Sivaramakrishnan, M., & Burke, M. (1982) *J. Biol. Chem. 257*, 1102–1105.
Sutoh, K. (1982) *Biochemistry 21*, 3654–3661.
Trayer, H. R., & Trayer, I. P. (1985) *FEBS Lett. 180*, 170–174.
Ueno, H., Kato, T., & Morita, F. (1985) *J. Biochem. (Tokyo) 97*, 1785–1793.
Wagner, P. D., & Giniger, E. (1981) *Nature (London) 292*, 560–562.
Waller, G. S., & Lowey, S. (1985) *J. Biol. Chem. 260*, 14368–14373.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Weeds, A. G., & Taylor, R. A. (1975) *Nature (London) 257*, 54–56.
Weeds, A., & Pope, B. (1977) *J. Mol. Biol. 111*, 129–157.
West, J. J., Nagy, B., & Gergely, J. (1967) *J. Biol. Chem. 242*, 1140–1145.
Yamamoto, K., & Sekine, T. (1979) *J. Biochem. (Tokyo) 86*, 1863–1868.
Yamamoto, K., & Sekine, T. (1983) *J. Biochem. (Tokyo) 94*, 2075–2078.

Aminoacyl-tRNA–Elongation Factor Tu–Ribosome Interaction Leading to Hydrolysis of Guanosine 5′-Triphosphate†

Kazuhiko Takahashi,‡ Sandhya Ghag, and Stanislav Chládek*
Department of Chemistry, Michigan Cancer Foundation, Detroit, Michigan 48201
Received March 12, 1986; Revised Manuscript Received August 18, 1986

ABSTRACT: We investigated the elongation factor Tu (EF-Tu) dependent binding of Phe-tRNA and Phe-tRNAs with the nicks at positions 46, 37, and 17 to the *Escherichia coli* 70S ribosome–poly(U)–$tRNA^{Phe}$ complex. Binding of Phe-$tRNA_{1-45+47-76}$, Phe-$tRNA_{1-36+38-76}$, or Phe-$tRNA_{1-16+17-76}$ to the 70S ribosome has been found to be poly(U)·tRNA dependent and, similar to that of intact Phe-tRNA, is inhibited by the antibiotic thiostrepton. We have further found that, contrary to a previous report [Modolell, J., Cabrer, B., Parmeggiani, A., & Vazquez, D. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1796], the EF-Tu–ribosome GTPase mediated by Phe-tRNA is not inhibited by thiostrepton; rather, the drug stimulates the endogenous GTPase of the EF-Tu·70S ribosome. Phe-tRNA fragments 47–76, 38–76, and 17–76 all promote the EF-Tu·GTPase reaction in the presence of 70S ribosome–poly(U)–$tRNA^{Phe}_{yeast}$. Moreover, since the GTPase-promoting activities of both the short and long fragments are similar, it appears that the most important aminoacyl transfer ribonucleic acid (aa-tRNA) interaction with EF-Tu occurs alongside its 3′ quarter. Thiostrepton slightly stimulates the GTPase activity of these Phe-tRNA fragments. Although the Phe-$tRNA_{1-36+38-76}$ cannot bind to poly(U) during its binding to 70S ribosomes, its binding at high Mg^{2+} concentration occurs at the A site. Thus, most of the bound modified Phe-tRNA functions as the acceptor in the peptidyltransferase reaction. We interpret these results to mean that the GTP hydrolysis is triggered upon the initial contact of the ternary aa-tRNA·EF-Tu·GTP complex with a ribosomal domain which may not be identical with the A site at which thiostrepton and EF-G act. The aa-tRNA molecule can reach the A site only after hydrolysis of GTP and removal of EF-Tu·GDP from ribosomes.

During the elongation phase of protein synthesis, aminoacyl transfer ribonucleic acid (aa-tRNA)[1] enters the programmed ribosome in the form of a ternary aa-tRNA·EF-Tu·GTP complex. Binding of aa-tRNA to the ribosomal acceptor sites ensues, and in the course of this process, one molecule of GTP is hydrolyzed, and EF-Tu·GDP leaves the ribosome (Miller & Weissbach, 1977). While it is clear that the catalytic activity for GTP hydrolysis is effected by EF-Tu (Chinali et al., 1977) and binding of an aa-tRNA molecule, especially its 3′ terminus, to EF-Tu is a prerequisite for activation of EF-Tu GTPase, it is worth mentioning that this reaction can only occur (under physiological conditions) upon the contact of the ternary complex with a certain, but as yet undefined, ribosomal domain (Liljas, 1982). The other elongation factor, EF-G, binds to the ribosome in the step following peptide bond formation and effects the translocation of the newly formed peptidyl-tRNA from the A to the P site (Brot, 1977). This reaction is also accompanied by hydrolysis of one molecule of GTP, though catalysis by EF-G (de Veridittis et al., 1984) also requires interaction of this factor with the ribosome. While it has been clearly shown that the ternary aa-tRNA·EF-Tu·GTP complex and EF-G compete with each other for binding to the ribosome (Miller, 1972), it does not necessarily follow that the ribosomal domains responsible for the activation of the GTPases of both factors are identical. Thus, protein L11 seems to be involved in an interaction of the ribosome with

†This investigation was supported, in part, by National Science Foundation Grant PCM 82-07539 and by an institutional grant to the Michigan Cancer Foundation from the United Foundation of Greater Detroit.
‡Present address: Laboratory of Pharmaceutical Chemistry, Nagoya City University, Nagoya, Japan.

[1] Abbreviations: aa-tRNA, aminoacyl transfer ribonucleic acid; EF-Tu, elongation factor Tu; DTT, dithiothreitol; TEMED, tetramethylethylenediamine; TCA, trichloroacetic acid; A-Phe, 2′(3′)-*O*-L-phenylalanyladenosine (similar abbreviations are used for other oligonucleotide derivatives); BD, benzoyldiethylaminoethyl; A_{260} unit, quantity of material contained in 1 mL of solution which has an absorbance of 1.00 at 260 nm when measured in a 1-cm path-length cell; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Me_2SO, dimethyl sulfoxide. The fragments of $tRNA^{Phe}_{yeast}$ are designated by their terminal nucleosides, e.g., $tRNA^{Phe}_{yeast(38-76)}$; see also Figure 1.

0006-2960/86/0425-8330$01.50/0

EF-G, but apparently not playing any role in EF-Tu·GTPase (Stark & Cudliffe, 1979). This protein also plays a role in binding of the antibiotic thiostrepton to the ribosomal 50S subunit, or more precisely to 23S RNA (Thomson et al., 1979). Accordingly, thiostrepton strongly inhibits the binding of EF-G to ribosomes and associated GTPase (Cundliffe, 1979). This antibiotic also inhibits both the binding of aa-tRNA to the A site and was also claimed to inhibit the associated EF-Tu-catalyzed GTP hydrolysis (Modolell et al., 1971). In contrast and quite inexplicably, EF-Tu·70S ribosome GTPase, which is promoted by alcohol or short 3′-terminal fragments of aa-tRNA, is not inhibited but rather stimulated by thiostrepton, albeit in rather high concentrations (Ballesta & Vazquez, 1972; Compuzano & Modolell, 1980; Bhuta & Chladek, 1982). Apparently, thiostrepton, by contrast with its effect on EF-G binding, does not per se inhibit attachment of EF-Tu to the 50S ribosome.

In order to resolve this apparent controversy and to address the larger question as to the ribosomal domains that are involved in the activation of both factors, we studied the effect of thiostrepton on the binding of Phe-tRNA and its nicked analogues (with a missing nucleotide at certain points of the molecule) to 70S ribosome. In addition, we examined the effect of thiostrepton on the EF-Tu·GTPase reaction.

We report in this paper that EF-Tu·GTPase is activated before aa-tRNA can reach the A site. Accordingly, thiostrepton, acting on the A site, competes with aa-tRNA for the binding to this site, but it does not directly interfere with EF-Tu·GTPase.

Materials and Methods

General methods and sources of reagents and biochemical are the same as described in previous papers (Bhuta et al., 1982a; Bhuta & Chladek, 1982). EF-Tu·GDP was kindly supplied by Dr. D. L. Miller, New York State Institute for Developmental Disabilities, Staten Island, NY, or prepared according to Miller and Weissbach (1974). *Escherichia coli* ribosomes low in endogenous GTPase activity were prepared from *E. coli* MRE 600 (RNase 1⁻) (Grain Processing Corp., Muscatin, IA) according to Staehelin and Maglott (1971). Thiostrepton was a kind gift of Barbara Stearns, The Squibb Institute, New Brunswick, NJ. [γ-^{32}P]GTP was from New England Nuclear 1 (Ci/mmol). [^{3}H]Phe-tRNA$^{Phe}_{yeast}$ was prepared by charging of tRNA$^{Phe}_{yeast}$ (Figure 1) (Boehringer Mannheim Biochemicals, Indianapolis, IN) with [^{3}H]-phenylalanine (1 Ci/mmol; ICN Chemicals and Radiochemicals Division, Irvine, CA) using purified Phe-tRNA synthetase from yeast (generous gift of Professor M. Sprinzl, Department of Biochemistry, University of Bayreuth, Bayreuth, Germany) according to the method of Thang et al. (1971). The charging of tRNA$^{Phe}_{yeast}$, measured by trichloroacetic acid precipitable radioactivity, ranged from 1400 to 1500 pmol of [^{3}H]Phe/A_{260} unit. Ac[^{3}H]Phe-tRNA was prepared according to Haenni and Chapeville (1966) (specific activity 5 Ci/mmol). Disc gel electrophoresis was performed by the method of Philipsen et al. (1968) with a Bio-Rad Model 220 instrument. The gel system was as follows: reservoir solution, cacodylic acid (30 mM) and imidazole (62.5 mM) (pH 4.9); running gel, HCl (50 mM), imidazole (62.5 mM) (pH 6.4), 12% acrylamide, and 0.7% bis(acrylamide); spacer gel, HCl (50 mM), imidazole (50 mM) (pH 4.1), 5% acrylamide, and 0.25% bis(acrylamide). Gels contained urea (7 M), TEMED (3.3 M), and ammonium persulfate (0.05%). tRNA and tRNA fragment solutions contained 10% sucrose. Electrophoresis was run at 8 °C for 16 h with cca 160 V and 105 mA. Staining was performed with 1% lanthanum acetate and 2% acridine orange

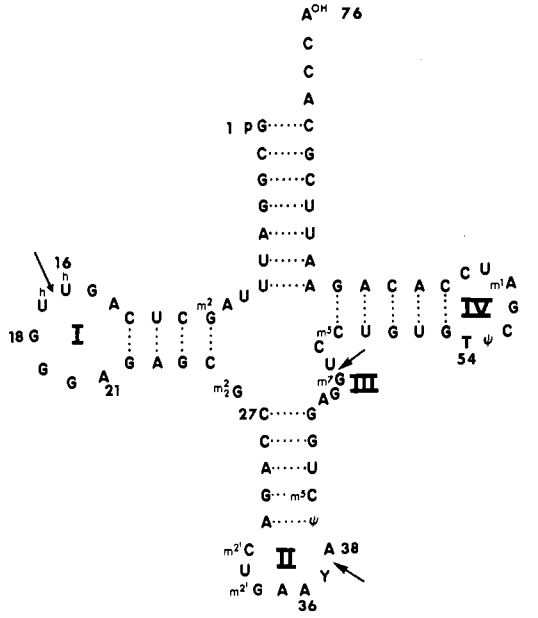

FIGURE 1: Structure of tRNA$^{Phe}_{yeast}$ [according to Rajbhandary et al. (1971)] showing sites of chemical cleavage.

in 15% acetic acid as described by Richards et al. (1965).

[^{3}H]Phe-tRNA Fragments. tRNA$_{1-16}$ and tRNA$_{17-76}$ were prepared as described by Werner et al. (1976) by splitting tRNA$^{Phe}_{yeast}$ in the presence of lead acetate. The mixture of fragments was charged with [^{3}H]Phe, fragments were separated by a DEAE-Sephadex A-25 column [using a linear gradient of 0.2–0.5 M NaCl in 7 M urea–HCl (pH 3.0)], and [^{3}H]Phe-tRNA$_{17-76}$ was further purified on a BD-cellulose column. The column was equilibrated with 20 mM CH_3CO-ONa (pH 5.0), eluted with a linear gradient of 0–1.0 M NaCl in 20 mM CH_3COONa (pH 5.0), and finally eluted with 1.5 M NaCl in 20 mM CH_3COONa (pH 5.0) containing 20% ethanol. Purity of fragments was checked by gel electrophoresis. The charging of tRNA$^{Phe}_{yeast(17-76)}$ with [^{3}H]Phe was approximately 1290 pmol of [^{3}H]Phe/A_{260} unit.

tRNA$_{1-36}$ and tRNA$_{38-76}$ were prepared as described by Thiebe and Zachau (1971) and fragments purified by standard procedure. The [^{3}H]phenylalanine charging of tRNA$_{38-76}$ was 1040 pmol of [^{3}H]Phe/A_{260} unit. [^{14}C]Phe-tRNA$_{38-76}$ was prepared analogously by charging with [^{14}C]Phe (specific activity 200 mCi/mmol), charging was 588 pmol of [^{14}C]-Phe/A_{260} unit. tRNA$_{1-45}$ and tRNA$_{47-76}$ were prepared according to Wintermeyer and Zachau (1975) and purified by standard procedures. The charging of tRNA$_{47-76}$ was 2570 pmol of [^{3}H]Phe/A_{260} unit.

Enzymatic Binding of [^{3}H]Phe-tRNA and Its Nicked Analogues to 70S Ribosomes. The enzymatic binding of the Phe-tRNA species to 70S ribosomes in the presence of tRNA$^{Phe}_{yeast}$ and poly(U) was performed essentially as described by Ringer et al. (1976). The binding was measured in the mixture obtained by the mixing of the Phe-tRNA·EF-Tu·GTP ternary complex pool (0.05 mL) and the ribosomal pool (0.05 mL). The ternary complex pool contained the following: Tris-HCl, 50 mM (pH 7.4); NH_4Cl, 80 mM; KCl, 80 mM; $MgCl_2$, 5 mM; DTT, 2 mM; phosphoenolpyruvate, 3 mM; pyruvate kinase, 2 IU; GTP, 0.01 mM; EF-Tu·GDP, 20 pmol; [^{3}H]Phe-tRNA or its analogues and thiostrepton (if any, dissolved in Me_2SO) at desired concentrations. The mixture without GTP, Phe-tRNA, and thiostrepton was first preincubated at 37 °C for 10 min and then, after addition of GTP, incubated at 37 °C for 5 min to convert EF-Tu·GDP to EF-Tu·GTP. Phe-tRNA and thiostrepton were then added, and

the mixture was incubated at 37 °C for 10 min to form the ternary complex (thiostrepton does not influence the formation of the ternary complex). The ribosomal pool contained the following, in a final volume of 0.05 mL: Tris-HCl (pH 7.4), 50 mM; NH_4Cl, 80 mM; KCl, 80 mM; $MgCl_2$, 5 mM; DTT, 2 mM; 70S ribosomes, 2 A_{260} units; poly(U), 10 μg; $tRNA^{Phe}_{yeast}$, 20 μg. The pool was preincubated at 37° C for 10 min, and the binding reaction was initiated by addition of the ribosomal pool to the ternary complex pool. After incubation at 0 °C for 10 min, the reaction was terminated by dilution with 2.0 mL of cold buffer which contained Tris-HCl (pH 7.4) (50 mM), NH_4Cl (80 mM), KCl (80 mM), and $MgCl_2$ (5 mM). The reaction mixture was immediately filtered through Gelman GN-6 filters (25 mm in diameter, 0.45-μm pore size); the filters were washed with 2.0 mL of cold buffer (3 times) and counted for radioactivity in toluene scintillation cocktail. The counts of [^{3}H]Phe-tRNA or its nicked analogues bound to ribosomes without EF-Tu·GTP were subtracted as the background.

Nonenzymatic Binding of [^{3}H]Phe-tRNA to 70S Ribosomes. The assay was essentially the same as described by Ringer et al. (1976). The ribosomal pool contained the following in a final reaction volume of 0.1 mL: Tris-HCl (pH 7.4), 50 mM; NH_4Cl, 80 mM; KCl, 80 mM; $MgCl_2$, 20 mM; poly(U), 10 μg; ribosomes, 3.8 A_{260} units; $tRNA^{Phe}_{yeast}$, 25 μg. The ribosomal pool was preincubated at 37 °C for 5 min, desired concentrations of thiostrepton were added, and the binding reaction was initiated by addition of [^{3}H]Phe-tRNA. After incubation at 37 °C for 15 min, the reaction was terminated, filtered, and counted as described for the enzymatic binding. The counts of [^{3}H]Phe-tRNA on the filter in the mixture without ribosomes were subtracted from the observed values as background.

Binding of 3'-Terminal Fragments of [^{3}H]Phe-tRNA to Ribosomes. The assay was carried out the same way as the assay for the nonenzymatic binding, except that the composition of the ribosomal pool was according to Bhuta et al. (1982b): The ribosomal pool contained the following in 0.1 mL: Tris-HCl (pH 7.4), 50 mM; NH_4Cl, 50 mM; KCl, 400 mM; $MgCl_2$, 40 mM; ribosomes, 4.1 A_{260} units; poly(U), 10 μg; $tRNA^{Phe}_{yeast}$, 50 μg; investigated fragment, 10 pmol; thiostrepton (if desired), 4×10^{-5} M.

Peptide Bond Formation between Ac[^{3}H]Phe-tRNA and [^{14}C]Phe-tRNA$_{1-36+38-76}$. The assay was carried in the following incubation mixture (0.025 mL total): Tris-HCl (pH 7.4), 50 mM; KCl, 80 mM; NH_4Cl, 80 mM; $MgCl_2$, 50 mM; DTT, 5 mM; ribosomes, 1.5 A_{260} units; poly(U), 4 μg; Ac-[^{3}H]Phe-tRNA, 25 pmol. The pool was preincubated for 5 min at 37 °C, [^{14}C]Phe-tRNA$_{1-36+38-76}$ in the amount indicated in Figure 7 was added, and the total mixture was then incubated for 30 min at 37 °C. The reaction was terminated by addition of 3 N NaOH (2.5 μL) and incubation for 20 min at 37 °C, 5 N HCl was added (0.1 mL), and the mixture was extracted with ethyl acetate (1.5 mL) according to Leder and Bursztyn (1966). The radioactive product (the dipeptide Ac[^{3}H]Phe[^{14}C]Phe) in 1.0 mL of ethyl acetate was counted as described previously (Quiggle et al., 1981).

Nonenzymatic Binding of [^{14}C]Phe-tRNA$_{1-36+38-76}$ to Ribosomes. The assay was carried out similarly as that of binding intact Phe-tRNA except that the $MgCl_2$ concentration was varied in the 15–60 mM range. Input of [^{14}C]Phe-tRNA$_{1-36+38-76}$ was 40.5 pmol.

EF-Tu·GTPase Assay. The [γ-^{32}P]GTP hydrolysis was carried out by mixing 0.05 mL of the EF-Tu·[γ-^{32}P]GTP·[^{3}H]Phe-tRNA (or its analogue) pool and 0.05 mL of the ribosomal pool as described by Bhuta et al. (1982a). The ternary complex pool contained the following: Tris-HCl (pH 7.8), 60 mM; NH_4Cl, 50 mM; $MgCl_2$, 10 mM; DTT, 2 mM; phosphoenolpyruvate, 3 mM; pyruvate kinase, 2 IU; $(NH_4)_2SO_4$, 45 mM from pyruvate kinase; EF-Tu·GDP, 20 pmol; EF-Ts, 0.11 pmol; [γ-^{32}P]GTP, 0.01 mM, specific activity 1 Ci/mmol; and the tested compound and thiostrepton (dissolved in Me_2SO) at the desired concentration, all in 0.05 mL. The mixture was incubated as described by Bhuta et al. (1982a), and conversion of EF-Tu·GDP to EF-Tu·[γ-^{32}P]GTP was monitored as described by Miller and Weissbach (1974). The ribosomal pool contained the following in final volume of 0.05 mL: Tris-HCl (pH 7.8), 60 mM; NH_4Cl, 150 mM; $MgCl_2$, 10 mM; DTT, 2 mM; 70S ribosomes, 2 A_{260} units; $tRNA^{Phe}_{yeast}$, 20 μg; poly(U), 10 μg. This pool was preincubated at 37 °C for 10 min, and the reaction was initiated by addition of the ribosomal pool to the ternary complex pool. The final volume was 0.1 mL. After incubation at 30 °C for 5 min, the reaction was terminated by addition of 0.01 M silicotungstic acid (0.05 mL) in 0.01 M H_2SO_4 followed by addition of 10 mM KH_2PO_4 (0.01 mL). After addition of 5% ammonium molybdate (0.5 mL) in 4 N H_2SO_4, the radioactive inorganic phosphate was immediately extracted with isobutyl alcohol–benzene (1:1 v/v) as described by Martin and Doty (1949). Radioactivity in 1 mL of the organic phase was determined as described by Bhuta and Chladek (1980). The counts of extracts without the test compounds were subtracted as the background from the observed values.

EF-Tu-Dependent Binding of [^{3}H]Phe-tRNA and Associated GTPase Assay. The simultaneous measurements of enzymic [^{3}H]Phe-tRNA binding to 70S ribosomes and [γ-^{32}P]GTP hydrolysis were carried out similarly as described by Modolell et al. (1971) and Bhuta et al. (1982) and in the previous experiment, except that the ternary complex pool contained in 0.04 mL the following: Tris-HCl (pH 7.7), 50 mM; NH_4Cl, 100 mM; $MgCl_2$, 9 mM; DTT, 2 mM; phosphoenolpyruvate, 3 mM; pyruvate kinase, 2 IU; $(NH_4)_2SO_4$, 45 mM from pyruvate kinase; EF-Tu·GDP, 20 pmol; [γ-^{32}P]GTP, 0.01 mM (specific activity 1 Ci/mmol); [^{3}H]Phe-tRNA, 10^{-7} M (specific activity 1 Ci/mmol); thiostrepton, 4×10^{-5} M (in Me_2SO). The mixtures were preincubated as a given assay for enzymic binding of Phe-tRNA (vide infra). The ribosomal pool contained in 0.04 mL the following: Tris-HCl (pH 7.7), 50 mM; NH_4Cl, 150 mM; $MgCl_2$, 9 mM; DTT, 2 mM; 70S ribosomes, 2 A_{260} units; $tRNA^{Phe}_{yeast}$, 20 μg; poly(U), 10 μg. This pool was preincubated as shown in the previous assay and the reaction initiated by addition of the ribosomal pool to the ternary complex pool. After incubation at 30 °C for 10 min, the reaction mixture was divided into two equal portions and assayed for [^{3}H]Phe-tRNA binding and GTPase as described in previous experiments. Binding of Phe-tRNA and GTPase was corrected with the values obtained from control reaction mixtures without EF-Tu or without Phe-tRNA, respectively.

Results

Binding of aa-tRNA to the Ribosomal A Site: Effect of Thiostrepton. It has been demonstrated that enzymatic binding of aa-tRNA to 70S ribosome–poly(U)–$tRNA^{Phe}_{yeast}$ complex is directed to the A site (deGroot et al., 1971). Figure 2 shows that such binding of Phe-$tRNA^{Phe}_{yeast}$ at 5 mM Mg^{2+} concentration is completely inhibited by thiostrepton at concentrations below 10^{-5} M. This agrees with results of Modolell et al. (1971), who observed the inhibition of Phe-tRNA and Ala-tRNA to ribosomes by this antibiotic at 10 mM Mg^{2+}. The nonenzymatic binding of Phe-$tRNA^{Phe}_{yeast}$ at high Mg^{2+}

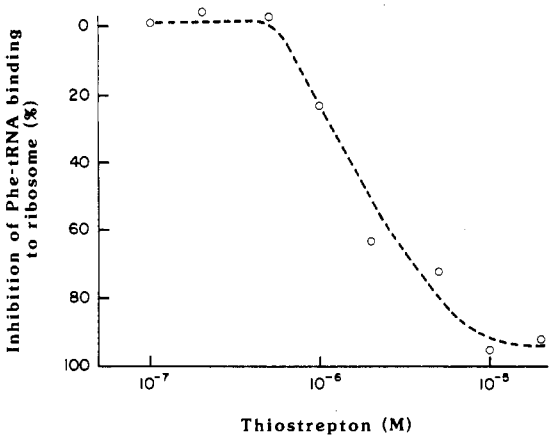

FIGURE 2: Effect of thiostrepton concentration on binding of Phe-$tRNA^{Phe}_{yeast}$ EF-Tu·GTP to the 70S ribosome–poly(U)–$tRNA^{Phe}_{yeast}$ complex. Concentration of Phe-tRNA was 1.2×10^{-6} M. In the absence of thiostrepton, 2.20 pmol of Phe-tRNA was bound. See Materials and Methods for details.

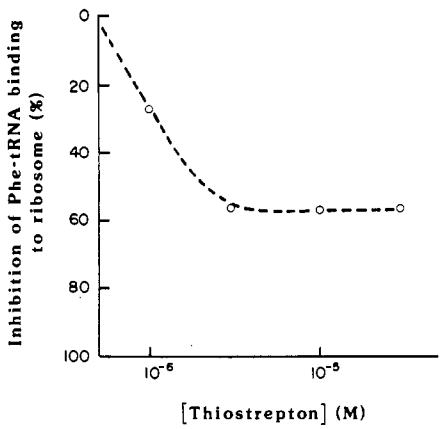

FIGURE 3: Effect of thiostrepton concentration on nonenzymatic binding of Phe-$tRNA^{Phe}_{yeast}$ to the 70S ribosome–poly(U)–$tRNA^{Phe}_{yeast}$ complex. Concentration of Phe-tRNA was 1.2×10^{-6} M. In the absence of thiostrepton, 1.75 pmol of Phe-tRNA was bound. See Materials and Methods for details.

concentrations is also inhibited by thiostrepton (Figure 3), although the inhibition is incomplete. Thus, thiostrepton is acting on the A site by competing with aa-tRNA.

EF-Tu·70S Ribosome GTPase Stimulated by Phe-tRNA: Effect of Thiostrepton. During enzymatic binding, Phe-tRNA stimulates EF-Tu·70S ribosome GTP hydrolysis (Miller & Weissbach, 1977), and this GTPase reaches almost stochiometric levels (Figure 4). A 10 mM Mg^{2+} (optimal) concentration was used for measuring Phe-tRNA-dependent EF-Tu·GTPase (Modolell et al., 1971; Inell et al., 1981). Contrary to the literature (Modolell et al., 1971), our data (see Figure 5) show that the Phe-tRNA-promoted GTPase is rather insensitive to thiostrepton in the 10^{-7}–10^{-6} M concentration range. The apparent stimulation of the GTPase, at high concentrations of thiostrepton, is evidently due to the increase of background (uncoupled) GTPase, independent of Phe-tRNA (see lower curve in Figure 5). This trend is confirmed in the experiment reported in Table I. Under these conditions, EF-Tu-dependent [^{3}H]Phe-tRNA binding to 70S ribosomes and the associated GTPase were measured simultaneously. In accord with data from the above experiments, the high concentration of thiostrepton strongly inhibits the enzymatic ribosomal binding of Phe-tRNA, while the associated GTPase is stimulated. This experiment shows that the differentiated effect of thiostrepton on binding and GTPase reaction is rather independent on variations of experimental

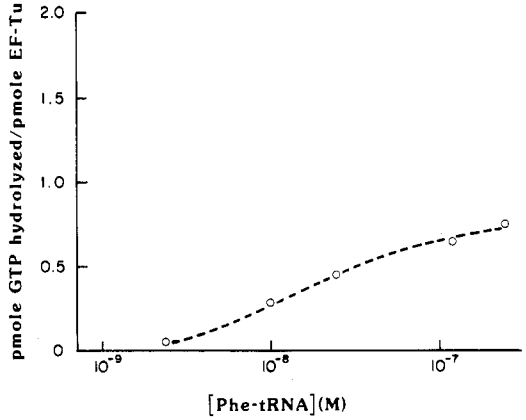

FIGURE 4: Phe-$tRNA^{Phe}_{yeast}$-promoted EF-Tu·GTP hydrolysis during binding of Phe-$tRNA^{Phe}_{yeast}$ EF-Tu·GTP to the 70S·poly(U)·$tRNA^{Phe}_{yeast}$ complex. See Materials and Methods for details.

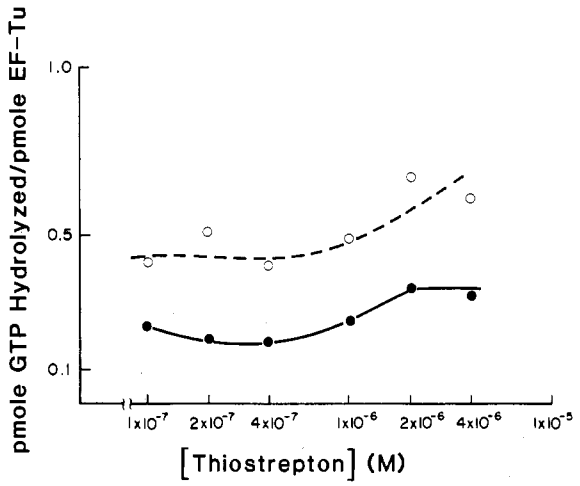

FIGURE 5: Effect of thiostrepton concentration on the EF-Tu·GTPase ribosome promoted by Phe-$tRNA^{Phe}_{yeast}$ (concentration of [^{3}H]Phe-tRNA = 5×10^{-8} M) (○). Effect of thiostrepton concentration on background EF-Tu·GTPase ribosome (in absence of Phe-tRNA) is also shown (●). For details, see Materials and Methods.

Table I: Effect of Thiostrepton on EF-Tu-Dependent Phe-tRNA Binding to 70S Ribosomes and Associated GTPase[a]

addition	[^{3}H]Phe-tRNA bound (pmol)	[γ-^{32}P]-GTP hydrolyzed (pmol)
[^{3}H]Phe-tRNA (10^{-7} M)	6.89	9.60
[^{3}H]Phe-tRNA (10^{-7} M) and thiostrepton (4×10^{-5} M)	0.86	21.57

[a] See Materials and Methods for further details.

conditions. It is of interest to note that comparable concentrations of the drug were stimulatory in EF-Tu·70S ribosome GTPase promoted by 3′-terminal fragments of aa-tRNA (e.g., C-C-A-Phe) or alcohol (Bhuta & Chladek, 1982). The reason for discrepancy between our data and those of Modolell et al. (1971) is not known. However, it should be noted that, in our experiments, we have used purified Phe-$tRNA^{Phe}_{yeast}$ and noted the effect of thiostrepton over a wide range of the antibiotic concentrations in experiments which were repeated many times.

Binding of 3′-Terminal Fragments of Phe-tRNA to 70S Ribosome. The binding of three aminoacyloligonucleotides derived from Phe-$tRNA^{Phe}_{yeast}$ with chain lengths of 30, 39, and 60 nucleotides (fragments 47–76, 38–76, and 47–76, respectively) was investigated at high Mg^{2+} concentration (Pestka,

Table II: Nonenzymatic Binding of 3′-Terminal Fragments of [^{3}H]Phe-tRNA to Ribosomes[a]

	pmol bound
Phe-tRNA$_{17-76}$	0.147
Phe-tRNA$_{38-76}$	0.603
Phe-tRNA$_{47-76}$	0.279

[a] Concentration of Phe-tRNA fragments was 10^{-7} M; see Materials and Methods for further details.

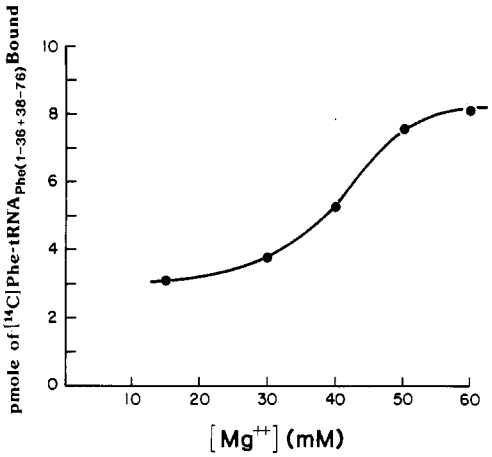

FIGURE 6: Effect of Mg^{2+} concentration on nonenzymatic binding of [^{14}C]Phe-tRNA$_{1-36+38-76}$ to ribosomes. See Materials and Methods for further details.

1971). These nonphysiological conditions are necessary for a significant binding of fragments to occur; therefore, the results are not directly comparable with binding of intact or nicked Phe-tRNAs which are measured at 5 mM Mg^{2+} concentration. As can be seen from Table II, all three fragments bind rather poorly. It is possible that these long 3′-terminal fragments, unlike "nicked" Phe-tRNAs (vide infra), cannot assume the necessary tertiary structures to allow them to interact properly with the ribosomal A site. This explanation would be in agreement with earlier observations of Hussain and Ofengand (1972), who noted low acceptor activity in the peptidyltransferase reaction of the 19-nucleotide-long fragment of Tyr-tRNA, which, in fact, is much lower than that of the 3′-tetranucleotide fragment A-C-C-A-Tyr.

Binding of Nicked Phe-tRNAs to the Acceptor Site of 70S Ribosome. Enzymatic binding of Phe-tRNA$_{1-36+38-76}$ to 70S ribosomes has been noted previously. Moreover, the binding was claimed to be relatively independent of Mg^{2+} concentration and "nonfunctional" since this Phe-tRNA analogue failed to compete with native Phe-tRNA (Thang et al., 1971). It was also shown that several nicked aa-tRNAs are able to form ternary complexes with EF-Tu·GTP as determined by the nitrocellulose filter method (Thang et al., 1971; Krauskopf et al., 1972). When we investigated nonenzymatic binding of Phe-tRNA$_{1-36+38-76}$, the dependence of this binding on Mg^{2+} concentration was noted, although the maximum binding occurs at nonphysiological levels of Mg^{2+} (Figure 6). However, even under these conditions, Phe-tRNA$_{1-36+38-76}$ binds to the A site, virtually all bound molecules engaged in peptide bond formation with prebound Ac[^{3}H]Phe-tRNA (Figure 7) to form the dipeptidyl-tRNA. Thus, Phe-tRNA$_{1-36+38-76}$ with a single nucleotide missing next to the anticodon triplet is capable of functional interaction with the A site.

Next, we investigated the enzymatic binding of three nicked Phe-tRNAs to the ribosome with breaks or nucleotides missing in different positions of the nucleotide chain. Since all these nicked tRNAs are chargeable by the appropriate synthetase, it might be expected that their teritary structure is quite similar

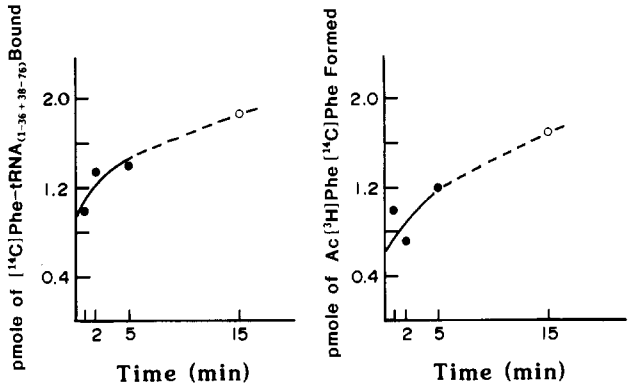

FIGURE 7: Time course of nonenzymatic binding of [^{14}C]Phe-tRNA$_{1-36+38-76}$ to ribosomes (left panel) and peptide bond formation with Ac[^{3}H]Phe-tRNA and [^{14}C]Phe-tRNA$_{1-36+38-76}$ on ribosome (right panel). For details, see Materials and Methods.

Table III: Enzymatic Binding of "Nicked" [^{3}H]Phe-tRNAs to Ribosomes[a]

	pmol bound		+thiostrepton (pmol bound)	
	−poly(U)	+poly(U)	−poly(U)	+poly(U)
Phe-tRNA$_{1-16+17-76}$	0.209	0.435		0.022
Phe-tRNA$_{1-36+38-76}$	0.232	0.491	0.179	0.389
Phe-tRNA$_{1-45+47-76}$	0.113	0.233		0.084
Phe-tRNA		0.997		

[a] Concentration of nicked Phe-tRNA was 10^{-7} M; concentration of thiostrepton was 4×10^{-5} M. See Materials and Methods for further details.

to the intact tRNA. However, the population of active species may be quite low due to the unfavorable entropic factors. Additionally, the nicks, in positions 17, 37, and 46 of tRNA may or may not be of importance for the interaction of modified Phe-tRNA species with either EF-Tu or the ribosomal A site. Indeed, EF-Tu-dependent binding of these nicked Phe-tRNAs is relatively inefficient, albeit significant enough to note that it is poly(U)·tRNA$^{Phe}_{yeast}$ dependent and sensitive to thiostrepton (Table III). Although the binding of Phe-tRNA$_{1-36+38-76}$ is not expected to be poly(U) dependent (Thang et al., 1971), it appears that tRNA$^{Phe}_{yeast}$, prebound to the P site of the ribosomal complex in the presence of poly(U), stimulates the binding of nicked Phe-tRNA to the A site. Assuming direct competition between thiostrepton and aa-tRNA at the A site, one may infer that there is some part of the tRNA molecule, different from both the C-C-A terminus and anticodon, which binds to that part of the ribosomal A site that interacts with thiostrepton. The sensitivity of binding of Phe-tRNA species with breaks in the dihydrouridine loop [Phe-TNA$_{1-17+18-76}$] or the extra loop [Phe-tRNA$_{1-46+47-76}$] to thiostrepton may indicate that neither loop plays a role in the interaction of the tRNA molecule with the thiostrepton site on the 50S subunit. In this connection, it is noteworthy that Thang et al. (1971) observed resistance of binding of Phe-tRNA$_{1-36+38-76}$ to another antibiotic, tetracycline [in the presence of poly(U)], which normally inhibits the codon–anticodon interaction. On the other hand, we have not observed similar resistance to thiostrepton inhibition with this nicked Phe-tRNA, although binding of the latter is inhibited only partially by thiostrepton. Moreover, EF-Tu-dependent binding of all three nicked Phe-tRNAs to 70S ribosomes produces the GTPase reaction, which is not inhibited by thiostrepton (results not shown).

EF-Tu·70S Ribosome GTP Hydrolysis Promoted by 3′-Terminal Fragments of Phe-tRNA. The Phe-tRNA fragments 17–76, 38–76, and 47–76 promoted the EF-Tu·70S

Table IV: EF-Tu–Ribosome–GTPase Reaction in the Presence of 3′-Terminal Fragments of [^{3}H]Phe-tRNA[a]

	pmol of GTP hydrolyzed/pmol of EF-Tu	
	−thiostrepton	+thiostrepton
Phe-tRNA$_{17-76}$	0.235	0.929
Phe-tRNA$_{38-76}$	0.129	0.163
Phe-tRNA$_{47-76}$	0.256	0.269

[a] Concentration of fragments was 10^{-7} M; concentration of thiostrepton was 4×10^{-5} M. See Materials and Methods for further details.

ribosome GTPase, and this hydrolysis is stimulated by high concentrations of thiostrepton (Table IV). It is interesting to note that the length of the nucleotide chain is not an important factor in the stimulatory activity of the fragments; the activity of the fragment 30 nucleotides long is similar to that of 60 nucleotides long [see also Parlato et al. (1983)]. Literature data, obtained by independent methods, also indicate that the most important interaction of aa-tRNA with EF-Tu occurs along the 3′-terminal quarter of the tRNA molecule and extending to the extra loop (Wikman et al., 1982).

Discussion

Present results, together with previous communications from our laboratory (Bhuta et al., 1982a; Bhuta & Chladek, 1982), clearly show that EF-Tu·70S ribosome GTP hydrolysis is not inhibited by thiostrepton, whether it is promoted by intact aa-tRNA, its 3′-terminal fragments, or alcohol. In this respect, GTPases catalyzed by EF-Tu and EF-G are dissimilar. The stimulatory effect of thiostrepton on EF-Tu·70S ribosome GTPase can be observed only at high concentrations of the antibiotic, which may be the result of the antibiotic acting on the other low-affinity site or via an indirect effect. The functional difference between EF-Tu and EF-G GTPases probably relates to the fact that the factors may interact with dissimilar sites of 50S subunit during the GTPase triggering process. The role of protein L11, in EF-G-dependent GTPase but not in EF-Tu-dependent GTPase (Stark and Cundliffe, 1979), may be an indication of topological differences between binding sites of the two factors. Our suggestion is in agreement with recent results of Langer and Lake (1986), using immunoelectron microscopy, who identified the binding site of EF-Tu on 30S subunits as different from that of EF-G, being more exposed to cytoplasm.

Thiostrepton binds to 23S RNA, and this binding is strongly enhanced by L11 (Thompson et al., 1979). The methylation of adenosine 1067 of *E. coli* 23S RNA is associated with antibiotic resistance; the methylated nucleotide residue is located within the thiostrepton and L11 binding sites in domain II of 23S RNA (Thompson et al., 1982). Since binding of EF-Tu to the ribosome is insensitive to thiostrepton, it appears that this part of 23S RNA, together with L11, does not constitute a binding site for EF-Tu, although it must be a constituent of the A site, alternatively interacting with either aa-tRNA or EF-G. Therefore, initial contact of the ternary complex with the ribosomal entity (as yet unidentified), which may be termed as an entry site (recognition site) (Culp et al., 1969; Lake, 1977; Johnson et al., 1977), leads to GTP hydrolysis and subsequent EF-Tu·GDP removal from the ribosome. Only then is the aa-tRNA molecule able to reach the A site and be fully accommodated. Therefore, it is very possible that proofreading of aa-tRNA takes place either prior to or after GTP hydrolysis and before aa-tRNA enters the peptidyltransferase reaction from the A site (Thompson & Stone, 1977).

Thiostrepton and EF-G both inhibit binding of aa-tRNA to the A site (Modolell et al., 1971; Miller, 1972; vide supra), though it is not known which part of the aa-tRNA molecule is involved in the competition. Assuming the direct effect of EF-G or thiostrepton, it is conceivable that a part of the tRNA in question base pairs with a stretch of 23S RNA adjacent of Ado$_{1067}$. Our results with the binding of nicked Phe-tRNAs indicate that such a stretch of aa-tRNA is probably not located in dihydrouridine or extra loops, since binding of Phe-tRNAs with breaks in these loops is still thiostrepton sensitive. Interaction of the anticodon loop with the 23S RNA domain can probably be excluded on topological grounds, since the codon–anticodon interaction takes place in the cleft of the 30S subunit (Lake, 1985) which seems to be spacially separated from the thiostrepton binding site on the 50S subunit (Liljas, 1982). Therefore, it is interesting to speculate that the common T-Ψ-C(A) nucleotide sequence of loop IV of tRNA may base pair to the conserved G-A-A$_{1070}$ sequence of 23S RNA located next to the site of thiostrepton resistance (Branlant et al., 1981; Noller, 1984). This possibility is attractive, since it neatly explains thiostrepton inhibition of aa-tRNA binding to the A site and resistance of EF-Tu GTPase to the same antibiotic as observed here. Competition between EF-G and A site bound peptidyl-tRNA (pretranslocation intermediate) could then be visualized as a part of the translocational mechanism. However, it was previously suggested that the tRNA common T-Ψ-C(A) sequence base pairs with the invariant C-G-A-A sequence of 5S RNA [Forget & Weissman, 1967; Erdmann et al., 1973; but also see Pace et al. (1982)] or with the C-G-U^m-A-C sequence of 16S RNA (Helk & Sprinzl (1985). In the absence of additional data, it is difficult to resolve this discrepancy, although it may be pointed out that a particular sequence of tRNA may interact with different ribosomal domains during the peptide chain elongation process. However, the putative interaction of the T-Ψ-C loop of tRNA with the sequence of 16S RNA (Helk & Sprinzl, 1985) would appear difficult to achieve in light of severe geometrical constraints placed by the interaction of the anticodon loop of tRNA with Cyt$_{1900}$ of 16S RNA (Ehresmann et al., 1984).

Acknowledgments

We are indebted to Margaret Litowich for skillful technical assistance with peptidyltransferase experiments and Drs. David L. Miller, New York State Institute for Developmental Disabilities, Staten Island, NY, and Jerome P. Horwitz, Michigan Cancer Foundation, for critical reading of the manuscript.

Registry No. GTPase, 9059-32-9; thiostrepton, 1393-48-2.

References

Ballesta, J. P., & Vazquez, D. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3058.

Bhuta, P., & Chladek, S. (1980) *FEBS Lett. 122*, 113.

Bhuta, P., & Chladek, S. (1982) *Biochim. Biophys. Acta 698*, 167.

Bhuta, P., Kumar, G., & Chladek, S. (1982a) *Biochemistry 21*, 899.

Bhuta, P., Kumar, G., & Chladek, S. (1982b) *Biochim. Biophys. Acta 696*, 208.

Branlant, C., Krol, A., Machatt, M. A., Pouyet, J., Ebel, J.-P., Edwards, K., & Kossel, H. (1981) *Nucleic Acids Res. 9*, 4303.

Brot, N. (1977) in *Molecular Mechanisms of Protein Synthesis* (Weissbach, H., & Pestka, S., Eds.) pp 375–411, Academic Press, New York.

Chinali, G., Wolf, H., & Parmeggiani, A. (1977) *Eur. J. Biochem. 75*, 55.

Compuzano, S., & Modolell, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 905.
Culp, W., McKeehan, W., & Hardesty, B. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 388.
Cundliffe, E. (1979) in *Antibiotics* (Hahn, F. E., Ed.) Springer-Verlag, Heidelberg and New York.
deGroot, N., Panet, A., & Lapidot, Y. (1971) *Eur. J. Biochem. 23*, 523.
deVeriditis, E., Parlato, G., Massulo, M., & Bocchini, V. (1984) *Abstracts of Papers*, EMBO–NATO Workshop on Function, Structure and Genetics of Procaryotic and Eucaryotic Elongation Factors, Thinerval-Grignon, France.
Ehresmann, C., Ehresmann, B., Millon, R., Ebel, J.-P., Nurse, K., & Ofengand, J. (1984) *Biochemistry 23*, 429.
Haenni, A. L., & Chapenville, F. (1966) *Biochim. Biophys. Acta 114*, 135.
Hussain, A., & Ofengand, J. (1972) *Biochem. Biophys. Res. Commun. 49*, 1588.
Inell, E., Sander, G., & Parmeggiani, A. (1981) *Biochemistry 20*, 6852.
Johnson, A. E., Fairclough, R. H., & Cantor, C. R. (1977) in *Nucleic Acid–Protein Recognition* (Vogel, H. J., Ed.) pp 469–490, Academic Press, New York.
Krauskopf, M., Chen, C.-M., & Ofengand, J. (1972) *J. Biol. Chem. 247*, 892.
Lake, J. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1903.
Lake, J. A. (1985) *Annu. Rev. Biochem. 54*, 507.
Langer, J. A., & Lake, J. A. (1986) *J. Mol. Biol. 187*, 617.
Leder, P., & Bursztyn, H. (1966) *Biochem. Biophys. Res. Commun. 25*, 233.
Liljas, A. (1982) *Prog. Biophys. Mol. Biol. 40*, 161.
Martin, T. B., & Doty, D. M. (1949) *Anal. Chem. 21*, 965.
Miller, D. L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 752.
Miller, D. L., & Weissbach, H. (1974) *Methods Enzymol. 30*, 219.
Miller, D. L., & Weissbach, H. (1977) in *Molecular Mechanisms of Protein Synthesis* (Weissbach, H., & Pestka, S., Eds.) pp 324–374, Academic Press, New York.
Modolell, J., Cabrer, B., Parmeggiani, A., & Vazquez, D. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1796.
Noller, H. F. (1984) *Annu. Rev. Biochem. 53*, 119.
Parlato, G., Pizzano, R., Picone, D., Guesnet, T., Fasano, O., & Parmeggiani, A. (1983) *J. Biol. Chem. 258*, 995.
Pestka, S. (1971) *Methods Enzymol. 20*, 502.
Philipsen, P., Thiebe, R., Wintermeyer, W., & Zachau, H. G. (1968) *Biochem. Biophys. Res. Commun. 33*, 922.
Quiggle, K., Kumar, G., Ott, T. W., Ryu, E. K., & Chladek, S. (1981) *Biochemistry 20*, 3480.
Rajbhandary, U. L., Chang, S. H., Stuart, A., Faulkner, R. D., Hoskinson, R. M., & Khorana, H. G. (1971) *Proc. Natl. Acad. Sci. U.S.A. 57*, 751.
Richards, E. G., Coll, J. A., & Gratzer, W. B. (1965) *Anal. Biochem. 12*, 452.
Ringer, D., Chladek, S., & Ofengand, J. (1976) *Biochemistry 15*, 2759.
Staehelin, T., & Maglott, D. R. (1971) *Methods Enzymol. 20*, 449.
Stark, M., & Cundliffe, E. (1979) *J. Mol. Biol. 134*, 767.
Thang, M. N., Springer, M., Thang, D. C., & Grunberg-Manago, M. (1971) *FEBS Lett. 17*, 221.
Thiebe, R., & Zachau, H. G. (1971) *Methods Enzymol. 20*, 179.
Thompson, J., Cundliffe, E., & Stark, M. (1979) *Eur. J. Biochem. 98*, 261.
Thompson, J., Schmidt, F., & Cundliffe, E. (1982) *J. Biol. Chem. 257*, 7915.
Thompson, R. C., & Stone, P. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 198.
Werner, C., Krebs, B., Keith, G., & Dirheimer, G. (1976) *Biochim. Biophys. Acta 432*, 161.
Wikman, F. P., Siboska, G. E., Petersen, H. U., & Clark, B. F. C. (1982) *EMBO J. 1*, 1095.
Wintermeyer, W., & Zachau, H. G. (1975) *FEBS Lett. 58*, 306.