

Interaction of dNTP, pyrophosphate and their analogs with the dNTP-binding sites of *E. coli* DNA polymerase I Klenow fragment and human DNA polymerase α

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The 3',5'-exonuclease center of the Klenow fragment of *E. coli* DNA polymerase I (FK) was selectively blocked by NaF. The latter was shown to forbid the binding of nucleotides and their analogs to the enzyme exonuclease center. In the presence of poly(dT)·r(pA)₁₀ template·primer complex and NaF, we observed AMP, ADP, ATP, PP_i and dATP to be competitive inhibitors of the FK-catalyzed DNA polymerization. The interactions of the nucleotides with FK and human DNA polymerase α were compared to reveal similarity of binding to the DNA polymerizing centers. Structural components of dNTP and PP_i playing key roles in forming complexes with pro- and eukaryotic DNA polymerases were identified.

Klenow fragment; Human DNA polymerase α ; Interaction with nucleotides

1. INTRODUCTION

According to the data of [1-3], the DNA polymerizing center of *E. coli* DNA polymerase I cannot interact with dNMP or NMP. This conclusion was based on two reasons: equilibrium dialysis of the enzyme complexes with nucleotides in the absence of template and primer [1]; and either absence of inhibition or activation of polymerization catalyzed by this enzyme in the presence of the nucleotides. However, the results of chemical and affinity modifications of both FK and DPH [4-6] testified to the effective formation of complexes between the center for DNA synthesis of these enzymes and varied ribo- and deoxyribonucleoside-5'-mono- and diphosphates. The data of [7] prove that the complex formed by the FK exonuclease center and NMP or NaF activates polymerization because the primer is preferentially coupled to the polymerizing center of the enzyme. Our results suggest competition between NMP and dNTP for the polymerizing center which is concealed, in the case of FK, by activation of polymerization due to the interaction of the nucleotide with the exonuclease center of the enzyme.

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Abbreviations: FK, Klenow fragment of *E. coli* DNA polymerase I; DPH, DNA polymerase α from human placenta

2. MATERIALS AND METHODS

Electrophoretically homogeneous FK (3.7×10^4 U/mg) and DPH (8.5×10^3 U/mg) were purified according to [8] and [9], respectively. All nucleotides and poly(dT) were from Nikti Bav (USSR); MgCl₂ and MnCl₂ were from Merck; phosphoroacetic acid and triphosphosphate from Serva; BSA from Koch Light; [³H]dATP (900 TBq/mol) from Izotop (USSR). Other reagents were of analytical grade.

Syntheses of d(pA)₁₀ and r(pA)₁₀ were described in [10,11]. Nucleotides and their analogs were thin-layer-chromatographed on F₂₅₄ Kieselgel 60 disks (Merck) prior to use as described in [7].

All analogs of pyrophosphate employed were synthesized according to the methods described in [12-15].

FK and DPH were tested for activity at 37°C as in [7]. Reaction mixtures (50-100 μ l) contained 50 mM Tris-HCl buffer (pH 7.5), 0.1-0.5 mg/ml BSA, 40 mM KCl, 1 mM EDTA, 5 mM MgCl₂ (10 mM for FK), 3 units of A₂₇₀/ml poly(dT) (1 unit of A₂₇₀/ml for FK); 20 μ M r(pA)₁₀ and [³H]dATP at various concentrations. The NaF concentration was varied between 0 and 10 mM. In the experiments on the polymerization rate it was 5 mM. A wide range of concentrations of the ligands was used.

K_m for dATP were determined using the Eisenthal and Cornish-Bowden linear plot [16]; K_i using dependencies I/V versus I and S/V versus I according to [17].

3. RESULTS AND DISCUSSION

A selective inhibitor of the FK exonuclease activity (NaF) [18], when added to the reaction mixture, prevents the formation of a complex between the 3'-end of the primer and the exonuclease center of the enzyme [7]. The effect of AMP and NaF is analogous: a 1.4-3-fold increase in the initial rate of polymerization. NaF decreases the effect of AMP. A typical inhibition curve was obtained for poly(dT)·r(pA)₁₀ complex at 5 mM concentration of NaF (Fig. 1). Based on the

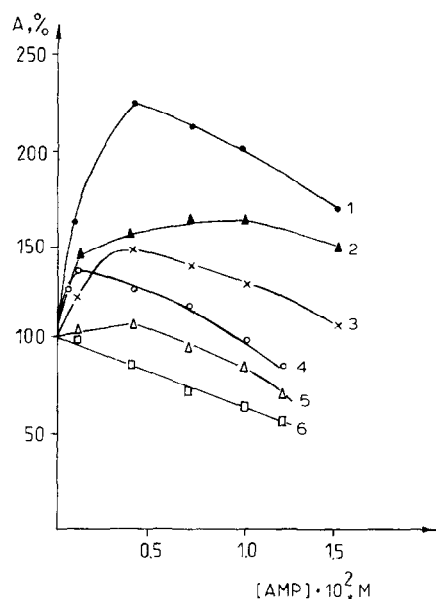


Fig. 1. Initial rate of FK-catalyzed polymerization versus concentration of AMP at various concentrations of NaF. Template: poly(dT); primers: d(pA)₁₀ – curves 1–3; and r(pA)₁₀ – curves 4–6. Without NaF – curves 1 and 4; at 1 mM NaF – 2 and 5; at 5 mM NaF – 3 and 6; 100% correspond to the rates of $2.5\text{--}5 \times 10^3$ (1–3) and $6.5\text{--}13 \times 10^3$ (4–6) cpm/min.

above results, we suggest the AMP-mediated activation of the enzyme to be a sum of two interactions: AMP + exonuclease center and AMP + dNTP-binding center of FK. Having blocked the FK exonuclease center with NaF (5 mM), one can reveal the inhibition of polymerization to be competitive towards dATP (Fig. 2). AMP can be a competitive inhibitor of the DNA synthesis when the exonuclease center is blocked with AMP. In this case, K_i is equal to 6.8 mM which is very close to the value obtained for AMP in the presence of NaF (7.9 mM).

Blocking of the FK exonuclease center with NaF makes it possible to study the interaction of the dNTP-binding site of FK with nucleotides, PP_i and their analogs irrespective of their interactions with the exonuclease center of the enzyme. Table I summarizes the results on the effect of several nucleotides and their analogs on the rate of FK-catalyzed polymerization (in the presence of 5 mM NaF) and DPH-catalyzed reactions.

Having compared the K_i for NMP, NDP and NTP, we showed that the effect of β -phosphate of nucleotides on the binding to FK and DPH is negligible. In the row AMP \searrow CTP \searrow carboxymethylphosphonyl-5'-AMP \searrow ATP, the nucleotide affinity for FK grows. The same regularity was observed for DPH. These data favour the formation of an additional contact between the γ -phosphate of nucleotides and enzymes, which is in agreement with [4]. It is likely that the carboxyl group can act to a certain extent as NTP γ -phosphate.

It is common knowledge that PP_i does not compete with dNTP for the active sites of *E. coli* DNA

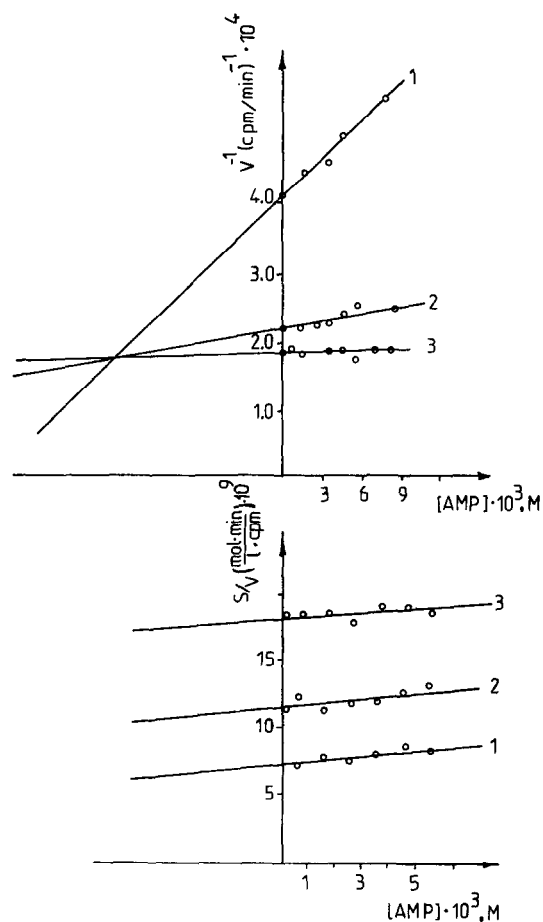


Fig. 2. Initial rate of FK-catalyzed polymerization versus concentration of AMP at various concentrations of dATP in the presence of 5 mM NaF for poly(dT) · r(pA)₁₀ template · primer complex in coordinates: (a) $1/V$ versus I ; and (b) S/V versus I . Curves 1, 2 and 3 correspond to 17, 50 and 100 μ M concentrations of dATP, respectively.

polymerase I and calf thymus DNA polymerase α [1,19]. However, this conclusion for the first enzyme was based on the results of equilibrium dialysis without template and primer. For the second enzyme, the type of inhibition of PP_i polymerization depended on the concentrations of the pyrophosphate. At certain concentrations, PP_i became a competitive inhibitor of DNA polymerization towards dNTP.

As shown in Table I, PP_i, PPP_i and their analogs are inhibitors of FK and DPH-catalyzed DNA polymerization competitive towards dATP when the exonuclease center of FK is blocked. Evidently, one of the PP_i phosphates can be replaced by carboxyl group. Simultaneous interaction of the two phosphates (or imitating them, carboxyls) with the enzyme within one and the same molecule of the ligand must be conditioned by a fixed distance between them. When this distance is shortened (in FPA instead of PAA) or extended (in the case of di- and trimethylenedicarbon acids), the type of inhibition changes. When ethyl groups are attached to the oxygen atoms of PP_i or PPA, the affinity is reduced, thus proving the importance of the PP_i molecule's negative charges in reactions with polymerases.

Table I

The K_i values for the ligands in the reactions of DNA polymerization catalyzed by Klenow fragment and DNA polymerase α from human placenta in the presence of poly(dT) template and r(pA)₁₀ primer

Ligand	FK		DPH	
	K_i^a (mM)	Type of inhibition ^b	K_i (mM)	Type of inhibition ^b
dATP	2.7×10^{-2}		1.7×10^{-2}	
ATP	0.14	competitive	0.49	competitive
AMP-5'-carboxymethylphosphonyl	0.38	competitive	0.76	competitive
CTP	0.53	competitive	0.18	competitive
ADP	3.0	competitive	2.5	competitive
AMP	7.8	competitive	3.3	competitive
Pyrophosphate	0.39	competitive	0.64	competitive
Inorganic triphosphate	0.36	competitive	0.76	competitive
Orthophosphate	10.0	non-competitive	14	competitive
Methylenediphosphonate (MDP)	0.24	competitive	1.2	mixed
Methylphosphinomethylenephosphonate	30	competitive	7.0	mixed
Simm. diethyl ester of MDP	51	competitive	46	mixed
Tetraethyl ester of MDP	58	competitive	930	competitive
Phosphonoformic acid (PFA)	2.5	non-competitive	0.047	mixed
Phosphonoacetic acid (PAA)	0.14	competitive	0.022	mixed
C-ethyl ester of PPA	3.2	competitive	0.6	mixed
Triethyl ester of PPA	280	competitive	330	mixed
Acetic acid	19	competitive	330	mixed
Methylenedicarbonate	6.6	competitive	—	—
Dimethylenedicarbonate	7.1	competitive	—	—
Trimethylenedicarbonate	8.2	competitive	—	—

^aThe error of constant calculation was within 20–50%

^bInhibition of polymerization with respect to dATP

^c K_m values for dATP

The observed non-competitive inhibition in PP_i analogs may be due to the existence in DPH of additional sites recognizing phosphate-bearing ligands and the enhanced affinity of the latter to these sites. The results obtained suggest that both phosphates of the PP_i molecule should make an equal contribution to the ligand affinity for the enzymes. This value is close to the K_d value of $2-3 \times 10^{-2}$ M.

Based on the sum of our data, we assumed that the β -phosphate group belonging to the PP_i fragment of dNTP did not react with the enzymes and formed an additional contact only after a free pyrophosphate molecule was formed.

In spite of the differences observed in the interactions of nucleotides, PP_i and their analogs with FK and DPH, these processes have a great deal in common.

REFERENCES

- [1] Englund, P.T., Huberman, J.A., Jovin, T.M. and Kornberg, A. (1969) *J. Biol. Chem.* 244, 3038–3044.
- [2] Quc, B.G., Downey, K.M. and So, A.G. (1978) *Biochemistry* 17, 1603–1606.
- [3] Huberman, J.A. and Kornberg, A. (1970) *J. Biol. Chem.* 245, 5326–5334.
- [4] Nevinsky, G.A., Doronin, S.V., Podust, V.N. and Lavrik, O.I. (1987) *Mol. Biol.* 21, 1070–1079.
- [5] Lavrik, O.I., Nevinsky, G.A., Potapova, I.A. and Khomov, V.V. (1988) *Mol. Biol.* 22, 485–492.
- [6] Lavrik, O.I., Nevinsky, G.A., Potapova, I.A., Tarusova, N.B. and Khalabuda, O.V. (1989) *Molek. Biol.* 23, 400–408.
- [7] Nevinsky, G.A., Potapova, I.A., Tarusova, N.B., Khalabuda, O.V. and Khomov, V.V. (1990) *Molek. Biol.* 24, 104–116.
- [8] Khomov, V.V., Zagrebelny, S.N., Legostaeva, G.A. and Oreshkova, S.R. (1987) *Prikl. Biokhim. Mikrobiol.* 23, 530–535.
- [9] Nevinsky, G.A., Podust, V.N., Levina, A.S., Khalabuda, O.V. and Lavrik, O.I. (1987) *Bioorg. Khim.* 13, 357–368.
- [10] Levina, A.S., Nevinsky, G.A. and Lavrik, O.I. (1985) *Bioorg. Khim.* 11, 358–369.
- [11] Veniaminova, A.G., Levina, A.S., Nevinsky, G.A. and Podust, V.N. (1987) *Mol. Biol.* 21, 1378–1385.
- [12] Cade, J.A. (1959) *J. Chem. Soc.*, 2266–2272.
- [13] Rozovskaya, T.A., Chenchik, A.A., Tarusova, N.B., Bibilashvili, R.Sh. and Khomutov, R.M. (1981) *Mol. Biol.* 25, 1205–1223.
- [14] Hubchinson, D.W., Naylor, M., Semple, G. and Cload, P.A. (1985) *Biochem. Soc. Trans.* 13, 752–753.
- [15] Cade, J.A. (1959) *J. Chem. Soc.* 2272–2275.
- [16] Dixon, W. and Webb, E. (1979) *Enzymes*, Longman Group.
- [17] Cornish-Bowden, E. (1978) in: *Principles of Enzyme Kinetics*, pp. 260–266, Mir, Moscow.
- [18] Mikhailov, V.S., Ataeva, D.O., Murlyev, K.A. and Atrazhev, A.M. (1989) *Mol. Biol.* 23, 306–314.
- [19] Chang, L.M.S. and Bollum, F.J. (1973) *J. Biol. Chem.* 248, 3398–3404.