INHIBITION OF THE PEPTIDYL TRANSFERASE A-SITE FUNCTION BY 2'-*O*-AMINOACYLOLIGONUCLEOTIDES

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SUMMARY

New "non-isomerizable" analogs of the 3'-terminus of AA-tRNA, C-A(2'Phe)H, C-A(2'Phe)Me, C-A(2'H)Phe and C-A(2'Me)Phe, were tested as acceptor substrates of ribosomal peptidyl transferase and inhibitors of the peptidyl transferase A-site function. The 3'-*O*-AA-derivatives were active acceptors of Ac-Phe in the peptidyl transferase reaction, while the 2'-*O*-AA-derivatives were completely inactive. Both 2'- and 3'-*O*-AA-derivatives were potent inhibitors of peptidyl transferase catalyzed Ac-Phe transfer to puromycin. The results indicate that although peptidyl transferase exclusively utilizes 3'-*O*-esters of tRNA as acceptor substrates, its A-site can also recognize the 3'-terminus of 2'-*O*-AA-tRNA.

INTRODUCTION

Since the discovery of the direct involvement of 2'(3')-*O*-aminoacyl-tRNA in protein biosynthesis, the exact location of the aminoacyl residue on the terminal adenosine unit has been the subject of considerable interest (1). Because of the extremely rapid 2' $\rightleftarrows$ 3' migration of the aminoacyl group of the *cis*-diol of the terminal adenosine unit (2,3), it is almost impossible to determine the exact position of the aminoacyl residue in native aminoacyl-tRNA. Moreover, it now appears that it is not a single isomer of aminoacyl-tRNA that is employed throughout the various stages of protein biosynthesis.

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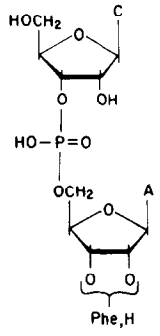
Abbreviations: C-APhe, cytidylyl(3'-5')-2'(3')-*O*-*L*-phenylalanyladenosine (I); C-A(2'Phe)H, cytidylyl(3'-5')-3'-deoxy-2'-*O*-*L*-phenylalanyladenosine (IIa); C-A(2'Phe)Me, cytidylyl(3'-5')-3'-*O*-methyl-2'-*O*-*L*-phenylalanyladenosine (IIb); C-A(2'H)Phe, cytidylyl(3'-5')-2'-deoxy-3'-*O*-*L*-phenylalanyladenosine (IIIa); C-A(2'Me)Phe, cytidylyl(3'-5')-2'-*O*-methyl-3'-*O*-*L*-phenylalanyladenosine (IIIb); $tRNA^{ox-red}$, tRNA which has been oxidized with periodate, reduced with borohydride (6); A^{ox-red}_{Phe}, *L*-phenylalanyl derivative of oxidized-reduced adenosine, either "2'" or "3'"-isomer (for correct nomenclature of the latter, see ref. 5).

On the basis of the structure and activity of puromycin as a 3'-analog of aminoacyl-tRNA and its 2'-analog (the latter was found to be inactive in inhibiting polypeptide synthesis), it has been suggested that 3'-*O*-aminoacyl-



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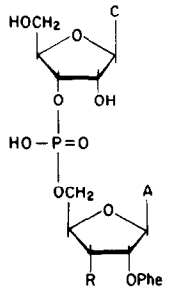
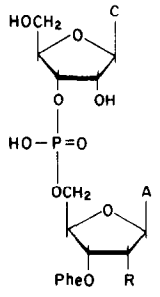


II a; R=H
b; R=OCH3



III a; R=H
b; R=OCH3

tRNA is the active isomer in protein biosynthesis (4). More specifically, we have recently reported that the 3'-isomer of $A_{Phe}^{ox\text{-}red}$, a "non-isomerizable" analog of 3'-*O*-aminoacyl-tRNA, is an active acceptor in the peptidyl transferase reaction and that its 2'-isomer is virtually inactive (5). Studies by Ofengand and Chen (6) and Sprinzl and Cramer (7) have shown that 2'-*O*-aminoacyl-tRNA is formed by the AA-tRNA synthetase reaction, thus indicating that 2' → 3' transacylation must occur after enzymic aminoacylation and before peptide bond formation.

As part of our study of the involvement of the 3'-terminus of tRNA in the various stages of protein biosynthesis, we have been interested to learn whether or not a 2' → 3' transacylation is a required step in the overall ribosomal mechanism. In order to study this question, we have synthesized several aminoacyloligonucleotides in which the aminoacyl residue is "fixed" at the 2'- or 3'-hydroxyl. These compounds, C-A(2'Phe)H (IIa), C-A(2'Phe)Me (IIb), C-A(2'H)Phe (IIIa) and C-A(2'Me)Phe (IIIb), were tested as acceptor substrates for peptidyl transferase and as inhibitors of peptidyl transferase

catalyzed Ac-Phe transfer to puromycin. Our results show that although only 3'-*O*-aminoacyl derivatives (IIIa, IIIb) can act as acceptors at the peptidyl transferase center, both 2'- and 3'-*O*-aminoacyl derivatives are potent inhibitors of peptidyl transferase, indicating that the 3'-terminus of 2'-*O*-aminoacyl-tRNA can be recognized by the peptidyl transferase A-site.

MATERIALS AND METHODS

Three times NH_4Cl-washed ribosomes from *E. coli* MRE-600 cells (General Biochemicals) and Ac-(^{3}H)Phe-tRNA, specific activity 0.44 nmoles phenylalanine/mg tRNA, were prepared as previously described (5).

The chemical synthesis of the 2'- and 3'-*O*-aminoacyldinucleoside phosphates used in this study will be described elsewhere.

Peptidyl transferase activity was measured as before (5). Details of the assay conditions are given in the figure legends.

RESULTS AND DISCUSSION

As can be seen from Fig. 1, only the 3'-*O*-aminoacyl derivatives C-A(2'H)Phe (IIIa) and C-A(2'Me)Phe (IIIb) are acceptors of Ac-Phe in the peptidyl transferase reaction using the Ac-Phe-tRNA·poly(U)·70 S ribosome system. No activity has been observed with the corresponding 2'-*O*-aminoacyloligonucleotides C-A(2'Phe)H (IIa) and C-A(2'Phe)Me (IIb) over a wide range of concentration. We have observed the same trend with the analogous adenosine derivatives (results to be published elsewhere). The decreased acceptor activity of C-A(2'Me)Phe (IIIb) relative to C-APhe (I) (8) (at 1×10^{-5} M less than 50% of that of C-APhe) may be attributed to steric hindrance by the 2'-*O*-methyl group. These results are consistent with the report of Hussain and Ofengand (9) who showed that 2'-$A_{Phe}^{ox\text{-}red}$, prepared from $tRNA^{ox\text{-}red}$ by enzymic aminoacylation and digestion, was inactive in the peptidyl transferase reaction, whereas a mixture of the 2'- and 3'-isomers of $A_{Phe}^{ox\text{-}red}$ prepared by chemical synthesis was active. Our recent report proved that 3'-$A_{Phe}^{ox\text{-}red}$ is indeed the active acceptor (5). We therefore

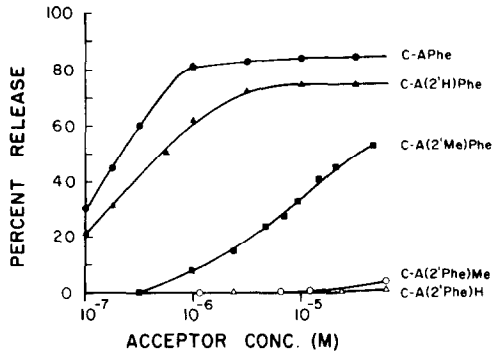


Figure 1. 2'- and 3'-*D*-*L*-Phenylalanyldinucleoside phosphate dependent release of the Ac-(^{3}H)Phe residue from Ac-(^{3}H)Phe-tRNA in the peptidyl transferase reaction. Each reaction mixture contained in 0.10 ml: 50 mM Tris-HCl (pH 7.4), 100 mM NH_4Cl, 10 mM $MgCl_2$, 3.7 A_{260} units of ribosomes, 10 µg poly(U), and 0.12 A_{260} units of Ac-(^{3}H)Phe-tRNA (2000 cpm). The reaction was initiated by addition of the acceptor compounds at the concentrations indicated. Following incubation at 37° for 30 min, the reaction was terminated by the addition of 2.0 ml of 2.5% CCl_3COOH at 4°. After 15 min at 4°, the entire reaction mixture was filtered through a HAWP-Millipore membrane which was then washed with three 2.0 ml portions of cold 2.5% CCl_3COOH. After the membranes were dried, the radioactivity was determined in a 4.5 g PPO/100 mg dimethyl-POPOP/1 liter toluene scintillation mixture. The amount of Ac-(^{3}H)Phe residue transferred from Ac-(^{3}H)Phe-tRNA to the acceptor was determined as the difference between radioactivity retained on the filter after incubation without acceptor and that retained after incubation with an acceptor. It was expressed as the percentage of the radioactivity of Ac-(^{3}H)Phe-tRNA added to the experimental mixture.
Symbols: ●, C-APhe (I); △, C-A(2'Phe)H (IIa); ○, C-A(2'Phe)Me (IIb); ▲, C-A(2'H)Phe (IIIa); ■, C-A(2'Me)Phe (IIIb).

conclude that peptidyl transferase possesses specificity for the 3'-isomer of AA-tRNA, although this fact *per se* does not necessarily mean that the 3'-isomer of AA-tRNA is required for all steps of protein biosynthesis at the ribosomal stage.[1]

Binding of the acceptor terminus of AA-tRNA (e.g., CCA-AA) to the peptidyl transferase A-site should precede the peptide bond forming step (11),

[1] Recently, Fraser and Rich (10) speculated that translocation of newly-formed peptidyl-tRNA (which is the 3'-isomer) from the A-site to P-site may be associated with 3' → 2' transacylation.

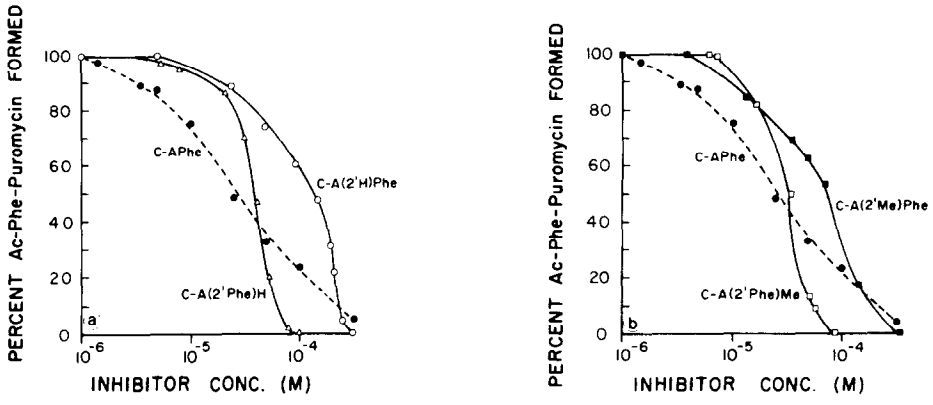


Figure 2. Ac-(^{3}H)Phe-puromycin formation as a function of 2'- and 3'-*O*-*L*-phenylalanyldinucleoside phosphate concentrations. The reaction mixtures were identical to those described in Figure 1 except that 1.0 X 10^{-4} M puromycin was also present. Compounds IIa, IIb, IIIa and IIIb were present at the concentrations indicated on the abscissa and ribosomes were added last to initiate the reactions. After 30 min at 37° the reactions were terminated (with 0.1 ml of 0.1 M $Be(NO_3)_2$ and 0.3 M NaOAc (pH 5.5) saturated with $MgSO_4$) and Ac-(^{3}H)Phe-puromycin was extracted with 1.5 ml of ethyl acetate, as described by Monro et al. (14). The radioactivity of 1.0 ml aliquots of the ethyl acetate extract was measured in 10 ml of 4.5g PPO/100 mg dimethyl-POPOP/0.25 liters 2-methoxyethanol/1 liter toluene scintillation mixture. Control experiments, in which puromycin was omitted, indicated that little or no radioactivity was extracted to ethyl acetate. Percent of Ac-(^{3}H)Phe-puromycin formed represents the amount of Ac-(^{3}H)Phe-puromycin formed in the presence of aminoacyldinucleoside phosphate relative to the amount formed in its absence. 100% Ac-(^{3}H)Phe-puromycin formed was equal to 1525 cpm.
(a)---, C-APhe (I); △, C-A(2'Phe)H (IIa); O, C-A(2'H)Phe (IIIa).
(b)---, C-APhe (I); □, C-A(2'Phe)Me (IIb); ■, C-A(2'Me)Phe (IIIb).

and if 2'-*O*-aminoacyl-tRNA can be bound we should be able to demonstrate the competition between puromycin, the standard acceptor substrate, and the 2'-*O*-aminoacyl derivatives, C-A(2'Phe)H (IIa) and C-A(2'Phe)Me (IIb).[2] This was indeed the case. Both 2'-*O*-aminoacyl derivatives, IIa and IIb, were excellent inhibitors of peptidyl transferase mediated Ac-Phe transfer to puromycin in the system containing Ac-Phe-tRNA as donor, poly(U), and 70 S ribosomes

[2] We had previously shown that 2'(3')-*O*-aminoacylnucleosides and 2'(3')-*O*-aminoacyloligonucleotides compete with puromycin in peptidyl transferase activity (12).

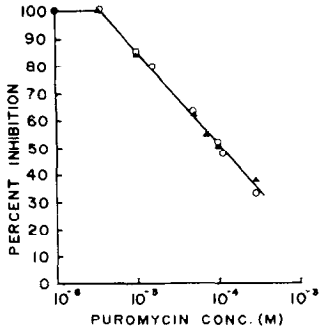


Figure 3. Reversal of C-A(2'Phe)H (IIa) and C-A(2'Phe)Me (IIb) inhibition of Ac-(^{3}H)Phe-puromycin formation in the peptidyl transferase reaction. Reactions were executed as in Figure 2 except that the concentrations of IIa and IIb were held constant (4.0 X 10^{-5} M and 3.4 X 10^{-5} M respectively) and the concentration of puromycin varied as indicated in the figure. Percent inhibition represents the difference in counts between reaction mixtures with both puromycin and the aminoacyldinucleoside phosphate present and that containing just puromycin.
O, C-A(2'Phe)H (IIa); ▲, C-A(2'Phe)Me (IIb).

(Fig. 2). Their inhibitory activity, 50% inhibition of Ac-Phe-puromycin formation at 4 X 10^{-5} M for IIa and 3.3 X 10^{-5} M for IIb, was similar to that of C-APhe (I) (50% inhibition at 2.5 X 10^{-5} M) which can exist as either the 2'- or 3'-isomer. Significantly, the inhibitory activity of the 3'-isomers (Fig. 2), C-A(2'H)Phe (IIIa) (50% inhibition at 1.4 X 10^{-4} M) and C-A(2'Me)Phe (IIIb) (50% inhibition at 8 X 10^{-5} M), was lower although these compounds are good acceptor substrates. In addition, C-A was found to be completely inactive as an inhibitor at a concentration up to 10^{-3} M (data not shown).[3] As can be seen from Fig. 3, increasing the concentration of puromycin overcomes the inhibitory effect of IIa and IIb, thus indicating that puromycin and the 2'-*O*-aminoacyloligonucleotides, IIa and IIb, probably act at the same ribosomal site. The above results indicate that 2'-*O*-aminoacyl derivatives may be recognized by the peptidyl transferase A-site. We therefore would like

[3] Recently, C-A was found to be a weak inhibitor of chloramphenicol binding to ribosomes (13).

to suggest that the same may hold true for the 3'-terminus of 2'-*O*-aminoacyl-tRNA.

Although the data presented here do not establish the presence of ribosomal 2' → 3' transacylation, they do show that 2'-*O*-aminoacyl-tRNA can bind to ribosomes, presumably to the A-site. It remains to be proven, however, that the isomer integrity of 2'-*O*-aminoacyl-tRNA is retained during EF-T_u dependent binding to ribosomes.

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