DERIVATIVES OF GUANOSINE TRIPHOSPHATE-PHOTOREACTIVE SUBSTRATES OF *ESCHERICHIA COLI* RNA POLYMERASE

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1. Introduction

Affinity modification by analogues of substrates is one of the main methods for investigating active sites of enzymes. The analogues of substrates used for this purpose must naturally preserve the ability to interact with the active centers, i.e., they must either be substrates or competitive inhibitors. γ-Anilide of adenosinetriphosphate (ATP) was shown to be a substrate of RNA-polymerase of *E. coli* [1]. Photoreactive γ-azidoanilide of ATP and γ-azidoanilide of GTP were also shown to be substrates of RNA polymerase [2] and the oligonucleotides synthesized from them contained photoreactive groups at the 5′-end and specifically modify β, β' and σ subunits of RNA polymerase [2]. If the substrate activity of γ-derivatives of nucleoside triphosphates is a general property, then by introducing an azide group into various positions, we can modify different sites of the RNA polymerase active centre allowing more thorough investigation of its topography. Here we demonstrate that the γ(ρ)-azidobenzylamide of guanosine triphosphate (I) [3] and γ-azidoguanosine triphosphate (II) [4] (fig.1) are substrates of RNA polymerase and that they can be used for photoaffinity modification of this enzyme.

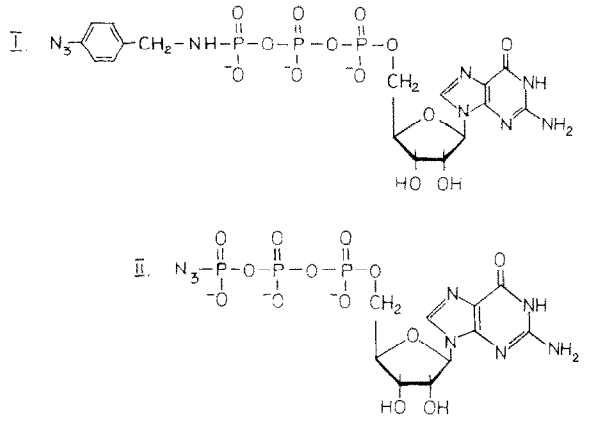

Fig.1. Structures of the γ-(azidobenzyl)amides of guanosine-5′-triphosphate (I) and γ-azidoguanosine-5′-triphosphate (II).

2. Materials and methods

RNA polymerase was isolated according to [5]. Alkaline phosphotase was from 'Worthington'. 4-Aminobenzylphthalimide was kindly given by Professor Z. A. Shabarova and Dr N. I. Sokolova. Guanosine triphosphate, 'Reanal', Hungary, was purified by column chromatography on DEAE-cellulose (Whatman DE-52) with the use of gradient elution (0.1–0.4 M) triethylammonium bicarbonate buffer (pH 7.5). γ-Azidoguanosine triphosphate (II) [4] was synthesized at Novosibirsk State University to our order by a method soon to be published. [α-^{32}P]-Uridinetriphosphate with spec. radioact. 350 Ci/mM was from the Radiochemical Centre, Amersham. DNA fragment phage λ *imm* 434 containing p_R and p_O promotors was isolated as in [6]. 4-Azidobenzylamine was synthesized similarly to 4-azidoaniline, as in [7]. The 4-azidobenzylamide of GTP (I) was obtained from 4-azidobenzylamine and the triethylammonium salt of GTP [3], which were condensed by diciclohexylcarbodimide by the common method of γ-amide triphosphate synthesis [8]. The γ-(4-azidobenzyl)amide of GTP (I) obtained was purified by chromatography on DEAE-cellulose using a gradient (0.05–0.5 M) of triethylammonium bicarbonate

(TEAB) (pH 7.5). Product I was eluted by 0.3 M TEAB, and the original GTP, by a 0.4 M buffer. Product I was characterized by ultraviolet spectra (λ_{min} 252 nm; λ_{max} 226 nm; $A_{252}/A_{280} = 2.30$; $A_{max}/A_{min} = 3.18$ at pH 7.0) by typical adsorption of the azide group within the infrared spectrum ($\nu = 2100\ cm^{-1}$), by stability with respect to the action of *E. coli* phosphomonoesterase and by chromatographic mobility in the system ethanol: IM ammonium bicarbonate (7 : 3) (R_F 0.2) which coincided with that described for [^{14}C]γ-(4-azidobenzyl)amide of GTP [3]. Ultraviolet irradiation of compound I ($\lambda > 290$ nm) decreases A_{252} and eventually its spectrum shifts to that of GTP.

For phosphomonoesterase treatment 30 μl solution containing 0.07 mM I or GTP were supplemented with 20 μl (0.11 u.a.) of phosphomonoesterase solution in 0.01 M Tris–HCl buffer (pH 8.2). The mixture was incubated for 1 h at 25°C, after which the phosphatase was inactivated by heating for 10 min at 65°C after supplementation with 8 μl 0.1 M EDTA solution.

For analysis of substrate activity in the RNA-polymerase action, the reaction mixture contained in 100 μl: 10 μg DNA phage λ, 4 μg RNA polymerase, 20 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 0.4 mM K_2HPO_4, 0.1 mM dithiothreitol (DTT), 0.1 mM [^{14}C]uridine triphosphate (UTP) (650 000 cpm/mmol) and 0.1 mM nucleoside triphosphate, as shown in the figure legends. At certain intervals acid-insoluble radioactivity was determined in 10 μl aliquots.

Photoaffinity modification of RNA polymerase was done as above [2]. The reaction mixture (20 μl) contained 1 μg *Eco*RI-G DNA fragment phage λ *imm* 434 [6], 4 μg RNA polymerase, 4 μg bovine serum albumin (BSA), 20 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 0.1 mM DTT. After 5 min incubation at 37°C this mixture was supplemented with 0.16 nmol [α-^{32}P]UTP and 2 nmol γ-azidoderivatized GTP in 5 μl and incubation was prolonged for another 5 min. The mixture was irradiated for 1 min, using the light of a SVD-12A mercury lamp focused with a quartz lens and directed through a BS-4 ($\lambda > 290$ nm) filter. The distance from the light source to the irradiated solution was 10 cm. The irradiated mixture was supplemented with 20 μl solution containing 10% glycerol, 3% SDS and 5% 2-mercaptoethanol, heated for 2 min at 90°C; RNA-polymerase subunits were separated in a gradient (4–30%) polyacrylamide gel, in the presence of SDS [2]. Radioactivity covalently bound to the subunits was tested for by autoradiography.

3. Results and discussion

From the data in fig.2 it can be concluded that γ-azidoderivatives of GTP are substrates of *E. coli* RNA-polymerase. To support this conclusion we compared the ability of GTP analogues and GTP itself to be included in RNA after being treated with *E. coli* alkaline phosphotase. As can be seen, after such treatment GTP practically loses its inclusion ability, while both analogues preserve quite noticeable activity, though not so great as that of GTP itself. Thus, apparently, analogues I and II can be used as substrates for the photoaffinity modification of the RNA polymerase active centre, which is responsible for the interaction with nucleoside triphosphates. To prove this assumption, we experimented using the scheme in [2]. DNA-fragment phage λ *imm* 434 *Eco*RI-G contains two promotors p_R and p_O [9], that programme RNA synthesis whose 5′-terminus nucleotide is G, which is followed by a uridine residue. Using this fragment as template plus two nucleoside triphosphates GTP and UTP it is possible to stimulate the synthesis of short oligonu-

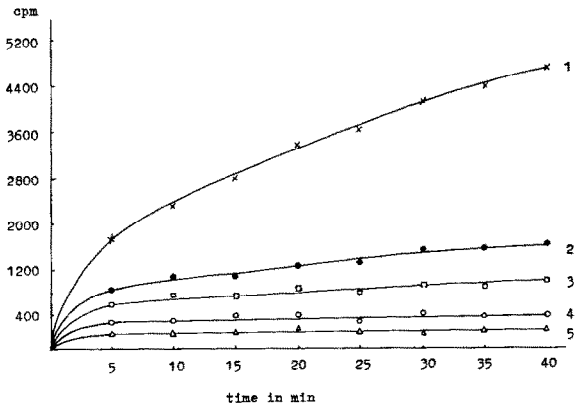

Fig.2. Kinetics of RNA synthesis when the γ-(4-azidobenzyl)-amide of GTP and γ-azidoguanosinetriphosphate are used as substrates. The reaction was done as in section 2. The following triphosphates were present in the mixtures: (1) GTP, ATP, CTP, [^{14}C]UTP; (2) II or I (3), treated with phosphomonoesterase, ATP, GTP, [^{14}C]UTP; (4) GTP treated with phosphomonoesterase, ATP, CTP, [^{14}C]UTP; (5) ATP, CTP, [^{14}C]UTP.

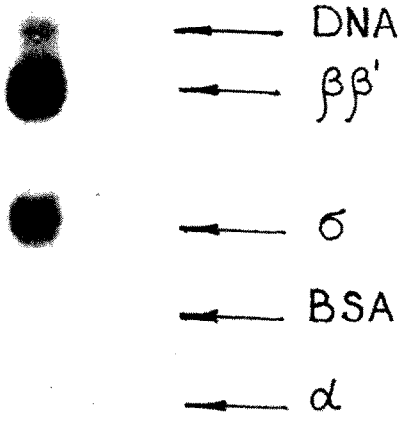

Fig.3. Autoradiographs of electrophoregrams of separated RNA polymerase subunits after irradiation of the reaction mixtures, containing I and [α-^{32}P]UTP. For details see section 2.

cleotides pppGpU . . . If, instead of GTP, we use γ-azidocompounds of GTP and [α-^{32}P]UTP, then the oligonucleotides, synthesized N_3pppG[^{32}p]U . . ., will combine properties of photoreactivity and radioactivity. That is why the detection of radioactivity associated with RNA polymerase subunits after irradiation of the reaction mixture must unambiguously testify to the modification of subunits by the reaction products. We successfully employed the scheme with the γ-azidoanilides of GTP and ATP [2]. Fig.3 shows the results of experiments with analogue I. As can be seen with the γ-azidobenzylamide of GTP β and/or β′ and σ subunits appear to be labeled after irradiation, as well as with the γ-azidoanilide of GTP [2], but in addition, yet another labeled product is observed with a mobility corresponding to the *Eco*RI-G fragment. Presumably, this analogue, unlike the γ-azidoanilide of GTP, also modifies DNA in the transcriptional complex. A number of control experiments show that modification occurs within the transcriptional complex. The strategy of these controls does not differ from that used in [2].

(i) The albumin present in the mixture is not labeled by the reaction product.

(ii) The destruction of the complex before irradiation leads to none of the subunits containing radioactivity.

The efficiency of modification of RNA polymerase by the γ-azide of GTP is much smaller. Presumably, this is because of the absence of the aromatic nucleus connected with N_3 group. The identification of the subunits which are modified is only preliminary in this case. However, the major site of attack is, probably, the σ-subunit.

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