent-labeled aa-tRNAs given in Table I. The values in parentheses indicate the 67% confidence interval.

was competent to bind GDP but unable to participate in ternary complex formation.

Determination of K_d Values for Unmodified Nonfluorescent Aminoacyl-tRNAs. The affinities of nonfluorescent unmodified aa-tRNAs for EF-Tu·GTP can be determined by their ability to compete with the fluorescent-labeled aa-tRNAs for binding to the protein. Thus, we titrated a mixture of unmodified nonfluorescent aa-tRNA and fluorescent-labeled aa-tRNA with EF-Tu·GTP. As expected, at the end of the titration, when EF-Tu·GTP was in excess, the total fluorescence change was the same in a sample containing only Phe-tRNA^{Phe}–F^8 at 3.2 nM as in a sample containing Phe-tRNA^{Phe}–F^8 at 3.2 nM and Phe-tRNA^{Phe} at 14.8 nM (Figure 5). However, in the early stages of the titration when EF-Tu·GTP was limiting, a smaller rate of increase in emission intensity was observed in the latter sample due to competition between Phe-tRNA^{Phe}–F^8 and Phe-tRNA^{Phe} for the available EF-Tu·GTP.

The ternary complex dissociation constants for three unmodified aa-tRNAs under different conditions are listed in Table II, along with the 67% confidence intervals. These K_d values were obtained by nonlinear least-squares analysis of the combined data from all of the titrations with a particular pair of aa-tRNAs, as described under Experimental Procedures. The same K_d values were obtained when competition experiments were done with a range of concentrations of the unmodified aa-tRNA (see Experimental Procedures). The confidence intervals for the titrations in Table II are larger than for the titrations in Table I because fewer data points were used in the analysis of the competition experiments. In addition, because the K_d values were lower than anticipated, the data points were asymmetrically disposed about the K_d; the consequences of such asymmetry are discussed by Johnson & Frasier (1984).

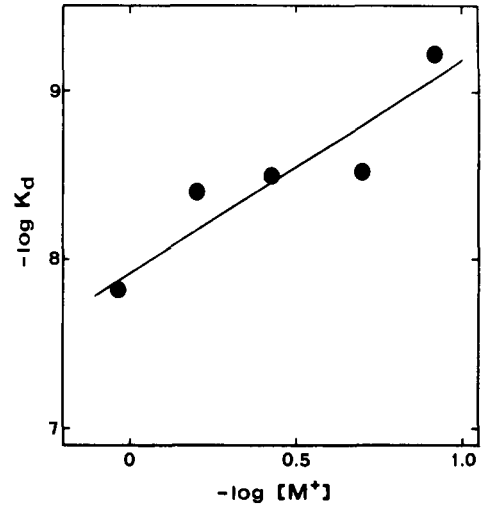

FIGURE 6: Ionic strength dependence of the ternary complex dissociation constant. Titrations were carried out in the presence of 50–1000 mM K^+ as described under Experimental Procedures. [M^+] represents the total concentration of monovalent cations in the samples. Each of the K_d values was obtained from the nonlinear least-squares best fit of the combined data from two or three separate experiments. The straight line was generated by a linear least-squares regression analysis of the data.

In principle, the K_d value found for a particular unmodified nonfluorescent aa-tRNA should be independent of the nature of the fluorescent-labeled aa-tRNA used in the competition experiments. Since Phe-tRNA^{Phe}–F^8 and Val-tRNA^{Val}–F^8 differ in their affinity for EF-Tu·GTP (Table I), we investigated whether or not competition experiments using these two fluorescence probes would yield the same K_d value for Phe-tRNA^{Phe}. As can be seen from the data in Table II, the K_d values obtained for Phe-tRNA^{Phe} with two different spectral probes were very similar. The similarity is particularly noteworthy because the value obtained for the K_d of an unmodified aa-tRNA ternary complex is very sensitive to the value used in the calculations for the K_d of the fluorescent aa-tRNA ternary complex. Thus, the K_d values determined by our approach appear to be independent of the spectral probe used in the titration.

On the basis of the data shown in Tables I and II, Phe-tRNA^{Phe}–F^8 has a lower affinity for EF-Tu·GTP than does Phe-tRNA^{Phe}. This suggests that the fluorescein dye somehow interferes with the protein–nucleic acid interaction. Such an effect by the dye would have to be allosteric, since EF-Tu does not cover this region of the aa-tRNA when in the ternary complex (Adkins et al., 1983). However, Adkins et al. (1983) observed some heterogeneity in Phe-tRNA^{Phe}–F^8 preparations, and it is possible that the fluorescent-labeled aa-tRNA solutions contain molecules with a reduced capacity to bind to or interact with EF-Tu·GTP.

Ionic Strength Effects. The electrostatic contribution to the interaction between a particular protein and nucleic acid can be evaluated by examining the ionic strength dependence of the K_d (Record et al., 1976, 1978; Lohman et al., 1980). The ionic strength dependence of the ternary complex K_d is shown in Figure 6. The increase in K_d with increasing ionic

strength demonstrates that the affinity of EF-Tu·GTP for aa-tRNA does involve ionic interactions. It is also clear that there is a major nonelectrostatic component to the binding, since the K_d is quite low, even in 1 M KCl.

Under some circumstances, the number of ion pairs involved in a protein–nucleic acid interaction can be determined by using the analysis procedures detailed by Record et al. (1976) and Lohman et al. (1980). If we assume that no anions are displaced during the EF-Tu·GTP association with aa-tRNA and that Mg^{2+} effects are limited to electrostatic competition with K^+ and EF-Tu for phosphate binding, then, using the results of Lohman et al. (1980), we calculate from the data in Figure 6 that there are between 1.4 and 1.9 ion pairs involved in ternary complex formation. The 1.4 value is obtained if one assumes that the protein binds only to double-stranded RNA and that the ψ value of 0.89 found for poly(rA)·poly(rU) (Record et al., 1976) is appropriate. The 1.9 value is obtained by using the ψ value of 0.68 for single-stranded poly(rU). Unfortunately, it is not known to what extent EF-Tu binds single-stranded and double-stranded regions of the aa-tRNA (see Discussion).

The data in Figure 6 indicate the extent of K^+ ion release during ternary complex formation under our conditions (i.e., in the presence of Mg^{2+}). Hence, the number of ion pairs calculated above represents the *minimum* number of ion pairs involved in ternary complex formation. EF-Tu does not bind GTP in the absence of Mg^{2+} (Ravel & Shorey, 1969; Leupold et al., 1983), thereby precluding a determination of the ionic strength dependence of ternary complex formation in the absence of divalent cations. In addition, numerous studies have shown that the conformation of tRNA is sensitive to $[Mg^{2+}]$ [e.g., Crothers & Cole (1978)], and consistent with such observations, we have found that the fluorescence of $tRNA^{Phe}$–F^8 is dependent upon the Mg^{2+} concentration (J. K. Abrahamson and A. E. Johnson, unpublished data). Thus, the above assumption that the only role of Mg^{2+} is to compete with K^+ and EF-Tu for binding to the tRNA phosphates is probably not justified.

The formation of the ternary complex at high ionic strength was confirmed by protection assays that showed an EF-Tu-dependent reduction of aminoacyl ester bond hydrolysis even at 1 M KCl (data not shown).

Temperature Dependence of K_d. The ternary complex dissociation constants for Phe-$tRNA^{Phe}$–F^8 in buffer A were determined to be 1.4 nM at 15 °C and 5.4 nM at 25 °C, according to the same procedures described under Experimental Procedures, except that the time of individual scans was reduced in order to minimize deacylation during the experiment. The best-fit K_d values were obtained as described (Experimental Procedures) by using deacylation rate constants (k) of 2.5×10^{-3} min^{-1} and 7.2×10^{-3} min^{-1} at 15 and 25 °C, respectively, determined as described under Experimental Procedures. A van't Hoff plot ($\ln K_a$ vs. $1/T$) of the K_d values at 6, 15, and 25 °C indicates that the $\Delta H°$ of this interaction is −16 kcal/mol of complex.

Discussion

The mechanism by which the ribosomal complex enhances the discrimination between aa-tRNAs that are correctly and incorrectly base paired to the mRNA codon is unknown. An essential prerequisite to elucidating that mechanism is the characterization, both thermodynamic and kinetic, of the interactions between the various macromolecules and multicomponent complexes that participiate in the recognition process. We have investigated the formation of the aa-tRNA·EF-Tu·GTP ternary complex by monitoring a fluorescence change that accompanies the association of Phe-$tRNA^{Phe}$–F^8 with EF-Tu·GTP (Adkins et al., 1983; Figure 1). This approach allowed us to examine the protein–nucleic acid interaction by direct means and at equilibrium.

The importance of examining this interaction under equilibrium conditions is demonstrated by comparing the K_d values obtained spectroscopically and those obtained by other techniques. The ternary complex dissociation constant at low temperature for *E. coli* Phe-$tRNA^{Phe}$ was estimated to be 1–10 nM (Miller et al., 1973) or 72 nM (Arai et al., 1974) by filter assay, and 79 nM (Tanada et al., 1982) or 100 nM (Derwenskus & Sprinzl, 1983) by nuclease protection assays. Although variations in the ionic strengths and temperatures of the incubations make direct comparisons impossible, it is apparent that, in general, these methods underestimated the strength of the ternary complex with Phe-$tRNA^{Phe}$, shown by our results to have a K_d of 0.13 nM at 6 °C in a buffer similar to those used in the other studies. At 25 °C, the value for the Phe-$tRNA^{Phe}$ ternary complex K_d determined by protection assay was 20 nM (Pingoud & Urbanke, 1980), while the K_d for Phe-$tRNA^{Phe}$–F^8 at 25 °C was found to be 5.4 nM (see Results). Since the K_d of Phe-$tRNA^{Phe}$ is less than that of Phe-$tRNA^{Phe}$–F^8 (Tables I and II), the 20 nM figure considerably underestimates the affinity of Phe-$tRNA^{Phe}$ for EF-Tu·GTP. This underestimation is to be expected, since only the spectroscopic measurements were obtained under equilibrium conditions. On the other hand, the nonequilibrium methods do provide an indication of the relative affinities of various aa-tRNAs for EF-Tu·GTP: while the K_d for Lys-$tRNA^{Lys}$ is 6.6-fold higher than the K_d for Phe-$tRNA^{Phe}$ by spectroscopy (Table II), it is 3.2- to 4.3-fold higher by nuclease protection experiments (Louie et al., 1984) and 12.5-fold higher by aminoacyl bond protection experiments (Pingoud & Urbanke, 1980). These differences may result from the differences in the experimental conditions.

It is also important to emphasize that the spectroscopic method described in this paper does not require the modification of an aa-tRNA to determine the K_d of its ternary complex. Instead, the affinity of an unmodified aa-tRNA for EF-Tu·GTP is determined by its ability to compete with a fluorescent-labeled aa-tRNA for binding to the protein. The identity of the fluorescent aa-tRNA is irrelevant: the fluorescent aa-tRNA only provides a mechanism for monitoring the extent of association of the unmodified aa-tRNA with the protein. The approach described in this paper is therefore a general approach for the determination of the binding affinity of a nucleic acid for EF-Tu·GTP.

Our experiments demonstrate that EF-Tu·GTP has a high affinity for aa-tRNA, and it is appropriate to consider why the binding between aa-tRNA and EF-Tu·GTP is so tight. Since the intracellular concentrations of EF-Tu and aa-tRNA are in excess of 100 μM (Ingraham et al., 1983), K_d values on the order of 1 μM would be sufficient to ensure ternary complex formation. However, the actual K_d values are approximately 100-fold lower. This suggests that the strong affinity of EF-Tu·GTP for aa-tRNA is required for something besides ternary complex formation. It is our hypothesis that the tight association between the protein and the aa-tRNA is necessary to take full advantage of the discriminatory mechanisms of the ribosomal complex. Stated most simply, because of the high affinity of EF-Tu·GTP for the aa-tRNA, the aa-tRNA will be released from the protein and will be free to bind to the ribosome only after the correct codon–anticodon interaction triggers a mechanism to disrupt the ternary complex. This mechanism would presumably involve the hydrolysis

of the GTP to GDP and the formation of the EF-Tu·GDP complex, which has a low affinity for aa-tRNA.

Hopfield (1974) and Ninio (1975) have proposed that the recognition process is a two-stage mechanism involving "proofreading". In this scheme, an initial codon–anticodon-mediated discrimination of various ternary complexes is followed by a second discriminatory stage (=proofreading) that is initiated by the hydrolysis of GTP and the dissociation of EF-Tu·GDP in those ternary complexes that successfully complete the first stage. There is some experimental evidence that supports this hypothesis (Thompson & Stone, 1977; Thompson et al., 1981; Yamane et al., 1981; Ruusala et al., 1982; Andersson & Kurland, 1983). If this is indeed the mechanism, then the tight binding between EF-Tu·GTP and aa-tRNA may be required to ensure that the ternary complex remains intact during the first stage of the recognition process. This would minimize the chances of premature release of the factor from the aa-tRNA and the possible passage of aa-tRNA into the proofreading stage prior to completion of the first stage. In effect, the high affinity of EF-Tu·GTP for aa-tRNA would reduce translational errors by ensuring that maximum discrimination was achieved during the first stage of the recognition process.

The importance of the solvent milieu on the ternary complex K_d is clearly shown by our results (Tables I and II; Figure 6). It is particularly noteworthy that the protein–nucleic acid binding is much tighter in a commonly used Hepes-based in vitro milieu than in the more physiological polymix milieu. This difference does not result from a difference in solvent pH (Table I) but rather from the difference in solvent ionic strength: the K_d in polymix is approximately the K_d expected in a Hepes-buffered solution of the same ionic strength (Figure 6). In any event, the differences in the K_d obtained in polymix compared with that obtained in other solvent systems will have to be considered when data from the different solvent systems are compared.

Recent experiments have shown that the acceptor arm of various aa-tRNAs is protected by EF-Tu·GTP from digestion by the double-strand-specific cobra venom ribonuclease (Boutorin et al., 1981). These data, together with other nuclease protection studies, have been interpreted to mean that the EF-Tu binds to the aa-tRNA along its acceptor arm (Jekowsky et al., 1977; Boutorin et al., 1981; Wikman et al., 1982). An interaction between EF-Tu and tRNA lacking the 3′-terminal C-C-A has also been reported (Picone & Parmeggiani, 1983). Since EF-Tu must bind to all elongator aa-tRNAs, no matter what the base sequence, one might expect that the putative binding along the acceptor arm would involve salt bridges between the protein and the phosphates on the aa-tRNAs. This view would appear to be supported by the fact that the ternary complex K_d values are nearly the same for yeast and *E. coli* Phe-tRNA^{Phe} (Table II; Pingoud & Urbanke, 1980; Tanada et al., 1982), despite the considerable difference in their acceptor arm sequences (Sprinzl et al., 1978).

Our data indicate that there are a minimum of two salt bridges involved in ternary complex formation. However, as detailed under Results, the actual number of ionic interactions cannot be determined. There also appears to be a substantial nonelectrostatic component of the binding between EF-Tu and aa-tRNA. Using protection assays, Pingoud et al. (1977) observed an ionic strength dependence of the Tyr-tRNA^{Tyr} ternary complex K_d that was very similar to that of Figure 6, and they also concluded that there was a considerable nonelectrostatic contribution to the binding energy. The interaction between EF-Tu·GTP and aa-tRNA can be further characterized in terms of enthalpy and entropy changes. At 6 °C in buffer A, the $\Delta G°$ for the Phe-tRNA^{Phe}–F^8 ternary complex is −11.6 kcal/mol, with a $\Delta H°$ of −16 kcal/mol and a $\Delta S°$ of −16 cal mol^{-1} deg^{-1}.

Although the exact nature of the aa-tRNA structural changes that elicit the changes in fluorescence emission has not been identified, we have now shown that two different aa-tRNAs behave similarly when confronted with EF-Tu·GTP. The emission intensities of both Phe-tRNA^{Phe}–F^8 and Val-tRNA$_1^{Val}$–F^8 increase when they associate with EF-Tu·GTP. The sensitivity of the s^{4}U-conjugated dye to the binding of EF-Tu·GTP (allosterically; Adkins et al., 1983) suggests that the region of the aa-tRNA near the s^{4}U is particularly susceptible to conformational changes, since aa-tRNAs with fluorescent moieties elsewhere have not exhibited spectral changes upon association with EF-Tu·GTP (Beres & Lucas-Lenard, 1973; Sprinzl & Faulhammer, 1978). This region has also been shown to be sensitive to the presence or absence of Mg^{2+} (Heerschap et al., 1983; Abrahamson and Johnson, unpublished data). Since the binding of Phe-tRNA^{Phe}–F^8 to ribosomes elicits an additional change in fluorescence emission (A. E. Johnson, unpublished data), it is possible that the aa-tRNA undergoes an additional conformational change upon binding to the ribosomal complex. Whatever the case, the additional spectral change will allow us to evaluate the thermodynamics and kinetics of the aa-tRNA interaction with the ribosomal complex and its dependence upon EF-Tu.

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