Role of the Aminoacyl End of Transfer RNA in the Allosteric Control of Guanosine Pentaphosphate Synthesis by the Stringent Factor–Ribosome Complex of *Escherichia coli*†

Gianni Chinali,‡ Richard Liou, and James Ofengand*

ABSTRACT: $tRNA^{Phe}$ of *E. coli* chemically modified at the 3′ end by oxidation ($tRNA_{ox}$) or by oxidation followed by reduction ($tRNA_{ox-red}$) and tRNAs lengthened, shortened, or with a base substitution at the 3′ end were tested for their ability to induce guanosine 5′-triphosphate 3′-diphosphate (pppGpp) synthesis in the *E. coli* ribosome–stringent factor system in order to study how this complex selects against aminoacyl-tRNA in favor of unacylated tRNA. Both the maximum rate (V_{max}) of the tRNA-induced reaction and the affinity (K_a) of the various modified tRNAs for the ribosomal complex were studied as a function of NH_4Cl, a known inhibitor. At 2–3 mM NH_4Cl, the relative V_{max} values for intact tRNA, tRNA with an extra C residue (tRNA-CpCpCpA), tRNA with the terminal adenosine replaced by cytidine (tRNA-CpCpC), and tRNA shortened by one cytidine (tRNA-CpA) were 100, 100, 69, and 20, respectively. All other tRNAs with shortened 3′ ends (-CpCp, -CpC, -Cp, -C) as well as tRNA-CpCpCp, $tRNA_{ox}$, and $tRNA_{ox-red}$ were inactive. At 83 mM NH_4Cl, the same trend was further emphasized. The relative V_{max} for tRNA-CpCpCpA, tRNA-CpCpC, and tRNA-CpA was 73, 37, and 0% of the control, respectively. Analysis of the inactive tRNAs by competition methods showed that they still retained sufficient binding affinity to have been active at the concentrations tested. Comparison of the relative K_a for the active tRNAs with their V_{max} showed that modification had a greater effect on V_{max} than on K_a and that NH_4Cl magnifies this difference. Normal tRNA showed a similar salt effect, the K_a of 10^7 M^{-1} being unchanged from 2 to 83 mM NH_4Cl, while the V_{max} decreased to 61%. We conclude that the main effect of 3′-end modifications of tRNA is to block the allosteric effector function of this molecule and that NH_4Cl also acts at the same site. Thus, the dominant feature of tRNA which determines its activity is the 3′ terminus. There is a strong specificity for an intact terminal adenosine which can only poorly be replaced by cytidine, and a full-length or extended 3′ end is also needed. Since both aminoacyl-tRNA and 3′-phosphate ended tRNA were inactive, there may be, in addition, specificity for an unblocked terminal ribose.

In *Escherichia coli,* the coupling of RNA and protein synthesis is accomplished primarily by means of the regulatory molecule, (p)ppGpp.[1] This guanine nucleotide, made under certain conditions of decreased protein synthesis, inhibits the synthesis of rRNA, tRNA, and some mRNA species by blocking the action of RNA polymerase (cited in Chinali et al., 1978). (p)ppGpp is synthesized when unacylated tRNA, but not aminoacyl-tRNA, interacts with the ribosome–stringent factor complex in a codon-dependent manner at the ribosomal A site (reviewed in Cashel, 1975). The catalytic center for this activity resides in the stringent factor, a protein of 77 000 daltons (Pedersen & Kjeldgaard, 1977), since it can carry out the synthesis of pppGpp from GTP and ATP in the absence of tRNA, mRNA, and ribosomes, although only at a much slower maximum rate (Sy et al., 1973; Pedersen & Kjeldgaard, 1977).

Ribosomes and tRNA are strong positive allosteric effectors of this enzyme, and certain purified ribosomal proteins as well as tRNA fragments can function as weak allosteric effectors (cited in Chinali et al., 1978). Several modifications of intact tRNA have also been investigated. At the aminoacyl end, periodate oxidation (Haseltine and Block, 1973; Pedersen et al., 1973; Lund et al., 1973) or removal of the 3′-terminal adenosine residue (Pedersen et al., 1973; Lund et al., 1973) rendered the tRNA inactive. Elsewhere in the molecule, the substitution of uridine for ribothymidine in a mutant tRNA had no effect, but the similar change of N_2-dimethylguanosine in yeast tRNA to guanosine (Phillips & Kjellin-Straby, 1967) inactivated the tRNA (Lund et al., 1973). In view of the discrimination by stringent factor between unacylated and acylated tRNA, and the results of the preceding paper (Chinali et al., 1978) showing that the TΨCG sequence as such is not a dominant factor in determining the allosteric effector activity of tRNA, we undertook a detailed investigation of the requirements at the 3′ end for retention of effector activity. Some of this work has been reported in preliminary form (Chinali et al., 1976).

After most of this work was completed, Sprinzl & Richter (1976) described a similar study of 3′-end modified tRNAs which parallels some aspects of the present work.

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‡ Present address: Cattedra di Chimica, II Facolta di Medicina e Chirurgia, Universita di Napoli, via Sergio Pansini 5, Napoli, Italy.

[1] Abbreviations used are: tRNA-CpCpA, native phenylalanine transfer RNA; tRNA-CpCp, tRNA missing the 3′-terminal adenosine; tRNA-CpC, tRNA missing the terminal AMP; tRNA-Cp, tRNA-CpC missing the 3′-terminal cytidine; tRNA-C, tRNA-Cp missing the 3′-terminal phosphate; tRNA-CpA, tRNA-C to which has been added a 3′-terminal AMP; tRNA-CpCpC, native tRNA with the 3′-terminal adenosine replaced by cytidine; tRNA-CpCpCp, tRNA-CpCpC bearing an additional 3′-terminal phosphate; tRNA-CpCpCpA, tRNA-CpCpC bearing an additional 3′-terminal AMP; $tRNA_{ox}$, native tRNA which has been oxidized with periodate; $tRNA_{ox-red}$, native tRNA which has been first oxidized with periodate and then reduced with borohydride; pppGpp, guanosine 5′-triphosphate 3′-diphosphate; (p)ppGpp, mixture of pppGpp and guanosine 5′-diphosphate 3′-diphosphate; BAP, bacterial alkaline phosphatase; A*, C*, radioactive adenosine or cytidine residues; A_{260} unit, the amount of material in 1 mL giving an absorbance at 260 nm of 1.0 in a 1-cm path cell.

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Materials and Methods

Materials. Poly(U) and *E. coli* $tRNA^{Phe}$ (11–1200 pmol/A_{260} unit) were obtained from Boehringer (Mannheim, West Germany), [^{14}C]phenylalanine, [α-^{32}P]GTP, [^{14}C]ATP, and [^{14}C]CTP were from New England Nuclear (Boston, Mass.), and snake venom phosphodiesterase (SVPD) and bacterial alkaline phosphatase (BAP) were from Worthington Biochemicals. CpA was a product of Miles Laboratories. Loose couple tRNA-free ribosomes were prepared as described (Chinali et al., 1978). Charcoal-treated lysine was prepared by mixing a 1.1 M solution of lysine (pH 6.5) with 0.1 volume of 14% (w/v) acid-washed Norit A for 10 min at room temperature. The charcoal was removed by centrifugation.

Stringent Factor. Two different preparations were used. For the experiments of Figures 1–3, a 1 M NH_4Cl ribosomal wash of *E. coli* ribosomes was precipitated with 50% saturated $(NH_4)_2SO_4$ and extracted four times with 3 volumes of 35% saturated $(NH_4)_2SO_4$ solution. The residual precipitate (200 mg of protein) containing most of the stringent factor activity was fractionated on a 1.5 × 30 cm column of phosphocellulose P11 (Whatman) (Cochran and Byrne, 1974). The stringent factor activity, eluting between 0.35 and 0.4 M NH_4Cl, was applied directly to a hydroxylapatite column (1.2 × 20 cm, Hypatite C, Clarkson, Ill.), and eluted with a 160-mL linear gradient of 10 to 90 mM KPO_4 (pH 7.1) in 0.4 M NH_4Cl, 1 mM dithiothreitol, and 10% glycerol. The stringent factor was precipitated with 65% saturated $(NH_4)_2SO_4$, dialyzed overnight against 20 mM Tris-HCl (pH 7.8), 125 mM NH_4Cl, 1 mM dithiothreitol, 10% glycerol, and stored frozen in small aliquots in liquid N_2. The specific activity varied from 400 to 1100 units/mg, where one unit of enzyme is defined as the amount which catalyzes the conversion of 1 nmol of GTP to pppGpp in 1 min at 30 °C in the standard assay containing 10 mM NH_4Cl, ribosomes (40 A_{260} units/mL), poly(U) (0.1 mg/mL), and $tRNA^{Phe}$ (1–2 μM).

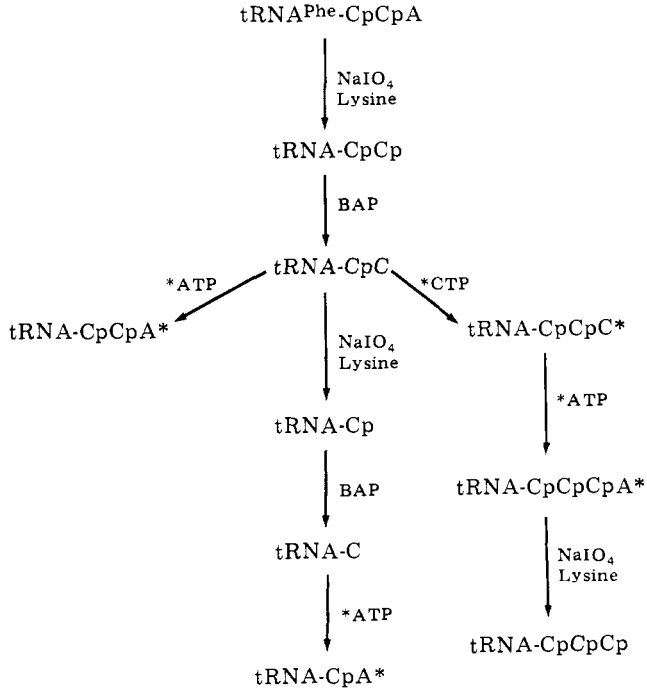
For the remaining experiments, an alternative method was used (Chinali et al., 1978). The last step of this procedure removes small amounts of contaminating tRNA-nucleotidyl transferase which elutes just before the stringent factor. Therefore, the stringent factor peak was divided into several parts for pooling and only those fractions were used which showed minimal tRNA-nucleotidyl transferase activity. Per unit of stringent factor activity the preparation used contained less than 2×10^{-5} unit of tRNA-nucleotidyl transferase when the unit of activity is defined in the same way as for stringent factor activity and assayed under the same conditions (see Methods below for details).

Other Enzymes. Phenylalanyl-tRNA synthetase (approximately 50% pure) was prepared as described (Chinali et al., 1978). tRNA-nucleotidyl transferase was prepared from mixed synthetase (Muench and Berg, 1966) by DEAE-cellulose and Sephadex G-200 chromatography. The mixed synthetase, in buffer I [20 mM Tris (pH 8.4), 10 mM $MgCl_2$, 7 mM mercaptoethanol, 0.1 mM EDTA, 5% glycerol], plus 25 mM KCl was applied to a DEAE-cellulose column (Whatman DE-52) and eluted with a linear KCl gradient from 50 to 350 mM in buffer I. The activity peak was pooled, precipitated by the addition of 0.35 g of ammonium sulfate per mL, and redissolved in buffer I plus 50 mM KCl for gel filtration on Sephadex G-200 in the same buffer. The peak fractions were pooled, concentrated with Aquacide II (Calbiochem), and adjusted to 50% glycerol in buffer I plus 50 mM KCl, and stored at −20 °C.

$tRNA^{Phe}_{ox}$ and $tRNA^{Phe}_{ox\text{-}red}$ were prepared by a modification of previously published procedures (Ofengand et al., 1974; Ofengand and Chen, 1972). $tRNA^{Phe}$ (15–60 A_{260} units/mL) in 0.1 M NaOAc (pH 5.5), 10 mM $Mg(OAc)_2$ was mixed with 0.05 volume of 0.1 M $NaIO_4$ and incubated for 30 min in the dark. Oxidation was stopped by the addition of 0.01 volume of 1 M rhamnose and further incubation for 5 min. $tRNA^{Phe}_{ox}$ was precipitated by the addition of 2 volumes of ethanol, dissolved in 0.1 M KOAc (pH 5), and reprecipitated with 2 volumes of cold ethanol. For reduction, the precipitate of $tRNA^{Phe}_{ox}$ was dissolved in 20 mM $Mg(OAc)_2$ (approximately 30A_{260} units/mL) and an equal volume of 0.2 M KPO_4 (pH 7.1) containing 0.5% $NaBH_4$ and 0.2% HOAc (final pH 7.5) was added. After 2 h at 23 °C in the dark, an additional 1 volume of reducing solution was added and incubation continued for an additional hour. $tRNA^{Phe}_{ox\text{-}red}$ was isolated by precipitation ($\frac{1}{10}$ volume of 2 M KOAc (pH 5) plus 2 volumes of ethanol) and washed by one reprecipitation from 0.2 M KOAc (pH 5) with 70% ethanol. $tRNA^{Phe}_{ox}$ and $tRNA^{Phe}_{ox\text{-}red}$ were desalted by dialysis in 2 mM KOAc (pH 5), 2 mM $MgCl_2$. $tRNA^{Phe}_{ox}$ was completely inactive in the acylation reaction, while $tRNA^{Phe}_{ox\text{-}red}$ could be acylated with phenylalanine to 35–40% of the control $tRNA^{Phe}$ when assayed at low salt concentration and to 70–75% when assayed in the presence of 1.5 M $(NH_4)_2SO_4$. These levels and the effect of $(NH_4)_2SO_4$ are similar to those previously reported (Ofengand et al., 1974).

$tRNA^{Phe}$ with Other Modified Ends (Scheme I). (1) Removal. The terminal adenosine or cytidine was removed by oxidation of $tRNA^{Phe}$ as indicated above followed by β-elimination of the 3′-terminal oxidized nucleoside by incubation with 20–100 A_{260} units/mL for 30 min at 47 °C with 0.5 M charcoal-treated lysine, 50 mM sodium cacodylate buffer (pH 6.4) (Uziel, 1975). At the end of the incubation, the mixture was made 0.1 M in KOAc (pH 5) and the tRNA precipitated

SCHEME I.[a]



[a] Periodate–lysine cleavage, removal of phosphate with alkaline phosphatase, and resynthesis with tRNA-nucleotidyl transferase were all performed as described under Materials and Methods. Characterization of the products is given in Table I. A* and C* designate radioactive residues. For the preparation of tRNA-CpCpCpA*, a parallel incubation of tRNA-CpC with unlabeled CTP was performed, and after isolation this product was incubated with [^{14}C]ATP.

at −25 °C by addition of 2.5 volumes of ethanol. The tRNA precipitate was dissolved in 0.1 M KOAc (pH 5) and reprecipitated with ethanol. In some instances, the tRNA was desalted by gel filtration of the reaction mixture. The 3′-terminal phosphate was removed with bacterial alkaline phosphatase. tRNA (10–60 A_{260} units/mL) was incubated at 37 °C with bacterial alkaline phosphatase (BAP) in 10 mM $MgCl_2$, 25 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol. In each case, a preliminary titration with BAP was performed to ensure complete removal of the 3′-terminal phosphate. The assay tested the ability to reincorporate AMP residues by adding aliquots to a second incubation mixture containing excess tRNA-nucleotidyl transferase, 0.1 mM [^{14}C]ATP, 50 mM glycine (pH 9.2), 10 mM $MgCl_2$, and 1 mM dithiothreitol. After incubation for 5–10 min at 37 °C, the samples were precipitated with cold 5% Cl_3AcOH, filtered, and counted. The preparative phosphatase reaction was stopped by extraction two times with redistilled phenol equilibrated with 0.1 M KOAc (pH 5). The tRNA was recovered by precipitation with ethanol as above, and freed of salts by dialysis against 2 mM KOAc (pH 5), 2 mM $MgCl_2$ before subsequent steps of periodate–amine cleavage, AMP or CMP incorporation, or assay for stimulation of (p)ppGpp synthesis.

(2) Resynthesis. Readdition of either CMP or AMP residues was performed by incubation with tRNA-nucleotidyl transferase in a reaction mixture containing 50 mM glycine (pH 9.2), 10 mM $MgCl_2$, 1 mM dithiothreitol, 7–10 A_{260} units/mL of tRNA appropriately modified at the 3′-end, and 0.05 mM [^{14}C]CTP or 0.1 mM [^{14}C]ATP at 37 °C for a time sufficient to reach a plateau of incorporation of radioactive nucleotide. Radioactivity was measured by 5% cold Cl_3AcOH precipitation of aliquots onto millipore filters and scintillation counting. For isolation, the reaction mixture was extracted two times with redistilled phenol saturated with 0.1 M KOAc (pH 5), the phenol phase was washed, the pooled aqueous phases were adjusted to 0.1 M KOAc (pH 5), and the tRNA was precipitated with 2.5 volumes of ethanol at −25 °C for 30 min. The tRNA was then dialyzed overnight against 2 mM KOAc (pH 5), 2 mM $Mg(OAc_2)$.

Methods

The assay for (p)ppGpp synthesis was as described (Chinali et al., 1978) in 25 μL with 0.22 A_{260} unit of loose couple ribosomes, 0.1–0.2 unit of stringent factor, tRNA, and NH_4Cl or KCl as indicated at 30 °C for 60 min or as specified. In all cases, the reaction was proportional to time.

Assay for tRNA-nucleotidyl transferase activity was as described above for the readdition of CMP residues, except that 0.5 mM nonradioactive ATP was added to block multiple addition of CMP residues. After incubation at 37 °C, aliquots were precipitated with cold 5% Cl_3AcOH, collected on millipore filters, and counted. For assay of activity in the stringent factor under (p)ppGpp synthesis conditions, a 25-μL reaction mixture containing 50 mM Tris-HCl (pH 7.8), 2 mM NH_4Cl, 20 mM $MgCl_2$, 2 mM dithiothreitol, 4 mM ATP, and 20–80 times the amount of stringent factor normally used was incubated with 0.8 μM tRNA-CpC for 30 min at 30 °C, after which 15 μL of the same buffer was added containing in addition 33 mM KCl, 17 μM [^{3}H]phenylalanine, and 0.4 unit (nmol/min) of Phe-tRNA synthetase. After an additional 15 min at 30 °C, the incorporation of phenylalanine onto regenerated tRNA-CpCpA was measured by cold 5% Cl_3AcOH precipitation onto millipore filters and counting.

Phe-tRNA synthetase activity was measured as previously described (Ofengand et al., 1974). Protein concentration was determined according to Lowry et al. (1951). Concentration of ribosomes and tRNA was measured by UV absorption. One A_{260} unit of ribosomes (in 10 mM Mg^{2+}) and of poly(U) (in water) was taken to correspond to 25 pmol and 33 μg, respectively.

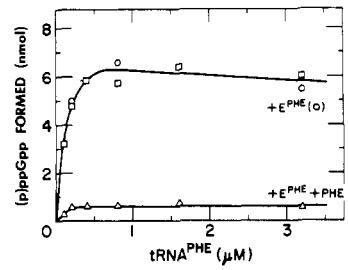


FIGURE 1: (p)ppGpp stimulatory activity of Phe-tRNA^{Phe}. The standard reaction mixture contained 40 mM NH_4Cl, tRNA^{Phe} as indicated, and, when present, 0.085 unit (nmol of Phe charging/min) of phenylalanyl-tRNA synthetase and 0.2 mM phenylalanine. tRNA^{Phe} alone (□), tRNA^{Phe} plus phenylalanyl-tRNA synthetase (○), tRNA^{Phe} plus phenylalanyl-tRNA synthetase and phenylalanine (△). Two partial reaction mixtures were prepared: one, containing tRNA^{Phe}, ATP, GTP, synthetase, and, when present, phenylalanine, was preincubated for 15 min at 30 °C; a second mixture containing ribosomes, poly(U), and stringent factor was kept at 0 °C for 10–15 min. Reaction was started by the addition of 10 μL of the second mixture to 15 μL of the first one. (p)ppGpp synthesis was assayed after 1 h incubation at 30 °C. The final concentrations of other components were as indicated under Materials and Methods.

Results

Requirement for Unacylated tRNA. The dependency of (p)ppGpp synthesis on unacylated tRNA is illustrated in Figure 1. Unacylated tRNA preincubated with or without a purified phenylalanyl-tRNA synthetase was equally active, 50% of the maximum rate being reached in both cases at 0.085 μM tRNA, while the addition of phenylalanine to the preincubation medium was sufficient to abolish the ability to induce synthesis of (p)ppGpp. The continued presence of excess synthetase and amino acid during the (p)ppGpp synthesis assay ensured that any loss of Phe-tRNA by chemical deacylation would be quickly regenerated. The small amount of apparent activity is probably due to an aminoacyl-tRNA hydrolytic activity in the ribosome or stringent factor preparation in excess of the reacylation capacity, as it did not increase in proportion to the added tRNA which had been acylated during the preincubation period. In any case, the activity was very small, corresponding to only ca. 0.01 μM in active tRNA. The inability of aminoacylated forms of tRNA to induce (p)ppGpp synthesis has been reported previously (Pedersen et al., 1973; Haseltine and Block, 1973). Pedersen et al., using a mixed aminoacyl-tRNA, observed only a partial block of activity probably because of partial deacylation, while Haseltine and Block examined only *N*-acetylphenylalanyl-tRNA.

The ability of stringent factor to distinguish between aminoacylated and unacylated forms of tRNA prompted us to examine in detail other modifications at the 3′-end in order to clarify the molecular basis for this selectivity.

Activity of tRNA$^{Phe}_{ox}$ and tRNA$^{Phe}_{ox\text{-}red}$. Periodate-oxidized unfractionated tRNA (Pedersen et al., 1973) or purified tRNA^{Phe} (Haseltine and Block, 1973) was stated to be unable to induce (p)ppGpp synthesis, although no data were presented, and Lund et al. (1973) while confirming this result with a time course did not present any tRNA-dependent concentration curves. We, therefore, reexamined this modification in detail, including a study of the effect of subsequent reduction of the terminal dialdehyde to the 2′,3′ primary alcohol.

Confirming the earlier reports, we found that at a high salt concentration (80 mM NH_4Cl) similar to that used previously

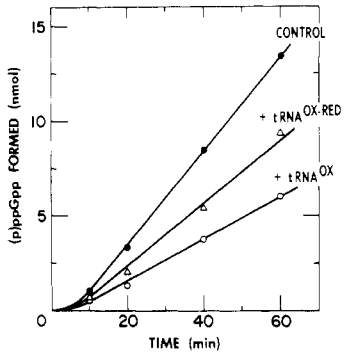


FIGURE 2: Kinetics of $tRNA^{Phe}$-dependent synthesis of (p)ppGpp in the presence or in the absence of $tRNA^{Phe}_{ox}$ or $tRNA^{Phe}_{ox-red}$. The reaction mixtures (50 μL) contained 83 mM NH_4Cl and 1 μM $tRNA^{Phe}$ (●), 1 μM $tRNA^{Phe}$ plus 4.5 μM $tRNA^{Phe}_{ox-red}$ (△), or 1 μM $tRNA^{Phe}$ plus 5.6 μM $tRNA^{Phe}_{ox}$ (○). Concentrations of all the other components and incubation temperature were the same as indicated under Materials and Methods. At the indicated time intervals, 7-μL fractions were withdrawn and analyzed for (p)ppGpp synthesis. The values on the abscissa refer to the amounts of (p)ppGpp formed in the total volume (50 μL) of the reaction mixture.

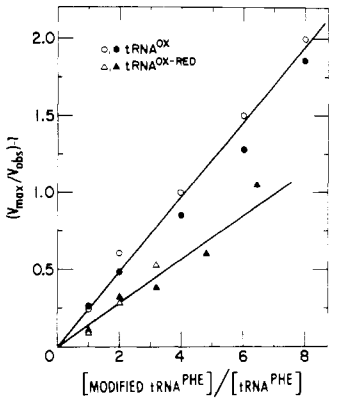


FIGURE 3: Competition of $tRNA^{Phe}_{ox}$ and $tRNA^{Phe}_{ox-red}$ with untreated $tRNA^{Phe}$ at 83 mM NH_4Cl. Reaction mixtures contained 0.6 (closed symbols) or 1.2 μM (open symbols) untreated $tRNA^{Phe}$ and $tRNA^{Phe}_{ox}$ (circles) or $tRNA^{Phe}_{ox-red}$ (triangles) as indicated. V_{max} and V_{obsd} represent the amount of (p)ppGpp synthesized in 60 min in the absence and in the presence, respectively, of modified tRNA. Under these conditions, reaction was linear for at least 75 min. Both modified and untreated tRNAs were added to the reaction mixture simultaneously. Other components and assay were as described under Materials and Methods. The analysis is described in the text.

by others periodate-oxidized tRNA was completely inactive (7 nmol of (p)ppGpp synthesized by a saturating amount of control tRNA; <0.2 nmol synthesized by 0.5–6 μM $tRNA_{ox}$). Oxidized-reduced tRNA was also completely inactive (<0.1 nmol synthesized over the same concentration range). However, as the salt concentration was reduced, activity began to appear approaching 44% of the control for $tRNA_{ox}$ and 24% for $tRNA_{ox-red}$ at 3 mM NH_4Cl.

This activity, quantitatively the same with two different stringent factor preparations and two separate tRNA preparations, could not, however, be reproduced in later experiments using a more purified stringent factor. In these experiments, $tRNA_{ox}$ and $tRNA_{ox-red}$ were only 3–5% active at 1–2 μM tRNA (the previous apparent saturation concentration). We were able to generate approximately half of the expected activity by the addition of excess tRNA-nucleotidyl transferase to the (p)ppGpp synthesis assay mixture but could not detect any exchange (<0.3%) of the terminus of $tRNA_{ox}$ with ATP by an aminoacylation assay when the tRNA and tRNA-nucleotidyl transferase were incubated under the (p)ppGpp synthesis assay conditions but without stringent factor and ribosomes. Two percent exchange would have been needed to account for the activity originally observed. We suspect that the activity found at low salt was due to contamination of the stringent factor with tRNA-nucleotidyl transferase which is known to be suppresible by high salt concentration plus a small amount of 3′-exonuclease in either the stringent factor or ribosomes which removed a small fraction of the modified ends of the tRNA allowing repair with ATP (see also Kucan and Chambers, 1972). A maximum of 8 and 4% exchange of $tRNA_{ox}$ and $tRNA_{ox-red}$, respectively, would have been sufficient.

In order to show that the modified tRNAs were bound to the ribosome–stringent factor complex even though they completely failed to stimulate stringent factor, the ability to compete with control $tRNA^{Phe}$ was examined. This assay was preferred to one in which the physical binding of radioactive tRNA was measured, since the competition assay measures only binding to the correct site (strictly speaking, it measures any binding that is capable of *blocking* the correct site). Lund et al. (1973) had previously tested $tRNA_{ox}$ for competition with negative results, apparently using a 1:1 mixture of control and oxidized tRNA, although no details were given.

The competitive behavior of $tRNA_{ox}$ and $tRNA_{ox-red}$ at 83 mM NH_4Cl is shown in Figures 2 and 3. Figure 2 shows that when a 5.6- ($tRNA_{ox}$) or a 4.5-fold excess ($tRNA_{ox-red}$) over control tRNA was added to a complete mixture, the rate of (p)ppGpp formation was partially decreased. It is important to note that the rate was inhibited and remained at a constant value from the first time point measured, indicating that the observed inhibition was not due to a slow progressive inactivation of the ribosome binding site, for example, by the failure of modified tRNA to be released, but to a true equilibrium between modified and control tRNA for limiting binding sites. (The concentration of control tRNA was 1 μM, four times the saturation value.)

In order to show directly the competitive nature of the inhibition, the concentration of both the control and modified tRNAs was varied. Assuming that only the equilibrium amount of control tRNA bound to the ribosome–stringent factor complex determines the rate of (p)ppGpp formation and that binding of modified tRNA to the ribosome–factor complex does not induce (p)ppGpp synthesis, the results should obey the relation $(V_{max}/V_{obsd}) - 1 = (K_{mod}/K_{con}) \times ([tRNA^{mod}]/[tRNA^{con}])$ (Ofengand and Henes, 1969) as long as tRNA is present in saturating amounts. V_{max} and V_{obsd} are defined in the legend to Figure 5; "mod" and "con" refer to modified and control tRNA, respectively and K is the association constant for the binding site. The concentration of free tRNA was approximated by the concentration of tRNA added, since the tRNAs were present in large excess over the number of binding sites. The slope of the line obtained is thus a measure of the relative affinity of the modified tRNA for the stringent factor–ribosome complex.

Two points are evident from the results (Figure 3). First, the inhibition is truly competitive, since doubling the control tRNA concentration at a constant $tRNA^{mod}$ concentration decreased the inhibition by the expected amount. Both concentrations of control tRNA were saturating, as verified in each experiment. Second, $tRNA_{ox}$ is a stronger competitor than $tRNA_{ox-red}$. The association constants relative to the control tRNA were 0.24 for $tRNA_{ox}$ and 0.14 for $tRNA_{ox-red}$.

This result means that the maximum concentration of $tRNA_{ox}$ and $tRNA_{ox-red}$ tested, 6.0 μM, which was inactive corresponds to control tRNA concentrations of 1.4 and 0.8 μM

TABLE I: Characterization of tRNAs Modified by Terminal Addition.[a]

Modified tRNA	Nucleotide incorp (nmol/A_{260} unit)	Nucleotide released (nmol/A_{260} unit)
-CpCpA*	1.49	1.48
-CpCpC*	1.57	1.34
-CpCpCpA*	1.55	1.54
-CpA*	1.31	1.27

[a] $tRNA^{Phe}$ (1195 pmol of Phe acceptance/A_{260} unit) was treated according to Scheme I. Periodate-amine cleavage, phosphate removal, and resynthesis with tRNA-nucleotidyl transferase were all performed as described under Materials and Methods. The amount of nucleotide incorporated was determined by cold Cl_3AcOH precipitation of the purified tRNA onto millipore filters. Nucleotide released was determined by treatment of an aliquot of each radioactive tRNA with 4 mM $NaIO_4$, 100 mM cacodylate (pH 6.5) for 3 min at 47 °C, followed by addition of an equal volume of 1 M lysine (pH 6.5) and incubation for 30 min at 47 °C (Uziel, 1975). Residual radioactivity bound to tRNA was determined by Cl_3AcOH precipitation and millipore filtration. The amount of nucleotide released was calculated by difference.

(see also Table II) and should, therefore, completely occupy the ribosomal binding site. The failure to induce (p)ppGpp synthesis is therefore attributed solely to the failure of the effector function of the modified ends. This result could come about either because the presence of an intact adenosine is needed as a positive effector or the modified ends act as negative effectors. In order to explore this aspect further, we examined tRNAs with lengthened and shortened 3′ ends.

Preparation of $tRNA^{Phe}$ with Shortened, Lengthened, or Substituted 3′ Ends. The variously modified tRNAs used in this section were prepared by combining the periodate–lysine–phosphatase cleavage method of Uziel (1975) with the resynthesis capabilities of tRNA-nucleotidyl transferase (Deutscher, 1973) as outlined in Scheme I. The time course of each step was monitored as described under Materials and Methods. Complete removal of the terminal A to give tRNA-CpCp and then tRNA-CpC was shown by the stoichiometric readdition of A residues, all of which were terminal since they could be quantitatively released again by a second treatment with periodate–lysine (Table I). Since the tRNA-CpCp preparation was completely devoid of activity (Table II), no intact tRNA could have escaped the treatment. tRNA-CpCpC was made by the addition of CTP in the absence of ATP. Although only one residue of CTP was added, after which a stable plateau value was reached, the data of Table I, line 2, shows that only 86% of the CTP incorporated was terminal, since 14% could not be released even when the treatment time with each reagent was doubled. This result cannot be due to the presence of contaminating amounts of ATP during the incubation, producing tRNA-CpCpCpA, since in that case the subsequent addition of labeled ATP (line 3) would also be less than stoichiometric. Taking the average of lines 1 and 3, 1.52 nmol/A_{260} unit, as the number of chains per A_{260} unit, 1.34 C* nmol/A_{260} unit released in line 2 means 0.18 nmol/A_{260} unit did not react at all, while 1.57 − 1.34 = 0.23 nmol/A_{260} unit gives the amount which was doubly labeled by the addition of two C residues. Lack of material precluded a second cycle of periodate–lysine treatment to verify this conclusion. Thus, it is most likely that the true composition of tRNA-CpCpC and those tRNAs derived from it is 73% tRNA-CpCpC, 15% tRNA-CpCpCpC, and 12% tRNA-CpC. Other examples of the addition of extra C residues by tRNA-nucleotidyl transferase in the absence of ATP are known (Kirschenbaum & Deutscher, 1976). However, the addition of more than one A residue has never been described, and here also the addition of A to tRNA-CpCpC was all terminal (line 3). The quantitative removal of A to generate tRNA-CpCpCp was verified by the full activity of tRNA-CpCpCpA and complete lack of activity of tRNA-CpCpCp (Figure 4 and Table II).

tRNA-Cp and tRNA-C were made as indicated. The

TABLE II: Relative Activity of tRNAs Modified at the 3′ Terminus for (p)ppGpp Synthesis and as Competitors of Unmodified tRNA.

tRNA species	Stimulatory act.[a] 2–3 mM NH_4Cl	Stimulatory act.[a] 83 mM NH_4Cl	Competitor act.[b]	Association constant[c] ($K_a \times 10^{-6}$) (M^{-1}) 2–3 mM NH_4Cl	Association constant[c] ($K_a \times 10^{-6}$) (M^{-1}) 83 mM NH_4Cl	V_{max}[d] (M^{-1}) 2–3 mM NH_4Cl	V_{max}[d] (M^{-1}) 83 mM NH_4Cl	Max tRNA in control equiv (μM)[e] 2–3 mM NH_4Cl	Max tRNA in control equiv (μM)[e] 83 mM NH_4Cl
-C	0 (2.0)	0 (1.2)	7.3		1.5	0	0	>0.27	0.16
-Cp	0 (4.8)	0 (1.2)	24.0[g]	0.50		0	0	0.20	
-CpC	2[f]	<1 (1.2)	9.9		1.1		0		0.12
-CpA	4	<1 (0.35)	7.3	2.7	1.5	20	0		0.05
-CpCp	0 (4.3)	0 (2.3)	24.0[g]	0.50		0	0	0.18	
-CpCpC	35	28		6.5	7.4	69	37		
-*CpCpA*	100	100		12.0	12.0	100	100		
-CpCpCp	0 (1.6)	0 (0.48)	28.0		0.39	0	0	>0.06	0.02
-CpCpCpA	100	79		12.0	12.0	100	73		
$-CpCpA_{ox}$	0 (6.0	0 (6.0)	4.2		2.6	0	0		1.4
$-CpCpA_{ox-red}$	0 (6.0)	0 (6.0)	7.1		1.6	0	0		0.8

[a] Calculated as the slope of the linear part of the tRNA concentration curves of Figure 4 and from unpublished results. The value for tRNA-CpCpA is set at 100. Values in parentheses are the maximum concentration tested. [b] Calculated as the reciprocal slope of the lines of Figure 3 and similar data not shown. The value is the number of moles of competitor required to complete equally with 1 mol of control tRNA for a limited number of binding sites. Reactions were at 83 mM MH_4Cl, except as noted. [c] For tRNA species with a V_{max} of 0, calculated from the slope of the lines of Figure 3 or similar data not shown, multiplied by the K_a for control tRNA. For active tRNA species, calculated from the activity curves of Figure 4 and other similar data as 1/[tRNA] at $V_{max}/2$. [d] Calculated as the maximum rate of synthesis relative to tRNA-CpCpA from the curves of Figures 4 and 5 and unpublished results. V_{max} for tRNA-CpCpA at 83 mM NH_4Cl is 61% of V_{max} at 2 mM. [e] Calculated as the maximum tRNA concentration tested (values in parentheses in columns 2 and 3) divided by the value in column 4. For the two cases in which the values in column 2 were measured at 2–3 mM NH_4Cl while the values in column 4 were obtained at 83 mM NH_4Cl, it was assumed that the true column 4 value at 2–3 mM NH_4Cl would be equal or lower (i.e., more active) as was the case with tRNA-CpA and tRNA and tRNA-CpCpC. [f] Uncorrected for any conversion to tRNA-CpCpA by contaminant tRNA-nucleotidyl transferase. [g] Measured at 3 mM NH_4Cl.

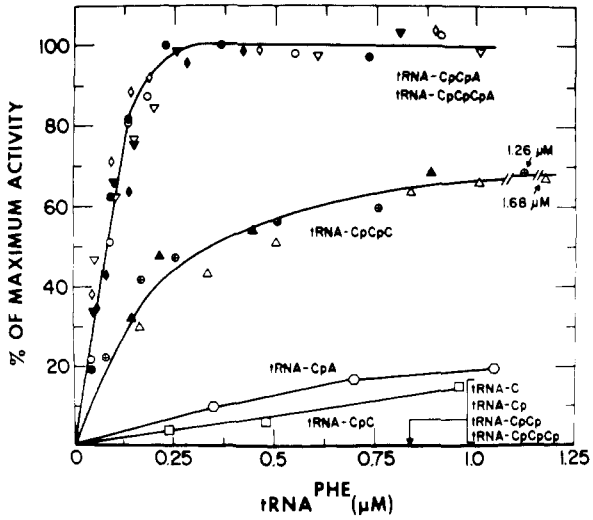


FIGURE 4: Activity of 3′-end modified $tRNA^{Phe}$ as a function of tRNA concentration. Reaction mixtures contained 2 mM NH_4Cl and 0.14 (expt 1) or 0.09 (expt 2) unit of stringent factor and tRNA as indicated, and were incubated for 60 or 40 min, respectively. Other conditions and assay procedures were as described under Materials and Methods. The results are expressed as percent of the maximal activity, which was 8.5 nmol (p)ppGpp synthesized (expt 1) or 3.3 nmol (expt 2). Open symbols, expt 1; closed symbols, expt 2. Control tRNA (◇, ◆); tRNA-CpCpA* (○, ●); tRNA-CpCpCpA* (▽, ▼); tRNA-CpCpC (△, ▲); tRNA-CpCpC* (⊕); tRNA-CpA (○); tRNA-CpC (□). The tRNA preparations are defined in Scheme I. The data for the other tRNAs indicated on the figure are omitted for clarity but were all completely inactive up to 2 (tRNA-C), 4.8 (tRNA-Cp), 4.3 (tRNA-CpCp), and 1.6 μM (tRNA-CpCpCp).

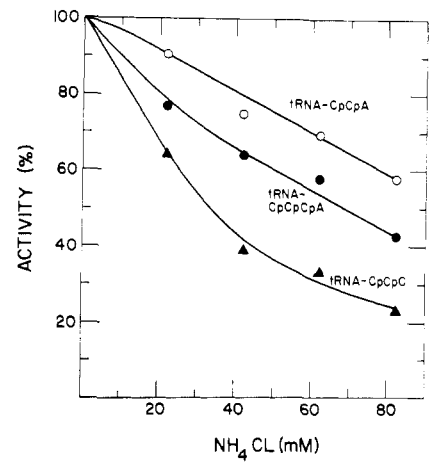


FIGURE 5: Effect of NH_4Cl on activity of control and modified $tRNA^{Phe}$ species. Plot of the maximal activity of tRNA-CpCpA (○), tRNA-CpCpCpA (●), and tRNA-CpCpC (▲) as a function of NH_4Cl concentration. Activity was measured in the standard assay using an excess of tRNA [0.4 μM for tRNA-CpCpA and tRNA-CpCpCpA, and 1.4 μM for tRNA-CpCpC], 0.3 unit of stringent factor (40 min incubation at 30 °C), and NH_4Cl as indicated. Activity is expressed as percent of the activity of each $tRNA^{Phe}$ species at 2 mM NH_4Cl which was 97% for tRNA-CpCpCpA and 78% for tRNA-CpCpC of the activity of tRNA-CpCpA.

readdition of A to make tRNA-CpA was not quite quantitative, only 85% of theory being observed, but it was all terminal. However, since the 15% of intermediates were completely inactive (Figure 4), they did not interfere with the subsequent measurements.

Activity of $tRNA^{Phe}$ with Modified Ends. The activity of the various tRNAs at 2 mM NH_4Cl is shown in Figure 4. Two independent experiments with different amounts of stringent factor are shown in this figure, as indicated. Untreated tRNA, resynthesized tRNA-CpCpA, and the extended tRNA-CpCpCpA were fully active. Since this latter preparation probably contains 15% tRNA-CpCpCpCpA, it appears that even such an extended chain is active. Nevertheless, there is specificity for a terminal A, since tRNA-CpCpC was considerably less active. (The data have been plotted assuming tRNA-CpCpCpC has the same activity as tRNA-CpCpC and that tRNA-CpC is inactive.) Furthermore, all three nucleotides are necessary, since tRNA-CpA or tRNA-CpC was only slightly active. In fact, even the slight activity of tRNA-CpC may be due to a residual level of tRNA-nucleotidyl transferase which can resynthesize tRNA-CpCpA during the (p)ppGpp assay (see also Sprinzl & Richter, 1976). tRNA-C, missing both terminal nucleotides, was completely inactive, as were all three tRNA species ending in a 3′-terminal phosphate. The effect of the terminal phosphate is particularly striking in the case of tRNA-CpCpCp in comparison to the activity of tRNA-CpCpC.

In experiments not shown, we attempted to replace the missing ends by noncovalent addition of the appropriate nucleotides, since Černá (1975) and Krayevsky et al. (1976) were successful in activating the peptidyl transferase P site in this manner. However, neither at 1 nor 0.1 mM nucleotide was there any restoration of activity. The combinations tested were tRNA-CpCp plus adenosine or cytidine, tRNA-Cp plus CpA, and tRNA-C plus CpA. CpA (0.5 mM) by itself was also inactive.

Ability of tRNAs with Modified Ends to Bind to the Ribosome–Stringent Factor Complex. The binding activity of the modified tRNAs was tested by competitive inhibition of control tRNA stimulation of (p)ppGpp synthesis exactly as described in Figure 3. tRNA-CpCp and tRNA-Cp were tested at 3 mM NH_4Cl, while tRNA-C, tRNA-CpA, tRNA-CpC, and tRNA-CpCpCp were studied at 83 mM NH_4Cl. In all cases, the inhibition was competitive. The reciprocal slopes of the lines obtained by analysis as in Figure 3 are given in Table II which summarizes the relative stimulatory and competitor activity as well as the derived K_a and V_{max} values for all of the modified tRNAs. The last column tabulates the maximum tRNA concentrations tested which gave no activity, expressed in terms of their equivalent control tRNA concentrations. These values were obtained using the relative affinities calculated from the competition experiments. Comparison of these values with the tRNA-CpCpA curve of Figure 4 shows clearly that considerable activity would have been expected for all of the tRNAs, if only binding ability were considered. The 2 and 83 mM NH_4Cl conditions are equivalent for this comparison because the K_a for control tRNA at both salt concentrations is the same (Table II; see also Figure 1 and Table II of Chinali et al., 1978).

We conclude that, while the various modifications tested certainly affect the ability to form a tRNA–stringent factor–ribosome complex (a maximum 28-fold reduction in affinity was found for the 3′-phosphate ended tRNAs), the main effect is to reduce the V_{max} to a vanishingly small value. That is, it is mainly the allosteric stimulatory activity which is lost.

Effect of NH_4Cl on the Activity of the Different Modified tRNAs. Since the well-known inhibitory effect of NH_4Cl (Sy et al., 1973) was found to affect only the V_{max} of control tRNA (Table II), we did a similar analysis of its effect on tRNA-CpCpC and tRNA-CpCpCpA, the two other modified tRNAs which possessed some activity. Each of the tRNAs was present in excess so that the V_{max} effect could be studied. The results (Figure 5) are expressed as a percentage of the reaction rate with each tRNA in the absence of added NH_4Cl in order to show the relative sensitivity to inhibition by NH_4Cl. The tRNAs behave differently. tRNA-CpCpA, like tRNA-

CpCpCpA, is relatively poorly inhibited by NH_4Cl, while tRNA-CpCpC is more easily inhibited as the NH_4Cl concentration is increased.

Discussion

Although stringent factor activation by ribosome-bound tRNA has been known to be specific for the unacylated form of tRNA for several years, there has been little attempt to investigate the molecular mechanism involved, despite the importance of this discriminatory ability for the proper regulation of (p)ppGpp synthesis. In this work, we have studied tRNAs modified chemically at the 3′-end, $tRNA_{ox}$ and $tRNA_{ox-red}$, as well as tRNAs lengthened, shortened, or with a base substitution at this end. By systematic variation of the tRNA concentration, we have been able to compare the effects of each modification on the maximum velocity of the reaction as well as on the affinity for the binding site. Furthermore, by measuring the binding activity (by competition methods) as well as the ability to induce (p)ppGpp synthesis, we have been able to determine approximate binding constants even for those tRNAs with zero activity. We interpret affinity as a measure of the ability to be recognized by the ribosome–stringent factor complex and V_{max} as a measure of the allosteric effector capacity of the modified tRNA. Analysis of both parameters as a function of NH_4Cl concentration has allowed us to deduce the site of action of NH_4Cl.

The data summarized in Table II illustrates several points. First, the only fully active modified tRNA was tRNA-CpCpCpA, a tRNA extended by one nucleotide but ending in A, and even this tRNA was a little less active at high salt. By contrast, tRNA-CpCpC, a full length tRNA with the terminal A replaced by C, had a reduced activity both with regard to K_a and V_{max} at both low and high salt. This strong preference for a terminal A residue means that in order to place the A of tRNA-CpCpCpA rather than the penultimate C at the correct site the three terminal C residues must fold up so as to occupy the space normally taken by only two of them.

The inability of the ribosome–stringent factor complex to discriminate between a −CpCpA and a −CpCpCpA end is in contrast to other situations in which tRNA is recognized. Aminoacyl-tRNA synthetases can aminoacylate tRNA-CpCpCpA (Rether et al., 1974) but, in general, do so less well (Kirschenbaum & Deutscher, 1976). Formation of the ternary complex with EF-Tu is severely affected (Thang et al., 1972), and, while factor-dependent ribosomal A- and P-site binding seems to be little affected, the rate of the peptidyl transferase reaction is also severely decreased (Thang et al., 1974).

Second, the strong preference for a normal adenosine end is also indicated by the complete loss of activity at both high and low salt upon oxidation or oxidation–reduction. Although the V_{max} was zero, the affinity was only decreased fourfold after oxidation and sevenfold after subsequent reduction. Sprinzl & Richter (1976) also found $tRNA_{ox}$ and $tRNA_{ox-red}$ to be inactive. Affinity constants cannot be compared, since competition assays were not done by these workers.

The loss of activity upon ribose modification was not due to an inhibitory effect of the modified ribose because removal of the terminus altogether in the form of tRNA-CpC or tRNA-C did not give an active molecule at either low or high salt. The low activity of tRNA-CpC at low salt could be almost entirely accounted for by the regeneration of tRNA-CpCpA by residual tRNA-nucleotidyl transferase in the stringent factor (unpublished results; see also Sprinzl & Richter, 1976). On the other hand, tRNA-CpA had some intrinsic activity at low salt but none at high salt. Since all three tRNAs, tRNA-CpC, tRNA-CpA, and tRNA-C, had similar binding affinities at high salt (Table II), it is reasonable to suppose that their affinities were also similar at low salt. The stimulatory activity difference between tRNA-CpA and tRNA-CpC or tRNA-C at low salt is therefore likely to be due to large differences in V_{max} which reflect the presence or absence of an intact terminal A residue.

Third, all three tRNAs ending in 3′-phosphate were completely inactive at low or high salt. The effect of a 3′-phosphate is most readily noted by comparison of tRNA-CpCpCp with its dephosphorylated form, tRNA-CpCpC. The K_a at 2 mM NH_4Cl for tRNA-CpCpCp can be estimated to be 0.8×10^6 M^{-1} from the value of 0.4×10^6 M^{-1} measured at 83 mM NH_4Cl, by analogy with tRNA-CpA. The K_a at 2 mM NH_4Cl for tRNA-CpCpC, 6.5×10^6 M^{-1}, is eight times this value. However, the effect on V_{max} is much more striking. Despite the fact that tRNA-CpCpCp was tested at a sufficiently high concentration (equivalent to 0.12 μM control tRNA), to have given 80% reaction according to the curve of Figure 4, it was completely inactive. tRNA-CpCpC, on the other hand, could stimulate up to 69% of the control activity at a saturating tRNA concentration. Thus, the main effect of phosphorylation of the 3′-hydroxyl is to abolish the effector activity (V_{max} effect).

Sprinzl & Richter (1976) also found that tRNA-CpC, tRNA-CpCp, and tRNA-C were inactive at 40 mM NH_4Cl. They further observed that, while tRNA whose terminal adenosine was replaced by 3′-deoxyadenosine or 3′-aminoadenosine was inactive, substitution with 2′-deoxyadenosine retained full activity. Replacement of the terminal A with formycin gave a tRNA with about 15% of control stimulatory activity. It is not known, however, if the inhibitory effects observed were due to a failure to bind the ribosome or to a failure to induce stringent factor.

The importance of NH_4Cl in (p)ppGpp synthesis has been appreciated ever since Sy et al. (1973) showed that the ribosomal system was inhibited progressively by increasing concentrations of NH_4Cl, while the ribosome-free system (using 20% methanol) was either unaffected or stimulated up to 200 mM NH_4Cl. This result shows that the intrinsic catalytic center of stringent factor is not the site of action of NH_4Cl, but that it must inhibit some aspect of the ribosome-dependent reaction. Recently, Richter et al. (1975) showed that NH_4Cl did not prevent binding of stringent factor to ribosomes, a reaction preceding, and independent of, the binding of tRNA. At 20 mM Mg^{2+}, the concentration used in this work, 40 and 200 mM NH_4Cl only inhibited 9 and 27%, respectively. Therefore, NH_4Cl must inhibit either the binding of tRNA to the ribosome–stringent factor complex or its effector function. The data presented in this and the preceding paper shows clearly that the main effect is on V_{max}, the allosteric effector function. Thus, the K_a for unmodified tRNA is unaffected by 83 mM NH_4Cl, but the V_{max} is decreased to 61% (Table II, Figure 5; see also Chinali et al., 1978). With modified tRNAs, the effect is even more striking. The V_{max} values of tRNA-CpCpCpA and tRNA-CpCpC drop 2.2- and 3.1-fold on shifting from 2 to 83 mM NH_4Cl with no change in the corresponding K_a values, while the V_{max} of tRNA-CpA goes from 20% to zero with only a twofold decrease in K_a.

The same conclusion was also reached about the site of inhibition of the various 3′-end modifications studied in this paper, namely, that their main effect is to block the effector function of the tRNA molecule. NH_4Cl which also acts at this site serves to potentiate the discriminatory ability of the stringent factor. We suggest that aminoacyl-tRNA may be selected against by the same mechanism. Thus, it is likely that the affinity of aminoacyl-tRNA for the ribosomal A site–

stringent factor complex in the absence of elongation factor may not differ appreciably from that for unacylated tRNA, 10^7 M^{-1}, but the presence of the aminoacyl residue, like that of a 3′-phosphate group, completely blocks the stimulatory activity of tRNA. Since elongation factor directed binding of aminoacyl-tRNA occurs to the same or to an overlapping site in the presence of stringent factor (Richter et al., 1975), it is not too surprising that *E. coli* has evolved a mechanism for selection which does not rely on the exclusion of aminoacyl-tRNA but rather on the specific recognition of the aminoacyl end of the tRNA.

Sprinzl & Richter (1976) have interpreted their results with 2′- and 3′-deoxyadenosine-substituted tRNAs in terms of a specific requirement for a free 3′-hydroxyl on the terminal nucleotide of tRNA. Our results do not contradict this hypothesis, except that the results with tRNA-CpCpC show that there is also a rather strong specificity determinant in the nature of the terminal nucleotide.

Finally, it should be noted that a class of (p)ppGpp synthesis-defective mutants exists, *rel* C (Parker et al., 1976) which has an altered ribosomal protein, L11. These mutant ribosomes can bind stringent factor (Christiansen & Nierhaus, 1976) but are unable to synthesize (p)ppGpp. It remains to be seen whether they are defective in binding tRNA or in the effector activity of the mutant ribosome-tRNA complex. In that context, it is interesting that L11 comprises part of the peptidyl transferase center (Brimacombe et al., 1976) which must interact with the aminoacyl end of tRNA when it is bound in the ribosomal A site.

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