Effects of Nucleotide- and Aurodox-Induced Changes in Elongation Factor Tu Conformation upon Its Interactions with Aminoacyl Transfer RNA. A Fluorescence Study†

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ABSTRACT: The effects of GDP and of aurodox (*N*-methylkirromycin) on the affinity of elongation factor Tu (EF-Tu) for aminoacyl-tRNA (aa-tRNA) have been quantified spectroscopically by using Phe-tRNA^{Phe}-Fl^{8}, a functionally active analogue of Phe-tRNA^{Phe} with a fluorescein dye covalently attached to the s^{4}U-8 base. The association of EF-Tu·GDP with Phe-tRNA^{Phe}-Fl^{8} resulted in an average increase of 33% in fluorescein emission intensity. This spectral change was used to monitor the extent of ternary complex formation as a function of EF-Tu·GDP concentration, and hence to obtain a dissociation constant, directly and at equilibrium, for the EF-Tu·GDP-containing ternary complex. The K_d for the Phe-tRNA^{Phe}-Fl^{8}·EF-Tu·GDP complex was found to average 28.5 μM, more than 33 000-fold greater than the K_d of the Phe-tRNA^{Phe}-Fl^{8}·EF-Tu·GTP complex under the same conditions. In terms of free energy, the $\Delta G°$ for ternary complex formation at 6 °C was −11.5 kcal/mol with GTP and −5.8 kcal/mol with GDP. Thus, the hydrolysis of the ternary complex GTP results in a dramatic decrease in the affinity of EF-Tu for aa-tRNA, thereby facilitating the release of EF-Tu·GDP from the aa-tRNA on the ribosome. Aurodox (200 μM) decreased the K_d of the GDP complex by nearly 20-fold, to 1.46 μM, and increased the K_d of the GTP complex by at least 6-fold. The binding of aurodox to EF-Tu therefore both considerably strengthens EF-Tu·GDP affinity for aa-tRNA and also weakens EF-Tu·GTP affinity for aa-tRNA. As a result, aurodox (and by extension kirromycin) may interfere with EF-Tu–ribosome interactions both/either prior to GTP hydrolysis by interfering with ternary complex recognition and binding by the ribosome and/or following GTP hydrolysis by retarding the release of EF-Tu·GDP from the ribosome. Yet the relatively small magnitude of the aurodox effect upon the affinity of EF-Tu·GDP for aa-tRNA suggests that the strong antibiotic-dependent retardation in EF-Tu·GDP dissociation is mediated primarily by ribosome–EF-Tu interactions. The replacement of GTP by GDP had no detectable effect upon the magnitude of the spectral change that accompanied ternary complex formation, while aurodox caused a small reduction in the size of the spectral change obtained with EF-Tu·GDP. Thus, the EF-Tu-mediated conformational change in the hinge region of the aa-tRNA that is detected by the fluorescent probe appears to be nucleotide-independent and appears to be only slightly altered by the binding of aurodox to EF-Tu·GDP.

The binding of aminoacyl-tRNA (aa-tRNA)[1] to the ribosome during protein biosynthesis in prokaryotes is catalyzed by elongation factor Tu (EF-Tu) (Miller & Weissbach, 1977a,b; Kaziro, 1978). When associated with GTP, this nonribosomal protein can bind to each aa-tRNA, and the resulting ternary complex then diffuses to the ribosomal complex to begin the codon-mediated recognition and binding process. The binding of an aa-tRNA to the ribosome is accompanied by the hydrolysis of GTP in the ternary complex to GDP, and the subsequent dissociation of EF-Tu·GDP from the aa-tRNA and the ribosomal complex. Since a thorough understanding of the ternary complex is a prerequisite to understanding its participation in the aa-tRNA recognition and binding process, many different aspects of the structure and function of the ternary complex have been examined (Miller & Weissbach, 1977a,b; Kaziro, 1978; Johnson et al., 1986).

For example, both the conformation and the functional state of EF-Tu have been shown to be dictated by the binding of small ligand molecules to the protein. GTP (K_d ~300 nM) and GDP (K_d ~3 nM) each bind tightly to EF-Tu (Miller & Weissbach, 1977a), but the structures of EF-Tu·GTP and EF-Tu·GDP have been shown to differ by using several techniques [reviewed in Miller and Weissbach (1977b) and Kaziro (1978)]. Since only EF-Tu·GTP forms a tight complex with aa-tRNA and facilitates the binding of aa-tRNA to ribosomes (Miller & Weissbach, 1977b; Kaziro, 1978), the nucleotide-dependent change in EF-Tu structure alters its affinity for aa-tRNA and may alter its ability to interact with the ribosomal complex. The former was demonstrated by Pingoud et al. (1982), who used a deacylation protection assay to show that aa-tRNA does associate with EF-Tu·GDP, but

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

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much more weakly than with EF-Tu·GTP.

Two other small ligands that alter the functional state of EF-Tu are antibiotics: kirromycin and aurodox, the equally effective N-methylated derivative of kirromycin [reviewed in Parmeggiani and Swart (1985)]. The association of kirromycin or aurodox with EF-Tu (K_d <1 μM; Pingoud et al., 1978; Eccleston, 1981) inhibits protein synthesis, but the exact mechanism of action of the antibiotics is uncertain. Two proposals have been put forth to explain their mode of action. Wolf et al. (1977) observed that the protein–antibiotic complex remains bound to the ribosome after EF-Tu-mediated GTP hydrolysis and suggested that kirromycin induces a conformation in the GDP-bound form of the protein that is similar to that of EF-Tu·GTP. Then, after GTP hydrolysis, the affinity of EF-Tu·GDP for aa-tRNA and perhaps for the ribosome would remain high, and the rate of dissociation of EF-Tu from the ribosome would be decreased. Since elongation factor G (EF-G) and EF-Tu cannot bind to the ribosome simultaneously [for references, see Miller and Weissbach (1977a)], the interaction of EF-G with the ribosome and therefore chain elongation would be inhibited when kirromycin is present. Alternatively, Pingoud et al. (1978) proposed that kirromycin alters the structure of EF-Tu·GTP and thus the topology of the ternary complex, thereby inhibiting the recognition and binding of aa-tRNA by the ribosome prior to GTP hydrolysis. Evidence for antibiotic-dependent conformational changes in both EF-Tu·GDP and EF-Tu·GTP have been presented by several groups [e.g., Chinali et al. (1977) and Douglass and Blumenthal (1979); reviewed in Parmeggiani and Swart (1985)].

A unique approach to investigating the interactions between the macromolecules of the ternary complex was introduced by Adkins et al. (1983), who used a fluorescent-labeled aa-tRNA to monitor ternary complex formation spectroscopically. The tRNA-bound probe provided an important perspective on this protein–nucleic acid association because two different aspects of the EF-Tu/aa-tRNA interaction could be examined: an EF-Tu-mediated change in aa-tRNA conformation and the affinity of EF-Tu for the aa-tRNA. Both of these protein-dependent properties are likely to be important for the function of EF-Tu, as we have discussed previously (Adkins et al., 1983; Abrahamson et al., 1985; Johnson et al., 1986).

The binding of EF-Tu·GTP to Phe-tRNA^{Phe}-Fl^{8}, a Phe-tRNA with a fluorescein dye covalently attached to its 4-thiouridine base, causes a conformational change in the aa-tRNA that can be detected spectroscopically (Adkins et al., 1983). Abrahamson et al. (1985) used this spectral change to measure the dissociation constant of the Phe-tRNA^{Phe}-Fl^{8} ternary complex directly and at equilibrium. The affinities of unmodified, and therefore nonfluorescent, aa-tRNAs for EF-Tu·GTP were determined by their abilities to compete with the fluorescent Phe-tRNA^{Phe}-Fl^{8} for binding to a limited amount of EF-Tu·GTP (Abrahamson et al., 1985). The ternary complex K_d values determined at equilibrium by this spectroscopic approach (Abrahamson et al., 1985) were substantially lower than those obtained by using various indirect, nonequilibrium techniques [e.g., Miller et al. (1973), Arai et al. (1974), Ofengand (1974), Pingoud et al. (1977, 1982), Pingoud and Urbanke (1980), Wagner and Sprinzl (1980), Knowlton and Yarus (1980), Tanada et al. (1981, 1982), Louie et al. (1984), and Louie and Jurnak (1985)].

Since the molecular mechanism by which EF-Tu is released from the aa-tRNA and the ribosome involves the nucleotide-dependent conformational change in the protein, it is important to quantify the difference between the EF-Tu·GTP and EF-Tu·GDP interactions with aa-tRNA. Furthermore, since aurodox and kirromycin may interfere with this process, it is necessary to determine the magnitude of the antibiotic's effect upon those interactions. Previous investigations have shown that EF-Tu·GDP has a lower affinity for aa-tRNA than EF-Tu·GTP (Miller & Weissbach, 1977a; Pingoud et al., 1982) and that aurodox both strengthened the GDP-containing complex and weakened the GTP-containing complex [reviewed in Parmeggiani and Swart (1985)]. However, since these studies employed indirect methods to monitor ternary complex formation, their results cannot be compared directly to K_d values that have been determined spectroscopically. We have therefore used Phe-tRNA^{Phe}-Fl^{8} to quantify, at equilibrium, the affinity of EF-Tu for aa-tRNA in the presence of GDP, GTP, and/or aurodox, and also to investigate the effects of both GDP and aurodox on the EF-Tu-dependent conformational change in the aa-tRNA.

Experimental Procedures

tRNA. Escherichia coli tRNA^{Phe} (Subriden RNA, Rolling Bay, WA) was reacted with 5-(iodoacetamido)fluorescein (Molecular Probes, Eugene, OR), and tRNA^{Phe}-Fl^{8}, the tRNA species with the fluorescent dye covalently attached to s^4U-8, was purified as described elsewhere (Johnson et al., 1982; Abrahamson et al., 1985). The tRNAs were aminoacylated as before (Johnson et al., 1982) either in the presence (tRNA^{Phe}) or in the absence (tRNA^{Phe}-Fl^{8}) of 1 mM dithiothreitol by using *E. coli* S-100 enzymes prepared according to Johnson et al. (1976). The [^{14}C]Phe-tRNA^{Phe}-Fl^{8} and [^{14}C]Phe-tRNA^{Phe} samples used in most experiments were aminoacylated to 1380 and 1370 pmol of Phe/A_{260} unit of tRNA, respectively, at a specific activity of 999 dpm/pmol of Phe. Transfer RNA concentration was determined in 50 mM Hepes (pH 7.6), 10 mM $MgCl_2$, and 50 mM NH_4Cl (unless noted otherwise, this buffer was used throughout this study) by using $\epsilon_{260} = 6.25 \times 10^5$ M^{-1} cm^{-1}. Samples of aa-tRNA were stored in 5 mM $MgCl_2$ and 1 mM potassium acetate (pH 5.0) in small aliquots at −75 °C.

EF-Tu. Crystalline EF-Tu·GDP was purified from *E. coli* B cells as described by Miller and Weissbach (1974). EF-Tu·GDP concentrations were calculated by using ϵ_{280} = 41 600 M^{-1} cm^{-1} (Miller & Weissbach, 1977b). 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.

Aurodox. Aurodox (X5108), the N-methylated derivative of kirromycin (Parmeggiani & Swart, 1985), a gift of Hoffmann-La Roche, Nutley, NJ, was dissolved in 100% ethanol to 100 mM and stored at −20 °C. The absorbance of aurodox at 490 nm is negligible (Eccleston, 1981), so it caused no inner filter effect in our experiments.

Determination of aa-tRNA Concentration. The total amount of aa-tRNA present in a titration sample was determined from aliquots taken directly from the cuvette and counted in a Triton-based scintillation cocktail. Net ^{14}C counts were used to calculate the total A_{260} units of tRNA in the cuvette from the previously determined specific activity (pmol of Phe/A_{260} unit of tRNA) of the stock aa-tRNA. In order to determine the amount of Phe covalently attached to tRNA (=aa-tRNA) in a sample, aliquots (10–100 μL) were taken from each 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, separately, on a Sephadex G-25 gel filtration column (0.7 cm i.d. × 20 cm) at 4 °C. Fractions

(250 μL) were collected directly into scintillation vials and counted. The fraction of [^{14}C]Phe 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 radioactivity had been subtracted.

Deacylation. The rate of aa-tRNA deacylation in the absence of EF-Tu was determined by using a control cuvette containing Phe-tRNA. The cuvette was placed in the fluorimeter and a mock titration was performed, with additions of solvent whenever the titration samples received EF-Tu-containing titrant. Mixing was done exactly as in the titration cuvettes. At both the start and end of the titration, aliquots were taken for analysis by gel filtration chromatography as above. The deacylation rate constant at 6 °C for Phe-tRNA was found to be 1.1×10^{-3} min^{-1} in standard buffer.

Fluorescence Spectroscopy. All fluorescence measurements were made on an SLM 8000 spectrofluorimeter, a photon-counting instrument with two holographic gratings in the excitation light path and one in the emission light path (SLM-Aminco, Urbana, IL). Temperature was maintained at 6 °C in all experiments and was monitored by using a temperature probe (Fisher Scientific) 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. The excitation and emission wavelengths were 490 and 520 nm, respectively, in all experiments. The band-pass was 2 or 4 nm for the excitation light and 4 nm for the emission light. To avoid photodegradation of the samples, shutters were kept closed except when taking fluorescence readings.

Titrations (230 or 500 μL total volume) were performed in 4 mm × 4 mm quartz microcuvettes. In order to obtain reproducible results with these microcells, extra care had to be taken both in mixing the samples in the cuvettes and in washing the cuvettes. A 2 mm × 2 mm magnetic Teflon-coated stirring bar (flea) was placed in each cuvette, and the solution was mixed by stroking the outside face of the cuvette numerous times with a large magnetic stir bar, causing the flea to move up and down through the entire solution along the inside face of the cuvette. The flea remained in the cuvette during spectral measurements, out of the light path at the bottom of each cuvette. Some titrations (2.3 mL total volume) were performed in 1 cm × 1 cm quartz fluorescence cuvettes.

The fluorescence emission from a cuvette containing a solution of disodium fluorescein (8 nM) in 0.1 N KOH was monitored periodically during experiments as a check on the stability of the lamp signal. During most (>90%) experiments, the measured fluorescein emission from this sample did not vary more than 1% over the course of the experiment (4–8 h).

Titrant Solutions. Immediately prior to a titration, the EF-Tu was incubated for 20 min at 37 °C with pyruvate kinase (Sigma; 20 μg/mL), 10 μM GTP, and 1.0 mM PEP to convert residual GDP to GTP. The protein used in EF-Tu·GDP experiments was treated the same, except that incubations contained pyruvate kinase and 10 μM GDP, but no PEP. For the titrations done in the presence of aurodox, the EF-Tu preincubation also contained either 0.2% (v/v) ethanol or 0.2 mM aurodox and 0.2% (v/v) ethanol. After the preincubations, the EF-Tu titrant solutions were kept on ice until used.

Titrations. All samples contained pyruvate kinase at 20 μg/mL. For EF-Tu·GTP experiments, the samples also contained 10 μM GTP, 1.0 mM PEP, and typically 3.4 nM Phe-tRNA^{Phe}-Fl^8. For EF-Tu·GDP experiments, the samples contained 10 μM GDP and typically 75 nM Phe-tRNA^{Phe}-Fl^8. For the experiments with aurodox, the samples contained either 0.2% (v/v) ethanol or 0.2 mM aurodox and 0.2% (v/v) ethanol.

At the beginning of each titration, the fluorescence emission intensity of a sample was monitored until it was stable. This initial value, typically reached after 2–3 h [cf. Abrahamson et al. (1985)], was designated F_0. The Phe-tRNA^{Phe}-Fl^8 was then presumed to have reached a conformational equilibrium with respect to the temperature and buffer conditions in the cuvette. At that time an aliquot was removed to determine the extent of aminoacylation, 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 10–15 min before emission intensity readings were taken. The signal of a fluorescein-free blank (i.e., a sample that contained Phe-tRNA^{Phe} instead of Phe-tRNA^{Phe}-Fl^8) was subtracted from each measured sample signal, and then the net emission was corrected for dilution due to the addition of titrant.

Determination of K_d. K_d values were determined by performing a nonlinear least-squares fit of the data as described by 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). EF-Tu concentrations were determined by absorbance, and filter binding data showed that that the EF-Tu was 100% active in binding GDP. 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

EF-Tu-Dependent Changes in aa-tRNA Conformation. When EF-Tu·GTP binds to Phe-tRNA^{Phe}-Fl^8, there is a substantial increase in the emission intensity of the fluorescein dye (Adkins et al., 1983). This is caused by a change in Phe-tRNA conformation that is allosterically induced by the binding of the protein to the nucleic acid. Since the conformation of EF-Tu·GTP differs from that of EF-Tu·GDP (Miller & Weissbach, 1977b), we wanted to determine whether the binding of EF-Tu·GDP to Phe-tRNA^{Phe}-Fl^8 elicited a conformational change in the tRNA that could be detected by the fluorescent probe, and, if so, whether association with EF-Tu·GDP induced the same structural change in Phe-tRNA as did association with EF-Tu·GTP.

When EF-Tu·GDP was added to a solution containing Phe-tRNA^{Phe}-Fl^8, an increase in fluorescein emission intensity was observed (Figure 1) that did not involve a change in the wavelength of maximum emission (data not shown). In contrast, no fluorescence change was observed when unacylated tRNA^{Phe}-Fl^8 was titrated with up to 100 μM EF-Tu·GDP (Figure 1). This showed that the fluorescence-detected interaction of tRNA with EF-Tu·GDP required an aminoacylated tRNA at protein concentrations below 100 μM and that the fluorescence change did not arise from a nonspecific protein–nucleic acid association. The slight rise in emission intensity observed at EF-Tu·GDP concentrations above 100 μM in the tRNA^{Phe}-Fl^8 titration may result from the association of (some of) the unacylated tRNA with the protein, but we have not investigated this further. Thus, Figure 1 provides spectroscopic evidence that a ternary complex can form between EF-Tu·GDP and aa-tRNA at high concentrations of macromolecules. These data also show that when the complex does form, the binding of EF-Tu·GDP to the aa-tRNA induces a conformational change in the tRNA molecule.

Table I: EF-Tu Dependence of Phe-tRNA^{Phe}-Fl^8 Emission Intensity and Ternary Complex Dissociation Constants[a]

nucleotide	n[b]	aurodox[c]	ethanol[d]	E_b/E_f	K_d (nM)	$\Delta G°$[e] (kcal/mol)
GDP	6[f]	−	−	1.33 ± 0.03	28 500 ± 7 800	5.8
GDP	3	+	+	1.28 ± 0.01	1 460 ± 180	7.4
GDP	1	−	+	1.36	29 700	5.8
GTP	4	−	−	1.36[g]	0.85[g]	11.5
GTP	3	+	+	1.34 ± 0.01	22.2 ± 1.7	9.7
GTP	3	−	+	1.33 ± 0.03	3.3 ± 0.5	10.8

[a] Experiments done in standard buffer as described under Experimental Procedures. [b] The number of separate experiments is given by n. [c] Final aurodox concentration was 200 μM. [d] Final ethanol concentration was 0.2% (v/v). [e] These are the $\Delta G°$ values at 6 °C for the dissociation of the ternary complex, and hence all are positive. [f] This includes one titration done in the presence of 100 μM GDP. [g] Taken from Abrahamson et al. (1985).

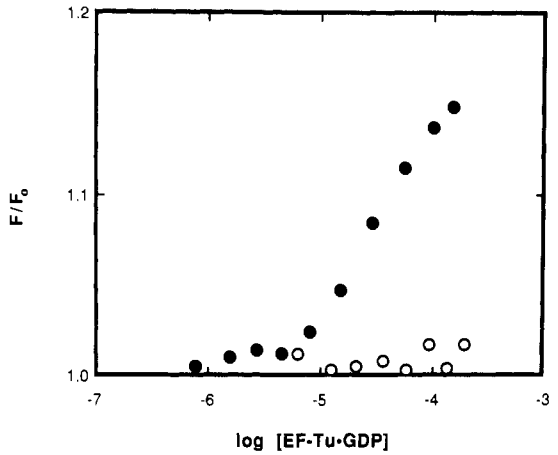

FIGURE 1: EF-Tu·GDP dependence of Phe-tRNA^{Phe}-Fl^8 and tRNA^{Phe}-Fl^8 fluorescence. EF-Tu·GDP was titrated into samples containing Phe-tRNA^{Phe}-Fl^8 (●) or tRNA^{Phe}-Fl^8 (○) at initial concentrations of 75 and 157 nM, respectively. Experiments were performed at 6 °C in standard buffer as described under Experimental Procedures.

Magnitude of the Spectral Change. If the conformation of Phe-tRNA^{Phe}-Fl^8 is the same in its complexes with EF-Tu·GDP and EF-Tu·GTP, then one would expect the fluorescent probe to have the same emission properties in the two complexes. The fluorescein emission intensity per free Phe-tRNA^{Phe}-Fl^8 molecule (E_f) is solvent- and pH-dependent (Abrahamson et al., 1985), so the emission intensities per Phe-tRNA^{Phe}-Fl^8 bound to EF-Tu·GDP or to EF-Tu·GTP (E_b) were compared in the same solvent (standard buffer). A polymix-type solvent (Jelenc, 1980) was not chosen for these titrations because the interaction between EF-Tu·GDP and Phe-tRNA^{Phe}-F^8 was weak and the K_d for Phe-tRNA^{Phe}-Fl^8·EF-Tu·GTP was 5-fold lower in standard buffer than in polymix (Abrahamson et al., 1985).

Because the amount of protein required to push Phe-tRNA^{Phe}-Fl^8·EF-Tu·GDP complex formation to completion (relative to the aa-tRNA) was prohibitive, the maximum emission intensity of Phe-tRNA^{Phe}-Fl^8 in the EF-Tu·GDP titrations was estimated by extrapolation of a double-reciprocal plot of the data, $F_0/(F - F_0)$ vs $1/[\text{EF-Tu·GDP}]$. Since the maximum emission intensity obtained in this way was not corrected for the deacylation of the Phe-tRNA^{Phe}-Fl^8 that occurred during the course of the experiment, the actual E_b is higher than the estimated value. However, the deacylation effect was small, as shown by comparing the estimated and observed final intensity values for EF-Tu·GTP titrations in which a well-defined plateau was reached. When the EF-Tu·GTP data of Abrahamson et al. (1985) were analyzed by using the double-reciprocal approach, the extrapolated value of E_b differed from the measured value by less than 1%.

The average E_b value for Phe-tRNA^{Phe}-Fl^8 bound to EF-Tu·GDP was calculated to be $1.33E_f$ (Table I), while the average E_b value for the EF-Tu·GTP complex was previously found to be $1.36E_f$ (Abrahamson et al., 1985). The similarity in the magnitudes of these changes in emission intensity indicates that EF-Tu·GTP and EF-Tu·GDP induce similar conformational changes in Phe-tRNA^{Phe}-Fl^8 upon forming ternary complexes.

Affinity of Phe-tRNA^{Phe}-Fl^8 for EF-Tu·GDP. The ternary complex dependent spectral change was used to determine the extent of Phe-tRNA^{Phe}-Fl^8 association with EF-Tu·GDP in a sample as a function of protein concentration (Figure 1). This spectroscopic approach allowed us to monitor ternary complex formation at equilibrium, and thereby to determine directly the affinity of EF-Tu·GDP for Phe-tRNA^{Phe}-Fl^8, as was done previously for the GTP-containing ternary complex (Abrahamson et al., 1985). Titrations of Phe-tRNA^{Phe}-Fl^8 with EF-Tu·GDP (Figure 1) showed that the average K_d for the Phe-tRNA^{Phe}-Fl^8·EF-Tu·GDP complex at 6 °C was 28 500 nM (Table I). Since this K_d value was more than 33 000-fold higher than that obtained with EF-Tu·GTP under the same conditions (Abrahamson et al., 1985), the nucleotide-dependent structural change in the EF-Tu clearly alters its affinity for aa-tRNA to a striking extent. As shown by the $\Delta G°$ values in Table I, changing the EF-Tu-bound nucleotide from GTP to GDP reduced the binding energy between the protein and the aa-tRNA by 5.7 kcal/mol at 6 °C.

EF-Tu was purified as EF-Tu·GDP, and on the basis of the association constant between EF-Tu and GDP (Miller & Weissbach, 1977a), every EF-Tu molecule should have been bound to GDP in these titrations. This appeared to be the case, because the same ternary complex K_d value was obtained at both 10 and 100 μM GDP.

Effect of Aurodox on the EF-Tu-Dependent Conformational Change in aa-tRNA. In order to assess whether the binding of aurodox to EF-Tu·GDP or to EF-Tu·GTP would block the ability of either to effect a conformational change in aa-tRNA, we determined the magnitude of the Phe-tRNA^{Phe}-Fl^8 emission intensity change upon ternary complex formation in the presence of 200 μM aurodox and either GTP or GDP. Judging from the K_d values for kirromycin association with various EF-Tu species (Pingoud et al., 1978; Eccleston, 1981), essentially all of the EF-Tu in the sample should be associated with aurodox in our samples.

Because ethanol was used to solubilize the antibiotic, control experiments were necessary in which ethanol alone was added to the samples. Titrations done in the presence of 0.2% (v/v) ethanol and in the absence of aurodox showed that ethanol had little effect on the E_b/E_f values for Phe-tRNA^{Phe}-Fl^8 association with the GTP and GDP forms of EF-Tu (Table I), and hence on the EF-Tu-induced conformational changes in the aa-tRNA.

Aurodox did not alter the extent of the fluorescence change (E_b/E_f) for Phe-tRNA^{Phe}-Fl^8 binding to EF-Tu·GTP but did reduce slightly the E_b/E_f for EF-Tu·GDP association with the

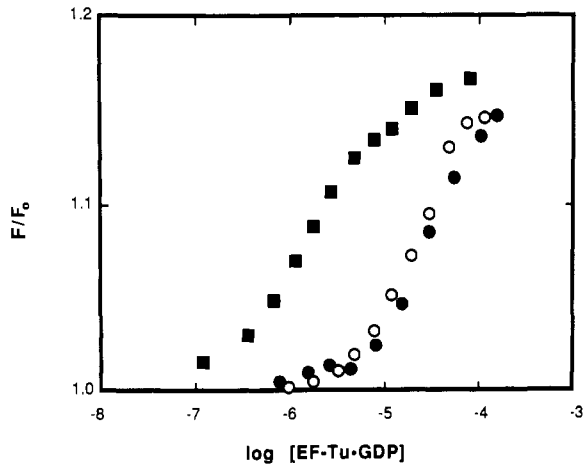

FIGURE 2: Effects of aurodox on the formation of ternary complex containing EF-Tu·GDP. Phe-tRNA^{Phe}-Fl^8 (initially 75 nM in these titrations) was titrated with EF-Tu·GDP in standard buffer containing no addition (●), 0.2% (v/v) ethanol (○), or 200 μM aurodox and 0.2% (v/v) ethanol (■).

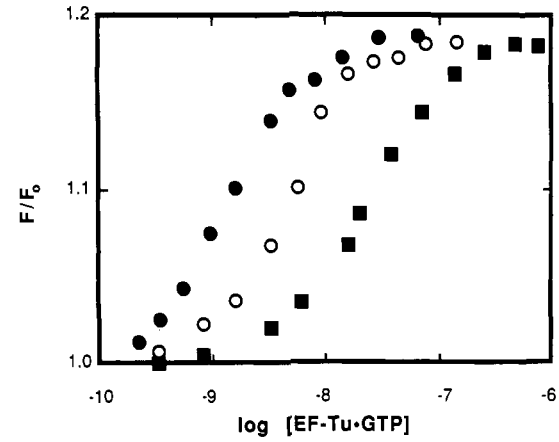

FIGURE 3: Effects of aurodox on the formation of ternary complex containing EF-Tu·GTP. Phe-tRNA^{Phe}-Fl^8 (initially 3.4 nM in these titrations) was titrated with EF-Tu·GTP in standard buffer containing no addition (●), 0.2% (v/v) ethanol (○), or 200 μM aurodox and 0.2% (v/v) ethanol (■).

aa-tRNA (Table I). This indicates that aurodox binding to EF-Tu does not alter the ability of EF-Tu·GTP to elicit a structural change in the aa-tRNA but does slightly alter the ability of EF-Tu·GDP to effect a conformational change in the aa-tRNA.

Effect of Aurodox on Ternary Complex Formation. Phe-tRNA^{Phe}-Fl^8 was titrated with EF-Tu·GDP in the presence of 200 μM aurodox, and less EF-Tu·GDP was required to form the ternary complex in the presence of aurodox than in its absence (Figure 2). This effect was aurodox-dependent, because the K_d for the Phe-tRNA^{Phe}-Fl^8·EF-Tu·GDP ternary complex at 6 °C was the same in the presence and absence of ethanol (Table I). Hence, the binding of aurodox to EF-Tu·GDP increases the protein's affinity for aa-tRNA, as shown by the nearly 20-fold reduction in the ternary complex K_d and the 1.6 kcal/mol change in $\Delta G°$ (Table I).

Phe-tRNA^{Phe}-Fl^8 was also titrated with EF-Tu·GTP in the presence of 200 μM aurodox. In this case, the binding of the antibiotic to the protein decreased its affinity for the aa-tRNA (Figure 3). Since ethanol itself also had a significant effect on ternary complex formation with EF-Tu·GTP (Figure 3), the magnitude of the aurodox-dependent change in affinity is determined by comparing the K_d value of the aurodox-containing sample with that of the ethanol-containing control sample. The data summarized in Table I show that aurodox significantly weakens EF-Tu·GTP binding to aa-tRNA, since the ternary complex K_d is increased by 6.7-fold relative to the control sample.

Discussion

The structure and function of EF-Tu are dictated by the small ligands that bind to the protein. A change in the ligands bound to EF-Tu (e.g., GTP to GDP, or the addition of aurodox) dramatically alters its interactions with aa-tRNA, EF-Ts, and perhaps the ribosome, as noted in the introduction. In this paper, we have focused on the ligand dependence of the interaction between EF-Tu and aa-tRNA. Since the spectroscopic probe employed in our experiments allowed us to monitor aa-tRNA conformation and to quantify the affinity of EF-Tu for aa-tRNA, we have therefore characterized the effects of GTP, GDP, and aurodox upon those aspects of the protein–nucleic acid interaction.

Does the hydrolysis of EF-Tu-bound GTP to GDP cause a change in the Phe-tRNA conformation? The emission intensity of the fluorescent probe, and hence its environment at the hinge region of the tRNA, was nearly the same when Phe-tRNA^{Phe}-Fl^8 was bound to EF-Tu·GTP and to EF-Tu·GDP (Table I), so the nucleotide-dependent structural change in EF-Tu does not appear to initiate a conformational change in the aa-tRNA. Since the E_b/E_f value for the GDP-containing complex had to be determined by extrapolation, it is possible that the aa-tRNAs in the GTP- and GDP-containing ternary complexes do not have exactly the same conformation. Yet any difference in structure near the s^{4}U-8 is not likely to be large since the difference between the two E_b/E_f values is small.

It has been proposed by many that changes in aa-tRNA conformation are involved in the mechanism of protein chain elongation on the ribosome [e.g., Kurland et al. (1975)]. It has also been proposed that the factor-dependent recognition and binding of aa-tRNA by the ribosome is a two-stage or "proofreading" process, with the second stage initiated by the EF-Tu-mediated hydrolysis of GTP (Hopfield, 1974), and there is considerable experimental evidence to support such a model [e.g., Thompson and Stone (1977) and Ruusala et al. (1982)]. It would therefore be reasonable to ask whether the GTP hydrolysis event induces an EF-Tu-dependent change in aa-tRNA conformation that initiates or accompanies the proofreading step. Since our results were obtained in the absence of ribosomes, the experiments reported here do not directly address that possibility. But the similarity of aa-tRNA conformation in ternary complexes containing either GTP or GDP suggests that the EF-Tu-mediated hydrolysis of the ternary complex GTP does not, by itself, trigger an allosteric structural change in the aa-tRNA. Instead, any structural changes in the aa-tRNA following GTP hydrolysis are more likely to occur following the dissociation of EF-Tu·GDP, since then the aa-tRNA would be able to relax from its EF-Tu-bound conformation (Adkins et al., 1983; Johnson et al., 1986) to its conformation when free in solution. Although this speculation ignores any influence that the ribosome may have on the aa-tRNA conformation, it does make the point that the EF-Tu-bound conformation of the aa-tRNA constitutes a higher energy state than that of the EF-Tu-free aa-tRNA and hence that the release of EF-Tu·GDP could, in principle, power a conformational change in the aa-tRNA.

In contrast, the conversion of GTP to GDP does have a tremendous effect upon the affinity between EF-Tu and aa-tRNA: the K_d of the GDP-containing ternary complex is more

than 33 000-fold higher than that of the GTP-containing ternary complex (Table I). In the absence of any direct ribosome–EF-Tu interactions, this would mean that the hydrolysis of the GTP in a ribosome-bound ternary complex would cause the dissociation rate of the protein from the aa-tRNA to be increased by as much as 33 000-fold. Although the magnitude of this change in rate is likely to be modulated by ribosomal interactions, the size of this effect is so large that it is easy to understand why the efficient release of EF-Tu from the aa-tRNA on the ribosome first requires GTP hydrolysis. Following the dramatic GDP-dependent weakening of the affinity between the EF-Tu and aa-tRNA, the rate of EF-Tu·GDP dissociation will then be governed by its residual affinity for the aa-tRNA and ribosome. [Thompson et al. (1986) have concluded that the rate of EF-Tu·GDP release is determined primarily by EF-Tu–ribosome interactions.]

The above nucleotide-dependent difference in K_d values for *E. coli* Phe-tRNA is 4 orders of magnitude, considerably greater than the 100-fold difference in the corresponding K_d values reported earlier by Pingoud et al. (1982) for yeast Phe-tRNA. This discrepancy probably results primarily from the difference in the two methods used to monitor ternary complex formation. In our experiments, the extent of ternary complex formation was determined spectroscopically, and therefore directly and at equilibrium, whereas Pingoud et al. (1982) measured the EF-Tu-dependent decrease in the rate of aa-tRNA deacylation to monitor ternary complex formation.

The free energy of formation of the GDP ternary complex at 6 °C was 5.7 kcal/mol less than that of the GTP ternary complex (Table I). Thus, the conversion of GTP to GDP and the resultant structural change in EF-Tu reduced the binding affinity of the protein for aa-tRNA substantially. The magnitude of the nucleotide-dependent change in the $\Delta G°$ of ternary complex formation indicates that the conformational change in EF-Tu disrupted a large fraction of the specific individual interactions and/or contacts that, together, mediate and stabilize the association of the protein and the aa-tRNA.

EF-Tu·GDP can still distinguish between aa-tRNA and unacylated tRNA (Figure 1), which indicates that the nucleotide-dependent change in EF-Tu conformation does not destroy the aminoacyl binding site of EF-Tu. Yet this region of EF-Tu is altered to some degree, because the difference in K_d values for EF-Tu·GTP binding to unacylated and aminoacylated tRNAs (>2000-fold; Johnson et al., 1986) is larger than that for EF-Tu·GDP, based on the 10- to 20-fold difference estimated by extrapolation of one titration with $tRNA^{Phe}$-Fl[8] taken to 280 μM EF-Tu·GDP (data not shown). In addition, it is clear that the nucleotide-dependent change in EF-Tu structure also affects, at least in terms of affinity for aa-tRNA, the region of EF-Tu that binds directly to the tRNA and contributes the bulk of the total binding energy for the ternary complex (Johnson et al., 1986).

Aurodox, the methylated form of kirromycin, acts as an antibiotic by binding to EF-Tu and interfering with its function. Several groups have reported (Chinali et al., 1977; Pingoud et al., 1978, 1982; Duisterwinkel et al., 1981) that aurodox weakens the binding between EF-Tu·GTP and aa-tRNA and/or that aurodox strengthens the binding between EF-Tu·GDP and aa-tRNA. Our fluorescence studies have confirmed and quantified each of these conclusions. The magnitude of the antibiotic's effect on the protein–nucleic acid interaction, determined spectroscopically, is considerable: the GDP ternary complex K_d was decreased by nearly 20-fold, while that of the GTP ternary complex was increased by at least 6.7-fold (relative to the ethanol-containing control) (Table I). These changes are considerably larger than those reported previously (5- and 2-fold, respectively; Pingoud et al., 1978), a difference that presumably results from the different techniques that were employed in the two studies.

The binding of aurodox to EF-Tu will clearly reduce the dissociation rate of EF-Tu·GDP from the ribosome-bound aa-tRNA following GTP hydrolysis and would thereby retard chain elongation. However, a 20-fold decrease in the K_d of the aa-tRNA·EF-Tu·GDP ternary complex does not seem sufficient to explain the strong aurodox inhibition of protein synthesis and the failure of EF-Tu·GDP to dissociate from the ribosome following GTP hydrolysis (Wolf et al., 1977). Thus, it appears likely that aurodox also alters the EF-Tu–ribosome interactions following GTP hydrolysis, presumably by inducing a more EF-Tu·GTP-like structure in the newly formed EF-Tu·GDP that strengthens any interaction between the EF-Tu and the ribosome.

It is also possible that the binding of aurodox to the GTP-containing ternary complex interferes with the recognition and binding process at the ribosome prior to GTP hydrolysis by significantly weakening the affinity of EF-Tu for aa-tRNA (Table I) and/or altering the conformation of the EF-Tu. Either or both of these effects may interfere with, for example, the proofreading process. In the absence of a detailed molecular description of the ribosome–ternary complex interaction, one cannot assess the relative importance of the aurodox (kirromycin) effects on EF-Tu·GTP and EF-Tu·GDP. It is possible that the mechanism of action of aurodox or kirromycin inhibition involves a combination of effects that occur both before and after GTP hydrolysis, and that the proposals by Wolf et al. (1977) and Pingoud et al. (1978) discussed in the introduction are complementary and thus are not exclusionary alternatives.

From an energetic standpoint, aurodox altered the free energy change associated with ternary complex formation by 1–2 kcal/mol for both EF-Tu·GTP and EF-Tu·GDP (Table I). These aurodox-dependent changes in $\Delta G°$ are small enough to be accomplished by an aurodox effect that is relatively localized on EF-Tu and limited in scope and hence alters only a small fraction of the total EF-Tu interactions with aa-tRNA. However, our data do not rule out an aurodox-dependent change in EF-Tu conformation that was far reaching, yet small in magnitude, and therefore caused many protein–nucleic acid interactions to each be slightly suboptimal.

The binding of aurodox to EF-Tu has a small effect upon the conformation of EF-Tu·GDP-bound aa-tRNA and no detectable effect upon that of EF-Tu·GTP-bound aa-tRNA (Table I). This indicates that aurodox-dependent changes in ternary complex conformation are localized primarily in the protein. As has been noted by others, the binding of aurodox to EF-Tu·GDP appears to alter the protein conformation so as to create an EF-Tu structure that is intermediate between the GDP and GTP forms, at least in terms of the strength of its binding to aa-tRNA. This is demonstrated by the fact that aurodox both decreases by 20-fold the K_d of aa-tRNA·EF-Tu·GDP and also increases by at least 6.7-fold the K_d of aa-tRNA·EF-Tu·GTP (Table I). These results also show that the aurodox (kirromycin) binding site on EF-Tu is conformationally coupled to the protein's aa-tRNA binding site.

In summary, the striking changes in EF-Tu function that are mediated by its association with small ligands are effected primarily through their alteration of the EF-Tu and its affinity for aa-tRNA. The small ligands have little observable effect upon the aa-tRNA conformation (detected at the hinge region) once it has associated with EF-Tu. Finally, our results suggest

that aurodox or kirromycin inhibition of protein biosynthesis may involve a combination of effects that influence ternary complex interactions with the ribosome both before and after GTP hydrolysis.

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