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The efficiency of dNTP complex formation with human placenta DNA polymerase α as demonstrated by affinity modification

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The interaction of deoxyribonucleoside 5'-mono-, di- and triphosphates with human placenta DNA polymerase α was examined. Dissociation constants of enzyme complex formation with dNMP, dNDP and dNTP were determined from the data on enzyme affinity modification by imidazolide of dTMP. The basic role of the primary template-primer interaction with the enzyme in dNTP complex formation is shown. The template-dependent nucleotide interaction does not occur in the case of dNMP and dNDP in comparison with dNTP. The significant contribution of the γ-phosphate of dNTP in this process is demonstrated.

DNA polymerase α; Affinity labeling; dNTP selection; (Human placenta)

1. INTRODUCTION

DNA replication processes in cells catalyzed by DNA polymerases (EC 2.7.7.7) are characterized by extreme accuracy [1–3]. In recent years significant advances have been achieved in the study of these enzymes [1,3,4]. However, details of the specific interaction of dNTP with DNA polymerases remain as yet unclear. Some attempts have been made [5–9] to clarify the character of dNTP and dNMP interaction with DNA polymerase I from *E. coli* with the aid of classical methods. These approaches do not allow, however, evaluation of dissociation constants for dNTP in the presence of the template and the primer and, besides, require large amounts of homogeneous preparations of proteins. Meanwhile, quantitative characteristics of complex formation can be obtained by affinity modification [10,11]. In the absence of foreign proteins that cause substrate degradation in enzyme preparations this method requires neither large amounts nor homogeneity of enzyme preparations. As shown, imidazolides of dNTP and dNMP modify selectively the dNTP-binding sites of DNA polymerases only in the presence of the template and primer. Complementary and non-complementary dNTPs protect the enzyme against the action of nucleotide analogs with different efficiency [12,13]. Here, for the first time, the affinity modification method has been used to analyze quantitatively the efficiency of interaction of deoxyribonucleoside mono-, di- and triphosphates with human placenta DNA polymerase α.

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Abbreviations: Im-dNMP, imidazolide of dNMP; Im-dNTP, imidazolide of dNTP; BSA, bovine serum albumin

2. MATERIALS AND METHODS

DNA polymerase α preparations (7.3 S) from human placenta (spec. act. 8.5×10^3 U/mg) were obtained as in [14]. One unit corresponds to

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1 nmol [^{3}H]dNTP incorporated into acid-insoluble materials in 1 h at 37°C. DNA from salmon sperm, dNTP, dNMP, NTP, poly(dA) and poly(dT) were from Nikti Bav (USSR), BSA from Koch Light, and $MgCl_2$ and $MnCl_2$ from Merck. [^{3}H]dATP and [^{3}H]dTTP (22×10^3 Ci/mol) were from Izotop (USSR). Im-dNMP and γ-*N*-methylamides of dNTP were synthesized as in [13]. Other reagents used were analytical grade. The activity of DNA polymerase α was determined at 37°C as in [14]. The reaction mixture (70 μl) contained 50 mM Tris-HCl buffer, pH 7.5, 0.1–0.5 mg/ml BSA, 2 A_{260} activated DNA, 5 mM $MgCl_2$, 30 mM KCl, 20 μM dNTP: dATP, dCTP, dGTP, dTTP (dATP or dTTP 1×10^3–3×10^3 Ci/mol). The polymerization reaction was started by adding 0.02–0.05 units DNA polymerase. Further treatment of the reaction mixture was done as in [14].

Modification of DNA polymerase α by Im-dTMP was carried out at 37°C as in [13]. The reaction mixture (70 μl) contained 50 mM Tris-HCl buffer, pH 7.5, 0.5 mg/ml BSA, 1.2 mM $MnCl_2$, 10 μM β-mercaptoethanol, 0.3 A_{260}/ml poly(dA), 0.1–0.32 μM d(pT)$_{15}$ and 0.1–0.5 units DNA polymerase. The concentration of Im-dTMP and dCMP was varied over the range 0.01–0.5 mM. During incubation aliquots were taken from the reaction mixture every 5–10 min and added to the mixtures for determining enzyme activity in DNA polymerization. Values of dissociation constants of the enzyme complexes with imidazolides of dNMP (K_X) and with their competitive ligands (dNMP, dNDP, dNTP) (K_Y) were determined as in our first contribution in this series.

The polymerization reaction in the presence of poly(dA) template and d(pT)$_{15}$ primer was used to determine K_M and K_i values for dNTP as in [13].

3. RESULTS AND DISCUSSION

As shown in [12,13], imidazolides of dNTP and dNMP do not inactivate DNA polymerase α from human placenta and the Klenow fragment of DNA polymerase I from *E. coli* in the absence of the template and the primer or in the presence of either template or primer. A noticeable inactivation was achieved upon adding the poly(dA) template and d(pT)$_{15}$ primer at saturating concentrations. Based on these results an assumption was made concerning the determining role of the template and primer in formation of dNTP-binding sites of DNA polymerases.

All experiments on affinity modification of enzyme by Im-dTMP were carried out with the use of saturating concentrations of the poly(dA) template and d(pT)$_{15}$ primer. The K_X value of the enzyme-reagent complex (150 μM) and the maximum modification rate (5.3×10^{-2} min^{-1}) were found from the linear dependence of k_{app} of enzyme inactivation on concentration of Im-dTMP (fig.1). Various dNMP, dNDP, dNTP and their analogs effectively protected the enzyme against inactivation by Im-dTMP. The protective effects of the nucleotides were enhanced on increasing their concentrations. The K_Y values of the enzyme-nucleotide complexes found using the protective effects (as in fig.2) are listed in table 1. The polymerization reaction in the presence of the poly(dA) template and d(pT)$_{15}$ primer was used to determine K_M for the complementary dTTP and K_i for the non-complementary dNTP (table 1). As was found, γ-methylamides of dTTP and dATP do not show substrate properties, and the K_Y values for these ligands scarcely differ from K_Y for dTTP and dATP. ddTTP that terminates DNA synthesis showed practically the same affinity to the enzyme as dTTP. From a comparison of K_i and K_Y for dNTP and their analogs it follows that the use of the affinity reagent and the polymerization reaction make it possible to obtain affinity characteristics for the same site of the enzyme, namely for its active center. Note that K_M for the complementary dTTP is 50-times as high as the corresponding K_Y.

Nucleotides having the same base but different sugar residues reveal very close affinities to DNA polymerase (see table 1). dTTP, dATP and their γ-methylamides also show similar affinities to the enzyme. Clearly enough, a sugar residue of the nucleotides and one of the negatively charged oxygen atoms of the dNTP γ-phosphate group make negligible contribution to the efficiency of enzyme complex formation with the nucleotides.

The values of K_Y for dNMP and the corresponding dNDP are also nearly the same. Therefore, the γ-phosphate group of the nucleotides makes no appreciable contribution to their enzyme affinity. Nevertheless, the dNMP affinity increases in the order: dCMP, dTMP,

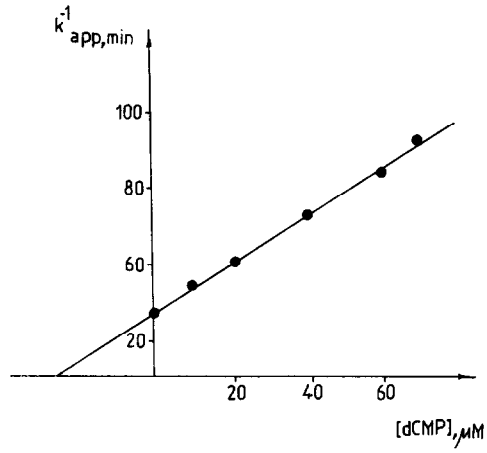

Fig.1. Dependence of k_{app} of inactivation rate of DNA polymerase α on concentration of Im-dTMP in inverse coordinates.

dGMP, dAMP. As can be seen in fig.3, the dependence ΔG of dNMP binding on retention time of the corresponding nucleosides on the reversed-phase sorbent is linear. Consequently, γ-phosphate groups of various dNMPs make approximately the same contribution to the efficiency of its complex formation with the protein. The difference in affinity of dNMP to the dNTP-binding

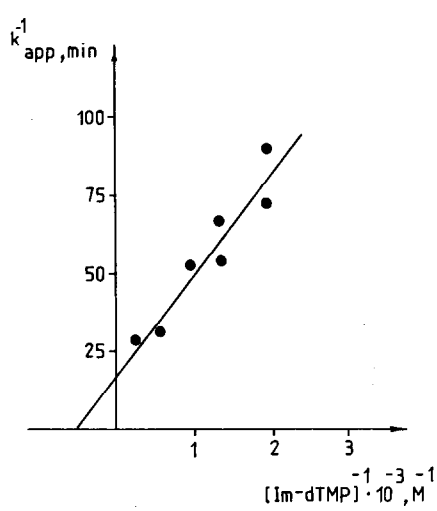

Fig.2. Dependence of inverse values of k_{app} of inactivation rate of DNA polymerase α by Im-dTMP (0.13 mM) on dCMP concentration.

Table 1

Values of K_Y of DNA polymerase α complexes with nucleotides in the presence of the poly(dA) template and $d(pT)_{15}$ primer

Nucleotide	K_Y (K_i or K_M)[a] (μM)	$-\Delta G$ (kcal/mol)
KH_2PO_4	10	7.1
dCMP	15	6.8
dTMP	9 (23)	7.2
dGMP	7 (6)	7.3
dAMP	3 (7.8)	7.8
dCDP	11	7.0
dTDP	7	7.3
dGDP	6	7.4
dCTP	7 (15)	7.3
CTP	3	7.8
dTTP	0.06(3.1)[b]	10.2
TTP	0.05	10.4
ddTTP	0.03	10.7
γ-Methylamide of dTTP	0.03	10.7
dGTP	0.3 (0.64)	9.3
GTP	0.8	8.7
dATP	0.8	8.7
ATP	0.7	8.7
γ-Methylamide of dATP	0.3	9.3

[a] Error of K_Y, K_i and K_M estimation was within 20–50%

[b] K_M is given for dTTP, K_i (in parentheses) for all other cases

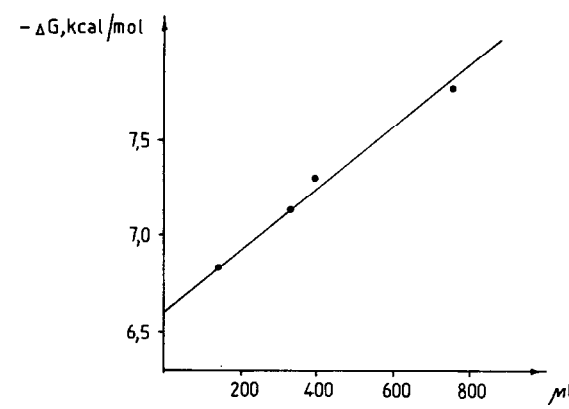

Fig.3. Dependence of ΔG of the complex formation between DNA polymerase α and dNMP on the difference between elution volumes of deoxynucleosides and orthophosphate in reversed-phase chromatography. A column of Lichrosorb RP-18 (Merck) (2 × 60 mm) was used. The column was washed with water and deoxynucleosides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

site of DNA polymerase is caused mainly by the difference in efficiency of hydrophobic interactions of bases of these nucleotides with the enzyme. The Gibbs' energy, ΔG, of binding of the phosphate group of dNMP to the enzyme is close to -6.6 to -6.7 kcal/mol, as can be estimated from fig.3.

When passing from dTMP and dTDP to dTTP, the affinity of the nucleotides increases ~150-times. No such increase in affinity is observed for dNTP which is non-complementary to the template.

Thus in the presence of the template and the primer, nucleoside 5'-mono-, di- and triphosphates are capable of binding to the dNTP-recognizing site of the enzyme independently of whether 2'- and 3'-oxy groups are present or absent in sugar residues and whether the template is complementary. Note that ΔG (-6.6 kcal/mol) of the enzyme binding with the dNMP phosphate group is close to ΔG (-6.8 to -6.9 kcal/mol) of the formation of Me^{2+}-dependent electrostatic contact and a hydrogen bond between DNA polymerase and phosphate groups of the oligonucleotide template (see the previous paper). This is the evidence in favour of the formation of the same type of contacts between the enzyme and the dNMP phosphate group.

Hydrophobic interactions of nucleotide bases and interactions of their phosphate groups with the enzyme seem to be primary and determine the complex formation between the enzyme and the triphosphate chain of dNTP. dNMP and dNDP cannot participate in complementary interactions with the template. A slight increase in affinity of non-complementary dNTP in comparison with the corresponding dNMP indicates, probably, the formation of a weak contact between the dNTP γ-phosphate group and the enzyme. It is not excluded, however, that these contacts in the case of complementary dNTP will be necessary for interaction between dNTP and the template. At the stage of the template-controlled 'adjusting' of complementary dNTP to its reactive state the interaction of terminal γ-phosphate with the active center leads to an increase in affinity of 2.5 orders of magnitude in comparison with a non-complementary dNTP. For reactive dNTP analogs, namely imidazolides, these contacts may result in phosphorylation of nucleophilic protein groups that have approached a terminal phosphate [12].

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