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Roles of the Conserved Carboxylic Residues in the Active-Site of 5'-3' Exonuclease of *Taq* DNA Polymerase

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Abstract *Taq* DNA polymerase from *Thermus aquaticus* has been shown to be very useful in a polymerase chain reaction. *Taq* DNA polymerase has a domain at the amino terminus (residues 1 to 290) that has 5'-3' exonuclease activity and a domain at the C-terminus that catalyzes the polymerase reaction. *Taq* DNA polymerase is classified into the Pol I family, which is represented by *E. coli* DNA polymerase I. The alignment of amino acid sequences for the 5'-3' exonuclease domains of the Pol I family DNA polymerases shows ten highly conserved carboxylic amino acids. Crystallographic studies suggested that six of the carboxylic amino acids are clustered within a 7 Å radius by chelating three metal ions in the active site. Those six carboxylic residues are mutagenized to alanines in order to better understand their function. All six carboxylic residues, Asp18, Glu117, Asp119, Asp120, Asp142, and Asp144, are crucial for catalysis of 5'-3' exonuclease.

Key words: *Taq* DNA polymerase, PCR, 5'-3' exonuclease, crystal structure

Taq DNA polymerase from *Thermus aquaticus* is very useful in a polymerase chain reaction (PCR). It shows an optimum reaction temperature of 75°C and maintains activity for about one hour at 94°C. The high optimum polymerization temperature of 75°C demonstrates unique advantages when comparing *Taq* DNA polymerase with mesophilic DNA polymerases, such as *E. coli* DNA polymerase I [13, 25, 28, 29]. Not only is *Taq* DNA polymerase highly useful commercially for PCR applications, it is also important in studying DNA replication. *Taq* DNA polymerase is apparently homologous to *E. coli* DNA polymerase I, which has long been used for DNA replication studies. *Taq* DNA polymerase has a domain at its amino terminus (residues 1 to 291) that has 5'-3' exonuclease activity, a non-functional 3'-5' exonuclease domain (residues 292-423), and a domain at its C-terminus (residues 424-832) that catalyzes the polymerase reaction [23].

All nucleic acid polymerases can be grouped into six families on the basis of amino acid homology — Pol I, Pol α, Pol β, DNA-dependent RNA polymerase, reverse transcriptase, and RNA-dependent RNA polymerase [15]. *Taq* DNA polymerase is classified into the Pol I family, which is represented by *E. coli* DNA polymerase I [7].

A comparison of a Klenow fragment (KF) with the corresponding parts of *Taq* DNA polymerase indicates that the polymerase domains are nearly identical, whereas the 3'-5' exonuclease domains differ extensively [22]. Unlike *E. coli* DNA polymerase I, the intervening domain of *Taq* DNA polymerase (the 3'-5' exonucleas domain) has lost the editing activity of 3'-5' exonuclease [21, 24]. High resolution structural data from crystallographic studies have been published on the polymerase and 3'-5' exonuclease domains of KF, and the reaction mechanism of 3'-5' exonuclease was nearly identified [1, 2, 8, 9, 11, 26, 27].

However, little was known about the structural basis of 5'-3' exonuclease activity until the structure of *Taq* DNA polymerase was published [19]. Thereafter, the crystal structure of T5 5' exonuclease clarified the structural features of the 5'-3' exonuclease domain [3]. The alignment of amino acid sequences for the 5'-3' exonuclease domains of the Pol I family DNA polymerases shows six highly conserved sequence motifs containing ten conserved acidic amino acid residues [12]. Six of these residues (Asp18, Glu117, Asp119, Asp120, Asp142, and Asp144) cluster within a sphere of a 7 Å radius by chelating three divalent metal ions in the active site of 5'-3' exonuclease of *Taq* DNA polymerase [19]. There are several basic amino acids in addition to the carboxylic amino acids in the putative active site of 5' exonuclease, such as Arg74, Lys82, and Arg85, which might be involved in binding the DNA substrate [3].

Several amino acids will prove interesting in a site-directed mutagenesis study to elucidate the roles of amino

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acids in the active site. The first ones are the six carboxylic amino acids involved in chelating metal ions. In this paper, we report the results of functional studies by site-directed mutagenesis for those six carboxyl amino acids in the active site.

MATERIALS AND METHODS

Materials

The mutagenesis kit and *Taq* DNA polymerase were obtained from Bioneer Co. (Korea). All other enzymes were purchased from Promega Co. (U.S.A.). The radioactive compound of [γ-^{32}P]ATP for DNA labeling was purchased from Amersham International (U.S.A.).

Plasmid Constructions

Site-directed mutagenesis by the PCR method was conducted to obtain the mutated 5'-3' exonuclease using pDS1 plasmid as described previously [18, 20]. The mutant *Taq* DNA polymerase genes was sequenced to confirm that the mutation was conducted correctly. The thermal DNA sequencing method was used to perform the DNA sequencing as described previously [14]. The mutant *Taq* DNA polymerase gene on the pDS1 plasmid was transformed into an *E. coli* DH5α host cell for protein overexpression.

Purification of Mutant *Taq* DNA Polymerase

E. coli DH5α carrying the pDS1 plasmid was incubated at 37°C and 250 rpm. *E. coli* DH5α in 3,000 ml LB medium was cultured overnight with 80 µg/ml ampicillin. The induction of gene expression and the purification of the enzyme were performed as described previously [10, 18, 20]. The two differences were that a Q-sepharose column (Pharmacia, U.S.A.) was substituted for FPLC mono Q as the final purification step, and the host cell was *E. coli* DH5α. The purified protein was pure enough to use for the assay of 5'-3' exonuclease activity.

Assay of 5'-3' Exonuclease Activity

The pUC18 DNA was digested with *Xma*I restriction enzyme and labeled at the 5'-terminus with [γ-^{32}P]ATP by T4 polynucleotide kinase. The labeled DNA at the 5'-phosphate position was purified by using the Gene Clean Kit (Bio101 Company, U.S.A.) and the unbound radioactive nucleotides were discarded. The 5'-3' exonuclease reaction was performed at 72°C for one hour in a 50 µl reaction mixture containing 25 mM Tris-HCl, pH 8.0, 1 mM-mercaptoethanol, 2 mM $MgCl_2$, 0.5-µl labeled pUC18 DNA as the substrate, and 7 µl of *Taq* DNA polymerase enzyme. The concentrations of the labeled DNA and *Taq* DNA polymerase in a reaction mixture were varied to achieve a linear dose response depending on the assay conditions. The mixture was cooled on ice with the addition of 2 µl of 60 mM EDTA to stop the reaction. Fifty two µl aliquots were spotted on a 2.3-cm diameter DE-81 Whatman filter paper and dried in a heat block for 10 min. The dried filter was washed twice with 0.5 M Na_2HPO_4, pH 7.0, for 15 min and then washed with 70% ethanol for 5 min. The radioactivity was counted with a Beckmann liquid scintillation counter, model LS 6500 (U.S.A.).

RESULTS

The Roles of Carboxylic Amino Acids in an Active Site

The alignment of the 5'-3' exonucleases of the Pol I family DNA polymerases indicates that ten carboxylic amino acid residues are particularly conserved [12]. The crystal structure of the 5'-3' exonuclease in *Taq* DNA polymerase demonstrates that six of those residues appear to be involved in chelating three divalent metal ions, as shown in Fig. 1 [19]. All the carboxylic amino acids are clustered within a 7 Å radius. It has been proposed that those six carboxylic amino acids probably perform similar roles in the reaction mechanism, in the same way that the catalytic four carboxylic amino acids play crucial roles in chelating two metal ions in the reaction mechanism of 3'-5' exonuclease

Fig. 1. The active site of the 5'-3' exonuclease showing the positions of three metal ion ligands as positioned in the apoenzyme [19].
The metal positions will presumably be adjusted slightly for the DNA substrate to generate optimal interactions.

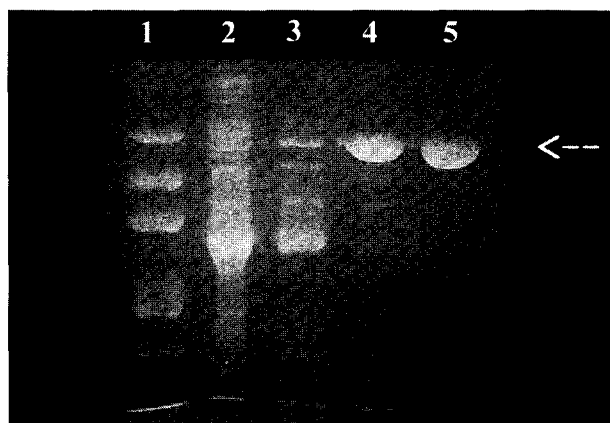

Fig. 2. SDS-PAGE of purified mutant *Taq* DNA polymerase from *E. coli* DH5α cells.
Taq DNA polymerase is indicated by an arrow at 94 kDa. Lane 1, molecular weight marker; Lane 2, non-induced cell extract; Lane 3, induced cell extract; Lane 4, supernatant after heat treatment; Lane 5, Q-sepharose column fraction.

[1, 2]. The exact roles of those six carboxylic amino acids cannot be postulated, since we do not yet know the structures of the substrate or product complexed with the 5'-3' exonuclease domain. However, site-directed mutagenesis can be used to test whether the six carboxylic amino acids are actually involved in catalysis by chelating the three divalent metal ions or by other means, as suggested in Fig. 1.

Expression and Purification of Mutant *Taq* DNA Polymerase

We conducted site-directed mutagenesis by using the PCR method on *Taq* DNA polymerase DNA to obtain mutated 5'-3' exonuclease. The *Taq* DNA polymerase was overexpressed in *E. coli* DH5α by induction with 0.2 mM IPTG at 0.5 O.D. units at the 550 nm wavelength. A large amount of protein was produced for wild-type and mutant *Taq* DNA polymerases. Our purification procedure was simplified compared to those previously reported [4, 10]. The procedure eliminated the PEI step and completed purity by a Q-sepharose column step. The purification was examined by SDS-PAGE, as shown in Fig. 2. The protein looked nearly pure after the Q-sepharose chromatography. It yielded about 30 mg out of a 3,000 ml flask culture.

Comparison of 5'-3' Exonuclease Activity for Mutant *Taq* DNA Polymerases

The conserved six carboxylic amino acids in the active site were mutated to alanine in order to determine which ones are involved in chelating metal ions for the mechanism of phosphoryl transfer, as described previously. We examined the 5'-3' exonucleolytic activities for the six mutant *Taq* DNA polymerases as described in Table 1. The mutants of E117A, D120A, and D142A showed a dramatic decrease

Table 1. The relative activities of the 5'-3' exonucleases for wild-type and mutant *Taq* DNA polymerases.
The assays were performed 10 times under the same conditions and averaged to minimize errors.

Taq DNA polymerase	Specific activity of 5'-3' exonuclease (Δcpm/μg/min)	Relative activity (%)
Wild-type	97.2±6.4	100.0
D18A	10.7±3.6	11.0
E117A	4.1±2.2	4.2
D119A	11.9±4.8	12.2
D120A	5.3±2.0	5.5
D142A	3.9±2.1	4.0
D144A	14.6±2.8	15.0
D142A/D144A	19.5±0.7	20.0

Values are means±standard deviations, based on n=10.

of 5'-3' exonuclease activity to 4.2%, 5.5%, and 4.0% respectively, compared to the wild-type *Taq* DNA polymerase. In addition, the mutants of D18A, D119A, D144A, and D142A/D144A (double mutant) showed big decreases of the 5'-3' exonuclease activity down to 11.2%, 12.2%, 15.0%, and 20.0% of the value of wild-type *Taq* DNA polymerase. The six residues can be classified roughly into two groups in terms of activity; one group consists of E117A, D120A, and D142A, which reduce 5'-3' exonuclease activity very dramatically, and the other group composed of D18A, D119A, and D144A, which decrease the activity less significantly. It is rather difficult to use the present assay system to distinguish the precise difference in the activities of the mutant *Taq* DNA polymerases within each group. However, it can be concluded that not only Glu117, Asp120, and Asp142 are crucial for maintaining 5'-3' exonuclease activity but also Asp18, Asp119, and Asp144 play significant roles in the catalysis of 5'-3' exonuclease. The accumulated evidence of mutational and structural studies suggest that Asp18, Glu117, Asp119, Asp120, and Asp142 might be involved either in chelating metal ions I, II, and III, as shown in Fig. 1, or in assisting other carboxylic amino acids to chelate those three metal ions.

DISCUSSION

The mechanism of phosphoryl transfer is becoming apparent in several enzymes where two divalent metal ions are employed for a catalytic reaction. There are several enzymes which follow the same path, such as alkaline phosphatase, pyrophosphatase, and RNase H. In addition, the mechanism of phosphoryl transfer is evident for the 3'-5' exonuclease and polymerase domain of KF; this is supported by structural and mechanical studies [5, 6, 16, 17, 30]. Previous structural evidence and biochemical studies indicate that the same mechanism might be

applicable for the 5'-3' exonuclease of *Taq* DNA polymerase. The two-metal ion mechanism is apparently a general process for nucleotide synthesis and hydrolysis in the pol I family of DNA polymerases.

In contrast, there are six carboxylic amino acids in the active site cleft of 5'-3' exonuclease which may be involved in chelating two divalent metal ions in performing the phosphoryl transfer catalysis, and one more metal ion such as metal III in the 5'-3' exonuclease of *Taq* DNA polymerase whose function has yet to be characterized, as was described previously [3, 12, 19]. In addition, several conserved positively-charged amino acids are positioned in the vicinity of the active site [3, 20]. However, another structural study for T4 5' exonuclease suggested that only two metal sites were observed, 8.1 Å apart, contradicting the report that two metal ions (metals I and II) were separated by about 5 Å and one additional metal ion (metal III) was separated by about 10 Å from the metals I and II sites in *Taq* DNA polymerase [3, 19]. The site-directed mutagenesis study provides the supportive evidence that the six carboxylic amino acids Asp18, Glu117, Asp119, Asp120, Asp142, and Asp144 are key residues in catalysis, either by chelating metal ions I, II, and III as shown in Fig. 1 or assisting other carboxylic amino acids in chelating those three metal ions, as suggested previously [1].

The additional metal ion III may be involved in the 5'-3' exonuclease reaction in a way different from 3'-5' exonuclease and polymerase catalysis in DNA synthesis, which correlates with the fact that the 5'-3' exonuclease of *Taq* DNA polymerase functions as a structure-specific endonuclease when it detects the specific features of DNA substrate for catalysis. It is unique compared to other exonucleases [24]. The additional metal ion was not detected in the case of T4 5'exonuclease, which might be quite difficult since the active site cannot form the exact conformation without the DNA substrate [3]. The geometry of the metal ion binding sites is not fixed and may cause a low-level metal occupancy which is undetectable on an electron density map. T4 5' exonuclease may also follow the catalytic pathway in a different way than *Taq* DNA polymerase.

This study provides biochemical evidence of the overall functions of the carboxylic amino acids of the active site cleft in the 5'-3' exonuclease domain of *Taq* DNA polymerase. A more complete understanding of the mechanism of 5'-3' exonucleases clearly requires structural information about DNA-bound 5'-3' exonuclease. This could lead to a better explanation for the biochemical studies that were performed for this report.

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