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Elongation Factor T_u·Ribosome Dependent Guanosine 5′-Triphosphate Hydrolysis: Elucidation of the Role of the Aminoacyl Transfer Ribonucleic Acid 3′ Terminus and Site(s) Involved in the Inducing of the Guanosinetriphosphatase Reaction†

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ABSTRACT: We have studied the interaction between *Escherichia coli* $tRNA^{Phe}$·poly(U)·70S ribosome·EF-T_u·GTP complex and 2′(3′)-*O*-(aminoacyl)nucleosides and -oligonucleotides (analogues of the AA-tRNA 3′ terminus) which triggers GTP hydrolysis. The results show that the binding of effectors (3′-terminal fragments of AA-tRNA) to an EF-T_u site, in the presence of ribosomes, triggers the GTPase. The affinity of effectors for the enzymatic complex depends greatly on their structure, e.g., K_a decreasing as the length of the oligonucleotide chain increases. Thus, K_a for Phe-$tRNA^{Phe}$ is approximately 1000-fold lower than that of C-C-A-Phe. On the other hand, the K_m for GTP is much less affected by the chain length, with the K^{GTP}_m in the presence of C-C-A-Phe being only 5-fold higher than that in the presence of Phe-tRNA. It follows that the aminoacylated C-C-A sequence of AA-tRNA is the most critical domain of tRNA for promotion of EF-T_u-dependent GTPase. The EF-T_u site that binds the 3′ terminus of AA-tRNA has the following requirements for interaction with the effectors: (i) it binds the side chain of the aminoacyl residue; (ii) it recognizes the entire C-C-A sequence of the AA-tRNA 3′ terminus [with the first (Ado) and third (Cyt) residues being most critical]; (iii) it is stereospecific but displays a surprising degree of flexibility, since it can functionally accommodate a substituent in lieu of the α hydrogen of the aminoacyl residue; (iv) it has stringent requirements for the recognition of the 3′-terminal ribose moiety of the effector. The GTP hydrolysis triggered by A-Gly, C-A-Gly, and C-C-A-Gly was strongly stimulated by thiostrepton, which is known to bind to the 50S ribosomal subunit. Since thiostrepton enhances the binding of fragments to EF-T_u and since it is also known to inhibit the EF-T_u-dependent binding of AA-tRNA to the 70S ribosome and the associated GTP hydrolysis, it follows that the antibiotic probably interferes with ribosomal binding of some AA-tRNA domain other than the 3′ terminus or anticodon. Thus, this unidentified portion of AA-tRNA, by virtue of its binding to the ribosome, plays a role in the promotion of EF-T_u GTPase in addition to the crucial role of the AA-tRNA 3′ terminus. Collectively, these results provide an insight into the highly coordinated chain of events leading to GTP hydrolysis by EF-T_u with active participation of AA-tRNA and ribosomes.

Elongation factor T_u (EF-T_u)[1] promotes the mRNA directed binding of AA-tRNA to the ribosome via a ternary AA-tRNA·EF-T_u·GTP complex. In the course of this binding reaction, one molecule of GTP is hydrolyzed, EF-T_u dissociates from the ribosome in the form of a binary EF-T_u·GDP complex, whereupon AA-tRNA enters the ribosomal acceptor site. At this site, the AA-tRNA molecule is able to participate in the peptide bond formation step as an acceptor (Miller & Weissbach, 1977). Thus, GTP hydrolysis appears to be directly involved in releasing EF-T_u from the ribosome, thereby allowing AA-tRNA to attain a reactive A site configuration. It has been proposed (Thompson & Stone, 1977) that GTP hydrolysis is also implicated in the proofreading process, which is required to maintain a high fidelity of translation. It is

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[1] Abbreviations: AA-tRNA, aminoacyl transfer ribonucleic acid; EF-T_u, elongation factor T_u; Tris, tris(hydroxymethyl)aminomethane; PEP, phosphoenolpyruvate; DTT, dithiothreitol; Me_2Gly, α-aminoisobutyric acid; cyclo-Leu, cycloleucine (1-amino-1-carboxycyclopentane); A-Phe, 2′(3′)-*O*-L-phenylalanyladenosine; similar abbreviations are used for other nucleoside and oligonucleotide derivatives; 3′-dA-3′-NH-Phe, 3′-deoxy-3′-L-phenylalanylamidoadenosine; 2′-dA-2′-NH-Phe, 2′-deoxy-2′-L-phenylalanylamidoadenosine; A(2′Me)Phe, 2′-*O*-methyl-3′-*O*-L-phenylalanyladenosine; A(3′Me)Phe, 3′-*O*-methyl-2′-*O*-L-phenylalanyladenosine; C-2′-dA-Lys, cytidylyl-(3′–5′)-2′-deoxy-3′-*O*-L-lysyladenosine; C-3′-dA-Lys, cytidylyl-(3′–5′)-3′-deoxy-2′-*O*-L-lysyladenosine; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid.

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obvious that if such an editing process were to take place, it must be implemented prior to de facto irreversible peptide bond formation.

Therefore, for elucidation of the detailed mechanism of the AA-tRNA selection, knowledge of the molecular mechanism of GTP hydrolysis is very important. Although the overall process is well documented (Miller & Weissbach, 1977), the detailed molecular mechanism of GTP hydrolysis is largely unknown. Since EF-T_u alone can hydrolyze GTP in the presence of the antibiotic kirromycin, the active site for GTP hydrolysis is located on EF-T_u and not on the ribosome. Nevertheless, AA-tRNA and ribosomes strongly stimulate the kirromycin-dependent EF-T_u·GTPase (Chinali et al., 1977). Since the models of the 3′ terminus of AA-tRNA, such as 2′(3′)-*O*-(aminoacyl)nucleosides and -oligonucleotides, can stimulate EF-T_u·GTPase in the presence of aurodox (methylkirromycin), it was concluded that the binding of the 3′-terminal region of AA-tRNA to EF-T_u triggers the hydrolysis of GTP (Bhuta & Chládek, 1980). Thus, the recent observation that 2′(3′)-*O*-L-phenylalanyladenosine initiates GTP hydrolysis on the EF-T_u·ribosome complex (Campuzano & Modolell, 1980) may be explained as a result of the binding of the effector (A-Phe) to EF-T_u in the presence of ribosomes.

In order to study the detailed molecular mechanism of EF-T_u·ribosome GTPase, we have used well-defined 2′(3′)-*O*-(aminoacyl)oligonucleotides (models of the 3′ terminus of AA-tRNA) as inducers of GTP hydrolysis. By use of the antibiotic thiostrepton, additional information regarding the AA-tRNA–EF-T_u–ribosome interactions leading to GTP hydrolysis was obtained.

Materials and Methods

PEP, pyruvate kinase (EC 2.7.1.40), DTT, Alumina, EDTA, poly(uridylic acid), and tris(hydroxymethyl)aminomethane were obtained from Sigma Chemical Co., St. Louis, MO. Ammonium chloride, potassium chloride, magnesium chloride and other reagents were of analytical grade and obtained locally. [γ-^{32}P]GTP (25 Ci/mmol) was purchased from ICN Chemical and Radiochemical Division, Irvine, CA. tRNA from *Escherichia coli* strain W was obtained from Sigma Chemical Co.; [^{14}C]Phe-tRNA (0.78 nmol of [^{14}C]phenylalanine/mg of tRNA) was prepared according to Chládek et al. (1974). $tRNA^{Phe}$ was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Membrane filters, Metrical type GN-6, were purchased from Gelman Sciences, Inc., Ann Arbor, MI. EF-T_u·GDP was kindly supplied by Dr. David Miller, Roche Institute of Molecular Biology, Nutley, NJ. Thiostrepton was a gift from Barbara Stearns, The Squibb Institute, New Brunswick, NJ. Thiostrepton was dissolved in Me_2SO, and its concentration was calculated by using a molecular weight of 1650 (Bodanszky et al., 1964). The (aminoacyl)nucleosides and -oligonucleotides were prepared as described (Bhuta et al., 1981; Kumar et al., 1982; Bhuta & Chládek, 1980; Chládek et al., 1974), and the samples for assays were prepared as described by Bhuta & Chládek (1980). Ribosomes low in endogenous GTPase activity were prepared from *E. coli* according to Staehelin & Maglott (1971).

GTPase Assay. The [γ-^{32}P]GTP hydrolysis was carried out by mixing 0.05 mL of EF-T_u·GTP·AA-nucleoside/oligonucleotide complex and 0.05 mL of the ribosomal pool. The EF-T_u·GTP·AA-oligonucleotide complex contained the following: Tris-HCl, 50 mM, pH 7.2, at 37 °C; NH_4Cl, 80 mM; KCl, 80 mM; $MgCl_2$, 10 mM; DTT, 2 mM; PEP, 6 mM; pyruvate kinase, 2 IU; [γ-^{32}P]GTP (1100 cpm/pmol), 0.01 mM; EF-T_u·GDP, 25 pmol; the test compounds at the desired concentration in 0.05 mL. This mixture was incubated at 37 °C for 15 min to convert EF-T_u·GDP to EF-T_u·[γ-^{32}P]-GTP·AA-nucleoside/oligonucleotide complex. The conversion of EF-T_u·GDP to EF-T_u·[γ-^{32}P]GTP was monitored according to Miller & Weissbach (1971) and was always more than 90%. The ribosomal pool contained, in a final volume of 0.05 mL, the following: Tris-HCl, 50 mL, pH 7.2, at 37 °C; NH_4Cl, 50 mM; DTT, 2 mM; ribosomes, 2.0 A_{260} units; poly(uridylic acid), 10 μg; $tRNA^{Phe}$, 20 μg. The pool was incubated at 37 °C for 10 min to form the 70S ribosome·poly(U)·$tRNA^{Phe}$ complex. GTP hydrolysis was initiated by addition of the ribosomal pool. The incubation temperature and time are given in the figure legends. After the appropriate length of incubation, the reactions were stopped by addition of 0.1 mL of 1 M perchloric acid, and the $^{32}P_i$ was extracted, as described earlier (Bhuta & Chládek, 1980). The appropriate blank values of 0.03–0.08 pmol of GTP, hydrolyzed in the absence of test compounds, have been subtracted from the data presented. Each curve represents an average of three experiments. The K_m for GTP was determined from double-reciprocal plots of Figure 3, and the slopes and intercept were calculated by using linear regression analysis.

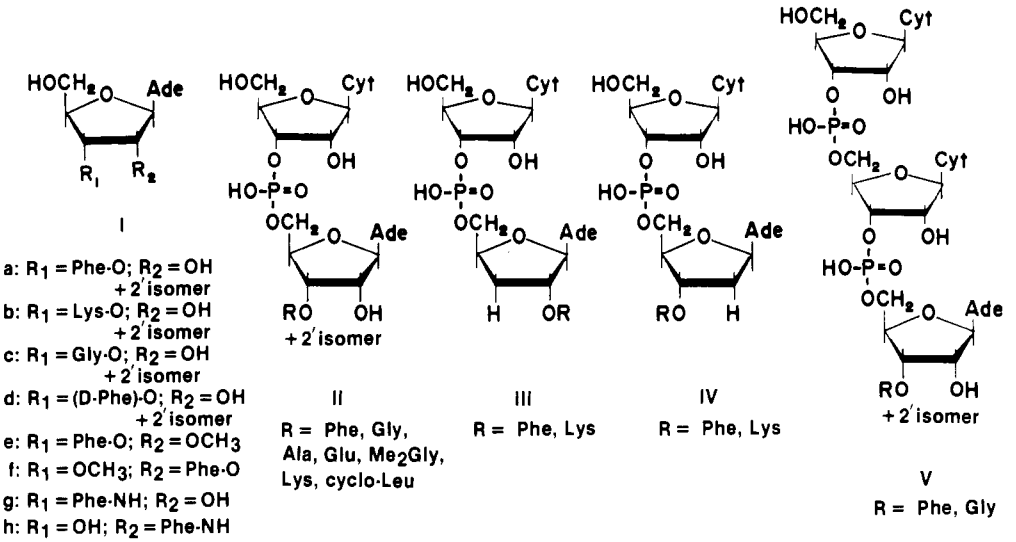
Results

As outlined earlier, the role of the 3′ terminus of AA-tRNA in EF-T_u·GTPase is conveniently studied by employing the AA-tRNA 3′-terminal fragments as promoters of the hydrolytic reaction (Campuzano & Modolell, 1980; Bhuta & Chládek, 1980; Parlato et al., 1981). We have prepared a series of such fragments [2′(3′)-*O*-(aminoacyl)nucleosides and -oligonucleotides, Chart I) by an unambiguous chemical synthesis (Bhuta et al., 1981; Kumar & Chládek, 1981 and references therein). These fragments were used in this study to delineate mechanism of the EF-T_u-dependent GTPase in the presence of ribosomes. We have used the $tRNA^{Phe}$·poly-(U)·70S ribosome·EF-T_u·GTP system, in which the P site is occupied by deacylated $tRNA^{Phe}$ (de Groot et al., 1971). In this system, 2′(3′)-*O*-(aminoacyl)nucleosides and -oligonucleotides can simulate the presence of the 3′ terminus of AA-tRNA and promote the EF-T_u-catalyzed ribosome-dependent GTPase, even in the absence of other functional parts of the tRNA molecule.

Figure 1a shows the EF-T_u·GTPase induced by several 2′(3′)-*O*-(aminoacyl) derivatives of adenosine. Our results with A-Phe confirm those of Campuzano & Modolell (1980), namely, that A-Phe strongly promotes the EF-T_u-dependent GTPase. However, in our system, the reaction may be detected at ~0.2 μM A-Phe as opposed to the 100-fold greater concentration reported by Campuzano & Modolell (1980). The reason for this difference is not known at this time, but it could be related to the presence of $tRNA^{Phe}$·poly(U) on the P site in our system or the result of using different ribosome preparations. It is also worthy to note that in the nonribosomal system (in the presence of aurodox) A-Phe-dependent stimulation of EF-T_u·GTPase was also detected at approximately 0.3 μM A-Phe (Bhuta & Chládek, 1980). Additionally, it may be seen (Figure 1a) that the other two "natural" 2′(3′)-*O*-(aminoacyl) derivatives of adenosine, A-Lys and A-Gly, also induce the EF-T_u-dependent GTPase in the same concentration range as A-Phe, although the extent of GTP hydrolysis strongly depends on the nature of the aminoacyl residue.

The remaining 2′(3′)-*O*-(aminoacyl)nucleosides tested as inducers of EF-T_u·GTPase were inactive. The inactivity of A(D-Phe) shows the requirement for the L configuration of the amino acid. This is in agreement with the observation of Pingoud & Urbanke (1980), who showed that D-Tyr–tRNA

Chart I



was unable to form a stable ternary complex with EF-T_u·GTP. Specificity for the aglycon portion of the inducer is clearly demonstrated by the apparent inactivity of U-Phe. It has been known that puromycin does not induce EF-T_u·GTPase (Campuzano & Modolell, 1980), and it can be seen (Figure 1a) that the same is true for both 2′- and 3′-phenylalanylamidodeoxyadenosines. Thus, it appears that the active site which binds the inducers and presumably located on EF-T_u (Bhuta & Chládek, 1980; see below) does not recognize the amido linkage of puromycin and related compounds. This is in full agreement with results of Sprinzl et al. (1977), who showed that the modified AA-tRNAs terminating with 2′- or 3′-(aminoacyl)amidodeoxyadenosine do not bind to EF-T_u·GTP. Also, very little, if any, activity was observed with nonisomerizable 2′- and 3′-*O*-phenylalanyl derivatives of 3′- or 2′-*O*-methyladenosine. Thus, the methyl substituent on the hydroxyl group neighboring the aminoacyl residue renders both compounds inactive, and no information could be obtained in regard to isomer specificity of EF-T_u·GTPase (see below).

Parts b and c of Figure 1 show the promotion of GTP hydrolysis by various 2′(3′)-*O*-(aminoacyl) derivatives of C-A, the "natural" 3′-terminal dinucleotide sequence of tRNA. It is clearly evident that the C-A derivatives of all natural amino acids tested (Gly, Ala, Phe, Lys, and Glu) induce GTP hydrolysis, albeit to different degrees. It is interesting to note that similar differences between various aminoacyl derivatives (Figure 1a–c) were observed in the nonribosomal kirromycin-dependent system (Bhuta & Chládek, 1980) and, also, in the formation of ternary AA-tRNA·EF-T_u·GTP complexes with AA-tRNAs differing in the aminoacyl residues (Pingoud & Urbanke, 1980; Wagner & Sprinzl, 1980; Knowlton & Yarus, 1980). Further, it was shown that C-A-Phe interacts more strongly with EF-T_u·GTP than do C-A-Asp or C-A-Pro (Ringer & Chládek, 1975) and that C-A-Phe protected EF-T_u against the sulfhydryl reagent more effectively than did C-A-Leu (Jonák et al., 1980). These results are well supported by the literature pertinent to the interaction of intact AA-tRNA with EF-T_u (see above) but are difficult to reconcile with the claim of Parlato et al. (1981) that the nature of the aminoacyl residue of the effector (3′-terminal fragments of AA-tRNA) does not significantly influence EF-T_u·GTPase in the presence of kirromycin.

A control experiment has further shown that the mixture of C-A and Phe, prepared by mild alkaline hydrolysis of C-A-Phe, does not promote the EF-T_u-dependent GTPase. Quite surprisingly, two C-A derivatives of unnatural amino acids lacking α hydrogen, such as α-aminoisobutyric acid and cycloleucine (1-amino-1-carboxycyclopentane), are both active in triggering GTP hydrolysis [Figure 1c; also see Bhuta & Chládek (1980) for a similar observation in the nonribosomal aurodox-containing system].

The nonisomerizable 3′- and 2′-*O*-(aminoacyl) derivatives C-2′-dA-Phe, C-2′-dA-Lys, C-3′-dA-Phe, and C-3′-dA-Lys, in which the neighboring hydroxyl group is replaced by hydrogen and, therefore, 2′,3′-transacylation (Griffin et al., 1966) is precluded, were also investigated in order to study possible isomer specificity of the GTPase reaction. Unfortunately, no significant activity was observed with any of these models (Figure 1c) even in the presence of the antibiotic thiostrepton, which stimulates the GTPase activity induced by 2′(3′)-*O*-(aminoacyl)oligonucleotides (see below). Thus, with all modified nonisomerizable derivatives used in this work (Chart I and Figure 1a,c), as in the aurodox-dependent system in the absence of ribosomes (Bhuta & Chládek, 1980), no conclusion may be drawn at this time concerning isomer specificity of the reaction due to the inherent imperfection of the model compounds that were used.

In view of relatively small differences in activities between A-Phe and C-A-Phe and analogous glycyl derivatives (Figure 1), the effects of the nucleotide sequence of the effectors on EF-T_u·GTPase were compared by using the initial rates of GTP hydrolysis (Figure 2 and Table I).

It is apparent that the joining of a Cp residue to A-Phe or A-Gly does not result in a large increase for K_a for C-A-Phe and C-A-Gly, although a moderate increase of V_{max} is evident. On the other hand, the trinucleotide derivatives of C-C-A-Phe and C-C-A-Gly have considerably higher affinities for EF-T_u than their dinucleotide counterparts, yet much lower than the intact Phe-$tRNA^{Phe}$. A considerable difference in the activities of C-C-A-Phe and C-C-A-Gly, though less pronounced than that between A-Phe and A-Gly, again reflects stronger binding of the side chain of the aromatic amino acid vs. glycine (see above) to EF-T_u. This comparison of activities of adenosine and C-A derivatives is also in agreement with our previous results from a nonribosomal system (Bhuta & Chládek, 1980). On the other hand, they differ from the recent findings of Parlato et al. (1981), who have detected significant differences in K_as between A-Phe and C-A-Phe in a kirromycin-containing system in the presence or absence of ribosomes. The reasons for the discrepancy between our results and those of Parlato et al. (1981) are not clear, but it is of some concern that no characterization of the compounds tested by Parlato et al.

Table I: Kinetic Parameters of Induced EF-T_u·GTPase and Association Constants of the AA-tRNA 3′-Terminal Fragments

compd	K_a[a] (M)	V_{max}[b]	K_a[a,c] (M)	V_{max}[b,c]	K^{GTP}_m (M)	V^{GTP}_{max}[b]
A-Phe	1.06×10^{-5}	0.25			2.82×10^{-6}	0.6
A-Gly	1.2×10^{-5}	0.1	1.6×10^{-6}	0.2		
C-A-Phe	1.06×10^{-5}	0.31			2.0×10^{-6}	1.6
C-A-Gly	1.2×10^{-5}	0.28	1.7×10^{-6}	0.48		
C-C-A-Phe	2×10^{-6}	0.48			1.1×10^{-6}	4.0
C-C-A-Gly	7×10^{-6}	0.42	1×10^{-6}	0.76		
Phe-$tRNA^{Phe}$	1.0×10^{-9}				0.2×10^{-6} [d]	

[a] K_a is the concentration of tested compounds where 50% of maximum GTP hydrolysis occurs. [b] V_{max} is picomoles of GTP hydrolyzed per picomole of EF-T_u at 30 °C. [c] In the presence of 10^{-4} M thiostrepton. [d] According to Chinali et al. (1977).

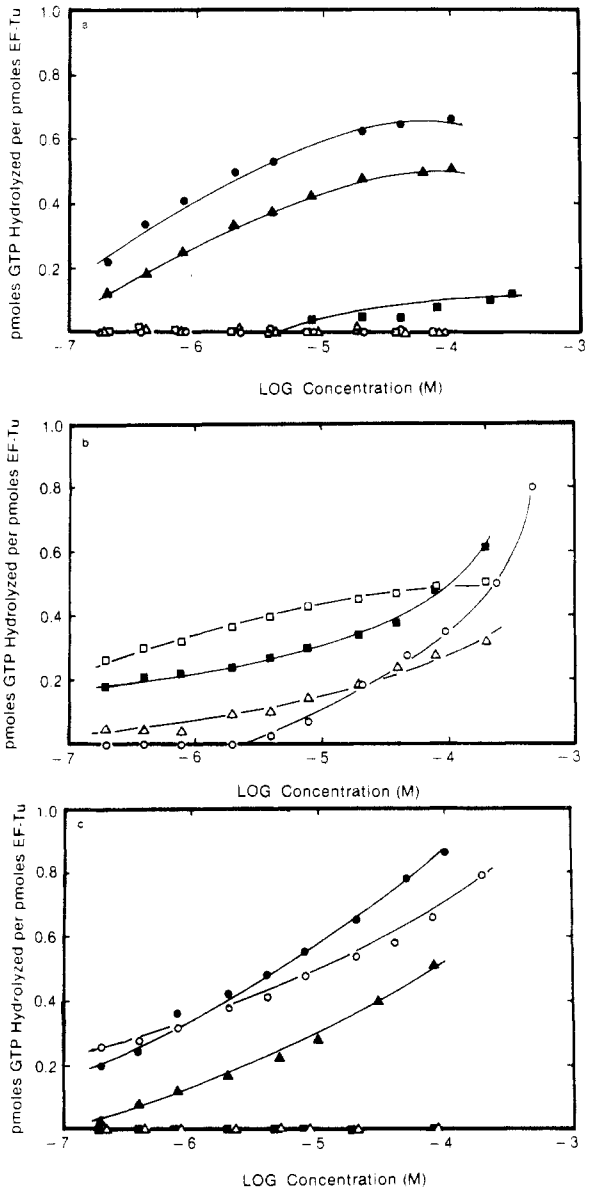


FIGURE 1: Effect of different (aminoacyl)nucleosides and -oligonucleotides on the EF-T_u·ribosome GTP hydrolysis. (a) A-Phe (●); A-Lys (▲); A-Gly (■); U-Phe or A(D-Phe) (○); 3′-dA-3′-NH-Phe or 2′-dA-2′-NH-Phe, (△); A(3′Me)Phe or A(2′Me)Phe (□); (b) C-A-Gly (■); C-A(Me_2Gly) (□); C-A-Ala (△); C-A(cyclo-Leu) (○); (c) C-A-Phe (●); C-A-Lys (○); C-A-Glu (▲); C-2′-dA-Lys (△); C-3′-dA-Lys (□); C-2′-dA-Phe or C-3′-dA-Phe, (■). Incubation was at 37 °C for 10 min. For details, see Materials and Methods.

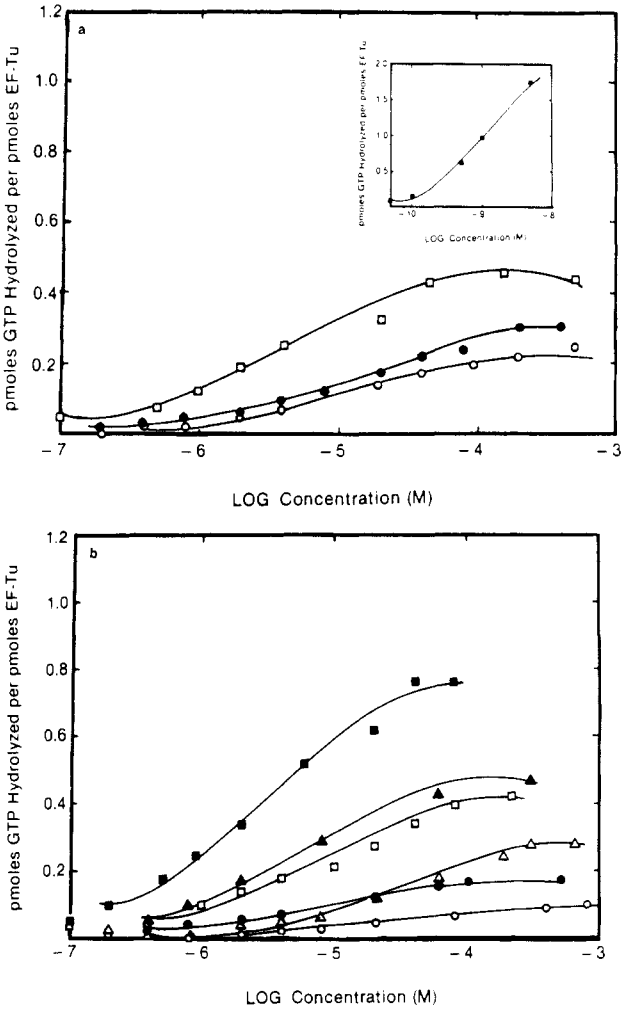


FIGURE 2: Effect of (aminoacyl)nucleosides and -oligonucleotides on the initial rate of EF-T_u·ribosome GTP hydrolysis. (a) A-Phe (○); C-A-Phe (●); C-C-A-Phe (□); (b) A-Gly (○); C-A-Gly (△); C-C-A-Gly (□); A-Gly plus 10^{-4} M thiostrepton (●); C-A-Gly plus 10^{-4} M thiostrepton (▲); C-C-A-Gly plus 10^{-4} M thiostrepton (■). Incubation was at 30 °C for 5 min during which the rate of GTP hydrolysis was linear within the time of incubation. Inset shows the effect of Phe-$tRNA^{Phe}$ concentration on GTP hydrolysis. For details see Materials and Methods.

(1981) was reported. Jonák et al. (1980) have reported that protection of the sulfhydryl groups of EF-T_u·GTP against the alkylation with TPCK increased in the following order: A-Phe < pA-Phe < C-A-Phe. It appears, however, that this indirect assay may not be ideally suited to the exact determination of affinities of ligands.

The effect of the GTP concentration on the hydrolytic reaction, in the presence of three effectors (A-Phe, C-A-Phe, and C-C-A-Phe), was also investigated (Figure 3), and the K_m for [γ-^{32}P]GTP was determined by using the double-reciprocal plot (plot not shown). It may be seen that the K_m for GTP is decreasing in the following order of inducers: A-Phe > C-A-Phe > C-C-A-Phe (Table I). Even though the change in K_m's is relatively small, it should be noted, however, that the K_m for the GTPase reaction associated with enzymatic binding of Phe-tRNA, as reported by Chinali et al. (1977), is only 5-fold lower than that in the presence of C-C-A-Phe.

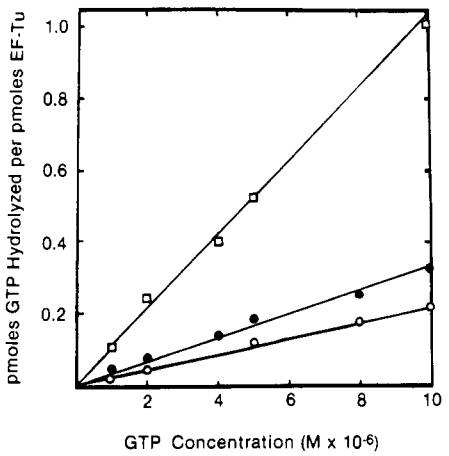


FIGURE 3: Effect of [γ-^{32}P]GTP concentration on the rate of EF-T_u·ribosome GTP hydrolysis induced. A-Phe (10^{-4} M) (○); C-A-Phe (10^{-4} M) (●); C-C-A-Phe (10^{-4} M) (□). Incubation was at 30 °C for 5 min. For details, see Materials and Methods.

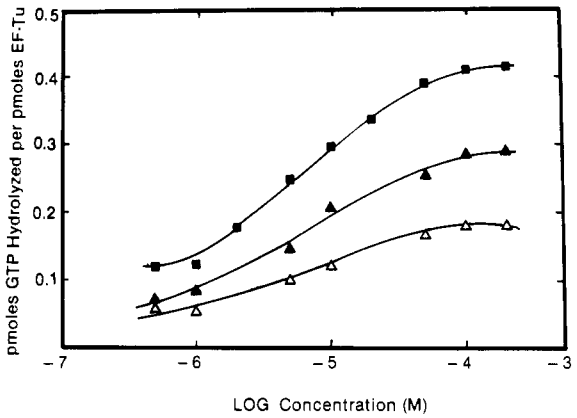


FIGURE 4: Effect of thiostrepton concentration on the EF-T_u·ribosome GTP hydrolysis induced by (aminoacyl)nucleosides and -oligonucleotides. A-Gly (10^{-4} M) (△); C-A-Gly (10^{-4} M) (▲); C-C-A-Gly (10^{-4} M) (■). Incubation was at 30 °C for 5 min. For details, see Materials and Methods.

Therefore, the aminoacylated C-C-A terminus of AA-tRNA plays the most critical role in promotion of the hydrolytical reaction.

The antibiotic thiostrepton was reported to stimulate the uncoupled EF-T_u·ribosome GTPase (in the absence of AA-tRNA and in the presence of ethanol; Ballesta & Vazquez, 1972) or A-Phe induced EF-T_u·ribosome GTPase (Campuzano & Modolell, 1980). We have investigated the effect of thiostrepton concentrations on the GTPase activity induced by A-Gly, C-A-Gly, and C-C-A-Gly. In all three cases, the saturation is reached at approximately 10^{-4} M concentrations of thiostrepton (Figure 4). The effect of thiostrepton on the K_a for these three inducers was measured in the initial rate conditions (Figure 2b and Table I). It can be clearly seen that in the presence of thiostrepton the K_a's of A-Gly, C-A-Gly, and C-C-A-Gly are increased by an order of magnitude and the corresponding V_{max}s are approximately doubled. It follows that thiostrepton, by binding to an appropriate site located on the 50S subunit (Highland et al., 1975a), significantly increases the affinity of the EF-T_u site for the 3′ terminus of AA-tRNA.

Discussion

The GTP hydrolysis is the result of a highly synchronized process involving EF-T_u, the ribosome, and AA-tRNA. In this report, we attempt to elucidate the role played by the 3′ terminus of AA-tRNA and possibly other domains of the tRNA molecule in the promotion of GTP hydrolysis.

There is a considerable body of evidence in support of the fact that the aminoacylated 3′ terminus of AA-tRNA interacts with EF-T_u in the ternary AA-tRNA·EF-T_u·GTP complex (Clark et al., 1980). The results presented here, in conjunction with our recent findings (Bhuta & Chládek, 1980; also confirmed by Parlato et al., 1981), provide convincing evidence that it is the binding of the AA-tRNA 3′ terminus to the EF-T_u site in the presence of ribosomes which triggers GTP hydrolysis. In fact, our results show that the GTPase activity of the EF-T_u·ribosome complex, in the presence of C-C-A-Phe (the 3′-terminal sequence of Phe-tRNA), approaches that of intact Phe-tRNA, even though the Phe-tRNA molecule is much more tightly bound than C-C-A-Phe.

This study allows us to present a reasonably detailed description of the EF-T_u site which binds the 3′ terminus of AA-tRNA. Our findings strongly support the existence of a region (locus) on EF-T_u which binds the side chain of the aminoacyl residue favoring the nonpolar amino acids, as suggested by Pingoud & Urbanke (1980). Further, it was interesting to observe that two C-A derivatives esterified with unnatural amino acids (without α hydrogen), C-A(cyclo-Leu) and C-A(Me_2Gly), are capable of a functional interaction with the EF-T_u site and trigger the GTP hydrolysis. It is apparent that the EF-T_u site probably possesses considerable flexibility in order to accommodate the α substituent of the aminoacyl residue [but see also Pingoud & Urbanke (1980)]. It is noteworthy that C-A(Me_2Gly) displays higher activity than C-A-Ala and C-A-Gly. This can probably be explained by the increased lypophilicity of the aminoacyl moiety of C-A-(Me_2Gly) relative to other derivatives. On the other hand, the EF-T_u site appears to be stereospecific since A(D-Phe) was unable to promote GTP hydrolysis. Therefore, the specificity of EF-T_u for L-aminoacyl-tRNA (Pingoud & Urbanke, 1980), together with similar specificities of the peptidyltransferase A and P sites (Bhuta et al., 1981; Quiggle et al., 1981) presents a formidable barrier for incorporation of D-amino acids into proteins.

The importance of the specific nucleotide residues at the C-C-A terminus of AA-tRNA for the interaction with the EF-T_u site is indicated by the inability of uridine to replace the terminal adenosine and by the large increase of inducing activity resulting from the addition of two cytidine 3′-phosphate residues to terminal adenosine.

At least two explanations may be invoked to account for these observations. First, it is possible that the EF-T_u binding region for the 3′ terminus of AA-tRNA possesses loci for binding not only the aminoacyl moiety (Pingoud & Urbanke, 1980) but also the base and phosphate residues of the C-C-A terminus, as similarly suggested for the peptidyltransferase active center (Chládek, 1980). Our observation that A-Phe cannot be replaced by U-Phe in triggering of GTPase, as well as that a replacement of the third (Cp) residue of the 3′ terminus in modified Met-tRNA by an Up residue results in a decrease of binding of the modified AA-tRNA to EF-T_u·GTP (Schulman et al., 1974), tends to support such a hypothesis. It was also observed that substitution at the penultimate Cp residue of Phe-tRNA^{Phe} did not prevent this AA-tRNA from interacting with EF-T_u·GTP (Sprinzl et al., 1978). Nevertheless, the role of the third residue of the C-C-A sequence of AA-tRNA appears to be more crucial than the penultimate one in binding to EF-T_u.

The second possible model of the interaction of the aminoacylated terminus of AA-tRNA with the EF-T_u site may involve the interaction of the amino acid residue and phosphate

backbone of the stacked C-C-A sequence of AA-tRNA with EF-T_u[2] (Kruse et al., 1980). It was found that EF-T_u protects the C-C-A end of AA-tRNA in the ternary complex against nuclease digestion (Jekowsky et al., 1977) and also that the complementary trinucleotide U-G-G binds to the C-C-A sequence of AA-tRNA complexed with EF-T_u·GTP (Kruse et al., 1980). Thus, it would appear that only the phosphate backbone of the AA-tRNA 3′ terminus interacts with EF-T_u while the base residues are left essentially free of contact. It should be pointed out, however, that the apparent specificity of the EF-T_u site for the adenosine residue, as observed here, is not easily explained by this hypothesis.

Therefore, our findings, related to the substrate specificity of an EF-T_u site responsible for inducing the GTPase reaction, do show excellent agreement with results of other authors from studies of ternary complexes containing modified AA-tRNAs (see above). Thus, it is very probable that the interaction of the 3′ terminus of AA-tRNA with the appropriate site on EF-T_u in the ternary AA-tRNA·EF-T_u·GTP complex is not significantly changed after binding of this complex to ribosomes triggers GTP hydrolysis.

It has been suggested that the ternary AA-tRNA·EF-T_u·GTP complex initially binds to a recognition (R) site of the mRNA programmed ribosome (Johnson et al., 1977; Lake, 1977). In the R configuration, AA-tRNA cannot react in the peptidyltransferase reaction as an acceptor, since GTP hydrolysis and subsequent EF-T_u·GDP release have not yet occurred [Johnson et al., 1977; see also Ringer et al. (1976)]. It is not clear whether the R site represents a separate topological entity on the ribosome (Johnson & Cantor, 1980). Nevertheless, it is apparent that at least a part of the R site, which binds the 3′ terminus of AA-tRNA (located on EF-T_u during its interaction with the ribosome), must be distinct from the A site of peptidyltransferase. We suggest that this site be called the R′ site, in analogy with the similar designation for the peptidyltransferase sites (A′ and P′; Harris & Symons, 1973). Our results provide insights as to the requirements of the R′ site, which may be compared with the substrate specificity of the A site of peptidyltransferase (Bhuta et al., 1981, 1982). The R′ site is similar to the A′ site in that it (i) probably binds the side chain of the aminoacyl residue of AA-tRNA in a similar way, (ii) also recognizes the complete C-C-A sequence of the AA-tRNA 3′ terminus, and (iii) is stereospecific. However, the specificity of the R′ site differs from that of the A′ site in that it has more stringent requirements for the recognition of the 3′-terminal ribose moiety of inducers, failing to functionally interact with compounds containing either deoxyribose, aminodeoxyribose, and *O*-methylribose moieties, and the R′ site is able to functionally interact with aminoacyl derivatives with a substituent in lieu of α hydrogen of the aminoacyl residue.

We have used the antibiotic thiostrepton to further elucidate the roles of different sites in the AA-tRNA·EF-T_u·GTP·70S ribosome complex, which are involved in hydrolysis of GTP. Thiostrepton[3] is known to inhibit the enzymatic and nonenzymatic AA-tRNA binding to the ribosomal A site and GTP hydrolysis associated with the enzymatic binding (Modolell et al., 1971). On the other hand, the antibiotic has no effect on the binding of 2′(3′)-*O*-(aminoacyl)oligoribonucleotides (analogues of the 3′ terminus of AA-tRNA) to the acceptor site of peptidyltransferase (Pestka, 1970). It was also noted, albeit yet largely unexplained, that thiostrepton stimulates the A-Phe-induced GTPase in the EF-T_u·GTP·ribosome complex (Campuzano & Modolell, 1980). We observed here that thiostrepton significantly stimulates the activity of A-Gly, C-A-Gly, and C-C-A-Gly, as inducers of EF-T_u·GTPase.

One interpretation of the similar stimulatory effect of thiostrepton that is observed in the presence of either A-Gly or C-C-A-Gly may be ascribed to the possibility that the bound antibiotic causes much tighter binding of the entire C-C-A sequence to the R′ site. The stimulatory effect of thiostrepton, on the EF-T_u·GTPase induced by the 3′-terminal fragments of AA-tRNA, via its binding to the 50S ribosomal subunit, can be thus explained by the existence of a liaison between the thiostrepton ribosomal binding site (T), the binding site of the aminoacylated C-C-A-sequence on EF-T_u (R′), and the active center for GTP hydrolysis on EF-T_u (G).[4]

The simplest explanation for the differentiated effect of thiostrepton on the GTPase reaction promoted by either AA-tRNA or its 3′-terminal fragments rests on the hypothesis that the R and A sites have an overlapping region, sensitive to thiostrepton, which binds some portion of AA-tRNA. The binding of this yet unknown AA-tRNA domain, distinct from the 3′ terminus or anticodon (Yamane et al., 1981), to a ribosomal locus, apparently, plays a role in promoting the GTPase reaction, in addition to the aforementioned crucial role of the 3′ terminus of AA-tRNA. In this connection, it should be noted that Ringer et al. (1976) suggested that the T-ψ-C-G sequence of loop IV of AA-tRNA may play some role in inducing the EF-T_u·GTPase via its binding to the ribosome. Although it is not possible, at the present time, to identify any other parts of the AA-tRNA molecule besides the 3′ terminus, as participating in EF-T_u·GTPase; our results point to the possible involvement of another tRNA domain in this reaction.

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[2] A defined structure of the AA-tRNA C-C-A end involving stacking interactions is probably necessary for EF-T_u AA-tRNA recognition, since the disruption of the stacking interactions (e.g., by an oxidation–reduction of the terminal adenosine) leads to the loss of binding of AA-tRNA to EF-T_u (Chinali et al., 1974; Maelicke et al., 1974). Similarly, the aforementioned decrease of the binding affinity to EF-T_u of Met-tRNA, which has the third Cp residue replaced by Up, may be explained by the disruption of the base-stacked structure which could change the orientation of the phosphate backbone of the C-C-A terminus and alter the ability of the modified AA-tRNA to interact with EF-T_u (Schulman et al., 1974).

[3] It is known that thiostrepton binds stoichiometrically to the 50S ribosomal subunit, and the protein L11 has been implicated in its binding (Highland et al., 1975a,b).

[4] The second possibility that the binding of thiostrepton to the ribosome exerts a direct effect on the GTPase site on EF-T_u appears to be less likely. Nevertheless, it was observed that thiostrepton stimulates the uncoupled GTPase reaction (under nonphysiological conditions, in the presence of ethanol) when the occupation of the R′ site with the AA-tRNA 3′ terminus is not apparently required (Ballesta & Vazquez, 1972; but see also Sedláček et al., 1974).

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