Unexpected behavior of DNA polymerase Mu opposite template 8-oxo-7,8-dihydro-2′-guanosine

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INTRODUCTION

Genomic DNA is susceptible to damage caused by reactive oxygen species, which are generated as byproducts of normal cellular metabolism, or from exogenous sources like ionizing radiation (1). Examples of DNA damage include removal/modification of sugar/base moieties and phosphodiester bond breakage (2), which have been associated with human cancers and diseases if left unrepaired (3). Repair of DNA double-strand breaks (DSBs), the most cytotoxic forms of DNA damage (4), can be complicated by the presence of clusters of missing bases or oxidized base lesions near break sites (5,6). DNA repair enzymes attempting to remove damaged bases are impeded by the proximity of DNA ends, and ligation of the ends is likewise inhibited by the presence of the damaged bases (7). Given the decreased repair efficiency of complex DSBs, these lesions have been shown to linger up to 19 hours after irradiation (8–10). Lesion persistence has been associated with biological consequences of radiation exposure, leading to loss of genetic information at break sites and chromosomal rearrangements/translocations (11).

Nonhomologous end-joining (NHEJ) is the favored pathway for DSB repair in nonreplicating cells, or during G1 phase of the cell cycle (12). In this pathway, broken DNA ends are recognized and bound by the Ku70/80 heterodimer in conjunction with the DNA-dependent protein kinase catalytic subunit. Additional processing factors, including the DNA Ligase IV/XRCC4 complex, XLF, the Artemis nuclease and gap-filling polymerases are recruited to process the ends, depending on their sequence and structure (13). The Family X polymerases (Pol) λ, μ, and terminal deoxynucleotidyl transferase (TdT) have been shown to associate with the NHEJ complex via their N-terminal BRCT domains and play specific roles in resolving DSBs. Pols λ and μ participate in classical NHEJ of DNA double-strand breaks, and also play critical roles in V(D)J recombination, a specialized pathway that relies on NHEJ for assembly of mature antigen-specific receptor genes (14,15). TdT, a primarily template-independent polymerase, contributes to sequence diversity for immunoglobulin gene maturation during V(D)J recombination (16).

8-oxo-7,8-dihydro-2′-guanosine (8OG) represents a unique threat to genomic stability and has been identified as the predominant DNA lesion subsequent to ionizing radiation (17). The 8OG base can exist in either the anti or syn conformation, correctly pairing with cytidine or mispairing with adenine, respectively—a characteristic known as dual coding potential (18,19). If unrepaired, mutagenic insertion of adenine nucleotides opposite 8OG can result in accumulation of guanine to thymine transversion events (20,21).
Because 8OG lesions populate the DNA damage clusters near complex DSB ends (5), the NHEJ repair machinery is likely to encounter such substrates in the course of its physiological function. There is evidence that the ligation step in NHEJ is unusually tolerant of mispairs and lesions at strand break termini, including 8OG (22,23). Much less is known about the impact of damage on polymerase activity during NHEJ, however. We therefore characterized behavior of Pol μ when encountering a template 8OG in the context of a DNA single-strand break, using *in vitro* steady-state kinetic assays. These results show that, in contrast to Pols β or λ, Pol μ exhibits a strong preference for mutagenic insertion opposite the template 8OG. Cellular NHEJ assays with double-strand break substrates containing a template 8OG demonstrate that endogenous Pol μ can engage this type of complex DSB, and commonly fills the gap with adenine nucleotides. The filled product can then be ligated by DNA LiggIV during NHEJ. We present an extensive characterization of 8OG bypass by Pol μ using X-ray crystallography, illustrating that 8OG is easily accommodated in the Pol μ active site in either the *syn* or *anti* conformations, with no apparent distortion of either the protein or the DNA substrates, in contrast with similar structures of Family X siblings Pols β or λ (24–26). Using these structures, we identified an active site residue (Gln441) unique to Pol μ that contributes to its strong mutagenic preference for adenine insertion opposite the template 8OG. These results indicate that Pol μ bypasses 8OG-containing substrates using either ribo- or deoxyriboadenosine nucleotides, suggesting such behavior could contribute to its physiological function during NHEJ.

**MATERIALS AND METHODS**

**Expression and purification of human Pol μ constructs**

Full-length (Met1-Ala494) or the catalytic domain (Pro132-Ala494) crystallization variant of human Pol μ (hPol μ Δ2, ΔPro398-Pro410 of Loop2 and replaced by Gly410) were expressed from the pGEXM vector in Rosetta2 (DE3) cells, as previously described (27). Individual amino acid substitution mutants were also generated in the full-length construct using site-directed mutagenesis and were purified similarly to the wildtype protein, lacking only the ion exchange step. All mutants behaved indistinguishably from wildtype during purification.

**Steady-state kinetics for single-nucleotide incorporation**

DNA substrates used for steady-state measurements of single nucleotide incorporation were prepared by hybridizing a 5′-Cy3-labeled 14-nucleotide primer (5′-GTCAGACTGAGTA-3′) and a 14-nucleotide 5′-phosphorylated downstream primer (5′-pGCCGGACGGACGGAG-3′) to a 29-mer template (5′-CTCCGTCGTCCGGCGTAAGTCTGACG-3′) containing either canonical guanine or 8OG (indicated in bold) to create a single-nucleotide (1-nt) gapped substrate. Reaction mixtures (20 μl) contained 50 mM Tris, pH 7.5, 1 mM dithiothreitol, 4% glycerol (w/v), 0.1 mg/ml bovine serum albumin, 5 mM MgCl2, 200 nM DNA and Pol μ wildtype or mutants Q441N or Q441A. The reactions were initiated by adding either dATP, ATP, dCTP or CTP and the mixtures were incubated at 37°C for 3-4 min. Detailed reaction conditions are indicated in Supplementary Table S1. The reactions were quenched by adding an equal volume of 99% formamide (v/v), 10 mM EDTA and 0.1% (w/v) bromophenol blue. The products were resolved on 16% (v/v) denaturing polyacrylamide gels, imaged using a Typhoon Imager (GE Healthcare), and quantitated using ImageQuant. The data were fit to the Michaelis–Menten equation using nonlinear regression in Kalaidagraph software version 3.6 (Synergy Software, http://www.synergy.com).

**Cell lines**

WT (C57BL/6) or Polm−/− murine embryonic fibroblast (MEF) cells (generously provided by Dr L. Blanco) were derived from E14.5d embryos and immortalized by introduction of SV40 large T-antigen as described previously (28). These lines were confirmed by qPCR to be free of mycoplasma contamination. MEF cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma), 100 U/ml penicillin, at 37°C and 5% CO2.

**Cell-based extrachromosomal nonhomologous end-joining assays**

Substrates were generated by ligation of ~15 bp double-stranded DNA caps (Supplementary Table S2) containing desired overhang sequences to a 280 bp core fragment digested with BssHII-HFv2 (New England Biolabs), with the caps in 3-fold excess. Substrates were then purified using a QiaQuick PCR purification kit (Qiagen), and resolved on a native acrylamide gel to ensure substrate preparations were free of detectable un- appended core or excess cap. Extrachromosomal DNA substrates (20 ng) were co-electroporated with pMAX-GFP plasmid (600 ng) into the MEF cell lines (2) transfection) using a 1350 V, 30 ms single pulse in a 10 μl volume (Neon, Invitrogen) as described previously (28). Each electroporation was reproduced in triplicate. Following transfection, cells were incubated at 37°C for 30 min in antibiotic-free DMEM media with 10% (v/v) FBS. Total cellular DNA was harvested from each sample using a QIAamp DNA mini kit (Qiagen). NHEJ repair products were recovered by PCR, using primers (5′-CTTACGGTTTTATCCCTGACTATCG-3′ and 5′-GCAAGGTAGCCAGTCTGAGATG-3′) to amplify head-to-tail junctions, and the fraction with different structures was assessed by digestion of the amplification products with restriction enzymes diagnostic for addition of a single A (NruI) and ligation, or addition of a single C (BssHII) and ligation. Digestion products were resolved on a 6% (w/v) non-denaturing polyacrylamide gel, visualized using a Typhoon Imager (GE Healthcare). The intensities of digested and undigested bands were quantified using ImageQuant.
Crystallization of hPol μ A2 with a 1-nt gapped DNA substrate

The following DNA oligonucleotides were used to generate the 1-nt gapped DNA substrate: template (5′-CGGGCGTACG-3′), containing either a canonical guanine or 8-oxo-7,8-dihydroguanine at the residue indicated in bold), upstream primer (5′-CGTA-3′), and a 5′-phosphorylated downstream primer (5′-pGCGC-3′), and were prepared for crystallization as previously described (27). The annealed DNA was mixed in a 4:1 molar excess to hPol μ A2 (~11 mg/ml) to generate the binary complex and then incubated on ice at 4°C for 1 h. Ternary complex crystals were obtained by addition of a nonhydrolyzable incoming nucleotide, followed by a further incubation at 4°C for 1 h. Details of the crystallization, cryoprotection, and soaking conditions for each reported structure are included in Supplementary Table S3. The crystals were flash frozen in liquid nitrogen and placed into a stream of nitrogen gas cooled to –173°C for data collection.

Structure solution and refinement

Data were collected on a rotating Cu anode x-ray generator and indexed, integrated, and scaled using either HKL2000 (29) or HKL3000 (30). The crystal structure of the 1-nt gapped pre-catalytic ternary complex and an incoming nonhydrolyzable analog (incoming dUpNpp opposite template dA, PDB ID code 4M04 (27)) was used as the starting model for refinement for all structures, and the same Rfree test reflections were used to avoid potential model bias. All structures were refined by iterative cycles of manual model building and refinement in COOT (31,32) and Phenix (33). TLS (Translation/Libration/Screw) vibrational motion refinement was used for all structures. Data collection and refinement statistics are listed in Supplementary Table S4. Ramachandran statistics were generated using MolProbity (34).

RESULTS

Pol μ exhibits a strong preference for inserting dA opposite a template 8OG

In order to explore the capabilities of Pol μ in lesion bypass of a template strand 8OG, we first determined its kinetic parameters for nucleotide insertion in vitro using a 1-nt gapped DNA substrate under steady-state conditions (Figure 1 and Supplementary Table S5). Under these reaction conditions, Pol μ efficiently incorporates dCTP opposite the canonical template dG. Selectivity against dATP misinsertion is robust, with greater than 2600-fold preference for dCTP versus dATP. However, with an oxidized 8OG template base, Pol μ incorporates dATP opposite the 8OG with nearly the same efficiency as dCTP opposite the canonical dG (1.5 × 10⁻² and 2.4 × 10⁻² s⁻¹ × μM⁻¹, respectively). In contrast, dCTP is inserted opposite 8OG with 50-fold lower preference than dATP. This difference in nucleotide incorporation preference appears to be largely driven by a 35-fold decrease in binding affinity for dCTP (Kd of 4.7 μM versus 167 μM opposite canonical versus oxidized guanosine bases, respectively), along with a slightly lower (~2-fold) catalytic rate (Supplementary Table S5). By comparison, the binding affinity for dATP opposite 8OG is only slightly (~2.5-fold) decreased, while the catalytic rate is roughly equivalent to that of dCTP opposite canonical dG.

Pol μ productively engages a complex DSB containing a template 8OG during NHEJ

A cell-based system was then employed to assess whether Pol μ repairs a complex DSB with a template 8OG proximal to the break with the same mutagenic dATP insertion preference as determined in the kinetic assays. For this assay, we used an extrachromosomal substrate with ends (Figure 2A) that align 2-bp of complementary sequence to generate single nucleotide gaps in both top and bottom strands. Ligation of the top strand requires synthesis opposite an 8OG template base, while ligation of the bottom strand is blocked due to the presence of a 5′-terminal abasic site (Tetahydrofuran; THF). Alternatively, ends can be joined through polymerase-independent mechanisms, by ligation after the overhang sequence has been degraded by a nuclease. This substrate was electroporated into a mouse embryonic fibroblast (MEF) cell line derived from wildtype mice, from a Polμ−/− littermate, or from a variant of the Polμ−/− line engineered to overexpress mouse Pol μ. The endogenous cellular NHEJ machinery was allowed to repair the break, and subsequent end-joining products were interrogated for repair fidelity. Gap-filling synthesis using adenosine nucleotide triphosphates opposite the template 8OG, followed by ligation, generates an NruI restriction site (+A product, Figure 2B), while gap-filling synthesis using cytosine nu-
Cleotides, followed by ligation, generates products that are instead cleaved by BssHII (+C product, Figure 2B). Products resistant to both diagnostic enzymes involve nucleolytic degradation of overhangs. Comparison of the restriction digestion of repair products from wildtype MEFs indicates that the gap in the DNA substrate is filled by contributions of both +A and +C products (Figure 2B and C). The majority of +A products are lost when using Polm −/− MEFs and +C products increase to compensate, presumably through action of Pol λ or another cellular polymerase. Overexpression of wildtype Pol μ in Polm −/− knockout cells was sufficient to generate even higher levels of +A product than observed even in wildtype MEFs. These results indicate that in these cells, gap-filling using adenosine nucleotides opposite the template 8OG is largely Pol μ-dependent, and that the filled products can be ligated by DNA Ligase IV during NHEJ (35). Moreover, the fraction of +C product slightly increased upon loss of Pol μ, implying that in wildtype cells, most if not all of Pol μ activity involved addition of adenosine nucleotides opposite 8OG.

Pol μ is structurally predisposed for insertion of adenosine nucleotides opposite template 8OG

To understand Pol μ’s predilection for inserting mutagenic adenine nucleotides opposite a template 8OG, the crystal structure of its catalytic domain (Pro132-Ala494) was first solved in binary complex with a 1-nt gapped DNA substrate containing an 8OG lesion on the template strand (Figure 3A). The binary complex with the 8OG-containing DNA substrate (PDB ID code: 6P1M, Supplementary Tables S3–S4) is observed in the ‘closed’ conformation and is nearly indistinguishable from the previously published binary complex structure (PDB ID code: 4LZG (27)) with an undamaged DNA substrate (RMSD of 0.15 Å over 303 Cα atoms). This suggests that the presence of the oxidized base does not overly disrupt either the protein or DNA substrate structure (Figure 3B). Within the hPol μ.A2 active site, the unpaired template 8OG base is observed solely in the syn conformation (Figure 3C), which is opposite to the canonical anti conformation seen in undamaged DNA (PDB ID code: 4LZG (27)). The 8OG’s predisposition toward the syn conformation in Pol μ could make binding an incoming adenosine nucleotide energetically more favorable than that of the correct cytosine nucleotide, and is consistent with the apparent binding affinities determined in the steady-state kinetic assays (Supplementary Table S5). The syn conformation observed in the Pol μ binary complex is similar to that observed for Pol λ (PDB ID code: 5IIO (25)), and is in direct contrast to the mixture of syn and anti conformations observed for Pol β (Figure 3D, PDB ID code: 3RJE (24)). It has been hypothesized that the O8 atom would clash with its 5'-phosphate oxygens in the anti conformation (24,25), which is consistent with the observed 2 Å shift of the backbone phosphate, and the rotation of its non-bridging oxygens, away from the 8OG base in Pol β.

One possible explanation for the preference of the 8OG syn conformation in the Pol μ active site is that the side chain of Gln441 exhibits a departure from its canonical conformation in previously published binary or pre-catalytic ternary complex structures (27), instead bending toward the 8OG base and forming a putative hydrogen bonding interaction with the O8 oxygen atom (2.9 Å, Figure 3C). Additional stabilizing interactions might be provided by a long-distance hydrogen bonding interaction between the N2 atom of the 8OG base and its non-bridging OP1 phosphate oxygen (3.3 Å), and between the N2 atom and a neigh-
Figure 3. Pol $\mu$ is structurally predisposed for mutagenic adenine incorporation opposite 8OG. (A) Wildtype hPol $\mu$Δ2 was crystallized in either binary (no incoming nucleotide) or pre-catalytic ternary (with incoming nonhydrolyzable nucleotide, as indicated) complex with a 1-nt gapped DNA substrate containing either a canonical or 8-oxo-7,8-dihydroguanine (red). Post-catalytic product complexes could be obtained by soaking binary or pre-catalytic ternary complex crystals with a hydrolyzable nucleotide, as indicated (Supplementary Table S3). (B) Global superposition of the hPol $\mu$Δ2 binary complex with undamaged (protein in dark gray, DNA in light gray) or 8OG-containing (protein in dark green, DNA in light green, 8OG in red) 1-nt gapped DNA substrates. The subdomains of the catalytic domain are labeled, and the location of the Loop2 deletion is indicated by a black asterisk. (C) The template 8OG nucleotide (light green) is observed in the syn conformation in the hPol $\mu$Δ2 binary complex, as indicated by the 2$F_o$-$F_c$ electron density map (gray mesh, contoured at 1σ). The side chain of Gln441 (dark green) makes a putative hydrogen bond (dashed line) with the O8 atom, in an altered conformation from that usually found in previously published hPol $\mu$Δ2 binary or ternary complex crystal structures (gray, PDB ID code 4LZG (27)). Other stabilizing interactions include putative hydrogen bonds between the N2 atom of the 8OG base and the non-bridging OP1 atom of its 5′-phosphate, or with a neighboring water molecule (red sphere). (D) The template 8OG in binary complex with Pol $\beta$ (PDB ID code 3RJE (24)) is observed in a mixture of syn (yellow) and anti (white) conformations, with the position of the latter resulting in a rearrangement of the phosphate backbone (measured distance between phosphorus atoms, magenta). 2$F_o$-$F_c$ electron density for the 8OG (residue T6) and preceding template base (residue T5) is shown as a gray mesh (contoured at 1σ).

boring water molecule. In keeping with the apparent structural rigidity of Pol $\mu$ (27), there are no other observed side chain rearrangements near the template 8OG, suggesting that the oxidized base is easily accommodated within the active site.

Pol $\mu$ deoxyribonucleotide insertion preference opposite template 8OG is subtly guided by structural and energetic interconversions

Given the predisposition for binding of 8OG-containing DNA substrates in a syn conformation, and its putative role in mutagenic adenine insertion, we next undertook a structural survey of template 8OG-nucleotide pairings to investigate the possibility of nucleotide-induced conformational changes. Crystals of the hPol $\mu$Δ2 pre-catalytic ternary complex bound to the 8OG-containing 1-nt gapped DNA substrate were initially grown in the presence of a nonhydrolyzable dCMPNPP analog. After crystallization, the dCMPNPP could be removed and replaced by soaking with either nonhydrolyzable dAMPNPP, or with hydrolyzable dCTP or dATP to generate post-catalytic product complexes (Supplementary Table S3). The dCMPNPP:8OG ternary complex (PDB ID code 6P1P, Supplementary Tables S3-S4) displays Watson-Crick base pairing between the incoming dCMPNPP and the template 8OG, which is now observed exclusively in the anti conformation, rather than the syn conformation seen in the binary complex (Figure 4A and B). The side chain of Gln441 no longer assumes the conformation found in the binary complex (Figure 4A, ...
Figure 4. Analysis of deoxycytidine incorporation opposite template 8OG. (A) Superimposed ribbon diagrams of pre-catalytic ternary complexes of hPol μΔ2 with DNA substrate containing a template 8OG (protein in orange, DNA in light orange, dCMPNPP in magenta, Mg2+ ions in green) versus with a DNA substrate containing a canonical template guanine (protein in dark gray, DNA and dCMPNPP in light gray). The Glu441 side chain is drawn in stick form each complex (8OG in orange, dCMPNPP in magenta, Mg2+ ions in green), which would clash with the position of the incoming nucleotide. Superposition of the dCMPNPP:8OG ternary complex with a complex containing a canonical dCMPNPP:dG base pair (PDB ID code 6P1V, Supplementary Tables S3 and S4) shows that the two structures are nearly identical globally (RMSD of 0.06 Å over 312 Ca atoms), and within the active site. In contrast to the proposed existence of a clash between the O8 atom of the template 8OG base and its 5′-phosphate oxygens in an *anti* conformation, the 8OG is accommodated with identical backbone geometry, when compared to the position of the DNA substrate containing the canonical guanine residue (Figure 4B). The O8 atom is positioned 3.3 Å from the bridging O5′ oxygen, and 3.1 Å from the non-bridging OP1 atom on the 5′-phosphate.

In both the dCMPNPP:8OG and the dCMPNPP:dG ternary complexes, all atoms are present and assembled in a catalytically relevant conformation poised for catalysis. The interatomic distances between the primerterminal 3′-OH and the α-phosphate on the incoming dCMPNPP are 3.5 and 3.6 Å for the 8OG and canonical dG template complexes, respectively. Replacement of the nonhydrolyzable dCMPNPP in the pre-catalytic template 8OG ternary complex crystals with hydrolyzable dCTP allows the incorporation reaction to proceed in *crystallo*, forming the post-catalytic product complex (PDB ID code 6P1Q, Supplementary Tables S3-S4, and Figure 4C). The reaction likely proceeds through a mechanism similar to when an unmodified template base is present, resulting in inversion of the α-phosphate and a shift of 1 Å toward the 3′-OH (27). The 3′-OH simultaneously shifts toward the α-phosphate by 1.1 Å, allowing the new phosphodiester bond to form. Two magnesium ions remain in the active site, as does the pyrophosphate leaving group. After dC incorporation, there were no observed overt global movements of protein subdomains, active site side chains, or substrate DNA residues (RMSD of 0.11 Å over 288 Ca atoms, compared to the pre-catalytic ternary complex), suggesting that the presence of the oxidized template 8OG is not unduly disruptive within the Pol μ active site.

Given the preference of Pol μ for inserting adenosine opposite a template 8OG, we next sought to determine the structure of the dAMPNPP:8OG pre-catalytic ternary complex (PDB ID: 6P1N, Supplementary Tables S3 and S4) by removing the dCMPNPP from the dCMPNPP:8OG ternary complex crystals and replacing it with nonhydrolyzable dAMPNPP. The ease with which the dCMPNPP was replaced should be noted, since the equivalent displacement does not occur with an equivalent dCMPNPP:dG ternary complex, even with extended soaking in high concentrations of dAMPNPP. This result is consistent with decreased binding affinity of dCTP opposite 8OG (Supplementary Table S5) and could be indicative of decreased stability for dCTP:8OG pairing, as compared to the canonical dCTP:dG interaction (19).

Global comparison of the dAMPNPP:8OG and dCMPNPP:8OG ternary complexes reveals that the two structures are nearly identical (RMDS of 0.07 Å over 304 Ca atoms) (Figure 5A). Though the initial crystallized dCMPNPP:8OG ternary complex contained 8OG observed in the *anti* conformation (Figure 4B), the oxidized base adopts
the syn conformation in the dAMPNPP:8OG ternary complex (Figure 5B). This is a surprising occurrence in crystallo, since interconversion between the two different conformations requires 180° rotation around the glycosidic bond and represents an unlikely feat due to inevitable steric clashes during the transformation, with its neighboring base (−1 position) and the Arg442 side chain. Arg442 stacks over the templating base, regardless of its identity, and makes two putative hydrogen bonds with the non-bridging 5′-phosphoryl oxygens (Figure 5C). Gln441 adopts the same conformation as in the dCMPNPP:8OG ternary complex, which is different from the rotamer observed in the binary complex that would clash with the incoming nucleotide (Figure 5A, green). The 8OG in the syn conformation base pairs with the incoming dAMPNPP via two hydrogen bonds along its Hoogsteen face, while the Watson-Crick face protrudes into the major groove (Figure 5B). This protrusion causes no disruption within the active site since the major groove there is solvent exposed. In the minor groove, the end of the Arg445 side chain is rotated very slightly toward the 8OG syn and lies within hydrogen bonding distance (3.2 Å) of the O8 atom, which is not possible when the 8OG adopts the anti conformation. Minor groove hydrogen bonding interactions with Arg445 and its structural equivalents in other Family X polymerases are thought to serve as a fidelity checkpoint (25,36,37), which has been circumvented in the Pol μ dAMPNPP:8OG complex.

The pre-catalytic dAMPNPP:8OG ternary complex was converted to the post-catalytic product complex in crystallo by replacement of the nonhydrolyzable dAMPNPP with dATP (PDB ID code 6P1O, Supplementary Tables S3-S4). Similar to the post-catalytic dCMP:8OG complex (RMSD of 0.07 Å over 294 Cα atoms), the reaction proceeded to completion with no movement or distortion of the DNA substrate, or of the active site side chains. A partial occupancy pyrophosphate remains in the active site, and both the metal A and metal B sites are occupied (Figure 5D). Thus, the mutagenic da:8OG pair is accommodated without obvious structural disruption of the nascent base pair binding site of Pol μ.
Pol μ competently incorporates ribonucleotides opposite template 8OG

Pol μ exhibits weak discrimination against incorporation of ribo- rather than deoxyribonucleotides (38,39), which could have advantageous consequences in the biological context of DNA double-strand break repair during phases of the cell cycle when dNTP concentrations are low (40–42). We therefore investigated the capability of this polymerase to insert ribocytidine or riboadenosine opposite a template 8OG. The initial dCMPNPP:8OG crystals were soaked with either nonhydrolyzable AMPNPP or CMPCPP to obtain pre-catalytic ribonucleotide insertion complexes (PDB ID codes: 6P1R and 6P1T, respectively, Supplementary Tables S3 and S4, and Figure 6). That the AMPNPP could efficiently replace the dCMPNPP in the initial ternary complex is notable, since Roettger et al (2004) hypothesized that only correctly paired NTPs could compete against dNTPs for binding since mismatched NTP insertion is considerably less efficient than for mismatched dNTPs (43). CMPCPP less effectively dislodged the dCMPNPP, as indicated by an occupancy of 0.70 for the O2’ atom.

Both the AMPNPP:8OG and the CMPCPP:8OG complexes are nearly indistinguishable from their pre-catalytic deoxyribonucleotide insertion counterparts (RMSD of 0.05 Å over 310 Ca atoms and 0.08 Å over 308 Ca atoms, respectively). AMPNPP pairs with the template 8OG in the syn conformation via two hydrogen bonds along its Hoogsteen face (Figure 6A, magenta inset), while the CMPPN pairs with the template 8OG in the anti conformation in canonical Watson-Crick geometry (Figure 6B, magenta inset). The AMPNPP:8OG complex displays some heterogeneity at the primer-terminus (Figure 6A, yellow inset), which has been modeled in two conformations—the predominant conformation is catalytically relevant, positioning the 3’-OH 3.6 Å away from the α-phosphate on the incoming AMPNPP.

In the less-occupied conformation, the primer terminal nucleotide is flipped ~180° from its original position, disrupting canonical Watson-Crick hydrogen bonding but maintaining base stacking interaction with the base pair in the −2 position, while leaving the 3’-OH entirely inaccessible for catalysis. Binding of the ribonucleotide causes minimal distortion of active site residues—limited to increased mobility of the primer terminus and the side chains of Trp434 and Val420—and is consistent with the behavior of these residues in structures containing incoming ribonucleotides pairing with undamaged template residues (40). Unlike in replicative polymerases, where ‘gate’ residues discriminate against 2’-OH containing nucleotides through Van der Waals interactions with aromatic side chains (44), Pol μ employs putative short-range hydrogen bonds between the 2’-OH and an active site backbone carbonyl (Gly433), with interatomic distances of 2.2–2.6 Å (Figure 6A and B, cyan inset). In both the AMPNPP:8OG and CMPCPP:8OG pre-catalytic ternary complexes, and in that of a reference CMPCPP:dG structure (PDB ID code: 6P1W, Supplementary Tables S3–S4), the 2’-hydroxyls are largely accommodated by subtle movements of the Gly433 carbonyl (0.2–0.9 Å), relative to its position in the dNTP-bound states (Figure 6, cyan inset). Both pre-catalytic ternary complexes contain two magnesium ions in the active site and display catalytically relevant conformation shown in pink (occupancy = 0.6), and the noncatalytic flipped conformation shown in white (occupancy = 0.4).
Physically relevant geometry nearly indistinguishable from the CMPCPP:dG reference structure with an undamaged template (RMSDs of 0.09 Å over 293–308 Ca atoms).

Figure 7. In crystallo incorporation of ATP opposite template 8OG is more efficient than that of CTP. Ribbon diagrams of the hPol μΔ2 post-catalytic product complexes for incorporation hydrolyzable ATP (A, protein in dark red, DNA in pink) or CTP (B, protein in dark blue, DNA in light blue, unreacted CMPCPP in yellow, pyrophosphate in orange) opposite template 8OG. Na+ ions in the metal B sites are shown as purple spheres. The hydrolyzable ribonucleotide stocks used for these soaks contain an unknown metal contaminant with anomalous scattering, which now occupies the metal A site in both structures. Given the observed coordination geometry and ionic distances, this metal has been modeled as Mn^{2+} (pink spheres).

Though insertion of ribonucleotides ATP and CTP was less efficient (<10-fold) than the equivalent deoxyribonucleotides, Pol μ exhibited remarkably similar ratios of adenosine versus cytidine incorporation (50-fold and 45-fold for deoxyribo- versus ribonucleotide) opposite 8OG (Figure 1 and Supplementary Table S5). Reduced efficiency of ribonucleotide insertion can be predominantly attributed to a decrease in the catalytic rate for CTP (>8-fold) and binding affinity for ATP (6-fold). However, when the relative catalytic efficiencies for ribo- and deoxyribonucleotide insertion are normalized based on endogenous concentrations (42), insertion of ribonucleotides hypothetically becomes more likely than their dNTP counterparts, and that of adenine nucleotides more likely than cytidine (Figure 1B).

Pol μ’s nucleotide insertion preference during template 8OG bypass is subtly influenced by Gln441

Using the crystal structures of Pol μ with an 8OG-containing substrate, we next attempted to probe the contributions of active site residues to nucleotide insertion preference. Although active site architecture of Family X polymerases is largely conserved, Gln441 in Pol μ differs in the other family members. In Pols β and λ, the equivalent residues (Asn279 and Asn513, respectively) function as ‘fidelity checkpoints’, interrogating the minor groove for correct hydrogen bonding. To determine whether Gln441 influences nucleotide insertion preference opposite the template 8OG, this residue was altered to either alanine or the Family X conservative mutation (asparagine). The Q441A and Q441N mutations yielded enzymes with negligibly decreased overall catalytic activity, as measured by dCTP insertion opposite canonical template dG (Supplementary Table S5). With template 8OG, dCTP incorporation was nearly indistinguishable from the wildtype, while dATP was inserted less efficiently than the wildtype (<2-fold). This modestly decreased the dATP:dCTP insertion ratio from 50-fold for the wildtype enzyme to 26- or 20-fold for Q441N or Q441A, respectively. Therefore, Gln441 and its putative hydrogen bond to the template 8OG may influence nucleotide base selectivity, however its presence is not the sole determining factor within the microenvironment of the Pol μ active site.

DISCUSSION

The Family X polymerases are involved in multiple DNA repair processes (45), including single- and double-strand breakage events secondary to UV and ionizing radiation (1). As a result, these enzymes may encounter a myriad of oxidative base damage, with lesions often clustered in close proximity to the broken ends (5,17). Their capabilities in engaging and bypassing these lesions must therefore be explored, from both structural and kinetic standpoints. Just as there is a correlation between template-dependence and protein movement (45), there also exists a gradient in accommodation of damaged or noncanonical substrates (15). Pol β, which participates solely in single-strand break repair, has a DNA substrate requirement for an intact template strand (45). During the course of its catalytic cycle,
Pol β undergoes large-scale movements of DNA substrate, protein subdomains and active site amino acid side chain rearrangements, all of which are thought to function as critical fidelity checkpoints to ensure faithful DNA synthesis (46). In contrast, Pol μ is the only known enzyme capable of repairing DNA double-strand breaks lacking sequence complementarity by polymerizing from an unpaired primer terminus. As such, Pol μ functions as a structurally rigid scaffold that can accommodate a wide variety of DNA end structures (27,47). Pol λ represents an intermediate between these extremes, since it can perform both single- and double-strand break repair although requiring pairing of its 3′-primer terminus, and it undergoes only small-scale structural rearrangements of DNA substrate and active site side chains for catalysis (15,48).

The function of these Family X polymerases upon encountering an oxidized guanine nucleotide in the templating position in the nascent base pair binding site is yet another example of a behavioral gradient. Pol β exhibits a slight preference in vitro (~2-fold) for correct insertion of incoming dCTP opposite a template 8OG, versus dATP (49). Pol λ appears to insert dCTP and dATP opposite template 8OG with relatively equal efficiency (25,49). Pol μ, in contrast, shows a strong (50-fold) preference for mutagenic insertion of dATP versus dCTP opposite a template 8OG (Figure 1). The unique behaviors of each enzyme can be correlated with the extent to which the presence of the template 8OG causes distortion of the DNA substrate when bound in the polymerase active site (Figure 8). Pol β, with the most discrimination against mutagenic insertion, struggles to reach a catalytically competent closed conformation with bound incoming dA (26,50) (Figure 8D, inset). Once closed, 8OG-containing DNA substrates exhibit a gross distortion of the downstream template backbone beginning immediately 3′ of the 8OG base and proceeding toward the 5′-end of that strand. Backbone distortions are observed in both the dCMP(CF2)PP:8OGuni and dAMPcpp:8OGuni structures (24) (Figure 8A and D, respectively), but differ slightly in each case (Figure 8G). In these structures, the 5′-phosphate is rotated sharply away (3.3–3.4 Å) from its canonical position in a ddCTP:dG reference structure (51). It was hypothesized that these rearrangements resulted from putative steric clashes between the O8 and O5′ atoms in the 8OGuni conformation, and between the N2 and O1 atoms in the 8OGsyn conformation. In each case, the backbone distortions are transmitted from the 8OG downstream of its position.

For Pol λ, the template backbone is distorted to a lesser extent, beginning immediately 5′ of the 8OG (Figure 8B and E). As with Pol β, 5′-phosphate of the 8OG is rotated away from the base (3.0–3.1 Å), as compared to its position in a dUMPNNP:da reference structure (PDB ID code: 2PFO (52)). Though a putative clash between the O8 oxygen on the 8OGsyn was hypothesized as the reason for the phosphate rearrangement, the backbone distortions observed in the Pol λ ddCTP:8OG or dATP:8OG structures (25) are nearly identical (<0.6 Å differences in this region of the template strand) (Figure 8H). In Pol μ, neither the dCMPNPP:8OG and dAMPNPP:8OG structures show apparent backbone distortions, and they are nearly indistinguishable from either the dCMPNPP:dG reference structure (Figure 8C and F) or each other (Figure 8I). It is perhaps surprising that there are no significant structural differences between the Pol μ 8OG structures, given its strong (50-fold) preference for inserting dATP versus dCTP opposite template 8OG.

Though Family X polymerases β and λ apparently struggle to discriminate against insertion of dATP opposite template 8OG at the initial nucleotide binding and incorporation steps, they have developed methods for excluding these mutagenic events from perpetuating in the genome by disfavoring extension from such mispairs (25,53). Pol λ accomplishes this discrimination largely through a hydrogen bond between the template 8OG, once translocated to the –1 position, and the side chain of Glu529. This hydrogen bond involving the N2 atom is only possible when the 8OG adopts the anti conformation. Loss of this hydrogen bond by mutation of Glu529 to alanine increases extension from the da:8OGsyn mispair by 3-fold (25). Glu529 is conserved in all known orthologs of Pol λ and is also found in Pol β (Glu295), but not in Family X siblings Pol μ (Ser468) or TdT (Asn474). Since TdT is a largely template-independent enzyme (16) and Pol μ predominantly fills gaps—regardless of their size—with a single incorporation event (47), this extension discrimination mechanism is likely not utilized by these enzymes.

The syn conformation of the template 8OG in the Family X polymerase structures is thought to be stabilized by a putative hydrogen bond with the side chain of a minor groove arginine residue (Arg283, Arg517 and Arg445 in Pols β, λ, and μ, respectively). A similar hydrogen bond is not possible when the 8OG adopts the anti conformation. When Arg283 was altered by a conservative mutation to lysine in Pol β, the resulting discrimination against dATP incorporation opposite template 8OG was significantly improved (from <2-fold to 15-fold preference of dCTP versus dATP) (50). This arginine residue occupies a similar position in all closed ternary complex structures of the Family X polymerases. In this configuration, the arginine side chain makes a hydrogen bond with all possible Watson-Crick base pairs and represents a fidelity checkpoint for correct minor groove geometry. As such, the hydrogen bond between this arginine and the syn conformation of the 8OG (Figure 5B) is an example of how the canonical fidelity checkpoints can be ‘hijacked’ by suboptimal base pairing scenarios.

Though Pol μ’s penchant for adenine insertion opposite the template 8OG might appear disadvantageous, it may play an essential physiological role during NHEJ. Given the fact that DNA double-strand breaks, especially when compounded by the added complexity of clustered oxidative damage, are severely genotoxic, swiftly and efficiently repairing them is of paramount importance. Since Pol μ has also been shown to efficiently incorporate ribonucleotides during NHEJ (40,41), its proclivity for adenine insertion in the context of oxidative DNA damage should be considered in a new light. NHEJ is the favored DNA DSB repair pathway in nonreplicating cells and during G1 phase of the cell cycle (12), when ribonucleotide concentrations are considerably higher than those of the corresponding deoxyribonucleotides (42). Given that cellular ATP concentrations are in the millimolar range, well above the threshold of Pol μ’s nucleotide binding affinity (Km of 72 μM, Supple-
Gradient of DNA backbone distortions in Family X polymerases as a consequence of guanosine oxidation. Stick representation of distortions (or lack thereof) in the template strand in pre-catalytic ternary complex structures of Pol β (top row), Pol λ (middle row), and Pol μ (bottom row), with either incoming nonhydrolyzable cytidine analogs (A–C, magenta, PDB ID codes 3RJI (24), 5IIJ (25), and 6P1P) or nonhydrolyzable deoxyadenosine (D–F, cyan, PDB ID codes 3RJF (24), 5III (25), and 6P1N; inset: Pol β ternary complex in green with dAMP:8OG in open conformation PDB ID code 1MQ2 (26)) opposite template 8OG in comparison to a correctly paired reference structure (gray, PDB ID codes 1BPY (51), 2PFO (52), and 6P1V).

(H–I) Superpositions of the same template DNA region in pre-catalytic ternary complex structures of incoming nonhydrolyzable dC (magenta) versus dA (cyan) opposite a template 8OG. The naming convention for base pairing is that of incoming nucleotide:template nucleotide, as is used elsewhere in this manuscript.

DATA AVAILABILITY

Structures have been deposited in the Protein Data Bank (https://www.rcsb.org/) under IDs: 6P1M, 6P1N, 6P1O, 6P1P, 6P1Q, 6P1R, 6P1S, 6P1T, 6P1U, 6P1V, 6P1W.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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