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Guanosine Triphosphate and Guanosine Diphosphate as Conformation-Determining Molecules. Differential Interaction of a Fluorescent Probe with the Guanosine Nucleotide Complexes of Bacterial Elongation Factor Tu†

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ABSTRACT: Tritium exchange studies have recently provided evidence that conformational differences between EFTu–GTP and EFTu–GDP may account for the differential binding of AA-tRNA by EFTu–GTP (Printz, M. P., and Miller, D. L. (1973), *Biochem. Biophys. Res. Commun. 53*, 149). These conformational differences have been further characterized by studying the interaction of the fluorescent dye 1-anilino-8-naphthalenesulfonate with EFTu–GTP and EFTu–GDP. EFTu–GTP enhances the fluorescence of 1-anilino-8-naphthalenesulfonate to a greater extent than does EFTu–GDP. When EFTu–GTP is complexed with Phe-tRNA, however, its interaction with 1-anilino-8-naphthalenesulfonate increases the fluorescence of the dye only as much as EFTu–GDP does. Titration of a solution of the dye with excess protein shows that both EFTu–GTP and EFTu–GDP produce the same fluorescence enhancement, about 200-fold, for the tightest bound dye. Equilibrium dialysis binding measurements indicate that EFTu–GTP binds three molecules of the sulfonate dye with an apparent $K_{diss} \simeq 2 \times 10^{-5}$ M, whereas EFTu–GDP binds two molecules with an apparent $K_{diss} \simeq 5\text{–}8 \times 10^{-5}$ M. Both complexes have at least one other population of more weakly bound dyes. It would appear from these data that differences in conformation between EFTu–GTP and EFTu–GDP are centered chiefly in a region of EFTu–GTP sensitive to AA-tRNA binding. However, further analysis of the fluorescence data indicates that somewhat more extensive conformational differences exist between the two nucleotide complexes of EFTu. Slope changes in the curve of the titration of 1-anilino-8-naphthalenesulfonate by EFTu–GTP and in Scatchard plots of the fluorescence data indicate cooperativity in the fluorescence yield and thus interaction of the dye binding sites on EFTu–GTP. EFTu–GDP gives no evidence of site interaction.

Nucleoside triphosphates perform three distinct functions in organisms. They may be reagents or intermediates in the synthesis or degradation of cellular components, where the formation of a phosphate ester intermediate is a favorable pathway for removing or adding the elements of water. The synthesis and degradation of glycogen are examples of this function. In contrast to these reactions, nucleoside triphos-

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phates also promote cellular processes where phosphate ester formation seems to play no obligatory role. Among the examples of this function are motile and contractile processes. A third and possibly related function of these compounds is to control biochemical reactions, as CTP regulates the activity of aspartate transcarbamoylase.

The role of GTP in the binding of aminoacyl-tRNA (AA-tRNA)[1] to ribosomes bears a resemblance both to the function of ATP in motile systems and to the regulatory role of CTP. In the process of protein biosynthesis in prokaryotes, GTP promotes the binding of AA-tRNA to ribosomes in the presence of mRNA and elongation factor Tu (EFTu).[2] Aminoacyl-tRNA and EFTu–GTP readily form the ternary complex AA-tRNA–EFTu–GTP (Weissbach *et al.*, 1970), which interacts with the mRNA–ribosome complex. As a result of this interaction, AA-tRNA is bound to the ribosome, the GTP in the ternary complex is hydrolyzed, and EFTu–GDP, which does not bind to AA-tRNA, is released.

The EFTu–GDP complex is very stable, having a dissociation constant in the range of 10^{-8}–10^{-9} M, whereas EFTu–GTP is about 100-fold less stable. The EFTu–GDP complex dissociates very slowly by itself; however, another protein, EFTs, catalyzes the exchange of GTP for GDP, thus completing the cycle of reactions in the binding process.

The details of this function of EFTu remain undetermined. There is some evidence that it alters the structure of the ribosome (Chuang and Simpson, 1971). Other possibilities, at present unproved, are that the protein alters the structure of AA-tRNA, or provides additional binding sites for interaction of the ternary complex with the ribosome. Whatever the function of EFTu in peptide chain elongation, the specificity of its interactions seems to be determined by which guanosine nucleotide is bound to it. Thus, the dissociation constant for AA-tRNA from EFTu–GTP is 10^{-8} M or less (Miller *et al.*, 1973), whereas EFTu–GDP does not interact with AA-tRNA to a measurable extent; the dissociation constant of the hypothetical AA-tRNA–Tu–GDP complex must be greater than 10^{-4} M.

We have postulated that this difference in reactivity is due to conformational differences between EFTu–GTP and EFTu–GDP. A previous study of tritium exchange rates provided evidence to support this concept (Printz and Miller, 1973). Tritiated EFTu–GTP exchanged some of its peptide bond hydrogens considerably more rapidly than EFTu–GDP did, and at certain times in the exchange process EFTu–GDP possessed about 50% more unexchanged hydrogens than EFTu–GTP, suggesting that GDP induces a tightening of at least a portion of the tertiary structure of EFTu. In an effort to localize the conformational differences between the two complexes, and to relate them to their differential interaction with AA-tRNA, we have examined the binding of a fluorescent dye, 1-anilino-8-naphthalenesulfonate, to the complexes of EFTu. Although this molecule has been shown to interact with a large number of proteins, the number of fluorescent binding sites per protein is usually small (Stryer, 1965; Daniel and Weber, 1966; Brand, 1970). Furthermore, the fluorescence yield of the resulting protein–dye complex depends markedly upon the properties of the dye binding site, and is thought to increase with the hydrophobicity of the binding site (Brand and Gohlke, 1972).

[1] The following abbreviations are used: AA- (or Phe-) tRNA, aminoacyl- (or phenylalanyl-) tRNA; EFTu, EFTs, and EFG, elongation factors Tu, Ts, and G; GMP–PCP, guanylyl methylene diphosphonate.

[2] For a review on the process of peptide chain elongation, see Lucas-Lenard and Lipmann (1971).

Materials and Methods

Preparation of EFTu Complexes. GDP, GTP, and dithiothreitol were obtained from Calbiochem. Methylenediphosphonic acid was a product of Miles Laboratories. GMP–PCP was prepared by the morpholidate method (Moffatt and Khorana, 1961) and was crystallized as the disodium salt. The disodium salt of 1-anilino-8-naphthalenesulfonate, obtained from K & K Laboratories, was converted to the magnesium salt, treated with Norite, and recrystallized from water (Stryer, 1965). The molar extinction coefficient of the purified material was found to be 4.9×10^3 at 350 nm. Thin-layer chromatography revealed a single fluorescent component. Purified Phe-tRNA from *Escherichia coli* (1600 pmol/A_{260}) was prepared from a tRNA mixture (Schwarz-Mann) by benzoylated DEAE-cellulose chromatography (Gillam and Tener, 1971). The preparation of homogeneous EFTu as the EFTu–GDP complex has been described elsewhere (Miller and Weissbach, 1970, 1973). The EFTu–GMP-PCP complex was prepared by passing EFTu–GDP (10 mg) through a 90 × 15 cm column of Bio-Gel P-4 polyacrylamide gel equilibrated with 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM GMP-PCP. One pass through the column removed 80–90% of the GDP, whereas two passes removed 95–97% of the GDP.

To ensure that small differences in the protein preparations did not influence the results, both EFTu–GDP and EFTu–GTP were formed from a common intermediate, EFTu–GMP-PCP, by adding a small excess of the appropriate nucleotide. Since GMP-PCP is relatively loosely bound to EFTu, it is readily displaced by GTP or GDP. The extent of conversion of EFTu–GMP-PCP to EFTu–GTP or EFTu–GDP was determined by a Millipore filter assay using the appropriate tritium-labeled nucleotide.

Fluorescence Measurements. All fluorescence measurements were made at 4° using an Aminco-Bowman spectrophotofluorometer with a ratio attachment. The instrument was routinely standardized with a solution of quinine sulfate (12 ppb) in 0.1 N H_2SO_4. The excitation and emission spectra of all the complexes tested were found to be very similar, and an excitation wavelength of 350 nm and an emission wavelength of 470 nm were used for all titrations (the emission maxima for the complexes were approximately 480 nm, but the lower wavelength was used to minimize fluorescence of the free dye in aqueous solution).

All titrations were performed in 0.5-cm cuvets in a buffer of 50 mM Tris–10 mM $MgCl_2$–1 mM dithiothreitol, (pH 7.4). The solution to be titrated (250 μl) was added to the cuvet, the fluorescence was measured, and then microliter increments of titrant were added. After each addition of titrant the solution was stirred with a polyethylene rod and the fluorescence was remeasured after it arrived at a constant value (in titrations of the protein–nucleotide complexes the final fluorescence was achieved immediately; when Phe-tRNA was being added to quench fluorescence, final readings were taken after approximately 2 min).

Three types of titrations were performed: (1) titrations of one of the EFTu complexes with 1-anilino-8-naphthalenesulfonate (the sulfonate dye) to determine the extent of fluorescence enhancement; for these titrations the complex was present in the buffer solution at an initial concentration of 1.0×10^{-6} M in the presence of a threefold excess of nucleotide; the sulfonate solution used for titration was usually 2×10^{-3} M in sulfonate dye; for titrations in the early region of the curve a titrant solution of 2×10^{-4} M sulfonate dye was used; (2)

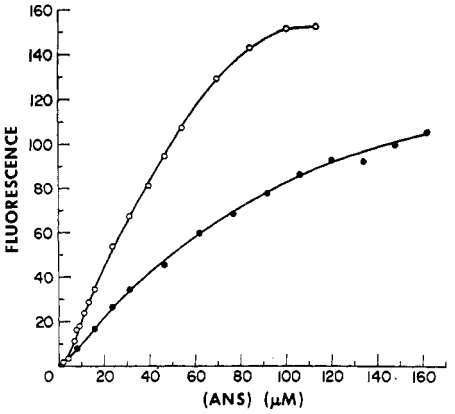

FIGURE 1: Titration of EFTu–GTP and EFTu–GDP by 1-anilino-8-naphthalenesulfonate (ANS): (○) EFTu–GTP; (●) EFTu–GDP. Fluorescence is in arbitrary units.

titration of the sulfonate with an EFTu complex to determine the fluorescence yield of the most tightly bound dye and its affinity for the protein; the dye was present in the buffer solution at an initial concentration of 2×10^{-6} M, to which increments of EFTu complex were added from a solution containing 1×10^{-4} M protein and 3×10^{-4} M nucleotide; (3) titration of a dye–EFTu complex solution with Phe-tRNA to measure its quenching effect; a titration in the early region of the curve was performed as described in 1, followed by incremental additions of microliter amounts of a 6×10^{-5} M solution of Phe-tRNA.

Blank corrections were made as follows. In all titrations corrections were made for dilution (which never exceeded 10%) and absorbance, which reached 0.25 at 350 nm at the highest concentration of dye used. In type 1 titrations correction was made for free dye by the method of Thompson and Yielding (1968). In type 2 titrations an additional correction was made for the contribution of the protein–complex solution to the fluorescence. Finally, the contribution of the Phe-tRNA solution to the measured fluorescence was also corrected for in type 3 titrations.

Equilibrium Dialysis. Equilibrium dialysis was performed at 4° in cells manufactured by Technilab Instruments. The usual buffer solution (0.5 ml) containing a range of 1-anilino-8-naphthalenesulfonate concentrations (4×10^{-5} to 1×10^{-3} M) were placed in both chambers of the dialysis cells. A fixed protein–nucleotide complex concentration of 3×10^{-5}–1.0×10^{-4} M was included in the solution on one side of each cell. After 5 hr, a time at which control experiments showed dialysis to be complete, the optical density at 350 nm of each chamber was measured, and the value was corrected for the protein contribution. The solutions on both sides of the cell contained an amount of GDP or GTP three times the protein concentration.

Results

Fluorescence of Dye-Tu-GDP and Dye-Tu-GTP. The conformational difference between the two complexes first observed by tritium exchange studies was reflected also in their interaction with the sulfonate dye. The titration curves obtained by adding small increments of a 1-anilino-8-naphthalenesulfonate solution to either EFTu–GDP or EFTu–GTP are shown in Figure 1. Upon addition of dye to either complex, the wavelength of maximum emission shifted from approximately 530 nm (dye in aqueous solution) to 480 nm, similar to the dye in ethanol. Although the excitation and

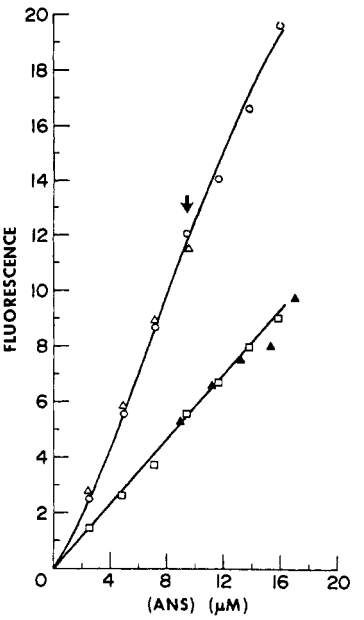

FIGURE 2: Effect of Phe-tRNA upon the fluorescence of dye–EFTu–GTP; comparison with dye–EFTu–GDP. At the point in the titration of EFTu–GTP by 1-anilino-8-naphthalenesulfonate (ANS) marked by an arrow, an equimolar amount of Phe-tRNA was added to the EFTu–GTP solution being titrated: (○) control titration of EFTu–GTP by 1-anilino-8-naphthalenesulfonate (ANS); no Phe-tRNA added during titration; (△) titration of EFu–GTTP before addition of Phe-tRNA; (▲) titration of EFTu–GTP after addition of Phe-tRNA; (□) control titration of EFTu–GDP, no Phe-tRNA added during titration.

emission spectra of the two dye–EFTu complexes were identical (data not shown), dye–EFTu–GDP consistently gave considerably less fluorescence than the dye–EFTu–GTP complex throughout the titration.

Effect of Phe-tRNA on the Fluorescence of Dye-EFTu-GTP and Dye-EFTu-GDP. In an attempt to determine whether this difference could be related to specific differences on the surface of EFTu–GTP and EFTu–GDP, we measured the effect of Phe-tRNA on the fluorescence of the protein–dye complexes. Figure 2 shows that a stoichiometric addition of Phe-tRNA to EFTu–GTP midway through sulfonate dye titration caused the fluorescence of the solution to decrease to a value corresponding to an identical concentration of EFTu–GDP. Further increments of dye produced a titration curve similar to the EFTu–GDP titration run as a control. In a parallel experiment, addition of the same amount of Phe-tRNA to EFTu–GDP produced no net change in fluorescence.

The specificity and extent of the fluorescence-diminishing effect of Phe-tRNA was tested by adding increments of Phe-tRNA to a solution of EFTu–GDP or EFTu–GTP pretitrated with 1-anilino-8-naphthalenesulfonate. As Figure 3A shows, a sharp decrease in fluorescence was observed with EFTu–GTP, which leveled off as the Phe-tRNA/(EFTu–GTP) ratio approached unity. As the Phe-tRNA/(EFTu–GTP) ratio was further increased, the fluorescence again decreased, leveling out a second time as the Phe-tRNA/(EFTu–GTP) ratio approached 3. The addition of Phe-tRNA to dye–EFTu–GDP (Figure 3A) caused only a gradual decrease in fluorescence at high concentrations of Phe-tRNA. Deacylated Phe-tRNA had no net effect upon the fluorescence of either dye–protein–nucleotide complex. It would seem that Phe-tRNA has two modes of interaction with EFTu, a specific stoichiometric interaction with the form of EFTu that binds GTP, and a nonspecific interaction that occurs at higher Phe-

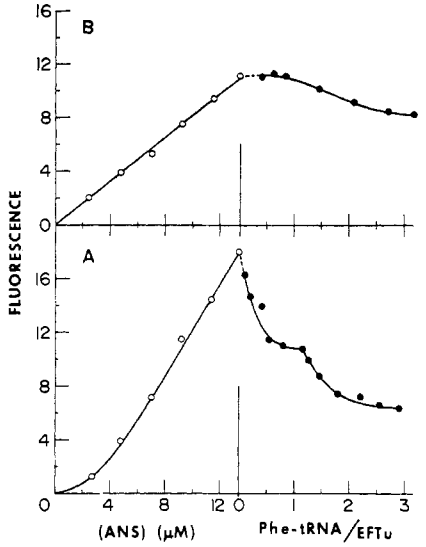

FIGURE 3: Ability of Phe-tRNA to quench fluorescence of dye–EFTu–GDP and dye–EFTu–GTP: (A) EFTu–GTP; (B) EFTu–GDP; (○) titration by 1-anilino-8-naphthalenesulfonate (ANS); (●) fluorescence of titrated solution after incremental additions of Phe-tRNA.

tRNA/EFTu ratios and differentiates much less strongly between the two forms of EFTu.

Quantitation of 1-Anilino-8-naphthalenesulfonate Binding. On the basis of the titration curves, it would appear that sulfonate binding is a sensitive indicator of the conformational differences between the two EFTu complexes that provides for the very selective binding of AA-tRNA by EFTu–GTP. In the absence of further quantitative information, however, it is not possible to conclude whether the conformational difference between the two forms of EFTu is confined to a local area directly involved in AA-tRNA binding, or if a major conformational difference exists with the identity of the EFTu–GDP and AA-tRNA–EFTu–GTP titration curves being a matter of coincidence.

Quantitation of the fluorescence results requires the fluorescence yield(s) of the bound dyes. If all of the bound dyes are approximately equivalent, the fluorescence yield can be obtained by titrating a solution of dye with excess protein (Weber and Young, 1964). Double reciprocal plots of such data are shown for the two forms of EFTu in Figures 4 and

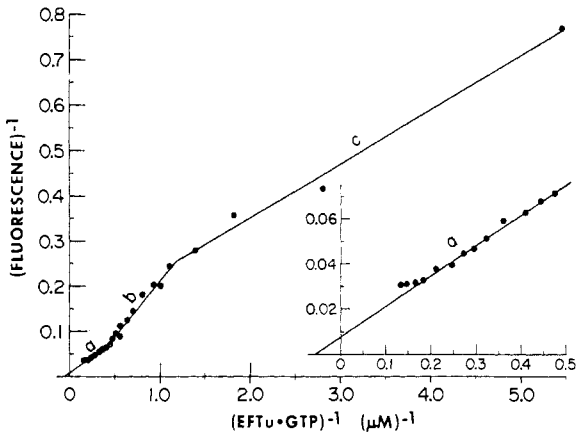

FIGURE 4: Double reciprocal plot of titration of 1-anilino-8-naphthalenesulfonate (ANS) by EFTu–GTP; 0.97 × 10^{-4} M EFTu–GTP containing 2 × 10^{-6} M dye was added in small increments to 2 × 10^{-6} M dye.

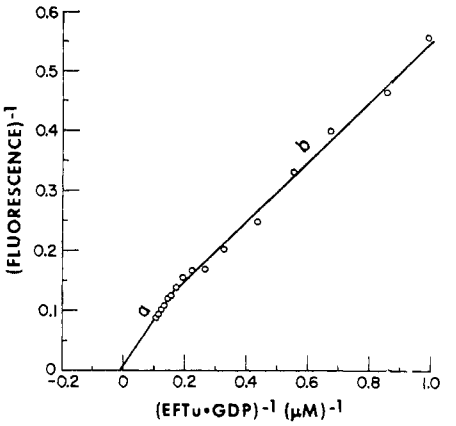

FIGURE 5: Double reciprocal plot of titration of 1-anilino-8-naphthalenesulfonate (ANS) by EFTu–GDP; 0.43 × 10^{-4} M EFTu–GDP containing 2 × 10^{-6} M dye was added in small increments to 2 × 10^{-6} M dye.

5. Both curves show slope changes as the protein concentration increases. In the absence of other information, there are several possible explanations for this type of behavior. The biphasic nature of the EFTu–GDP curve could result from two populations of binding sites having the same fluorescence yield and differing affinities for the dye, or alternatively two populations differing in both characteristics. The EFTu–GTP curve is still more complex, having at least two slope changes in the binding region of interest. Some complexity was expected because of the sigmoidal nature of the early region of the 1-anilino-8-naphthalenesulfonate titration curve (Figure 1). This type of curve usually reflects cooperativity of binding, and the increase in slope in Figure 4 designated as region b is consistent with this interpretation. However, the same type of behavior would be seen if the second population of dye binding sites had a higher fluorescence yield than the first.

Although no simple analysis can be made of regions b and c, in the region of large excess protein (region a) both reciprocal curves extrapolate to the same intercept and give a fluorescence yield for the most tightly bound dye molecule of 70/μM in arbitrary units, about a 200-fold increase over the fluorescence of 1-anilino-8-naphthalenesulfonate alone in aqueous solution at 470 nm. The apparent dissociation constants determined from the respective reciprocal plots differ; however, extrapolation to the 1/[EFTu] intercept yields K_{diss} = 1.7 × 10^{-5} M for EFTu–GTP and 8.0 × 10^{-5} M for EFTu–GDP. When these fluorescence yield values are used to treat the titration data of the two forms of EFTu by the method of Scatchard *et al.* (1956), the curves shown in Figure 6 result. These curves are subject to the same difficulties in interpretation as the double reciprocal plots. The convex nature of the curves is to be expected if cooperativity exists among the 1-anilino-8-naphthalenesulfonate binding sites, as analyzed and discussed by Cassman and King (1972). However, the same type of curve could result from varying fluorescence yields.

Because of the apparent complexities of the fluorescence binding data, equilibrium dialysis measurements were used to obtain binding information by an independent method, although the amount of EFTu complex required prohibited the extensive use of this technique. The results from these experiments for both forms of EFTu are shown in Figure 7. EFTu–GDP binds a large number of 1-anilino-8-naphthalenesulfonate molecules very weakly. Clearly differentiated from these sites are the two tight-binding sites with an apparent K_{diss} =

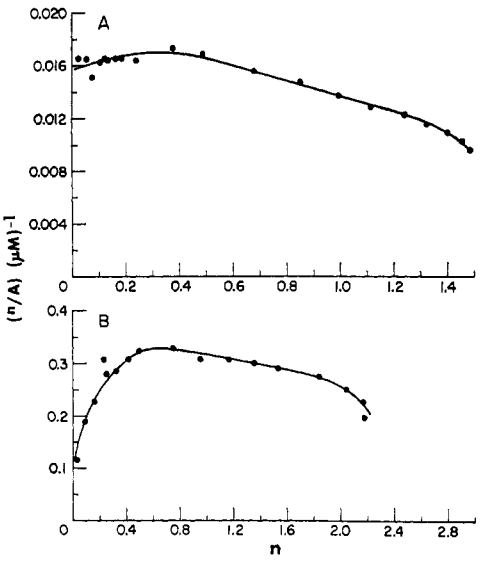

FIGURE 6: Scatchard plots, fluorescence data of 1-anilino-8-naphthalenesulfonate (ANS) titration of EFTu-GTP and EFTu-GDP: n, number of moles of dye bound per mole of EFTu, calculated on the basis of a micromolar fluorescence yield of 70: A, concentration of free dye; (A) EFTu-GDP; (B) EFTu-GTP.

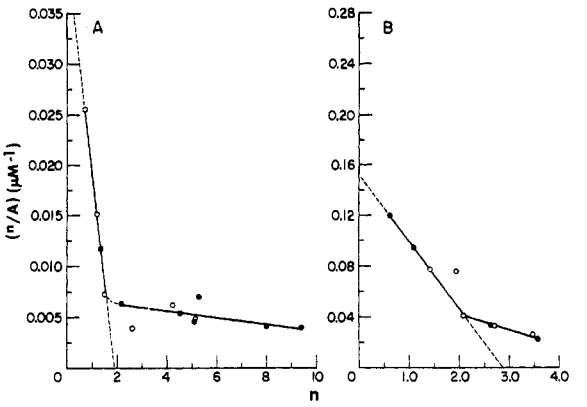

FIGURE 7: Scatchard plots, equilibrium dialysis of EFTu-GDP and EFTu-GTP with 1-anilino-8-naphthalenesulfonate (ANS); n, number of moles of dye bound per mole of EFTu, calculated on the basis of a molar extinction coefficient of 4.9×10^3: A, free dye; (A) EFTu-GDP; (○) [EFTu-GDP] = 9.9×10^{-6} M; (●) [EFTu-GDP] = 7.9×10^{-5} M. (B) EFTu-GTP; (○) [EFTu-GTP] = 3.0×10^{-5} M; (●) [EFTu-GTP] = 2.9×10^{-5} M.

4.7×10^{-5} M. Equilibrium dialysis of dye-EFTu-GTP yields three dye molecules bound tightly ($K_{diss} = 1.9 \times 10^{-5}$ M) and at least two additional molecules bound much less tightly.

Interaction of EFTs with EFTu-GDP and EFTu-GTP. The function of EFTs seems to be to facilitate the replacement of GDP with GTP on EFTu by first displacing GDP, forming EFTu-EFTs; EFTs is then displaced by GTP. *In vitro* the EFTu-EFTs complex can be formed from either EFTu-GDP or EFTu-GTP. It was of interest to determine the nature of the EFTu-EFTs complex in terms of its ability to bind 1-anilino-8-naphthalenesulfonate. When EFTs was added to EFTu-GDP and the solution titrated with dye (Figure 8) the resulting titration curve was equal to the sum of the individual EFTu-GDP and EFTs titration curves, indicating no net effect of EFTs upon the dye-binding properties of EFTu-GDP. When EFTs was added to a partially titrated solution of EFTu-GTP, the net fluorescence of the complex dropped, and further titration produced a curve roughly superimposable upon a control EFTu-GDP titration curve, as would be required by the thermodynamics of the system. To the extent that 1-anilino-8-naphthalenesulfonate binding is an indication of conformation, EFTu in EFTu-EFTs is similar to that form which binds GDP

That EFTs diminishes the fluorescence of dye-EFTu-GTP is required by the previous observations that the fluorescence of dye-EFTu-GTP is greater than that of dye-EFTu-GDP, and EFTs does not alter the fluorescence of dye-EFTu-GDP; therefore, these observations constitute a test of the consistency of the system. That EFTu in the EFTu-EFTs complex resembles EFTu-GDP rather than EFTu-GTP might have been expected, since EFTu when bound to EFTs should be in a form that does not bind AA-tRNA. Furthermore, displacement of GDP by EFTs would be facilitated if little or no conformational change were involved.

Discussion

Interaction of Dye with EFTu-GDP. The equilibrium dialysis data and the fluorescence data can be rationalized if one assumes a difference in fluorescence yield between the two populations of binding sites. Thus, there are two sites of $K_{diss} = 5\text{–}8 \times 10^{-5}$ M with micromolar fluorescence yield of 70, corresponding to region a of the double reciprocal plot, and a very large number of other sites, corresponding to region b, which both bind and fluoresce much more weakly. The convex nature of the fluorescence Scatchard plot could arise from the fact that a constant, high fluorescence yield was used to calculate "n" whereas a decreasing, composite value of the fluorescence yield would be more valid.

Interaction of Dye with EFTu-GTP. The equilibrium dialysis data indicate that there are three equivalent dyes bound tightly ($K_{diss} = 1.9 \times 10^{-5}$ M) plus at least one other population of less tightly bound dyes. The fluorescence data indicate that the second dye molecule that interacts with EFTu-GTP has, in effect, a higher fluorescence yield than the first dye. In order to satisfy both the equilibrium dialysis data for site equivalency and the fluorescence data for site difference, it is necessary to postulate a kind of cooperativity of fluorescence, such that regardless which site is first occupied, giving rise to

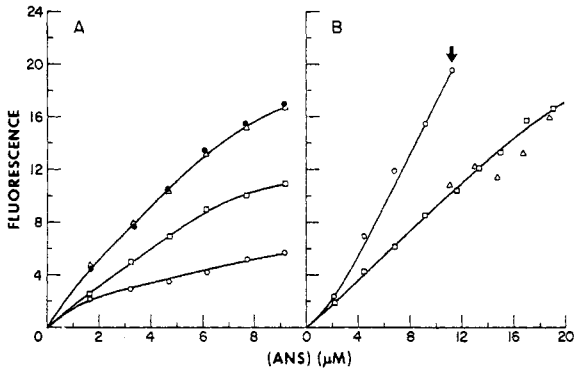

FIGURE 8: Effect of EFTs on the fluorescence of dye-EFTu-GDP and dye-EFTu-GTP: (A) titration of EFTu-GDP by dye in the presence and absence of EFTs: (□) EFTu-GDP, 1.3×10^{-6} M; (○) EFTs, 2×10^{-6} M (control curve); (△) summation of previous two curves; (●) EFTu-EFTs (EFTu-GDP and EFTs were combined prior to titration at the same concentrations as when titrated separately). (B) Effect of EFTs on the titration curve of dye-EFTu-GTP. At arrow, the solution being titrated was made 2×10^{-6} M in EFTs: (○) EFTu-GTP; (□) EFTu-GDP (control curve); (△) EFTu-GTP after addition of EFTs and correction for EFTs enhancement of fluorescence.

region a of Figure 4, the second and third sites will have an apparent higher fluorescence yield. This would result in the slope increase seen in region b of the double reciprocal plot and in the fluorescence Scatchard plot. The final decrease in slope of region c of Figure 4 would be due to the decreased fluorescence yield of the more weakly bound dyes indicated somewhat incompletely in the equilibrium dialysis data.

Conformational Differences between EFTu–GTP and EFTu–GDP as Evidenced by 1-Anilino-8-naphthalenesulfonate Binding. Although binding of AA-tRNA appears to cancel the difference between the two complexes in terms of their overall interaction with 1-anilino-8-naphthalenesulfonate, it is not possible to conclude that their conformational difference is localized to the AA-tRNA binding site. The observed differences in dissociation constant and fluorescence yield of the bound dye molecules could, however, be due to small perturbations in basically similar sites. Indeed, circular dichroic (CD) studies of EFTu–GDP and EFTu–GTP show no detectable differences in conformation (data not shown).

The assumption throughout this work has been that differential binding of the sulfonate by the two complexes truly reflected differences in conformation. The other possibility, that differential binding is due to selective binding to the nucleotides, is highly unlikely. Both complexes show a high and identical specificity for guanosine; close analogs such as the di- and triphosphates of inosine and xanthosine show no affinity for EFTu. Thus, the guanosine moiety is probably bound to the protein and is unavailable for interaction with the dye in both complexes. The additional phosphate moiety of GTP is also not likely to cause enhanced binding of the anion 1-anilino-8-naphthalenesulfonate.

Function of EFTu. Whereas the results from these experiments and the tritium exchange studies support the view that GTP induces EFTu to assume a conformation that selectively binds AA-tRNA, little is known about subsequent functions of EFTu–GTP. Whether the interaction of EFTu–GTP with AA-tRNA causes a significant alteration in the structure of the tRNA is uncertain. Nmr studies of the base-pair hydrogen bonds in AA-tRNA show that interaction with EFTu–GTP does not change the extent of base pairing in tRNA (C. Hilbert *et al.*, submitted for publication); however, changes in the tertiary structure of AA-tRNA are still possible. Although details of the reaction of the ternary complex with the ribosome remain unclear, it appears that the conformational change accompanying the hydrolysis of GTP to GDP allows EFTu to be removed from the ribosome, freeing the aminoacyl group for peptide bond formation. GDP is then displaced by EFTs with no apparent conformation change in EFTu; the cycle of reactions is complete when GTP interacts with the EFTu–EFTs complex to change the conformation of EFTu into its AA-tRNA binding form.

EFTu–GTP as a Model for the Function of Nucleoside Polyphosphates. The role of GTP in the function of EFTu resembles the role of other nucleoside polyphosphates in the function of motile protein systems and allosteric enzymes. As examples, GTP is an essential effector for CTP synthetase when glutamine is the nitrogen donor (Levitzki and Koshland, 1972) and CTP is an allosteric inhibitor of aspartate transcarbamoylase. These effects are thought to be transmitted to the active site by conformational changes induced by the allosteric ligand; however, other explanations must be considered. In the case of aspartate transcarbamoylase, CTP may inhibit the enzyme by a steric effect rather than a conformational alteration (Warren *et al.*, 1973). The validity of this proposal can be tested; however, it is unlikely that this hypothesis can be extended to explain allosteric acceleration. Fluorescence studies have previously identified conformational changes induced by nucleotide allosteric effectors. In a study of the binding of 1-anilino-8-naphthalenesulfonate to phosphofructokinase, the allosteric effector AMP greatly decreased the fluorescence of the dye–phosphofructokinase complex.

Nevertheless, these enzymes are usually multisubunit complexes, and sometimes, as is true of aspartate transcarbamylase, the regulatory and catalytic sites are on different types of subunits. These properties complicate studies of conformational changes. Similarly, the proteins involved in motility, such as actomyosin, the dynein–tubulin complex of cilia, and the EFG–ribosome–mRNA complex are multiprotein aggregates where the primary function of ATP or GTP is extremely difficult to identify, although there is evidence that the triphosphates induce different conformational states than the diphosphates do (Schaub and Watterson, 1973; Cheung, 1969; Werber *et al.*, 1972).

Motile systems could be related to allosteric systems in that both processes could be initiated by a conformational change induced by a nucleoside triphosphate (Hill, 1969). The former process could be reversed by the dissociation of the inducer, whereas the motile system could be rendered unidirectional and irreversible by hydrolysis of the inducer together with additional interactions between the components after the hydrolytic step. This idea becomes more attractive with the demonstration that a nucleoside triphosphate can promote a significant conformational change upon binding to a relatively small, simple protein of one polypeptide chain.

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Reversible Equilibrium in the Reaction between Ribosomes and the Dissociation Factor of *Escherichia coli*†

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ABSTRACT: Various features of the dissociation of free ribosomes of *Escherichia coli* by initiation factor IF_3 have suggested that the reaction is readily reversible. This reversibility is now demonstrated: ribosomes that have come to equilibrium with the factor rapidly shift their dissociation, to the expected value, when the concentration of the reactants is changed. A similar reequilibration is observed on raising or lowering the concentration of Mg^{2+}, which evidently influences the equilibrium constant. Moreover, when labeled ribosomes were added to an equilibrated mixture it could be shown that the first set of ribosomes partly reassociated and the second set dissociated, both reaching the same equilibrium value.

The ribosome dissociation factor (DF)[1] of *Escherichia coli* (Subramanian *et al.*, 1969), subsequently identified with initiation factor IF_3 (Sabol *et al.*, 1970; Subramanian and Davis, 1970), causes net dissociation of free ribosomes by forming a complex with the 30S subunit (Subramanian *et al.*, 1968; Parenti-Rosina *et al.*, 1969; Sabol and Ochoa, 1971). Since free ribosomes are normally in equilibrium with a low concentration of free subunits (Infante and Baierlein, 1971), the complexes can conceivably be formed either by interaction of DF with the free 30S subunits or by direct attack on the 70S ribosomes (Davis, 1971). But whichever the actual sequence, the overall reaction appears to involve a rapidly reversible, Mg^{2+}-dependent equilibrium rather than a stoichiometric titration, since the amount of dissociation by added IF_3 varies strikingly with Mg^{2+} concentration and is far less than a molar equivalent (Subramanian and Davis, 1970).

Such an equilibrium would be consistent with the observed rapid exchange of subunits between "heavy" and "light" labeled ribosomes mixed shortly after runoff (Subramanian and Davis, 1971), though it is not certain how much of this exchange involves interaction of the particles with DF. On the other hand, it has been reported that free ribosomes previously isolated from a sucrose gradient failed to exchange subunits with ribosomal particles being released from polysomes in their presence, and they also failed to compete effectively with these particles for dissociation ("anti-association") by a limited supply of DF (Kaempfer, 1970, 1971, 1973). Since the discrepancy has significant bearing on the ribosome cycle (Subramanian and Davis, 1973) it seemed important to test directly for the reversibility of the DF reaction.

In order to approximate physiological conditions we used a crude preparation of initiation factors as DF; key experiments were confirmed with partly purified or with pure IF_3. The results will show that freshly released ribosomes reach a reversible equilibrium with DF and subunits, and the subunits exchange rapidly with subsequently added differentially labeled ribosomes. Since this work was completed Sabol *et al.* (1973) have demonstrated reversibility with labeled IF_3 rather than labeled ribosomes: the IF_3 binds reversibly to free 30S subunits and does not bind to 70S ribosomes.

Materials and Methods

Bacterial Preparations. Strain MRE600 of *E. coli*, lacking RNase I, was grown in minimal medium A (Davis and Mingioli, 1950) supplemented with 0.2% glucose and 0.2% Casamino acids as previously described (Beller and Davis, 1971).

Runoff ribosomes were prepared by pelleting from lysates of slow-cooled cells (Beller and Davis, 1971); NH_4Cl-washed ribosomes were prepared according to the method of Iwasaki *et al.* (1968), with modifications, from an S30 extract in TNMD buffer (10 mM Tris-HCl (pH 7.8), 60 mM NH_4Cl, 10 mM $Mg(Ac)_2$, 2 mM dithiothreitol) with 0.5 μg/ml of DNase (Worthington). In this procedure the ribosomes were repelleted, dissolved in TNMD buffer + 20% glycerol to an A_{260} of 200–500, and stored at 0°.

Crude DF was prepared from the 1 M NH_4Cl supernatant (Iwasaki *et al.*, 1968) by slow addition of $(NH_4)_2SO_4$ to 70% saturation; the precipitate was stored at −70°. For use in DF assays part of the precipitate was dissolved, at 10–20 mg of protein/ml, in TKM_5D (10 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM $Mg(Ac)_2$, 2 mM dithiothreitol), and was dialyzed against the same buffer for 1–2 hr. Partly purified IF_3 was

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[1] Abbreviations used are: DF, dissociation factor; IF, initiation factor(s); TKMD and TNMD, buffers described under Materials and Methods.