PYRIDOXAL 5' PHOSPHATE: A SELECTIVE INHIBITOR OF ONCORNAVIRAL DNA POLYMERASES

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SUMMARY:

Pyridoxal 5' phosphate at concentrations < 0.5 mM inhibits polymerization of deoxynucleoside triphosphate catalysed by variety of DNA polymerases isolated from type C RNA tumor viruses, as well as E. coli, but does not affect the polymerase associated RNase H activity. Both phosphate and aldehyde groups of pyridoxal phosphate are essential for the inhibition which appears to be mediated through the reversible Schiff base.

INTRODUCTION:

During the characterization of purified reverse transcriptases from a variety of mammalian type C RNA tumor viruses, we discovered that inorganic phosphate was a strong inhibitor of catalysis of DNA synthesis (1 and submitted for publication). This observation led us to examine various phosphate containing compounds which may serve as anti-oncornaviral reverse transcriptase agents. During this search we observed that pyridoxal 5'-phosphate (PyP)* was a strong inhibitor of all of the reverse transcriptases examined. The specificity of this inhibition is shown by the fact that neither pyridoxal nor pyridoxamine phosphate are effective inhibitors. The inhibitory effect of PyP on the prokaryotic as well as eukaryotic RNA polymerase(s) has been fairly well documented (2-4). However, this is the first time that the effect of pyridoxal phosphate on DNA polymerases has been demonstrated. Furthermore, the inhibition of DNA polymerases appear to be highly specific since it can be conveniently monitored in high Tris buffer background unlike that observed with RNA polymerase(s). The

* Abbreviations used: Pyridoxal 5' phosphate - PyP; AMV - Avian Myeloblastosis virus; RLV - Rauscher leukemia virus; FeLV - Feline leukemia virus; SSV - Simian sarcoma virus.

result of this investigation and possible implications are reported in this communication.

MATERIALS AND METHODS:

(a) Materials: All radioactive deoxyribonucleoside triphosphates were obtained from Amersham-Searle, Inc. Unlabelled triphosphates and template-primers were products of P.L. Biochemicals, Inc. The molar ratio of template to primer was 1:1 in case of poly(rA)·$(dT)_{10}$. Pyridoxine HCl, pyridoxal, pyridoxal 5' phosphate, pyridoxamine and pyridoxamine 5' phosphate were obtained from Sigma Chemical Company. All other chemicals were of analytical grade.

(b) Enzymes: Avian Myeloblastosis Virus (AMV) and Rauscher Leukemia Virus (RLV), Feline Leukemia Virus (FeLV) and Simian Sarcoma Virus (SSV) DNA polymerases used in these studies were highly purified (> 95% as judged by SDS-polyacrylamide gel electrophoresis) using affinity chromatography on polycytidylate agarose columns (5). The details of the purification procedures used to obtain these enzymes together with their characterization will appear elsewhere. Briefly, the concentrated virus suspension (approximately 2-5 mg protein) is treated with high salt and non-ionic detergent in the presence of dithiothreitol to solubilize the virion-associated RNA dependent DNA polymerase. The soluble extract, after dilution with buffer, is passed through a Pasteur pipette column of poly rC-agarose, washed and the enzyme is eluted either with a linear salt gradient or 0.4 M salt. Enzyme is stored in a buffer containing 1% serum albumin, 20 mM Tris pH 7.8, 5 mM dithiothreitol and 20% glycerol at -70°. Under these conditions enzymes are quite stable. Homogeneous DNA polymerase I from E. coli was generously provided by Dr. L.A. Loeb of Fox Chase Institute for Cancer Research.

(c) DNA Polymerase Assays: Enzyme reactions were carried out in a final volume of 100 microliters and contained the following components: 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 50 µg bovine serum albumin, 20 µM tritiated TTP, adjusted to a final specific activity of 1,000 cpm per picomole, and 0.5 µg of template-primer. The type of divalent cation (Mg^{2+} or Mn^{2+}) and its concentration (6) is given in the legend to tables.

Incubations, unless indicated otherwise, were carried out at 37°C for 30 minutes and were terminated by addition of 5% (w/v) trichloroacetic acid containing 0.01 M sodium pyrophosphate. The acid insoluble material was collected on Whatman GF/B filters, washed extensively with TCA-containing Na-pyrophosphate, water, and finally ethanol, dried and counted in toluene-based scintillation fluid.

(d) RNase H Assay: The substrate used for detection of RNase H activity was øX174 DNA-(^{3}H)-RNA hybrid synthesized as described by Verma (7) with the following modifications. The reaction mixture contained, in a final volume of 1.0 ml, 80 mM Tris-HCl, pH 7.8, 50 mM KCl, 10 mM DTT, 10 mM $MgCl_2$, 100 µg of øX174 DNA (Miles Laboratories), 1 mM each ATP, CTP, and UTP, 20µM of (^{3}H)-GTP (3,000 cpm per picomole) and approximately 100 µg of homogeneous E. coli RNA polymerase. After 1.5 hr of incubation at 37°C, the reaction mixture was placed in a 70°C water bath for 15 min, and quickly cooled in an ice bath. RNA-DNA hybrids were purified by isopycnic banding in cesium sulfate (8), and desalted by chromatography on a Sephadex G-50 column. The reaction mixture for assay of RNase H activity contained, in a volume of 0.1 ml 20 mM Tris-HCl, pH 7.8, 10 mM DTT, 150 mM KCl, 0.02% (w/v) bovine serum albumin, 12 picomole of øX174 DNA-(^{3}H)-RNA hybrid (as total nucleotides), and 1 mM Mn^{++}. Reactions were initiated by the addition of enzyme fraction and were incubated for various times at 37°C. Reactions were terminated by the addition of trichloroacetic acid, and acid-insoluble counts determined as described in the DNA polymerase assay.

TABLE I

Effect of Pyridoxal 5' phosphate on the Synthetic (polymerization) and Nucleolytic (RNase H) Activity of DNA Polymerase(s) from AMV, RLV, FeLV and SSV and E. coli DNA Polymerase I

Enzyme Source	Poly rA·dT_{10}-directed TTP incorp. (pmole)		Degradation of ^{3}H-RNA:øX174 DNA hybrid (acid insoluble cpm)	
	Control	+ PyP	Control	+ PyP
None	--	--	9,800	9,800
AMV	68	11.0	3,650	3,691
RLV	20.9	3.1	6,200	6,150
RLV*	120.0	16.8	600	620
FeLV	43.2	6.8	5,860	5,920
FeLV*	88.0	12.6	5,660	5,700
SSV	6.9	0.9	7,120	7,060
E. coli DNA pol.I	170.0	30.0	4,130	4,180

For AMV and E. coli enzyme, $MgCl_2$ was used at 5mM, while for the remaining enzymes $MnCl_2$ was the divalent cation at the final concentration of 1mM.

* In this experiment NP·40 disrupted virions were used as an enzyme source in place of purified enzyme.

RESULTS:

Effect of Pyridoxal 5' phosphate on DNA Polymerase and RNase H: Effect of pyridoxal 5' phosphate on the polymerase and RNase H activity of various DNA polymerases from oncornaviruses and E. coli is shown in Table 1. It is clear from the table that all of the polymerases tested are severely inhibited (∿ 80-85%) as measured by TTP incorporation but are totally unaffected when their associated RNase H activity was monitored. Furthermore, the inhibition was unaffected by the type of divalent cation used. A typical time course of this inhibitory effect of PyP is described in Fig. 1 using RLV and FeLV DNA

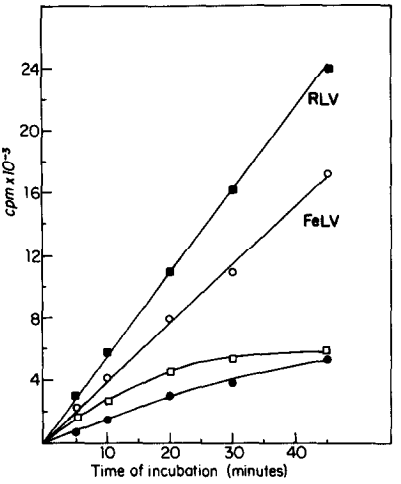

Fig. 1: Time course of TTP incorporation in the presence and absence of 0.2 mM pyridoxal 5' phosphate of a reaction catalysed by RLV and FeLV DNA polymerase and directed by poly(rA)·$(dT)_{10}$. RLV control (■—■); RLV in the presence of PyP (□—□); FeLV control (o—o); FeLV in the presence of PyP (●—●).

TABLE 2

Effect of Different Pyridoxine Derivatives on the poly rA·dT_{10}-Directed Synthesis Catalyzed by Various DNA Polymerases.

Enzyme Source	pmole TTP Incorporation in the Presence of				
	None (Control)	Pyridoxal 5'phosphate (0.2mM)	Pyridoxal (2mM)	Pyridoxamine or Pyridoxine (2mM)	Pyridoxamine 5'phosphate (2mM)
AMV	38	9.6	36.0	38.1	36.0
RLV	30	6.6	28.0	29.6	26.5
FeLV	42	9.2	39.8	41.5	38.0
SSV	12	3.1	10.1	11.0	10.3
E. coli DNA polymerase I	80	23.1	77.0	79.3	76.6

Assays were as described in legend to Table 1.

polymerase as representatives of reverse transcriptases.

Specificity of Inhibition: In order to determine the specificity of inhibition by pyridoxal phosphate, four other related compounds were tested for their inhibitory effect on the various DNA polymerases. The results are summarized in Table 2. Of the different pyridoxine compounds only pyridoxal 5' phosphate is effective whereas pyridoxal, pyridoxine, pyridoxamine or pyridoxamine 5' phosphate at 10 times the concentration of PyP exhibit very little inhibitory effect.

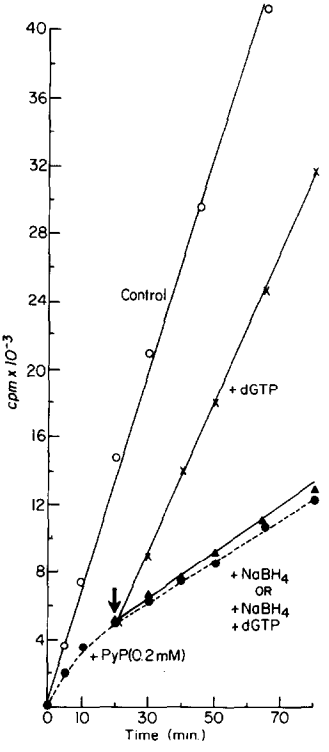

Fig. 2: Kinetics of reversal of inhibition of dTTP incorporation by pyridoxal phosphate. Rates of control reaction are shown by (o——o) while the rates of synthesis in the presence of PyP (0.2 mM) is denoted by (●——●). At 20 min (position shown by arrow) the reaction mixture containing PyP was divided in 3 parts. One part received dGTP at the final concentration of 1 mM (x——x), the second part received $NaBH_4$ to the final concentration of 1 mM (▲——▲) and to the third part both dGTP and $NaBH_4$ (final concentration 1 mM each) were added (▲——▲). Aliquots of various reaction mixtures were removed at desired time and synthesis of poly(dT) monitored as described in Materials and Methods. The addition of both $NaBH_4$ and dGTP to control reaction (absence of PyP) either individually or in combination has no effect on the incorporation of dTTP.

Kinetic Study, Reversal of Inhibition and Nature of Binding: As shown in Fig. 1 rates of DNA synthesis under our assay conditions are linear for at least 1 hr in the absence of PyP. Addition of PyP prior to or following initiation of synthesis (data not shown) results in an immediate reduction in the rate of DNA synthesis. On the other hand, addition of excess deoxyribonucleoside triphosphate to a reaction mixture containing PyP promptly reduces the inhibitory effect (Fig. 2). It, therefore, appears that the binding of PyP to enzyme protein is quite reversible. In order to understand the nature of binding of PyP to enzyme protein, sodium borohydride was added to a reaction proceeding in the presence of PyP. A subsequent addition of deoxyribonucleoside triphosphate failed to reverse the inhibition implying a permanent binding (irreversible) between PyP and enzyme protein via the Schiff base.

DISCUSSION:

A specific inhibitory effect of PyP on the catalysis of polymerization reaction but not on the nucleolytic function of type C reverse transcriptases indicates that the two sites are mechanistically independent. Brewer and Wells (9) have previously demonstrated similar mechanistic independence based on inhibition of RNase H activity of AMV reverse transcriptase in the presence of fluoride ions. The specificity of inhibition expressed by pyridoxal phosphate is remarkable in that both aldehyde and phosphate groups are required for inhibition. A similar specificity of inhibition has also been shown to exist for prokaryotic as well as eukaryotic RNA polymerases (2-4). The apparent mode of inhibition in this case was attributed to Schiff base formation between the aldehyde group of PyP and the ε-amino group of lysine, presumably located at the active center of these enzymes. The inhibition of DNA polymerases reported herein, also appears to be mediated through reversible Schiff base formation between PyP and lysine and/or arginine residue(s) present at the substrate binding site. Furthermore, analogous to the RNA polymerase system, substrate triphosphates are also shown to reverse the inhibitory effect of PyP at least partly (Fig. 2). However, the

affinity of lysine/arginine residue(s) present at the active site of DNA polymerases seems to be much greater than that shown by RNA polymerases for the following reason: Tris buffer (up to 100 mM) used in the reaction mixture or addition of 20 mM lysine does not protect the enzyme against PyP inhibition. A protective effect of these compounds (due to the availability of free NH_2 groups to form Schiff base with PyP) has been demonstrated in the RNA polymerase system. The evidence for Schiff base formation at the active site is provided by the fact that addition of $NaBH_4$ to the enzyme-PyP complex prevents the normal reversal of inhibition by subsequent addition of deoxyribonucleoside triphosphate (Fig. 2). The presence of lysine residue at the catalytic site of E. coli DNA polymerase I has been shown by Jovin et al. (10). Recently, Salvo et al. (11), using affinity labeling techniques, have demonstrated the presence of both lysine and arginine at the active center of the same enzyme. Similarly, the occurrence of arginine residues at the active site of several oncornaviral DNA polymerases has been reported by Borders et al. (12). The affinity of PyP for these residues present at the active site of DNA polymerases provides a useful reagent for the dissection of the structure and probably the sequence of amino acids at the active site of these enzymes.

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