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The algorithm of estimation of the K_m values for primers of various structure and length in the polymerization reaction catalyzed by Klenow fragment of DNA polymerase I from *E.coli*

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DNA synthesis at primers $d(pT)_n$, $d(pA)_n$, $d(pC)_n$, and $d(pG)_n$ in the presence of corresponding complementary templates and at hetero-oligoprimers complementary to M13 phage DNA was investigated. The values of both $-\log K_m$ and $\log V_{max}$ increased linearly if homo-oligoprimers contained less than 10 nucleotides. The lengthening of $d(pT)_n$ and $d(pA)_n$ primers by one mononucleotide unit ($n=1$–10) resulted in the 1.82-fold decrease of the K_m values. The incremental decreases of K_m for $d(pC)_n$ and $d(pG)_n$ were equal to about 2.46. The enhancement of the homo- and hetero-oligonucleotide primers' affinity to the enzyme due to one Watson-Crick hydrogen bond between complementary template and primer is about 1.35 times. This allows to calculate the K_m values for primers of various structure and length up to 10 units. The objective laws of the K_m and V_{max} values changes for primers containing more than 10 nucleotides were analyzed.

Klenow fragment; Primer; K_m calculation, algorithm of

1. INTRODUCTION

The mechanism of binding and elongation of homo-oligoprimers $d(pT)_n$, $(pA)_n$, $(pU)_n$ and $(pA)_n$ in the case of human DNA polymerase α, DNA polymerase I from *E.coli* and its FK, and DNA polymerases from archaebacteria has been investigated [1–7]. dNMP, NMP and dNTP were shown to be the minimal primers of DNA polymerases [2–5,7]. The dependences of $-\log K_m$ ($-\Delta G°$) vs the number of the primer nucleotide units (n) are linear up to $n=9$–10. The results may be described by a common equation: $K_m(n)=K_m(1)\cdot f^{1-n}$, where $K_m(1)$ is the K_m value for dNMP or NMP ($n=1$), and f is a factor of the affinity enhancement with the increase of the primer length by a unit. The coefficient f was found to be 1.82 for mesophilic DNA polymerases (polymerization at 30°C) [2,4,7] and 2.22 for DNA polymerases from archaebacteria (60°C) [5]. In the present work we have investigated the dependences of the K_m values on n for $d(pC)_n$, $d(pG)_n$ and hetero-oligoprimers complementary to single-stranded DNA of M13 phage in the case of FK. A common calculation algorithm of the K_m values for primers of various structure and length has been found.

Abbreviations: FK, Klenow fragment of *E. coli* DNA polymerase I; V, V_{max} of polymerization reaction

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2. MATERIALS AND METHODS

Electrophoretically homogeneous FK with a specific activity of $6\cdot10^4$ units/mg was obtained according to [8]. Poly(dN), dNMP and dNTP were from NIKTI BAV(USSR), BSA from Koch Light, $MgCl_2$ from Merck, [^{3}H]dNMP and [^{3}H]dNTP from Izotop (USSR), NaF of high purity from Reakhim (USSR). Other compounds used were analytical grade.

The synthesis, characterization and methods of purification to homogeneity have been reported in [1–4]. Homogeneous M13 phage DNA was a gift from Dr. S.V. Mamaev (our Institute).

The polymerase activity of FK was determined at 30°C. The reaction mixture (50–150 μl) contained 50 mM Tris-HCl buffer (pH 7.5), 0.2–0.5 mg/ml BSA, 10–50 μM EDTA and 6 mM NaF. Other conditions are listed in table 1. The reaction was started by adding of FK. Further treatment of the reaction mixtures was done as in [2–4] using the method of acid insoluble precipitates for $d(pN)_{6-300}$ and reversed-phase chromatography for $d(pN)_{1-6}$ primers.

The K_m and V values were determined according to Eisenthal and Cornish-Bowden [9]. Errors in K_m and V were within 20–40%.

3. RESULTS AND DISCUSSION

The optimal conditions for copying poly(dT), poly(dA), poly(dC) and poly(dG) have been found (table 1). NaF was used for selective inhibition of 3′-5′-exonuclease activity of FK according to [10]. NaF was shown to have no effect on K_m for templates, primers and dNTP and to increase the V values for the

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Table 1

Optimal concentrations of the components of the mixtures for the polymerization reaction catalyzed by Klenow fragment of *E. coli* DNA polymerase I

Components	Template · primer complex			
	Poly(dG) · d(pC)$_{10}$	Poly(dC) · d(pG)$_{10}$	Poly(dA) · d(pT)$_{10}$	Poly(dT) · d(pA)$_{10}$
Template, $A\lambda_{max}$/ml*	0.5	0.7	0.4	0.4
Primer, μM	2.2	0.08	1.6	6.0
dNTP, μM**	15 (dCTP)	15 (dGTP)	15 (dTTP)	15 (dATP)
$MgCl_2$, mM	1.0	2.0	2.5	10.0
KCl, mM	10	10	3	3
FK, U/ml	100–200	0.5–2.4	1–2.4	2–5

*The saturated concentrations of templates and primers which do not inhibit the polymerization reaction. The lengths of the templates were: 100–700; 100–500; 50–400; 50–150 nucleotides for poly(dT), poly(dA), poly(dC), poly(dG), respectively.

**[^{3}H]dNTPs were with a specific activity of 0.2–20 · 10^{14} Bq/mol. For the synthesis on M13 phage DNA (0.3 A_{260}/ml) the conditions of poly(dA) · d(pT)$_{10}$ were used; 15 μM ^{3}H-labelled dNTP: dATP, dCTP, dGTP and dCTP were with a specific activity of 1–4 · 10^{13} Bq/mol.

polymerization reaction by 10–100% [10,11]. A comparison of polymerization reactions with various templates in the presence of NaF revealed the following ratio of the initial rates of the synthesis: 100% for poly(dC), 67% for poly(dA), 10% for poly(dT), and 0.5% for poly(dG).

According to [6], K_m for primers negligibly differ from K_d. This allows us to take K_m as a measure of affinity of primers to FK and to calculate the $\Delta G°$ values as $-RT \ln K_m$.

The K_m and V values for various primers were determined using the initial rates of polymerization. The dependences of $-\log K_m$ (K_m were determined using base concentration of primers) and relative V values for d(pT)$_n$ vs the number of nucleotide units (n) are given in fig.1. The increase of these values is observed up to $n = 9$–10. If the molar extinction coefficients were used for determination of primer concentration, similar dependences of the K_m values for d(pT)$_n$, d(pA)$_n$, d(pC)$_n$, and d(pG)$_n$ were linear up to $n = 9$–10 (fig.2). The coefficients of the primer affinity enhancement with the increase of the oligonucleotide length by a unit were found equal to 1.82 for d(pA)$_n$ and d(pT)$_n$ and 2.46 for d(pC)$_n$ and d(pG)$_n$. Taking into account 2 and 3 hydrogen bonds of Watson-Crick base pairing for A · T and G · C, the increase of the primer affinity due to one hydrogen bond was estimated as a factor of 1.35 ($\sqrt{1.82} = \sqrt[3]{2.45} = 1.35$). The K_m change for homo-oligoprimers ($n = 1$–10) may be described by the equation: $K_m(X) = K_m(10) \cdot 1.35^m$, where $K_m(10)$ is the K_m value for d(pN)$_{10}$, and m is the difference in the number of hydrogen bonds formed by template with d(pN)$_{10}$ and a shorter primer X. Experimentally determined K_m and V for heterooligoprimers complementary to M13 phage DNA are given in table 2. The experimental K_m value for heterodecanucleotide was used for calculation of the K_m values of hetero-d(pN)$_{3-9}$ primers according to the equation: $K_m(X) = K_m(10) \cdot 1.35^m$. The data of table 2 show that experimental and calculated K_m values for d(pN)$_{3-9}$ primers are the same within the accuracy of their determination. This suggests the same efficiency of one hydrogen bond formation of A · T and G · C pairs in the case of homo- and heteroduplexes of templates and primers and allows us to calculate K_m for primers of different structure and length. The calculated K_m value for dTMP (1.1 μM) in the case of M13 phage DNA (see table 2) is about 35 times lower than the experimental K_m value (39 μM) for dTMP determined with poly(dA) template (fig.2). For analysis of reasons of the increase of the primer 3′-terminal nucleotide affinity in the case of heteropolynucleotide template in comparison with homopolymeric template, it is necessary to take into account a number of other facts. The rate of polymerization reaction depends on the template used and decreases by a factor of 200 when passing from poly(dC) to poly(dG). The affinity of minimal primers dNMP also depends on the homopolymeric templates used and increases in the order: dAMP ≤ dCMP < dTMP < dGMP (fig.2). As seen from fig.2, the absolute K_m values for primers d(pA)$_n$ and d(pT)$_n$ depend on the composition of the reaction mixture. In contrast to heterooligonucleotides, the V values for various dNMP are different and equal to 7, 12, 25, and 35% for dCMP, dGMP, dAMP, and dTMP, respectively, in comparison with 100% for corresponding d(pN)$_{10}$ (table 2, fig.3).

The duplexes with the G · C pair were found to be drastically polymorphic [12]. In contrast, some A · T polymers were all found to be in B-forms, with some variations within the B-genus [12]. According to the X-ray data, FK can accommodate double-stranded B-DNA [13]. One could believe that in the case of heterologous DNA, there is more optimal adaptation to the B-form DNA conformation necessary for FK than

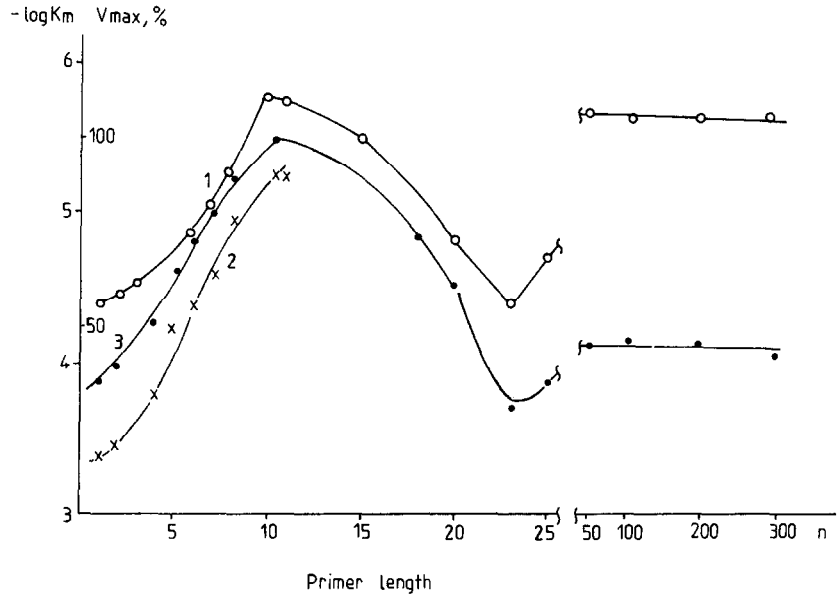

Fig.1. The dependences of $-\log K_m$ (1) and relative values of V_{max} (2,3) on the length (n) of d(pT)$_n$ primers. K_m values were calculated using base concentrations of the primers; 2, in the absence of NaF; 3, in the presence of 6 mM NaF. K_m values were the same either with or without NaF.

in the case of homopolymeric duplexes. Such DNA conformation could supposedly provide a higher affinity of the primer to the enzyme and an optimal orientation of the primer 3'-end for catalysis of polymerization reaction. According to [3,4,7] the decrease of (pU)$_n$ and [d(pT)$_n$]pU affinity to DNA polymerases in comparison with d(pT)$_n$ primers takes place not due to the decrease of the efficiency of the primers' complementary interaction with the template. The decrease is a result of about 10-fold reduction of the affinity of the riboprimer 3'-end to DNA polymerases. The enhancement of the mobility of the 3'-terminus results in an increase of the V value for (pU)$_n$ by a factor of 15 [3,4].

According to [2–5,7] only 3'-terminal nucleotide of d(pT)$_n$ primers interacts with DNA polymerases by electrostatic contact of the phosphate group ($\Delta G° = -1.2$ kcal/mol) and one or two hydrogen bonds of nucleotide ($\Delta G° \approx -5.0$ kcal/mol). All other nucleotide units of

Table 2

The K_m and V values for hetero-oligoprimers of various length (n) complementary to M13 phage DNA

Primer (3'-5')	n	K_m(nM)		Experimental V values, (cpm/min)·10^{-3}
		Experimental values	Calculated values*	
dTMP	1	-	1100	3.5
d(TTG)	3	190 ±30	245	3.7
d(TTGC)	4	105 ±20	100	3.7
d(TTGCA)	5	60 ±10	55	3.7
d(TTGCAG)	6	21 ± 2	22	3.7
d(TTGCAGC)	7	8.3± 2	9.0	3.7
d(TTGCAGCA)	8	3±2(4.5±1.3)***	5.0	3.8
d(TTGCAGCAC)	9	2±0.3(2.5±0.5)	2.0	4.0
d(TTGCAGCACT)	10	1.1±0.3(2.0±0.4)	-	3.8
d(TTGCAGCACTGA)	12	1.2±0.3	1.36**	3.6
d(TTGCAGCACTGACC)	14	1.9±0.5	1.54	3.5
d(TTGCAGCACTGACCC)	15	2.1±0.4	1.75	3.4

* Calculated using the equation: $K_m(X) = 1.1$ nM·$(1.35)^m$, where 1.1 nM is the K_m value for decanucleotide, and m is the difference in the number of hydrogen bonds formed by DNA with decanucleotide and with one of the analyzed X-primers.

** Calculated using the equation: $K_m(n) = 1.1$ nM·$(1.13)^{n-10}$.

*** K_m (in parentheses) were determined using only [^{3}H]dGTP instead of 4 [^{3}H]dNTPs. The primers were complementary to the following region of DNA: (5')CGTTTTACAACGTCGTCGTGACTGGG(3')

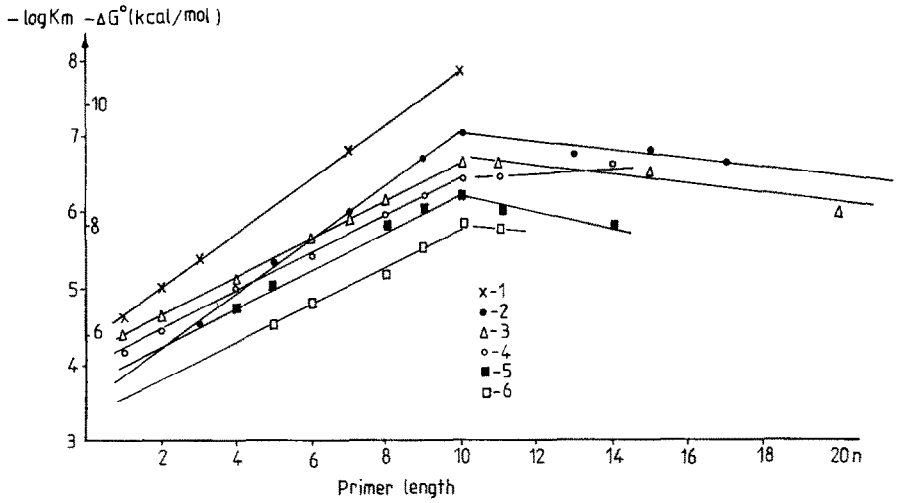

Fig.2. The dependences of $-\log K_m$ ($-\Delta G°$) on the length (n) of the primers: d(pG)$_n$ (1), d(pC)$_n$ (2), d(pT)$_n$ (3,4), d(pA)$_n$ (5,6); curves 4 and 5 are from [3,4] and were obtained using a different composition of the reaction mixture. The molar extinction coefficients were used for determination of the primers' concentrations and K_m values.

the primer interact only with the template. The efficiency of such primer 3'-end nucleotide contacts with FK supposedly depends on the structure of the template-primer complex. We interpret the above data by assuming that the orientation of the 3'-end of homologous primers is not optimal for catalysis and that they bind with the enzyme not so productively as heteroprimers.

We proposed [2-5,7] that only 9-10 nucleotide units of a primer interact with the binding cleft of FK. The interaction between template and primer outside the enzyme was supposed to lead to an increase of the affinity and V values for primers with $n > 10$. As follows from the data of figs. 1 and 2 as well as from table 2, if $n = 11$-20 the decrease of the primers' affinity to FK is practically independent on the primer structure. In all the cases investigated, the changes of K_m may be described approximately by the equation: $K_m(n) = K_m(10) \cdot 1.13^{n-10}$, where n is the number of the primer mononucleotide units; 1.13 is a factor of the affinity decrease with an increase of the primer length by a unit. The K_m and V values for primers with $n > 20$ are determined only for d(pT)$_n$ (fig.1.). One can see that d(pT)$_{22-23}$ have a minimal affinity to FK and minimal V values. d(pT)$_{50-300}$ primers have nearly the same affinity to FK (as calculated using base concentration) as d(pT)$_{10-11}$ and 1/2 V values.

The decrease in the affinity and V values for d(pN)$_{11-18}$ primers was also found for other DNA polymerases [2-5,7]. Therefore this phenomenon might be common for many DNA polymerases. The elucidation of its causes needs further investigation.

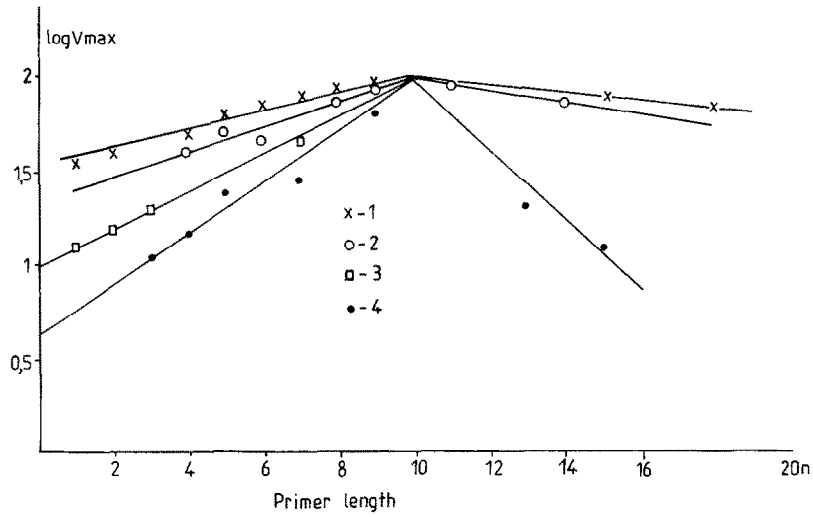

Fig.3. The dependences of the log V_{max} values on the length (n) of the primers: d(pT)$_n$ (1), d(pA)$_n$ (2), d(pG)$_n$ (3), d(pC)$_n$ (4).

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