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The novel function of a short region K^{253}xRxxxD^{259} conserved in the exonuclease domain of hyperthermostable DNA polymerase I from *Pyrococcus horikoshii*

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Abstract The DNA polymerase gene of the hyperthermophile *Pyrococcus horikoshii* was successfully overexpressed after removing an intein. The importance of an amino acid sequence around a highly conserved Asp was studied by site-directed mutagenesis. The results indicated that Lys253, Arg255, and Asp259 form a novel functional motif, K^{253}xRxxxD^{259} (outside known motifs Exo I, II, and III), that is important not only for exonuclease activity but also for polymerizing activity, confirming functional interdependence between the polymerase and exonuclease domains. The short loop region, $K^{253}G^{254}R^{255}$, probably contributes to binding to DNA substrates. Moreover, the negative charge and the side-chain length of D259 might play a supporting role in coordinating the conserved Mg^{2+} to the correct position at the active center in the exonuclease domain.

Key words DNA polymerase · Site-directed mutagenesis · Exonuclease · *Pyrococcus horikoshii*

Introduction

Pyrococcus horikoshii is a hyperthermophilic archaeon. It was isolated from a hydrothermal vent in the Okinawa Trough in the Pacific Ocean (Gonzalez et al. 1998). It grows optimally at about 100°C. The whole genome sequence of this archaeon has been analyzed by the National Institute of Technology and Evaluation, Japan (Kawarabayasi et al. 1998). The discovery of this archaeon provides us with invaluable research materials to elucidate the physiology of the hyperthermophilic archaeon. Many thermostable enzymes from this archaeon have been studied (Matsui et al. 1999, 2000a,2000b; Ishikawa et al. 1998). DNA polymerase from *Pyrococcus horikoshii* (Pho DNA polymerase) contains six conserved regions: motifs Exo I, II, and III in the 3′-5′ exonuclease domain, and motifs A, B, and C in the polymerase domain, which are indicative of family B DNA polymerases (Braithwaite and Ito 1993; Kunkel and Wilson 1998; Brautigam and Steitz 1998). Because this enzyme plays a significant role in DNA replication and repair in hyperthermophiles and has a potential application in polymerase chain reaction (PCR) amplification, studies on the structure–function relationship of this hyperthermostable enzyme should receive more attention.

Previous reports have suggested functional interdependence between the polymerase and exonuclease domains in family B DNA polymerases (Gibbs et al. 1991; Southworth et al. 1996). Those mutation sites were mostly selected either in the polymerase domain (Komori and Ishino 2000) or in the known motifs of the exonuclease domain (Soengas et al. 1992). The work presented here identifies a novel conserved region outside the reported motifs in the exonuclease domain and supports the functional interdependence between the polymerase and exonuclease domains.

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Materials and methods

Strains, plasmids, media, and chemicals

The hyperthermophilic archaeon *Pyrococcus horikoshii* was deposited in the Japan Collection of Microorganisms (JCM no. 9974). Its genomic DNA was isolated and then used as a template for Pho DNA polymerase gene cloning (Kawarabayasi et al. 1998). Plasmids pET-15b and pET-11a

Table 1. Primers used to clone wild-type DNA polymerase gene

Primer name	Sequence
Primer new 1	5′-TTTTGTCGTCTTACATATGATCCTGGATGCTGATTATATTACCGAAG AT GGCAAACCGATTATTCGTATCCTTAAAAAAGAAAATGGTGA-3′
Primer L	5′-TTTTGGTACCTTTGGATCCTTAAGCATATTAAGACTTCTTGACCTT-3′
Primer 2	5′-CTATCAAGATCTTAGCAAACAGTTATTACGGGTATTATGG-3′
Primer 3	5′-CCATAATACCCGTAATAACTGTTTGCTAAGATCTTGTATG-3′
Primer 4	5′-TTTTGGTACCTTTGGATCCTTAGGCATATTAAGACTT-3′
Primer 5	5′-TGGTTCCTCCTCAGAAAAGCGTATGAAAGGAACGAGCTAGC-3′
Primer 6	5′-GCTAGCTCGTTCCTTTCATACGCTTTTCTGAGGAGGAACCA-3′

were used as vectors, and ultracompetent *Escherichia coli* XL2-Blue MRF' was used as a host strain for cloning; *E. coli* BL21(DE3)-Codon Plus-RIL and *E. coli* HMS174(DE3)pLysS were used to express Pho DNA polymerase. The plasmids were obtained from Novagen (Madison, WI, USA) and the hosts from Stratagene (La Jolla, CA, USA). The *E. coli* cells were grown in 2×YT medium (1% Bacto Tryptone, 0.5% yeast extract, 0.5% NaCl; adjusted to pH 7.3 with NaOH). DNA polymerase from *Thermococcus litoralis* (Vent DNA polymerase) was purchased from New England Biolabs (Beverly, MA, USA). Ultrapure deoxynucleotide (dNTP) solution was obtained from Pharmacia Biotech, Uppsala, Sweden, and isopropyl-β-D-thiogalactopyranoside (IPTG) was from Takara Shuzo (Otsu, Shiga, Japan).

Protein sequences

The protein sequences used in this study were retrieved from the web site of the National Center for Biotechnology Information (http://www3.ncbi.nlm.nih.gov/Entrez/). Their accession numbers are as follows: Pho DNA polymerase from *Pyrococcus horikoshii* (O59610); Vent DNA polymerase from *Thermococcus litoralis* (P30317); DNA polymerase from *Pyrococcus* sp. GB-D (Deep Vent DNA polymerase) (Q51334); DNA polymerase from *Pyrococcus kodakaraensis* KOD1 (KOD DNA polymerase) (BAA06142); Pfu DNA polymerase (DNA polymerase from *Pyrococcus furiosus*) (P80061); DNA polymerase from *Thermococcus gorgonarius* (Tgo DNA polymerase) (P56689); DNA polymerase from *Thermococcus sp.* (CAA73475); *E. coli* DNA polymerase II (AAC73171); Poc DNA polymerase II from *Pyrodictium occultum* (B56277); DNA polymerase from *Archaeoglobus fulgidus* (O29753); T4 DNA polymerase (1NOYA); and DNA polymerase from bacteriophage RB69 (1WAJ).

Software for DNA and protein analysis

OLIGO4.0-s (National Biosciences, Plymouth, MN, USA), GENETYX-MAC 6.2.0 (Software Development, Tokyo, Japan) and GeneWorks 2.5.1 (IntelliGenetics, Mountain View, CA, USA) were used for PCR primer design, DNA sequence analysis, and alignment of amino acid sequences, respectively. Sequence alignment by the GeneWorks program was based on the unweighted pair group method with an arithmetic mean (Nei 1987). The modeled Pho DNA polymerase structure was retrieved from the Internet (http://www.expasy.ch/swissmod/SWISS-MODEL.html/) (Peitsch et al. 1995) using family B DNA polymerase of *T. gorgonarius* as a template (ExPDB code: 1TGOA.pdb). The protein structure figure was drawn with TURBO FRODO software (Roussel and Cambillaud 1991).

Construction of wild-type Pho DNA polymerase and its mutant genes

The wild-type Pho gene was prepared using the primers listed in Table 1 . One intervening sequence (1,380 bp) was deleted, and the mature Pho DNA polymerase was formed by ligation of two extein genes using PCR as follows: oligonucleotide primers new 1 and new 3, and primers 2 and 4, were designed to produce the genes of left and right exteins, respectively. Because primers 2 and 3 have partial overlapping sequences, the mature gene of DNA polymerase was formed by PCR using oligonucleotide primers new 1 and L. Oligonucleotide primers new 1 and 6, primers 5 and L, and primers new 1 and L were used to produce a site-directed mutation to eliminate the second *Nde*I site to ligate with vectors by unique *Nde*I and *Bam*HI sites. All PCR reactions were carried out on a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT, USA). The resulting plasmid pET-15b/Pho or pET-11a/Pho was first transformed to *E. coli* XL2-Blue MRF' (cloning host), and then introduced into expression host *E. coli* BL21(DE3)-CodonPlus-RIL and *E. coli* HMS174(DE3)pLysS.

Each mutant is constructed on the base of plasmid pET-15b/Pho by PCR because the *Nde*I site was removed in the wild-type Pho gene. The codon GAT of Asp259 was substituted by GAA (D259E), GGC (D259G), AAC (D259N), CAT (D259H), and AAA (D259K), respectively; the AAA of Lys253 and the AGA of Arg255 were converted to GAA (K253E) and GAT (R255D), respectively. The AAA of Lys253 and the AGA of Arg255 were all substituted by GAA in the K253E/R255E mutant. All the substituted

bases lead to the creation of codons preferred by *E. coli*. The nucleotide sequences of each mutant plasmid were examined by infrared fluorescence DNA sequencer (LI-COR 4200; Aloka, Tokyo, Japan).

Expression and purification of wild-type Pho DNA polymerase and its mutants

Pho DNA polymerase and its mutants were overexpressed and purified as follows: *E. coli* BL21(DE3) CodonPlus-RIL and *E. coli* HMS174(DE3)pLysS strains harboring pET-15b/Pho or pET-11a /Pho were propagated at 37°C overnight as seed cultures in 2×YT medium containing ampicillin (100 μg/ml). When the *E. coli* cells grew to OD_{600}=0.3–0.5, induction was done by adding 1 mM IPTG, and the cultures were grown at 25°C for 16 h to produce DNA polymerase. The induced cells were collected by centrifugation and stored at –20°C. The frozen cells were thawed and broken by sonication before heat treatment at 85°C for 30 min. Protein purification was carried out at room temperature as follows.

For His-tagged protein, after centrifugation the supernatant was loaded onto a 3-ml column of nickel resin (Novagen). Pho DNA polymerase was eluted with a 12-ml elution buffer containing 60 mM imidazole (Novagen). The protein solution was concentrated and equilibrated with a 0.1 M sodium phosphate buffer (pH 6.0) with Centriprep 30 (Amicon, Beverly, MA, USA), and then applied to a 5-ml HiTrap SP (Pharmacia Biotech) column. Elution was carried out using a NaCl linear gradient (0–5 M NaCl). Fractions of interest were collected and further purified by a HiTrap heparin column using 50 mM Tris-HCl buffer at pH 8.0 (0–1 M NaCl).

For native protein, the crude cell extract was first purified by a HiTrap heparin column as described; elution fractions were concentrated and applied to a HiLoad Superdex 200 column (Pharmacia). The protein was eluted with 100 mM Tris-HCl buffer (pH 8.0) containing 1.0 M NaCl.

Then, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with PhastGel Gradient 10–15 (Pharmacia Biotech) on PhastSystem (Pharmacia Biotech). Protein bands were made visible by Coomassie blue staining.

Protein concentrations were determined by Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA). N-terminal peptide sequencing was examined as follows: the pure protein was separated by 15% resolving gel in SDS-PAGE, and then transferred onto polyvinylidene difluoride (PVDF) membrane. The N-terminal sequence of Pho DNA polymerase was sequenced by Takara Shuzo using a protein sequencer PSQ-1 (Shimadzu, Kyoto, Japan).

The secondary structure of pure proteins was examined by far UV-CD spectroscopy using a circular dichroism (CD) spectrometer (model 62A DS; Shimadzu) and a quartz cuvette of 1.0-mm or 2.0-mm path length. Protein samples were dissolved in 50 mM Tris-HCl, pH 8.0, buffer containing 75 mM NaCl. An average of three scans was used to investigate the protein conformation.

Assay for DNA polymerase and exonuclease activity

DNA polymerase activity was determined by an acid-insoluble precipitant assay (Aposhian and Kornberg 1962). The 100-μl reaction mixture contained 70 ng purified Pho DNA polymerase or mutant protein, 0.25 mM dNTP, 8 mM $MgSO_4$, 0.1 μl [α-^{32}P]dATP (0.37 MBq/μl), 0.2 mg/ml heated salmon testes DNA or M13mp18 DNA and M13 universal primer (5:1 molar ratio), in standard reaction buffer. The standard reaction buffer contained 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), and 0.1% Triton X-100. ^{32}P-incorporation rate was monitored using a scintillation counter by the Cerenkov method (Gould et al. 1972) after reaction for 30 min at 75°C. One unit of enzyme activity is defined as the amount required to incorporate 10 nmol dNTP into an acid-insoluble form at 75°C in 30 min.

To visualize polymerizing activity, a chain elongation assay was performed using a single-strand DNA (M13mp18) annealed with a ^{32}P-labeled M13 universal primer (17-mer) as a substrate. The reaction mixture contained a standard reaction buffer, 40 μM dNTP, and 14 ng Pho DNA polymerase. After incubation at 60°C, an equal amount was sampled from the reaction mixture at 1 min and 5 min and loaded onto a 15% denaturing polyacrylamide gel containing 7 M urea and 1×TBE (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). The electrophoretic profiles were analyzed by autoradiography with a PhosphoImager (Bio-Rad, Hercules, CA, USA). The substrate was made as follows: 2 pmol M13 universal primer was first labeled by [γ-^{32}P]ATP with T4 polynucleotide kinase, and then mixed with M13mp18 at a molar ratio (1:2) in 20 mM Tris-HCl (pH 7.5) buffer including 200 mM NaCl. After boiling for 2 min, the mixture was cooled slowly to let the primer anneal with the template.

For 3′-5′ exonuclease activity assay, 17-mer oligo DNA (M13 universal primer) was labeled by [α-^{32}P]dATP at the 3′-terminus using terminal transferase, and incubated with each protein in the foregoing reaction condition without dNTPs. The amount of monomer product was measured by autoradiography.

Results and discussion

Sequence organization and gene cloning of Pho DNA polymerase

Pho DNA polymerase belongs to family B DNA polymerase because it contains characteristic motifs Exo I, II, and III in the exonuclease domain and A, B, and C in the polymerase domain (Uemori et al. 1995). There is a typical intervening sequence (intein, 460 amino acids) in motif B, which divides this protein into two exteins. This structure is similar to one intein found in Deep Vent DNA polymerase (Xu et al. 1993). In contrast, it is different from *Pyrococcus furiosus* (Uemori et al. 1997) and *Sulfolobus solfataricus*

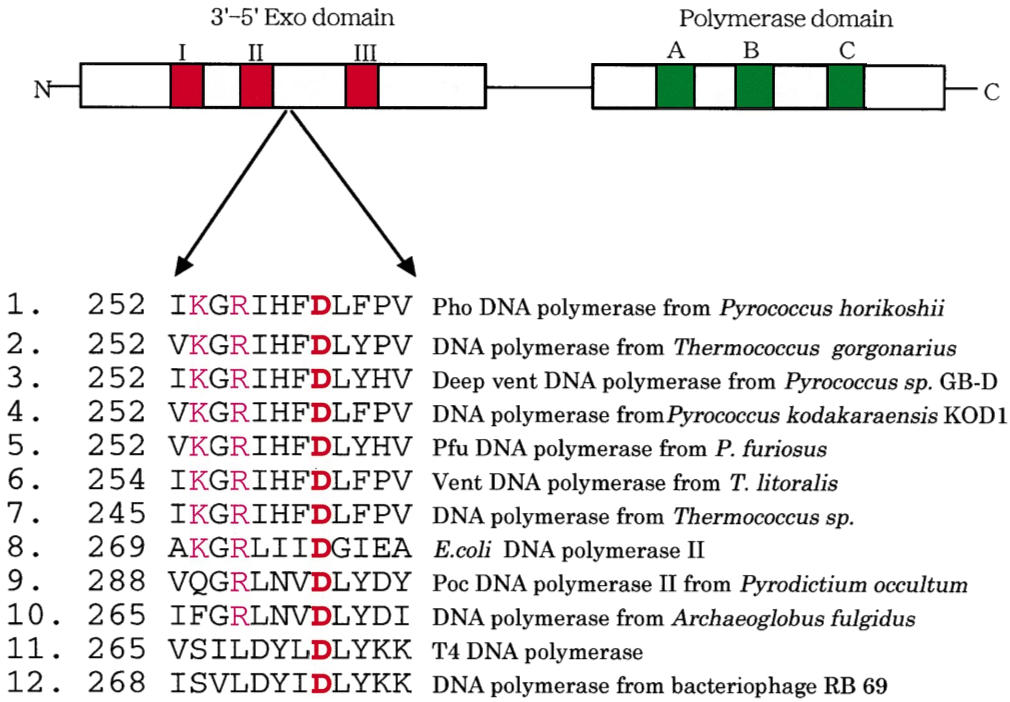

Fig. 1. The functionally highly conserved region found in the exonuclease domain. Pho DNA polymerase contains conserved motifs Exo I, II, and III (*red*) in the 3′-5′ exonuclease domain, and motifs A, B, and C (*green*) in the polymerase domain in family B DNA polymerases (Uemori et al. 1995). The conserved region of Pho DNA polymerase that was studied is located between motifs Exo II and Exo III in the exonuclease domain. The alignment was carried out as described in Materials and methods. *Numbers* before a sequence alignment indicate the amino acid position relative to the N-terminal end of each DNA polymerase. The aspartic residue conserved in all the compared DNA polymerases is indicated in *red letters*; the lysine and arginine residues conserved in one part of the compared DNA polymerases are indicated in *pink letters*

DNA polymerase (Pisani et al. 1992), which do not have any inteins. It is also different from *Thermococcus litoralis* (Vent) DNA polymerase (Kong et al. 1993) and KOD DNA polymerase (Takagi et al. 1997), which contain two inteins. This intein starts at Ser493 and ends at dipeptide His-Asn952; the first amino acid of the extein following the intein is Ser953. This intein is spliced out by itself to form the mature protein when protein processing starts after translation (Perler 1998; Shao and Kent 1997). In the cloning of Pho DNA polymerase from *Pyrococcus horikoshii* genome DNA, the mature DNA polymerase gene was introduced into plasmid pET-11a or pET-15b after removing the intervening DNA sequence (1,380 bp) of the in-frame intein by PCR. Pho DNA polymerase has strong similarities with other thermophilic DNA polymerases (Vent, Deep Vent, KOD, Pfu, Tgo, etc.) in mature and intein sequence alignments. The identities of amino acid residues are more than 75%, compared with most commercially available DNA polymerases. This result suggests they are very close in the phylogenetic tree and have many common features.

Searching for the conserved Lys 253, Arg 255, and Asp 259

By amino acid sequence homology, a highly conserved aspartic residue was found among distinct sources of DNA polymerases from bacteria, eukarya, and archaea. The amino acid sequences around this conserved Asp are listed in Fig. 1. Lys 253, Arg 255, and Asp259 were selected to investigate the function of polymerase and exonuclease domains in Pho DNA polymerase.

Expression and purification of wild-type DNA polymerase and its mutants

The expression of Pho DNA polymerase in *E. coli* BL21(DE3) or *E. coli* HMS174(DE3) was poor, whereas it expressed well in *E. coli* BL21(DE3)-CodonPlus-RIL and HMS174(DE3)pLysS. The protein was purified completely (more than 95.5% by HPLC). The N-terminal peptide sequence (27 amino acid residues) is identical to that deduced from the DNA sequence. Pho DNA polymerase consists of 774 amino acids. The molecular weight determined by SDS-PAGE is in accordance with that estimated from the amino acid sequence (90 kDa). Mutant proteins were expressed and purified by the same process as the wild-type protein.

The DNA polymerase activity assay showed that the half-life of this enzyme is more than 16.5 h at 90°C and 2 h at 105°C. The CD spectrum did not change greatly when this protein was heated at 90°C for 2.5 h, suggesting that it is as thermostable as other thermophilic enzymes from ther-

mophilic Archaea (Matsui et al. 1999, 2000a, 2000b; Ishikawa et al. 1998). The CD spectra of mutants were identical to that of the wild-type protein, except for the mutant D259H, in which a sharp peak was observed at 228 nm. The overall structures of mutants, except D259H, were almost the same as that of the wild type, but the secondary structure of D259H mutant was very different (data not shown).

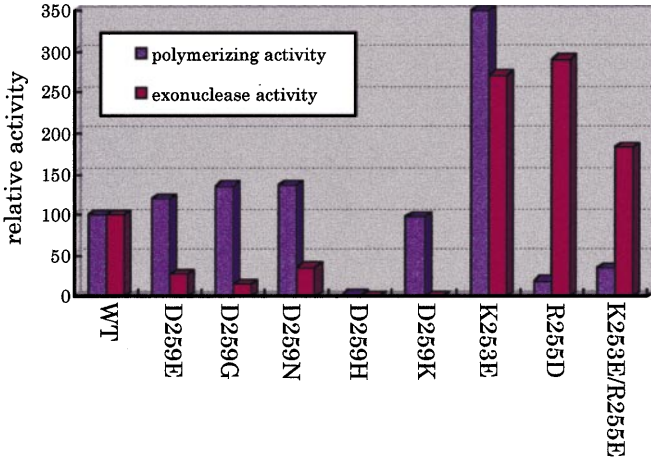

Fig. 2. Effect of mutations on DNA polymerizing and 3′-5′ exonuclease activities. The activities of the wild-type Pho DNA polymerase are expressed as 100. The DNA polymerase activity was measured by the acid-insoluble method (Aposhian and Kornberg 1962). The 3′-5′ exonuclease activity was assayed with 3′-^{32}P-labeled M13 universal primer

Polymerase activity assay for mutants

The effect of each mutant on polymerizing and 3′-5′ exonuclease activities was investigated using purified proteins. As shown in Fig. 2, the polymerase activities of most mutants changed in comparison with that of the wild-type protein. The D259H mutation abolished almost all the polymerizing activity as a result of the structural change suggested by the far UV-CD spectrum. Other mutations at Asp259 changed the polymerizing activity slightly. These results indicate that the substitution of D259 did not affect the polymerizing activity significantly because Asp259 is located far from the active center of the polymerase domain (Fig. 3). The K253E mutant increased the polymerase activity by 3.5 fold, but the R255D and K253E/R255E mutants decreased the activity to 19.4% and 35.4% of the wild-type protein activity, respectively. The decreased polymerizing activity of K253E/R255E indicates that this mutant activity was affected mainly by the substitution in Arg255, not in Lys253. This result suggests that Arg255 contributes more to polymerizing activity than Lys253.

Observing the electrostatic surface potential of Tgo DNA polymerase (Hopfner et al. 1999), the loop $K^{253}G^{254}R^{255}$ seems to be involved in cleft T, which is responsible for binding to the single-strand template DNA. The

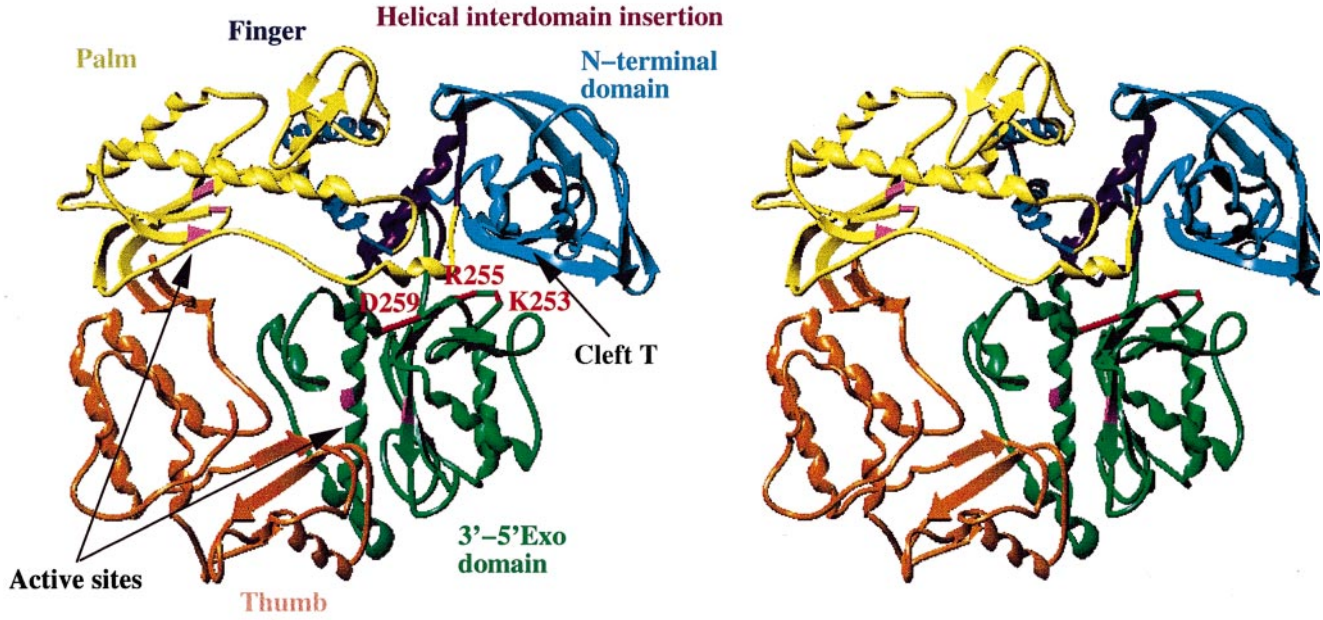

Fig. 3. Stereoview for the modeled structure of Pho DNA polymerase using Tgo DNA polymerase as a template. Because Pho DNA polymerase has a very high homology with Tgo DNA polymerase from *Thermococcus gorgonarius* (amino acid identities are 82%), the molecular structure of Tgo DNA polymerase is used to model that of Pho DNA polymerase. The modeled molecule has the same overall domain topology as Tgo DNA polymerase, which is composed of five domains: N-terminal domain (*light blue*), 3′-5′ exonuclease (*green*), helical interdomain insertion (*purple*), palm (*yellow*), fingers (*blue*), and thumb (*orange*). Critical residues are indicated by different colors. *Magenta moieties*, polymerase active sites (D405, D540, D542) and 3′-5′ exonuclease active sites (D141, E143, D215, D315); *red moieties*, the mutated residues (D259, K253, R255)

positive charge residue Arg255 probably contributes to binding with the DNA substrate. To evaluate the activity change using another method, dNTP incorporation ability was assayed by primed single-strand M13mp18 as a substrate. The reaction products of the mutants were analyzed by 15% denaturing gel electrophoresis. The relative activity of each mutant was comparable to that measured by the acid-insoluble method (Aposhian and Kornberg 1962) (data not shown).

Exonuclease activity assay for mutants

3′-5′ Exonuclease activity was also measured with purified mutant proteins. As shown in Fig. 2, the exonuclease activity of K253E, R255D, and K253E/R255E mutants increased 2.7, 2.9, and 1.8 fold, respectively, compared with wild-type protein activity. The Asp259 substitution affected the exonuclease activity more strongly than the polymerizing activity. When Asp259 was replaced by positively charged residues, the exonuclease activity of D259K decreased drastically to 0.58% compared with that of the wild-type DNA polymerase. The acidic and neutral residue substitutions D259E, D259G, and D259N showed a moderate decrease of the polymerizing activity.

Based on the protein modeling study, Pho DNA polymerase has the same overall architecture and domain topology as Tgo DNA polymerase (Hopfner et al. 1999) (see Fig. 3). It looks ring shaped with a small hole in the center. The residues Asp404, Asp 540, and Asp542 in the palm domain form the polymerase active sites. The residues Asp141, Glu143, Asp215, and Asp315 in the exonuclease domain form the exonuclease active sites. The acidic residues bind with two Mg^{2+} to form the active center in each domain. Asp259 is in proximity to the active center of the exonuclease domain and could be involved in binding to the conserved Mg^{2+}. Exonuclease inactivation of D259K mutant and lower exonuclease activities of other mutations at Asp259 support this hypothesis (see Fig. 2). The Asp259 is further from the conserved Mg^{2+} than D141 and E143 in the exonuclease domain; the distance calculated between Asp259 and Mg^{2+} is 11.54 Å using TURBO FRODO software (Roussel and Cambillaud 1991). Asp259 does not seem to interact with the conserved Mg^{2+} directly, but it might play a supporting role in coordinating Mg^{2+} to the correct position in the exonuclease active center. The negative charge and the side-chain length of Asp259 might be critical in this function, because the exonuclease activity decreased when this residue was replaced in the mutants (Fig. 2).

Taking the foregoing results into account, the conserved segment KxRxxxD, coxnsisting of Lys253, Arg255, and Asp259, is a potential region outside the motifs Exo I, Exo II, and Exo III in the exonuclease domain. The results also show that the replacement of one residue in one domain affects the activity of the other domain. The polymerizing and exonuclease activities are interdependent.

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