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Construction of Two Metal-ion Binding Sites to Improve the 3'-5'Exonuclease Activity of *Taq* DNA Polymerase

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Abstract *Taq* DNA polymerase from *Thermus aquaticus* is very useful in the polymerase chain reaction. *Taq* DNA polymerase is classified in the pol I family, represented by *E. coli* DNA polymerase I. The three-dimensional structural alignment of 3'-5'exonuclease domains from the pol I family DNA polymerases explains why *Taq* DNA polymerase does not carry out proofreading in polymerase chain reactions. Three sequence motifs, Exo I, II, and III, must exist to carry out 3'-5'exonuclease activity for proof-reading by a 3'-5'exonuclease reaction, but these are abolished in *Taq* DNA polymerase. The key catalytic module in 3'-5'exonuclease is two metal ions chelated by four active-site carboxylic amino acids. *Taq* DNA polymerase was mutagenized to construct the catalytic module in the active site. The circular dichroism technique supported the formation of the catalytic module, and the radioactive assay showed that the 3'-5'exonuclease activity doubled in the mutant *Taq* DNA polymerase.

Key words: *Taq* DNA polymerase, two-metal ion mechanism, 3'-5'exonuclease, PCR

Taq DNA polymerase from *Thermus aquaticus* is very useful in a polymerase chain reaction (PCR). It shows an optimum reaction temperature at 75°C and maintains activity for about one hour at 94°C. The high optimum polymerization temperature at 75°C provides unique advantages when comparing *Taq* DNA polymerase with mesophilic DNA polymerase, such as *E. coli* DNA polymerase I [14, 26, 30, 31]. Not only is *Taq* DNA polymerase highly useful commercially for PCR applications, but 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 nonfunctional 3'-5'exonuclease domain (residues 292–423), and a domain at its C-terminus (residues 424–832) that catalyzes the polymerase reaction [24].

Research into *Taq* DNA polymerase commenced when it became a commercially useful DNA polymerase. One objective was to improve the processivity and fidelity of *Taq* DNA polymerase, primarily because *Taq* DNA polymerase is the most popular enzyme in PCR. Another goal was to find a new thermophilic DNA polymerase to substitute for *Taq* DNA polymerase, which was mostly undertaken by biotech companies. Their efforts have launched several thermophilic DNA polymerases into the market, such as *Pfu* DNA polymerase (Stragene Company), Vent DNA polymerase (New England Biolab), and *Bca* DNA polymerase (Takara Shuzo Company). *Taq* DNA polymerase presently has a major disadvantage compared to the other thermophilic DNA polymerases, in that it demonstrates lower proof-reading activity due to the deficiency of 3'-5'exonuclease activity. *Taq* DNA polymerase does not carry a noticable 3'-5'exonuclease activity, which may be due to the shorter 3'-5'exonuclease domain, even though there is a corresponding domain [12]. High fidelity DNA synthesis is a very important factor for a thermophilic DNA polymerase to be a preferred enzyme for a PCR reaction [9]. DNA polymerization fidelity is influenced by several factors, but the presence of 3'-5'exonuclease activity is one of the most critical elements. The presence of 3'-5'exonuclease activity will remove mismatched nucleotides and thereby reduce the error rate. The *Taq*, *Pfu*, and Vent DNA polymerases are currently the most widely used thermophilic DNA polymerases. Their fidelities for DNA synthesis vary; the error rates of *Taq*, *Pfu*, and Vent DNA polymerases are reported at 1.8×10^{-5}, 0.2×10^{-5}, and 1.2×10^{-5}, respectively [12]. The disadvantage of the

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lower 3'-5'exonuclease activity of *Taq* DNA polymerase has been a point of contention for other thermophilic DNA polymerases which share the market.

All DNA polymerases can be grouped into six families, pol I, pol α, pol β, DNA-dependent RNA polymerase, reverse transcriptase, and RNA-dependent RNA polymerase, on the basis of amino acid homology [16]. *Taq* DNA polymerase is classified into the pol I family, represented by *E. coli* DNA polymerase I [5]. 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 [23]. Unlike *E. coli* DNA polymerase I, the intervening domain of *Taq* DNA polymerase has lost the editing activity of 3'-5'exonuclease [22, 25]. High resolution structural data from crystallographic studies have been published on the polymerase and 3'-5'exonuclease domains of KF, and even the reaction mechanism of 3'-5'exonuclease has nearly been identified [1, 2, 6, 7, 13, 27, 28]. An analysis was conducted (based on the reaction mechanism of 3'-5'exonuclease in KF) as to why *Taq* DNA polymerase carries just a minimal background level of 3'-5'exonuclease activity while other homologous DNA polymerases show higher values. Following this analysis, a study was undertaken to improve the proofreading activity of *Taq* DNA polymerase by constructing the catalytic module of a 3'-5'exonuclease activity in the active site by protein engineering.

MATERIALS AND METHODS

Materials

The mutagenesis kit and *Taq* DNA polymerase were obtained from Bioneer company (Taejon, Korea). All other enzymes were purchased from Promega company (U.S.A.). Radioactive compounds of γ-^{32}P[dCTP] for DNA labeling were purchased from Amersham International (Amersham, U.S.A.).

Plasmid Constructions

Site-directed mutagenesis was conducted to construct mutant 3'-5'exonuclease using pDS1 plasmid [19]. DNA sequencing was performed by the thermal DNA sequencing method using *Taq* DNA polymerase in order to confirm correct mutagenesis [15]. The subclone pDS1 plasmid for mutated DNA was used to transform *E. coli* DH5α, the host cell for protein overproduction.

Purification of Mutant *Taq* DNA Polymerase

E. coli DH5α carrying pDS1 plasmid was shaken at 37°C and 250 rpm. *E. coli* DH5α in a 300 ml of LB medium was cultured overnight with 80 μg/ml ampicillin. The induction of gene expression and the protein purification procedure performed basically as described previously [10, 20]. The slight differences were that a Q-sepharose open column was substituted for FPLC mono Q at the final purification step and the host cell was *E. coli* DH5α. The purified *Taq* DNA polymerase showed as a clean band on SDS-PAGE [21]. This recombinant protein was used for the 3'-5'exonuclease assay and circular dichroism (CD) experiments.

Assay of 3'-5'Exonuclease Activity

The vector pUC18 DNA was digested with *Xma*I restriction enzyme and labeled at the 3'-terminus with γ-^{32}P[dCTP] by *Taq* DNA polymerase to make two mismatched base pairs of C-C. The labeled DNA was purified by a Gene Clean kit (Bio101 Company, U.S.A.) and the unbound radioactive nucleotides were discarded. The 3'-5'exonuclease reaction followed at 72°C for one hour in a 50 μl reaction mixture containing 25 mM Tris-HCl, pH 8.0, 1 mM β-mercaptoethanol, 10 mM $MgCl_2$, 0.5 μg DNA substrate, and 7 μl of diluted *Taq* DNA polymerase enzyme depending on the assay condition. The reaction was stopped by cooling the mixture on ice with the addition of 2 μl of 60 mM ethylenediaminetetraacetic acid (EDTA). The 52 μl aliquot was 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 in a Beckmann liquid scintillation counter model LS 6500.

Circular Dichroism (CD) Measurements

]Taq DNA polymerase was dialyzed in Tris 20 mM, pH 8.0 and diluted to 2 μM concentration for the CD measurements. Mn^{+2}-dependent CD spectra were measured for 250 μM of *Taq* DNA polymerase by adding an aliquot of concentrated Mn^{+2} solution. The increments of the Mn^{+2} concentrations were 1 μM, and the highest Mn^{+2} concentration was 12 μM. The CD spectrum was collected in the range of 190 nm to 260 nm by a Jasco J-715 spectropolarimeter. The bandwidth was 2 nm and the spectra were averaged over at least eight scans in order to improve the signal-to-noise ratio. The spectra were obtained using a 0.1 cm cell to avoid too high an absorbance of the sample. All the CD experiments were conducted at 25°C and were controlled by a Jasco PTC-348 peltier.

RESULTS

Structural Comparison of 3'-5'Exonuclease Domain Between *Taq* DNA Polymerase and *E. coli* DNA Polymerase I

Taq DNA polymerase is highly homologous to *E. coli* DNA polymerase I in both its primary and tertiary

structures. Even though the two polymerases are very similar in terms of general sequence homology, there is a major difference in that *E. coli* DNA polymerase I carries 3'-5'exonuclease activity for proofreading while *Taq* DNA polymerase has just a background level of 3'-5'exonuclease activity, which causes higher error rate in PCR. *Taq* DNA polymerase carries a 3'-5'exonuclease domain from residues 292 to 423, which is much shorter than that of *E. coli* DNA polymerase I. We compared the 3'-5'exonuclease domain of *Taq* DNA polymerase with that of *E. coli* DNA polymerase I in three-dimensional structural alignment to determine why *Taq* DNA polymerase does not conduct 3'-5'exonuclease activity. The comparison indicated that the two structures are very similar in secondary and tertiary conformations even though there is not much sequence homology in amino acids, as shown in Fig. 1. However, there were two very distinct differences in the overall structures. One was the deletion of three loops with lengths between 8 to 27 residues (385~396, 406~413, and 472~496 in *E. coli* DNA polymerase I sequence) and one helix was replaced by a random coil (335~349 in *E. coli* DNA polymerase), as previously reported [20]. In *E. coli* DNA polymerase I, these loops pack together on one side of the 3'-5'exonuclease domain, as shown in Fig. 1. The other difference was that all four carboxylic amino acids (D424, D501, D355, and E357 in *E. coli* DNA polymerase I), known to be essential for the "two-metal ion mechanism" of 3'-5'exonuclease catalysis [1, 2], were replaced by residues incapable of binding the metal ions (L356, R405, G308, and V310) in the vestigial 3'-5'exonuclease domain of *Taq* DNA polymerase, as shown in Fig. 1. Those four carboxylates play roles to bind metals A and B for catalysis. Although the 3'-5'exonuclease catalytic site was destroyed and the size of the domain reduced, the contact with the polymerase domain and the distance between the polymerase and 3'-5'exonuclease domains remained similar in the two homologous polymerases [20].

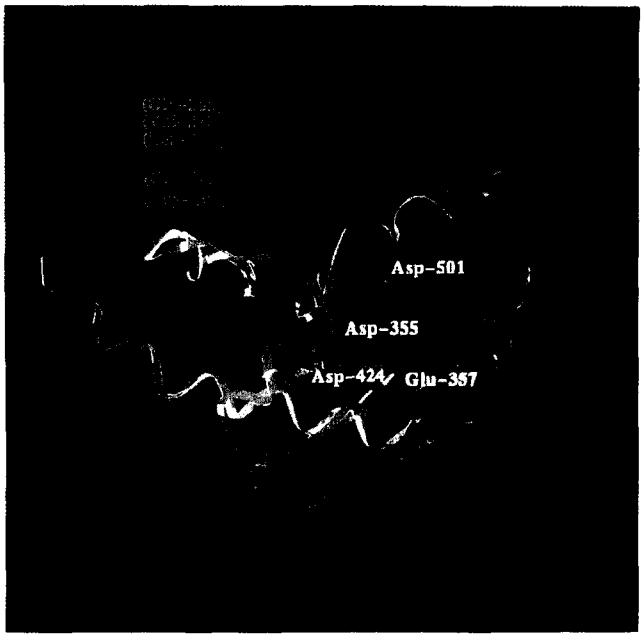

Fig. 1. Superposition of the 3'-5'exonuclease domains of *E. coli* DNA polymerase I and *Taq* DNA polymerase. *E. coli* DNA polymerase I is purple and Taq DNA polymerase is yellow.

Two divalent metal sites are labeled metals A and B, and four carboxylic amino acids are asigned to divalent metals, respectively. The four amino acids in the active site of *Taq* DNA polymerase matched the corresponding ones of *E. coli* DNA polymerase I.

Mutation of the Four Active-site Residues to Carboxylic Amino Acids

Taq DNA polymerase shows lower proofreading in PCR, due to the deficiency of 3'-5'exonuclease activity. The key requirement for the 3'-5'exonuclease domain to be active is that two metal ions must be chelated by four carboxylic amino acids in the active site. Any mutation of those four carboxylic amino acid residues reduces 3'-5'exonuclease activity to 10^{-3} to 10^{-5} times in *E. coli* DNA polymerase I [6, 7]. It is conceivable that the site-direct mutagenesis of L356, R405, G308, and V310 to functionally active carboxylates might raise the 3'-5'exonuclease activity to some extent. Therefore, the four presumed active-site residues, Gly308 (GGC), Val310 (GTG), Leu356 (CTG), and Arg405 (CGG), were mutated to Asp308 (GAC), Glu310 (GAG), Asp356 (GAC), and Asp405 (GAC). The mutagenic DNA fragment was digested by restriction enzymes *Kpn*I and *Bam*HI at base 479 and 1778 of the *Taq* DNA polymerase gene. We sequenced all 1305 bases by the thermal DNA sequencing method [15] in order to assure correct mutations. No random mutations were found and those four amino acids were correctly replaced with carboxylic amino acids (data not shown). The mutagenic fragment was ligated into the vector DNA, which contains the *tac* promotor as the gene expression system.

The wild-type and mutant *Taq* DNA polymerases were expressed in *E. coli* DH5α cells. Protein production was induced with 0.2 mM iso-propyl-1-thio-beta-galactopyranoside (IPTG) at $OD_{550} = 0.2$. A large amount of protein was overproduced for wild-type and mutant *Taq* DNA polymerases. The expression and purification procedures were reported previously [21]. *Taq* DNA polymerase was identified by SDS-PAGE analysis for the molecular weight, where mutant *Taq* DNA polymerase was only positioned as a single band in 94 kDa size (data not shown).

Circular Dichroism of *Taq* DNA Polymerase

The secondary structure of *Taq* DNA polymerase in the presence and absence of the Mn^{+2} ion is of interest

because the mutant *Taq* DNA polymerase acquired extra Mn^{+2} binding sites by inserting two divalent metal-ion sites by site-directed mutagenesis. CD spectra were collected for both wild-type and mutant *Taq* DNA polymerases in the 190~260 nm wavelength range. Figures 2a and 2b show the CD spectra of the wild-type and mutant *Taq* DNA polymerases in the absence and presence of 12 μM Mn^{+2}. The shapes and intensities of the CD spectra for both the wild-type and mutant proteins initially appear essentially the same in the absence of an Mn^{+2} ion, indicating that the insertion of four carboxylic amino acids did not result in a significant conformational change. In the presence of 12 μM Mn^{+2}, the wild-type *Taq* DNA polymerase shows a notable decrease in the CD intensity in the 200 nm~250 nm region (Fig. 2a), suggesting that the binding of the Mn^{+2} ion introduces a significant change in the protein's secondary structure. This decrease in the CD intensity is more pronounced (Fig. 2b) for mutant *Taq* DNA polymerase. In the mutant protein case, the decrease in the CD intensity is as much as 40% in the presence of 12 μM Mn^{+2}. Two reasons may be postulated for these observations - either the changes in the protein's secondary structure (represented by changes in the CD spectrum) induced per bound Mn^{+2} ion is larger for mutant *Taq* DNA polymerase, or more Mn^{+2} ions are bound to the mutant protein. Although these reasons cannot be clearly distinguished at this point, Mn^{+2} titration may provide more information.

The changes in the CD intensity at 209 nm with increasing Mn^{+2} concentrations are depicted for wild-type *Taq* DNA polymerase (Fig. 3a) and its mutant, to which four carboxylic amino acids were inserted (Fig. 3b). The carboxylic acids inserted in the mutant protein may provide additional Mn^{+2} ion binding sites. The 2 μM wild-type protein showed a gradual decrease in the CD intensity with increases in the Mn^{+2} concentration

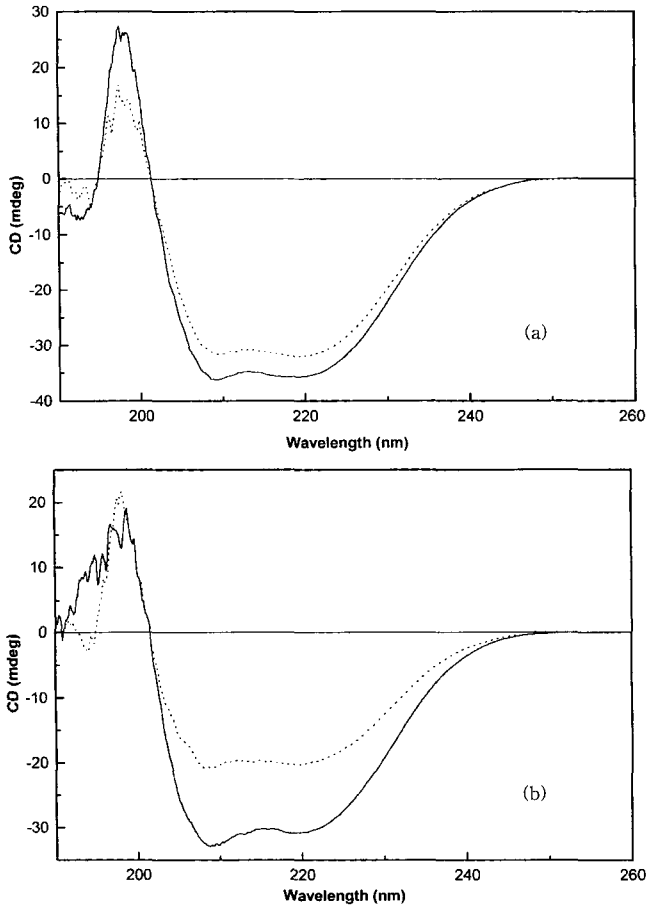

Fig. 2. The changes of CD spectra for wild-type and mutant *Taq* DNA polymerases.

The CD spectra of (a) wild-type and (b) mutant *Taq* DNA polymerases in the presence of 0 μM (solid line) and 12 μM (dotted line) Mn^{+2} concentrations. The protein concentration was 2 μM.

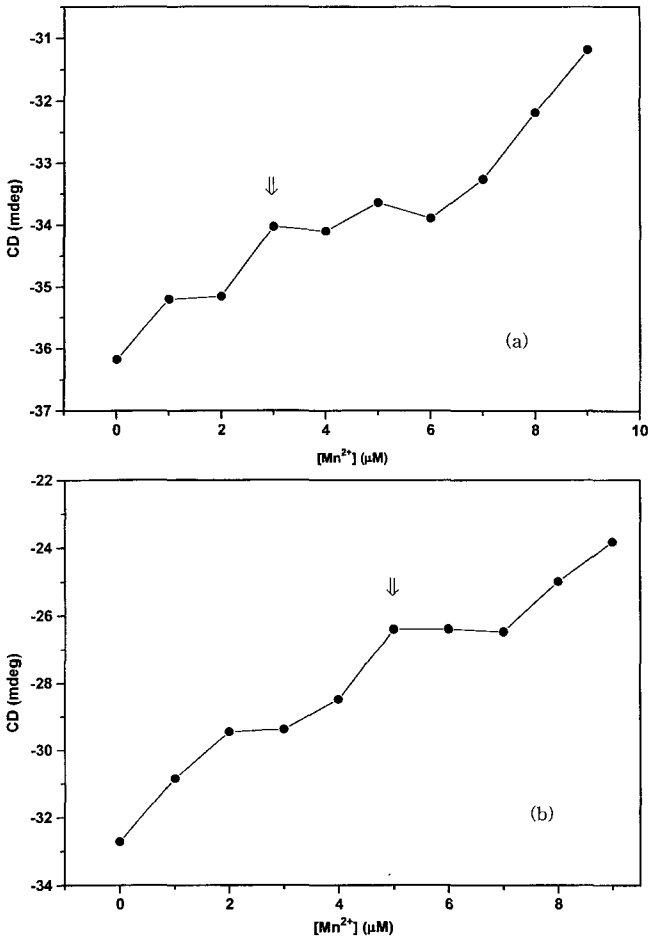

Fig. 3. The effect of Mn^{+2} concentration on the CD spectrum.

For wild-type *Taq* DNA polymerase (a), the first curve plateau is shown at 3 μM Mn^{+2}. For mutant *Taq* DNA polymerase (b), it comes at 5 μM Mn^{+2}. The CD spectra support that mutant *Taq* DNA polymerase possesses more Mn^{+2} binding sites than the wild-type.

and reached the first plateau at 3 μM at the Mn^{+2} concentration, while it occurred at 5 μM for the mutant protein, implying that the ratio of mutant to wild-type *Taq* polymerase for Mn^{+2} sites may be about 1:7. Wild-type *Taq* DNA polymerase possesses four or five Mn^{+2} binding sites for catalysis [20]. Considering that we have attempted to insert two more Mn^{+2} sites by protein engineering, the mutant *Taq* DNA polymerase probably contains six or seven sites. Therefore, the ratio of mutant to wild-type *Taq* polymerase for Mn^{+2} sites could be 1:5, which coincides approximately with the value obtained from the CD measurement. The significance of this coincidence is under further investigation in our laboratory.

Assay of the 3'-5'Exonuclease Activity for Mutant *Taq* DNA Polymerase

The 3'-5'exonucleolytic activity of mutant *Taq* DNA polymerase was compared to that of wild-type *Taq* DNA polymerase. While the 3'-5'exonuclease activity of wild-type *Taq* DNA polymerase showed 505 cpm/μg protein, the value of the mutant *Taq* DNA polymerase increased up to 1005 cpm/μg protein. The results of the 3'-5'exonuclease assays are summarized in Table 1. The insertion of the catalytic carboxylates into the active site doubled the 3'-5'exonuclease activity. Since many factors are involved in 3'-5'exonuclease reaction, the construction of those carboxylic amino acids alone could not bring up full activity, which could amount to the 3'-5'exonuclease activities as shown in *Pfu*, or Vent polymerases. However, it supports the idea that the key-catalytic module for 3'-5'exonuclease catalysis engineered in the active site, contributing to the improvement of the 3'-5'exonuclease activity to some extent.

DISCUSSION

According to biochemical and X-ray crystallographic studies, the 3'-5'exonuclease reaction is probably best described at a mechanistic level as a phosphoryl transfer mechanism. The phosphoryl transfer mechanism is becoming apparent in several enzymes where two divalent metal ions are involved. There are several enzymes which follow the same path, such as alkaline phosphatase, pyrophosphatase, and RNase H. The mechanism, which utilizes two divalent metal ions for catalysis, is evident in the 3'-5'exonuclease and polymerase domains of KF; this is supported by structural and mechanical studies. Accumulated structural evidence and biochemical studies indicate that this could be true for the 3'-5'exonuclease case [3, 4, 11, 17, 18, 32]. For the phosphoryl transfer mechanism, four active-site carboxylic amino acids are involved in chelating two divalent metal ions. These amino acid are Asp355, Glu 357, Asp424, and Asp501 in KF. In addition to those carboxylic amino acids, several amino acids such as Leu361, Phe473, Tyr497, Gln419, and Arg455 play significant roles in binding the substrates in KF. In general, DNA polymerases, which have a proofreading function due to 3'-5'exonuclease activity, possess three sequence motifs, Exo I, II, and III. The Exo I motif contains the core sequence DXE, where two carboxylic amino acids, such as Asp355 and Glu357, position in KF. The Exo II has the sequence NX2-3(F/Y)D, where Asn420 and Asp424 fit the case in KF. The Exo III motif has the sequence YX3D, corresponding to active-site residues Tyr497 and Asp501 in KF. The motifs are necessary to keep 3'-5'exonuclease in a fully active form for chemical catalysis and substrate binding [8]. However, those motifs are absent in *Taq* DNA polymerase, resulting in no proofreading by 3'-5'exonuclease activity [29].

Table 1. Activity measurements of the 3'-5'exonucleases of wild-type *Taq* DNA polymerase and mutant *Taq* DNA polymerase which contains four carboxylic amino acids in the active site. The values were averaged from 6 measurements. The standard deviations were 3.1% and 5.3% for the wild-type and mutant *Taq* DNA polymerases, respectively.

Taq DNA polymerase	3'-5'Exonuclease activity (Δcpm/μg protein)	Relative activity (%)
Wild-type	505.3	100
Mutant	1005.0	204

The mutagenesis inserted four catalytic carboxylic residues into the active site of *Taq* DNA polymerase so that it could chelate two divalent metal ions, presumed to be the critical catalytic module. It was anticipated that the 3'-5'exonuclease activity would be restorable to some extent if the inserted catalytic module was positioned in proper conformation in the active site of the *Taq* DNA polymerase. The CD spectroscopic analysis provided indirect evidence that this *Taq* DNA polymerase possesses additional Mn^{+2} binding sites. Subsequently, the direct radioactive assay showed that the 3'-5'exonuclease activity doubled, compared to that of wild-type *Taq* DNA polymerase. This evidence supported the theory that the catalytic module for 3'-5'exonuclease catalysis was in an active conformation.

If we simply assume that the 3'-5'exonuclease activity of mutant *Taq* DNA polymerase could be extrapolated to PCR fidelity, then mutant *Taq* DNA polymerase may show the error rate as 0.9×10^{-5}, which would be better than the 1.2×10^{-5} rate of Vent DNA polymerase. The PCR fidelity assay is underway to elucidate the efficacy of the construction of two metal-ion binding sites.

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