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STIMULATION OF *ESCHERICHIA COLI* ELONGATION FACTOR Tu-DEPENDENT GTP HYDROLYSIS BY AMINOACYL OLIGONUCLEOTIDES IN THE PRESENCE OF AURODOX

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1. Introduction

During protein synthesis in a procaryotic cell, elongation factor Tu plays an important role by promoting the binding of AA-tRNA to ribosomes. This binding is mediated through the formation of a ternary complex of AA-tRNA · EF-Tu · GTP, followed by the hydrolysis of GTP during the binding of AA-tRNA to the ribosomal A site. The binary complex EF-Tu · GDP is then released from the ribosome and is recycled through the formation of transient EF-Tu · Ts complex. After the displacement of EF-Ts by GTP, the EF-Tu · GTP can interact with another molecule of AA-tRNA [3].

The antibiotic kirromycin (or its derivatives, e.g., Aurodox) inhibits protein biosynthesis by virtue of its interaction with EF-Tu and effects practically all the EF-Tu-dependent reactions, presumably through a conformational alteration of EF-Tu [4,5]. This antibiotic enables EF-Tu alone to catalyze the hydrolysis of GTP in the absence of other components such as AA-tRNA and ribosomes which are otherwise required. The kirromycin-induced GTPase conserves many features of the physiological GTPase and is strongly stimulated by AA-tRNA and ribosomes. Therefore, the catalytic center of ribosome · EF-Tu · GTPase activity was assigned to EF-Tu [4].

Simple models of the 3′-terminus of AA-tRNA such as 2′(3′)-*O*-aminoacylnucleosides and aminoacyldinucleotides can specifically interact with EF-Tu and ribosomes in a way resembling that of AA-tRNA [6–8].

Here, we have studied the effect of simple analogs of the 3′-terminus of AA-tRNA on the EF-Tu GTPase in the presence of Aurodox. We find that these simple analogs can substitute for AA-tRNA in the stimulation of EF-Tu GTPase in the presence of aurodox. Thus, this novel finding underscores the role of 3′-terminus of AA-tRNA in promoting the EF-Tu-dependent GTP hydrolysis.

Abbreviations: EF-Tu, elongation factor Tu; A-Phe, 2′(3′)-*O*-L-phenylalanyladenosine; analogous abbreviations for other nucleoside and dinucleotide derivatives; C-2′-dA-2′-NH-Glu, cytidylyl (3′–5′) 2′-deoxy-2′-L-glutamylamidoadenosine; C-3′-dA-Lys, cytidylyl (3′–5′) 3′-deoxy-2′-*O*-(L-lysyl)adenosine, analogous abbreviations for isomeric aminoacyl derivatives; Me_2 Gly, α-aminoisobutyric acid. AA-tRNA, aminoacyl tRNA; Ac-AA-tRNA, *N*-acetyl aminoacyl tRNA, TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; PEP, phosphoenolpyruvate; DTT, dithiothrietol, TLC, thin-layer chromatography

Aurodox is the designation of antibiotic X-5108 and is *N*-methylated kirromycin [1]. It is identical to kirromycin in its effect on EF-Tu catalyzed GTP hydrolysis [2]

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2. Materials and methods

PEP, pyruvate kinase (EC 2.7.1.40), DTT, puromycin and Tris(hydroxymethyl)aminomethane were obtained from Sigma, St Louis, MO. Potassium chloride, ammonium chloride, magnesium chloride were of analytical grade. [γ-^{32}P]GTP (25 Ci/mmol) was purchased from ICN Chemicals and Radiochemicals Division, Irvine, CA. EF-Tu · GDP was kindly supplied by Dr David Miller, Roche Institute of Molecular Biology, Nutley, NJ. Aurodox was a gift from Dr H. Maehr, Hoffmann-LaRoche, Nutley, NJ. A stock solution was prepared in glass distilled water and stored in the dark at –80°C. A fresh solution was made every 2 months. Aurodox concentration was determined by weight (M_r 808) [1]. The aminoacyl nucleosides and aminoacyl nucleotides were prepared by the described

method ([9] and references therein). The samples were dissolved in distilled water just prior to assays and the pH was carefully adjusted to 6.5 with NaOH. 2′-dA-2′-NH-Phe and 3′-dA-3′-NH-Phe were obtained by catalytic hydrogenation of the corresponding *N*-benzyloxycarbonyl derivatives [10] and characterized by TLC, electrophoresis and positive reaction with ninhydrin.

GTPase reaction. The final 100 μl reaction mixture contained: 60 mM Tris–HCl (pH 7.8 at 37°C), 30 mM KCl, 30 mM NH_4Cl, 10 mM $MgCl_2$, 2 mM DTT, 3 mM PEP, 3 IU pyruvate kinase, 0.01 mM Aurodox, 60 pmol EF-Tu · GDP and 0.005 mM [γ-^{32}P]GTP adjusted to 1 Ci/mmol. EF-Tu · GDP was first converted to EF-Tu · GTP by incubation at 37°C for 10 min in the presence of buffer, PEP and pyruvate kinase [11] followed by the addition of [γ-^{32}P]GTP. 75 μl of this mixture were transferred to reaction tubes containing Aurodox, test compounds, and distilled water. The final volume of the reaction mixture was 100 μl. The concentrations of test compounds are indicated in the figures. After incubation at 37°C for 10 min, the reaction was stopped by adding 0.5 ml 0.01 M silicotungstic acid in 0.01 M H_2SO_4, followed by 0.5 ml 0.04 ammonium molybdate in 4 M H_2SO_4 and 100 nmol KH_2PO_4. The radioactive inorganic phosphate was extracted in 2.0 ml isobutanol:benzene as in [12]. Organic solvent (1 ml) was placed in a glass scintillation vial and the radioactivity was determined using 10 ml 3a70B cocktail (Research Products Int. Elk Grove Village, IL) in a liquid scintillation spectrometer.

3. Results and discussion

AA-tRNA (but not tRNA or Ac-AA-tRNA) as well as ribosomes stimulated the EF-Tu GTPase induced by kirromycin [4]. Simple analogs of the 3′-terminus of AA-tRNA such as 2′(3′)-*O*-aminoacyl nucleosides, nucleotides or dinucleotides can specifically interact with EF-Tu · GTP [6,8] and can induce EF-Tu GTPase in the presence of ribosomes [7]. Therefore, it was of considerable interest to study the effect of these analogs on the EF-Tu GTPase reaction induced by aurodox (a derivative of kirromycin) in the absence of ribosomes. We have shown here (fig.1) that in the most simplified system, four 2′(3′)-*O*-aminoacyl derivatives of cytidylyl(3′→5′) adenosine esterified with different aminoacids (Phe, Lys, Glu and Me_2Gly) indeed stimulate the GTP hydrolysis in the presence of EF-Tu and Aurodox. The efficiency of models as stimulators of EF-Tu GTPase in the presence of aurodox decreases in the order C-A-Phe > C-A-Lys > C-A-Glu > C-A-(Me_2Gly)*.

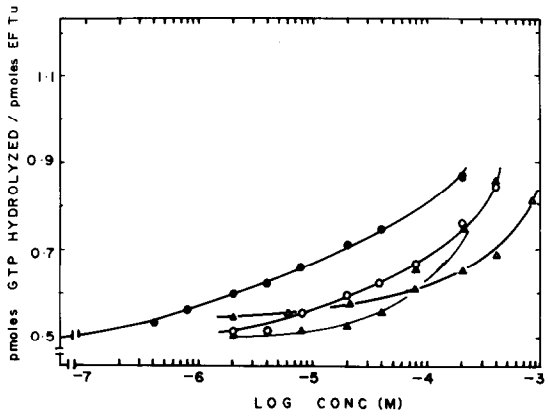

Fig.1. Effect of 4 different aminoacyldinucleotides on the EF-Tu · Aurodox induced GTP hydrolysis. The concentrations of C-A-Phe (●), C-A-Lys, (○), C-A-Glu (▲), and C-A-(Me_2Gly) (△) are indicated on the abscissa. In the presence of Aurodox alone, 0.5 pmol GTP/pmol EF-Tu were hydrolyzed and in the presence of Phe-tRNA^{Phe} (170 pmol) 3.26 pmol GTP/pmol EF-Tu were hydrolyzed.

Various AA-tRNAs have widely different affinities for EF-Tu in the formation of the AA-tRNA · EF-Tu · GTP complex [14]. These differences must be attributed to the nature of the amino acid present on the tRNA. Similarly, it was shown that C-A-Phe interacts more strongly with EF-Tu · GTP than C-A-Asp or C-A-Pro [6] and that C-A-Phe offered stronger protection to EF-Tu · GTP against the sulfhydryl reagent TPCK than CA-Leu [7]. Thus, these findings are in excellent agreement with the above order of stimulatory activities of C-A derivatives in EF-Tu · Aurodox-dependent GTPase. It is interesting to observe that the derivatives of α-aminoisobutyric acid [C-A-Me_2Gly)], which has a second α-substituent in lieu of hydrogen, can functionally interact with EF-Tu to stimulate the GTPase reaction. This finding is appar-

* An increase in the concentration of monovalent cations stimulates the kirromycin-dependent EF-Tu · GTP hydrolysis [13]. Since the amount of monovalent cations contributed by the test compounds is negligible in comparison to the concentration of monovalent ions in the assay, the stimulation of GTP hydrolysis observed cannot be due to the presence of monovalent ions in the test compounds

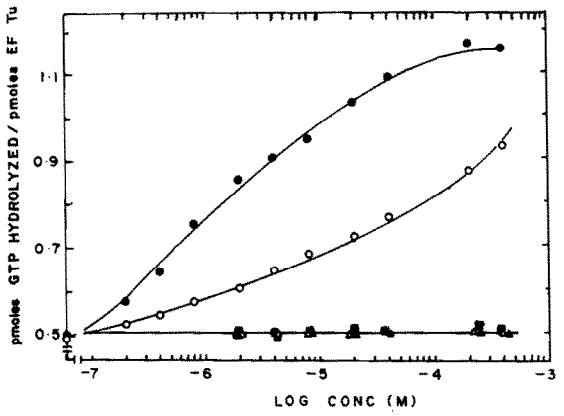

Fig.2. Effect of different aminoacylnucleosides on the EF-Tu · Aurodox-induced GTP hydrolysis: puromycin (●); A-Phe (○); U-Phe (▲); 2′-dA-2′-NH-Phe (△); 3′-dA-3′NH-Phe (■).

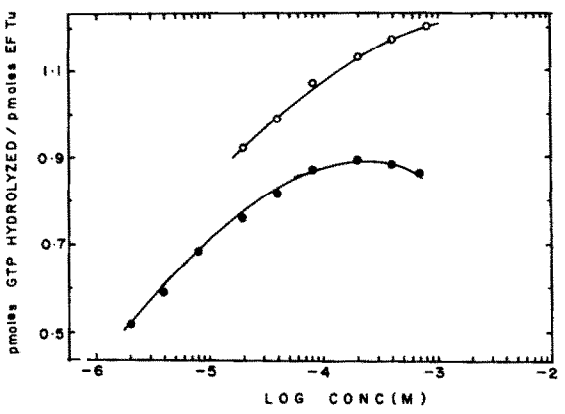

Fig.3. Effect of different concentration of puromycin and puromycin plus A-Phe on EF-Tu · Aurodox-induced GTP hydrolysis: puromycin (●); puromycin (0.2 mM); and A-Phe (○). EF-Tu · GDP was 40 pmol.

ently not due to the alteration of EF-Tu specificity as a result of aurodox binding, since the same compound also stimulates ribosome-dependent EF-Tu GTPase in the absence of Aurodox (P. B. S. C., unpublished.

Fig.2 shows the results relevant to the specificity of aminoacyl-nucleosides in the stimulation of EF-Tu GTPase in the presence of Aurodox. It may be readily seen that A-Phe, a 'natural' 3′-terminus of Phe-tRNA, stimulates the GTPase reaction similarly to C-A-Phe, and 'unnatural' U-Phe does not have any stimulating activity. Quite surprisingly, puromycin, possessing an 'unnatural' amido linkage, stimulates the GTPase reaction even more strongly than the 'natural' A-Phe. However, the 'unnatural' aminoacylamido linkage (e.g., in puromycin) should not be recognized by EF-Tu · GTP [6–8, 15–17]. Indeed, we have also observed that none of the two isomeric 2′-and 3′-phenylalanylamidoadenosines (2′-dA-2′-NH-Phe and 3′-dA-3′-NH-Phe) are active in the stimulation of GTPase reaction (fig.2). Thus, with regards to this structural specificity of EF-Tu · Aurodox GTPase, it seems that puromycin and A-Phe may react at different sites on EF-Tu after it is conformationally modified with aurodox. Further, at the saturating concentration of puromycin, addition of A-Phe results in an increased GTPase activity (fig.3). This stimulation is directly related to the concentration of A-Phe added. Thus, increased GTPase activity due to the addition of A-Phe can be attributed to the different binding sites on EF-Tu for A-Phe and puromycin. It would be of considerable interest to learn which moieties of the puromycin molecule distinguish it from A-Phe in binding to EF-Tu.

Fig.4 shows the results of experiments with 3 different isomeric pairs of 2′- and 3′-aminoacyldinucleoside phosphates in which the 3′-terminal is modified so that the 2′⇄3′ transacylation is impossible.

The comparison of two isomeric lysine derivatives, C-2′dA-Lys and C-3′-dA-Lys, show that the latter is marginally preferred. It should be pointed out that the lack of a second hydroxyl group in C-2′-dA-Lys

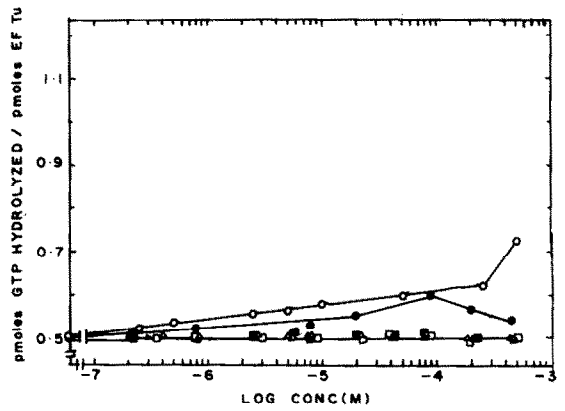

Fig.4. Effect of different isomeric pairs of aminoacyldinucleotides on GTP hydrolysis induced by EF-Tu · Aurodox complex: C-2′-dA-Lys (●); C-3′-dA-Lys (○); C-2′-dA-Phe (▲); C-3′-dA-Phe (△); C-2′-dA-2′-NH-Glu (■); C-3′-dA-3′-NH-Glu (□).

significantly reduces the extent of GTPase stimulation in comparison to C-A-Lys. Therefore, the differences between the two isomers should be cautiously interpreted, even more so because in the case of isomeric phenylalanine derivatives (C-2′-dA-Phe and C-3′-dA-Phe), neither isomer has any effect on the EF-Tu · Aurodox GTPase. Thus, it is possible that the vicinal hydroxyl group could be interacting with the appropriate binding site of EF-Tu or, alternatively, the absence of hydroxyl group may influence unfavorably the ribose pucker [18] and, consequently, the orientation of the aminoacyl group which is recognized by EF-Tu [19]. The inactivity of both isomers of glutamyl derivatives, C-2′-dA-2′-NH-Glu and C-3′-dA-3′-NH-Glu, is readily understandable in light of the above inactivity of the analogous phenylalanylamido derivatives of adenosine. Therefore, the information about isomer specificity of GTPase could not be obtained due to inherent imperfection of the models.

4. Conclusions

(1) The 3′ terminal region of AA-tRNA is sufficient to stimulate the EF-Tu-dependent GTP hydrolysis in the presence of Aurodox and in the absence of ribosomes. Therefore, the binding of this region to EF-Tu, even in the presence of ribosomes, is most probably responsible for GTP hydrolysis which is EF-Tu catalyzed.
(2) The EF-Tu GTPase is significantly influenced by the nature of aminoacyl residue of aminoacyldinucleotides.
(3) The EF-Tu fails to recognize modified aminoacyldinucleotides in which the hydroxyl group of the ribose is substituted by hydrogen.
(4) Puromycin can functionally bind to EF-Tu in the presence of Aurodox at a site which is probably different from that for the 3′-terminus of AA-tRNA.

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