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 concentra-
tions 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 reac-
tion 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% tri-
chloroacetic 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]Nucleo-
side triphosphates adsorbed onto Norite were eluted
with 1 M NH$_4$OH in 50% ethanol and analyzed by
paper chromatography using 2.5 M NH$_4$OH–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
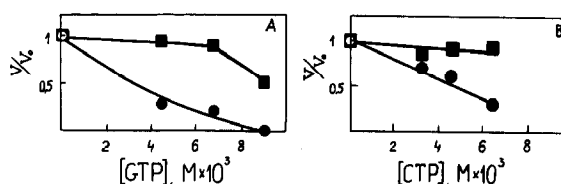5–10-fold decrease of the rate of chain elongation.

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$_i$] = 5×10^{-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 triphos-
phates and ribonucleoside diphosphates are listed in
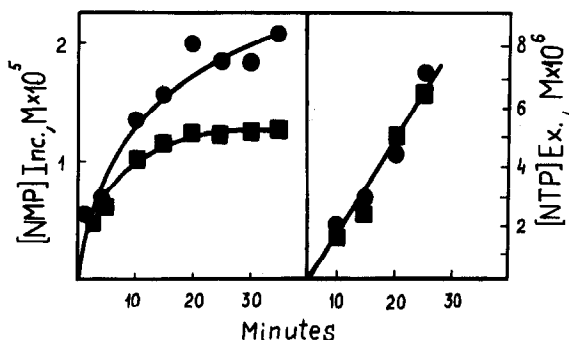table 1. Three of four deoxynucleoside triphosphates

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] = 3×10^{-4} M
(7.5×10^{-5} M of each NTP), [PP$_i$] = 1.5×10^{-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 ($\% \pm 10\%$)	
		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$_i$] = 3×10^{-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.
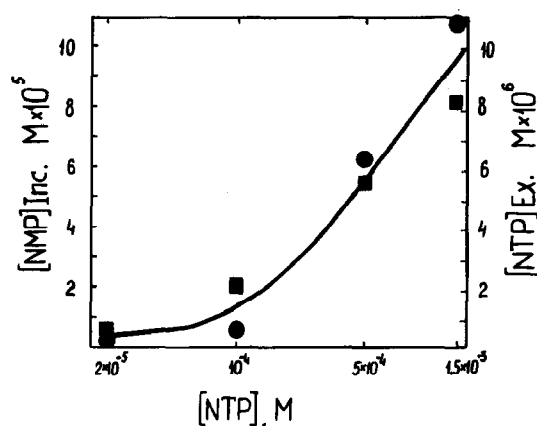

Fig.3. T7 DNA-directed RNA synthesis (●) and PP$_i$–NTP exchange (■) at varying NTP concentrations. The composition of the reaction mixture is given in section 2. [NTP] = 2×10^{-5} M (5×10^{-6} M of each NTP). 20 min after starting RNA synthesis PP$_i$ (1.5×10^{-4} M) and NTP were added up to final concentrations 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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