ATP γ-ANILIDATE; A SUBSTRATE OF DNA-DEPENDENT RNA-POLYMERASE OF *ESCHERICHIA COLI*

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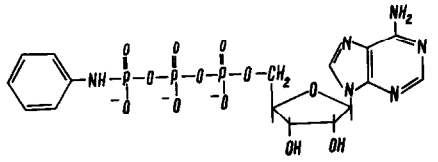
Received 7 October 1974

1. Introduction

It was found recently [1] that ATP γ-anilidate



inhibits the incorporation of ATP in the synthesis of RNA, catalyzed by DNA-dependent RNA-polymerase of *Escherichia coli* (EC 2.7.7.6). This finding suggested that the compound was either an inhibitor, or a substrate of the enzyme.

The present communication reports evidence which clearly shows that ATP γ-anilidate is a substrate of DNA-dependent RNA-polymerase.

2. Materials and methods

Highly purified RNA-polymerase of *E. coli* was kindly given by Dr N. M. Pustoshilova of this Institute; the purity of the enzyme according to its gel electrophoretic pattern was more than 90%. The specific activity of the enzyme was 15 000 units per mg (an activity unit is the amount of enzyme which incorporates 1 nmole of nucleotides into acid-insoluble fraction during one hr at 37°C in the assay mixture of Hinkle and Chamberlin [2]). The enzyme was stored in buffered 50% glycerol at −20°C. [^{14}C]ATP of specific radioactivity 3 mCi/mmole was given by Dr Pustoshilova. [^{3}H]GTP (2 Ci/mmole) was a preparation of Isotop (Moscow). [γ-^{32}P]ATP was synthesized by Dr V. E. Taurine (Institute of Organic Synthesis, Riga).

T7 phage DNA was obtained from phage by phenol deproteinisation [3]; denatured DNA was obtained by heating high molecular weight DNA (Olayne Reagents Plant) solution for 10 min at 100°C. Alkaline phosphatase of *E. coli* was isolated by Dr G. T. Babkina of this laboratory according to Garen and Levintahl [4]; the specific activity of the enzyme was 120 000 units per mg [4]. It was stored in buffer solution (0.01 M Tris–HCl pH 8, containing 5×10^{-3} M $MgCl_2$) at concentration 6 mg/ml at −20°C.

2.1. *Characteristics of the ATP γ-anilidate preparations*

The synthesis of unlabeled and labeled ATP γ-anilidate preparations was performed by condensation of ATP with aniline in an aqueous solution in the presence of a water-soluble carbodiimide; the details of this synthesis and of the purification procedures will be published elsewhere. The purity of the preparations was checked by micro-column chromatography on DEAE-cellulose [5]; the elution was performed with a linear gradient of NaCl in 7M urea–conc. HCl, pH 3.5, or in 7M urea–0.02 M Tris–HCl pH 7.5. At pH 7.5, ATP γ-anilidate has the same chromatographic mobility as ADP, at pH 3.5, the same as ATP. The chemical (and radiochemical, in case of labeled compounds) purity of the preparations was more than 95% as estimated by these techniques.

ATP γ-anilidate is labile at pH < 3.5. However, it is quite stable under the conditions involved in the RNA-polymerase reaction (in reaction mixtures containing either no DNA, or no enzyme).

2.2. *Kinetics of the synthesis of RNA*

The reaction mixtures were prepared according to [2] in the case of the synthesis on T7 DNA template, and according to [6] in the case of poly(A) synthesis on denatured DNA (see legends to figs). In the case of [^{14}C]- and [^{3}H]substrates, aliquots were applied onto Whatman 3MM paper discs; the discs were washed with cold trichloroacetic acid (5%, 3 X 15 min) followed by ether-ethanol (1:1) mixture (2 X 5 min) and ether. In the case of ^{32}P-labeled substrates, aliquots were precipitated with cold 5% trichloroacetic acid after adding albumin as carrier, and the precipitates collected on nitrocellulose ultrafilters (Synpore, ČSSR); the filters were washed according to [7]. The radioactivity of the solid samples was counted in toluene scintillation liquor using a Nuclear Chicago Mark II counter.

3. Results

Fig. 1 shows the stimulation of the incorporation of [^{3}H]GTP, CTP, and UTP caused by ATP γ-anilidate in the synthesis of RNA on T7 DNA template. It is seen, that the anilidate stimulates the incorporation about two times less strongly than does ATP.

Fig. 2 shows the kinetics of the incorporation of [^{14}C]ATP γ-anilidate into RNA, synthesized under the same conditions. The labeled, as well as the unlabeled preparation of the anilidate contained no detectable admixture of ATP; the anilidate being stable under the reaction conditions (see Materials and methods), this evidence leaves little doubt that the compound is in fact a substrate of RNA-polymerase.

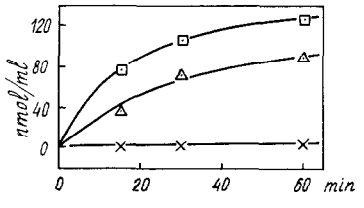


Fig. 1. Kinetics of the synthesis of RNA from [^{3}H]GTP, CTP, UTP and ATP (□) or ATP γ-anilidate (△) catalyzed by RNA-polymerase; native T7 DNA template. Reaction mixtures: 0.04 M Tris–HCl pH 7.9; 0.02 M $MgCl_2$; 0.01 M 2-mercaptoethanol; 1 mg/ml albumin; 0.3 mM T7 DNA; 4 X 10^{-4} M [^{3}H]GTP (diluted to 100 mCi/mmole); 4 X 10^{-4} M each CTP, UTP and ATP (or ATP γ-anilidate); 10 μg/ml RNA-polymerase. The reaction was run at 37°C; 20 μl aliquots were analyzed as described under Materials and methods. (X) Control experiment in the absence of ATP or ATP γ-anilidate. Ordinate; incorporation of [^{3}H]GMP into acid-insoluble fraction, nmole/ml.

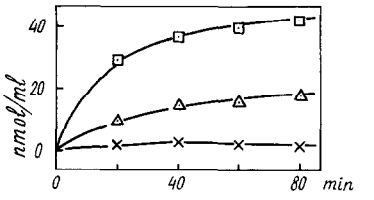


Fig. 2. Kinetics of the incorporation of [^{14}C]ATP and of [^{14}C]ATP γ-anilidate into RNA synthesized by RNA-polymerase on native T7 DNA template. Reaction mixtures: the same as in fig. 1, but unlabeled GTP instead of [^{3}H]GTP, and [^{14}C]ATP (or [^{14}C]ATP γ-anilidate) instead of the unlabeled compounds; 2 μg/ml RNA-polymerase. Ordinate: incorporation of [^{14}C]AMP into acid-insoluble fraction, nmole/ml. (□) [^{14}C]ATP, GTP, CTP, UTP; (△) [^{14}C]ATP γ-anilidate, GTP, CTP, UTP; (X), 4 X 10^{-5} M [^{14}C]ATP; GTP, CTP, UTP.

In order to obtain final proof of this, it was necessary to perform the reaction under conditions which would provide incorporation into RNA of a large fraction of the substrate present initially in the reaction mixture. For this reason, we studied the substrate properties of ATP γ-anilidate in reiterative synthesis of poly(A) on denatured DNA template (cf. [6,8]). Fig. 3 shows the kinetics of the synthesis of

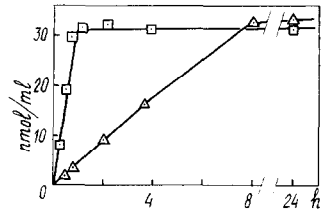
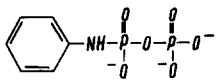


Fig. 3. Kinetics of the reiterative synthesis of poly(A) on denatured DNA template from [^{14}C]ATP (□) and from [^{14}C]ATP γ-anilidate (△). Reaction mixtures: 0.02 M Tris – HCl pH 7.9; 10^{-3} M $MnCl_2$; 50 μg/ml denatured DNA; 50 μg/ml RNA-polymerase; 6 X 10^{-5} M [^{14}C]ATP or [^{14}C]-ATP γ-anilidate. The reaction was run at 37°C; 10 μl aliquots were analyzed as described under Materials and methods. Ordinate: incorporation of [^{14}C]AMP into acid-insoluble fraction, nmole/ml.

poly(A) from ATP and from ATP γ-anilidate. It is seen that the rate of the incorporation of the anilidate is much smaller than that of ATP, but the final incorporation is the same and corresponds to consumption of approximately half of the substrate.

The normal product formed during the synthesis of RNA by RNA-polymerase is inorganic pyrophosphate. Obviously, when ATP anilidate is used, pyrophosphoric acid anilidate



should be formed instead. As suggested by micro-column chromatography, this product is in fact accumulated in the reaction mixture in the course of the reiterative synthesis run with [γ-^{32}P]ATP γ-anilidate. It is seen in fig. 4 that the reaction affords a compound whose chromatographic mobility at pH 7.5 is the same as that of the starting anilidate, but whose UV-spectrum is remarkably different from the later (A_{240} much greater than A_{260}, like in the spectrum of aniline).

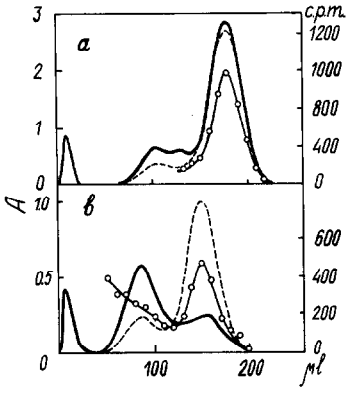


Fig. 4. Micro-column chromatography of reiterative synthesis reaction mixture aliquots. 20 μl of reaction mixture prepared as described in fig. 3 but with [γ-^{32}P]ATP γ-anilidate instead of the ^{14}C-compound was applied onto a 25 μl DEAE-cellulose micro-column. Elution was performed with 600 μl of linear gradient of NaCl (0 to 0.2 M) in 7M urea–0.02 M Tris–HCl pH 7.5 at a rate of 300 μl per hour; 12 μl fractions were collected and applied to Whatman 3MM paper discs for counting the radioactivity. The absorbance at 240 and 260 nm was recorded by means of the microspectrophotometer described in [5]. (– – – –) A_{240}; (——) A_{260}; (o——o) radioactivity, cpm/12 μl. a) At the beginning of the reaction; b) in 15 hr of reaction.

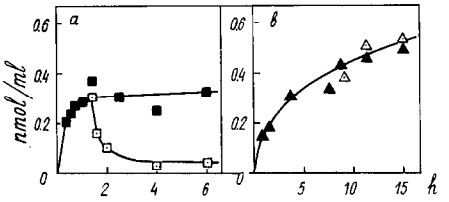


Fig. 5. Kinetics of the incorporation of ^{32}P into a acid insoluble fraction during the synthesis of poly(a) from [γ ^{32}P] ATP and from [γ-^{32}P]ATP γ-anilidate (b) and in the course of the subsequent action of *E. coli* alkaline phosphatase. Ordinate: incorporation of ^{32}P, nmole/ml. The reaction conditions were the same as those outlined in fig. 3 except that ^{32}P-preparations of ATP and ATP γ-anilidate were taken instead of ^{14}C ones; the concentration of the substrates was 1.25×10^{-4} M. At time intervals, corresponding to the first open squares and triangles on the curves, 50 μl aliquots of the reaction mixtures were removed, and 3000 units of phosphatase was added to them. (■, ⊡) Reaction mixture with ATP; (▲, △) reaction mixture with ATP γ-anilidate. Solid squares and triangles – before the addition of phosphatase, and in the residues of the reaction mixture after the removal of 50 μl aliquots. Open squares and triangles – after the addition of phosphatase.

The last question to be investigated was the ability of ATP γ-anilidate to incorporate into the 5′-termini of the newly synthesized RNA chains, i.e., its ability to initiate synthesis. The above data did not provide proof of this ability because the length of the synthesized chains was great, and a minute admixture of the native substrate could be sufficient for the initiation.

To demonstrate the initiative ability, we made use of the fact that the 5′-terminal triphosphate group may be removed by *E. coli* alkaline phosphatase [9]. Preliminary experiments showed that ATP γ-anilidate, unlike ATP, is absolutely stable to this enzyme.

We performed reiterative synthesis of poly(A) from [γ-^{32}P]ATP (fig. 5a) and from [γ-^{32}P]ATP γ-anilidate (fig. 5b), and, after some product accumulated, added alkaline phosphatase to portions of the reaction mixtures. It is seen that phosphatase rapidly removes the 5′-triphosphate terminal groupings of poly(A) synthesized from ATP, while the radioactivity of the polymer synthesized from [γ-^{32}P]ATP γ-anilidate does not drop after the addition of this enzyme. Hence, ATP γ-anilidate may act as initiator in the reaction catalyzed by RNA-polymerase.

4. Discussion

As far as we know, ATP γ-anilidate is the first active analog of ATP modified at the polyphosphate residue. The substrate activity of this compound opens the possibility of obtaining other similar analogs, particularly, reagents for affinity modification of RNA-polymerase, spin-labeled analogs, etc. Secondly, introduction of the hydrophobic phenyl residue into the 5′-termini of the transcription products may be of some interest for structural analysis, because ribonucleic acids which contain similar groupings may be easily separated from unmodified ones by chromatography on benzoyl-diethylaminoethyl cellulose [10].

Acknowledgements

The authors are thankful to Professor D. G. Knorre for his interest in this study, to Dr N. M. Pustoshilova for the gift of RNA-polymerase and of labeled ATP, to Dr G. T. Babkina for the preparation of phosphatase, to Dr V. A. Kurbatov for participation in the synthesis of ATP γ-anilidate and in the preparation of T7 DNA, and to Dr V. E. Taurine for the synthesis of [γ-^{32}P]ATP.

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