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Thermostable DNA Polymerase from *Thermus thermophilus* B35: Preparation and Study of a Modified Form of the Enzyme with High Affinity to ddNTP

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Abstract—The hybrid protein consisting of *Tte* DNA polymerase fragment and mutant *Taq* DNA polymerase (F667Y) fragment in the ratio 20 : 1 was constructed. Affinity of the modified enzyme (substitutions F669Y, V667I, and S692Q) to ddNTP was two orders higher than that of the wild type enzyme. The modified enzyme was used for sequencing DNA fragment with total deoxyguanosine and deoxycytidine content of 68%. In the polymerase chain reaction, the modified enzyme exhibits properties typical of the wild type *Tte* DNA polymerase.

Key words: thermostable DNA polymerase, site-directed mutagenesis, dideoxynucleoside triphosphate, DNA sequencing

Thermostable DNA polymerases are very important for molecular-biological studies. Application of these enzymes is closely related with their properties. Thermostable DNA polymerases are used for DNA amplification and sequencing by the polymerase chain reaction. These enzymes are especially important for sequencing DNA with GC-rich sites susceptible to formation of secondary structures. Use of thermostable DNA polymerases for DNA sequencing was for a long time limited by their low affinity to ddNTP [1]. It is known that DNA polymerases belonging to the same polymerase family (Pol I) markedly differ in their ability to discriminate deoxy- and dideoxyribose residues in nucleoside triphosphates. Thus, DNA polymerase of T7 phage incorporates deoxynucleotides only three times more efficiently than dideoxynucleotides [2, 3], whereas for DNA polymerase I from *Escherichia coli* and *Taq* DNA polymerase from *Thermus aquaticus* YT1, the incorporation rates of deoxy- and dideoxynucleotides differ hundreds and even thousands of times [1-4]. Using site-directed mutagenesis, it was shown that the observed difference in this property of DNA polymerases is defined by the only amino acid residue in the active site: replacement of Tyr526 for Phe in the DNA sequence of T7 polymerase drastically decreased efficiency of ddNTP incorporation, and vice verse, replacements of Phe for Tyr in the homologous positions of *E. coli* and *Taq* DNA polymerase I (762 and 667, respectively) caused the opposite result [5]. It was shown later that Phe667 is also important for fidelity of *Taq* DNA polymerase [6], and incorporation of chance mutations in the dNTP-binding site (A-motif, amino acid residues 605-617) produced mutant forms of *Taq* DNA polymerase which synthesize RNA [7]. Thus, change in the properties of enzymes by modification of the certain sites in encoding genes not only provides new instruments for molecular-biological studies but also clarifies the general regularities in functioning of these enzymes and structure–properties relationships.

Earlier we were first to isolate a thermostable *Tte* DNA polymerase from *Thermus thermophilus* B35 [8]. The gene encoding this enzyme was cloned into the expressing vector, the recombinant protein was isolated as homogeneous substance, and its properties were studied.

Abbreviations: *Tte* pol) DNA polymerase from *Thermus thermophilus* B35; ddNTP) dideoxynucleoside-5′-triphosphate; PCR) polymerase chain reaction.

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In particular, it was shown that the enzyme has a high temperature optimum and is able to catalyze PCR in the presence of formamide [9-11], which extends its use in clinical diagnostics. In this study, we obtained a modified form of *Tte* pol with increased affinity to ddNTP compared with the wild type enzyme and studied it properties.

MATERIALS AND METHODS

In this study the following materials were used: *E. coli* JM109 and *E. coli* BL21 strains; pUC19 [12] and pGTTE [9] plasmid DNA; the components of the culture medium, peptone, and yeast extract from Difco (USA); chromatographic sorbents: hexyl-agarose and heparin-Sepharose from Sigma (USA). Restriction endonucleases, DNA ligase of T4 phage, the Klenow fragment, and deoxyribonucleoside triphosphates were from SibEnzyme (Russia); reagents for electrophoresis and the main components of buffers were from Sigma; [^{3}H]dATP and [γ-^{32}P]ATP were from Izotop and Biosan (both Russia), respectively. Other reagents were of extra pure grade.

Construction, isolation, and restriction analysis of recombinant plasmid were performed according to the standard procedures [13]. Electroporation was performed using an EasyjecT Prima in a 2-mm cuvette at the electric field tension 12,500 V/cm according to the standard protocol from EquiBio (Great Britain) [14].

DNA sequencing. DNA for clone sampling with the gene modification was sequenced using the Klenow fragment according to the protocol from Promega (USA) [15].

Sequencing with the modified *Tte* pol was performed in 10 μl of the reaction mixture containing 60 mM Tris-HCl, pH 8.9 (25°C), 1.5 mM $MgCl_2$, 16% formamide, 25 mM KCl, 0.1% Tween-20, 30 μg/ml DNA, 0.7 μM primer ^{32}P-labeled at the 5′-end, 75 μM each dNTP; one of terminators 0.3 μM ddATP, 0.3 μM ddCTP, 0.3 μM ddGTP, or 0.4 μM ddTTP; and 1 U DNA polymerase. Amplification was performed according to the following procedure: denaturation, 150 sec, 84°C, 1 cycle; denaturation, 15 sec, 84°C; annealing, 20 sec, 39°C; polymerization, 20 sec, 64°C, 40 cycles. After addition of 3 μl of formamide with Phenol Red and xylene cyanole and evaporation to the volume 3 μl, the reaction products were completely applied on 5% polyacrylamide gel (acrylamide/bis-acrylamide 20 : 1) containing 7.5 M urea. Electrophoresis was performed in buffer containing 50 mM Tris, 48 mM serine, and 1 mM EDTA, pH 8.5. The autoradiograph was obtained using the Cyclon Storage Phosphor System from Packard BioScience Company (Japan).

Expression of mutant thermostable DNA polymerase in *E. coli* cells was detected by electrophoresis of crude cell lysates.

Growth of biomass and isolation of the wild type and mutant recombinant *Tte* pol were performed as described in [9].

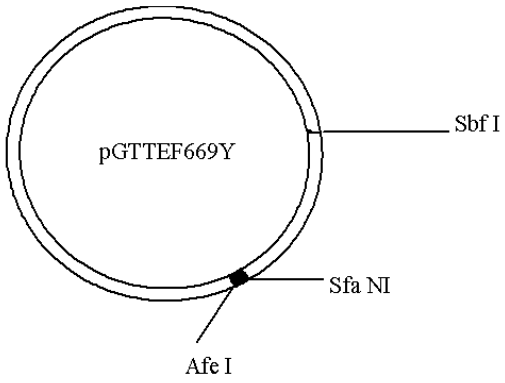
Radioactive label was incorporated into the 5′-end of the oligonucleotide as described in [13]. The labeled oligonucleotides were purified by electrophoresis in polyacrylamide gel as described in [16]. The primer and template were hybridized for 5 min at room temperature.

Elongation of the radioactively labeled primer with wild type or mutant *Tte* DNA polymerase was performed in 10 μl of reaction mixture containing the standard components of buffer (50 mM Tris-HCl, pH 9.0 (25°C), 50 mM NaCl, 5 mM $MgCl_2$), 1 μM 5′-^{32}P-labeled primer–template complex, 0.1 μM DNA polymerase, and 10 μM ddTTP or 100 μM ddTTP (in case of the wild type enzyme). Mixtures were incubated for 30 min at 70°C. The reaction was terminated by addition of 5 μl of 0.1% Bromophenol Blue and 50 mM EDTA in 90% formamide. Samples were heated for 5 min at 90°C. The reaction products were separated by electrophoresis in 20% polyacrylamide gel (acrylamide/bis-acrylamide 20 : 1) in the presence of 7 M urea. Electrophoresis was performed in 100 mM Tris-borate buffer, pH 8.3, containing 2 mM EDTA.

Amplification was performed in 50 μl of the reaction mixture containing 60 mM Tris-HCl, pH 8.9 (25°C), 1.5 mM $MgCl_2$, 25 mM KCl, 0.1% Tween-20, 3 μg/ml DNA, 0.2 μM primers, 50 μM each dNTP, and 20 U/ml DNA polymerase. Formamide concentration is given in figure captions. For a 1-kb DNA fragment with 52% (G + C) content, the procedure was as follows: denaturation for 3 min at 86°C, annealing for 1 min at 43°C, polymerization for 1 min at 60°C, 1 cycle; denaturation for 1 min at 80°C, annealing for 1 min at 43°C, polymerization for 1 min at 60°C, 5 cycles; denaturation for 1 min at 80°C, annealing for 1 min at 57°C, polymerization for 1 min at 60°C, 30 cycles. For a 0.8-kb DNA fragment with 68% (G + C) content, the procedure was as follows: denaturation for 3 min at 86°C, annealing for 1 min at 45°C, polymerization for 1 min at 60°C, 1 cycle; denaturation for 1 min at 82°C, annealing for 1 min at 45°C, polymerization for 1 min at 60°C, 30 cycles.

Kinetic parameters for dTTP and ddTTP in the reaction catalyzed by wild type and mutant (F669Y) *Tte* pol were determined in 10 μl of reaction mixture containing 50 mM Tris-HCl, pH 9.0 (25°C), 50 mM NaCl, 5 mM $MgCl_2$, 0.5 μM 5′-^{32}P-labeled primer–template duplex, 0.01 μM DNA polymerase, and 0.05-50 μM dTTP or ddTTP (in case of the wild type *Tte* pol ddTTP concentration was varied from 10 to 100 μM). Mixtures were incubated for 5 min at 70°C, and the reaction was terminated by addition of 5 μl of 0.1% Bromophenol Blue and 50 mM EDTA in 90% formamide. Samples were heated for 5 min at 90°C. The reaction products were separated by electrophoresis in 20% polyacrylamide gel (acrylamide/bis-acrylamide 20 : 1) in the presence of 7 M urea. Positions of the initial and one-step elongated primers in the gel were determined autoradiographically, then subsequent portions of the gel were cut and treated according

a



b

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pUC19fr         CAGGTCGACTCTAGAG GATCCGCCGGGCCTTCGTGGCCGAGGCGGGATGGGCG
ttepol          CCCCCTTGGGCCAGAG GATCCGCCGGGCCTTCGTGGCCGAGGCGGGATGGGCG
                               ************************************
                               ↑
                               B a m H I

pUC19fr         TTGGTGGCCCTGGACTATAGCCAGATAGAGCTCCGCGTCCTCGCCCACCTCTCCG
ttepol          TTGGTGGCCCTGGACTATAGCCAGATAGAGCTCCGCGTCCTCGCCCACCTCTCCG
                ************************************************************

pUC19fr         GGGACGAGAACCTGATCAGGGTCTTCCAGGAGGGGAAGGACATCCACACCCAGACCG
ttepol          GGGACGAGAACCTGATCAGGGTCTTCCAGGAGGGGAAGGACATCCACACCCAGACCG
                ************************************************************

pUC19fr         CAAGCTGGATGTTCGGCGTCCCCCCGGAGGCCGT G GAC CCC CTG ATG CGC
ttepol          CAAGCTGGATGTTCGGCGTCCCCCCGGAGGCCGT G GAC CCC CTG ATG CGC
                ********************************** * *** *** *** *** ***
                                                   ↑
                                                   S f a N I

pUC19fr         CGG GCG GCC AAG ACC ATC AAC  TAC GGG GTC CTC TAC GGC ATG
ttepol          CGG GCG GCC AAG ACG GTG AAC  TTC GGC GTC CTC TAC GGC ATG
                *** *** *** *** **  *  ***  * * **  *** *** *** *** ***

                                    V667I    F669Y
pUC19fr         TCG GCC CAC CGC CTC TCC CAG GAG CTA GCC ATC CCT TAC GAG
ttepol          TCC GCC CAT AGG CTC TCC CAG GAG CTT GCC ATC CCC TAC GAG
                **  *** **   *  *** *** *** *** **  *** *** **  *** ***

pUC19fr         GAG GCC CAG GCC TTC ATT GAG C GGGTACCGAGC
ttepol          GAG GCC TCG GCC TTC ATT GAG C GCTACTTCCAG
                *** ***  *  *** *** *** *** *
                         ↑
                S692Q    A f e I
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Fig. 1. Modification of *Tte* DNA polymerase. a) Recombinant plasmid with the modified *Tte* pol gene. The relative position of the recognition sites of restriction endonucleases used for obtaining three fragments for ligase cross-linking is shown. The gene fragment (Phe667Tyr) of the modified *Taq* DNA polymerase is shown by hatching. b) Comparison of the primary sequences of the modified and initial *Tte* pol gene fragments. The pUC19 sequence is underlined. Places of hydrolysis by restriction endonucleases are shown by arrows (the recognition sites are highlighted in bold). The sequence replaced in *Tte* DNA polymerase gene is gray-shadowed (divided into triplets for convenience). Coinciding nucleotides are marked by asterisks. The modified triplets in the nucleotide sequence causing amino acid replacements are framed (the numbers of amino acid residues in the *Tte* DNA polymerase gene are given).

to Cherenkov. Kinetic parameters were determined by the Michaelis–Menten equation using the Microcal Origin program from Microcal Software (USA).

RESULTS AND DISCUSSION

Since amino acid sequences of *Taq* and *Tte* DNA polymerases coincide by 87%, we supposed that modification of *Tte* pol in position homologous to F667 in the sequence of *Taq* DNA polymerase results in change in the enzyme properties with respect to dideoxynucleoside triphosphate sampling. Comparative analysis of amino acid sequences of the two enzymes showed that F667 of *Taq* polymerase is homologous to F669 pol. The gene of *Tte* polymerase with the F669Y mutation was obtained as depicted in Fig. 1a. Plasmid pGTTE DNA bearing the *Tte* pol gene was hydrolyzed in two ways: *Sbf*I and *Sfa*NI restriction endonucleases and *Afe*I and *Sbf*I restriction endonucleases, respectively. After electrophoretic separation, a 979-bp *Sbf*I-*Sfa*NI hydrolyzate fragment with sticky four-nucleotide 5′-protruding ends (the 989-1968 site of gene sequence) and a 4.5-kb *Afe*I-*Sbf*I hydrolyzate fragment (a vector sequence with 2090-2604 site at the *Afe*I-end and 1-983 site at the *Sbf*I-end) were eluted from the gel. Plasmid recombinant pUC19 DNA bearing the *Taq* DNA polymerase gene with F667Y mutation was hydrolyzed by *Sfa*NI and *Afe*I restriction endonucleases, and a 118-bp fragment with sticky four-nucleotide 5′-protruding end (the 1962-2084 site of *Taq* DNA polymerase gene sequence with the F667Y mutation) was isolated. The isolated DNA fragments were used in the ligase reaction with DNA ligase of T4 phage. After desalting by re-precipitation with ethanol, the products of the ligase reaction were used for transformation of *E. coli* JM109 cells by electroporation. Plasmid DNA was isolated from the sample clones. To find a clone containing recombinant plasmid bearing the *Tte* DNA polymerase gene with F669Y mutation, plasmid DNA samples were hydrolyzed by *Afe*I and *Bam*HI restriction endonucleases, and a 300-bp fragment with sticky four-nucleotide end bearing a modified gene site was incorporated into pUC19 plasmid via *Sma*I and *Bam*HI sites, respectively, with subsequent sequencing. Nucleotide sequence of the pUC19 site bearing incorporated fragment compared with the sequences of the corresponding site of *Tte* pol gene is presented in Fig. 1b. As can be seen, the analyzed recombinant DNA fragment is highly homologous and contains three modified codons compared with the wild type *Tte* pol gene; these codons cause amino acid replacements corresponding to F669Y, V671I, and S692Q. This recombinant plasmid named pTTEF699Y for the target replacement was used for transformation of *E. coli* BL21 cells for growth of biomass and subsequent isolation of the enzyme. Results of analysis of crude lysate of clone cells bearing pTTEF669Y recombinant plasmid before (*2*) and after IPTG induction (*1*) and of biomass obtained from this clone (*3*) are presented in Fig. 2. As shown, ITPG initiates expression of a polypeptide with molecular mass corresponding to the purified recombinant *Tte*

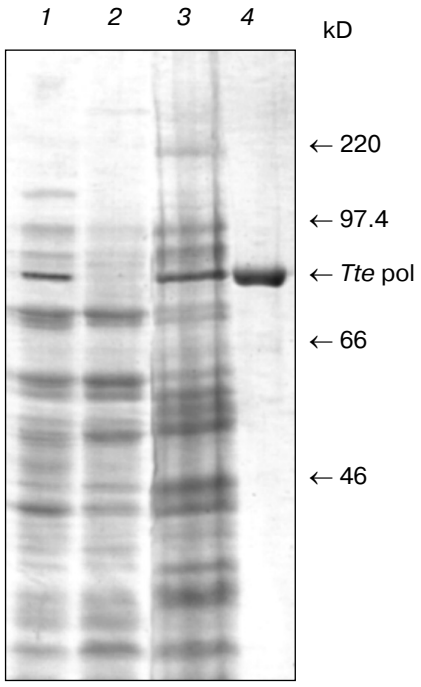

Fig. 2. Expression of *Tte* DNA polymerase (Phe669Tyr) in *E. coli* BL21 cells. The products were separated by electrophoresis in 12% polyacrylamide gel in the presence of SDS with subsequent staining with Coomassie Brilliant Blue. Lanes: *1*) after IPTG induction; *2*) before IPTG induction; *3*) expression of the modified *Tte* polymerase (in biomass); *4*) purified *Tte* polymerase.

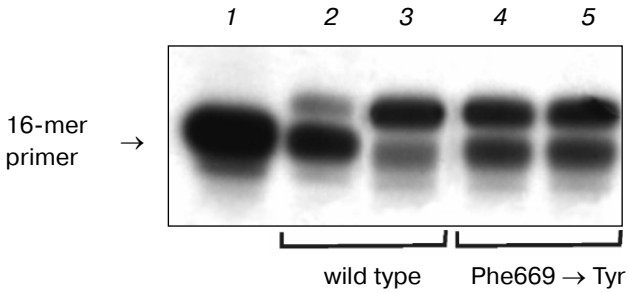

Fig. 3. Primer elongation in the presence of dTTP and ddTTP catalyzed by the wild type and modified *Tte* DNA polymerases. The 5′-^{32}P-labeled DNA duplex was incubated for 30 min at 70°C in the absence of the substrate (lane *1*), in the presence of ddTTP (lanes *2*, *4*) or dTTP (lanes *3*, *5*), the reaction products were separated by electrophoresis in 20% polyacrylamide gel under denaturing conditions.

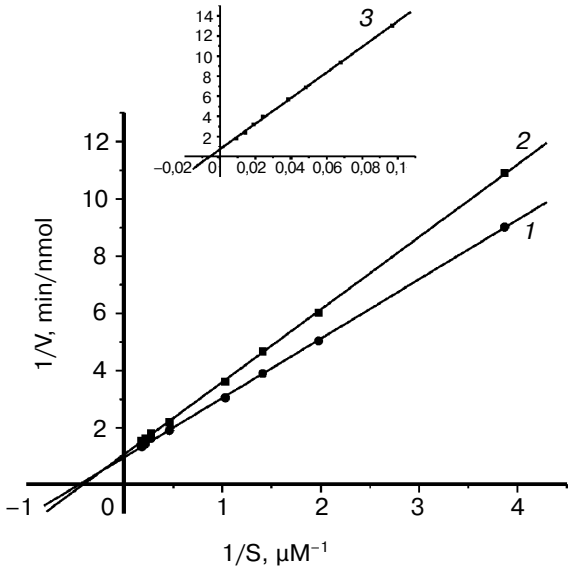

Fig. 4. Rate of primer elongation reaction catalyzed by the wild type and modified *Tte* DNA polymerases versus the substrate concentration in double-reciprocal coordinates: in the presence of dTTP (*1*) and ddTTP (*2*); in the presence of ddTTP for the wild type *Tte* pol (*3*, inset, in the same coordinates).

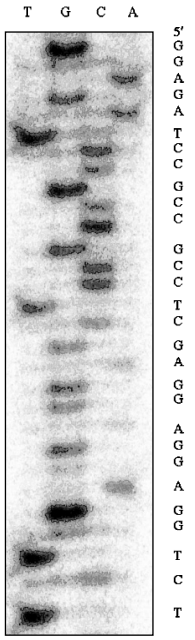

Fig. 7. Autoradiograph of the gel portion with the products of sequencing reactions. Lanes corresponding to a certain dideoxy terminator are designated atop by letters. The read sequence is at the right (from the top down).

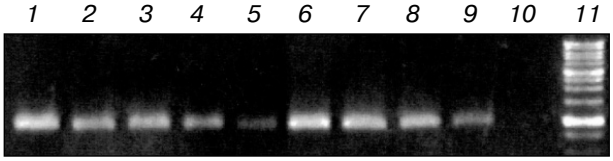

Fig. 5. Amplification of a 1-kb DNA fragment with 52% (G + C) content. In the presence of 10, 12, 14, 16, and 18% formamide: *Tte* (Phe669Tyr) (lanes *1-5*) and *Tte* (lanes *6-10*), respectively; "1 kb DNA markers" (0.25-10 kb) (lane *11*).

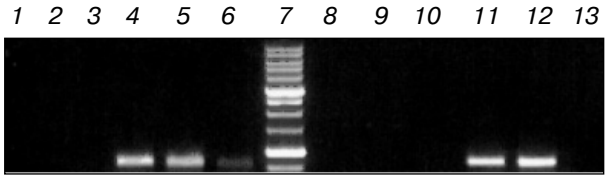

Fig. 6. Amplification of a 0.8-kb DNA fragment with 68% (G + C) content. In the presence of 10, 12, 14, 16, 18, and 20% formamide: *Tte* (Phe669Tyr) (lanes *1-6*) and *Tte* (lanes *8-13*), respectively; "1 kb DNA markers" (0.25-10 kb) (lane *7*).

pol (*4*). The hybrid protein as homogeneous pattern was isolated as described earlier for the wild type *Tte* pol [9].

Then we studied the substrate properties and determined kinetic parameters for dNTP and ddNTP in the reaction catalyzed by the mutant form of the enzyme designated as *Tte* (Phe669Tyr) compared with the wild type enzyme. The results of electrophoretic separation of the products of one-step elongation of the primer by wild type (lanes *2*, *3*) and mutant (lanes *4*, *5*) enzymes in the presence of dTTP (lanes *3*, *5*) and ddTTP (lanes *2*, *4*) are presented in Fig. 3. As can be seen, the mutant enzyme is able to use the terminating substrate for primer elongation almost as efficiently as the natural substrate, whereas for the wild type enzyme, a noticeable discrimination between these substrates is observed, and efficiency of synthesis in the presence of ddTTP is significantly lower even with excess ddTTP compared with dTTP. The rates of primer elongation by one nucleotide unit versus the substrate concentration in double-reciprocal coordinates are presented in Fig. 4. As shown, the K_m values determined from these plots are almost equal (~2 μM) for ddTTP and dTTP in the reaction catalyzed by the mutant enzyme (Fig. 4, lanes *1* and *2*). Earlier a similar K_m value was obtained for dTTP in the reaction catalyzed by the wild type *Tte* pol [10], whereas K_m for ddTTP in this case is ~200 μM (Fig. 4, inset). Thus, affinity of the mutant enzyme to ddNTP is two orders of magnitude higher than that of the wild type enzyme.

To study the possible effect of V667I and S692Q replacements, we used the mutant enzyme along with the initial one for DNA amplification by polymerase chain reaction. Figures 5 and 6 present the results of PCR on DNA templates with (G + C) content 52 and 68%, respectively. As shown, in the given temperature intervals both DNA polymerases demonstrate ability for such amplification of the fragments chosen. Both enzymes function within almost the same formamide concentration intervals and are usable for amplification of both usual and high-melting DNA fragments. Consequently, the difference between the initial and modified *Tte* pol related with V667I and S692Q replacements does not result in significant conformational changes.

The possibility for the modified *Tte* pol to be used in sequencing reactions at low concentrations of dideoxy terminators was checked as described in "Materials and Methods". Recombinant pUC19 bearing a *Tte* pol gene fragment with 68% (G + C) content was used as a template. The autoradiograph of the reaction products after their separation in the polyacrylamide gel under denaturing conditions according to the standard procedure is presented in Fig. 7. As proved, the modified *Tte* pol can be used in sequencing reactions.

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