CYCLIC ADENOSINE-5′-TRIMETAPHOSPHATE PHOSPHORYLATES A HISTIDINE RESIDUE NEARBY THE INITIATING SUBSTRATE BINDING SITE OF *ESCHERICHIA COLI* DNA-DEPENDENT RNA-POLYMERASE

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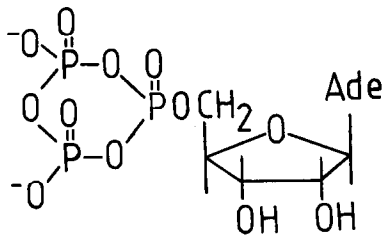
1. Introduction

In [1] it was demonstrated, that cyclic adenosine–5′-trimetaphosphate (ATMP):



Scheme 1

in the presence of a promoter template acts as an efficient affinity reagent towards the center of initiating substrate binding of DNA-dependent RNA-polymerase of *Escherichia coli.* It was found that ATMP inactivates RNA-polymerase, the enzyme being protected from inactivation by ATP. On the contrary, addition of [α-^{32}P]UTP causes acceleration of the inactivation by a factor of 10–20. Radioactivity of [α-^{32}P]UTP under these conditions is bound covalently by the β-subunit of the enzyme. These data suggested that the result of the affinity modification was phosphorylation of an amino acid residue by an oligonucleotide synthesized by RNA-polymerase from ATMP and [α-^{32}P]UTP.

The present studies was aimed at the determination of the length of this covalently-bound oligonucleotide and at elucidation of the nature of the phosphorylated amino acid residue.

* O. Filimonova, a student of Novosibirsk State University, participated in the synthesis of this compound

2. Materials and methods

The promoter-containing fragment *Bsp*I-1462 of T7 phage DNA which contains the promoters A_0, A_1, A_2, A_3 isolated as in [2] was kindly given by Dr Zaychikov. As suggested by the structure of this fragment [3], the 5′-terminal sequences of the RNA molecules which are synthesized by RNA-polymerase with this template are the following: pppGUUGG . . . (A_0); pppAUCG . . . (A_1); pppGCU . . . (A_2); pppAUGAAAC . . . (A_3). RNA-polymerase isolated by method [4] and having spec. act. 5000 units/mg was kindly given by Dr Smirnov; the preparation contained subunits in a ratio close to stoichiometric ($\alpha_2\beta\beta'\sigma$).

2.1. *Adenosine-5′-trimetaphosphate (ATMP)*

This was synthesized by a modified method of [5]. Dicyclohexylcarbodiimide (20 mg) and 10 μl 1 M pyridinium chloride dimethylsulfoxide solution were added to 10 mg ATP triethylammonium salt dissolved in 100 μl dry dimethylsulfoxide. In 1 h at 25°C, the yield of the cyclization was checked by reaction with ethylenediamine [5], the reaction mixture was decanted from the dicyclohexylurea precipitate, and the solution was diluted with methanol to the required concentration. The methanol solution obtained was used directly for the affinity modification. However, the derivatives of ATP were synthesized with the non-diluted dimethylsulfoxide solution.

2.2. *Bis-(γ-adenylyl-5′-triphosphoryl)hexamethylene 1,6-diamide**

Hexamethylene diamine (5 mg) in 200 μl water and 100 μl triethylamine were added to 700 μl ATMP

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solution in dimethylsulfoxide (section 2.1). The mixture was kept during 1 h at 25°C, the phosphate derivatives were precipitated with 3% NaI acetone solution and chromatographed on DEAE-cellulose in an LiCl gradient (0–0.5 M) pH 8.5. The peak of the substance with the longest retention time was evaporated to 2–3 ml, 2 ml methanol was added followed by 60 ml acetone. The precipitate formed was centrifuged, washed with acetone–methanol, 2:1, and dried in vacuo; yield 50%. The structure of the substance follows from the method of its synthesis, and from the fact that ATP (charge −4) is formed when the compound is subjected to acidic hydrolysis; an intermediate product (charge −2) is formed during such hydrolysis, namely the mono-substituted γ-derivative of ATP and hexamethylene diamine.

2.3. *Affinity modification of RNA-polymerase*

This was performed essentially as in [1]. The reaction mixture (65 μl) contained 0.07 M Tris–HCl (pH 8)–0.15 M KCl–1.5×10^{-4} M EDTA–15 mM $MgCl_2$–1.5×10^{-4} M mercaptoethanol, 25 pmol RNA-polymerase, 15 pmol the *Bsp*I-1460 promoter fragment of T7 DNA, and 0.015% sodium azide. It was kept for 5 min at 37°C, cooled to 20°C and supplemented by 3.5 μl 0.01 M methanolic ATMP solution. In 30 min incubation at 20°C, 30 μl (30 μCi) [α-^{32}P]UTP (500 Ci/mmol) solution in 50% ethanol was added. In 1 h, sodium dodecylsulfate (SDS) was added to 0.1% concentration. Gel-electrophoresis of an aliquot of the reaction mixture in SDS showed, in complete accordance with [1], that the radioactivity was bound exclusively by the β-subunit of RNA-polymerase.

The mixture was applied to a column (12 × 0.45 cm) with Sephadex G-50 'fine' equilibrated with 0.01 M Tris–HCl (pH 8)–0.1 M NaCl–0.1% SDS–0.015% NaN_3. The rate of elution was 1 ml/h, fraction vol. 180 μl. Complete separation of the modified enzyme from excess [α-^{32}P]UTP took place. The yield of the modified enzyme was 4×10^5 cpm, corresponding to an extent of modification of some 0.04 mol/mol*. In a control experiment with ATP taken instead of ATMP, no radioactivity was found in the polymer peak.

* The contradiction between the small extent of modification (4%) and the high extent of inactivation (70–100%), as found in [1], may be due to the presence of a large fraction of RNA-polymerase molecules active in the binding with promoters, but inactive in transcription

2.4. *Complete demodification of RNA-polymerase*

The modified radioactive enzyme obtained as in section 2.3 was treated by formic acid as in [6]. Acidic hydrolysis was performed in a reaction mixture which contained 10% formic acid and 0.05% SDS for 1 h at 40°C. The solution was evaporated to dryness to remove formic acid, the residue dissolved in 0.01 M Tris–borate (pH 8.3)–0.05 M NaCl and subjected to gel-filtration on Sephadex G-50 in the same buffer. The radioactivity was present only in the retained fraction. It was collected, supplemented by markers dGMP, ADP, CTP and bis-(γ-adenylyl-5′-triphosphoryl)hexamethylene 1,6-diamide, extracted with butanol to remove SDS, and subjected to micro-column chromatography with multiwavelength detection according to [7] on a 50 μl DEAE-cellulose column. Conditions of chromatography: rate 10 μl/min; 550 μl of a linear gradient (0–0.3 M) of potassium phosphate (pH 7.5) in 7 M urea; 25 μl fractions; absorbance monitored at 250, 260, 270, 280 and 290 nm for the identification of markers by absorbance ratio criterion. Radioactivity of fractions was also counted (see fig.1; the absorbance profile shown is only that measured at 260 nm).

2.5. *Alkaline hydrolysis of the radioactive product of acidic hydrolysis of modified RNA-polymerase*

An aliquot of the modified enzyme obtained as in section 2.3 was treated with formic acid as in section 2.4, except that after evaporation the residue was dissolved in 100 μl 10% piperidine, supplemented by 5 μl 10^{-2} M ATP, and left for 16 h in a stoppered test-tube. The mixture was extracted with butanol, diluted with water and analyzed in the same way as in section 2.4 (see fig.2).

2.6. *Kinetics of the demodification of RNA-polymerase*

The modified enzyme obtained as in section 2.3 (15 000 cpm) was placed into buffer of the following composition: 0.1 M triethylammonium acetate pH 4.9–0.05% SDS, and kept at 20°C. At time intervals, 30 μl aliquots were taken, neutralized by triethylamine, and subjected to gel-filtration on Sephadex G-50 fine (12 × 0.45 cm) in 0.01 M Tris–borate (pH 8.3)–0.05 M NaCl; 200 μl fractions were collected, and their radioactivity levels counted (fig.3).

2.7. *ATP γ-(N-methyl)amidate*

ATMP solution (100 μl) (section 2.1), was mixed with 40 μl triethylamine and 100 μl 2 M methanolic

methylammonium chloride. In 30 min the product was precipitated as sodium salt with 3% NaI acetone solution. The precipitate was washed with acetone, and dried in vacuo. The structure follows from the method of preparation, from the chromatographic behaviour (charge −3) and from the quantitative transformation into ATP caused by mild acidic hydrolysis.

2.8. *ATP γ-imidazolide*

Imidazole solution (100 μl, 1 M) was added to 100 μl 0.1 M ATMP solution (section 2.1). In 20 min, the product was isolated as in section 2.7; yield 90%. The structure follows from the method of synthesis, from the chromatographic behaviour (charge −3) and from the UV-spectrum ($A_{220}/A_{260} = 0.65$; cf. for ATP, $A_{220}/A_{260} = 0.40$) and from its quantitative transformation into ATP after mild acidic hydrolysis.

2.9. *Attempt of the synthesis of the γ-derivative of ATP and guanidine*

Methanolic solution (100 μl, 1 M) of guanidine was added to 100 μl 0.1 M ATMP solution (section 2.1), and the reaction mixture kept during 8 h. Analysis at different time intervals by micro-column liquid chromatography revealed that the only stable products are AMP (70%) and ADP (30%).

2.10. *Kinetics of the acidic hydrolysis of ATP γ-derivatives*

The reaction mixtures (100 μl) contained 2 A_{260} units of the γ-derivative, 0.1 M triethylammonium acetate (pH 4.9) and 0.05% SDS. The mixtures were incubated at 20°C, or 37°C. At time intervals, 10 μl aliquots were analyzed by micro-column chromatography (fig.4,5).

3. Results

It was found earlier [1] that the reaction of ATMP and [α-^{32}P]UTP with RNA-polymerase in the presence of promoter-containing templates results in covalent binding of radioactivity with the β-subunit of the enzyme. It was shown also, that no covalent binding takes place in the absence of ATMP (with ATP instead), or in the absence of promoter template. Hence, it was obvious that the covalently-bound radioactivity belongs to an oligonucleotide which was synthesized by RNA-polymerase.

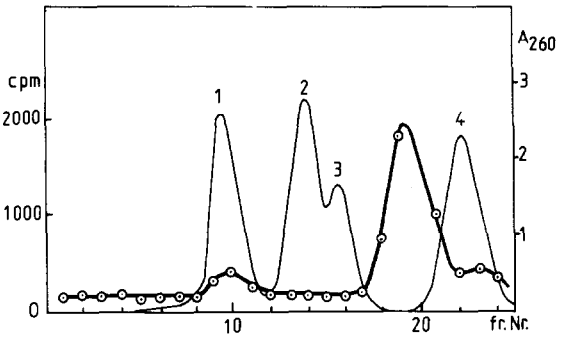


Fig.1. Micro-column liquid chromatography of the radioactive product obtained by demodification of RNA-polymerase modified with ATMP and [α-^{32}P]UTP (see section 2.4). Markers: (1) dGMP; (2) ADP; (3) CTP; (4) bis-(γ-adenylyl-5′-triphosphoryl)hexamethylene 1,6-diamide. The radioactive product has a charge −5. (——) A_{260}; (−⊙−⊙−) radioactivity.

The first task was determination of the length of this oligonucleotide. In order to fulfil this task, the modified enzyme was treated with 10% formic acid according to [6]. Gel-filtration showed, that such treatment results in quantitative demodification. It was therefore concluded that the oligonucleotide is bound to the protein by a phosphoamide bond.

The radioactive substance cleaved off the enzyme by formic acid was subjected to chromatography on DEAE-cellulose in the presence of markers (fig.1). It is seen, that the chromatographic behaviour of the radioactive product corresponds to a charge of −5, and consequently the product has the structure pppA$\overset{*}{p}$U (* = radioactive phosphorus atom). This structure is confirmed by the fact, that the radioactive substance is transformed into a product having a charge −6 (pppA$\overset{*}{p}$, see fig.2) by alkaline hydrolysis.

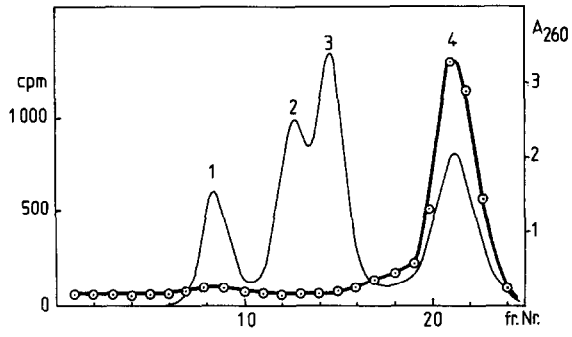


Fig.2. The same as in fig.1, but the product subjected to alkaline hydrolysis (section 2.5). The radioactive product (pppA$\overset{*}{p}$) has a charge −6.

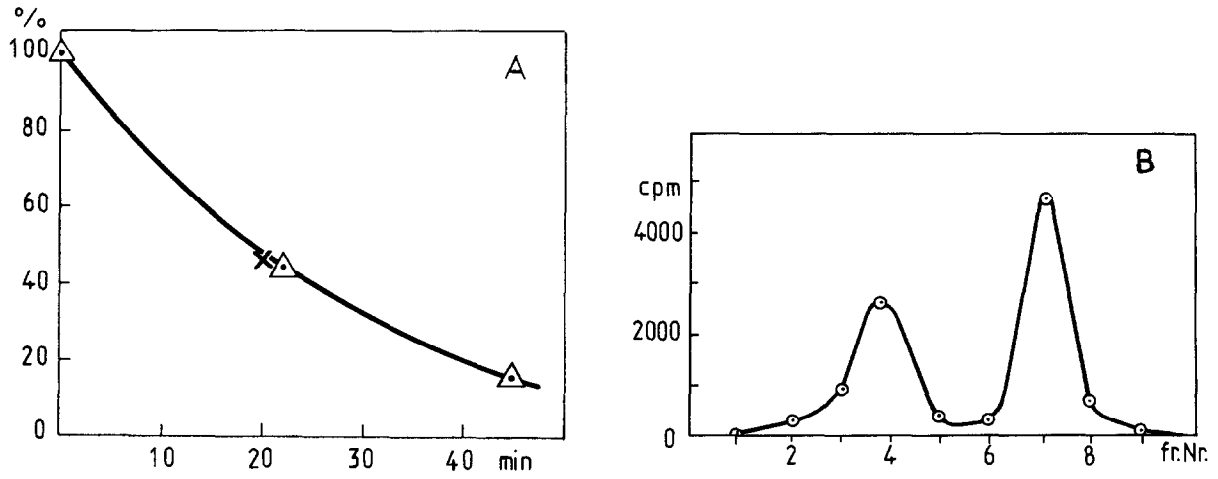


Fig.3. (A) Kinetics of the demodification of RNA-polymerase (section 2.6) at pH 4.9, 20°C (▲). Ordinate: extent of modification, or radioactivity of the first peak divided by the sum of radioactivities of the first and the second peaks in gel-filtration (%). (B) Gel-filtration pattern (see section 2.6) corresponding to 22 min of demodification. For comparison, shown in (A) is also the % of non-hydrolyzed $C_3H_4N_2$pppA after 20 min hydrolysis at pH 4.9, 20°C (×); see section 2.10, and fig.5.

It will be specially emphasized that the dinucleotide pppA$\overset{*}{p}$U is the only radioactive product of demodification.

The second task of this investigation was to elucidate the nature of the amino acid residue of the β-subunit to which the oligonucleotide pppA$\overset{*}{p}$U binds covalently due to the affinity modification. The solution of this problem was achieved by studying the kinetics of the demodification of RNA-polymerase in acidic media. It was found, that at pH 4.9 and 20°C the demodification proceeds with a half-life time of 20 min (fig.3). It is noteworthy that the demodification was performed in a denaturing solution which contained SDS, i.e., enzymatic acceleration of the hydrolysis although being possible was believed to be very improbable.

Lability of the covalent bond between the enzyme and the modifying oligonucleotide in a weakly-acidic medium strongly suggested that it is a phosphoamide rather than any other type of bond. Hence, the following candidates were left as the amino acid residues which could have bound covalently pppA$\overset{*}{p}$U: lysine; histidine; arginine; N-terminal residue. To study the kinetics of the phosphoamide bond hydrolysis, ATP methyl-γ-amidate (CH_3NHpppA; model of the derivative of lysine, or of an N-terminal amino acid residue); ATP γ-imidazolide ($C_3H_4N_2$pppA; model of the derivative of histidine) have been synthesized. Attempted synthesis of the derivative of guanidine (model of the arginine residue), as expected, failed to give any stable guanidine-containing phosphorylation product.

Fig.4 shows the result of 30 min hydrolysis of CH_3NHpppA at pH 4.9, 37°C. It is seen that even at this elevated temperature the hydrolysis yielding ATP is very slow, much slower than the reaction of the enzyme demodification (cf. fig.3). On the other hand, hydrolysis of $C_3H_4N_2$pppA proceeds at pH 4.9, 20°C

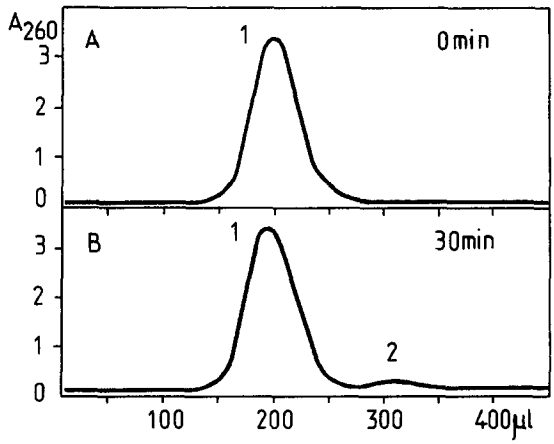


Fig.4. Micro-column chromatography of the reaction mixture (section 2.10) at zero time (A) and in 30 min (B) of hydrolysis of CH_3NHpppA at pH 4.9, 37°C. The conditions of chromatography: 50 µl column with DEAE-cellulose; 550 µl of a linear gradient (0–0.2 M) of potassium phosphate (pH 7.5) in 7 M urea; 10 µl/min: (1) CH_3NHpppA; (2) ATP.

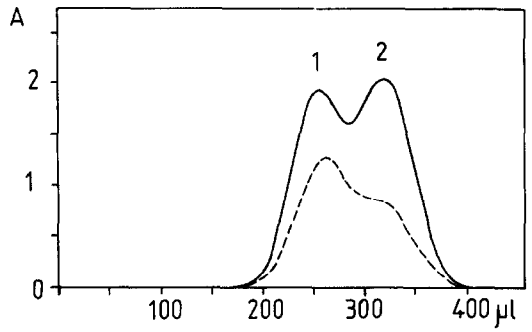


Fig.5. Micro-column liquid chromatography of the reaction mixture in 20 min of hydrolysis of $C_3H_4N_2$pppA at pH 4.9, 20°C: (——) A_{260}; (– – –) A_{220}; (1) $C_3H_4N_2$pppA; (2) ATP.

at exactly the same rate as that of the demodification (cf. fig.3,5). These kinetics strongly suggest that the modified residue is histidine.

4. Discussion

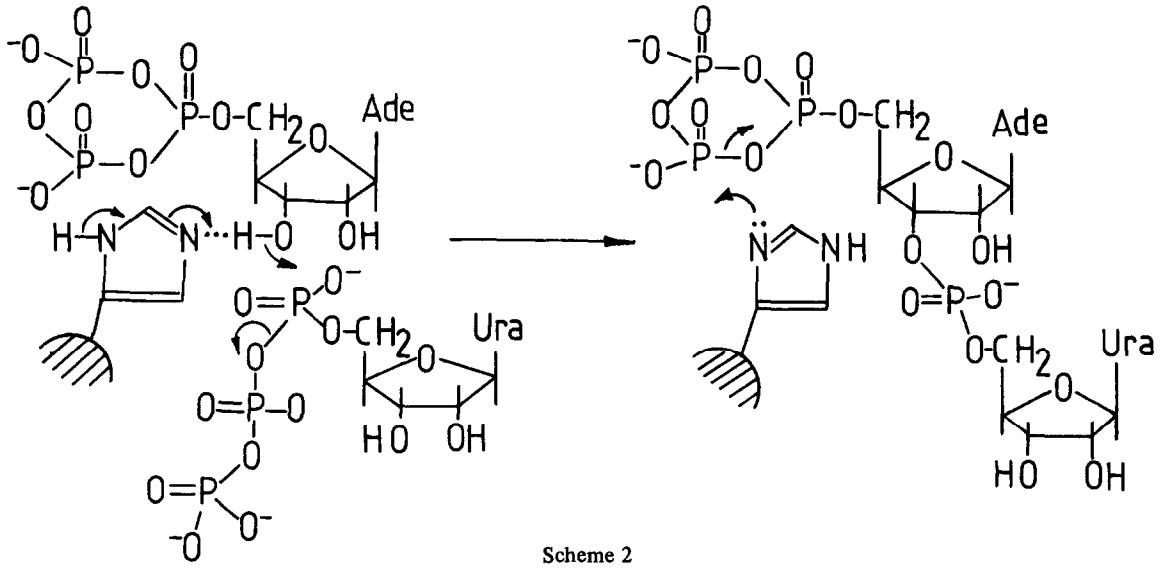
Hence, affinity modification of RNA-polymerase in the presence of a natural, promoter-containing template in a system which contained ATMP and [α-^{32}P]UTP leads to covalent binding of the dinucleotide pppA$\overset{*}{p}$U with a histidine residue of the β-subunit. The fact that the inactivation of RNA-polymerase by ATMP is greatly accelerated in the presence of UTP [1] suggests that the affinity modification may proceed by two alternative ways:

(i) ATMP phosphorylates a histidine residue nearby the center of the initiating substrate binding, and the enzyme uses subsequently the covalently-attached ATP molecule as the initiating substrate and adds to it the $\overset{*}{p}$U residue by the normal mechanism of the synthesis of phosphodiester bonds;

(ii) The enzyme at first uses ATMP as the initiating substrate and synthesizes from it and UTP a dinucleotide with a trimetaphosphate group at the 5′-end, which subsequently phosphorylates the histidine residue.

The dependence obtained in [1] of the initial rate of inactivation on the concentration of ATMP suggests, that the UTP-induced acceleration of the inactivation is much greater than that which could be explained by a decrease of the dissociation constant of the phosphorylating oligonucleotide compared with ATMP. Therefore, one has to assume, that the histidine residue which is modified becomes for some reason much more reactive after the synthesis of the phosphodiester bond of the oligonucleotide.

One may speculate, that RNA-polymerase has a histidine residue which helps in the ionization of the 3′-hydroxyl during the synthesis of the phosphodiester bond. Histidines with similar functions are known to be present in other enzymes [8]. Looking at a space-filling molecular model, one sees that this histidine would be very close to the trimetaphosphate residue of ATMP. However, the nucleophility of this residue should be low due to the hydrogen bond between it and the 3′-hydroxyl. The nucleophility should greatly increase after the formation of the phosphodiester bond:



Scheme 2

Acknowledgements

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