

The efficiency of interaction of deoxyribonucleoside-5'-mono-, di- and triphosphates with the active centre of *E. coli* DNA polymerase I Klenow fragment

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The interaction of deoxyribonucleoside-5'-mono-, di- and triphosphates with *E. coli* DNA polymerase I Klenow fragments was examined. Dissociation constants of the enzyme complex with nucleotides were determined from the data on the enzyme inactivation by adenosine 2',3'-riboepoxide 5'-triphosphate. The role of nucleotide bases, phosphate groups and sugar moieties in the complex formation of nucleotides with the enzyme was elucidated. The necessity of complementary interaction of nucleotides with templates for template-controlled 'adjusting' of complementary dNTP to its reactive state was found. The crucial role of the interaction of dNTP γ -phosphate with the enzyme in this process is discussed.

DNA polymerase I Klenow fragment; dNTP selection; Affinity inhibition; (*Escherichia coli*)

1. INTRODUCTION

The DNA replication process catalyzed by DNA polymerase (EC 2.7.7.7) is characterized by an extreme accuracy (10^{-4} – 10^{-8}) [1]. However, details of the specific interaction of dNTP and its selection by DNA polymerases are as yet unclear. The models of dNTP selection by DNA polymerases take into account a preliminary discrimination at the step of dNTP and the enzyme-specific complex formation [2,3]. The next steps, namely, the conformational adaptation of complementary dNTP to the active site of the enzyme [2–6] and catalytic formation of the phosphodiester bond, contribute to the accuracy of replication [5,6]. An additional correction can be provided by the excision of the error nucleotide either with pyrophosphorolysis or pyrophosphate exchange [7], 3'-5'-exonuclease activity [1,8] or more effective dissociation of incorrect primer from the complex with the enzyme [9].

Here, for the first time, the efficiency of the complex formation of FK with various nucleotides in the presence of template and primer was quantitatively analysed. The affinity modification was used for the evaluation of K_d values of the enzyme and nucleotide complexes, similarly to [2,3].

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Abbreviations: epATP, adenosine 2',3'-riboepoxide 5'-triphosphate; BSA, bovine serum albumin; FK, *E. coli* DNA polymerase I Klenow fragment; DPH, DNA polymerase α from human placenta

2. MATERIALS AND METHODS

Electrophoretically homogeneous FK (specific activity $4 \cdot 10^4$ units/mg) was obtained according to [10]. DNA from calf thymus and all nucleotides used were from NIKTI BAV (USSR), poly(dT) and BSA from Sigma, $MgCl_2$ from Merck, [3H]dNTP ($2.2 \cdot 10^4$ Ci/mol) from Izotop (USSR), and other reagents were of analytical grade.

Syntheses of d(pA)₁₀ [11] and epATP [12] were described earlier. The polymerase activity of FK and the enzyme inactivation were measured at 30°C. In both cases the mixtures (50 μ l) contained standard components: 50 mM Tris-HCl buffer (pH 7.5), 30–40 mM KCl, 10–25 mM $MgCl_2$, 5 mM NaF, 0.1 mg/ml (1 mg/ml for the enzyme inactivation) BSA. The mixture for measuring of the enzyme activity also contained 2 A_{260} /ml activated DNA, 20 μ M each of four dNTPs ([3H]dTTP with a specific activity of 300 Ci/mol). The polymerisation reaction was started by adding 0.1–0.5 units of FK. Further treatment of the reaction mixture aliquots was done as in [9].

The mixture for the FK inactivation contained the above standard components, 1 A_{260} /ml poly(dT), 3 μ M d(pA)₁₀ and various concentrations of epATP (0–40 μ M). The modification reaction was started by addition of 1–5 units of FK. During incubation, aliquots (5 μ l) were taken from the mixture every 5–10 min and added to the mixture for the determination of the FK activity.

The K_m and K_i values for nucleotides were determined using poly(dT), d(pA)₁₀ and NaF as described in [13].

Dissociation constants of FK complexes with epATP (K_x) and with ligands competitive to epATP (dNMP-dNTP) (K_y) were evaluated as in [2,3] according to the Kitz-Wilson method [14].

3. RESULTS AND DISCUSSION

Nearly complete inactivation of FK by epATP takes place in the presence of both template and primer, in agreement with [15,16]. In the polymerisation reaction, epATP initially serves as a substrate elongating the DNA by a nucleotidyl unit without the enzyme and primer covalent bond formation. The FK complex with

the duplex of template and primer terminated by epATP has an extremely low rate of dissociation ($4 \cdot 10^{-4}/s$) [16], which leads to apparent FK inactivation. All the data obtained [15,16] are evidence for the possibility that the K_x and K_y values can be determined by the Kitz-Wilson method [14].

All experiments on affinity inactivation of FK by epATP were carried out under saturated concentrations of poly(dT) template, d(pA)₁₀ primer and 5 mM NaF. NaF was used as a strong selective inhibitor of 3'-5'-exonuclease activity of FK [13,17].

The K_x value of FK epATP complex ($16 \mu M$) and the maximum inactivation rate ($4.4 \cdot 10^{-4}/s$) were evaluated from the linear dependence of k_{app} of FK inactivation on the concentration of epATP (fig.1). The K_x value for epATP is nearly the same as the dissociation constant ($21 \mu M$) estimated in [16]. The protective effects of various nucleotides against FK inactivation by epATP were used for the determination of K_y (K_d) values of these nucleotides. The K_y ($2.5 \mu M$) of the dATP complex with FK was practically the same as the K_y ($5 \mu M$) estimated in [5] by the method of presteady-state kinetics. The latter approach is considered as a direct method for K_d determination. Therefore, it gives the possibility to receive true K_d values from protective effects of nucleotides.

As can be seen in fig.3, the dependence of $-\Delta G^\circ$ of dNMP binding on retention time of the corresponding nucleotides on reversed phase sorbent is practically linear, like the same dependence for DPH [2,3]. The data of fig.3 show that the α -phosphate group of various dNMPs makes approximately the same contribution to the efficiency of its interaction with the proteins. The difference in affinity of dNMPs to the dNTP-binding site of the enzymes is caused mainly by the difference in the efficiency of hydrophobic interaction of dNMP bases with the binding site. Similar slopes of the dependences (fig.3) for FK and DPH indicate a similar

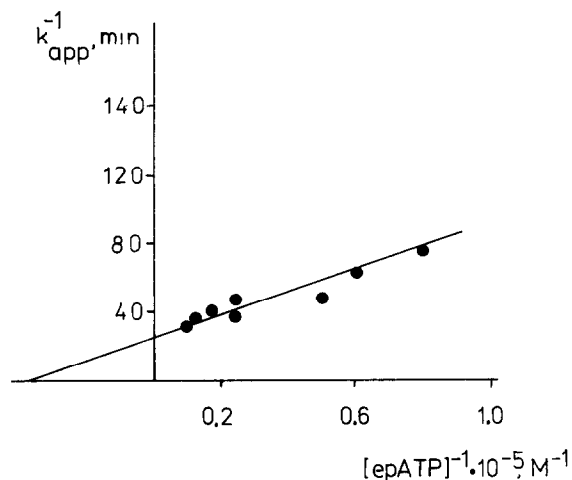


Fig.1. Dependence of the k_{app} of FK inactivation on the concentration of epATP in inverse coordinates.

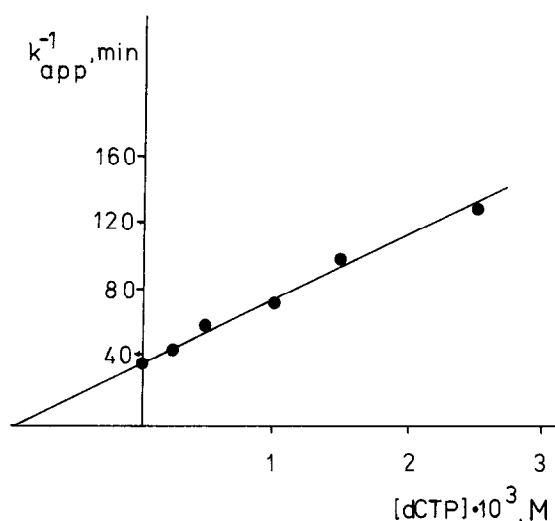


Fig.2. Dependence of the inverse values of the k_{app} of the inactivation rate of FK by epATP (0.4 mM) on dCTP concentration.

hydrophobicity of the active centres of both enzymes. K_y for dNMP and corresponding dNDP are nearly the same (table 1). Consequently, the β -phosphate group of the nucleotides makes no appreciable contribution to the enzyme affinity.

When passing from dAMP (dADP) to dATP complementary to template, the affinity of nucleotides increases by a factor of 5-6. The same results were obtained in an analogous analysis of DPH (these results will be published separately). However, according to [2,3], the affinity of dTTP in comparison with dTMP to DPH

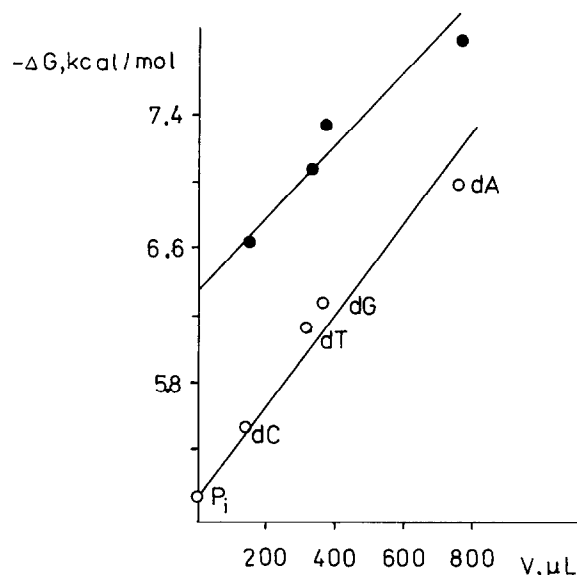


Fig.3. Dependence of $-\Delta G$ of the complex formation of FK (○) and human DNA polymerase α (●) [2,3] with dNMP on the difference between elution volumes of deoxynucleosides and orthophosphate in reversed phase chromatography. A column of Lichrosorb RP-18 (Merck) (2×60 mm) was used. The column was washed with water and deoxynucleosides were eluted with a 5% aqueous solution of methanol.

Table 1

Values of dissociation constants (K_x or K_y) of FK complexes with nucleotides and their analogues in the presence of poly(dT)-template and d(pA)₁₀-primer^a

Ligand	Dissociation constant (K_i or K_m) ^b (μ M)	
P _i	230	
PP _i	130	(140)
dCMP	100	
dTMP	37	
dGMP	30	
dAMP	12	
dGTP	100	
dADP	14	
dCTP	200	
dTTP	36	
dGTP	7	
dATP	2.5	(14) ^c
CTP	72	
UTP	30	
CTP	7	
ATP	4	(3.9)
epATP	16.2	(17.5)

^a All dissociation constants and K_i values (polymerisation reaction) were measured using 5 mM NaF

^b Error of the constants' estimation was within 20–50%

^c K_m is given for dATP, K_i (in parentheses) for all other cases

in the case of poly(dA) template is 150-fold higher. The data indicate a remarkable difference in the dNTP discrimination level in the case of various template-primer duplexes.

No such increase in the affinity is observed for non-complementary dNTP when compared with dNMP (table 1). The ratio (3–80) of the K_y values for complementary dATP to those for non-complementary dNTP corresponds to $\Delta\Delta G^\circ$ equal to $-(0.63 + 2.38)$ kcal/mol. ΔG° of A · T pairing of template and primer on FK and DPH was estimated to be -0.35 kcal/mol. Therefore the $-\Delta G^\circ$ value of the formation of additional contacts of dATP (most probably via γ -phosphate group) with FK may be estimated as 0.3–2.0 kcal/mol. The main regularities (including details) for the interaction of nucleotides with FK are the same as for DPH discussed earlier [2,3]. The results presented here and published earlier [2,3] testify to the existence of template-controlled change of the nucleotide-enzyme complex state to a reactive form which is necessary for catalysis. As was shown by us earlier [4], conformational changes of the dNTP molecule complementary to template following the complex formation of the en-

zyme with nucleotide in the case of imidazolides of dNTP lead to the inactivation of *E. coli* DNA polymerase I [4]. This conclusion has been confirmed by the investigation of the nucleotides' interaction with DPH by affinity modification [2,3] and by kinetic analysis [5,6]. The above results and the data published earlier [2,3] show that the stage of complex formation gives 10^{-1} – 10^{-2} accuracy of DNA replication. The correction of wrong nucleotides due to 3'-5'-exonuclease activity provides the additional accuracy of 10^{-1} – 10^{-2} [8]. The overall accuracy in DNA replication in vitro was estimated as 10^{-5} – 10^{-8} [1]. We suppose that the factor of 10^{-1} – 10^{-8} of the replication accuracy (10^{-5} – 10^{-4} in [6]) is provided due to the stage of conformational rearrangement of the dNTP molecule controlled by the template-primer in which the γ -phosphate group plays a key role.

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