NaF and mononucleotides as inhibitors of 3'-5'-exonuclease activity and stimulators of polymerase activity of *E. coli* DNA polymerase I Klenow fragment

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It has been shown that, in the absence of dATP in the poly(dT)·oligo(dA) template–primer complex, the rate of primer cleavage by the *E. coli* DNA polymerase I Klenow fragment equals 4% of polymerization rate, while in the presence of dATP it equals as much as 50–60%. NaF and NMP taken separately inhibit exonuclease cleavage of oligo(dA) both with and without dATP. The addition of NaF (5–10 mM) or NMP (5–20 mM) increases the absolute increment of polymerization rate 5–9-fold relative to the absolute decrement of the rate of nuclease hydrolysis of primer. This proves the assumption that not more than 10–20% of primer molecules, interacting with the exonuclease center of polymerase, are cleaved by the enzyme. Presumably, NaF and nucleotides disturb the coupling of the 3'-end of oligonucleotide primer to the exonuclease center of the enzyme. As the primers mostly form complexes with the polymerizing center, the reaction of polymerization is activated.

Klenow fragment; NaF; Nucleotide; Activation and inhibition of activity

1. INTRODUCTION

FK is known to have polymerase and 3'-5'-exonuclease activities. The distance between the binding site of the primer and FK exonuclease center is 20–30 Å [1]. It is worthwhile finding new ways of selective inhibition of the FK exonuclease activity as this enzyme is widely used in genetic engineering. Previously [2,3] it was found that NMP- and dNMP-induced inhibition of the exonuclease activity correlated with the stimulation of FK-catalyzed DNA polymerization.

We have studied the effect of dATP, NaF [4] and NMP on the ratio of the rates of two FK-catalyzed reactions: polymerization and exonuclease hydrolysis. NMP and NaF induce both acceleration of polymerization and deceleration of exonuclease cleavage of the primer. The former effect, however, is by far more pronounced.

2. MATERIALS AND METHODS

Electrophoretically homogeneous FK with a specific activity of 3.7×10^4 U/mg was obtained as in [5].

Poly(dT), dNMP and dNTP were from NIKTI BAV (USSR), BSA from Koch Light, $MgCl_2$ and PEI-cellulose discs from Merck. AC_{III} 300 aminosylchrome sorbent from Novosibirsk State University (USSR) and [^{3}H]dATP (900 TBq/mol) from Izotop (USSR). Other reagents were of analytical grade.

Syntheses of $d(pA)_{10}$ and $r(pA)_{10}$ are described in [6,7]. Nucleotides were TLC-purified as in [8]. Poly(dT) · [^{3}H]oligo(dA) was prepared by FK-catalyzed polymerization with $d(pA)_{10}$ primer. The incorporation of [^{3}H]dAMP into the primer was 10–20% of the maximal. Surplus [^{3}H]dATP (180 TBq/mol) was removed by gel filtration. The FK activity was determined at 37°C. Reaction mixtures (15–100 μl) contained 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 0.1 mg/ml BSA, 40 mM KCl, 1 A_{260}/ml poly(dT), 20 μM $r(pA)_{10}$ or 0.4 μM $d(pA)_{10}$, 20 μM [^{3}H]dATP (1.5 μM in the case of $d(pA)_{10}$). In experiments with poly(dT)·[^{3}H]oligo(dA), the concentration of poly(dT) was 0.2 A_{270}/ml and that of [^{3}H]oligo(dA) was 80 nM. 1.5 μM dATP was used for [^{3}H]dATP, 180 TBq/mol).

The reaction was initiated by the addition of 2–10 U/ml FK. The polymerising activity of the enzyme was measured according to the method of acid-insoluble precipitates as described in [8]. The exonuclease activity was determined by 3 methods: (i) by testing the accumulation of [^{3}H]dAMP by chromatography on F_{254} PEI cellulose discs as in [9], or (ii) by ion-exchange chromatography in AC_{III} 300 sorbent as in [10] and (iii) by measuring the decrease in the amount of label during trichloroacetic precipitation of poly(dT) · [^{3}H]oligo(dA) as in [8]. In these 3 tests, a maximal amount of [^{3}H]dAMP was found to be up to 10–20% of that in poly(dT) · [^{3}H]oligo(dA).

3. RESULTS AND DISCUSSION

It has been shown that, in the poly(dT) · [^{3}H]-oligo(dA) complex, the rate of exonuclease hydrolysis of primer by FK equals 4% of the rate of polymerization and increases up to 50–60% under addition of [^{3}H]dATP (see Table I). This is the evidence of a higher efficiency of the primer cleavage during polymeriza-

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Abbreviation: FK, Klenow fragment of *E. coli* DNA polymerase I

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Table I

The rates of incorporation of [^{3}H]dAMP into the primer and of accumulation of free [^{3}H]dAMP due to exonuclease hydrolysis

Template-primer complex	dNTP	Incorporation of [^{3}H]dAMP into the primer (cpm/min · 10^{-3})	Accumulation of [^{3}H]dAMP due to hydrolysis, (cpm/min · 10^{-3})	Rate of hydrolysis versus rate of synthesis (%)
1. Poly(dT) · oligo[^{3}H]d(pA)	-	-	0.21	
2. Poly(dT) · oligo[^{3}H]d(pA)	dATP	-	2.1	40*
3. Poly(dT) · oligo[^{3}H]d(pA)	[^{3}H]dATP	5.3	3.5	66
4. Poly(dT) · $d(pA)_{10}$	[^{3}H]dATP	8.5	1.0	12
5. Poly(dT) · $r(pA)_{10}$	[^{3}H]dATP	15.8	2.1	13

*This value was calculated based on equal rates of polymerization for complexes 2 and 3; the difference between the rates of hydrolysis for complexes 2 and 3 (26%) characterizes the hydrolysis of the elongated primer.

tion. As follows from Table I, the rate of [^{3}H]dAMP accumulation observed with dATP is about 60% of that observed with [^{3}H]dATP. Hence it appears that 50–60% of [^{3}H]dAMP is formed due to the hydrolysis of [^{3}H]oligo(dA) before its elongation, i.e. the rate of hydrolysis of the primer before its elongation is equal to 40% of the polymerization rate, while that of the elongated primer only equals 20% of the polymerization rate. This is in agreement with the data obtained for poly(dT) · $d(pA)_{10}$ and poly(dT) · $r(pA)_{10}$ complexes, where the rate of hydrolysis of the elongated primer was equal to 12–13% of the polymerization rate. The above facts may be explained by Paponikolau's hypothesis [11] that polymerases may have two states during polymerization; with higher and lower exonuclease activity. Predominant hydrolysis of the primer before elongation is presumably due to the competition between its 3′-end and dNTP for the polymerization site on the enzyme [10].

Fig. 1 shows the data of the AMP and NaF effects on the poly(dT) · [^{3}H]oligo(dA) complex hydrolysis in the absence of [^{3}H]dATP. Fig. 2 demonstrates the effect of these two substances on the FK-catalyzed polymerization and exonuclease hydrolysis in the presence of [^{2}H]dATP. In all cases the increase in AMP and/or NaF concentrations brings about an enhanced inhibition of hydrolysis and a 1.4–3-fold increase in the initial rate of polymerization. Virtually complete suppression of hydrolysis of adenylate primers in a complex with

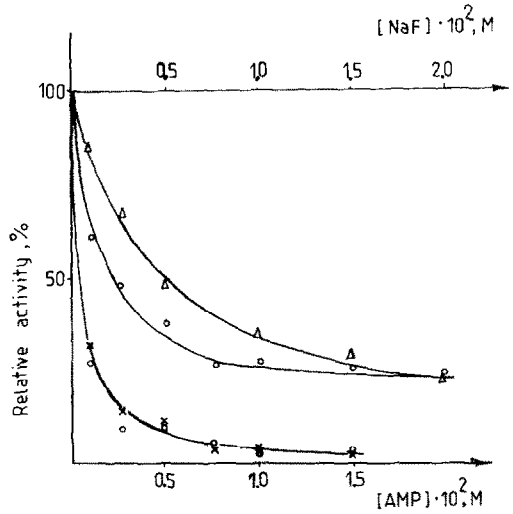

Fig. 1. Dependences of the initial rate of FK-catalyzed exonuclease hydrolysis on the concentrations of NaF (▵;○) and AMP (×;•) in the absence of dATP. The hydrolysis was tested by decrease of radioactivity in oligo[^{3}H]$d(pA)_{10}$ (○;•) and by accumulation of [^{3}H]dAMP (▵;x).

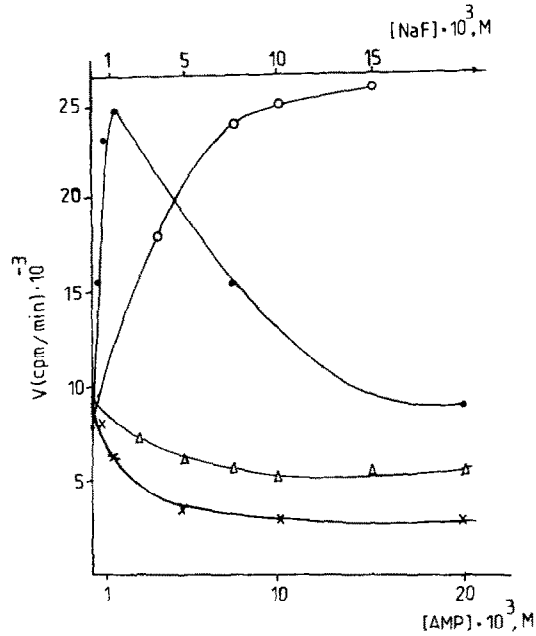

Fig. 2. Dependences of the initial rates of FK-catalyzed polymerization (•;○) and exonuclease hydrolysis (×; ▵) on the concentrations of NaF (○; ▽) and AMP (•;x) in the presence of dATP for the poly(dT) · oligo]^{3}H]d(pA) complex.

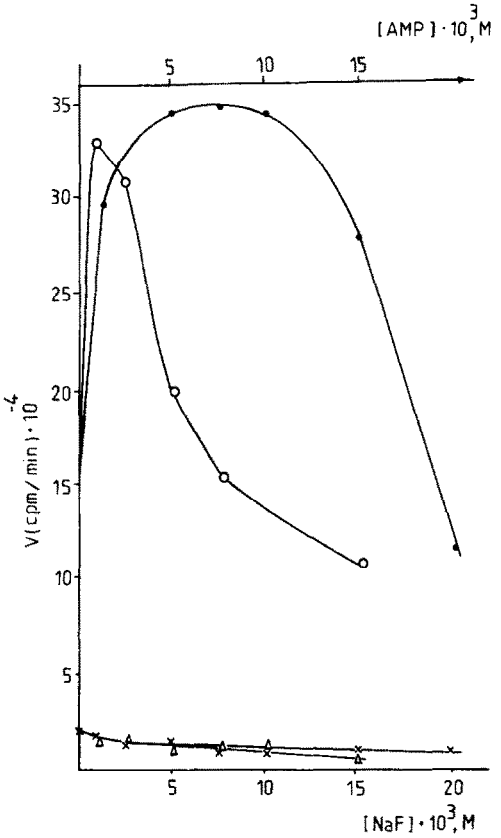

Fig. 3. Dependences of the initial rates of FK-catalyzed polymerization (○;●) and exonuclease hydrolysis (△;×) on the concentrations of NaF (●;×) and AMP (○;△) in the presence of dATP for the poly(dT)·r(pA)$_{10}$ complex.

poly(dT) is achieved at high concentrations of these ligands: 0.3–0.5 M NaF and 10–50 mM AMP both in the presence and absence of [^{3}H]dATP. The data of Figs 2 and 3 shows that, in the complexes poly(dT)·[^{3}H]oligo(dA) and poly(dT)·d(pA)$_{10}$, the rate of the label incorporation into the primer is increased 5–9-fold faster than the rate of [^{3}H]dAMP accumulation is decreased. The effect is even more pronounced in the complex poly(dT)·r(pA)$_{10}$ where this difference is 40-fold, which may be due to the fact that r(pA)$_{10}$ is not hydrolyzed [12]. The data suggest that the AMP- and NaF-mediated inhibition of the exonuclease cleavage of both initial and elongated primer goes the same way.

Highly concentrated NaF (30 mM) is known to inhibit numerous nucleotide- and polynucleotide-dependent enzymes. A selective inhibition of the FK exonuclease activity was first reported and described by Mikhailov et al. [4].

Our results are in agreement with the assumption made in [14] that there is a competition between polymerizing and exonuclease centers for the 3′-end of the primer which constantly 'migrates' between the centers. A 5–40-fold excess of the increase in the polymerization rate over decrease in the cleavage rate proves that not more than 2.5–20% molecules interacting with the exonuclease center is hydrolyzed during the life-time of the complex. The observed enhancement of polymerization, in our judgement, results from the NaF- and AMP-induced inhibition of complex formation between the 3′-end of the primer and exonuclease center when the primer is pushed towards the polymerizing center. The increased rate of polymerization is, therefore, a result of predominant complex formation with this center.

NaF is shown to have no effect on K_m for templates, primers and dNTP. Therefore it can be used for studying the interactions of these ligands with the FK polymerizing center under the conditions when their interaction with the exonuclease center of the protein does not affect polymerization.

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