Kinetic evidence for multiple binding sites of nucleoside triphosphates in Escherichia coli RNA polymerase

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KINETIC EVIDENCE FOR MULTIPLE BINDING SITES OF NUCLEOSIDE TRIPHOSPHATES IN *ESCHERICHIA COLI* RNA POLYMERASE

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1. Introduction

The kinetic behaviour of *E. coli* RNA polymerase follows the generally accepted model of the RNA polymerase reaction with a single common site where nucleoside triphosphates are bound and enter the elongation step [1–3]. For the single-site reaction the rates of the chain elongation, V_{el}, and of the PP_i–NTP exchange, V_{ex}, are determined by the following expressions:

$$V_{el} = K_1 \cdot [ES]$$

$$V_{ex} = K_2 \cdot [ES] \cdot [PP_i] \cdot \alpha$$

where [ES] is some intermediate enzyme form, α is the probability of ^{32}P-labelled NTP formation independent of the concentration of enzyme forms [4]. As it is seen from the above expressions, the single-site model predicts that competitive inhibitors, which do not influence the enzyme forms ratio, must inhibit the chain elongation and PP_i–NTP exchange to the same extent. However, we have found that in transcription of poly(dA–dT) and T7 DNA, non-complementary nucleoside triphosphates and deoxynucleoside triphosphates inhibit the chain elongation to a greater extent than the PP_i–NTP exchange.

Our results suggest that there are multiple binding sites for nucleoside triphosphates in *E. coli* RNA polymerase.

2. Materials and methods

2.1. *Materials*

RNA polymerase from *E. coli* (SBDT BAC, Novosibirsk) was purified additionally by chromatography on DNA–Sepharose as in [5]. The purity of the enzyme was estimated to be 80% by polyacrylamide gel electrophoresis in sodium dodecyl sulphate. RNA polymerase had a specific activity about 500 calf thymus DNA units/mg protein [6]. The synthesis of RNA without template was less than 1% and the PP_i–NTP exchange was less than 10% of those in the presence of the template.

T7 DNA was produced on a pilot plant of the Institute of Organic Chemistry, Novosibirsk. Poly-(dA-dT) and deoxynucleoside triphosphates were kindly supplied by Dr A. Romashchenko (Institute of Cytology and Genetics, Novosibirsk). Ribonucleoside triphosphates were purchased from Sigma Chemical Co. (USA) and were 95% purity from mono-, diphosphates and othergates ribonucleoside triphosphates estimated by paper or thin-layer chromatography on 'Silufol' UV-254 (Czechoslovakia). Ribonucleoside diphosphates were kindly supplied by Dr V. Rait (Novosibirsk State University). [^{14}C]ATP (0.5 Ci/mmol) was purchased from Amersham (England), sodium [^{32}P]pyrophosphate ($^{32}PP_i$) from Isotope (Leningrad).

2.2. *Transcription of DNA*

The reaction mixture contained 3×10^{-4} M T7 DNA as nucleotides; 4×10^{-4} M of each CTP, GTP, UTP and ATP ([^{14}C]ATP was used to assey polymerization); 2×10^{-2} M $MgCl_2$; 0.04 M Tris–HCl, pH 7.8; 0.01 M β-mercaptoethanol and 0.06–0.2 mg/ml *E. coli* RNA polymerase.

2.3. *Transcription of poly(dA-dT)*

The reaction mixture contained 5×10^{-5} M poly-

(dA-dT) as nucleotides; 4×10^{-4} M of each UTP and ATP ([^{14}C]ATP was used to assay polymerization); 2×10^{-2} M $MgCl_2$; 0.04 M Tris–HCl pH 7.8; 0.01 β-mercaptoethanol; 0.125 mg/ml *E. coli* RNA polymerase.

PP_i, rifampicin and substrate analogs at concentrations given in the figure legends were added to the reaction mixtures several minutes after the beginning of the reaction to exclude their influence on the initiation stage. The substrate analogs and PP_i were added with equimolar amounts of $MgCl_2$.

The mixtures were incubated at 37°C and the reaction was stopped by transferring 0.05 ml aliquots of the reaction mixture to 0.1 ml 0.1 M EDTA and 0.1 mg/ml yeast RNA as a carrier. Incorporation of radioactive nucleotide into the polynucleotide product was determined by precipitation with cold 5% trichloroacetic acid. The precipitates were collected on Millipore filters (1.5 μm), washed extensively with 5% trichloroacetic acid, dried and counted with Nuclear Chicago Mark II liquid scintillation counter.

The PP_i–NTP exchange was assayed by the amount of ^{32}P adsorbed by charcoal.

The reaction mixture was the same as that used in the polymerisation assay except for sodium [^{32}P]-pyrophosphate (4–12 mCi/mmol) and unlabelled nucleoside triphosphates. Aliquots (0.05 ml) of the reaction mixture were transferred to 0.1 ml 0.1 M EDTA and 0.5 ml charcoal suspension (Norite, 20 mg/ml) in 0.01 M sodium-pyrophosphate, pH 6.0. After 30 min at 20°C, the mixtures were filtered through Millipore filters (2.5 μm), washed extensively with unlabelled sodium-pyrophosphate, dried and counted with a flow counter 'Protoka'. [^{32}P]Nucleoside triphosphates adsorbed onto Norite were eluted with 1 M NH_4OH in 50% ethanol and analyzed by paper chromatography using 2.5 M NH_4OH–isobuturic acid (34:66, v/v).

3. Results

The inhibition of the chain elongation and PP_i–NTP exchange by noncomplementary nucleoside triphosphates GTP and CTP during the synthesis of RNA on poly(dA-dT) template is shown in fig.1. The presence of $6–8 \times 10^{-3}$ M GTP or CTP results in 5–10-fold decrease of the rate of chain elongation.

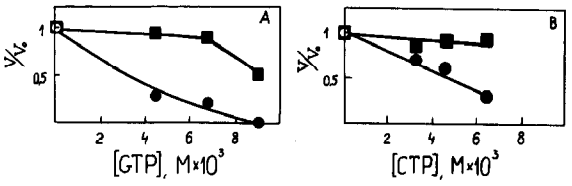

Fig.1. Relative effect of varying concentrations of GTP (A) and CTP (B) on poly(dA-dT)-directed poly(AU) synthesis (•) and PP_i–NTP exchange (▪). The composition of the reaction mixture is given in section 2. $[PP_i] = 5 \times 10^{-4}$ M.

Under the same conditions, the rate of PP_i–NTP exchange decreases though to a very small extent (about 20%). The high rate of PP_i–NTP exchange is due to PP_i–ATP and PP_i–UTP exchanges but not due to PP_i–GTP and PP_i–CTP exchanges as is revealed by chromatographic analysis of the PP_i–NTP exchange products.

The relation between the chain elongation and PP_i–NTP exchange during the transcription of natural template T7 DNA was studied using deoxynucleoside triphosphates and ribonucleoside diphosphates as inhibitors. The influence of ADP on the kinetics of the chain elongation and PP_i–NTP exchange during RNA synthesis directed by T7 DNA is shown in fig.2. ADP gives a 2-fold inhibition of the chain elongation without any influence on the PP_i–NTP exchange. The results of experiments with deoxynucleoside triphosphates and ribonucleoside diphosphates are listed in table 1. Three of four deoxynucleoside triphosphates

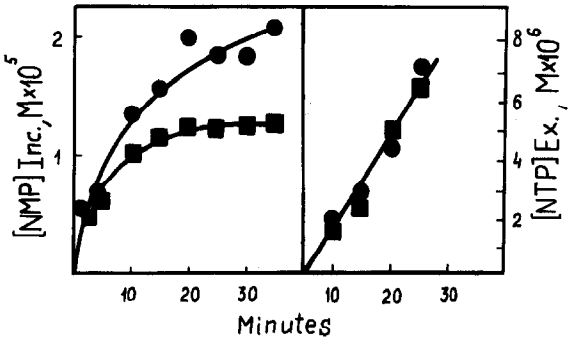

Fig.2. The time course of T7 DNA-directed RNA synthesis (A) and PP_i–NTP exchange (B) without inhibitors (•) and with ADP (4×10^{-3} M) (▪). The composition of the reaction mixture is given in section 2. $[NTP] = 3 \times 10^{-4}$ M (7.5×10^{-5} M of each NTP), $[PP_i] = 1.5 \times 10^{-4}$ M.

Table 1
Effect of substrate analogs on T7 DNA-directed reactions

Substrate analogs	Concentration of analogs ($M \times 10^3$)	Degree of inhibition (% ± 10%)	
		Synthesis of RNA	PP_i–NTP exchange
dATP	8	40	no inhibition
dCTP	6	30	no inhibition
dTTP	8	70	30
dGTP	3.5	50	60
ADP	6	70	no inhibition
GDP	6	40	40
CDP	4	30	40
UDP	7	slight inhibition	slight inhibition

Compositions of the reaction mixtures are in section 2. $[PP_i] = 3 \times 10^{-4}$ M, 20 mg/ml rifampicin

inhibit the chain elongation much more efficiently than the PP_i–NTP exchange. This effect was observed also with ADP, but not with the other nucleoside diphosphates which inhibit both processes with the same efficiency.

A high rate of PP_i–NTP exchange under the condition of inhibited chain elongation could be due to pyrophosphorolysis of the newly synthesized RNA chain. In this case the rate of the PP_i–NTP exchange should be independent of the concentration of nucleoside triphosphates in the reaction mixture. This is however not the case, as is seen from the same concentration dependence of the chain elongation and PP_i–NTP exchange (see fig.3). In these experiments PP_i and increasing concentrations of the four nucleoside triphosphates were added to the reaction mixture in 20 min after the beginning of the reaction with low concentration of nucleoside triphosphates. The concentration dependence of the PP_i–NTP exchange rate obtained in these experiments (see fig.3) shows that the nucleoside triphosphates (but not the ends of the transcripts) are substrates in the PP_i–NTP exchange reaction.

On the other hand, the pyrophosphorolysis of specially prepared RNA–DNA hybrids with RNA-polymerase *E. coli* was shown to produce [^{32}P]-nucleoside triphosphates at a rate much smaller than that of the PP_i–NTP exchange [7,8] and therefore cannot ensure the high level of PP_i–NTP exchange observed in our experiments.

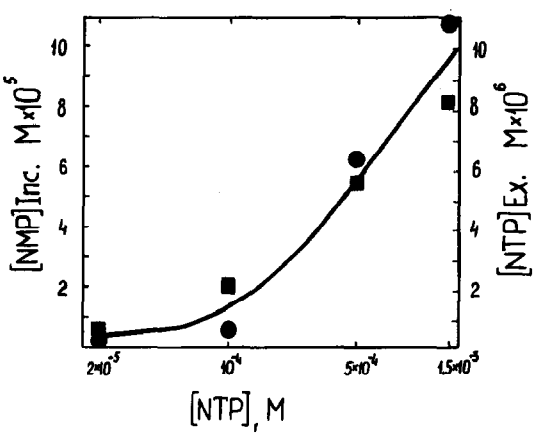

Fig.3. T7 DNA-directed RNA synthesis (•) and PP_i–NTP exchange (■) at varying NTP concentrations. The composition of the reaction mixture is given in section 2. [NTP] = 2×10^{-5} M (5×10^{-6} M of each NTP). 20 min after starting RNA synthesis PP_i (1.5×10^{-4} M) and NTP were added up to final concentrations 10^{-4} M, 5×10^{-4} m and 1.5×10^{-3} M. The extent of RNA synthesis and PP_i–NTP exchange were determined after 50 min incubation for each concentration of NTP.

4. Discussion

Our experiments show that the substrate analogs can inhibit the chain elongation much more effectively than the PP_i–NTP exchange during the transcription catalyzed by RNA polymerase from *E. coli*.

It follows from our data for noncomplementary

nucleoside triphosphates that three of the four dNTP and ADP do not fit the 'single binding site' model of RNA polymerase with T7 DNA as template [3].

Our results can be explained in two ways:

1. RNA polymerase may catalyze the PP_i–NTP exchange and phosphodiester bound formation in two different catalytic sites.

2. RNA polymerase may have one catalytic site, but multiple binding sites for substrates or their analogs.

The fact that RNA polymerase *E. coli* does catalyze the PP_i–NTP exchange at the same catalytic site where the phosphodiester bond is formed was established by the following experimental evidence:

(i) Formation of the phosphodiester bond is necessary for PP_i–NTP exchange [8–11].

(ii) Incorporation of $^{32}PP_i$ proceeds only into complementary nucleoside triphosphates during the transcription ([10], our results).

(iii) Similar dependence of the rates of PP_i–NTP exchange and synthesis on the concentrations of nucleoside triphosphates during the chain elongation in transcription on DNA template (fig.3).

(iv) Equal inhibition of the chain elongation rate and the PP_i–NTP exchange rate by some nucleoside diphosphates during transcription on DNA template (table 1).

To explain our data we suppose that RNA polymerase has multiple binding sites for substrates or their analogs. Only one of these has a catalytic property and binds true substrates better than the other site(s). Experiments with deoxynucleoside triphosphates and ADP show that the strength of the binding is determined not only by the nitrogen base but also by the sugar-phosphate moiety.

The noncatalytic binding sites show a small accuracy in binding of a true substrate as compared to catalytic sites, and the competition between substrates and their analogs determines the kinetics of chain elongation. This means that the $K_m/K_i \sim 10^{-2}$ ratio obtained [1] from the data on inhibition of chain elongation by noncomplementary nucleoside triphosphates is determined by the competition in the noncatalytic sites.

It is not clear which monomer residue of the template is in the noncatalytic site(s) and determines whether the given nucleoside triphosphate is a true substrate or not. As shown [1,2], binding of only one nucleoside triphosphate is sufficient for the chain elongation by one monomer residue. This means that binding of a true substrate(s) in a noncatalytic site(s) does not influence the chain elongation. At the same time, when bound at the noncatalytic site(s), the substrate analogs block the chain elongation by a competitive mechanism, at least for noncomplementary nucleoside triphosphate [1]. Perhaps, the substrate analogs block the translocation of RNA polymerase along the template.

In conclusion, let us point out that the multiple binding site model of RNA polymerase is consistent with affinity labelling data obtained for RNA polymerase of *E. coli* [12,13] and with similar models for other template-directed enzymes [14,15].

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