Effect of DNA and bivalent metal ions on the interaction of thermostable DNA polymerase *Tte* with dNTPs

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The interaction of DNA polymerase *Tte* from *Thermus thermophilus* B35 with dUTP analog containing a fluorescein residue bound to C(5) of the base (Flu-dUTP) was studied by fluorescence titration. The dissociation constants of the enzyme—substrate complexes in the absence and in the presence of a DNA duplex containing an extended template and bivalent metal ions and the kinetic parameters of polymerization by DNA polymerase *Tte* in the presence of Flu-dUTP were determined.

Key words: DNA replication, fidelity of DNA synthesis, thermostable DNA polymerase *Tte*, fluorescent derivatives of dNTP.

The DNA biosynthesis performed by DNA polymerases underlies the vital activity of the cell. The specificity of DNA synthesis during replication is an important factor for the genetic stability of an organism. Therefore, detailed analysis of the mechanism of specific selection of dNTP bases by the enzyme including evaluation of the role of the primer—template complex and bivalent metal ions in this process is a key point in the investigation of DNA polymerases and replication. Thermostable DNA polymerases are important subjects for investigating the mechanisms ensuring the fidelity of DNA replication, as they are highly specific with respect to DNA synthesis at extremely high temperatures. In addition, thermostable DNA polymerases are of high applied importance because they are used in methods based on DNA amplification by a polymerase chain reaction. This is why the fidelity of DNA synthesis is the major requirement to these enzymes.

To study this problem, we chose a new enzyme, thermostable DNA polymerase *Tte* (*Tte*-pol) from the strain *Thermus thermophilus* B35 discovered in the thermal spring water. Previously,[1] the reaction of *Tte*-pol with dNTP in the presence of bivalent metal ions was studied using kinetic approaches, which made it possible to elucidate the influence of various factors on the substrate affinity for the enzyme and on the rate constant for the catalytic DNA synthesis. Fluorescent dNTP and oligonucleotide derivatives are widely used to study the mechanisms of functioning of DNA polymerase and other DNA and/or dNTP-binding proteins, in particular, to determine quantitative characteristics of enzyme—substrate interactions.[2—6] Here we report a fluorescent titration study of the interaction of DNA polymerase *Tte* with a dUTP analog containing a fluorescein residue bound to the heterocycle C(5) atom in the absence and in the presence of a primer—matrix complex and bivalent metal ions, Mg^{2+} and Mn^{2+}. In addition, the kinetic parameters of the one-step primer elongation by DNA polymerase *Tte* were measured using dNTP and the fluorescent analog Flu-dUTP as the substrate.

Experimental

The following chemicals were used: γ-[^{32}P]-ATP with a specific activity of 4000 Ci mmol^{-1} (Biosan, Russia), T4 polynucleotide kinase (5000 au mL^{-1}) (SibEnzim, Russia), Sigmamarker High Range molecular mass markers, dATP, dUTP, reagents for electrophoresis, and the main buffer components (Sigma, USA). The other reagents were commercial chemicals (special purity or chemical pure grade) produced in Russia.

Recombinant DNA polymerase *Tte* was isolated from *E. coli* BL21 according to a previously described procedure.[7]

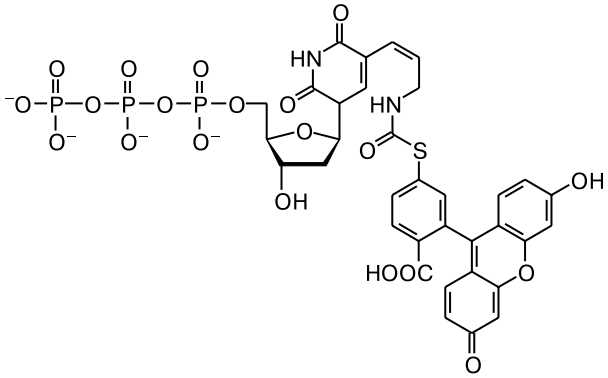
The synthesis and the photochemical properties of the fluorescent analog Flu-dUTP, 5-[*N*-(fluorescein-5-ylthiocarbonyl)-3-*trans*-aminoprop-1-enyl]-2´-deoxyuridine-5´-triphosphate, have been studied previously.[8]

Partial DNA-duplexes **I** (20/12-mer) and **II** (20/10-mer) were designed using deoxyribooligonucleotides synthesized at the Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the RAS, on a Biosset ASM 800 automatic synthesizer by the standard phosphoramidite method:

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 5, pp. 1268—1272, May, 2005.

1066-5285/05/5405-1306

20-mer, 5′-TTGTTCCAATGATAGCCCCT-3′; 12-mer, 5′-AGGGGCTATCAT-3′; 10-mer, 5′-AGGGGCTATC-3′.



5′-TTGTTCCAATGATAGCCCCT-3′ **I**
TACTATCGGGGA*-5′

5′-TTGTTCCAATGATAGCCCCT-3′ **II**
CTATCGGGGA*-5′

Radioactive labeling of the 5′-end oligonucleotide was carried out as described previously.[9] The labeled oligonucleotides were purified by electrophoresis in polyacrylamide gel (PAAG) followed by electroelution.[10]

The kinetic characteristics of the primer elongation in duplexes **I** or **II** in the presence of dATP or Flu-dUTP by *Tte*-pol were determined by alternating variation of the concentrations of the major components. The reaction mixtures (75 μL) contained various concentrations of the enzyme (0.1—500 nmol L^{-1}) and dATP or Flu-dUTP (50—0.1 μmol L^{-1}), 0.5 μ*M* 5′-[^{32}P]-labeled primer·template complex **I** or **II**, and a buffer containing 50 m*M* Tris-HCl (pH 8.6, 25 °C), 50 m*M* KCl, and 6 m*M* $MgCl_2$. The reactions were carried out at 70 °C. Aliquots (5 μL) were withdrawn at definite intervals. The reactions were terminated by ice-cooling of the sample to 0 °C and by adding a loading buffer containing 0.05% Bromophenol Blue, 90% formamide, and 50 m*M* EDTA in the initial concentration. The samples were heated for 2 min on a water bath at 95 °C. The reaction products were analyzed by electrophoresis in 20% PAAG (acrylamide—*N*,*N*′-methylenebisacrylamide, 19 : 1) with 7 *M* urea on vertical 15×30×0.04 cm plates in 100 m*M* Tris-borate buffer, pH 8.3, at 1000 V. The positions of the labeled oligonucleotides were determined by autoradiography. The bands corresponding to the elongation products and the initial primer were cut out and the content of the radioactive isotope was determined according to Cherenkov using a 1211 Racbeta LKB Wallac scintillation counter. The results were processed using a Microcal Origin 6.1 program (Microcal Software, USA) in terms of the enzymatic Michaelis—Menten kinetic scheme.[11]

The inhibition constants for the dATP primer elongation in duplex **I** by DNA polymerase *Tte* in the presence of Flu-dUTP were determined by varying the concentration of Flu-dUTP. The mixtures (10 μL) contained 5 n*M* of the enzyme, 3.5 μ*M* of dATP, Flu-dUTP (500—1 μmol L^{-1}), and 0.5 μ*M* of radioactive 5′-[^{32}P]-labeled primer·template complex **I** in a buffer containing 50 m*M* Tris-HCl (pH 8.6, 25 °C), 50 m*M* KCl, and 6 m*M* $MgCl_2$. The reaction was carried out at 70 °C for 10 min and terminated by ice-cooling to 0 °C. Then 2 μL of the loading buffer containing 0.05% Bromophenol Blue, 90% formamide, and 50 m*M* EDTA was added. The samples were heated for 2 min on a water bath at 95 °C. The reaction products were analyzed as described above.

The dissociation constants of the complexes enzyme·Flu-dUTP, enzyme·DNA·Flu-dUTP, enzyme·Flu-dUTP·M^{2+}, and enzyme·DNA·Flu-dUTP·M^{2+} were determined by fluorescent titration. The measurements were carried out on a MPF-4 spectrofluorimeter (Hitachi, Japan) at a slit width of 4 nm. The experiments were performed in a buffer containing 50 m*M* Tris-HCl (pH 8.6, 25 °C) and 50 m*M* KCl at an excitation wavelength of 492 nm and an emitted wavelength of 515 nm, corresponding to a fluorescence maximum. A solution of Flu-dUTP (0.5 μmol L^{-1}) was titrated by aliquots of the enzyme or an enzyme—DNA mixture (1 : 1) in such a way that the solution volume increased by not more than 0.5% in each titration point. After addition of every next portion of the enzyme, the samples were incubated for 2 min to attain the equilibrium, and then the fluorescence intensity of the solution was measured. The results were processed by the Microcal Origin 6.1 program (Microcal Software, USA) by the single-ligand binding model. The influence of Mg^{2+} or Mn^{2+} cations on the complexation was studied in a similar way.

Results and Discussion

Substrate properties of Flu-dUTP in the *Tte*-pol-catalyzed reaction. The fluorescent dUTP derivative, Flu-dUTP, was used as either the substrate or a competitive inhibitor in the one-step primer elongation by DNA polymerase *Tte* in duplexes **I** and **II**, respectively.

The Michaelis constants (K_m) for dNTP and Flu-dUTP are presented below.

Substrate	K_m/μmol L^{-1}
Flu-dUTP	24.62±5.26
dATP	3.67±0.43
dTTP	2.05±0.09

It follows from the obtained data that the fluorescent analog is the substrate for *Tte*-pol, although it has a K_m value an order of magnitude higher than the native dNTP, which is indicative of its affinity to the enzyme and/or the enzyme—substrate complex. In addition, Flu-dUTP is a competitive inhibitor of the primer elongation (duplex **II**) in the presence of dATP with K_i ~56.5 μmol L^{-1}.

The effect of metal ions on the stability of Flu-dUTP·*Tte*-pol complexes. Functioning of DNA polymerases is known[13,14] to require a bivalent metal ion, which acts as a cofactor in the polymerase reaction; *in vivo* this is normally Mg^{2+}. Most of DNA polymerases studied are able to perform catalysis in the presence of Mn^{2+} ions and some other bivalent ions, although with a much lower efficiency.[15—18] A mechanism of the catalysis has been proposed for DNA synthesis involving two Mg^{2+} ions, one promoting deprotonation of the 3′-OH group of the primer, thus facilitating the attack by the α-phosphate on the 3′-O^- group, and the other promoting the formation

of the pentacovalent transition state at the α-phosphate and release of pyrophosphate.[18] The maximum activity of DNA polymerase is manifested, most often, in the Mg^{2+} concentration range of 5—10 mmol L^{-1};[19] in particular, in the case of *Tte*-pol,[1] at 6 mmol L^{-1}. It has not been determined exactly which of the reaction steps depends most appreciably on the concentration of bivalent ions. The results of our earlier kinetic studies suggest that in the case of *Tte*-pol, the substrate affinity for the enzyme and the step of the catalytic transformation of the substrate are unlikely to depend on the bivalent ion concentration, and only the affinity of the primer·template complex for the enzyme changes.[1] Later, it has been shown by fluorescent anisotropy[19] that the dependence of dissociation constants (K_d) on the Mg^{2+} concentration for the complex of thermostable DNA polymerase *Taq* with primed DNA is linear in the range of 0—10 mmol L^{-1}.

In this work, we studied the effect of bivalent ions on the dNTP binding to the enzyme both in the presence and in the absence of DNA using fluorescence quenching technique. The fluorescence quenching curves for Flu-dUTP during the titration by *Tte*-pol in the presence of various concentrations of Mg^{2+} (0—10 mmol L^{-1}) are presented in Fig. 1. It can be seen that on the addition of the enzyme, the intensity of the fluorescent signal drops; however, the variation curves do not reach a plateau. The initial sections of the curves (up to the enzyme concentration of 0.5 μmol L^{-1}) are well described by an equation corresponding to a hyperbolic dependence characterizing the binding isotherm (Fig. 2), whereas a further increase in the enzyme concentration results in an almost linear decrease in the fluorescence of the solution. In our opinion, the decrease in the fluorescence at saturating concentrations of the enzyme is nonspecific and is attributable to an increase in the amount of hydrophobic components of the solution following the increase in the concentration of the hydrophobic biopolymer not bound to the ligand. The K_d values of the Flu-dUTP complexes with *Tte*-pol, found from the initial sections of the titration curves, are summarized in Table 1. It can be seen from the Table that the lowest K_d value is observed at 6 mmol L^{-1} of Mg^{2+}; in the concentration range of 3—10 mmol L^{-1}, K_d changes insignificantly. These features correlate well with the data on the influence of Mg^{2+} on the enzyme activity obtained earlier.[1] The effect of Mn^{2+} ions on the stability of the enzyme—substrate complex was analyzed in a similar way. Since the dependence of the enzyme activity on the concentration of Mn^{2+} shows an optimum at 0.5 mmol L^{-1}, we varied the Mn^{2+} concentration around this value (0.1—1.2 mmol L^{-1})[1]. It can be seen from Table 1 that in the concentration range chosen, the dissociation constant of the Flu-dUTP complex with *Tte*-pol is minimum at 0.1 *mM* Mn^{2+} concentration. Perhaps, in the presence of low concentrations of

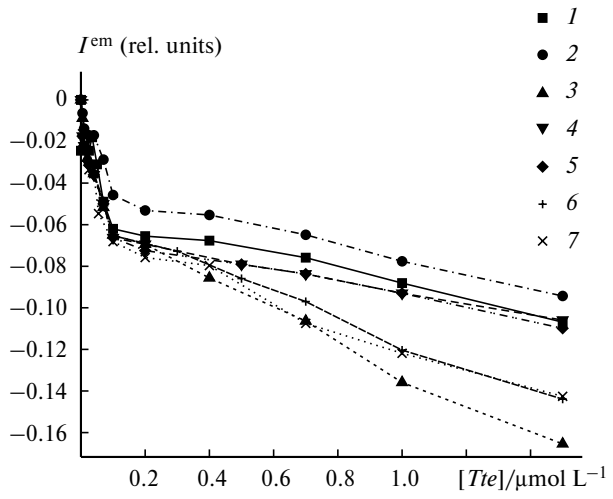


Fig. 1. Intensity of Flu-dUTP fluorescence *vs.* *Tte*-pol concentration in the absence of Mg^{2+} (*1*) and in the presence of Mg^{2+} in a concentration of 0.1 (*2*), 1.5 (*3*), 3 (*4*), 6 (*5*), 8 (*6*), and 10 mmol L^{-1} (*7*).

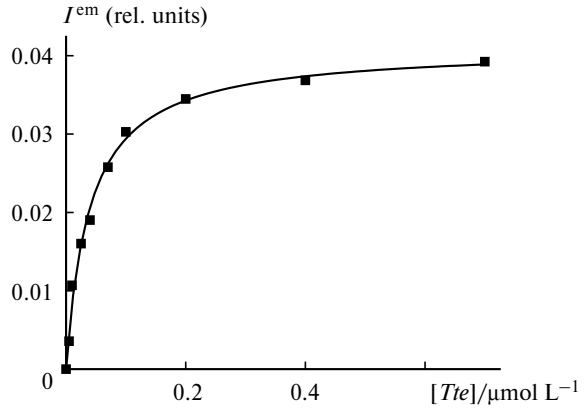


Fig. 2. Binding isotherm of *Tte*-pol with Flu-dUTP in the presence of 6 m*M* of Mg^{2+}.

Table 1. Effect of the concentration (*C*) of bivalent metal ions on Flu-dUTP binding to the enzyme in the absence and in the presence of DNA (in parentheses)

Ion	*C*/mmol L^{-1}	K_d/nmol L^{-1}
Mg^{2+}	0	58±11 (74±10)
	0.1	74±16 (47±5)
	1.5	79±16 (42±9)
	3	45±9 (51±8)
	6	36±6 (12±3)
	8	53±8 (15±4)
	10	39±6 (53±12)
Mn^{2+}	0	58±11
	0.1	14±3
	0.5	33±8
	0.9	38±8
	1.2	35±4

transition metal ions, the nucleotide is bound most strongly to the enzyme to form a nonproductive complex, whereas the formation of a productive complex requires a higher Mn^{2+} concentration. It was found[15] that in the presence of Mn^{2+} ions, several types of enzyme—substrate complexes are formed, probably differing in the structure of the ion coordination sphere. Generally, a comparison of the K_d values of the complexes with Mg^{2+} and Mn^{2+} ions shows that the substrate—enzyme binding is stronger with the Mn^{2+} ion than with Mg^{2+}. An increase in the enzyme affinity of dNTP can entail a decrease in the selection accuracy with respect to nucleotides, which is observed for most DNA polymerases in the presence of Mn^{2+} ions and some other bivalent cations.[20,21]

Binding of Flu-dUTP to *Tte*-pol in the presence of DNA. According to a generally accepted mechanism of functioning of DNA polymerase, the reaction of the enzyme with DNA is the first step, necessary for the subsequent specific dNTP selection.[19] Therefore, we studied the reaction of Flu-dUTP with *Tte*-pol in the presence of DNA. For this purpose, *Tte*-pol was incubated with DNA-duplex **II** in the enzyme : DNA ratio of 1 : 1, and the resulting solution was used to titrate Flu-dUTP. The titration curves obtained at different Mg^{2+} concentrations are presented in Fig. 4. It can be seen that the curves for the dependence of fluorescence quenching of the analog on the concentration of the enzyme—DNA complex consist of at least two regions corresponding to concentration ranges of 0—0.5 and 0.5—1 μmol L^{-1}. As in the case of complexation of *Tte*-pol with Flu-dUTP in the absence of DNA, the initial regions of the curves are well described by a hyperbolic law. This is indicative of the formation of a specific enzyme—substrate complex. The presence of the second regions in the fluorescence quenching curve can be attributed to the formation of nonspecific complexes due to the aggregation of protein molecules. This effect might be attenuated at higher temperatures optimal for *Tte*-pol function; however, measurements under these conditions are impracticable. The dissociation constants of the complexes calculated from the initial regions of the titration curves are summarized in Table 1. It can be seen that both in the presence and the absence of DNA, the strongest binding of Flu-dUTP to the enzyme is attained at Mg^{2+} concentration of 6 mmol L^{-1}; however, in the presence of DNA, the stability of the complexes increases.

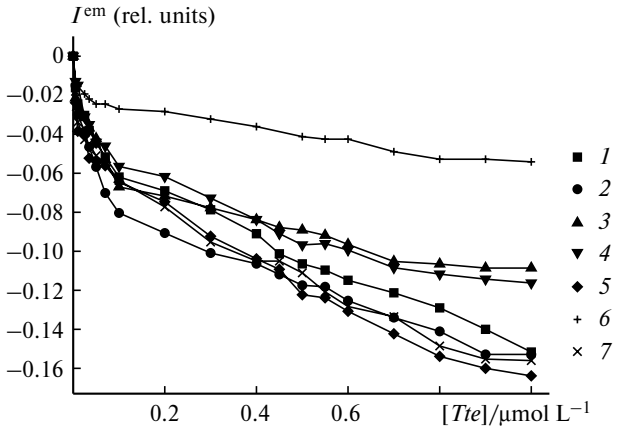


Fig. 3. Fluorescence intensity of Flu-dUTP *vs.* concentration of the *Tte*-pol·DNA complex in the absence of Mg^{2+} (*1*) and in the presence of Mg^{2+} at concentrations of 0.1 (*2*), 1.5 (*3*), 3 (*4*), 6 (*5*), 8 (*6*), and 10 mmol L^{-1} (*7*).

Thus, fluorescent titration can be used to estimate comparative characteristics of the complexation of polymerase with dNTP in the presence and in the absence of DNA or metal ions in the study of mechanisms of protein—nucleic acid interactions of DNA polymerases. We showed that thermophilic DNA polymerase *Tte* reacts with dNTP both in the absence and in the presence of DNA. Despite the similarity of complexation behavior in the presence of different ions, the formation of *Tte*-pol complexes with dNTP in the presence of DNA is more efficient.

It is noteworthy that although Flu-dUTP we used as the fluorophore is a substrate for DNA polymerase *Tte*, it can be used as a dNTP analog for investigating the thermodynamic characteristics of binding only in a rather narrow concentration range due to the high hydrophobicity of the fluorescent fragment. However, its substrate properties allow one to introduce a fluorescent group into specific positions of DNA and to use these derivatives for investigating the mechanism of DNA replication.

The authors are grateful to S. V. Dezhurov for the synthesis of the dUTP fluorescent derivative.

This work was financially supported by the Russian Foundation for Basic Research (Projects No. 04-04-48171 and No. 02-04-48404).

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Received April 20, 2004;
in revised form September 23, 2004