Enzymatic Binding of Aminoacyl Transfer Ribonucleic Acid to Ribosomes: The Study of Binding Sites of 2′ and 3′ Isomers of Aminoacyl Transfer Ribonucleic Acid†

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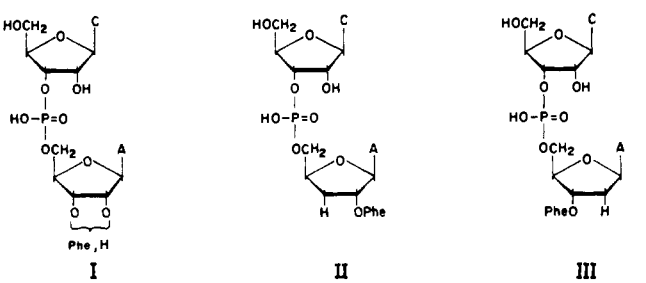
ABSTRACT: The mechanism of enzymatic binding of AA-tRNA to the acceptor site *Escherichia coli* ribosomes has been studied using the following aminoacyl oligonucleotides as models of the 3′ terminus of AA-tRNA: C-A-Phe, C-A-(2′-Phe)H, and C-A(2′H)Phe. T-ψ-C-Gp was used as a model of loop IV of tRNA. The EF-T dependent binding of Phe-tRNA to ribosomes (in the presence of either GTP or GMPPCP) and the GTPase activity associated with EF-T dependent binding of the Phe-tRNA were inhibited by C-A-Phe, C-A(2′Phe)H, and C-A(2′H)Phe. These aminoacyl oligonucleotides inhibit both the formation of ternary complex EF-T_u·GTP·AA-tRNA and the interaction of this complex with the ribosomal A site. The uncoupled EF-T_u dependent GTPase (in the absence of AA-tRNA) was also inhibited by C-A-Phe, C-A(2′Phe)H, and C-A(2′H)Phe, while nonenzymatic binding of Phe-tRNA to the ribosomal A site was inhibited by C-A-Phe and C-A(2′-Phe)H, but not by C-A(2′H)Phe. The tetranucleotide T-ψ-C-Gp inhibited both enzymatic binding of Phe-tRNA and EF-T dependent GTP hydrolysis. However, inhibition of the latter reaction occurred at a lower concentration of T-ψ-C-Gp suggesting a specific role of T-ψ-C-Gp loop of AA-tRNA in the GTPase reaction. The role of the 2′ and 3′ isomers of AA-tRNA during enzymatic binding to ribosomes is discussed and it is suggested that 2′ → 3′ transacylation in AA-tRNA is a step which follows GTP hydrolysis but precedes peptide bond formation.

In a previous publication for this laboratory, the interaction between *E. coli* elongation factor T_u·GTP complex and 2′(3′)-*O*-aminoacyl dinucleoside phosphates corresponding to the nucleotide sequence of the aminoacyl terminus of AA-tRNA[1] was reported (Ringer and Chládek, 1975). We concluded that this interaction is analogous to that between the aminoacyl end of AA-tRNA and EF-T_u·GTP and that EF-T_u is specific for 2′-AA-tRNA. We have also observed that the 3′ terminus of 2′-AA-tRNA can be recognized by the peptidyltransferase A site, or by a site which is closely related (Ringer and Chládek, 1974a; Ringer et al., 1975). In a related report, Chinali et al. (1974) showed that Phe-tRNA-C-C-3′dA (nonisomerizable 2′-AA-tRNA) was enzymatically bound to the ribosome and stimulated EF-T_u dependent GTP hydrolysis. Other experiments have shown that 3′-AA-tRNA is the preferred (Chinali et al., 1974) if not exclusive (Hussain and Ofengand, 1973; Chládek et al., 1973, 1974) acceptor of the peptide chain in the peptidyltransferase reaction. Consequently, we suggested that these results can be accounted for by a ribosome catalyzed 2′ → 3′ transacylation (Chládek et al., 1974; Ringer et al., 1975; Ringer and Chládek, 1975).

In this contribution we discuss investigations of the role of both the 2′ and 3′ isomers of AA-tRNA during enzymatic and nonenzymatic binding of AA-tRNA to the ribosomes using convenient models of the 3′ termini of 2′- and 3′-AA-tRNA (Scheme I). We also report on the role of the tRNA common sequence T-ψ-C-G in enzymatic binding of AA-tRNA since it has been shown that this sequence inhibits binding to part of the ribosomal acceptor site (Ofengand and Henes, 1969; Shimizu et al., 1970; Richter et al., 1973; Erdmann et al., 1974). Since the enzymatic binding of AA-tRNA to ribosomes is accompanied by EF-T_u dependent hydrolysis of GTP, we have studied the role of GTP hydrolysis in the "fixation" of the 3′ terminus of AA-tRNA on the acceptor site of ribosomes. We suggest in this paper that enzymatic binding of AA-tRNA to the ribosomal A site is followed by the transacylation of the

Scheme I



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[1] Abbreviations used are: AA-tRNA, aminoacyl transfer ribonucleic acid; tRNA-C-C-3′dA, tRNA with 3′-deoxyadenosine incorporated at the 3′ end; tRNA-C-C-2′dA, tRNA with 2′-deoxyadenosine incorporated at the 3′ end; A-Phe, 2′(3′)-*O*-L-phenylalanyladenosine; A(2′Phe)H, 3′-deoxy-2′-*O*-L-phenylalanyladenosine; A(2′H)Phe, 2′-deoxy-2′-*O*-L-phenylalanyladenosine (analogous abbreviations are used for dinucleotide derivatives); EF-T, elongation factor T (mixture of EF-T_u and EF-T_s); GMPPCP, guanylyl-5′-methylene diphosphonate; DEAE, diethylaminoethyl; Tris-HCl, tris(hydroxymethyl)aminomethane.

TABLE I: Inhibition of Nonenzymatic Binding of Phe-tRNA to Ribosomes by 2′- and 3′-*O*-L-Phenylalanyl Dinucleoside Phosphates.[a]

Inhibitor	% Inhibition at Concn (M) of Inhibitor		
	1×10^{-4}	7×10^{-5}	5×10^{-5}
C-A-Phe (I)	19	7	0
C-A(2′Phe)H (II)	49	26	8
C-A(2′H)Phe (III)	2	0	0

[a] The details of procedure are given in Materials and Methods. Zero percent inhibition equals 17.5 pmol of [^{14}C]Phe-tRNA bound to ribosomes.

aminoacyl residue between the 2′ and 3′ positions on the tRNA 3′ terminus and that the transacylation is allowed by GTP hydrolysis and release of EF-T_u. Part of these results has appeared in abstract form (Chládek and Ringer, 1975).

Materials and Methods

2′(3′)-*O*-Aminoacyl dinucleoside phosphates I–III were prepared by chemical synthesis as previously described (Chládek et al., 1974); T-ψ-C-Gp was prepared by enzymatic degradation of yeast tRNA (Ofengand and Henes, 1969). Ribosomes were prepared from late log phase *E. coli* MRE 600 (RNase 1$^-$) cells (General Biochemicals) and were washed either three or five times by centrifugation in 0.5 M NH_4Cl as described previously by Ravel and Shorey (1971). [^{14}C]-Phe-tRNA (0.4 nmol of [^{14}C]phenylalanine per mg of tRNA) was prepared as described previously (Chládek et al., 1974). EF-T (a mixture of EF-T_u and EF-T_s) was prepared from the 150 000*g* supernatant of *E. coli* MRE 600 (RNase 1$^-$) cells (General Biochemicals) by ammonium sulfate fractionation and DEAE-Sephadex chromatography as described by Leder (1971). Purified EF-T_u·GDP and EF-T_s were prepared as described (Miller and Weissbach, 1974) and were the kind gifts of Dr. D. L. Miller. [γ-^{32}P]GTP (specific activity 16.2 Ci/mmol) and [^{3}H]GTP (specific activity 5.28 Ci/mmol) were obtained from New England Nuclear, stripped *E. coli* tRNA from General Biochemicals, and guanylyl-5′-methylene diphosphonate (GMPPCP) from Boehringer Corp., Mannheim, Germany.

Inhibition of [^{14}C]*Phe-tRNA Binding to Ribosomes.* (a) Nonenzymatic Binding to the A Site. The binding of [^{14}C]-Phe-tRNA to ribosomes in the presence of deacylated tRNA was performed basically as described by Suzuka et al. (1965), except that ribosomes were first pre-incubated in the presence of poly(U) and tRNA to prevent the nonenzymatic binding of Phe-tRNA to the ribosomal P site (Watanabe, 1972). The inhibition of [^{14}C]Phe-tRNA binding to ribosomes by 2′- and 3′-*O*-L-phenylalanyl dinucleoside phosphates I–III was measured in the following reaction mixture (0.1 ml final reaction volume): 0.05 M Tris-HCl (pH 7.4), 0.08 M KCl, 0.08 M NH_4Cl, 0.02 M $MgCl_2$, 10 μg of poly(U), 3.5 A_{260} units of three times washed ribosomes, and 100 μg of tRNA. Mixtures were preincubated for 5 min at 37 °C and binding was initiated by the addition of 0.1 A_{260} unit of [^{14}C]Phe-tRNA (20 pmol), and the inhibitors I–III at the concentration given (see Table I). Reaction mixtures were incubated for 15 min at 37 °C, terminated by dilution with 2.0 ml of the same cold buffer, and filtered on HAWP-Millipore membranes (25 mm diameter, 45 Micropore size) which were washed by the same buffer (3 × 2.0 ml). The membranes were then dried and counted as previously described (Chládek et al., 1974). The inhibition of [^{14}C]Phe-tRNA binding was determined as the difference in the ribosome [^{14}C]Phe-tRNA complex retained on the Millipore membrane in the absence and in the presence of inhibitor compounds. The difference was expressed as a percentage of the radioactivity bound to ribosomes in the absence of inhibitors.

(b) Enzymatic Binding. The enzymatic binding of [^{14}C]-Phe-tRNA to ribosomes in the presence of deacylated tRNA was performed essentially as described by Chinali et al. (1974). The inhibition of [^{14}C]Phe-tRNA binding to ribosomes by 2′- and 3′-*O*-L-phenylalanyl dinucleoside phosphates I–III was measured in the following reaction mixture (0.2 ml final reaction volume): 0.05 M Tris-HCl (pH 7.4), 0.08 M KCl, 0.08 M NH_4Cl, 0.005 M $MgCl_2$, 0.001 M 2-mercaptoethanol, 20 μg of poly(U), 2.5 A_{260} units of ribosomes, and 100 μg of tRNA. This mixture was preincubated for 5 min at 37 °C, and then 0.2 A_{260} unit of [^{14}C]Phe-tRNA (40 pmol), 40 μg of EF-T, 2 μM GTP, and the inhibitors I–III at the concentration given were added. Reactions were initiated by the addition of GTP, incubated for 10 min at 4 °C, and terminated as described for the nonenzymatic binding (Figures 1a and 3).

The enzymatic binding of [^{14}C]Phe-tRNA in the presence of GMPPCP was performed as just described for binding in the presence of GTP with the exception that 0.01 M $MgCl_2$ was employed in place of 0.005 M $MgCl_2$ and 0.5 mM GMPPCP was used instead of GTP. The inhibition by T-ψ-C-Gp of GTP and GMPPCP dependent binding of [^{14}C]-Phe-tRNA to ribosomes was performed under the same conditions, with the exceptions that ribosomes, poly(U), tRNA, and T-ψ-C-Gp at the concentrations given were preincubated for 5 min at 37 °C and EF-T, GTP or GMPPCP, [^{14}C]Phe-tRNA, and 2-mercaptoethanol were added prior to the second incubation for 10 min at 4 °C (Figures 1b and 3).

Inhibition of the Formation of EF-T_u·GTP·AA-tRNA Ternary Complex by C-A-Phe (I). The formation of the ternary complex EF-T_u·GTP·AA-tRNA was measured by the method of Gordon (1967) and Sedláček et al. (1974). This procedure gives a clear separation of EF-T_u·GTP or EF-T_u·GTP·AA-tRNA from unbound GTP. The column of Sephadex G-25 (1 × 25 cm) was equilibrated with 0.05 M Tris-HCl (pH 7.4), 0.08 M KCl, 0.08 M NH_4Cl, 0.01 M $MgCl_2$. Flow rate was 3 ml/h. The reaction mixtures (total volume 0.2 ml) were: (1) 0.05 M Tris-HCl (pH 7.4), 0.08 M NH_4Cl, 0.08 M KCl, 0.01 M $MgCl_2$, 0.001 M 2-mercaptoethanol, 2 μM [γ-^{32}P]-GTP (specific activity 1000 Ci/mol), and 50 μg of EF-T. After incubation for 10 min at 4 °C, the mixture was applied to the column and eluted with equilibration buffer; (2) the reaction mixture was the same as in 1, except that 0.1 A_{260} unit of [^{14}C]Phe-tRNA was added prior to incubation; (3) the same as in 2, except that 5×10^{-6} M C-A-Phe (I) was added at the end of the first 10 min of incubation and the mixture was allowed to incubate for another 10 min at 4 °C (Figure 2).

Inhibition of EF-T_u Dependent GTP Hydrolysis. (a) "Coupled" Conditions. Inhibition of GTP hydrolysis associated with enzymatic binding of Phe-tRNA by 2′- and 3′-*O*-L-phenylalanyl dinucleoside phosphates (I–III) and T-ψ-C-Gp was assayed by a modification of the method described by Chinali et al. (1974). Reaction mixtures containing 0.05 M Tris-HCl (pH 7.4), 0.03 M KCl, 0.03 M NH_4Cl, 0.01 M $MgCl_2$, 0.001 M 2-mercaptoethanol, 20 μg of poly(U), 3–4 A_{260} units of five-times washed ribosomes, and 200 μg of tRNA were preincubated for 5 min at 37 °C and 120 pmol of EF-T_u·GDP, 6 pmol of EF-T_s, 0.1 A_{260} unit of [^{14}C]Phe-tRNA (20 pmol), the inhibitors at the given concentrations, and 2 μM [γ-^{32}P]-

TABLE II: Inhibition of EF-T_u Dependent [γ-^{32}P]GTP Hydrolysis ("Coupled Conditions") by 2′- and 3′-*O*-L-Phenylalanyl Dinucleoside Phosphates.[a]

Inhibitor	% Inhibition at Concn (M) of Inhibitor		
	2.5×10^{-5}	5×10^{-6}	1×10^{-6}
C-A-Phe (I)	100	60	29
C-A(2′Phe)H (II)	100	69	33
C-A(2′H)Phe (III)	91	47	19

[a] The experiments were performed as described in Materials and Methods with purified EF-T_u and EF-T_s which were completely free of endogenous GTPase activity and with five-times washed ribosomes. One hundred percent of [γ-^{32}P]GTP hydrolyzed in the absence of inhibitor is equal to 15.8 pmol (value corrected for 11.5 pmol of [γ-^{32}P]GTP hydrolysis in the absence of [^{14}C]Phe-tRNA).

TABLE III: Inhibition of "Uncoupled" EF-T_u Dependent [γ-^{32}P]-GTP Hydrolysis by 2′- and 3′-*O*-L-Phenylalanyl Dinucleoside Phosphates.[a]

Inhibitor	% Inhibition at Concn (M) of Inhibitor		
	2.5×10^{-5}	5×10^{-6}	1×10^{-6}
C-A-Phe (I)	97.5	64.6	25.5
C-A(2′Phe)H (II)	98.7	70.8	30.4
C-A(2′H)Phe (III)	97.8	45.2	15.10

[a] The experiments were performed as described in Materials and Methods using purified EF-T_u and EF-T_s and five-times washed ribosomes. One hundred percent of [γ-^{32}P]GTP hydrolyzed in the absence of inhibitors is equal to 240 pmol; 10 pmol of [γ-^{32}P]GTP was hydrolyzed in absence of EF-T_u and EF-T_s; 8.51 pmol of [γ-^{32}P]GTP was hydrolyzed in absence of methanol.

GTP (specific activity 300 Ci/mol) were added to a final volume of 0.2 ml. Reactions were initiated by the addition of GTP, incubated for 10 min at 4 °C, and analyzed by the extraction procedure as described by Martin and Doty (1949). Samples were counted using a counting solution for aqueous samples (3a70B, Research Products International). Blank values of GTPase were measured in the absence of [^{14}C]Phe-tRNA. The results given in Table II and Figure 4 are calculated as a percentage of the amount of Phe-tRNA dependent [γ-^{32}P]GTP hydrolyzed in the presence of inhibitor relative to the amount of [γ-^{32}P]GTP hydrolyzed in the absence of inhibitor.

(b) "Uncoupled" Conditions. The inhibition of uncoupled GTP hydrolysis by 2′- and 3′-*O*-L-phenylalanyl dinucleoside phosphates I–III and T-ψ-C-Gp was performed basically by the method of Ballesta and Vazquez (1972). The reaction conditions were the same as described for "coupled" GTP hydrolysis except that the addition of [^{14}C]Phe-tRNA was omitted and methanol was added to a final concentration of 20% (v/v). The reactions were initiated by the addition of [γ-^{32}P]GTP, the mixtures were incubated for 10 min at 4 °C, and the extent of the formation of P_i was measured as described in the assay for "coupled" GTP hydrolysis. The results were calculated in the same manner as for "coupled" conditions (Table III).

The Assay of Interaction of C-A-Phe (I) and [^{14}C]Phe-tRNA with EF-T_u·GTP in the Presence of Methanol. The interaction of C-A-Phe (I) and [^{14}C]Phe-tRNA with EF-T_u·GTP was measured by a procedure similar to that of Ringer and Chládek (1975). The reaction mixture (total volume 0.2 ml) contained: 0.05 M Tris-HCl (pH 7.4), 0.03 M KCl, 0.03 M NH_4Cl, 0.01 M $MgCl_2$, 0.001 M 2-mercaptoethanol, 120 pmol of EF-T_u·GDP, 6 pmol of EF-T_s, 2 μM [^{3}H]GTP (specific activity 300 Ci/mol), 20% (v/v) methanol (where indicated), and 0.1 A_{260} unit of [^{14}C]Phe-tRNA (20 pmol) or 2.5×10^{-5} C-A-Phe (I) where indicated. Reactions were initiated by the addition of GTP and after 10 min of incubation at 4 °C the reaction mixtures were analyzed by the Millipore membrane filtration technique (Ringer and Chládek, 1975) (Table IV).

TABLE IV: The Interaction of C-A-Phe (I) and [^{14}C]Phe-tRNA with EF-T_u·GTP in the Presence of Methanol.[a]

Addition	Disappearance of EF-T_u·[^{3}H]GTP Complex from Millipore Membrane (%)	
	$-CH_3OH$	$+CH_3OH$
None	0	0
[^{14}C]Phe-tRNA (20 pmol)	24	31
C-A-Phe (I) (2.5×10^{-5} M)	35.5	3

[a] The EF-T_u·[^{3}H]GTP complex was formed from EF-T_u, EF-T_s, and [^{3}H]GTP as described in Materials and Methods. One hundred percent of EF-T_u·[^{3}H]GTP complex retained by the Millipore membrane in the absence of C-A-Phe or [^{14}C]Phe-tRNA is equal to 10 pmol (in the absence of methanol) or 16 pmol (in the presence of methanol).

Results

Inhibition of AA-tRNA Binding to Ribosomal A Site by Compounds I–III. (a) Nonenzymatic Binding. At high Mg^{2+} concentration and in the presence of deacylated tRNA, nonenzymatic binding of AA-tRNA to ribosomes is known to be directed to the acceptor site (Seeds et al., 1967; Ofengand and Henes, 1969; Igarashi and Kaji, 1970; de Groot et al., 1971; Watanabe, 1972). We find that C-A(2′Phe)H (II) and, less efficiently, C-A-Phe (I) inhibit the poly(U) dependent binding of Phe-tRNA to the acceptor site (Table I). The 3′ ester C-A(2′H)Phe (III), which is known to be an excellent acceptor in the peptidyltransferase reaction, is a very poor inhibitor. We have also confirmed that nonenzymatically bound Phe-tRNA can participate in dipeptide formation with Ac-Phe-tRNA as donor molecule (data not shown). The observed inhibition is not due to peptide bond formation between compounds I or II and Phe-tRNA since no dipeptide was found in an incubation mixture which was subjected to alkaline hydrolysis and standard electrophoretical analysis (Chládek et al., 1974) (data not shown). Thus, inhibition of nonenzymatic binding of AA-tRNA by compounds I and II must be caused solely by interaction of I and II with the ribosomal A site.

(b) Enzymatic Binding. The ternary complex EF-T_u·GTP·AA-tRNA binds to the acceptor site of ribosomes at low (5 mM) Mg^{2+} concentration (for a recent review, see Lucas-Lenard and Lipmann, 1971) and in the presence of deacylated tRNA (de Groot et al., 1971). This binding is very strongly inhibited by compounds I–III (Figure 1a).[2] The effect of all

[2] It has been recently reported by Hecht et al. (1974), that enzymatic binding of Phe-tRNA to ribosomes is inhibited by Phe-tRNA-C-C-3′dA and Phe-tRNA-C-C-2′dA.

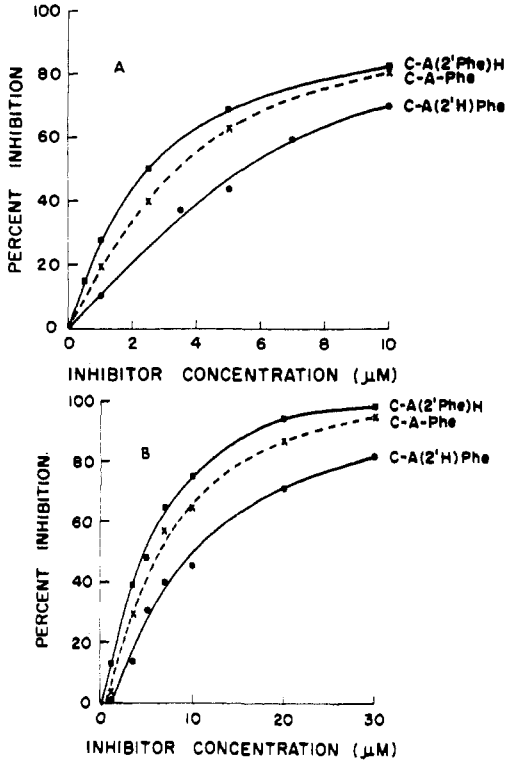

FIGURE 1: Inhibition of enzymatic binding of Phe-tRNA to ribosomes by 2′- and 3′-*O*-L-phenylalanyldinucleoside phosphates. The details are given under Materials and Methods. (a) GTP dependent binding, zero percent of inhibition equals 8 pmol of [^{14}C]Phe-tRNA bound to ribosomes; (b) GMPPCP dependent binding, zero percent inhibition equals 11 pmol of [^{14}C]Phe-tRNA bound to ribosomes. (■) C-A(2′Phe)H (II); (X) C-A-Phe (I); (●) C-A(2′H)Phe (III).

TABLE V: Summary of Data from Figures 1a and 1b.[a]

Compound	A	B	C	D	E
C-A-Phe (I)	$>10^{-4}$	3.5×10^{-6}	6×10^{-6}	3.5×10^{-6}	3.2×10^{-6}
C-A(2′ Phe)H (II)	1×10^{-4}	2.5×10^{-6}	5×10^{-6}	2.1×10^{-6}	2.5×10^{-6}
C-A(2′H)Phe (III)	$\gg10^{-4}$	5.2×10^{-6}	1×10^{-5}	5.2×10^{-6}	5.1×10^{-6}

[a] Tables I, II, and III show the concentrations (M) of aminoacyl oligonucleotides I–III at which 50% inhibition occurs in systems (A) nonenzymatic binding of AA-tRNA; (B) enzymatic binding of AA-tRNA (with GTP); (C) enzymatic binding of AA-tRNA (with GMPPCP); (D) EF-T_u dependent GTPase coupled conditions; (E) EF-T_u dependent GTPase, uncoupled conditions.

three compounds is similar; 50% inhibition of enzymatic binding occurs at 3–5 μM while >30 times this amount was required to inhibit nonenzymatic binding. Especially noteworthy is the effect of compound III, which was practically inactive in the inhibition of nonenzymatic binding. The results presented in Figure 2 show that at least part of the inhibition of enzymatic binding of AA-tRNA by compounds I or II may be caused by their interaction with the EF-T_u·GTP·AA-tRNA ternary or EF-T_u·GTP binary complex since a large excess of C-A-Phe (I) causes the displacement of Phe-tRNA from the preformed ternary complex EF-T_u·GTP·Phe-tRNA (>58% decrease of ternary complex is attained at 5×10^{-6} M C-A-Phe). However, since compound III does not interact with

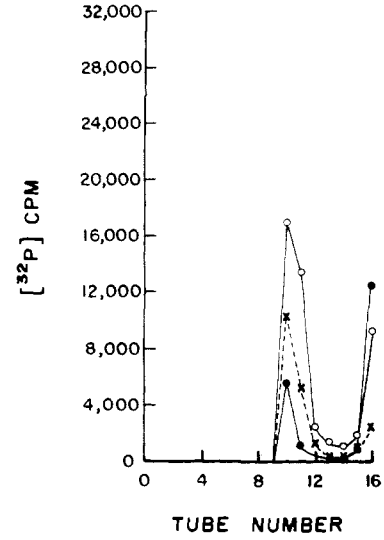

FIGURE 2: Inhibition of EF-T·[γ-^{32}P]GTP·[^{14}C]Phe-tRNA complex formation by C-A-Phe (I). The analysis of the reaction mixture was performed by gel filtration. Reaction mixtures containing EF-T·[γ-^{32}P]GTP and EF-T·[γ-^{32}P]-GTP·[^{14}C]Phe-tRNA as described in Materials and Methods were analyzed by Sephadex G-25 filtration after incubation in the presence of 5×10^{-6} M C-A-Phe: (X) binary complex EF-T·[γ-^{32}P]GTP; (○) ternary complex EF-T·[γ-^{32}P]GTP·[^{14}C]Phe-tRNA; (●) ternary complex plus 5×10^{-6} M C-A-Phe.

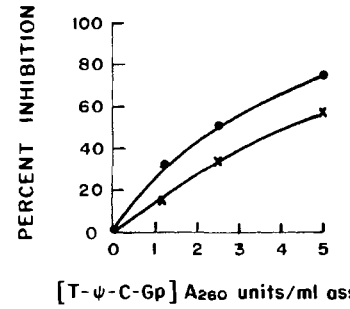

FIGURE 3: Inhibition of enzymatic binding of Phe-tRNA to ribosomes by T-ψ-C-Gp. The assay conditions are given under Materials and Methods; (X) GTP stimulated binding, zero percent inhibition equals 4.5 pmol of [^{14}C]Phe-tRNA bound to ribosomes; (●) GMPPCP stimulated binding, zero percent inhibition equals 6.2 pmol of [^{14}C]Phe-tRNA bound to ribosomes.

EF-T_u·GTP under conditions where compounds I and II do interact (Ringer and Chládek, 1975), its only target of inhibition should be the peptidyltransferase A site. Again, it was shown that inhibition of enzymatic binding of AA-tRNA was not due to peptide bond formation between compounds I–III and Phe-tRNA (data not shown).

Enzymatic binding of AA-tRNA to ribosomes also occurs if the analogue of GTP, GMPPCP, which cannot be enzymatically hydrolyzed, is used; but the bound AA-tRNA cannot participate in peptide bond formation (Lucas-Lenard et al., 1969; Skoultchi et al., 1969). As is clear from Figure 1b, the GMPPCP mediated enzymatic binding of AA-tRNA to the ribosomes is also inhibited by the aminoacyl oligonucleotides I–III, though less efficiently. Since these compounds interact with the peptidyltransferase A site (Ringer and Chládek, 1974a; Ringer et al., 1975) as analogues of the AA-tRNA aminoacyl end, the most plausible explanation for the inhibition is that AA-tRNA, enzymatically bound to ribosomes in the presence of GMPPCP, has its aminoacyl end at the peptidyltransferase A site.

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pounds I–III. Approximately one molecule of GTP is hydrolyzed per molecule of AA-tRNA bound enzymatically to ribosomes (for review, see Lucas-Lenard and Lipmann, 1971). Thus, this hydrolysis should also be inhibited when compounds I–III inhibit enzymatic binding. Table II shows that coupled GTPase activity is inhibited by compounds I–III at concentrations similar to those for the inhibition of enzymatic binding.

EF-T_u dependent GTP hydrolysis can also be observed uncoupled from the requirement for AA-tRNA and mRNA by the addition of 20% methanol (Ballesta and Vazquez, 1972; but see also Sander et al., 1975). As shown in Table III, uncoupled GTP hydrolysis was inhibited by compounds I–III with an efficiency equal to that under coupled conditions. The effect of compounds I–III on uncoupled GTP hydrolysis must be due to their binding to the acceptor site of peptidyltransferase,[3] rather than to any effect on EF-T_u since 20% methanol abolishes the ability of EF-T_u·GTP to interact with C-A-Phe (I). As shown in Table IV, AA-tRNA interacts with EF-T_u·GTP normally in the presence of 20% methanol in agreement with the finding of Ballesta and Vazquez (1972) that methanol only slightly affects the enzymatic binding of AA-tRNA to ribosomes, but the effect of C-A-Phe (I) is lost in the presence of alcohol.

It is worth noting that, in all of the systems employed, the 2′ ester II displayed the highest activity, the 3′ ester III the lowest, and the isomerizable 2′(3′) ester I had an intermediate activity. While the higher activity of the 2′ ester as compared with the 3′ ester may be ascribed to their differences in reactivity toward EF-T_u and perhaps to the differences in affinities to peptidyltransferase A site (as is seen from differences in inhibition of nonenzymatic AA-tRNA binding), the "natural" 2′(3′) ester C-A-Phe (I) should behave as either isomer because of fast equilibration between both positions (Griffin et al., 1966). The lower activity of I compared with II may be caused by the actual lower concentration of the 2′ ester of I in the equilibrium mixture (about 33%) or by a decrease in total concentration due to the greater lability of the aminoacyl bond in I as compared with II or III (Sprinzl and Cramer, 1973). Selective participation of the 2′ ester of I in the interaction with EF-T_u presumably does not influence significantly the equilibrium because of the large excess of C-A-Phe (I) compared with that of EF-T_u.

Inhibition of AA-tRNA Binding to Ribosomal A Site and EF-T_u Dependent GTP Hydrolysis by T-ψ-C-Gp. Figure 3 shows the inhibition of enzymatic binding of Phe-tRNA by the common tetranucleotide T-ψ-C-Gp at 5 mM Mg^{2+}.[4] This nucleotide sequence appears to provide another binding site for the anchoring of AA-tRNA to the ribosomal A site (Ofengand and Henes, 1969; Shimizu et al., 1970; Richter et al., 1973). It is interesting to note that both types of enzymatic binding, i.e., in the presence of either GTP or GMPPCP, were inhibited by the tetranucleotide T-ψ-C-Gp. We have also confirmed the T-ψ-C-Gp inhibition of GTP hydrolysis associated with EF-T_u dependent binding of AA-tRNA to ribosomes reported by Richter et al. (1973) who, however, used 20 mM Mg^{2+} and did not examine the concentration dependence of the reaction. The results are shown in Figure 4. It is interesting to note that at a concentration of 0.5 A_{260} unit/ml,

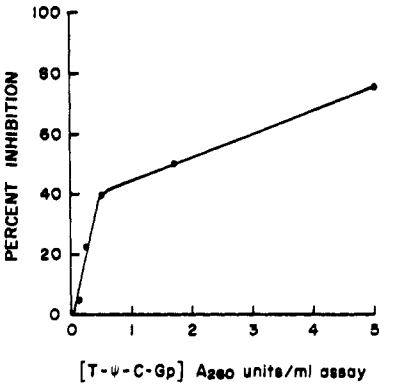

FIGURE 4: Inhibition of EF-T_u dependent [γ-^{32}P]GTP hydrolysis by T-ψ-C-Gp. Reaction conditions are given under Materials and Methods; experiments were performed with purified EF-T_u and EF-T_s and five-times washed ribosomes. One hundred percent of [γ-^{32}P]GTP hydrolyzed in the absence of T-ψ-C-Gp is equal to 15.8 pmol (value is corrected for 11.5 pmol of [γ-^{32}P]GTP hydrolyzed in the absence of [^{14}C]Phe-tRNA).

T-ψ-C-Gp inhibits 40% of the coupled GTPase activity, but only 7% of the enzymatic binding. While the reason for this differential effect of T-ψ-C-Gp is not clear, it may suggest a specific role of the T-ψ-C-Gp loop of tRNA in inducing the GTPase reaction. This effect may be more sensitive to low concentrations of T-ψ-C-Gp than is the overall binding reaction. The uncoupled GTPase (in the presence of 20% methanol) is inhibited only very weakly by T-ψ-C-Gp (data not shown). This is in agreement with the fact that AA-tRNA is not required for GTPase under uncoupled conditions (Ballesta and Vazquez, 1972) and that T-ψ-C-Gp does not interact with EF-T_u (Jerez et al., 1969). We have also examined the possibility that compounds of the types I–III alone or in the presence of T-ψ-C-Gp would be able to simulate the presence of AA-tRNA on the ribosomal A site and stimulate EF-T_u dependent GTP hydrolysis. However, in no case was a significant amount of GTP hydrolyzed (data not shown).

Discussion

It is now generally accepted that at least three regions of AA-tRNA are involved in its interaction with the ribosomal acceptor site: the anticodon loop, the "common" tetranucleotide sequence T-ψ-C-G, and the aminoacylated 3′-terminal sequence C-C-A. We report here that aminoacyl dinucleoside phosphates I–III, which are analogues of AA-tRNA termini, inhibit the enzymatic binding of AA-tRNA to the ribosomal A site and the GTP hydrolysis associated with this binding. Compound I, and presumably also II, interferes both with the association of the 3′ end of AA-tRNA with EF-T_u·GTP (Figure 2) and with the site of attachment (peptidyltransferase A site) of the 3′ end of AA-tRNA on the ribosome (Figure 1 and Table I). The 3′ ester III, which does not interact with EF-T_u·GTP (Ringer and Chládek, 1975), must inhibit only the latter step. It was surprising to find that C-A(2′H)Phe (III), which is an excellent acceptor in the peptidyltransferase reaction (Ringer and Chládek, 1974b), is a very weak inhibitor of the nonenzymatic binding of AA-tRNA to the ribosomal A site, a reaction performed at high Mg^{2+} concentration.[5] Under somewhat different ionic conditions, practically no differences were observed in the inhibition of C-A-C-C-A-Phe binding to the peptidyltransferase A site with the same pair

[3] It is known that alcohol does not unfavorably influence the interaction of compounds such as I and III with peptidyltransferase A site since these compounds are acceptors under fragment reaction conditions (Ringer and Chládek, 1974c).

[4] Inhibition of binding of AA-tRNA to ribosomes in the presence of EF-T_u at 20 mM Mg^{2+} was observed by Richter et al. (1973).

[5] It should also be kept in mind that inhibition of nonenzymatic binding of AA-tRNA by compounds I and II occurs at much higher inhibitor concentration than inhibition of enzymatic binding of AA-tRNA.

of inhibitors II and III (Ringer et al., 1975). It is not known whether this phenomenon may reflect a conformational change in the ribosome due to the higher Mg^{2+} concentration (5 mM for enzymatic and 20 mM for nonenzymatic binding), as was recently suggested by Ginzburg and Zamir (1975), or some unknown ribosomal control mechanism.

We have recently suggested (Chládek and Ringer, 1975; Ringer and Chládek, 1975) that 2′-AA-tRNA participates in formation of the ternary complex EF-T_u·GTP·2′-AA-tRNA, and that it is bound as such to the ribosomal A site. The correct isomer for the peptidyltransferase reaction, 3′-AA-tRNA, is then supplied by a ribosome-catalyzed transacylation. Since the transformation of EF-T_u·GTP to EF-T_u·GDP affects the conformation of EF-T_u in such a way that EF-T_u·GDP is no longer recognized by AA-tRNA and is released from ribosomes (Printz and Miller, 1973; Arai et al., 1974), GTP hydrolysis and the subsequent EF-T_u·GDP release may allow the 2′ → 3′ transacylation of AA-tRNA. An independent line of evidence (Ringer et al., 1975, and manuscript in preparation) suggests that the ribosomal binding sites for the aminoacyl termini of 2′- and 3′-AA-tRNA are not exactly identical, differing in binding loci (Rychlík et al., 1970) for the side chains of amino acids. Since binding of the C-A sequence of either 2′- or 3′-AA-tRNA should occur to the same ribosomal locus, both isomers II and III would be expected to inhibit binding of the 3′ terminus of either 2′- or 3′-AA-tRNA to the peptidyltransferase A site.

The aminoacyl oligonucleotides I–III also strongly inhibit the enzymatic binding of AA-tRNA in the presence of GMPPCP, the nonhydrolyzable analogue of GTP. This suggests that the 3′ terminus of AA-tRNA, enzymatically bound without GTP hydrolysis, is already in the peptidyltransferase A site. It was previously thought (Lucas-Lenard et al., 1969) that this AA-tRNA does not participate in the peptidyltransferase reaction because it is not properly fixed to the ribosomal A-site in the absence of GTP hydrolysis. Also in the absence of GTP hydrolysis, EF-T_u is not released from ribosomes (Lucas-Lenard et al., 1969). Thus, it seems possible that in the absence of GTP hydrolysis the bound AA-tRNA is conserved as the 2′ isomer and, in the continuous presence of EF-T_u on the ribosomes, transacylation to the 3′ isomer does not occur. This interpretation is supported by the observation that Phe-tRNA-C-C-3′dA enzymatically bound to the A site shows a similar behavior to Phe-tRNA bound in the presence of GMPPCP. In both cases, the stability of ribosomal complexes is decreased and bound AA-tRNA is readily exchangeable with free Phe-tRNA (Shorey et al., 1971; Chinali et al., 1974).[6]

The inhibitory effect of aminoacyl oligonucleotides I–III on EF-T_u dependent GTPase activity associated with AA-tRNA binding seems to be explainable on the same basis as the inhibition of enzymatic binding of AA-tRNA, that is, an interference with the formation of EF-T_u·GTP·AA-tRNA (only in the case of I and II) and as an interference with the peptidyltransferase A site. We also observed that compounds I–III inhibited uncoupled EF-T_u dependent GTPase (Ballesta and Vazquez, 1972), even though the presence of AA-tRNA is not required for this reaction. Since C-A-Phe (I) does not appear to interact with EF-T_u·GTP in the presence of methanol (Table IV), we must assume that the inhibitory effect of compounds I–III on the uncoupled GTPase is caused exclusively by their interaction with the ribosome, presumably at the peptidyltransferase A site. These observations seem to indicate the existence of a functional link between the peptidyltransferase A site and the GTPase center, both of which are localized to the 50S subunit. Indeed, it is possible to visualize such a functional link on the basis of studies of ribosomal structure (Hsiung et al., 1974; Hsiung and Cantor, 1974; Schrier and Möller, 1975). Alternatively, the compounds I–III, when bound to the peptidyltransferase A site, may block the functional attachment of EF-T_u to ribosomes.

A common tetranucleotide sequence T-ψ-C-G has been reported by several groups of investigators (Ofengand and Henes, 1969; Shimizu et al., 1970; Richter et al., 1973; Erdmann et al., 1974) to be involved in the interaction of AA-tRNA with the ribosomal A site, possibly through Watson–Crick pairing with the corresponding sequence of 5S RNA (Forget and Weissman, 1967; Jordan, 1971; Erdmann et al., 1973). We have confirmed and extended the observations of Richter et al. (1973) that T-ψ-C-Gp inhibits enzymatic binding of AA-tRNA to ribosomes and the GTPase associated with this binding. The reason for the differential inhibition of the coupled GTPase and the enzymatic binding of AA-tRNA by T-ψ-C-Gp is not clear at the present time although it is reminiscent, in the reverse way, of the failure of tetracycline to inhibit the coupled GTPase reaction while blocking the binding of AA-tRNA to the ribosomal A site (Gordon, 1969; Lucas-Lenard et al., 1969; Skoultchi et al., 1969). In an analogous way, the preferential inhibition of GTPase by T-ψ-C-Gp suggests that this loop of AA-tRNA may be involved in inducing GTPase activity. It is of interest to point out that the specific 5S RNA protein complexes (in *E. coli* L18 and L25) do bind to T-ψ-C-Gp and were reported to possess a GTPase activity (Erdmann et al., 1973; Gaunt-Klopper and Erdmann, 1975). It is also noteworthy that T-ψ-C-Gp inhibits both GMPPCP- and GTP-dependent enzymatic binding of AA-tRNA with similar efficiency. Since T-ψ-C-Gp is known to interact exclusively with 50S ribosomes (Richter et al., 1973) and not with EF-T_u (Jerez et al., 1969), the latter result suggests that the T-ψ-C-G loop of AA-tRNA is properly bound to its locus at the ribosomal A site even in the absence of GTP hydrolysis.

In contrast to T-ψ-C-Gp and tetracycline, compounds I–III inhibited both overall binding of AA-tRNA and GTPase equally well as a function of inhibitor concentration even though the GTPase activity presumably reflects interaction of only part of the tRNA with the 50S subunit, and the site blocked is presumably the peptidyltransferase A site. This rather surprising result suggests the hypothesis that compounds I–III, acting as analogues of AA-tRNA, trigger in some way a "closure" of the ribosomal A site to prevent further binding of tRNA or AA-tRNA. This effect would then explain the much higher affinity of AA-tRNA than unacylated tRNA for the A site and might also explain the potent inhibition of tRNA-dependent ppGpp synthesis by compounds II and III (Chinali, Chládek, and Ofengand, unpublished results), as well as the effects on the uncoupled GTPase reaction cited above.

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[6] The confirmation of this hypothesis would require the isolation and identification of 2′-AA-tRNA bound enzymatically with GMPPCP to ribosomes. Because of rapid 2′ ⇄ 3′ migration of the aminoacyl residue in solution (Griffin et al., 1966), this is virtually impossible, unless the bound AA-tRNA could be chemically derivitized at the free hydroxyl group (supposedly 3′).

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