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Insights into the effect of minor groove interactions and metal cofactors on mutagenic replication by human DNA polymerase β

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Abstract

DNA polymerases accommodate various base pair conformations in the event of incorrect insertions. In particular, Watson-Crick-like dG:dTTP base pair has been observed at the insertion site of human DNA polymerase β (pol β). A potential factor contributing to the diverse conformations of base pair mismatches is minor groove interactions. To gain insight into the effect of minor groove interactions on base pair conformations, we generated an Asn279Ala pol β mutant that cannot make minor groove contacts with incoming nucleotide. We conducted structural and kinetic studies of Asn279Ala pol β in complex with incoming dTTP and templating dG or O6-methyl-dG. The crystal structure of the Asn279Ala pol β -G:T complex showed a wobble dG:dTTP base pair, indicating that the previously observed Watson-Crick-like dG:dTTP conformation was induced by the minor groove contact. In contrast, O6-methyl-dG, an analog of the enol tautomer of guanine, formed a Watson-Crick-like base pair with dTTP in the absence of the minor groove contact. These results suggest that the Watson-Crick-like G:T base pair at the insertion site is formed by the rare enol tautomers of G or T, whose population is increased by the minor groove hydrogen bond with Asn279. Kinetic studies showed that Asn279Ala mutation decreased dG:dTTP misincorporation rate 6-fold in the presence of Mg^{2+} but increased the rate 3-fold in the presence of Mn^{2+} , highlighting the effect of minor groove interactions and metal ions on promutagenic replication by pol β .

INTRODUCTION

DNA polymerases play a critical role in preserving genome integrity by incorporating the correct nucleotide from a pool of structurally similar nucleotides during DNA repair and replication [1]. Kinetic and structural studies of DNA polymerases that incorporate correct nucleotides have provided important mechanistic insights into how these enzymes efficiently and accurately catalyze nucleotidyl transfer [2–5]. Several factors, such as inter-nucleobase hydrogen bonding, shape complementarity, dNTP binding affinity, the induced-fit

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Author Contribution

M.K conducted protein expression, kinetic experiments, and structure determination experiments and wrote the paper; S.L. designed the experiments and wrote the paper.

Competing Interests

The authors declare that there are no competing interests.

mechanism, intrinsic proofreading activity, and minor groove interactions, have been identified as contributors to the replication fidelity of DNA polymerases [6,7].

Recent structural studies of the incorporation of incorrect nucleotides by DNA polymerases have provided insights into how these enzymes increase their replication fidelity by deterring misincorporation [8–13]. Crystal structures have shown that most DNA polymerases allow only Watson-Crick base pairing for correct insertions, whereas they accommodate varying base pair conformations for mismatches in the binding pocket. For example, the A-family DNA polymerase *Bacillus stearothermophilus* DNA polymerase I fragment (BF) accommodates wobble dG:dTTP and dA:dCTP base pairs in the presence of active-site Mg^{2+} [11,14,15,16]. The B-family DNA polymerase RB69 induces wobble dG:dTTP and dT:dGTP base pairs in the active site [12]. In the catalytic pocket of the Y-family DNA polymerases *Sulfolobus solfataricus* Dpo4 and human pol η , dT:dGTP adopts staggered and wobble base pair conformations, respectively [10,17]. In contrast, in the active site of the X-family DNA polymerase pol λ , Watson-Crick-like dT:dGTP base pairs have been observed in the presence of active-site Mg^{2+} [18]. Interestingly, BF shows a metal-dependent conformational transition for dA:dCTP mismatches [11]: Substituting the active-site Mn^{2+} for Mg^{2+} triggers a wobble-to-Watson-Crick-like conformational transition for dA:dCTP via a rare tautomerization mechanism. Recently, we have reported that the X-family DNA polymerase β (pol β) induces the formation of a staggered dG:dTTP base pair in the presence of active-site Mg^{2+} and a Watson-Crick-like dG:dTTP base pair in the presence of Mn^{2+} (Figure 1) [19]. Unlike BF, pol β accommodates a staggered dA:dCTP base pair in the presence of either Mg^{2+} or Mn^{2+} [20].

What explains the wide variety of mismatch base pair conformations observed in DNA polymerase active sites? A potential contributing factor is minor groove hydrogen bonding interactions, which vary widely among DNA polymerases [11,21]. Crystal structures of DNA polymerases in complex with duplex DNA and an incoming nucleotide show significant differences in specific hydrogen bonding interactions between the minor groove edge of the DNA and the amino acid residues of the protein [2,4, 11,22]. Minor groove contacts in the catalytic pocket of DNA polymerases contribute to the replication fidelity and the catalytic efficiency of these enzymes [23–28]. In addition, minor groove interactions can modulate deletion error rates [29], base substitution specificity [24], misincorporation rates [30], mismatch extension [14,30] and lesion bypass [26]. However, the effect of minor groove hydrogen bonding interactions on mismatched base pair conformations in the catalytic pocket remains poorly understood.

We selected pol β as a model DNA polymerase to gain insights into the effect of minor groove interactions on the conformations of base pair mismatches. Pol β , an error-prone enzyme that lacks intrinsic 3'-to-5' exonuclease activity, is the smallest mammalian DNA polymerase and has been extensively used as a model enzyme to study the replication-fidelity and nucleotidyl-transfer mechanisms of DNA polymerases [31]. Pol β plays a critical role in the base-excision-repair pathway by filling in short nucleotide gaps [31]. During correct nucleotide insertion, Tyr271, Arg283, and Asn279 make hydrogen bond contacts with the minor groove edges of the primer terminus, templating base, and incoming nucleotide, respectively [22,32]. Disrupting the minor groove hydrogen bonding to the

primer terminus by using a Tyr271Phe mutation has minimal effects on catalytic efficiency and fidelity [33]. However, introducing an Arg283Lys mutation to disturb minor groove contact to the templating base drastically decreases both the catalytic efficiency and the replication fidelity of the enzyme [25,33–35]. Intriguingly, the disrupted minor groove interactions in the Asn279Ala mutant leads to a decrease in replication fidelity for non-gapped DNA, whereas replication fidelity increases 5-fold for short-nucleotide-gapped DNA [33, 36].

To evaluate the effect of minor groove interactions and metal ions on promutagenic replication by pol β , we conducted kinetic studies of Asn279Ala pol β for correct and incorrect insertions in the presence of Mg²⁺ or Mn²⁺. In addition, we determined four ternary complex structures of Asn279Ala pol β . The four structures contained the mutant enzyme in complex with dG:dTTP in the presence of the active-site Mg²⁺ or Mn²⁺, dG:dCTP in the presence of Mg²⁺, and O6MeG:dTTP in the presence of Mn²⁺. These studies provide new insights into how minor groove hydrogen bonding interactions and metal ions affect promutagenic replication by DNA polymerases.

EXPERIMENTAL

Preparation of the Asn279Ala pol β mutant

The Asn279Ala mutant pol β construct was generated by using oligonucleotide-directed mutagenesis, according to the manufacturer's protocol (QuikChange Mutagenesis System, Stratagene). A pair of complementary mutagenic oligonucleotides, (forward) 5'-CACTGGGAGTGATATTTTCGCTAAGAATATGAGGGCTCATG-3' and (reverse) 5'-CATGAGCC CTCATATTCTTAGCGAAAATATCACTCCCAGTG-3', were designed to introduce a mutant codon (underlined) encoding the Asn279Ala mutation in the pol β sequence. PCR was performed using a *pET32* vector containing human POL B as a template, the mutagenic primers, dNTP mixture, and *Pfu* turbo DNA polymerase in the manufacturer's buffer under the following conditions: 95 °C for 2 min, followed by 28 cycles of 95 °C for 30 s, 50–65 °C for 1 min, and 68 °C for 8 min. *DpnI* was then added to the reaction mixture and incubated for 30 min at 37 °C to digest the methylated parent plasmid. The mixture was used to transform competent XL-10 Gold *Escherichia coli* cells, and the colonies were selected on LB agar plates supplemented with kanamycin (30 μ g/mL). The plasmids were purified from an overnight culture, and the desired DNA sequence was confirmed by DNA sequencing (DNA Core Facility, The University of Texas at Austin). The Asn279Ala pol β mutant was expressed and purified according to previously described protocols [19], with minor modifications.

Steady-state kinetics of nucleotide insertion opposite the templating G

The steady-state kinetic parameters for insertion opposite the templating guanine by pol β were measured as described previously [20]. Briefly, the DNA oligonucleotides for the kinetic assays (upstream primer, 5'-FAM-CTGCAGCTGATGCG-3', downstream primer, 5'-phosphate/CGTACGGATCCCCGGGTAC-3', and template, 5'-GTACCCGGGGATCCGTACGGCGCATCAGCTGCAG-3') were synthesized by Midland Certified Reagent company (Midland, TX) and Integrated DNA Technologies (Coralville,

IA). They were purified by the manufacturers and confirmed by MALDI-TOF mass spectrometry. Each oligonucleotide was annealed in hybridization buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) to prepare a DNA substrate for pol β containing a single-nucleotide gap opposite guanine. Enzyme activities were determined using a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl₂ or MnCl₂, 80 nM single-nucleotide gapped DNA, and different concentrations of incoming dNTP.

To prevent end-product inhibition and substrate depletion from interfering with accurate velocity measurements, reaction times and enzyme concentrations (either the wild type or Asn279Ala pol β mutant) were optimized for each experiment (less than 20% insertion product formed). The reactions were initiated by the addition of the enzyme and stopped with gel-loading buffer (95% formamide with 20 mM EDTA, 45 mM Tris-borate, 0.1% bromophenol blue, and 0.1% xylene cyanol). The quenched samples were separated on 18 or 20% denaturing urea polyacrylamide gels. The gels were analyzed using a PhosphorImager (Molecular Dynamics) to quantify product formation. The k_{cat} and K_{m} were determined by fitting the reaction rates at various dCTP concentrations to the Michaelis-Menten equation. Each experiment was repeated at least three times to measure the average of the kinetic results. The efficiency of nucleotide insertion was calculated as $k_{\text{cat}}/K_{\text{m}}$. The relative frequency of dNTP incorporation opposite the templating guanine was determined as $f = (k_{\text{cat}}/K_{\text{m}})_{[\text{dNTP:dG}]} / (k_{\text{cat}}/K_{\text{m}})_{[\text{dCTP:dG}]}$.

Co-crystallization and structure determination of binary and ternary pol β -DNA complexes

To obtain the binary complex of Asn279Ala pol β -DNA complex, pol β was incubated with a single-nucleotide gapped DNA containing a 16-mer template (5'-CCGAC[G or O6-methylguanine]GCGCATCAGC-3'), a complementary 10-mer upstream primer (5'-GCTGATGCGC-3'), and a 5-mer downstream primer (5'-pGTCCG-3'). Subsequently, a 10-fold molar excess of nonhydrolyzable dTMPNPP or dCMPNPP (Jena Bioscience) was added to the binary complex. Ternary Asn279Ala pol β -DNA complex co-crystals with nonhydrolyzable dNTP analogs paired with templating G or O6-methylguanine (O6MeG) were grown in a buffer solution containing 50 mM imidazole, pH 7.5, 14–23% PEG3400, and 350 mM sodium acetate as described previously [19]. The diffraction data were collected at 100 K at the beamline 5.0.3 at the Advanced Light Source, Lawrence Berkeley National Laboratory. All diffraction data were processed using HKL2000. The structures were solved by molecular replacement using a ternary complex structure with a closed conformation (PDB ID 1BPY) as the search model [22]. The model was built using COOT and refined using Phoenix, and the figures were generated using PyMOL.

RESULTS

The Asn279Ala mutation of pol β modulates the catalytic efficiency of the enzyme

To assess the effect of the Asn279Ala mutation on catalytic efficiency, we first determined the kinetic parameters for single nucleotide incorporation opposite the templating dG by pol β in the presence of active-site Mg²⁺ or Mn²⁺ (Table 1). DNA containing a single-nucleotide gap and a 5'-phosphorylated downstream primer was used for the kinetic study because this type of DNA has been shown to be an ideal substrate for pol β -mediated

nucleotidyl transfer [37]. Mn^{2+} was chosen because this metal ion increased the misincorporation rate and promote the formation of a closed conformation [38].

A comparison of the kinetic parameters between Asn279Ala pol β and wild-type pol β indicated that the Asn279Ala mutation significantly affected catalytic efficiency. In the presence of Mg^{2+} , the Asn279Ala mutation yielded an ~3-fold increase in the catalytic efficiency for dG:dCTP (924.1 vs. 353.3 $s^{-1}\mu M^{-1}$) compared with wild-type pol β . This result contrasted with previous work in which an Asn279Ala pol β mutant showed a 17-fold decrease in catalytic efficiency for correct base pairing when non-gapped DNA was used [36], thus highlighting the substrate dependence of the catalytic efficiency in Asn279Ala pol β . The enhanced catalytic efficiency for correct nucleotide insertion resulted from an increased k_{cat} rather than a decreased K_m . The Asn279Ala mutation decreased the insertion efficiency for dG:dTTP ~2-fold (0.021 vs. 0.049 $s^{-1}\mu M^{-1}$) compared with the wild-type enzyme, owing to an increased K_m for the incorrect nucleotide (110.9 vs. 56.1 μM). The increased catalytic efficiency for correct nucleotide insertion and the decreased efficiency for incorrect insertion decreased the dG:dTTP misincorporation frequency (F_{ins}) ~6-fold (2.3×10^{-5} vs. 1.4×10^{-4}) compared with wild-type pol β (Table 1).

Published reversion-frequency studies with Asn279Ala and wild-type pol β have shown that the mutation increases the replication fidelity ~5-fold [33], which is consistent with our observed 6-fold decrease (2.3×10^{-5} vs. 1.4×10^{-4}) in the error rate for dG:dTTP incorporation by Asn279Ala pol β . Together, the results suggest that the increased replication fidelity of the Asn279Ala pol β mutant [33] is a consequence of increased catalytic efficiency for correct insertion and reduced incorrect insertion efficiency. The decreased misincorporation rate for Asn279Ala pol β is interesting because previous studies of pol β with mutations at Tyr265, Ile260, Lys289, Lys280, and Arg283 have shown an increase in the misincorporation rate [24, 33, 36, 39–41].

The effect of metal cofactor on promutagenic replication of pol β

When Mn^{2+} was used as metal cofactor in place of Mg^{2+} , the Asn279Ala mutation increased the dG:dTTP misincorporation frequency 3-fold (4.4×10^{-3} vs. 1.6×10^{-2} , Table 1), exhibiting the opposite phenotype. In the case of wild-type pol β , replacing Mg^{2+} with Mn^{2+} did not significantly change the correct nucleotide insertion efficiency (353.3 vs. 383.7 $s^{-1}\mu M^{-1}$) but increased the efficiency for incorrect insertion by 34-fold (0.049 vs. 1.71 $s^{-1}\mu M^{-1}$), thereby greatly promoting misinsertion. The Mn^{2+} -mediated increase in misincorporation rate suggests significant conformational differences between the Mg^{2+} - and Mn^{2+} -bound mismatched (dG:dTTP) complexes. In the case of the Asn279Ala mutant, the use of Mn^{2+} decreased the efficiency of correct insertion by 12-fold (924.1 vs. 80.8 $s^{-1}\mu M^{-1}$) but increased the efficiency of incorrect nucleotide insertion by 60-fold (0.021 vs. 1.29 $s^{-1}\mu M^{-1}$), thereby dramatically (720-fold) increasing the misincorporation rate. The Mn^{2+} -induced decrease in correct insertion efficiency (dG:dCTP) mainly results from decreased k_{cat} (730 vs. 31.5).

The Asn279Ala mutation alters the protein and base-pair conformations in G:T mismatches

We solved the ternary structure of Asn279Ala pol β in complex with the dG:dTTP mismatch and Mg²⁺ to investigate the effects of Asn279-mediated minor groove interactions on the conformation of a G:T base pair. The Asn279Ala pol β -G:T-Mg²⁺ ternary structure, which was refined to 2.51 Å, shows that the Asn279Ala mutation significantly alters the protein and DNA conformations (Figure 2D). The mutant-G:T-Mg²⁺ ternary structure shows a semi-closed conformation, a coplanar G:T base-pair conformation, and one active-site metal ion (Figure 2A). This structure differs from the published wild-type pol β -G:T-Mg²⁺ ternary structure, which exhibits an open conformation, a pseudo-propeller twist base-pair conformation and two active-site metal ions (Figure 2D). The templating dG forms a wobble base pair with the incoming dTTP* (Figure 2C). The O6 and N1 of the templating dG engage in hydrogen bonding interactions with N3 and O2 of dTTP*, respectively (Figure 2B). Notably, Tyr271 forms a bifurcated hydrogen bond with N2 of the templating dG and O2 of the incoming dTTP*. The formation of a hydrogen bond network among the templating dG, incoming dTTP*, and Tyr271 stabilizes a wobble dG:dTTP base-pair conformation and a semi-closed conformation.

Minor groove contact by Asn279 is required for the formation of a Watson-Crick-like dG:dTTP base-pair conformation

The Asn279Ala pol β -G:T-Mg²⁺ structure with a semi-closed conformation does not represent a catalytically competent state. To gain structural insights into the pre-chemistry state of the Asn279Ala pol β -G:T complex, we determined the ternary structure of Asn279Ala pol β in complex with a dG:dTTP mismatch and Mn²⁺ (Figure 3). Using Mn²⁺ in place of Mg²⁺ has been shown to promote the formation of a closed pol β conformation [19,38]. The Asn279Ala pol β -G:T-Mn²⁺ structure, which was solved to 2.75 Å resolution, shows a closed conformation and two active-site metal ions rather than the semi-closed conformation and one active-site metal ion that were observed in the Asn279Ala pol β -G:T-Mg²⁺ structure. This result indicates that substituting Mn²⁺ for Mg²⁺ facilitated the formation of a closed conformation in addition to catalytic metal ion coordination (Figures 3B and 3D). The large conformational difference between the Asn279Ala pol β -G:T-Mn²⁺ and the Asn279Ala pol β -G:T-Mg²⁺ complexes is consistent with the ~60-fold difference in catalytic efficiency (Table 1). The overall structure of the Asn279Ala pol β -G:T-Mn²⁺ complex is essentially identical to that of the wild-type pol β -G:T-Mn²⁺ structure (RMSD = 0.356 Å, Figure 3E).

The Asn279Ala mutation significantly affects the base-pair conformation of dG:dTTP in the enzyme active site (Figures 3C and 3F). The major structural difference between the Asn279Ala pol β -G:T-Mn²⁺ and wild-type pol β -G:T-Mn²⁺ complexes is the base-pair conformation of the G:T mismatch. While the published wild-type pol β -G:T-Mn²⁺ structure shows a Watson-Crick-like G:T conformation in the active site (Figure 1), the Asn279Ala pol β -G:T-Mn²⁺ structure shows a wobble G:T base-pair conformation, thus signifying that the formation of a Watson-Crick-like G:T conformation requires hydrogen bonding between the O2 of dTTP and the NH₂ of Asn279. The hydrogen bonding between the O2 of dTTP and NH₂ of Asn279 appears to increase the population of the enol tautomeric or ionized

species of dTTP (Figure 1), thereby promoting the formation of a Watson-Crick-like G:T base pair.

The presence of a Watson-Crick-like G:T base pair in the active site of wild-type pol β , as opposed to the wobble base pair in the mutant, suggests that the catalytic pocket is ideally equipped to accommodate Watson-Crick or Watson-Crick-like conformations in a closed conformation. This result contrasts with the characteristics of other DNA polymerases, such as the high-fidelity DNA polymerases BF and RB69, which allow wobble-mismatched base pairs in a closed conformation [11,12]. Differences in the mismatched base-pair conformations among DNA polymerases may result from differences in the geometric selection mechanisms employed by these enzymes. While BF and RB69 enforce the A-DNA conformation in their catalytic sites, pol β promotes the B-DNA conformation, which is narrower than A-DNA [31]. The strict geometric selection of pol β may be largely influenced by the minor groove interactions of Asn279. Disrupting these interactions through the Asn279Ala mutation appears to mitigate the geometric restraints imposed by the enzyme active site, thereby allowing a relaxed wobble G:T base pair.

The Asn279Ala mutation does not affect the protein and nascent base-pair conformations for correct nucleotide insertion

To evaluate whether the minor groove hydrogen bonding by Asn279 also affects protein and base-pair conformations during correct nucleotide insertion, we determined the crystal structure of Asn279Ala pol β in complex with nonhydrolyzable dCMPNPP (hereafter dCTP*) opposite the templating dG in the presence of Mg²⁺. The Asn279Ala pol β -G:C ternary structure, which was refined to 2.7 Å, shows a closed conformation and a coplanar Watson-Crick base pair (Figure 4). The protein and base-pair conformations are essentially indistinguishable from those observed in the published pol β ternary structures with correct insertions, thus indicating that Asn279 has a minimal effect on the pol β ternary complex with correct base pairing. The Asn279Ala pol β -G:C structure indicates that the minor groove contact by Asn279 is not required for the formation of a closed pol β conformation.

Minor groove hydrogen bonding by Asn279 is not required for the formation of the Watson-Crick-like O6-methylguanine:dTTP base pair

The observation of a Watson-Crick-like G:T base pair in the presence of the minor groove contact with the incoming nucleotide in the pol β active site and the formation of a wobble G:T base pair in the absence of the minor groove contact strongly suggests that the formation of Watson-Crick-like G:T base pairs involves the ionization or enol tautomerization of dTTP, which is promoted by the minor groove hydrogen bonding with Asn279. To evaluate the possible involvement of ionized or tautomeric bases in Watson-Crick-like G:T base pairing, we determined the crystal structure of pol β in complex with O6-methylguanine (O6MeG) and incoming dTTP in the presence of active-site Mn²⁺. We chose O6MeG because it is a close analog of the enol tautomeric species of guanine and because an analog of the tautomeric species of dTTP was not commercially available. Active-site Mn²⁺ was used because this metal ion is known to facilitate the formation of a closed pol β conformation [20]. We expected O6MeG and dTTP to form a Watson-Crick-like base pair in the absence of the minor groove contact with dTTP. The cocrystal structure of

Asn279Ala pol β in complex with templating O6MeG and incoming dTTP* in the presence of Mn²⁺ was refined to 2.44 Å.

The structure of the Asn279Ala pol β -O6MeG:dTTP* complex shows a closed conformation and a Watson-Crick-like O6MeG:dTTP* base pair, which is virtually identical to that of the published wild-type pol β -O6MeG:dTTP* complex [20] (Figure 5A). The N1 and N2 positions of O6MeG form hydrogen bonds with the N3 and O2 positions of dTTP, respectively (Figures 5B and 5C). The O6-methyl group does not form a C-H...O hydrogen bond with O4 of dTTP. The base-pair geometry of O6MeG:dTTP* in the mutant pol β structure is indistinguishable from that in the wild-type pol β structure (Figure 5D). This result contrasts with the large conformational difference that was observed in the dG:dTTP base-pairing geometry of the pol β -G:T and Asn279Ala pol β -G:T complexes [20]. Unlike the Watson-Crick-like dG:dTTP base pair, the formation of the Watson-Crick-like O6MeG:dTTP base pair in the pol β active site does not require a minor groove contact with Asn279, probably because O6MeG, which is structurally analogous to the enol tautomeric form of guanine, can form a Watson-Crick-like base pair with the keto tautomer of dTTP (Figure 1). The formation of a Watson-Crick-like O6MeG:dTTP* base pair in the absence of the minor groove contact thus supports the hypothesis that the ionized or enol tautomeric species of guanine or thymine is involved in Watson-Crick-like G:T base pairing.

DISCUSSION

The effect of Asn279Ala mutation and Mn²⁺ on pol β and base pair conformations

Our structural studies reveal that the Asn279Ala mutation and metal cofactor replacement significantly alters conformations of pol β and dG:dTTP base pair (Figure 6). In the presence of Mg²⁺ and wild-type pol β , the nascent dG:dTTP base pair adopts a staggered conformation with an interbase hydrogen bond (between N1 of dG and O4 of dTTP) and the protein is in an open conformation. When Mn²⁺ is used as a cofactor, the mismatched base pair takes on Watson-Crick-like conformation and the protein now assumes a closed conformation, which is structurally competent for nucleotide incorporation. The Mn²⁺-induced conformational change of pol β and dG:dTTP base pair is consistent with the 35-fold increase in catalytic efficiency (Table 1). The introduction of Asn279Ala of pol β in the presence of Mg²⁺ results in the formation of wobble base pair with two interbase hydrogen bonds (between dG and dTTP) and a semi-open protein conformation, where only nucleotide-binding metal ion is present. Under the combined influence of Asn279Ala mutation and Mn²⁺ cofactor, the nascent dG:dTTP remains in the same wobble base pair but the protein undergoes semi-open to closed conformational reorganization. When Mn²⁺ is used, the catalytic efficiencies of dG:dTTP insertion for wild-type and Asn279Ala pol β are similar (1.71 vs. 1.29) despite the difference in base pair conformation (Watson-Crick-like vs. wobble), indicating that protein conformation contributes more to the catalytic efficiency than the base pair conformation. Overall, these results clearly show that Asn279Ala mutation and Mn²⁺ exert significant impact on conformations of pol β and dG:dTTP mismatch.

Our results provide insight into the mechanisms of manganese mutagenesis. The trace element Mn²⁺ is used as a cofactor for wide range of cellular enzymes such as superoxide

dismutase. Although Mg^{2+} is physiologically relevant cofactor for DNA polymerases, other metal ions such as Mn^{2+} can substitute for Mg^{2+} at higher concentrations: The higher level of Mn^{2+} , which can be arisen by disruption of Mn^{2+} homeostasis, is toxic and causes higher mutagenicity and a central nervous system disorder. When Mg^{2+} is used as a cofactor, pol β misinserts dTTP opposite templating dG about every 7,000 nucleotides incorporated (Table 1). The utilization of Mn^{2+} cofactor for nucleotide incorporation facilitates mutagenic replication, making an error for about every 230 nucleotides incorporated (Table 1). This manganese-promoted mutagenesis is likely caused, in part, by stabilization of mismatched nascent base pair in the active site.

Minor groove interactions may facilitate the formation of Watson-Crick-like mismatched base pairs in DNA polymerase active sites

In the absence of protein contacts, mismatched base pairs with Watson-Crick-like geometry are energetically unfavorable and exist transiently in low abundance [13,42, 43,44]. The enol tautomer of guanine has been calculated to be ~one million-fold less abundant than the keto tautomer in water [45]. Thus, the observation of Watson-Crick-like mismatched base pairs in the active site of various DNA polymerases indicates that the microenvironments within the active site of DNA polymerases promote the formation of Watson-Crick-like mispairs. The wild-type and Asn279Ala pol β -G:T structures provide insights into the effect of minor groove interactions on mismatched base pair conformations (Figure 6). The formation of a Watson-Crick-like dG:dTTP base pair in the presence of the minor groove hydrogen bond donor Asn279 and a wobble dG:dTTP base pair in the presence of Asn279Ala suggest that the minor groove contact with an incoming nucleotide promotes Watson-Crick-like G:T mispairs. In principle, Watson-Crick-like dG:dTTP base pairs can occur through either tautomerization or ionization. Hydrogen bonding to the minor groove edge of an incoming nucleotide may increase the population of the tautomeric or ionic form of the nucleotide, thereby facilitating the formation of Watson-Crick-like mismatches.

Minor groove interactions between DNA polymerase and an incoming nucleotide may contribute to the formation of a wide variety of mispair conformations at the insertion site. To date, mismatches with a Watson-Crick-like geometry have been observed in the structures of A- and X-family DNA polymerases. In the case of the A-family DNA polymerase BF, dA:dCTP adopts a wobble conformation in the absence of minor groove contact with the incoming nucleotide. In contrast, the same base pair adopts a Watson-Crick-like conformation in the presence of Mn^{2+} -induced minor groove contact [11], illustrating the effect of minor groove interactions on mispair conformations. In the case of the X-family DNA polymerase pol λ , dT:dGTP adopts a Watson-Crick-like conformation in the presence of minor groove contact with the incoming nucleotide [18]. The B-family DNA polymerase RB69, which does not engage in minor groove hydrogen bonding with the incoming nucleotide, accommodates wobble conformations for both dG:dTTP and dT:dGTP base pairs [12]. These observations highlight that minor groove contact with the incoming nucleotide may significantly influence mismatched base pair conformations.

A highly stringent geometric selection mechanism for pol β

Although pol β lacks an intrinsic proofreading exonuclease activity, this enzyme possesses several characteristics of high-fidelity DNA polymerases, such as an open-to-closed conformational change and a strict geometric selection mechanism [31]. Pol β uses a rigorous geometric selection mechanism to discriminate between correct and incorrect base pairs within the nascent base-pair binding pocket. Although many DNA polymerases, including the A-, B-, and Y-family DNA polymerases, allow a wobble G:T base pair with two hydrogen bonds in the incipient base-pair site, the X-family DNA polymerase pol β does not allow such base pair. Instead, this enzyme enforces a staggered G:T base pair with a hydrogen bond and adopts an open conformation in the presence of active-site Mg $^{2+}$. When the mutagenic Mn $^{2+}$ is used in place of Mg $^{2+}$, the enzyme induces the formation of a Watson-Crick-like G:T base pair and adopts a closed conformation [19]. In the case of A:C mismatches, even the presence of Mn $^{2+}$ does not induce the formation of a Watson-Crick-like mispair, and the protein adopts a semi-open conformation [19]. The observed conformational differences indicate that in the closed conformation, the nascent base-pair binding pocket of pol β is highly stringent and strongly discriminates between base pairs with Watson-Crick and non-Watson-Crick geometry. The rigid geometric constraints imposed by the pol β active site may enforce the B-DNA conformation rather than the A-DNA conformation, with a widened minor groove within the active site, thereby deterring the formation of a relaxed, non-Watson-Crick conformation in the pocket.

The minor groove contacts of pol β with the incoming nucleotide and templating base may contribute to the strict geometric selection of this enzyme. A DNA polymerase that engages in minor groove contacts with both the incoming nucleotide and templating base is rare. The A-, C- and Y-family DNA polymerases (e.g., BF, PolC, Dpo4, and pol η) make contact with only the minor groove edge of incoming nucleotide. On the other hand, the B-family DNA polymerase RB69 probes the minor groove edges of the replicating base pair via only Van der Waals interactions. In the case of the X-family polymerases pol β and pol λ , the minor groove edges of the incoming nucleotide and templating base are contacted by Asn and Arg residues, respectively. Pol β , which possesses some characteristics of high-fidelity DNA polymerases but lacks an intrinsic proofreading function, appears to increase its replication fidelity by extensively probing minor groove edges and imposing tight geometric constraints at the insertion site. The Asn279Ala-mediated abrogation of the minor groove contact with the incoming nucleotide alleviated the strict geometric selection of pol β , thereby allowing a wobble G:T base pair at the insertion site. This result highlights the effect of minor groove contact on the geometric selection mechanism and implies that perturbing minor groove interactions in other DNA polymerases may alter their geometric selection mechanisms.

The tight geometric constraints of the pol β active site may enable this enzyme to display a distinct mismatch discrimination strategy. First, pol β strongly discriminates between correct and incorrect base pairs when it is in an open conformation. In particular, in the open-conformation state, pol β does not allow coplanar wobble G:T and A:C base pairs. Instead, the enzyme induces staggered conformations for G:T and A:C mispairs. Second, pol β allows only Watson-Crick and Watson-Crick-like geometries when in the closed conformation, thereby precluding the formation of mispairs with non-Watson-Crick geometry at the

insertion site. In the closed conformation induced by active site Mn^{2+} , the G:T mismatch evades the mismatch discrimination of pol β by adopting a Watson-Crick-like geometry, but other mismatches cannot readily escape such discrimination. This result partially explains why the G:T mismatch is the major (~60%) spontaneous replication error made by pol β [46]. Third, the nascent base-pair binding pocket of pol β is highly sensitive to structural and electronic perturbations caused by nucleobase lesions, particularly alkylated lesions. For example, pol β incorporates dCTP opposite N7-methylguanine 300-fold less efficiently than opposite guanine [47]. In addition, although N7-methylguanine:T forms a Watson-Crick-like conformation in the absence of protein contact [48], pol β does not permit the formation of a Watson-Crick-like N7-methylguanine:T base pair at the insertion site and induces a staggered base-pair conformation [47]. The enzyme's active site architecture appears to sense the steric and/or electronic perturbation of the N7-methylguanine moiety. Fourth, while the A-family polymerase BF accommodates a coplanar relaxed O6-methylguanine:C base pair in a closed conformation [49], pol β enforces a staggered O6-methylguanine:C base pair and adopts an open conformation, even in the presence of the misincorporation-promoting Mn^{2+} [20]. In the case of the O6-methylguanine:T base pair, pol β fails to discriminate the mismatch in the closed conformation, allowing a Watson-Crick-like conformation [20]. These results highlight differences in the mismatch discrimination strategies used by DNA polymerases.

The effect of minor groove interaction on replication fidelity and catalytic efficiency

Crystal structures and studies with nucleoside analogs have indicated that minor groove interactions between DNA polymerases and the N3 of purines or O2 of pyrimidines may play important roles in probing for correct base pairs in the nascent base-pair binding pocket [23, 27]. In the case of pol β , minor groove contacts with the incoming nucleotide and templating base exert opposing effects on replication fidelity and catalytic efficiency. An alanine substitution at Arg283, which interacts with the minor groove edge of the incoming nucleotide, dramatically decreases both replication fidelity (~2,000-fold) and catalytic efficiency (~30,000-fold) [33]. On the other hand, substituting alanine for Asn279 reduces misinsertion efficiency and increases correct insertion efficiency. The abrogation of the minor groove contact with the incoming nucleotide by the Asn279Ala mutation increases the k_{cat} (~3-fold) for correct nucleotide incorporation but had minimal effects on K_m . The Asn279Ala mutation of pol β has been shown to increase the replication fidelity ~5-fold [33].

Asn279 of pol β has been shown to stimulate 8-oxo-dGTP incorporation opposite templating dA by stabilizing *syn* conformation of the oxidized nucleotide [50, 51]. While wild-type pol β more efficiently incorporates 8-oxo-dGTP opposite dA over dC (24:1), Asn279Ala mutation leads to the preferential 8-oxo-dGTP insertion opposite dC over dA (14:1), highlighting that minor groove interactions of Asn279 with *syn*-8-oxo-dGTP facilitates mutagenic replication.

An X-family DNA polymerase η from *Thermus thermophilus* (*Tth* PolX) contains Ser266, which is equivalent to Asn279 of pol β [52]. Mutation of Ser266 of *Tth* PolX into Asn, thereby mimicking pol β , greatly (~20-fold) accelerates 8-oxo-dGTP opposite dA compared to the wild-type enzyme, suggesting that the minor groove interaction at the nucleotide-

binding site promotes mutagenic 8-oxo-dGTP insertion opposite dA in the active site of the X-family DNA polymerases.

Minor groove interactions with the incoming nucleotide are observed in most DNA polymerases, including A-, C-, X-, and Y-family DNA polymerases. In the case of the Y-family DNA polymerase η , removal of the minor groove interaction with 3-deaza-dGTP decreases the catalytic efficiency for correct insertion ~120-fold and increases incorrect insertion ~12-fold, thereby reducing replication fidelity ~1,800-fold, thus illustrating the effect of the minor groove interaction with the incoming nucleotide on fidelity [26]. Determining whether the elimination or addition of minor groove contacts at the insertion sites of DNA polymerases significantly influences replication fidelity and mismatched base-pair conformations would be of interest. Such studies would provide insights into how DNA polymerases utilize insertion site-minor groove interactions to perform their functions in replication, DNA repair and translesion synthesis.

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Abbreviations

BF	<i>Bacillus stearothermophilus</i> DNA polymerase I fragment
O6MeG	O6-Methylguanine
Tth	<i>Thermus thermophilus</i>

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Figure 1.
Base-pairing patterns of G:T mismatches. G:T mismatches with wobble and Watson-Crick-like base pairing. Mismatched base pairs with a Watson-Crick-like geometry were observed in the active sites of wild-type pol β , pol λ and BF (11, 18, 19).

Figure 2.

Ternary structure of Asn279Ala polβ in complex with the dG:dTTP* mismatch in the presence of active-site Mg²⁺ (PDB ID 4Z6D). (A) Overall structure of Asn279Ala polβ with the templating dG paired with nonhydrolyzable dTMPNPP (dTTP*). The incoming dTTP* is shown in blue. The templating dG is shown in magenta, whereas the rest of templating strand bases are shown in yellow. The upstream and downstream primers are shown in orange. The α-helix N is shown in red. (B) Close-up view of the active site of the Asn279Ala dG:dTTP-Mg²⁺ ternary structure. The catalytic aspartates and amino acid residues involved in minor groove edge recognition are indicated. (C) Base pairing between the templating dG and the incoming dTTP*. The C1' distance and λ angles are shown. A 2F_o-F_c map contoured at 1σ around dG:dTTP* is shown. Distances for O6(dTTP*)-O6(dG), N1(dTTP*)-N1(dG), and O2(dTTP*)-N2(dG) are 3.8 Å, 3.9 Å, and 3.9 Å, respectively, indicating a wobble dG:dTTP* base pair. (D) Comparison of the active site of the Asn279Ala polβ-dG:dTTP*-Mg²⁺ structure (shown in green) with that of the published wild-type polβ-dG:dTTP*-Mg²⁺ structure (shown in blue, PDB ID 4PGQ) (19).

Figure 3.

Ternary structure of Asn279Ala pol β with the dG:dTTP* mismatch and active-site Mn $^{2+}$ (PDB ID 4Z6E). (A) Overall structure of the Asn279Ala pol β -dG:dTTP*-Mn $^{2+}$ ternary complex. Pol β adopts a closed conformation. (B) Close-up view of the active site of the Asn279Ala pol β -dG:dTTP*-Mn $^{2+}$ ternary structure. Mn $^{2+}$ ions are shown as cyan spheres. (C) dG:dTTP* base pairing observed in the Asn279Ala pol β active site. Distances for N1(dTTP*)-O6(dG) and O2(dTTP*)-N1(dG) are indicated by dashed lines. Distances for O6(dTTP*)-O6(dG), N1(dTTP*)-N1(dG), and O2(dTTP*)-N2(dG) are 3.8Å, 3.4Å, and 3.1Å, respectively. The λ angles for dG and dTTP* are 45.8° and 65.8°, respectively, indicating a wobble base pair geometry. A $2F_o - F_c$ map contoured at 1σ around dG:dTTP* is shown. (D) Comparison of the Asn279Ala pol β -dG:dTTP*-Mg $^{2+}$ (green) and the Asn279Ala pol β -dG:dTTP*-Mn $^{2+}$ (purple) structures. (E) Published ternary structure of wild-type pol β with the dG:dTTP* mismatch and active-site Mn $^{2+}$ (PDB ID 4PGX). Hydrogen bond contacts by Asn279 are indicated by dotted lines. O2 of dTTP* and the backbone carbonyl oxygen of Tyr271 engage in hydrogen bond interactions with Asn279. (F) Base pairing properties of dG:dTTP* in the published structure with the wild-type pol β and Mn $^{2+}$ (PDB ID 4PGX)¹⁹. The λ angles for dG and dTTP* are 50.1° and 56.2°, respectively, indicating Watson-Crick-like base pair geometry. A $2F_o - F_c$ map contoured at

1σ around dG:dTTP* is shown. Distances of N1(dTTP*)-O6(dG) and O2(dTTP*)-N1(dG) are 3.1 Å and 3.2 Å, respectively. (G) Comparison of the Asn279Ala pol β -dG:dTTP*-Mn²⁺ (green) and the pol β -dG:dTTP*-Mn²⁺ (black) structures. Both structures contain two Mn²⁺ ions at the insertion site. (H). Comparison of dG:dTTP base pairing in the active site of wild-type pol β (black) and Asn279Ala pol β (green). Hydrogen bonds are indicated by dashed lines.

Figure 4.

Ternary structure of Asn279Ala polβ with dG:dCTP* (PDB ID 4Z6C) in the presence of active-site Mg²⁺. (A) Overall structure of the Asn279Ala polβ-dG:dCTP* complex. (B) Close-up view of the active site of the Asn279Ala polβ-dG:dCTP* complex. The α-helix N adopts a closed conformation. (C) Base pairing of dG:dCTP* in the active site of Asn279Ala polβ. A 2F_o-F_c map contoured at 1σ around dG:dCTP* is shown. (D) Comparison of the active site structures of the Asn279Ala polβ-dG:dCTP*-Mg²⁺ (green) and wild-type polβ-dG:dTTP*-Mn²⁺ (blue) complexes.

Figure 5.

Structure of Asn279Ala polβ with the O6MeG:dTTP* mismatch in the presence of active site Mn²⁺ (PDB ID 4Z6F). (A) Overall structure of the Asn279Ala polβ-O6MeG:dTTP* complex. (B) Closeup view of the active site of the Asn279Ala polβ-O6MeG:dTTP* complex. The α-helix N adopts a closed conformation, and O6MeG:dTTP* adopts coplanar conformation. (C) Base pairing properties of O6MeG:dTTP* in the active site of Asn279Ala polβ. O6MeG:dTTP* forms a Watson-Crick-like base pair with two hydrogen bonds. A 2F_o-F_c map contoured at 1σ around O6MeG:dTTP* is shown. (D) Comparison of the active site structures of the Asn279Ala polβ-O6MeG:dTTP* (shown in green) and published wild-type polβ-O6MeG:dTTP* (shown in purple, PDB ID 4NXZ) complexes (20).

Figure 6.

The effect of Asn279Ala mutation and metal cofactor substitution on the conformations of polβ and base pair. Key hydrogen bonds involving Asn279 and base pairs are indicated in dotted lines. Mg²⁺ and Mn²⁺ are shown in green and cyan spheres, respectively.

Table 1

Steady-state kinetics for nucleotide incorporation opposite templating dG by wild-type or Asn279Ala pol β .

	template-dNTP (metal ion)	K_m (μM)	k_{cat} ($10^{-3}s^{-1}$)	k_{cat}/K_m ($10^{-3}s^{-1}\mu M^{-1}$)	F_{ins}
wild type polβ	G-C (Mg^{2+})	0.6 ± 0.10	212.0 ± 19.87	353.3	1
	G-T (Mg^{2+})	56.1 ± 4.59	2.77 ± 0.35	0.049	1.4×10^{-4}
	G-C (Mn^{2+})	0.08 ± 0.01	30.3 ± 1.51	383.7	1
	G-T (Mn^{2+})	11.2 ± 0.47	19.1 ± 0.78	1.71	4.4×10^{-3}
Asn279Ala polβ	G-C (Mg^{2+})	0.79 ± 0.04	730 ± 17.52	924.1	1
	G-T (Mg^{2+})	110.9 ± 5.29	2.38 ± 0.17	0.021	2.3×10^{-5}
	G-C (Mn^{2+})	0.39 ± 0.03	31.52 ± 0.90	80.8	1
	G-T (Mn^{2+})	7.93 ± 0.38	10.28 ± 0.55	1.29	1.6×10^{-2}

Table 2

Data collection and refinement statistics.

PDB code	Asn279Ala dG:dCTP* Mg ²⁺ (4Z6C)	Asn279Ala dG:dTTP* Mg ²⁺ (4Z6D)	Asn279Ala dG:dTTP* Mn ²⁺ (4Z6E)	Asn279Ala O6MeG:dTTP* Mn ²⁺ (4Z6F)
Data Collection				
Detector	Rigaku RAxis IV++ imaging plate area detector	Rigaku RAxis IV++ imaging plate area detector	Rigaku RAxis IV++ imaging plate area detector	3 × 3 CCD array (ADSC Q315R)
Wavelength	1.5418	1.5418	1.5418	0.97648
Space Group	P2 ₁	P2 ₁	P2 ₁	P2 ₁
Cell constants				
a (Å)	48.180	54.283	50.562	50.753
b	79.274	79.382	79.567	79.558
c	54.372	54.859	55.143	55.204
α (°)	90.00	90.00	90.00	90.00
β	105.96	110.18	107.64	107.70
γ	90.00	90.00	90.00	90.00
Resolution (Å) ^a	20-2.68 (2.73-2.68)	20-2.51 (2.55-2.51)	20-2.75 (2.80-2.75)	20-2.44 (2.48-2.44)
R _{merge} ^b (%)	0.110 (0.380)	0.138 (0.525)	0.111 (0.385)	0.125 (0.478)
<I/σ>	13.3 (2.22)	12.7 (2.04)	13.8 (2.46)	13.3 (2.00)
Completeness (%)	99.8 (98.4)	99.9 (99.7)	99.3 (93.9)	99.6 (96.9)
Redundancy	4.0 (3.4)	4.4 (4.2)	4.4 (3.9)	4.6 (4.1)
Refinement				
R _{work} ^c /R _{free} ^d (%)	20.4/27.2	19.3/27.1	18.1/24.8	19.5/24.8
Unique reflections	11111	15005	10785	15517
Mean B factor (Å ²)				
Protein	31.4	32.0	31.1	29.6
Ligand	30.3	38.6	27.9	28.2
Solvent	32.6	29.7	30.0	29.8
Ramachandran plot				
Most favored (%)	96.9	97.8	96.0	97.5
Additional allowed (%)	3.1	2.2	4.0	2.5
RMSD				
Bond lengths (Å)	0.005	0.004	0.004	0.004
Bond angles (°)	1.178	1.063	1.034	1.065

^aValues in parentheses are for the highest resolution shell.^b $R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum I$ where I is the integrated intensity of a given reflection.^c $R_{\text{work}} = \sum |F(\text{obs}) - F(\text{calc})| / \sum F(\text{obs})$.

$d_{R_{\text{free}}} = \Sigma |F(\text{obs}) - F(\text{calc})| / \Sigma F(\text{obs})$, calculated using 5% of the data.

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