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Pyrophosphate analogues in pyrophosphorolysis reaction catalyzed by DNA polymerases

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It is demonstrated here that rat liver DNA polymerase β catalyzes the pyrophosphorolysis reaction with pyrophosphate (PP_i) and its analogues. The substrate specificity of the PP_i-binding site of several DNA polymerases was investigated. It was discovered that the ability of DNA polymerases to utilize PP_i analogues instead of PP_i in the pyrophosphorolysis reaction was markedly restricted. Only imidodiphosphate and methylenediphosphonate were demonstrated as participating in this process. Oxodiphosphonate and phosphonoformate inhibited DNA synthesis, but probably not via the interaction with the PP_i-binding site of DNA polymerases.

Pyrophosphorolysis; DNA polymerase; Pyrophosphate analog

1. INTRODUCTION

DNA polymerases catalyze the elongation of DNA chains. This reaction is reversible for several DNA polymerases, the shortening of DNA chains taking place when PP_i (instead of deoxynucleoside 5′-triphosphates, dNTPs) is added to the reaction mixture, and dNTP being formed in this case. The PP_i-binding properties of these enzymes have practically not been investigated. It has been reported only that PP_i inhibits DNA chain elongation non-competitively towards dNTPs in the case of calf thymus DNA polymerase α and I from *E. coli*, whereas the inhibition is competitive for calf thymus DNA polymerase β [1].

Here, we describe an investigation of the substrate specificity of DNA polymerases α (from calf thymus), β (from rat liver), I Klenow fragment (I KF) from *E. coli* and AMV reverse transcriptase (RT) in the pyrophosphorolysis reaction. Several PP_i analogues differing in electronic and conformational properties were tested of which a number had been shown previously to be substrates of the pyrophosphorolysis reaction catalyzed by *E. coli* RNA polymerase [2,3].

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2. MATERIALS AND METHODS

The PP_i analogues used here were imidodiphosphate (I), methylenediphosphonate (II), phosphonoacetate (IX) and phosphonoformate (XI) (Sigma). Oxodiphosphonate (III) and derivatives of methylenediphosphonate and phosphonoacetate (IV–VIII, X) were prepared as in [4–6]. We used [α-^{32}P]dNTPs (1000–3000 Ci/mmol; Isotop, USSR), PP_i (tetrasodium salt; Sigma), dNTP (Sigma), Biogel A1.5m (Bio-Rad), PEI-cellulose plates (Merck) and DE-81 filters (Whatman). Isolation of M13mp10 phage DNA was according to [7], and that of DNA polymerase I KF from *E. coli* CJ 155, α, β and RT as described in [8–11], respectively. d(CCCAGTCACGACCT) was a kind gift from Dr A. Azhayev. Enzyme homogeneity as judged by data obtained on electrophoresis was 99% or greater. The ^{32}P-labeled (−)-chain of DNA was synthesized as in [8]. [^{32}P]DNA hydrolysis and pyrophosphorolysis by DNA polymerase I KF were carried out in an incubation mixture (5 μl) containing 50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 10 mM $MgCl_2$, 50 mM KCl, 0.5 mM mercaptoethanol, 10–20 μg/ml of [^{32}P]-DNA, 1.5–3 activity units of enzyme at 25°C and 2–4 mM PP_i

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Table 1

Substrate specificity of DNA polymerases in the pyrophosphorolysis reaction

Compounds	Symbol	DNA polymerases		*E. coli* RNA polymerase [2,3]
		I KF and α	β and RT	
$(HO)_2(O)PNHP(O)(OH)_2$	I	+	+	+
$(HO)_2(O)PCH_2P(O)(OH)_2$	II	–	+	+
$(HO)_2(O)PC(O)P(O)(OH)_2$	III	–	–	+
$(HO)_2(O)PCBr_2P(O)(OH)_2$	IV	–	–	
$(HO)_2(O)PCH(COCH_3)P(O)(O-H)_2$	V	–	–	
$(HO)_2(O)PCH(COOC_2H_5)P(O)(OH)_2$	VI	–	–	
$(HO)_2(O)PCH(CH_2COOC_2H_5)P(O)(OH)_2$	VII	–	–	–
$(CH_3)(OH)(O)PCH_2P(O)(OH)_2$	VIII	–	–	–
$(HO)_2(O)PCH_2COOH$	IX	–	–	+
$(HO)_2(O)PCHBrCOOH$	X	–	–	
$(HO)_2(O)PCOOH$	XI	–	–	

or analogue in the case of the pyrophosphorolysis reaction. Pyrophosphorolysis in the case of DNA polymerase α, β and RT was performed in an incubation mixture (5 μl) containing 50 mM Tris-HCl (pH 8.3; pH 7.5 for α), 10 mM $MgCl_2$ (5 mM for α), 5 mM mercaptoethanol, 100 mM KCl for β and RT, 10–20 μg/ml of [^{32}P]DNA, 2–4 mM (optimal concentration) PP_i or analogue and 2–3 activity units of enzyme at 25°C. All reactions were stopped by addition of 50 mM EDTA and chromatography on PEI-cellulose plates was according to [2,3,8].

3. RESULTS

The structures of PP_i analogues are shown in table 1. Fig.1 demonstrates the results obtained on the pyrophosphorolysis reaction catalyzed by RT:

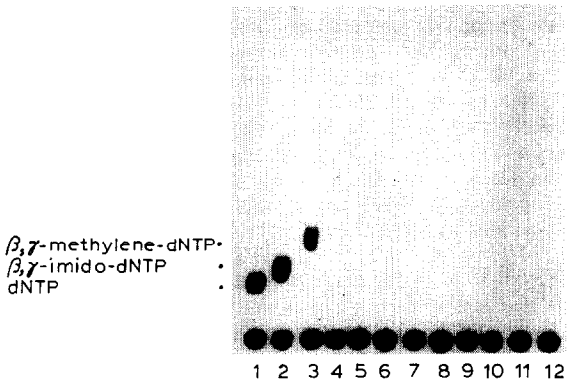

Fig.1. Pyrophosphorolysis of M13mp10 DNA by RT (3 h, 22°C) in the presence of PP_i (track 1) and its analogues I (2), II (3), III–XI (4–12); M13mp10 DNA, 10 μg/ml; PP_i and I–XI, 2 mM.

dNTPs and their analogues arising after the action of PP_i and PP_i analogues. Apart from the initial spots, new radioactive spots were found only for imidodiphosphate (I) and methylenediphosphonate (II). These spots comigrated with β,γ-imido-dATP and β,γ-methylene-dATP, respectively.

Although other PP_i analogues were inactive as regards pyrophosphorolysis, some were able to inhibit DNA synthesis effectively. Inhibition of DNA

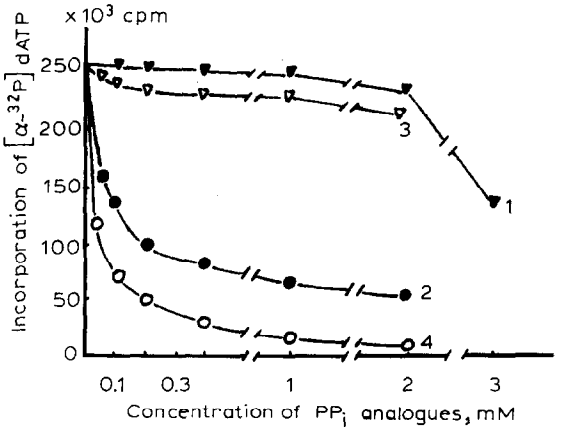

Fig.2. Inhibition of M13mp10 DNA elongation with RT by PP_i (curve 1), III (2), IX (3) and XI (4). DNA, 35 μg/ml; dATP, dGTP, dTTP, 100 μM each; [α-^{32}P]dCTP, 2 μM. Incubation mixture (5 μl) contained RT (1.5 activity units), 50 mM Tris-HCl (pH 8.3), 100 mM KCl, 10 mM $MgCl_2$ and 5 mM mercaptoethanol (15 min, 38°C). Reactions were stopped by addition of 50 mM EDTA, the reaction mixtures passed through DE-81 filters, and the filters washed with 0.2 M NaCl solution plus 5 mM EDTA.

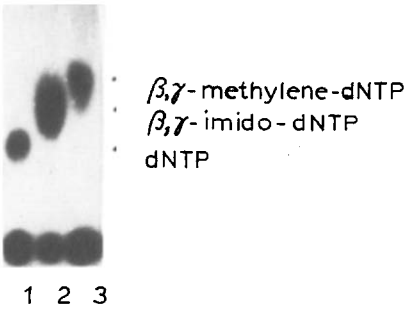

Fig.3. Pyrophosphorolysis of M13mp10 DNA by DNA polymerase β (3 h, 22°C) in the presence of PP_i (track 1) and its analogues I (2) and II (3); DNA, 10 μg/ml; PP_i, I, II, 2 mM.

chain elongation catalyzed by RT in the presence of PP_i and three analogues is depicted in fig.2. One can see that III (curve 2) and the well-known inhibitor of DNA synthesis XI (curve 4) inhibited the reaction at concentrations 50-times lower than those for PP_i (curve 1).

Fig.3 illustrates the results for DNA polymerase β. This enzyme also catalyzes the pyrophosphorolysis reaction with PP_i, I and II (not shown for inactive analogues). DNA polymerases α and I KF catalyze pyrophosphorolysis only with PP_i and I (not shown).

We studied the ability of substances inactive as substrates of pyrophosphorolysis to inhibit the pyrophosphorolysis reaction. As shown in fig.4, the quantity of products of the pyrophosphorolysis reaction remained practically unchanged even at a 5-fold excess in concentration of reagents II–V or IX over PP_i, however marked inhibition of 3′→5′-exonuclease hydrolysis was observed, depending on the concentration of analogues (tracks 4–6, 7–8, 9–11, 12–14, 15–17: corresponding to compounds II, III, IV, V, IX). Analogues III, IX and XI did not inhibit RT-catalyzed pyrophosphorolysis of DNA (not shown).

In addition to the spots of deoxymono- and triphosphates, an unknown byproduct resulting from the above reaction was found (fig.4). Its mobility corresponds to that of NDP. [^{32}P]Orthophosphate was obtained when the reaction mixture was incubated with alkaline phosphatase after pyrophosphorolysis. This unknown compound was observed solely in the case of pyrophosphorolysis, and did not appear during 3′→5′-exonuclease hydrolysis in the absence of PP_i. It was found during neither prolonged incubation of [α-^{32}P]dNTPs with enzyme nor pyrophosphorolysis catalyzed by RT or DNA polymerases α and β. The structure and mechanism of formation of this product remain unelucidated.

4. DISCUSSION

Here, we have shown that rat liver DNA polymerase β is able to catalyze pyrophosphorolysis of DNA. In other studies [12–14], this property has not been reported for polymerase β, perhaps because of the low specific activity of dNTPs (1–2 Ci/mmol) for DNA labeling, whereas in our

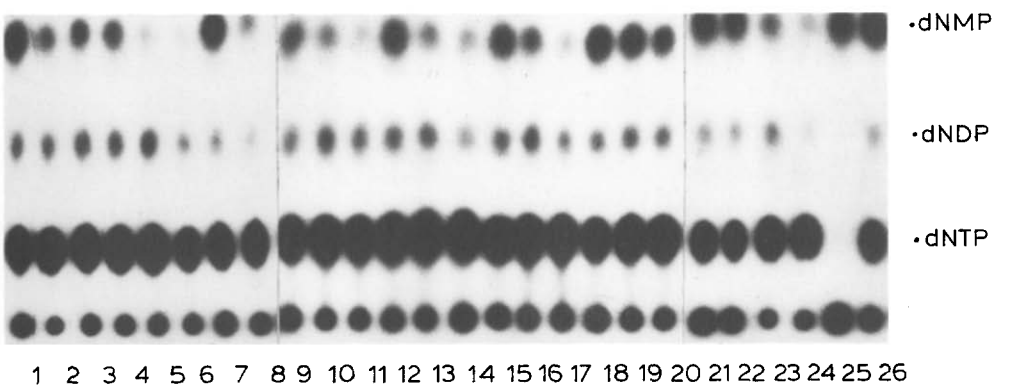

Fig.4. Pyrophosphorolysis of M13mp10 DNA by DNA polymerase I KF (5 h, 23°C) with simultaneous addition to the incubation mixture of 2 mM PP_i and of PP_i analogues: VIII (tracks 1–3), II (4–6), III (7–8), IX (9–11), IV (12–14), V (15–17), XI (18–20). DNA, 6 μg/ml; PP_i analogues, 2 mM (tracks 1,4,7, 9,12,15,18), 5 mM (2,5,8,10,13,16,19), 10 mM (3,6,11,14,17, 20); tracks 21–24 were PP_i controls: 2 mM (21,22), 5 mM (23), 10 mM (24); (25) incubation in the absence of PP_i and other analogues; (26) incubation in the presence of 2 mM PP_i and 50 mM KCl. Reference compounds are shown in the right-hand panel.

experiments dNTPs of specific activity 1000–3000 Ci/mmol were employed.

The substrate specificity of the four DNA polymerases investigated with respect to PP_i analogues is comparatively greater than that of *E. coli* RNA polymerase [2]. Among the DNA polymerases studied, the most specific are enzymes α and I KF catalyzing the pyrophosphorolysis reaction with PP_i and imidodiphosphate (I), the analogue bearing the greatest similarity with PP_i. Enzymes β and RT can involve PP_i, I and also methylenediphosphonate (II) in this reaction. Oxodiphosphonate (III) and phosphonoformate (XI) inhibit RT-catalyzed DNA synthesis more effectively than PP_i. Taking into account the fact that these analogues are neither substrates nor inhibitors of the pyrophosphorolysis reaction, it is possible that they do not interact with the PP_i-binding site, since their mechanism of inhibition is unknown.

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