Kinetic Studies of *Escherichia coli* Elongation Factor Tu–Guanosine 5′-Triphosphate–Aminoacyl-tRNA Complexes†

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ABSTRACT: A new method for measuring the dissociation rate of the *Escherichia coli* elongation factor Tu–GTP–aminoacyl-tRNA complex has been developed and applied to the determination of the dissociation rates of ternary complexes formed between *E. coli* EF-Tu–GTP and a set of *E. coli* aminoacyl-tRNAs. The set of aminoacyl-tRNAs includes at least one tRNA coding for each of the 20 amino acids as well as purified isoacceptor tRNA species for arginine, glycine, leucine, lysine, and tyrosine. The results reveal that the dissociation rates vary for each ternary complex. Tu–GTP–Gln-tRNA dissociates the slowest and Tu–GTP–Val-tRNA the fastest of all noninitiator ternary complexes at 4 °C, pH 7.4. The equilibrium dissociation constant for Tu–GTP–Thr-tRNA has been determined to be 1.3 (0.4) $\times 10^{-9}$ M under identical reaction conditions, and the absolute value of the equilibrium dissociation constant has been calculated for 28 ternary complexes from the relative equilibrium dissociation constant ratios previously measured [Louie, A., Ribeiro, N. S., Reid, B. R., & Jurnak, F. (1984) *J. Biol. Chem. 259*, 5010–5016]. The association rate of each ternary complex has been estimated from the ratio of the dissociation rate relative to the equilibrium dissociation constant. Tu–GTP–His-tRNA associates the fastest and Tu–GTP–Leu-tRNA$_1^{Leu}$ the slowest. By inclusion of Tu–GTP–Met-tRNA$_f^{Met}$ in the studies, evidence has been obtained that suggests that the initiator ternary complex does not function in the elongation cycle because the dissociation rate of the complex is very fast.

The primary function of elongation factor Tu in *Escherichia coli* is to recognize and transport noninitiator, codon-specified, aminoacyl-tRNAs to the ribosome during the elongation cycle of protein synthesis [for review, see Miller & Weissbach (1977) and Kaziro (1978)]. The mechanism by which EF-Tu[1] recognizes the correct type and form of the available tRNAs has been a longstanding research problem. It is now known that the GTP-induced conformation of EF-Tu forms a ternary complex with each noninitiator aminoacyl-tRNA as well as with the initiator fMet-tRNA$_f^{Met}$ species. The latter complex, however, is considerably less stable and is therefore not likely to be relevant in cellular functions (Tanada et al., 1981).

The most recent studies on the ternary complex have revealed the surprising result that EF-Tu–GTP does not bind to all noninitiator aminoacyl-tRNAs with equivalent affinities (Pingoud et al., 1977; Knowlton & Yarus, 1980; Wagner & Sprinzl, 1980; Pingoud & Urbanke, 1980; Tanada et al., 1981, 1982; Louie et al., 1984a). EF-Tu–GTP forms the strongest ternary complex with Gln-tRNA and the weakest with Val-tRNA (Louie et al., 1984a). Although the relative strengths of all noninitiator ternary complexes are not likely to affect in vivo protein synthesis significantly, the differences are related to the specific recognition of unique features of each aminoacyl-tRNA by EF-Tu–GTP. Several regions of tRNA, including the acyl linkage (Ravel et al., 1967; Weissbach et al., 1978), the CCA 3′-terminal sequence (Thang et al., 1972; Ofengand & Chen, 1972; Schulman et al., 1974; Sprinzl et al., 1977), the amino group substituent (Miller & Weissbach, 1977), and the acceptor stem helix (Jekowsky et al., 1977), are known to interact with the elongation factor. Of these, only the latter two regions are unique to each aminoacyl-tRNA, and several studies have definitively correlated these structural differences with the observed differences in equilibrium dissociation constants among noninitiator ternary complexes (Pingoud et al., 1977; Knowlton & Yarus, 1980; Wagner & Sprinzl, 1980; Pingoud & Urbanke, 1980; Tanada et al., 1981, 1982; Louie et al., 1984a). We have undertaken the present research to determine if the dynamic interactions, as defined by the association and dissociation rates of the ternary complex, would provide additional insights into the differential recognition of the noninitiator aminoacyl-tRNAs by EF-Tu–GTP. We have developed a new assay procedure to measure the dissociation rate of the ternary complex and have applied it to the study of complexes formed between EF-Tu–GTP and each of 28 naturally occurring *E. coli* aminoacyl-tRNAs. We have estimated the association rate for each ternary complex by appropriately combining our studies of the equilibrium dissociation constants and the dissociation rates and have analyzed the data in light of the differential structural features of the aminoacyl-tRNAs. By including Met-tRNA$_f^{Met}$ in our studies, we have obtained evidence that suggests a mechanism by which EF-Tu–GTP selectively discriminates against incorrect tRNA species during the elongation cycle.

Experimental Procedures

Materials

Unless otherwise noted, all standard biochemicals, including pyruvate kinase (PK) (EC 2.7.1.40), phosphoenolpyruvate (PEP), GDP, and GTP, were purchased from Sigma. Twenty-seven purified *E. coli* tRNA species were obtained as a gift from Susan Ribeiro and Dr. Brian Reid, Department of Chemistry, University of Washington, Seattle, WA. Purified yeast tRNA^{Phe} was purchased from Boehringer Mann-

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[1] Abbreviations: EF-Tu, protein synthesis elongation factor Tu from *Escherichia coli*; aa-tRNA, aa-tRNA^{aa} from *E. coli* where aa is given in terms of the standard three-letter notation for each amino acid; Tu–GTP–aa-tRNA, ternary complex between EF-Tu–GTP and aa-tRNA^{aa}.

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heim. Asn-tRNA^{Asn} was purified from unfractionated *E. coli* tRNAs purchased from Plenum Scientific Research, Inc., according to the EF-Tu–GTP affinity chromatographic method described previously (Louie et al., 1984b). All assay materials were purchased from sources described previously (Louie et al., 1984a).

Methods

Preparation of EF-Tu–GTP, Unfractionated Synthetases, and [^{14}C]Aminoacyl-tRNAs. The preparation of the major macromolecular components has been described previously in detail (Louie et al., 1984a). In order to obtain an estimate of the association rates, samples from the same preparation of [^{14}C]aminoacyl-tRNAs were used in both the ternary complex dissociation rate measurements and in the relative equilibrium dissociation constant study (Louie et al., 1984a). Although the specific activity of each [^{14}C]aminoacyl-tRNA listed in Table I is lower than that expected for a fully aminoacylated tRNA, extensive aminoacylation assays demonstrated that the lower value, with the exception of Asn-tRNA^{Asn}, was due to partial deacylation rather than to contamination by other tRNAs or fragments. For the determination of the absolute equilibrium dissociation constant of Tu–GTP–[^{14}C]Thr-tRNA, the tRNA^{Thr} was aminoacylated and repurified by EF-Tu–GTP affinity chromatographic methods (Louie et al., 1984b). The final specific activity of the [^{14}C]Thr-tRNA was 1662 pmol/absorbance unit at 260 nm, indicating that the tRNA was fully aminoacylated.

Determination of the Ternary Complex Equilibrium Dissociation Constant. The equilibrium dissociation constant for the ternary complex between EF-Tu–GTP and [^{14}C]Thr-tRNA was determined by the ribonuclease resistance assay under conditions similar to those described by Knowlton & Yarus (1980) but modified as described herein. The assay was performed at four concentrations of [^{14}C]Thr-tRNA and five concentrations of EF-Tu–GTP in a fixed volume of reaction buffer, which contained 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 150 mM NH_4Cl, 5 mM DTT, 0.02% (w/v) sodium azide, 3 mM PEP, 20 IU/mL PK, and 1×10^{-5} M GTP. Each of the 20 different sets of concentrations were assayed in duplicate. The final [^{14}C]Thr-tRNA concentrations surveyed were 4.8×10^{-9}, 8.8×10^{-9}, 26.2×10^{-9} and 54.3×10^{-9} M; the final EF-Tu–GTP concentrations were 8.7×10^{-9}, 13.0×10^{-9}, 21.7×10^{-9}, 30.4×10^{-9} and 43.5×10^{-9} M in a total reaction volume of 1.61 mL. Each reaction mixture was incubated at 4 °C for 30 min and then treated with 20 μL of pancreatic ribonuclease (1 mg/mL) for 15 s at 4 °C. The ribonuclease digestion was stopped by the rapid addition of 10 μL of bulk nucleic acid (10 mg/mL) and 3 mL of chilled 10% TCA. Precipitates were collected on nitrocellulose filters, washed thrice with chilled 10% TCA, and counted in Ultrafluor.

The equilibrium dissociation constant is given by

$$K_{diss} = [\text{uncomplexed EF-Tu–GTP}][\text{uncomplexed [}^{14}\text{C]Thr-tRNA}]/[\text{Tu–GTP–[}^{14}\text{C]Thr-tRNA}]$$

As shown previously (Knowlton & Yarus, 1980; Tanada et al., 1981, 1982; Louie et al., 1984a), the concentration of the ternary complex formed during the assay is measured directly from the ^{14}C radioactivity remaining on the filter. The concentration of the uncomplexed [^{14}C]Thr-tRNA is calculated from the difference between the total concentration of the [^{14}C]Thr-tRNA in the particular assay and the concentration that formed into ternary complex.

The activity of EF-Tu toward nucleotide exchange is retained longer than its ability to complex aminoacyl-tRNAs.

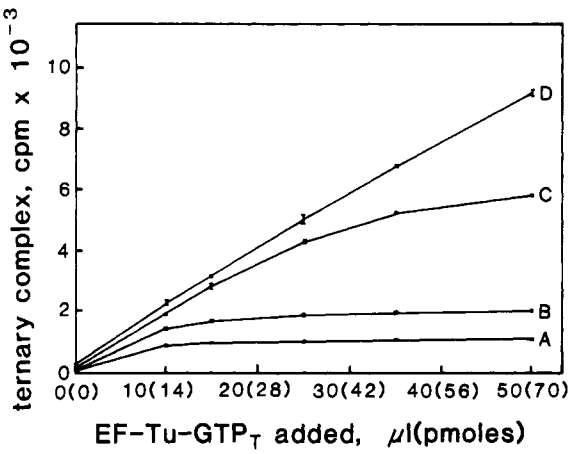

FIGURE 1: Dependence of ternary complex concentration on aminoacyl-tRNA and EF-Tu–GTP concentrations. A total of 14, 21, 35, 49, or 70 pmol of EF-Tu–GTP was incubated in the presence of 7.7 of (A), 14.1 (B), 42.3 (C), or 87.4 (D) pmol of [^{14}C]Thr-tRNA (143 cpm/pmol) in 1.61 mL of reaction buffer for 30 min at 4 °C. The reaction was terminated by the addition of 20 μg of pancreatic ribonuclease for 15 s, followed by the addition of 100 μg of bulk nucleic acid and 4 mL of 10% TCA. The precipitates were collected on nitrocellulose filters and counted. The residual ^{14}C radioactivity, after correction for background, is proportional to the concentration of EF-Tu–GTP–[^{14}C]Thr-tRNA at each set of EF-Tu–GTP and [^{14}C]Thr-tRNA concentrations.

Therefore, the standard assays (Miller & Weissbach, 1974) for determining the EF-Tu–GTP concentration are not reliable in quantitating the amount of protein that is actually available for complexation with aminoacyl-tRNA. A similar problem is encountered with the aminoacyl-tRNA which deacylates over time in storage. To circumvent the problems, the total concentrations of the stock solutions for EF-Tu–GTP and [^{14}C]Thr-tRNA were determined directly in the equilibrium dissociation constant assays but at extreme conditions in which one species was saturated with the other. For the Tu–GTP–[^{14}C]Thr-tRNA assays, the amount of radiolabeled ternary complex is plotted as a function of the EF-Tu–GTP volume in Figure 1. In the presence of excess EF-Tu–GTP (curves A and B), the maximal concentration of the ternary complex detected is assumed to be equivalent to the concentration of [^{14}C]Thr-tRNA capable of binding to EF-Tu–GTP. For the experiment, the initial stock concentration of active [^{14}C]Thr-tRNA was determined to be 8.74×10^{-6} M. The initial stock concentration of active EF-Tu–GTP was determined to be 2.8×10^{-6} M in a similar manner. In repeated experiments (not shown), the saturation method yielded more accurate and reproducible measurements of species concentrations active in ternary complex formation than any other independent assays for EF-Tu–GTP or aminoacyl-tRNA. Only those assays in which the uncomplexed species were within 10–90% of the total species concentration were used in the final calculations of the equilibrium dissociation constant for Tu–GTP–[^{14}C]Thr-tRNA. The standard error is given as the standard deviation from the mean value.

Determination of Ternary Complex Dissociation Rates. A new method termed the ribonuclease digestion rate assay was developed to determine the dissociation rate of each ternary complex under consideration. In this method, EF-Tu–GTP is incubated with [^{14}C]aminoacyl-tRNA until equilibrium with the ternary complex is attained. The equilibrium is subsequently perturbed by the addition of pancreatic ribonuclease, which selectively digests the uncomplexed [^{14}C]aminoacyl-tRNA but does not affect the ternary complex in the reaction mixture (Knowlton & Yarus, 1980; Tanada et al., 1981). The

removal of one component of the reaction prevents ternary complex reassociation, which would otherwise mask the normal dissociation process. The disappearance of the ternary complex as a function of time is monitored by the loss of TCA-precipitable radioactivity and is equivalent to the dissociation rate of the ternary complex.

For ternary complex formation, 91 pmol of EF-Tu–GTP in 650 μL of reaction buffer was added to 65–130 pmol of [^{14}C]aminoacyl-tRNA in 75 μL of 0.1 mM sodium thiosulfate and brought to a final volume of 6.5 mL with reaction buffer. After 15 min at 4 °C, 130 μL of pancreatic ribonuclease stock (5 mg/mL) was added. Aliquots of 1.0 mL were withdrawn after 0.5, 1, 2, 3, 4, and 5 min for the noninitiator ternary complexes and after 0.5, 1, 1.5, 2, 2.5, and 3 min for Tu–GTP–Met-tRNA$_f^{Met}$. Aliquots were then added to 10% TCA, and precipitates were processed as described previously. The amount of ternary complex remaining at each time interval was equivalent to the radioactivity present on the filters after correcting for background radioactivity that survived a blank reaction in which EF-Tu–GTP was omitted. The dissociation is described by

$$\text{EF-Tu–GTP–aa-tRNA} \xrightarrow{k_{off}} \text{EF-Tu–GTP} + \text{aa-tRNA}$$

and the disappearance of the ternary complex is given by the first-order rate law:

$$\frac{d[\text{Tu–GTP–aa-tRNA}]}{dt} = -k_{off}t$$

The natural logarithm of Tu–GTP–[^{14}C]aa-tRNA (in cpm) was plotted as a function of time (t), and the data were fitted to a straight line by a linear least-squares analysis. For each ternary complex, the correlation coefficient for all six data points was greater than 99%. The dissociation rate (k_{off}) of each ternary complex was determined from the slope; the standard deviation was derived from the standard error of the calculated slope and then doubled.

To validate the accuracy of the dissociation rates determined by the new method, the dissociation rate of the Tu–GTP–[^{14}C]Thr-tRNA complex was determined by an alternate method, in which excess unlabeled aminoacyl-tRNA, instead of ribonuclease, was added to the equilibrated mixture to prevent reassociation of the radiolabeled ternary complex. The assay was carried out as described in detail by Knowlton & Yarus (1980), but three reaction conditions were altered in order to permit a direct comparison of the ternary complex dissociation rates determined by the two independent methods. The assay was performed at 4 °C, rather than 37 °C, EF-Tu–GTP and [^{14}C]Thr-tRNA were equilibrated for 15 min rather than 5 min, and the reaction buffer described herein was used.

Optimization of the Ribonuclease Digestion Rate Assay. Preliminary experiments were conducted to optimize the reaction conditions of the ribonuclease digestion rate assay and to determine if any factors perturbed the first-order nature of the dissociation of the ternary complex.

To optimize the ribonuclease concentration, the rate assay was conducted at each of six different ribonuclease concentrations. For each assay, 161 pmol of EF-Tu–GTP was incubated with 230 pmol of [^{14}C]Thr-tRNA in a total volume of 11.5 mL of reaction buffer. After 15 min at 4 °C, 230 μL of a pancreatic ribonuclease stock solution at 0.2, 1, 5, 10, 20, or 50 mg/mL was added. Aliquots of 1.0 mL were withdrawn from each assay reaction after 1, 3, 5, 7, 10, 13, 15, 20, 25, 30, and 40 min, placed in 10 μL of bulk nucleic acid (10 mg/mL), and then added to 4.0 mL of prechilled 10% TCA. The precipitates were processed as described previously, and the natural logarithm of the ternary complex radioactivity was plotted as a function of time for each ribonuclease concentration. At the highest ribonuclease concentration, the slope was linear for a period of time that exceeded the half-life by a factor of 3. As the ribonuclease concentration was lowered in the reaction mixture, the slope deviated from linearity at earlier time intervals. Yet, at all ribonuclease concentrations tested, the initial slopes of the plots were equivalent to one another and linear for a time interval greater than the half-life of the ternary complex. Additional experiments were performed to clarify the effects observed at the lower ribonuclease concentration.

Two major changes occur as the ribonuclease digestion of the ternary complex reaction mixture is prolonged. First, the concentration of tRNA fragments produced by the ribonuclease digestion increases with time. To determine if the tRNA fragments effectively compete with the uncomplexed [^{14}C]Thr-tRNA for ribonuclease activity, excess unacylated tRNA was added to the rate assay, and the effects upon ternary complex dissociation were monitored. EF-Tu–GTP was incubated with [^{14}C]Thr-tRNA, and after 15 min at 4 °C, ribonuclease was added. Aliquots were withdrawn after 1, 4, 5.5, and 7 min, precipitated, and processed as described previously. After 7.5 min from the start of the ribonuclease digestion, a 600-fold excess of unfractionated, unacylated tRNAs was added. The removal and precipitation of aliquots continued after an additional 0.5, 2.5, 4.5, 6.5, and 9.5 min. Between 1 and 7 min, the slope was equivalent to the initial slopes previously observed. After the addition of excess tRNAs, the slope changed and the dissociation rate decreased, indicating that the presence of a high concentration of tRNA fragments effectively competed with the uncomplexed [^{14}C]-Thr-tRNA for ribonuclease activity. The experiment suggested that the nonlinearity of the slopes at lower ribonuclease concentrations was due to reassociation of the labeled ternary complex rather than to the perturbation of the first-order nature of the ternary complex dissociation in the presence of ribonuclease. At all ribonuclease concentrations tested, the initial slopes were linear and equivalent to the total slope at the highest ribonuclease concentration. Therefore, the initial linear slopes represent the dissociation rate of the ternary complex. In other experiments, the time interval and ribonuclease concentration at which the dissociation rate became nonlinear was dependent upon the aminoacyl-tRNA species used in ternary complex formation. At a ribonuclease stock concentration of 5 mg/mL, the dissociation rate of each ternary complex remained constant for a time period equivalent to or greater than the half-life of the complex.

The second major change that occurs as the ribonuclease digestion is prolonged is the increase in the concentration of the uncomplexed EF-Tu–GTP relative to uncomplexed aminoacyl-tRNA. To determine if the excess EF-Tu–GTP was competing with the ribonuclease for reassociation with the uncomplexed aminoacyl-tRNA, the rate assay was performed as described at each of four concentrations of EF-Tu–GTP. The slopes of the fitted lines were equivalent over the 5-fold range of elongation factor concentrations tested, indicating that excess EF-Tu–GTP does not compete with the ribonuclease for reassociation with uncomplexed [^{14}C]Thr-tRNA.

As a final check on the kinetic behavior of the ternary complex dissociation, the total reaction volume of the rate assay was varied from 0.65 to 6.5 mL of reaction buffer. The rate assay was carried out as initially described with the exception that aliquot volumes ranged from 0.1 to 1.0 mL to compensate

Table I: Kinetic Data for the Tu–GTP–Aminoacyl-tRNA Complexes at 4 °C

[^{14}C]aa-tRNA^{aa} (species)[a]	sp act. (pmol/A_{260})	dissociation rate ($\times 10^3$ s^{-1})	equilibrium dissociation constant ($\times 10^{10}$ M)	association rate ($\times 10^{-5}$ M^{-1} s^{-1})	k_{on}([^{14}C]aa-tRNA)/k_{on}([^{14}C]Phe-tRNA)
Gln	483	0.9 (0.1)	5.2 (2.0)	17.0 (7.0)	1.14 (0.20)
Asn	167	1.1 (0.1)	8.0 (3.1)	14.0 (6.0)	0.90 (0.15)
Thr	952	1.1 (0.2)	13.0 (4.0)	8.4 (3.7)	0.55 (0.14)
Pro	574	1.2 (0.2)	10.0 (4.0)	12.0 (5.0)	0.77 (0.17)
Trp	814	1.2 (0.5)	7.9 (3.1)	15.0 (9.0)	0.99 (0.44)
Ile	462	1.3 (0.1)	20.0 (8.0)	6.4 (2.5)	0.42 (0.07)
Leu (2)	1172	1.3 (0.1)	23.0 (9.0)	5.6 (2.2)	0.36 (0.06)
Leu (4)	1000	1.3 (0.1)	18.0 (7.0)	7.1 (2.8)	0.46 (0.08)
Gly (2)	1055	1.3 (0.3)	8.9 (3.5)	15.0 (7.0)	0.95 (0.26)
Gly (3)	789	1.6 (0.3)	9.1 (3.6)	18.0 (8.0)	1.15 (0.28)
Met (m)	508	1.6 (0.3)	8.9 (3.4)	18.0 (8.0)	1.18 (0.28)
Phe (yeast)	1154	1.6 (0.1)	9.4 (3.8)	17.0 (7.0)	1.11 (0.20)
Phe (*E. coli*)	1408	1.7 (0.1)	11.0 (5.0)	15.0 (6.0)	1.00 (0.19)
Cys	1331	1.8 (0.3)	10.0 (5.0)	17.0 (8.0)	1.14 (0.33)
Glu (2)	1110	1.9 (0.1)	34.0 (13.0)	5.7 (2.3)	0.37 (0.06)
Tyr (2)	832	2.0 (0.1)	13.0 (5.0)	15.0 (6.0)	1.00 (0.16)
Tyr (1)	590	2.0 (0.1)	13.0 (5.0)	15.0 (6.0)	1.00 (0.16)
Leu (1)	1218	2.0 (0.1)	58.0 (22.0)	3.4 (1.3)	0.22 (0.03)
Ser (1B)	1048	2.0 (0.1)	14.0 (5.0)	15.0 (6.0)	0.96 (0.17)
Asp	732	2.2 (0.5)	23.0 (9.0)	9.5 (4.3)	0.62 (0.17)
Ala (1B)	1508	2.4 (0.2)	23.0 (9.0)	10.0 (4.0)	0.67 (0.11)
Leu (5)	1100	2.8 (0.1)	60.0 (25.0)	4.6 (1.9)	0.30 (0.06)
Lys (1)	1263	2.9 (0.2)	34.0 (13.0)	8.6 (3.4)	0.56 (0.10)
Lys (2)	698	2.9 (0.2)	46.0 (18.0)	6.3 (2.5)	0.41 (0.07)
His	881	3.0 (0.1)	19.0 (5.0)	25.0 (10.0)	1.65 (0.24)
Arg (2)	1214	3.0 (0.1)	20.0 (8.0)	15.0 (6.0)	0.97 (0.14)
Arg (1)	1230	3.2 (0.1)	20.0 (8.0)	16.0 (6.0)	1.04 (0.15)
Val (1)	933	4.9 (0.2)	71.0 (28.0)	6.9 (2.8)	0.45 (0.08)
Met (f)	1290	49.0 (6.0)	1100.0 (460.0)	4.5 (2.0)	0.30 (0.07)

[a] Number or letter in parentheses denotes the designated tRNA species type.

for the change in total volume. The dissociation rate of the Tu–GTP–[^{14}C]Thr-tRNA complex remained the same at all reaction volumes tested. In the course of the ribonuclease digestion rate assay, 14 independent measurements of the dissociation rate of the Tu–GTP–Thr-tRNA complex were obtained and subsequently averaged to yield the final value.

Results and Discussion

Equilibrium Dissociation Constants for All Ternary Complexes. The equilibrium dissociation constant between *E. coli* EF-Tu–GTP and *E. coli* [^{14}C]Thr-tRNA was determined at 4 °C by application of the ribonuclease resistance assay as described under Methods. The results from 15 independent assays were averaged to obtain the final value of 1.3 (0.4) $\times$ 10^{-9} M for the Tu–GTP–[^{14}C]Thr-tRNA equilibrium dissociation constant at 4 °C.

In a previous study (Louie et al., 1984a), the relative affinities of a set of *E. coli* aminoacyl-tRNAs for *E. coli* EF-Tu–GTP were accurately determined under identical reaction conditions as the Tu–GTP–Thr-tRNA complex studies. By combining the observed ratios of equilibrium dissociation constants measured in the previous study and the equilibrium dissociation constant of Tu–GTP–Thr-tRNA determined in the present study, an absolute equilibrium dissociation constant for each ternary complex was calculated. The probable error in the equilibrium dissociation constant was estimated from the standard deviation of each variable according to the method of Margenau & Murphy (1955). The results are included in Table I. The results indicate that all ternary complexes are strong but that EF-Tu–GTP does exhibit a preference for different aminoacyl-tRNA species. Gln-tRNA forms the strongest complex and Val-tRNA the weakest of all noninitiator Tu–GTP–aminoacyl-tRNA complexes, with equilibrium dissociation constants ranging from 5.2 (2.0) $\times$ 10^{-10} M to 7.1 (2.8) $\times$ 10^{-9} M, respectively. The equilibrium dissociation constant for the Tu–GTP–Met-tRNA$_f^{Met}$ complex is 1.1 (0.5) $\times$ 10^{-7} M, approximately 50-fold weaker than the average dissociation constant for the noninitiator aminoacyl-tRNAs.

The equilibrium dissociation constants for several naturally occurring *E. coli* ternary complexes, including those with Glu-tRNA, Trp-tRNA (Knowlton & Yarus, 1980; Pingoud & Urbanke, 1980), fMet-tRNA$_f^{Met}$, Met-tRNA$_m^{Met}$ (Tanada et al., 1982), Lys-tRNA, Phe-tRNA, Tyr-tRNA (Pingoud & Urbanke, 1980), and Val-tRNA (Wittinghoffer et al., 1980), have been measured by several laboratories by different methods under different reaction conditions. The values range from 4 $\times$ 10^{-5} M for fMet-tRNA$_f^{Met}$ to 1.7 (0.5) $\times$ 10^{-8} M for Glu-tRNA. In general, the equilibrium dissociation constant reported herein for a particular ternary complex is smaller by approximately 100-fold, indicating that the ternary complex can be stronger than previously reported. Most of the discrepancies can be attributed to the different reaction conditions for the measurement of the ternary complex equilibrium dissociation constant, such as the lower temperature and the higher concentration of NH_4Cl in the buffers used herein. The latter condition has recently been shown to be very important in increasing the stability of the Tu–GTP–Val-tRNA complex (Antonsson & Leberman, 1982). Any remaining discrepancies are probably due to experimental difficulties in measuring the dilute concentrations of the individual components. In order to minimize the concentration errors and the effect upon the equilibrium dissociation constant calculation, the assay conditions were carefully designed to yield measurements of the total concentration of EF-Tu–GTP and aminoacyl-tRNA that were actually active in ternary complex formation directly within the assay. Furthermore, to obtain a reliable determination, the absolute equilibrium dissociation constant of Tu–GTP–Thr-tRNA was measured under multiple sets of concentration conditions, and all values are in good agreement. Therefore, although our equilibrium

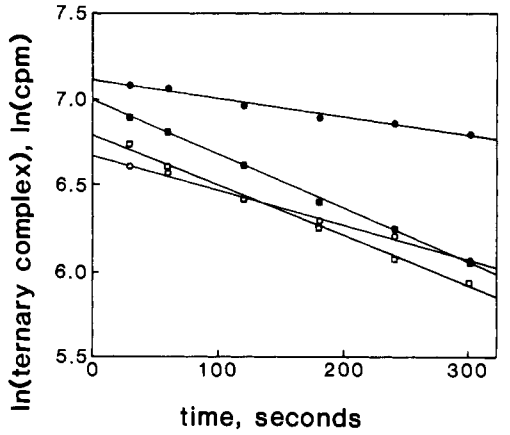

FIGURE 2: Dissociation rates of four ternary complexes. Experimental data for the determination of the dissociation rates of complexes formed between EF-Tu–GTP and Arg-tRNA$_1^{Arg}$ (162.9 cpm/pmol, ■), Arg-tRNA$_2^{Arg}$ (162.9 cpm/pmol, □), Leu-tRNA$_4^{Leu}$ (156.6 cpm/pmol, ●), or Leu-tRNA$_5^{Leu}$ (156.6 cpm/pmol, ○) are shown. Each ternary complex was equilibrated in 6.5 mL of reaction buffer in the presence of 91 pmol of EF-Tu–GTP and 26–169 pmol of [^{14}C]-aminoacyl-tRNA. After 15 min at 4 °C, 650 μg of ribonuclease was added. Aliquots of 1.0 mL were removed after 0.5, 1, 2, 3, 4, and 5 min and processed as described under Methods. The residual ^{14}C radioactivity, after correction for background, represents the amount of ternary complex remaining at the designated time interval. The best fit of the data was determined by a linear least-squares analysis, and the dissociation rate of each ternary complex was calculated from its slope.

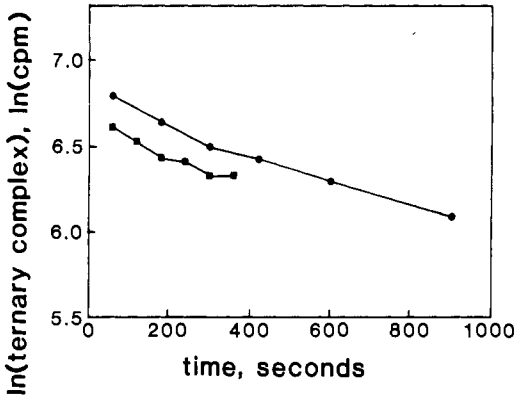

FIGURE 3: Determination of the dissociation rate of the Tu–GTP–Thr-tRNA complex by two methods. The dissociation rate of the Tu–GTP–Thr-tRNA complex was determined by the ribonuclease digestion rate assay (●) and the Knowlton & Yarus (1980) method (■). In each assay, 91 pmol of EF-Tu–GTP was incubated with 130 or 65 pmol, respectively, of [^{14}C]Thr-tRNA (113 cpm/pmol) in 6.5 mL of reaction buffer for 15 min at 4 °C. In the ribonuclease digestion rate assay (●), 65 μg of ribonuclease was added to the reaction mixture. Aliquots of 1.0 mL were removed after 1, 3, 5, 7, 10, and 15 min and processed as described under Methods. In the Knowlton & Yarus assay (■), 2600 pmol of unlabeled, unfractionated aminoacyl-tRNAs was added to the reaction mixture. After 1, 2, 3, 4, 5, and 6 min, aliquots of 1.0 mL were withdrawn, digested with 100 μg of ribonuclease for 15 s, precipitated in TCA, and processed as described under Methods. The dissociation rate of the Tu–GTP–Thr-tRNA complex was calculated from the slope of the line fitted to the data from each method by linear least-squares analysis.

dissociation constant for Tu–GTP–Thr-tRNA is smaller than that expected from other ternary complex studies (Knowlton & Yarus, 1980; Pingoud & Urbanke, 1980; Tanada et al., 1982; Wittinghofer et al., 1980), we believe it to be a reliable one and suitable for a standard on which to calculate the absolute equilibrium dissociation constants for all other ternary complexes.

Dissociation and Association Rates of the Ternary Complex. The dissociation rate of the ternary complex between *E. coli* EF-Tu–GTP and each aminoacyl-tRNA from a set of naturally occurring *E. coli* aminoacyl-tRNAs has been determined by a newly developed method termed the ribonuclease digestion rate assay. The set of purified aminoacyl-tRNAs included tRNAs coding for each of the 20 amino acids as well as isoacceptor species for arginine, glycine, leucine, lysine, and tyrosine. For comparison, *E. coli* Met-tRNA$_f^{Met}$ and yeast Phe-tRNA were also included.

To determine the dissociation rate of each ternary complex, EF-Tu–GTP and the chosen [^{14}C]aminoacyl-tRNA were equilibrated at 4 °C for 15 min. Subsequently, the uncomplexed [^{14}C]aminoacyl-tRNA was removed by ribonuclease digestion, and reassociation of the ternary complex was prevented. Several preliminary experiments demonstrated that, under the conditions of the assay, the ribonuclease does not affect the complexed aminoacyl-tRNA and that, as the uncomplexed aminoacyl-tRNA is selectively removed from the reaction, the dissociation of the ternary complex follows that expected for a first-order reaction. Examples of the data are shown in Figure 2. The complete listing of dissociation rates is presented in Table I in ascending order from the slowest dissociation rate to the fastest.

The final results indicate that the dissociation rate of most ternary complexes varies slowly but continuously over a 3.5-fold range at 4 °C from the slowest rate of 9.0 (0.1) × 10^{-4} s^{-1} for Gln-tRNA to 3.2 (0.1) × 10^{-3} s^{-1} for Arg-tRNA$_1^{Arg}$. There are two ternary complexes with dissociation rates that deviate from the normal range. The dissociation rate of 4.9 (0.2) × 10^{-3} s^{-1} for Tu–GTP–Val-tRNA is the fastest of all noninitiator ternary complexes and that of 4.9 (0.6) × 10^{-2} s^{-1} for Tu–GTP–Met-tRNA$_f^{Met}$ is 10-fold faster than all other complexes considered. With the exception of the leucine isoacceptors, the dissociation rates are equivalent within the experimental error for each pair of ternary complexes with isoacceptor tRNAs coding for glycine, arginine, tyrosine and lysine. The ternary complexes with Leu-tRNA$_1^{Leu}$ and Leu-tRNA$_5^{Leu}$ dissociate faster than the complexes with Leu-tRNA$_2^{Leu}$ and Leu-tRNA$_4^{Leu}$ by a factor of 2.

As an independent check on the validity of the ternary complex dissociation rates measured by the ribonuclease digestion rate assay, the dissociation rate of the Tu–GTP–Thr-tRNA complex was determined by an alternate assay proposed by Knowlton & Yarus (1980) and described under Methods. Because both rate methods were carried out under identical temperature and buffer conditions, the results can be compared directly and are shown in Figure 3. The Tu–GTP–Thr-tRNA dissociation rate of 1.0 × 10^{-3} s^{-1} determined by the latter method compares well with the rate of 1.1 (0.2) × 10^{-3} s^{-1}, which was averaged over 14 independent measurements of the ribonuclease digestion rate assay. Therefore, the new dissociation rate method yields comparable values to the only previously published method.

The rate of association of the ternary complex is too fast to be measured by standard laboratory techniques. Instead, a reasonable estimate of the association rate for each ternary complex was calculated from the dissociation rate and the equilibrium dissociation constant, both of which were determined for the same set of aminoacyl-tRNAs at 4 °C under identical assay conditions. The probable error in the association rate was estimated from the standard deviation of each variable according to the method of Margenau & Murphy (1955). The complete listing of association rates and the estimated errors is included in Table I. There is a 7.4-fold range in the association rate over the entire set of ternary complexes. The slowest association rate, 3.4 (1.3) × 10^5 M^{-1}

s^{-1}, is found for Tu–GTP–Leu-tRNA$_1^{Leu}$ and the fastest, 2.5 (1.0) × 10^6 M^{-1} s^{-1}, for Tu–GTP–His-tRNA. Surprisingly, the association rate of the ternary complex with Met-tRNA$_f^{Met}$ falls within the range of all noninitiator ternary complexes. With the exception of the complexes with Tu–GTP–Tyr-tRNA$_{1,2}^{Tyr}$, the association rates are not equivalent for the ternary complexes formed with other sets of isoacceptor aminoacyl-tRNAs. The association rates vary from 7% for the arginine isoacceptors to 69% for the leucine set.

Because the large standard deviations tend to blur the distinction among isoacceptors or other ternary complexes, a more reliable indicator of the real differences may be the ratio of association rates of one ternary complex relative to Tu–GTP–Phe-tRNA. Such a ratio is calculated directly from data that are less prone to experimental errors such as the relative equilibrium dissociation constant ratio and the observed dissociation rates of the two ternary complexes. The new ratio of association rates and the calculated error is included in Table I for each ternary complex. The data clearly indicate that the differences in the association rates among all complexes are real and cannot be attributed to experimental errors.

Regardless of the size of the estimated error, the ternary complex association rates fall within the range of 10^5–10^6 M^{-1} s^{-1}, rates that are generally considered to be slower than diffusion-controlled rates 10^8–10^9 M^{-1} s^{-1} (Koren & Hammes, 1976). The magnitude of the rates, therefore, suggests that the association of any ternary complex involves a correct orientation of EF-Tu–GTP and aminoacyl-tRNA. Furthermore, the small but real differences observed in the association rates over the range of all ternary complexes imply that the final alignment of the protein and each aminoacyl-tRNA involves recognition of features that are unique to each aminoacyl-tRNA. The largest differences in association rates are found among the leucine and methionine isoacceptors: Tu–GTP–Leu-tRNA$_4^{Leu}$ associates twice as fast as Tu–GTP–Leu-tRNA$_1^{Leu}$ and the ternary complex with Met-tRNA$_m^{Met}$ 4-fold faster than that with Met-tRNA$_f^{Met}$. Because these aminoacyl-tRNA sets have the same amino acid group, the association rate differences can be attributed solely to the differences in the acceptor stem helix sequences of the tRNA (Gauss & Sprinzl, 1983). The present study includes no examples of aminoacyl-tRNAs that have identical base pair sequences but different aminoacyl groups, therefore, it is not possible to estimate the relative effect of the aminoacyl substituent upon the association rate.

Contrary to the association rates, the observed dissociation rates of the noninitiator ternary complexes vary over a much narrower range. The narrow range suggests that once the ternary complex is formed between EF-Tu–GTP and each aminoacyl-tRNA, the stabilizing forces are nearly equivalent in all ternary complexes, thus minimizing the effects of any differences due to the aminoacyl group or the tRNA structure and reducing the possibility that EF-Tu–GTP would favor one type of noninitiator aminoacyl-tRNA over another in protein synthesis.

The most surprising result of the present study is the finding that Met-tRNA$_f^{Met}$ associates with EF-Tu–GTP at a rate comparable to that of all noninitiator aminoacyl-tRNAs. In previous investigations, Met-tRNA$_f^{Met}$ has been observed to form a very weak complex with EF-Tu–GTP, which does not function in the elongation cycle of protein synthesis (Tanada et al., 1982). Given the results of the present study, the cause of the weaker ternary complex formation can clearly be attributed to a much faster dissociation rate of the errant complex. The results suggest that EF-Tu–GTP initially recognizes all aminoacyl-tRNAs approximately equivalently but that proper selection of the noninitiator aminoacyl-tRNAs occurs by the rapid rejection of the initiator tRNA species. It is tempting to speculate that EF-Tu–GTP could recognize and bind to unacylated tRNAs as well but, without the enhanced stability arising from specific interactions with the aminoacyl group or linkage, would rapidly reject them. Ternary complexes between EF-Tu–GTP and unacylated tRNAs have indeed been detected (Shulman et al., 1974; Pingoud et al., 1982), but due to the lack of a radioactive aminoacyl label, it is not possible to determine the dissociation or association rates by the present methods.

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Registry No. Ribonuclease, 9001-99-4.

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Irreversible Binding of Phage φX174 to Cell-Bound Lipopolysaccharide Receptors and Release of Virus–Receptor Complexes†

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ABSTRACT: At 37 °C, binding of φX174 to the lipopolysaccharide receptors in the outer membrane of *Escherichia coli* C is followed by an irreversible ejection of its DNA. DNA ejection marks the beginning of the eclipse period in the infection cycle. Binding data with a φX mutant F*cs*70 at 15 °C, where the DNA ejection, or eclipse, rate is essentially zero, do not follow the law of mass action. This rules out a simple mechanism of reversible binding followed by irreversible DNA ejection. A more complex reaction model was devised to fit the data [Incardona, N. L. (1983) *J. Theor. Biol. 105*, 631–645]. It takes into account the fact that lipopolysaccharide-containing outer membrane fragments are continually released from infected *E. coli* cells, some of which have φX bound to them. In this paper the model is shown to fit the binding data for wild-type virus at 15 °C and to account for the nonlinearity observed at 37 °C in the pseudo-first-order binding kinetics and first-order eclipse kinetics for both mutant and wild-type virus. This leads to the conclusion that φX174 binding to cell-bound receptors is irreversible but binding to released receptors is reversible. The release of virus–receptor complexes from infected cells and the dissociation of these complexes were confirmed by electron microscopy. We propose that initially a single φX174 vertex interacts reversibly with *E. coli* lipopolysaccharide but dissociation from the cell is prevented by the subsequent interaction of additional vertices with adjacent receptor molecules.

Protein–protein and protein–nucleic acid interactions play a central role in stabilizing the nucleocapsid structure of most viruses, particularly in their extracellular form. In the case of φX174, the isometric single-stranded DNA phage, a specific set of noncovalent interactions must be rapidly broken when the phage binds to its LPS[1] receptor in the bacterial outer membrane and ejects its DNA into the periplasmic space. Since binding and DNA ejection occur with purified LPS (Incardona & Selvidge, 1973), these virus–receptor interactions can be used to characterize mutationally induced alterations in tertiary and quaternary structure of the viral capsid proteins that are involved in the delivery of the viral genome into the cell.

The icosahedral capsid φX has a single capsomere at each of the 12 vertices. If all capsomeres are identical, each would be composed of a single H protein surrounded by five molecules each of F and G proteins with the G and H proteins on the external surface of the capsid (Burgess, 1969; Edgell et al., 1969; Siden & Hayashi, 1974). The 60 J proteins are required for DNA packaging into preformed proheads, suggesting an internal location (Aoyama et al., 1981). Several, if not all, molecules of the gene H protein are injected along with the viral DNA (Jazwinski et al., 1975b), and *cs*70, a mutation located in gene F (Hutchison et al., 1972), leads to a defect in DNA injection (Incardona, 1974; Segal & Dowell, 1974). Thus, at least two of the four structural proteins are involved in DNA penetration of the outer-membrane.

Newbold & Sinsheimer (1970a,b) developed a technique for measuring the kinetics of the partial DNA ejection or eclipse reaction for φX bound to intact cells. We analyzed the temperature dependence of the reaction rate in terms of Arrhenius kinetic theory for wild-type phage and two types of mutants, those having amino acid substitutions in the capsid proteins and those having deleted or inserted sequences in the DNA (Incardona & Müller, 1985). However, it became clear that the reversibility of the binding step could be altered by these mutations. Therefore, a study of the binding of φX174

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[1] Abbreviations: LPS, lipopolysaccharide; EDTA, ethylenediaminetetraacetic acid; cs, cold sensitive; am, amber; SVB, starvation buffer; PFU, plaque-forming unit; Tris, tris(hydroxymethyl)aminomethane.

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