RNA POLYMERASE: POTENT COMPETITIVE INHIBITION BY D-RIBOSE-5-TRIPHOSPHATE AND OTHER PENTOSE POLYPHOSPHATES

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Received February 22,1977

SUMMARY

Ribose and deoxyribose 5' pyro and triphosphates are shown to be potent substrate competitive inhibitors for an in vitro transcription system containing either calf thymus or T_7 DNA as template, and the E. coli RNA polymerase [E.C.2.7.7.6]. Each of these analogues gave K_I values ($\sim$25 μM) essentially the same as the K_m values ($\sim$15 μM) for the substrates. In contrast the ribose and deoxyribose 5' monophosphates, ribonucleosides, deoxynucleoside mono and triphosphates were not significant inhibitors. The data are consistent with the interpretation that the enzyme binds the substrate primarily through the 3'-endo ribose polyphosphate moiety.

INTRODUCTION

An important aspect of the transcription mechanism is the substrate specificity of the RNA polymerase. Studies of the substrate binding characteristics of the enzyme have utilized nucleotide analogs (1-5), affinity labels (6,7), and fluorescent probes (8). Equilibrium dialysis studies (9) and kinetic studies (10) have shown that the binding of the 5' terminal nucleotide to the polymerase enzyme is purine specific and has a K_m value of 150 μM, whereas the binding of all subsequent nucleotide substrates added during the elongation phase is essentially base nonspecific in that their K_m values are nearly identical ($\sim$15 μM). Our present studies have been concerned with the contribution of the pentose polyphosphate moiety of the substrate to the binding event. Accordingly, we have synthesized the series of pentose

Abbreviations

RP_3, RP_2, RP	D-Ribose-5-tri-, pyro-, and monophosphate
dRP_3, dRP_2, dRP	D-2-Deoxyribose-5-tri-, pyro-, and monophosphate
RP_3*	[α^{32}P] D-Ribose-5-triphosphate
NTP, NMP	Ribonucleoside tri- and monophosphates
dNTP, dNMP	2-Deoxyribonucleoside tri- and monophosphates

ISSN 0006-291X

polyphosphate compounds ribose-5-triphosphate, ribose-5-pyrophosphate, 2-deoxy ribose-5-triphosphate and 2-deoxy ribose-5-pyrophosphate and measured their inhibition constants (K_i) in kinetic experiments employing two different templates. In each case the analog competes for the substrate binding site so effectively that it essentially accounts for the total substrate interaction with the enzyme. Although ribose-5-triphosphate binds tightly to the enzyme it does not appear to be an alternate substrate since [α^{32}P]-ribose-5-triphosphate was not incorporated into the polymeric product.

EXPERIMENTAL

Materials. Ribonucleoside mono and triphosphates were obtained from Schwarz/Mann Biochemicals; 2-deoxyribonucleoside mono and triphosphates, from Calbiochem; [5,6-^{3}H]UTP and [^{32}P]P_i from New England Nuclear Corp.; and calf thymus DNA from Sigma Chemical Co. T_7 DNA was the kind gift of Dr. W. C. Summers. The E. coli RNA polymerase was the gift of Dr. C. W. Wu (Burgess procedure (11) specific activity of 720 n moles of ^{3}H-GMP incorporated/20 mins/mg enzyme using calf thymus DNA).

Synthesis. Ribose-5-monophosphate was converted to the pyro and triphosphate by phosphorylation with P_i using dicyclohexylcarbodiimide (12). The products were separated and purified by column chromatography to yield the chromatographically pure (TLC; 1-propanol, NH_4OH, H_2O; 55·20·25) pyro and triphosphate compounds.

2-deoxyribose-5-monophosphate was prepared by acid depurination of 2'-d-GMP (13) and then phosphorylated as above to yield the corresponding 2-deoxyribose-5-pyro and triphosphate compounds.

[^{32}P]ribose-5-monophosphate was prepared by acid hydrolysis of [α^{32}P]-5'-ATP (14) and then phosphorylated as above to yield the corresponding [α^{32}P] ribose-5-triphosphate. The specific activity of the compound was 3800 cpm/n mole.

Kinetic Assays. Assay reaction mixtures (0.1 ml) contained 40 mM tris·HCl (pH 7.9), 10 mM $MgCl_2$, 0.1 mM EDTA, 150 mM KCl, 0.1 mM dithiothreitol, 0.8 mM K_2HPO_4, 0.68 µg bovine serum albumin, 15 µg calf thymus DNA, 1.2 µg polymerase and 200 µM each of ATP, GTP, CTP were incubated for 5 minutes at 37°C. The inhibitor was then added to a final concentration of 25-40 µM; incubation was continued for an additional 5 minutes at which time the reaction was started with the addition of [^{3}H]UTP (5.5 x 10^3 cpm/n mole) over the concentration range 10-100 µM. Aliquots of 25 µl were removed at one minute intervals, spotted on nitrocellulose filters (Whatman 3 MM) and placed in cold 5% trichloroacetic acid. The filters were washed two more times in 5% trichloroacetic acid, once with ethanol and finally with diethyl ether. The filters were dried and then counted in a Beckman LS 100 liquid scintillation counter using a toluene based cocktail. Reaction protocols using T_7 DNA as template contained 40 µM tris·HCl (pH 7.9), 5 µM $MgCl_2$, 0.1 µM EDTA, 50 mM KCl, 15 mM mercaptoethanol, 3.2 µg T_7 DNA and 0.4 µg RNA polymerase.

Product Analysis for [α^{32}P] Ribose-5-Triphosphate Incorporation. The reaction components and conditions were varied (Table I) to investigate the incorporation of ribose-5-triphosphate into thepolymeric product. Reactions were run for

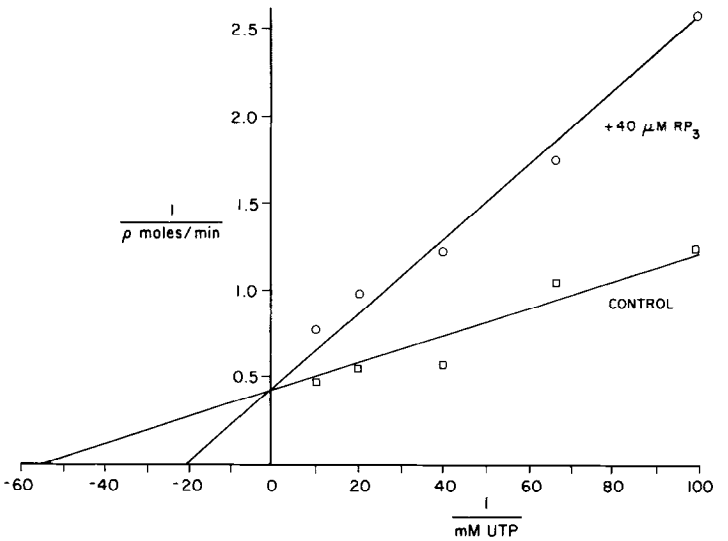

Figure 1. Lineweaver-Burk plot for the inhibition of E. coli RNA polymerase with T_7 DNA by ribose 5-triphosphate. See Methods for reaction protocol. Open squares (□) represent control in the absence of inhibitor. Open circles (○) represent reaction in the presence of 40 μM ribose 5-triphosphate. K_m^{UTP} = 17.4 μM and K_i^{RP3} = 21.3 μM.

3-5 hours using T_7 DNA as template. Aliquots of 50-75 μl were applied to either a Sephadex G-10, G-15 (1 x 16 cm) or Biogel P30 (1 x 12 cm) column and eluted with 0.2 M NaCl. Five drop fractions were collected and examined for labeled polymer (^{32}P and ^{3}H). Alternatively, aliquots were applied to TLC-cell-300 PEI plates and chromatographed with 1.5 M KH_2PO_4 and autoradiographed for ^{32}P containing oligo nucleotides.

RESULTS

Kinetic Inhibition Studies. The rate of incorporation of ^{3}H-UTP into polymer was measured as a function of [UTP] and analyzed by plotting $\frac{1}{v}$ or $\frac{1}{s}$ as shown in Figure 1. The intercept V_M is common to the plots with and without inhibitor present. The data are summarized in Table I for all the kinetic studies. The K_i values obtained with either calf thymus or T_7 DNA as template were similar and close to the K_m values of the substrate NTP's as reported previously (15) and confirmed by our studies. Since the 5-monophosphates of both ribose and 2-deoxyribose were not inhibitory at a concentration of 3 mM

Table I. Kinetic Constants for Inhibitors

	T_7 DNA	Calf Thymus DNA	
	K_i, μM	K_i, μM	K_m, μM
Initiation			
ATP, GTP	--	--	150[a]
Elongation			
NTP	--	--	15[a]
UTP	--	--	13
RP_3	21	28	--
RP_2	8	31	--
dRP_3	26	28	--
dRP_2	38	33	--
dNTP	--	2800	--
RP, dRP, dNMP, NMP	> 5000		

[a]Data from Anthony, et al. (15).

it appears that at least a pyrophosphate linked to the 5 carbon of the pentose is essential for binding.

It is curious that both the 2-deoxyribo and ribopolyphosphates are equally effective with regard to binding to the enzyme, particularly since dNTP's exhibit little or no inhibition of the polymerase. The possibility exists that the enzyme can distinguish between the pentose structures when a C_1 base substituent is present (see Discussion).

[α^{32}P] Ribose-5-Triphosphate Incorporation Studies. Since ribose-5-triphosphate binds to the enzyme substrate binding site nearly as well as the substrate itself, it was of interest to ascertain whether or not it was incorporated into the polymeric product. A variety of conditions were investigated with the result that no α^{32}P labeled ribose-5-triphosphate was incorporated into product (see Table II). The ^{3}H labeled product (from ^{3}H-UTP) was clearly separated from the reaction components in the void volume and never contained a ^{32}P

Table II. Protocols for [α^{32}P]-RP_3 Incorporation Studies[a]

	Components	Variant Conditions
1.	E + DNA + NTP + RP_3*	--
2.	E + DNA + ATP + GTP + RP_3*	--
3.	E + DNA + ATP + GTP + UTP + RP_3*	--
4.	E + DNA + ATP + RP_3*	--
5.	E + DNA + ATP + GTP + CTP + RP_3*	50°C
6.	E + DNA + ATP + GTP + CTP + RP_3*	-KCl
7.	E + DNA + NTP + RP_3*	-Mg + Mn
8.	E + DNA + NTP + RP_3*	pH 5.6
9.	E + ATP + RP_3*	-Mg + Mn
10.	E + DNA + NTP + RP_3*	Mn + 50°

[a]For experimental details see Methods.

label. Examination of the reaction mixture by a TLC autoradiographic procedure using 1.5 M KH_2PO_4 as solvent failed to detect any ^{32}P labeled components other than the initial compounds.

DISCUSSION

The substrate binding site of RNA polymerase during the elongation phase appears to be base indiscriminate in that the pentose polyphosphate moiety can account for most, if not all, of the binding interaction. This conclusion then would imply that the base discrimination observed for the transcription event must occur as an independent event perhaps prior to the binding of the substrate NTP's to the enzyme surface as suggested by Freese (16), or subsequent to binding as suggested by Rhodes (1).

The question of pentose discrimination must now be addressed in that dNTP's are neither usual substrates (except in systems where Mn^{++} replaces Mg^{++})

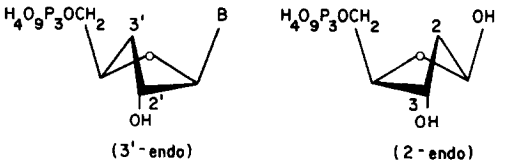

Figure 2. Spatial arrangement of the hydroxyl substituents of NTP and ribose-5-triphosphate.

or inhibitors for the reaction whereas 2-deoxy ribose-5-triphosphate is a potent competitive inhibitor for the reaction. A possible explanation for this observation is the suggsstion that binding involves a 2-OH substituent on the pentose in the 3-endo conformation or an hydroxyl with an equivalent spatial conformation. In Figure 2 the spatial arrangement of the hydroxyl substituents of NTP and ribose-5-triphosphate are contrasted in their suggested preferred conformations.

The pentose polyphosphate is suggested (to exist and/or bind) in the 2-endo conformer whereas the pentose moiety of a nucleoside triphosphate (exists and/or binds) in the 3'-endo conformer. Note that the 2-OH of the 3-endo conformer is in essentially the same spatial position as the 3-OH of the 2-endo conformer.

According to this suggestion NTP's bind to the polymerase using the available 2-OH of the 3'-endo conformer of the pentose, dNTP's do not bind to the polymerase to any large extent since the pentose moiety is again in the 3'-endo conformation and there is no suitable OH for binding. On the other hand, 2-deoxyribose-5-triphosphate binds through its 3-OH since the pentose moiety is in the 2-endo conformation. Ribose-5-triphosphate is also in the 2-endo conformation and binds to the enzyme through its 3-OH substituent. Neither of the pentose triphosphates is incorporated into product since catalysis requires the 3-endo conformation. Cordycepin triphosphate (3'-deoxy ATP) binds through its 2-OH of a 3'-endo conformer, forms a phosphodiester bond with the nascent RNA and then terminates the chain.

Pentose analogs of NTP and ribose-5-triphosphate are currently under study in our laboratory to seek further information concerning this aspect of the mechanism of RNA polymerase.

Acknowledgments

We thank Drs. G. Rose and R. F. Colman for fruitful discussion and critical advice. This work was supported in part by a grant from the Eli Lilly Co.

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