Structural Mechanism of Ribonucleotide Discrimination by a Y-Family DNA Polymerase

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Introduction

Loss of ribonucleotide discrimination by DNA polymerases leads to replicative stress and genome instability.¹ The ability to differentiate between deoxyribonucleotides (dNTP) and ribonucleotides (NTP) (Fig. 1a) is an essential function of all DNA polymerases that must be highly specific, in part, due to high cellular NTP concentrations.²,³ Most DNA polymerases utilize bulky side-chain residues in their active sites to discriminate against NTPs.⁴ The identity of this residue is typically glutamine for A-family DNA polymerases⁵,⁶ and tyrosine or phenylalanine for Y- and B-family DNA polymerases⁷⁸–¹⁰ (Fig. 1b). X-ray crystallographic studies have revealed that these residues create a stacking interaction with the deoxyribose sugar of an incoming dNTP and form a hydrogen bond between the backbone amine group and the 3′-OH group of the deoxyribose sugar moiety.¹¹–¹⁵ Mutating each of these residues to a smaller amino acid generally results in a loss of NTP discrimination.⁵,⁷,⁹,¹⁶,¹⁷ It has been hypothesized that these bulky active-site residues prevent NTP incorporation by clashing with the 2′-OH group of the ribose and have thus been coined ‘steric gates.’⁷,¹⁸ Although the steric gate residue is clearly involved in NTP discrimination, no structural studies have been reported on steric gate mutants of DNA polymerases. Thus, exactly how dNTP/NTP selectivity is achieved by a DNA polymerase still remains elusive.

The Y-family DNA polymerases are specialized to bypass DNA lesions during DNA replication in a process known as translesion DNA synthesis.¹⁹
These enzymes have a similar domain organization to all other DNA polymerases, consisting of a Finger, Thumb, and Palm domain. In addition, the Y-family polymerases contain a unique fourth domain, known as the Little Finger or Pad/Wrist Domain. The Y-family polymerases have evolved open, solvent-accessible active sites, which accommodate bulky and distorting DNA lesions. Consequently, these polymerases can accommodate an incoming nucleotide in different conformations, which allows permissive base-pairing to facilitate translesion DNA synthesis. Remarkably, the solvent-accessible active sites of the Y-family polymerases, which have minimal contacts to incoming nucleotides, are still highly discriminatory against NTPs. Although the steric gate residue is likely responsible for NTP discrimination, structural investigations are required to fully understand how a single active-site residue in the permissive environment can induce such a high sugar selectivity.

In order to elucidate the structural role of the steric gate residue in the active site of a Y-family DNA polymerase, we generated a single point mutation (Y12A) in Dpo4, a model Y-family polymerase from the archaeon Sulfolobus solfataricus. Here, we report that mutating this conserved residue in Dpo4 creates a defect in ribonucleotide discrimination and produces a pseudo-DNA/RNA polymerase. In addition, the Y12A mutation leads to low incorporation efficiencies of both dNTPs and NTPs. Crystal structures were obtained for the Dpo4 Y12A ternary complexes, incorporating either dATP or ATP opposite template dT, demonstrating the first structure of a DNA polymerase incorporating a ribonucleotide. These results reveal for the first time how a Y-family polymerase structurally discriminates against ribonucleotides, and advance our understanding of the general enzymatic mechanism of the Y-family DNA polymerases.

Results

Impaired ribonucleotide discrimination by Dpo4 Y12A

To test the ability of the Dpo4 Y12A mutant to discriminate against ribonucleotides, primer extension assays were performed with the wild-type and mutant Dpo4 proteins. Wild-type Dpo4 incorporates dNTPs with high efficiency, extending the primer strand to the end of the DNA template (Fig. 2a). In contrast to dNTP incorporation, wild-type Dpo4 incorporates virtually no NTPs and is thus highly discriminatory against incoming ribonucleotides (Fig. 2b). Compared to wild-type Dpo4, the Y12A mutant is able to incorporate NTPs, the efficiency of which is lower than that of its dNTP incorporation (Fig. 2a). Although the Dpo4 Y12A mutant is able to incorporate NTPs and has therefore lost the ability to discriminate against ribonucleotides (Fig. 2b). The Dpo4 Y12A mutant also incorporates dNTPs, but the efficiency of incorporation is dramatically reduced compared to the wild-type Dpo4 (Fig. 2a). Although the Dpo4 Y12A mutant is able to incorporate NTPs, the efficiency of its NTP incorporation is lower than that of its dNTP incorporation (Fig. 2). The Y12A mutant prefers to incorporate the matched dATP or ATP opposite template dT with minimal misincorporation of the other nucleotides, similar to the wild-type Dpo4 (Fig. 2c and d). The Dpo4 Y12A functional assays are consistent with recently published kinetic studies on this steric gate mutant.

Crystal structures of the Y12A mutant

In order to establish a structural basis for the acquired function of ribonucleotide incorporation,
we crystallized the Dpo4 Y12A mutant in ternary complex with a DNA substrate and either dATP or ATP. The DNA substrate contains a 2′,3′-dideoxy-terminated 13-mer primer (ddC) and an 18-mer template with a thymine base 5′ to the template–primer junction (see Materials and Methods). Incoming dATP or ATP was incubated with the DNA substrate and co-crystallized with the Dpo4 Y12A mutant. Both nucleotides were hydrolyzed to dADP and ADP, similar to dideoxynucleotides in the wild-type Dpo4 ternary crystal structures. The resulting two ternary crystal structures are referred to as Y12A-dADP and Y12A-ADP according to the identity of the incoming nucleotide in the active site. Both Y12A-dADP and Y12A-ADP structures belong to space group P21212 and diffracted X-rays to 2.65- and 2.40-Å resolutions, respectively (Table 1). The structures have been refined with Ca²⁺ as the active-site metal ion (Fig. 3), similar to previous Dpo4 ternary crystal structures. The absence of a metal ion at the A site is likely due to the high mobility of ions at this position. The Dpo4 Y12A structures are virtually identical to previously solved wild-type Dpo4 crystal structures (Fig. 3a). Comparing all Ca²⁺ atoms between the two Y12A structures and wild-type Dpo4 (PDB: 2AGQ) gave root-mean-square deviations between 0.30 and 0.36 Å. In addition, the active-site residues contacting the replicating base pair are positioned identically between the two Y12A structures and the wild-type Dpo4 structure. Therefore, the Y12A mutation does not induce any disturbance in the protein structure except for the side-chain difference at the mutation site. The major difference is observed in the positioning of the incoming nucleotides (Fig. 3). The bases of incoming dADP and ADP are positioned nearly identical to that of dATP in the wild-type Dpo4 structure (Fig. 4a). However, the sugar groups of both dADP and ADP are slightly shifted towards the minor groove of the DNA to fill the space left by the aromatic site chain of tyrosine 12 (Fig. 5). In addition, the α and
β phosphates are positioned similarly to the wild-type Dpo4 structure with ddADP (PDB: 1JX4) and differently from dATP in the wild-type Dpo4 ternary structure (PDB: 2AGQ) (Fig. 5) due to the hydrolysis of the γ-phosphate.11,35

Positions of incoming nucleotides in the Y12A mutant

The Dpo4 Y12A mutant, which has lost the ability to discriminate against ribonucleotides, induces subtle but distinct conformational changes on both incoming dADP and ADP nucleotides. Y12A-dADP and Y12A-ADP structures have the template thymine base in a standard anti conformation, positioned identically to wild-type Dpo4 (Fig. 3). The adenine base of incoming dADP/ADP of Y12A forms a Watson–Crick base pair with the template thymine base (Fig. 4). Thus, the bases of incoming dADP and ADP are positioned identical to those of dATP and ddADP in the Dpo4 wild-type structures (Fig. 5). However, the sugar groups of both dADP and ADP are shifted towards the minor groove of the DNA in Y12A (Fig. 5), since the stacking interaction with the sugar ring is lost due to tyrosine being mutated to alanine at position 12. The mutation makes room for the sugar group to move towards the minor groove. The sugar and phosphates of dADP/ADP are

Table 1. Summary of crystallographic data

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ᵃ AU, asymmetric unit.
ᵇ Data for the highest-resolution shell are in parentheses.

Fig. 3. Comparison of wild-type Dpo4 and Dpo4 Y12A ternary complexes. (a) Superposition of wild-type Dpo4 (2AGQ, grey), Y12A-dADP (Blue), and Y12A-ADP (pink). (b) Close-up view of the incoming nucleotides and template base from the side and top views. The backbone atoms are shown in ribbon diagram; the incoming nucleotides are shown in ball and stick; and surface representation of 2AGQ is shown in grey. Active-site metal ions are shown as green spheres.
positioned similarly to the wild-type Dpo4 structure with ddADP (PDB: 1JX4), instead of the dATP in the wild-type Dpo4 structure (PDB: 2AGQ) (Fig. 5). The 2′-OH of ADP is projected into the open pocket created by the Y12A mutant (Fig. 4a). The electron density is poor for the ribose sugar of the ADP (Fig. 4b), indicating a high degree of mobility of the nucleotide. The 3′-OH of the sugar groups shifts ~1 Å compared to that of dATP in the wild-type Dpo4 structure (Fig. 5a and b). This likely contributes to the hydrolysis of the γ-phosphate. The 3′-OH of the sugar in dADP/ADP still makes a hydrogen bond with the amide group of the A12 residue, similar to wild-type Dpo4 (Fig. 4). However, the ADP sugar has a weaker stabilizing H-bond (3.1 Å) than the one formed between the dADP sugar and the A12 backbone amide (2.8 Å). The weak H-bond leaves the ADP ribose a loose contact to Dpo4. Both ribose and deoxyribose are in a C3′-endo pucker. Thus, instead of sugar puckering, the mobility of the ribose group likely contributes to the destabilization of ADP in the Dpo4 active site.

Discussion

The steric gate and ribonucleotide discrimination

Our structural observations have finally verified the long-standing hypothesis that the steric gate residue blocks NTP incorporation by clashing with the 2′-OH group. When the Dpo4 steric gate residue Tyr12 is mutated to Ala, the 2′-OH group of the ribose sugar can be spaciously accommodated in the polymerase active site, which allows the formation of a productive ternary complex. In the presence of Tyr12, the 2′-OH group of the ribose sugar would clash with the steric gate residue within ~1.5 Å, preventing NTP binding and incorporation (Fig. 5a). These results are consistent with the pre-steady-state kinetic analysis demonstrating that the wild-type Dpo4 incorporates a dNTP with 9200-fold greater efficiency than a corresponding NTP, while the efficiency difference is only 5-fold from the Dpo4 Y12A mutant. Thus, the Dpo4 Y12 residue directly blocks NTP incorporation by clashing with its ribose sugar. A similar clashing mechanism likely occurs in the closely related archaeal Y-family polymerase Dbh from Sulfolobus acidocaldarius, which also displays a loss of ribonucleotide discrimination upon mutating its conserved steric gate residue Phe12. Interestingly, DNA polymerases pol β and pol λ from the X-family utilize the protein backbone for ribonucleotide discrimination rather than a specific steric gate residue. Nevertheless, NTP clashing appears to be a general mechanism of all DNA polymerases.

Although DNA polymerases are highly discriminatory against ribonucleotides, a small percentage of NTPs are inevitably incorporated into the genome during DNA replication. Such events could convert portions of the duplex from B-form to A-form, which may alter the rate of replication and interfere with a wide range of B-form DNA binding proteins. In addition, ribonucleotide incorporation may induce a high frequency of dNTP misincorporations due to misaligned primer termini and altered coding potential of NTP bases. Thus, NTP incorporation by DNA polymerases would likely be a significant contributor to genome instability. Indeed, organisms have evolved repair proteins, such as human RNase H1 to remove incorporated NTPs within the genome. In contrast to the deleterious effects of widespread ribonucleotide incorporation into the genome, it has been suggested that site-specific NTP incorporation by DNA polymerases may have biological roles in DNA repair processes such as non-homologous end joining.

The steric gate and the efficiency and fidelity of nucleotide incorporation

In addition to discrimination against ribonucleotides, the steric gate residue in Dpo4 also influences...
nucleotide incorporation efficiency. The Y12A mutation in Dpo4 causes significant reductions in activity for dNTP and NTP incorporation. Pre-steady-state kinetics reveal a 3- to 19-fold decrease in dNTP incorporation efficiency between Dpo4 wild type and the Y12A mutant. Similarly, NTP incorporation efficiency is reduced by 3- to 30-fold compared to dNTPs for the Y12A mutant. These decreased activities can be rationalized by comparing the ternary structures of wild-type Dpo4, Y12A-dADP, and Y12A-ADP. The Y12A mutation creates a more open and permissive active site, which likely destabilizes the binding of incoming nucleotides by abolishing the sugar group stacking interaction with the steric gate residue and allowing greater conformational heterogeneity in the polymerase active site. In addition, the mobility of the NTP ribose likely induces greater destabilization compared to a dNTP ribose. The mobility is demonstrated by the poor density of the ADP sugar moiety in Y12A-ADP. Thus, it is plausible that the sugar group must be stabilized and positioned adjacent to the steric gate residue for efficient catalysis to occur. Interestingly, the dADP and ADP nucleotides in Y12A-dADP and Y12A-ADP are positioned identically to ddADP in a previous wild-type Dpo4 structure (PDB: 1JX4). The ddADP nucleotide also shifts its sugar group towards the minor groove of the DNA due to the absence of the 3′-OH group (Fig. 5c and d). Consequently, the incorporation efficiency of ddNTPs is significantly reduced compared to dNTPs for both Y-family polymerases and high-fidelity polymerases. Thus, the stability of the nucleotide sugar group appears critical for...
nucleotide incorporation efficiency. Furthermore, mutation of the catalytic residues (D105A/E106A) abolishes all enzymatic activity and indicates that Dpo4 uses the same active site for both nucleotide incorporation and γ-phosphate hydrolysis. The side reaction of γ-phosphate hydrolysis could be a contributing factor to the reduced nucleotide incorporation efficiency.

Interestingly, the nucleotide incorporation fidelity is maintained for the Dpo4 Y12A mutant. In addition, nucleotide incorporation specificity is maintained between dNTPs and NTPs for the Dpo4 Y12A mutant. Previous work has shown the NTP incorporation fidelity of Dpo4 Y12A to be in the range of 10−3−10−4, which is identical to the dNTP incorporation fidelity of wild-type Dpo4. Thus, the steric gate residue of Dpo4 has little influence on nucleotide selection in spite of its critical function in positioning and stabilizing incoming nucleotides. This result is consistent with the structural observations that incoming nucleotide selection by Y-family DNA polymerases is determined by the hydrogen bonding and base stacking potentials of the base of an incoming nucleotide and not of the sugar moiety.

Sugar positioning and γ-phosphate hydrolysis

The γ-phosphates of both dATP and ATP nucleotides in the Y12A structures have been hydrolyzed and eliminated. Interestingly, this phenomenon has been observed in different Y-family polymerase structures with incoming ddNTP nucleotides. It appears that sugar positioning of the incoming nucleotide is involved in γ-phosphate hydrolysis as both ddADP in Dpo4 and dADP in the Dpo4 Y12A mutant have identical sugar positions (Fig. 5d). As the sugar group shifts to the minor groove, the α-phosphate moves away from the γ′ end of the primer strand, and the rest of phosphate groups of the incoming nucleotides are more likely exposed to the catalytic site of Dpo4 for hydrolysis. Therefore, the sugar position likely contributes to γ-phosphate hydrolysis of ddNTP in wild-type Dpo4 and dNTP/NTP in the Dpo4 Y12A mutant by repositioning the phosphate groups in the active site. This unusual positioning of phosphate groups in the active site of Dpo4 may also be responsible for the primer degradation observed in the Dpo4-DNA binary complex. In the binary complex, the γ′-OH ends of primer strands are located at the position of incoming nucleotides, which would interact with the catalytic residues and be removed from the primer strands.

Conclusions

Here, we have described the first ternary structure of a Y-family DNA polymerase steric gate mutant in complex with DNA and an incoming ribonucleotide. We have validated the hypothesis that the steric gate residue prevents NTP incorporation by clashing with the ribose 2′-OH group. In addition, we have shown how the steric gate of Dpo4 is critical for stabilizing incoming nucleotides for optimal enzymatic efficiency. Lastly, we propose that shifted sugar positions may induce γ-phosphate hydrolysis of incoming nucleotides and cause a drop in nucleotide incorporation efficiency.

Materials and Methods

DNA and protein preparation

Oligonucleotides for crystallization were purchased from Keck Oligo, Inc., and were purified and desalted before use. The 16-nt primer (5′-GGGGAAAGCTC3′) containing a 2′,3′-dideoxy 3′ end (Cdd) was annealed to an 18-nt template (5′-TTCATGAGTCTTCCCCC′) and incubated with Dpo4 or Dpo4 Y12A (10 nM) and either all four dNTPs or rNTPs (2 mM) in the presence of 5 mM MgCl2. Crystals were obtained in 12.5% polyethylene glycol 3350+100 mM Ca(Ac)2+100 mM Hepes (pH 7.0) by incubating protein (0.2 mM) and DNA at 37 °C for 2 min in buffer containing 40 mM Tris (pH 8.0), 5 mM MgCl2, 250 μg/ml bovine serum albumin, 10 mM DTT, and 2.5% glycerol. Reactions were terminated with loading buffer containing 5% formamide, 20 mM ethylenediaminetetraacetic acid, and 0.025% bromophenol blue. Reactions were resolved on a 20% polyacrylamide gel containing 7 M urea. The gels were visualized with PhosphorImager.

Primer extension assays

The DNA substrate (10 nM) was incubated with either Dpo4 or Dpo4 Y12A (10 nM) and either all four dNTPs or rNTPs (20−100 μM) or individual dNTPs or rNTPs (100 μM) and reacted at 37 °C for 2 min in buffer containing 40 mM Tris (pH 7.0), 5 mM MgCl2, 250 μg/ml bovine serum albumin, 10 mM DTT, and 2.5% glycerol. Reactions were terminated with loading buffer containing 95% formamide, 20 mM ethylenediaminetetraacetic acid, and 0.025% bromophenol blue. Reactions were resolved on a 20% polyacrylamide gel containing 7 M urea. The gels were visualized with PhosphorImager.

Crystallization and structure determination

Ternary complexes were formed for Y12A-dADP and Y12A-ADP by incubating protein (0.2 mM) and DNA at 1:1.2 molar ratio with either dATP or ATP (2 mM) in the presence of 5 mM MgCl2. Crystals were obtained in 12.5% polyethylene glycol 3350+100 mM Ca(AC)2+100 mM Hepes (pH 7.0)+2.5% glycerol. Crystals were flash frozen in liquid nitrogen using a solution of 20% polyethylene glycol 3350+100 mM Ca(AC)2+100 mM Hepes (pH 7.0)+20% ethylene glycol as a cryoprotectant. The data were collected using a Rigaku-MSC RU-200 generator and a
mar345 image plate detector. The Y12A-dADP structure was processed with MOSFELM, while the Y12A-ADP structure was processed with DENZO and SCALEPACK. Both structures were solved by molecular replacement using PHASER with Dpo4 wild type (1JX4) as the search model. Refinement was performed using PHENIX. Model building was performed with COOT, as the search model. Refinement was performed using PHENIX.

Coordinates

The atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB) with accession codes 3PR4 and 3PR5 for the Y12A-dADP and Y12A-ADP structures, respectively.

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References


