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DNA polymerase I (Klenow fragment): role of the structure and length of a template in enzyme recognition

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The values of K_d and Gibbs energy (ΔG°) have been measured for complexes of the template site of DNA polymerase I Klenow fragment with the homo-oligonucleotides $d(pC)_n$, $d(pT)_n$, $d(pG)_n$ and $d(pA)_n$ and hetero-oligonucleotides of various structures and lengths. These parameters were evaluated from the protective effect of the oligonucleotide on enzyme inactivation by the affinity reagents $d(Tp)_2C[Pt^{2+}(NH_3)_2OH](pT)_7$ and $d[(Tp)_2C(Pt^{2+}(NH_3)_2OH)p]_3T$ of the template site. The present results and previously reported data [(1985) Biorg. Khim. 13, 357–369] indicate that the nucleoside components of the template form complexes as a result of their hydrophobic interactions with the enzyme. Only one template internucleotide phosphate forms an Me^{2+}-dependent electrostatic contact and a hydrogen bond with the enzyme. The 19–20-nucleotide fragments of the template appear to interact with the protein molecule.

DNA polymerase I; Klenow fragment; Protein-nucleic acid interaction

1. INTRODUCTION

The efficiency of oligothymidylates of various lengths and their derivatives ethylated on the internucleotide phosphates with human DNA polymerase α and KLF was quantitatively assayed in [1–5] using R_1 reagent. Only one template internucleotide phosphate was suggested to form an Me^{2+}-dependent electrostatic contact (ΔG = −1.2 kcal/mol) and a hydrogen bond (ΔG = −4.4 kcal/mol) with the enzymes. The interaction of mononucleoside units of oligothymidylates with enzymes is likely to be hydrophobic.

This study deals with the interaction of Klenow fragment with the oligonucleotides $d(pC)_n$, $d(pT)_n$, $d(pG)_n$ and $d(pA)_n$. Their affinities have been shown to increase in the order $d(pC)_n < d(pT)_n < d(pG)_n \approx d(pA)_n$, in agreement with the relative hydrophobicity of the nucleotide bases C < T < G ≤ A.

The bases C, T, G and A of homo-oligonucleotides were found to contribute to the affinity to almost the same extent as those of hetero-oligonucleotides of various lengths.

We propose a model for template recognition with DNA polymerase based on the electrostatic contact and hydrogen bond only of the internucleotide phosphates and on the hydrophobic interaction of the mononucleoside units of the template.

2. MATERIALS AND METHODS

Klenow fragment of DNA polymerase I of specific activity 3.7×10^4 U/mg and homogeneous according to electrophoresis was obtained as in [6]. dNTP, poly(dT) and poly(dA) were from Nikti Bav (USSR); $MgCl_2$ and $MnCl_2$ from Merck (FRG); BSA from Koch Light (England) and [^{3}H]dNTP (20×10^3–25×10^3 Ci/mol) from Izotop (USSR). Other reagents were analytical grade.

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Abbreviations: KLF, Klenow fragment; R_1, $d(Tp)_2C[Pt^{2+}(NH_3)_2OH](pT)_7$; R_2, $d[(Tp)_2C(Pt^{2+}(NH_3)_2OH)p]_3T$; BSA, bovine serum albumin

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All the oligonucleotides used were synthesized as in [1,2,7] and were homogeneous according to reverse-phase and ion-exchange chromatographic analysis [7]. The affinity reagent R_1 was obtained as in [5]. Similarly, we performed the synthesis, separation and characterization of reagent R_2. Atomic adsorption was used to show that about 3–3.5 Pt^{2+} residues were present per $d[(Tp)_2Cp]_3T$ molecule.

Enzyme activity was determined as in [1,2,4,5,7] during the course of inactivation experiments.

The enzyme was modified using R_1 and R_2 at 30°C. The incubation mixture (100 μl) contained 50 mM Hepes/KOH buffer (pH 7.0), 0.5 mg/ml BSA, 0.22 mM $MnCl_2$, 5 mM NaF and 2.5–10 units KLF.

To evaluate affinities, a wide range of concentrations of R_1 and R_2 were employed. Determination of K_d values for competitive ligands entailed their concentrations being fixed, equalling 2–10 μM. Aliquots were removed from the incubation mixture at intervals of 10 min and added to the reaction mixture for ascertainment of enzyme activity. Dissociation constants for the complexes were determined as in [1–5] using the method of Kitz and Wilson [9].

3. RESULTS AND DISCUSSION

Addition of NaF (5 mM) prevents the degradation of oligonucleotides by KLF 3′–5′-exonuclease activity [5]. NaF does not affect the K_m values for primers and template. In the presence of NaF in the mixture, reagent R_1 selectively modifies the template binding site of KLF according to the criteria for affinity modification. In the presence of $r(pA)_5$, the affinity of reagent for the enzyme increased 12-fold due to complementary interactions with the primer. Therefore, the affinity of oligonucleotides that are complementary to R_1 cannot be evaluated from their protective effects. Another reagent, namely R_2, was used to investigate the efficiency of the interaction of $d(pA)_n$ with the KLF template site. K_d values for the complexes between KLF and reagent were determined as being equal to 4.3 and 0.3 μM for R_1 and R_2, respectively. The difference between the K_d values for $d(pT)_n$ measured using R_1 and R_2 was negligible. Fig.1 shows the dependence of the values of the reciprocal of k_{app} for inactivation upon $d(pA)_6$ concentration for a fixed concentration of R_2. The above data led us to conclude that since complementary interactions were not observed, R_2 and $d(pA)_n$ must compete for the enzyme template site. Fig.2 shows the dependence of $-\log K_d(-\Delta G)$ on the number of nucleotide units of $d(pC)_n$, $d(pT)_n$, $d(pG)_n$ and $d(pA)_n$. The minimal ligand was orthophosphate. The most effective binding was found to occur with $d(pN)_n$ (n = 19–20). The dependence of ΔG^0 of the complex [enzyme · ligand] on n was linear for $n \leqslant 20$. Each mononucleotide unit of $d(pC)_n$, $d(pT)_n$, $d(pG)_n$ and $d(pA)_n$ displayed an increase in affinity to the template site by a factor (f) equal to 1.58, 1.78, 1.95 and 1.95 for the above oligonucleotides, respectively. These f values correspond to the decreases in ΔG^0 by −0.28, 0.35, −0.40 and −0.40 kcal/mol, respectively.

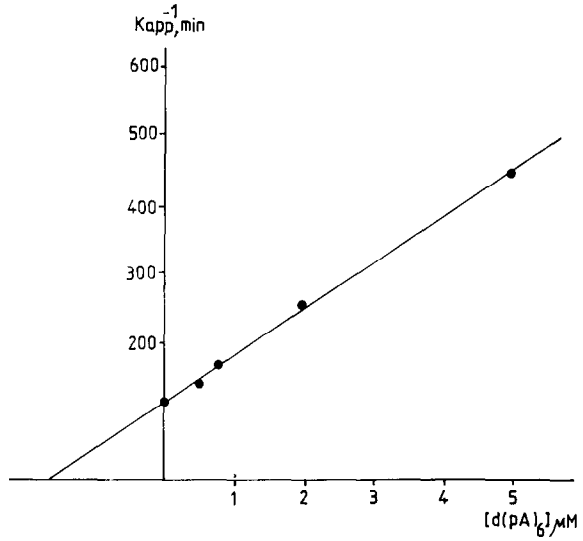

Fig.1. Dependence of the reciprocal values of k_{app} for the KLF inactivation rate on concentration of $d(pA)_6$ for a fixed concentration (10 μM) of the reagent $d[(Tp)_2C(Pt^{2+}(NH_3)_2OH]p]_3T$. KLF activity was assayed at 30°C in the course of inactivation experiments. The mixture (60 μl) contained 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 0.5 mg/ml BSA, 30 mM KCl, 10 μM EDTA, 30 μM dATP, dCTP, dGTP, dTTP (dNTP, 10–50 Ci/mol), and 2 A_{260}/ml DNA activated as in [8]. The reaction was initiated by adding 0.2–2.0 U enzyme, aliquots were withdrawn at 1–2 min from the reaction mixture and applied to the paper disks (FN 16). Subsequent treatment of disks was carried out as in [1,2,4,5,7].

For any of the homo-oligonucleotide series under study, K_d was transformed according to the equation:

$$K_d = 26\ \mu M \cdot (1/f)^n,$$

where 26 μM is the K_d value for orthophosphate, f represents the coefficient characterizing the increase in base affinity, n denotes the number of mononucleotide units (in oligonucleotides).

Although the affinities of dCMP and dAMP are

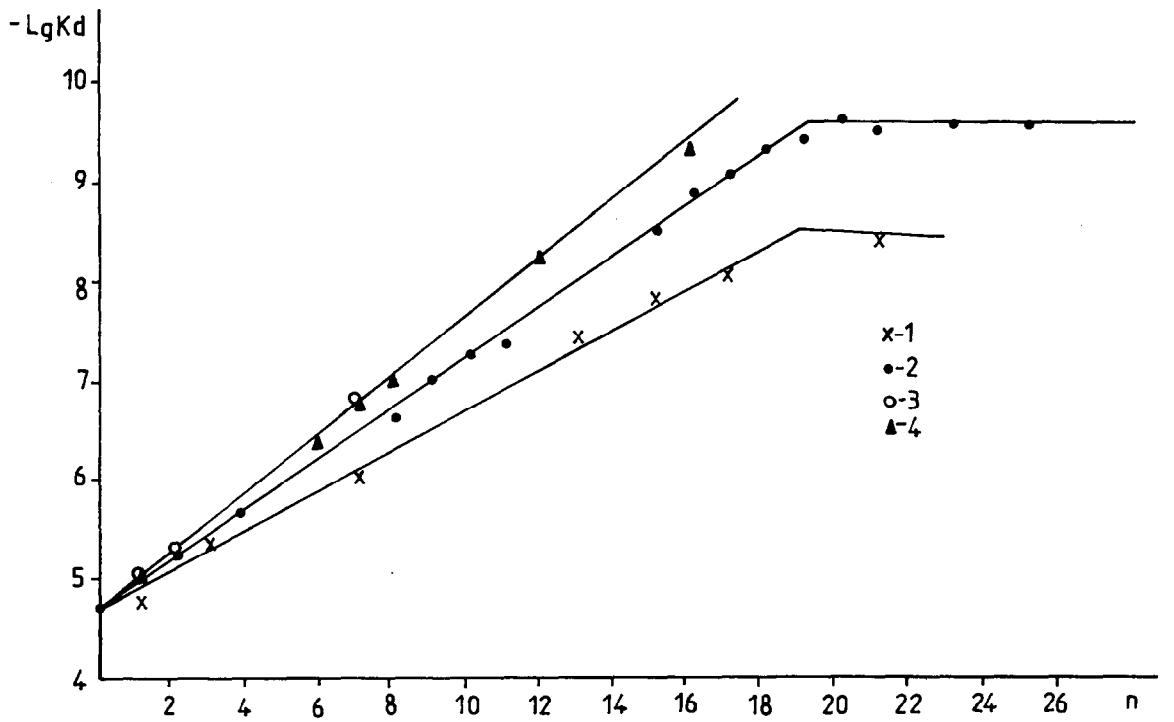

Fig.2. Dependence of $-\log K_d$ of several complexes, KLF·homo-oligonucleotide, on number of mononucleotide units (n). (1) $d(pC)_n$, (2) $d(pT)_n$, (3) $d(pA)_n$, (4) $d(pG)_n$.

very similar, the K_d values for $d(pC)_{20}$ and $d(pA)_{20}$ differ almost 50-fold. The fact that there is no change in affinity of $d(pN)_n$ for $n > 19$–20 means that only 19–20 template mononucleotides are involved in the interaction with the enzyme. The above suggestion is in good agreement with data on the protection of $d(pN)_{19}$ from degradation by the nuclease [10]. K_d values for complexes of KLF with various hetero-oligonucleotides were calculated according to the equation:

$$K_d = 26\,\mu M \cdot (1/1.58)^l \cdot (1/1.78)^m \cdot (1/1.95)^p \cdot (1/1.95)^q,$$

where l, m, p and q denote the respective numbers of bases C, T, G and A in hetero-oligonucleotides. The calculated and experimentally obtained K_d values are listed in table 1. These data do not appear to differ within the limits of the experimental accuracy, thus constituting evidence in favour of the equal contribution by all types of bases to the affinity of homo- and hetero-oligonucleotides.

Covalent binding of R_1 and R_2 to the enzyme points to hydrophilic amino acid residues being present at the enzyme template site. Moreover, the increase in affinity of the oligonucleotides in the order $d(pC)_n < d(pT)_n < d(pG)_n \approx d(pA)_n$ is in good agreement with the trend in hydrophobicity

Table 1

Dissociation constants of complexes formed by KLF and oligonucleotides of various lengths and structures

	K_d (μM)	
	Calculated[a]	Experimental[b]
d(pTpApApTpApCpGpA)	0.14	0.17
d(pCpTpGpCpGpTpTpApC)	0.12	0.13
d(pApApTpCpApTpGpGpTpCpApT)	0.013	0.015
$d(pCpA)_5$	0.07	0.06
$d(pGpA)_5$	0.024	0.015
$d(pGpA)_3$	0.4	0.45
$d(pGpT)_6$	0.011	0.009

[a] K_d values were calculated according to the equation:

$K_d = 26\,\mu M \cdot (1/1.58)^l \cdot (1/1.78)^m \cdot (1/1.95)^p \cdot (1/1.95)^q$,

where l, m, p, q represent the number of bases C, T, G, A in oligonucleotides

[b] Error does not exceed 20–30%

of the bases, C < T < G ⩽ A [3]. The energy gain from the transfer of a $d(pN)_n$ residue from H_2O to the template site of the enzyme ($\Delta G = -0.28$ to -0.40 kcal/mol) is comparable to that of transfer of T and A from H_2O to 6 M aqueous methanol ($\Delta G = -0.47$ to -0.88 kcal/mol) [11].

We could therefore consider it reasonable to conclude that the interaction of the template nucleoside units with the template site of KLF is hydrophobic in nature. The above interaction together with an Me^{2+}-dependent electrostatic contact and hydrogen bonding of an internucleotide phosphate group [2,5] quite likely represent the totality of the interactions of the DNA fragment with the template site of DNA polymerases in both pro- and eukaryotes.

The additional increase in template affinity is due to complementary interactions with the primer. At the same time, the distant electrostatic field arising from the cluster of positive charges that were detected in the DNA-binding enzyme site should not be neglected [12], since this factor may be of significance during certain stages of DNA recognition.

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