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RNA Polymerase: Potent Linear Competitive Inhibition by D Arabinose-5-Triphosphate Compared to Non-inhibition by 5' Ara-ATP

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ABSTRACT

D-arabinose-5-triphosphate is a potent linear competitive inhibitor (K_i = 60 μM) for substrates of E. coli RNA polymerase [E.C.2.7.7.6] in an in vitro transcription system employing ΔD111 DNA as template. In contrast the corresponding nucleotide triphosphate (5' Ara-ATP) is without effect on the system. These facts coupled with other kinetic studies support the suggestion that the pentose triphosphate moiety of the normal substrates are bound at the catalytic site of the polymerase in the 3' endo ribose conformation and require the 2' hydroxyl in the axial position.

INTRODUCTION

Kinetic studies concerning the specificity of substrate and inhibitor binding interactions at the catalytic surface of the E. coli RNA polymerase have suggested that the conformation of the pentose moiety is critical. Tight binding of substrates or competitive inhibitors requires either a 3 hydroxyl of the 2 endo conformation or a 2 hydroxyl of the 3 endo conformation where the configuration of the interactive hydroxyl is the same configuration as ribose. Productive catalysis of the bound pentose moiety is suggested to be constrainted to the 3 endo conformation where the 2 hydroxyl has the configuration of ribose. These initial studies (1,2) were reported for 5-ribose and 2 deoxy ribose polyphosphate analogues. This report is concerned with kinetic studies of the arabinose analogues, β,D-arabinofuranosyl-5-triphosphate (Ara-5-P_3) and 9-β,D arabinofuranosyl 5' triphosphate (Ara -ATP).

Ara-ATP and other analogues (Ara-CTP and Ara-GTP) have been shown to be potent inhibitors of DNA polymerase from various sources (3-7). Ara-CTP and Ara-ATP have been reported to inhibit E. coli RNA polymerase (8,9) by acting as premature chain terminators. The presence of degradation products such as Ara-5-P_3 and Ara-5-P_2 are common in many commercial preparations and are difficult to remove except by HPLC purification. The presence of these degradation products and their effect on the kinetics of various enzymes may be a complication in the comparison of results. A possible mode of

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therapeutic action in the case of Ara-NTP's may result from their degradation in vivo to Ara-5-P_3 or Ara-5-P_2 in which case they may function as potent inhibitors of RNA polymerase.

MATERIALS AND SYNTHESIS

Nucleoside triphosphates (HPLC purified) were obtained from I.C.N.; D arabinose-5-phosphate from Sigma Chemical Co. and [5,6 ^{3}H]UTP from New England Nuclear Corporation. The ΔD111 (a T_7 mutant containing the A_1 promoter) DNA was extracted from ΔD111 (obtained from J. Dunn, Brookhaven National Lab.) and purified through a cesium chloride gradient centrifugation. The RNA polymerase was isolated from E. coli K_{12} and purified by the method of Zillig (10). The enzyme had a specific activity of 300 mU/mg and had an σ/E mole ratio of 0.6.

D arabinofuranosyl-5-triphosphate was synthesized from D arabinofuranosyl-5-monophosphate by the water soluble carbodiimide condensation employing H_3PO_4 according to the method of Horecker, et al., for the phosphorylation of D-ribose-5'-triphosphate from D-ribose-5-monophosphate (11). The product peak isolated from the Dowex-1-Cl chromatography gave a single spot on TLC (acetate backed cellulose sheets, solvent N-propanol, NH_4OH, H_2O; 50:20:25). The rf values were: Ara-5-P (0.84) and Ara-5-P_3 (0.41) when visualized with an anisidine/phthalic acid spray reagent. A sample of Ara-5-P_3 was subjected to mild acid hydrolysis and analyzed for labile phosphate (12) and orcinol positive pentose (13) with the following results: mole ratio of labile phosphate/orcinol pentose = 2.03. A sample of the mild acid hydrolysis was chromatographed (as above) and showed a single spot corresponding to Ara-5-P.

KINETIC ASSAYS

An assay reaction mixture was prepared so that when 80λ of the mixture was diluted to a final volume of 100λ, the reaction was: 40 mM tris•HCl (pH 7.9), 0.1 mM EDTA, 7.5 mM β mercapto ethanol, 50 mM KCl, 10 mM $MgCl_2$, 0.4 mM in ATP, GTP, CTP and contained 1.2 μg ΔD111 DNA, and 0.56 μg of E. coli holoenzyme (σ/E = 0.6). The final reaction mixture also contained [5,6 ^{3}H]UTP (12-100 μM final concentration having 200 cpm/p mole) and either Ara-5'-ATP (at 0 or 50 or 500 μM final concentration) or Ara-5-P_3 (12-100 μM final concentration) as appropriate for a particular kinetic experiment. The reaction was initiated by combining the 80λ reaction mixture (preincubated at 37° for 5 minutes) with the 20λ increment containing UTP and the Ara derivative. The reaction was incubated for 10 minutes at 30°C and terminated by the addition of 1.0 ml of ice cold 10% trichloroacetic acid (TCA).

The samples were stored in ice until filtered through glass filter discs prewashed with cold 10% TCA. The discs containing the precipitate were washed three times each with 3.0 ml volumes of cold 10% TCA and finally with 2.0 ml of 95% ethanol, dried for 15 minutes at 100°C and then counted in a toluene based cocktail in a scintillation counter.

RESULTS AND DISCUSSION

The incorporation of [^{3}H]UMP into acid insoluble polymer during a 10 minute incubation was measured as a function of UTP concentration (over a concentration range 12-100 μM) with or without a constant concentration of Ara-5'-ATP (50 or 500 μM) and analyzed. No inhibition was observed over this range of Ara-5'-ATP concentration.

The inhibition of the incorporation of [^{3}H]UMP into acid insoluble polymer was also measured as a function of Ara-5-P_3 (over a concentration range 12-100 μM) at different fixed concentrations of [^{3}H]UTP. The data was analyzed

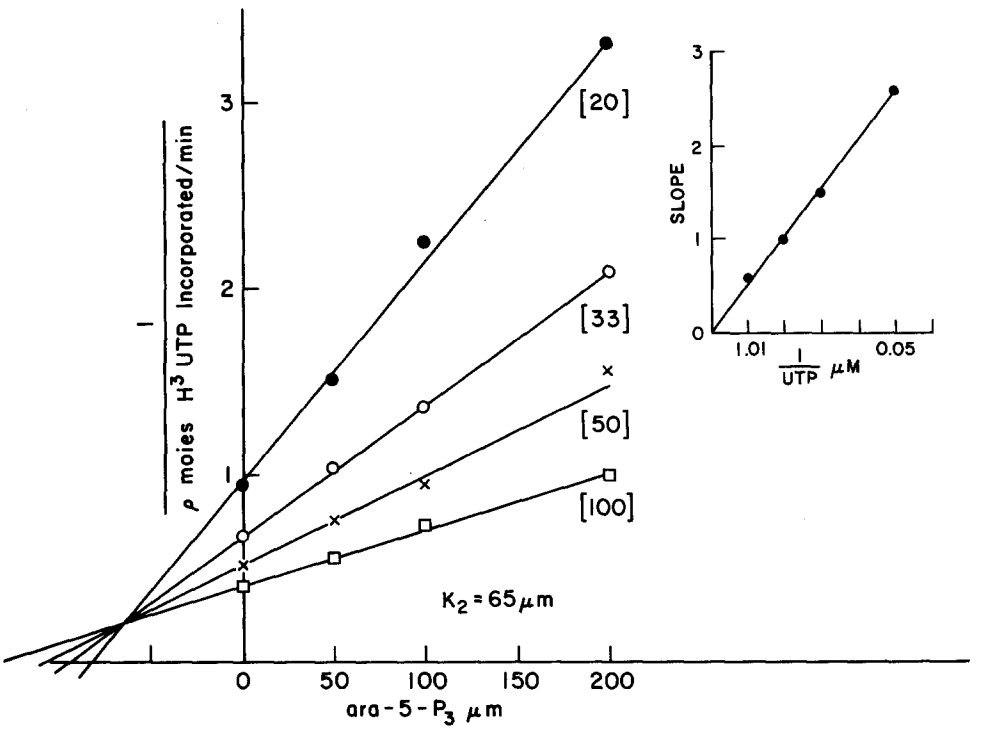
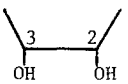
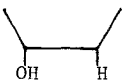
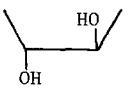
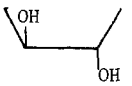
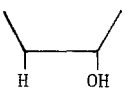
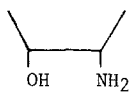
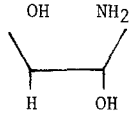


Figure 1. Inhibition by Ara-P_3. a) Dixon plot where [ ] is the fixed μM concentration of UTP. b) Insert for slope (a) vs. 1/[UTP].

by plotting 1/v vs. [Ara-5-P_3] at each concentration of [^{3}H]UTP as shown in Figure 1. A secondary plot of slope vs $[UTP]^{-1}$ was linear (14) therefore Ara-5-P_3 is a linear competitive inhibitor having a K_i = 60 μM.

The data presented in Table 1 support the hypothesis of Sylvester and Dennis (1) that the binding of the pentose polyphosphate moiety of nucleotide triphosphates (substrates as well as inhibitors) accounts for the major part of the interaction between these compounds and the E. coli polymerase. In detail the specificity of binding of the pentose polyphosphate moiety requires that either the 3 hydroxyl of the 2-endo conformation (see Figure 2), or the 2 hydroxyl of the 3 endo conformation, have the same configuration as ribose. Productive catalysis (formation of a phosphodiester bond between the bound 5 triphosphate and the pendant 3 hydroxyl of the growing RNA polymer) is suggested to be constrained to the 3 endo conformation where the 2 hydroxyl having the configuration of ribose is the interactive hydroxyl for binding. (For instance, [1-^{14}C] D ribose 5 triphosphate (2-endo) is not a substrate for the enzyme but is a potent competitive inhibitor.) The model assumes that the absence of the bulky base in position 1 of the pentose restricts the binding of the pentose-5-triphosphate series to the 2-endo conformation whereas the

Table 1. Kinetic Inhibition Studies Summary for Pentose-5-Triphosphates and/or Nucleopentose-5'-Triphosphates

D Pentose	2 and 3 hydroxyl conformation	K_i μM	
		Pentose•P_3	Nucleopentose•P_3
Ribose (1)		21	13 (K_m)
2d Ribose (1)		26	2800
Arabinose		60	>5000
Xylose (15)		--	37
3d Ribose (16)		--	18
2d 2 NH_2 Ribose (17)		--	>2000
3d 3 NH_2 Ribose (17)		--	2

presence of the bulky base in the nucleopentose-5-triphosphate series restricts the binding to the 3' endo conformation. Conformational restraints on binding are not to be confused with solution equilibria for these pentose conformations as a function of the position 1 substituents.

The D ribose-5-triphosphate is a potent competitive inhibitor as is 2d ribose-5-triphosphate since both possess the interactive 3 hydroxyl of the 2 endo conformation in the ribose configuration (axial). In contrast, 2'd

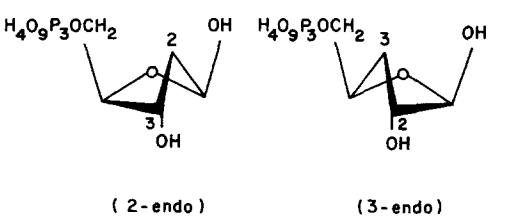


Figure 2. Two conformations of pentose moiety considered for binding at catalytic site.

nucleopentose triphosphate is neither a substrate nor effective inhibitor since the 3 endo conformation requires an axial 2' hydroxyl, and it is absent. Arabinose-5-triphosphate is an excellent competitive inhibitor having an axial 3 hydroxyl of the 2 endo conformation. In contrast, the Ara-ATP is not an effective inhibitor since the 3-endo conformation does not possess an axial 2' hydroxyl. Xylo-ATP is again a potent competitive inhibitor since it possesses an axial 2 hydroxyl of the 3'-endo conformation. The 3'd ATP (cordicepin triphosphate) is not only bound tightly, but is also a substrate (functions as a chain terminator) since it is bound in the 3 endo conformation using its axial 2' hydroxyl. Consistent with the observation that ribose-5-diphosphate is a potent competitive inhibitor (K_i = 26 μM), the diphosphate analogue of 3'd ribose (3d ADP) is not a substrate but is a good competitive inhibitor of the reaction (K_i = 37 μM in our studies, K_i = 106 μM in Bruzel (16)). 3d ADP is not a chain terminator as is the triphosphate. The 3' amino ATP is a good inhibitor of the enzyme whereas the 2 amino ATP is an ineffective inhibitor for the same reason as stated above.

The therapeutic effectiveness of Ara 5-NTP's has been suggested to be the inhibition of DNA polymerase at various stages and perhaps the inhibition of RNA polymerase. The presence of contaminants in non-HPLC purified commercial preparations of Ara-ATP and Ara-CTP (namely Ara-5-P_3 ~ 5-9%) indicate that caution be exercised in interpretation of the kinetic inhibition data. Our results with Ara-5-P_3 suggest that this agent might also be an effective therapeutic agent for neoplasias and viral infection in that transcription is effectively inhibited. Ara-5-P_3 may also prove to be an effective inhibitor of DNA polymerases.

ACKNOWLEDGEMENTS

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