Roles of Residues Arg-61 and Gln-38 of Human DNA Polymerase η in Bypass of Deoxyguanosine and 7,8-Dihydro-8-oxo-2′-deoxyguanosine*

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Background: Arg-61 and Gln-38 of human DNA polymerase (hpol) η play important roles in the catalytic reaction.
Results: Mutations R61M or Q38A/R61A dramatically disrupt the activity of hpol η.
Conclusion: Polarized water molecules can mimic and partially compensate for the missing side chains of Arg-61 and Gln-38 in the Q38A/R61A mutant.
Significance: The positioning and positive charge of Arg-61 synergistically contribute to the activity of hpol η, with additional effects of Gln-38.

Like the other Y-family DNA polymerases, human DNA polymerase η (hpol η) has relatively low fidelity and is able to tolerate damage during DNA synthesis, including 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxoG), one of the most abundant DNA lesions in the genome. Crystal structures show that Arg-61 and Gln-38 are located near the active site and may play important roles in the fidelity and efficiency of hpol η. Site-directed mutagenesis was used to replace these side chains either alone or together, and the wild type or mutant proteins were purified and tested by replicating DNA past deoxyguanosine (G) or 8-oxoG. The catalytic activity of hpol η was dramatically disrupted by the R61M and Q38A/R61A mutations, as opposed to the R61A and Q38A single mutants. Crystal structures of hpol η mutant ternary complexes reveal that polarized water molecules can mimic and partially compensate for the missing side chains of Arg-61 and Gln-38 in the R61A and Q38A/R61A mutant. The combined data indicate that the positioning and positive charge of Arg-61 synergistically contribute to the nucleotidyl transfer reaction, with additional influence exerted by Gln-38. In addition, gel filtration chromatography separated multimeric and monomeric forms of wild type and mutant hpol η, indicating the possibility that hpol η forms multimers in vivo.

Y-family DNA polymerases are characterized by their low fidelity and ability to tolerate a wide range of lesions during DNA synthesis. Due to their relatively large active sites and loss of 3′→5′ exonuclease domains compared with replicative DNA polymerases, they readily introduce mutations into the genome. These enzymes are able to insert dNTPs opposite and beyond DNA lesions, therefore rescuing blocked replication forks and releasing the arrested cell cycle (1–6).

Human DNA polymerase η (hpol η) belongs to the DNA polymerase Y family (5). It plays a crucial role in bypassing cyclobutane pyrimidine dimers, which arise as a result of UV exposure. Defects in hpol η bypass of cyclobutane pyrimidine dimers in xeroderma pigmentosum variant patients results in increased rates of skin and internal cancers (7–9). hpol η contributes to replication past DNA lesions induced by platinum chemotherapeutic drugs, leading to escape of the arrested cell cycle and therefore proliferation of cancer cells (10–17). Besides translesion synthesis, there is evidence that hpol η is involved in homologous recombination by interaction with Rad51 and extension of the D-loop (18–20). hpol η also contributes to somatic hypermutation in B cells by introducing mutations in specific DNA stretches embedded within antigen-binding sites, thereby causing structural changes and potentially elevating the antigen-binding ability (21–27).

hpol η and its yeast homolog Rad30 also play roles in the bypass of 7,8-dihydro-8-oxoguanine (8-oxoG) (28–30), one of the most common DNA lesions introduced by oxidative stress. 8-oxoG is generated at a rate of ≪1000 molecules/cell/day and prefers the syn conformation, which facilitates formation of a Hoogsteen pair with dATP and introduces mutations into the genome (31–33). hpol η replicates past 8-oxoG mainly by inserting dCTP, scaffolding the dCTP:8-oxoG pair in the Watson-Crick geometry, although the dATP misincorporation rate is 280-fold higher in the bypass of 8-oxoG compared with unmodified G (30).

Crystal structures of the catalytic core of hpol η (amino acids 1–432) and its yeast homolog with unmodified or lesion-containing oligonucleotides have been published (10, 11, 21, 28, 30, 34–39). These studies provide a better understanding of the
catalytic mechanism and have yielded valuable information regarding the design of potential drugs against hpol η in the hope of blocking tumor proliferation. The residue Arg-61, located in a short loop region, is highly conserved. Its dynamic movement contributes to the catalytic activity of the enzyme. X-ray crystal structures reveal that after binding the DNA sub- strate in the absence of an incoming dNTP, the positively charged guanidino moiety of Arg-61 is projected toward the nucleobase of the 5’-overhanging, single-stranded region of template DNA of the template-primer duplex (38). However, following binding of dNTP and divalent metal ions, the side chain of Arg-61 undergoes a rotation and interacts with the α-phosphate of the incoming dNTP. Interestingly, the side chain of Arg-61 also moves during phosphodiester bond formation to allow enough space for a third divalent metal ion, which interacts with both the α-phosphate and the oxygen, bridging the α- and β-phosphates. Thus, Arg-61 can adopt different conformations during each stage of the catalytic reaction cycle.

In addition, the reported structures have also indicated that another highly conserved residue near the active site, Gln-38, may play an important role in stabilizing the template base. The side chain of Arg-61 also moves during phosphodiester bond formation to allow enough space for a third divalent metal ion, which interacts with both the α-phosphate and the oxygen, bridging the α- and β-phosphates. Thus, Arg-61 can adopt different conformations during each stage of the catalytic reaction cycle.

Site-directed mutagenesis was used in this study to further investigate the roles of Arg-61 and Gln-38 in the catalytic activity. Wild-type and mutant forms of hpol η were analyzed and crystallized with unmodified DNA or DNA containing 8-oxoG. The combined results of the structural and biochemical analysis indicate that the positioning and positive charge of the Arg-61 synergistically contribute to the nucleotidyl transfer reaction in addition to the hydrogen bonding effect of Gln-38 with the template base.

When both Arg-61 and Gln-38 were replaced by Ala, polarized water molecules could mimic and partially compensate for the missing wild-type side chains.

Experimental Procedures

Materials—All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA), and some were purified by HPLC. dNTPs were from New England Biolabs (Ipswich, MA). PreScission protease was from GE Healthcare. Polyethylene glycol monomethyl ether 2000 (for crystalliza- tion) was from Hampton Research (Aliso Viejo, CA).

Wild-type and mutant hpol η derivatives were expressed in Escherichia coli BL21 (DE3) pLysS cells, followed by purification through a HiTrap HP column (5 ml; GE Health- care). After removal of the N-terminal His tag by PreScission protease (GE Healthcare), the proteins were purified through a Mono S column (GE Healthcare), eluted with the buffer containing 20 mM sodium MES (pH 6.0), 3 mM dithiothreitol (DTT), 0.1 mM EDTA, 10% glycerol (v/v), and ~350 mM KCl (using a gradient of 250–500 mM KCl). The proteins were used for activity assays after flash freezing in liquid nitrogen and storage at −80 °C. Proteins were further purified through a Superdex-75 10/300 GL column (GE Healthcare) in 20 mM Tris-HCl buffer (pH 7.5) containing 450 mM KCl and 3 mM DTT (35) to obtain crystals. In addition, the R61M mutant was analyzed with a Superdex-200 10/300 GL column (GE Health- care) with 20 mM Tris-HCl (pH 7.5) containing 450 mM KCl and 3 mM DTT as well as bovine thyroglobulin (Sigma-Aldrich) and bovine serum albumin (BSA).

Annealing and Extension Assays—Primer and template DNA were annealed (1:1 molar ratio) by heating to 95 °C for 5 min and slow cooling to room temperature: (a) 5’-6-carboxylfluorescein (FAM)-CGGGCTCGTAAGCGTCAT-3’ and 3’-GCCGGACATTGCATGATACT-5’; (b) 5’-FAM-CGGCTGTAAAGCTCGAT-3’ and 3’-GCCGGACATTGCATGATACT-5’; (c) 5’-FAM-CGGGCTCGTAAGCGTCAT-3’ and 3’-GCCGGACATTGCATGATACT-5’; (d) 5’-FAM-CGGGCTCGTAAGCGTCAT-3’ and 3’-GCCGGACATTGCATGATACT-5’; (e) 5’-AGGGCTCAT-3’ and 3’-TCGAGTAGTAC-5’; (f) 5’-AGGGCTCAT-3’ and 3’-TCGAGTAGTAC-5’; (g) 5’-FAM-CGGGCTCGTAAGCGTCAT-3’ and 3’-GCCGGACATTGCATGATACT-5’. DNA complexes a–d were used for extension assays; a–c and g were applied in steady-state kinetic assays; a and b were used in pre-steady-state kinetic assays; e and f were used for crystallization. For the extension assays, wild-type hpol η or mutant proteins (25 nM) were incubated with 5 μM FAM-labeled primer-template DNA complex in 40 mM Tris-HCl buffer (pH 7.5), 10 mM DTT, 0.1 mg/ml BSA, 5% glycerol (v/v), 5 mM MgCl2, 100 mM KCl, and 200 μM each dNTP. The reactions were conducted for 0, 2, 5, and 15 min at 37 °C. After stopping the reactions, the products were separated using 18% denaturing polyacrylamide gels (w/v) and visualized using a Typhoon system (GE Healthcare).

Steady-state Kinetics—Reactions were conducted by incubating hpol η (2–500 nM), 5 μM FAM-labeled primer-template DNA complex, 40 mM Tris-HCl (pH 7.5) containing 10 mM DTT, 0.1 mg/ml BSA, 5% glycerol (v/v), 5 mM MgCl2, 100 mM KCl, and various amounts of dCTP or dATP at 37 °C for 5 min. After quenching the reactions, products were separated using 18% denaturing polyacrylamide gels (w/v), visualized with a Typhoon system (GE Healthcare), and quantified using ImageJ software (National Institutes of Health). The data were fit to the (hyperbolic) Michaelis-Menten equation with Prism software (GraphPad, La Jolla, CA) (10, 30).

Pre-steady-state Kinetics—FAM-labeled primer-template DNA complex (1 μM) was mixed with wild-type (100 mM) or mutant (500 mM) hpol η, 40 mM Tris-HCl (pH 7.5), 10 mM DTT, 0.1 mg/ml BSA, 5% glycerol (v/v), and 100 mM KCl (Solution A).
Burst amplitude, representing the apparent concentration of 10 min, followed by the addition of 0.5 (1.2:1 molar ratio) were first incubated at room temperature for (30, 37, 40).

Data Bank (PDB) code 4O3N were used as the search model for R61M-GdCTP and Q38A/R61A-GdCTP, those with PDB code 4O3P were used for R61M-8-oxoGdCTP and Q38A/R61A-8-oxoGdCTP, and those with PDB code 4O3O were used for R61M-8-oxoGdATP. PHENIX (43) was applied for refinements, and ARP/wo ARP classic (44, 45) and COOT (46) were used for model building. Structural illustrations were generated with the program UCSF Chimera (47).

**Results**

**Primer Extension past G or 8-oxoG by Wild-type or Mutant hpol η**—Wild-type or mutant hpol η (25 nM), FAM-labeled primer-template DNA complex (5 μM), and a saturating concentration of all four dNTPs (200 μM each dNTP) were incubated at 37 °C for 0, 2, 5, and 15 min. The products were separated on denaturing polyacrylamide gels. In A, X represents G; in B, X represents 8-oxoG.

Solution B contained 1 mM dCTP and 10 mM MgCl₂. The two solutions were mixed rapidly with a KinTek RP-3 instrument (KinTek Corp., Austin, TX) at 25 °C for 0.005–5 s (the final reaction concentration for wild-type hpol η was 50 nM, and that for mutant hpol η was 250 nM) and stopped by the addition of 0.5 M EDTA. The products were separated with 18% denaturing polyacrylamide gels (w/v) and visualized with a Typhoon system (GE Healthcare). After quantification using ImageJ software, the results were fit to a burst equation using GraphPad Prism: $y = A(1 - e^{-k_{p}t}) + k_{sE}E_{o}$, where A is the burst amplitude, representing the apparent concentration of the active form of the enzyme, $k_{p}$ is the burst rate, $k_{sE}$ is the steady-state rate, and $E_{o}$ is the total enzyme concentration (30, 37, 40).

**Crystallizations**—The hanging drop vapor diffusion technique was used to obtain crystals. DNA complex and protein (1.2:1 molar ratio) were first incubated at room temperature for 10 min, followed by the addition of 0.5 μl of 1 mM CaCl₂ and concentration by centrifugation through a filter (10 kDa, Millipore). After the addition of 0.5 μl of 100 mM dNTP (New England Biolabs), the solution (1 μl) was mixed with an equal volume of reservoir solution (100 mM sodium MES (pH 6.0), 5 mM CaCl₂, and 15–22% (w/v) polyethylene glycol monomethyl ether 2000 (Hampton Research, Aliso Viejo, CA)). The mixture was equilibrated against 500 μl of the reservoir solution at 18 °C. Crystals were typically observed after overnight incubation (30, 35).

**X-ray Diffraction Data Collection and Structure Determination**—Crystals were mounted in nylon loops and swiped through a mixture of reservoir solution plus 25% glycerol (v/v), followed by flash-cooling in liquid nitrogen. Diffraction data were collected on the Sector 21-ID-G beamline at the Advanced Photon Source (Life Sciences Collaborative Access Team, Argonne National Laboratory, Argonne, IL). All collected data were integrated and scaled using HKL2000 (41). Structures were determined by molecular replacement phasing using the program Phaser MR (42). Coordinates with Protein Data Bank (PDB) code 4O3N were used in this study because its activity is similar to that of the full-length protein in vitro (10). In order to understand the mechanism of dNTP incorporation by hpol η, point mutations were introduced at Arg-61 and Gln-38. In the extension assays, wild-type hpol η bypassed both unmodified template G and 8-oxoG and fully extended the primer in 15 min, whereas mutants R61M and Q38A/R61A partially elongated the primer by inserting at most two nucleotides, indicating that these mutations strongly attenuate the activity of hpol η. In comparison, the mutations Q38A, Q38L, R61A, and R61K only slightly reduced the catalytic activity (Fig. 1, A and B).

**Steady-state Kinetics of dCTP or dATP Incorporation Opposite G or 8-oxoG by Wild-type and Mutant hpol η**—All hpol η mutants used in our study had attenuated efficiency for dCTP insertion opposite unmodified G compared with the wild-type enzyme. The Q38L, R61M, and Q38A/R61A mutants showed 22-, 19-, and 16-fold attenuation in enzyme efficiency, respectively, whereas Q38A, R61A, and R61K showed only small decreases. Even when these mutations were introduced, hpol η still maintained relatively high fidelity opposite G, and the dATP misincorporation frequency for each hpol η was <4% (Fig. 2A and Table 1). For dCTP incorporation opposite 8-oxoG (Fig. 2B and Table 2), the lowest catalytic efficiencies ($k_{cat}/K_{m}$) were observed for the Q38L, R61M, and Q38A/R61A mutants...
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The oligonucleotides used were 5′-FAM-CCGGCTCGTAAGCGTCAT-3′ and 3′-GCCCGAGCATTCGCAGTACT-5′.

**TABLE 1**

| hpol η | dNTP | 
|--------|------|
|        | Km  | kcat | kcat/Km | f* |
| WT*    | dCTP 1.3 ± 0.2 | 80 ± 1 | 0.005 ± 0.018 | 0.0010 |
| Q38A | dATP 92 ± 23 | 6.0 ± 0.6 | 7.6 ± 0.2 | 0.0011 |
|        | dCTP 5.2 ± 0.9 | 88 ± 5 | 17 ± 3 | 0.0036 |
| Q38L | dCTP 103 ± 13 | 14 ± 1 | 0.14 ± 0.02 | 0.0082 |
| R61A | dCTP 20 ± 5 | 55 ± 5 | 2.8 ± 0.7 | 0.0014 |
| R61K | dCTP 148 ± 36 | 15 ± 1 | 0.10 ± 0.03 | 0.0013 |
| R61M | dCTP 1.5 ± 0.2 | 21 ± 1 | 14 ± 2 | 0.0012 |
| Q38A/R61A | dCTP 49 ± 10 | 0.92 ± 0.09 | 0.019 ± 0.004 | 0.0013 |
| dCTP 8.7 ± 1.7 | 72 ± 3 | 8.3 ± 1.7 | 0.0013 |
| dCTP 73 ± 10 | 80 ± 0.4 | 1.11 ± 0.02 | 0.0013 |
| dCTP 1.3 ± 0.1 | 4.2 ± 0.2 | 3.2 ± 0.3 | 0.0013 |
| dATP 47 ± 13 | 0.14 ± 0.01 | 0.0030 ± 0.0009 | 0.0009 |
| dCTP 6.5 ± 2.2 | 26 ± 2 | 4.0 ± 1.4 | 0.0009 |
| dATP 78 ± 15 | 2.1 ± 0.1 | 0.027 ± 0.005 | 0.0068 |

* Misinsertion frequency: f = (kcat/Km)_{dATP} / (kcat/Km)_{dCTP}
  
* Steady-state kinetic data of dNTP incorporation opposite G by WT hpol η were from Ref. 30. The experimental conditions for WT and hpol η mutants were identical.

(i.e. 4.9, 1.4, and 2.2 µM⁻¹ min⁻¹ respectively, compared with 31 µM⁻¹ min⁻¹ for wild-type hpol η). In the study of steady-state kinetics of dNTP misinsertion opposite 8-oxoG, a second insertion band was always observed due to the next base T, 3′ to 8-oxoG on the template strand (Fig. 2C). To obtain catalytic efficiencies specifically for the insertion step opposite 8-oxoG, the two insertion bands were both included in the quantitation. We reported previously that the dATP insertion frequency was 280-fold higher for wild-type hpol η replicating past 8-oxoG than that for replicating past G (30). For each mutant, dATP was more frequently inserted opposite 8-oxoG than G. Interestingly, all mutants except R61K showed higher fidelity (inserting dCTP instead of dATP) than wild-type hpol η in bypassing 8-oxoG. The R61K mutant, consistent with a previous report (21), had a higher misincorporation frequency (0.60, compared with 0.28 for wild-type hpol η) (Fig. 2B and Table 2).

**TABLE 2**

| hpol η | dNTP | 
|--------|------|
|        | Km  | kcat | kcat/Km | f* |
| WT*    | dCTP 1.3 ± 0.2 | 80 ± 1 | 0.005 ± 0.018 | 0.0010 |
| Q38A | dATP 92 ± 23 | 6.0 ± 0.6 | 7.6 ± 0.2 | 0.0011 |
|        | dCTP 5.2 ± 0.9 | 88 ± 5 | 17 ± 3 | 0.0036 |
| Q38L | dCTP 103 ± 13 | 14 ± 1 | 0.14 ± 0.02 | 0.0082 |
| R61A | dCTP 20 ± 5 | 55 ± 5 | 2.8 ± 0.7 | 0.0014 |
| R61K | dCTP 148 ± 36 | 15 ± 1 | 0.10 ± 0.03 | 0.0013 |
| R61M | dCTP 1.5 ± 0.2 | 21 ± 1 | 14 ± 2 | 0.0012 |
| Q38A/R61A | dCTP 49 ± 10 | 0.92 ± 0.09 | 0.019 ± 0.004 | 0.0013 |
| dCTP 8.7 ± 1.7 | 72 ± 3 | 8.3 ± 1.7 | 0.0013 |
| dCTP 73 ± 10 | 80 ± 0.4 | 1.11 ± 0.02 | 0.0013 |
| dCTP 1.3 ± 0.1 | 4.2 ± 0.2 | 3.2 ± 0.3 | 0.0013 |
| dATP 47 ± 13 | 0.14 ± 0.01 | 0.0030 ± 0.0009 | 0.0009 |
| dCTP 6.5 ± 2.2 | 26 ± 2 | 4.0 ± 1.4 | 0.0009 |
| dATP 78 ± 15 | 2.1 ± 0.1 | 0.027 ± 0.005 | 0.0068 |

* Misinsertion frequency: f = (kcat/Km)_{dATP} / (kcat/Km)_{dCTP}

* Steady-state kinetic data of dNTP incorporation opposite 8-oxoG by WT hpol η were from Ref. 30. The experimental conditions were the same for WT and mutant forms of hpol η.

Pre-steady-state Kinetics of dCTP Incorporation Opposite G or 8-oxoG by Wild-type and Mutant hpol η—In the pre-steady-state kinetic analysis of dCTP incorporation opposite G, the burst rates for R61M and Q38A/R61A were 2.6- and 5.9-fold lower than that for wild-type hpol η (Fig. 3 and Table 3). A similar trend was observed for bypass of 8-oxoG, with 90 s⁻¹ for wild-type, 35 s⁻¹ for R61M, and 53 s⁻¹ for Q38A/R61A. In the bypass of G, the burst amplitudes of wild type were nearly 100% of the estimated enzyme concentration, but the burst amplitudes for R61M and Q38A/R61A were only 21 and 16%, respectively, suggesting the presence of either inactive enzymes or multiple
of dCTP opposite 8-oxoG; R61A incorporation of dCTP opposite G; incorporation opposite G and 8-oxoG.

For the bypass of 8-oxoG, the burst amplitudes were ~14% for each hpol η mutant and ~70% for wild-type hpol η. Notably, for each individual enzyme, the burst amplitude for incorporation opposite G was always larger than that for 8-oxoG, suggesting that non-productive forms are more prominent in the presence of 8-oxoG (48).

conformations in mutant-DNA-dNTP ternary complexes (48).

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R61M-G-dCTP and Q38A/R61A-G-dCTP complexes respectively, compared with 3.3 or 3.5 Å (two conformations) for wild-type hpol η (30).

Arg-61 of wild-type hpol η engages in favorable Coulombic interaction with the α-phosphate of the incoming dCTP. The electrostatic force was lost after mutating it to the hydrophobic Met, although the size of the side chain was similar to Arg.

Interestingly, when Arg-61 was mutated to Met. Interestingly, when Arg-61 was mutated to Ala, water molecules were lodged on top of dCTP. One of the water molecules was on top of the nucleobase of the dCTP, whereas the position of the other matched that of Nο of Arg-61 from the wild-type hpol η8-oxoG-dCTP complex, allowing it to donate a hydrogen bond to the α-phosphate of the incoming dCTP (Fig. 7).

In the minor groove of the wild-type ternary complex, Νο of Gln-38 was engaged in a hydrogen bond with N3 of 8-oxoG. After superimposition, a water molecule in the Q38A/R61A-G-dCTP complex, one water molecule was located above the base ring of dCTP and likely to form a lone pair-π or H-π interaction with the nucleobase (Fig. 6) (49).

In the minor groove, the Νο atom of Gln-38 connected with N3 of the template G through a hydrogen bond and a water molecule-bridged O* of Gln-38 and N2 of G, thus stabilizing the template G to form a base pair with the incoming dCTP. When Gln-38 was mutated to Ala, these interactions were disrupted, and the bridging water molecule was not present in the Q38A/R61A-G-dCTP structure (Fig. 6).

**X-ray Crystallography of Mutant hpol η Inserting dCTP Opposite 8-oxoG**—To investigate the roles of Arg-61 and Gln-38 in bypassing 8-oxoG, mutant hpol η was crystallized with dCTP opposite 8-oxoG in the presence of Ca²⁺. Fourier (2Fo - Fo) sum electron density maps are shown in Fig. 5. Similar to the structures depicting dCTP insertion opposite unmodified G, wild-type hpol η and the mutants provided a scaffold for dCTP pairing with 8-oxoG in the Watson-Crick geometry, with 8-oxoG in the anti conformation (Fig. 7). The distances between the primer terminal 3'-OH and the Pφ of dCTP were 3.2 or 3.4 Å in the complex with wild-type hpol η (30), 3.3 or 3.9 Å with the R61M mutant, and 3.9 Å with Q38A/R61A, respectively.

Similar to dCTP insertion opposite G by wild-type hpol η, the side chain of Arg-61 is involved in an electrostatic interaction with the α-phosphate of dCTP in the wild-type hpol η8-oxoG-dCTP complex, but this interaction was destroyed when replacing Arg-61 with Met. Interestingly, when Arg-61 was mutated to Ala, water molecules were lodged on top of dCTP. One of the water molecules was on top of the nucleobase of the dCTP, whereas the position of the other matched that of Νο of Arg-61 from the wild-type hpol η8-oxoG-dCTP complex, allowing it to donate a hydrogen bond to the α-phosphate of the incoming dCTP (Fig. 7).

In the minor groove of the wild-type ternary complex, Νο of Gln-38 was engaged in a hydrogen bond with N3 of 8-oxoG. After superimposition, a water molecule in the Q38A/R61A-G-dCTP complex was observed near the Νο of Gln-38 in the wild-type hpol η8-oxoG-dCTP complex. Further investigation revealed a hydrogen bonding interaction between this water and the N3 of 8-oxoG. In addition, in the wild-type hpol η8-oxoG-dCTP complex, a water molecule bridged both the Oφ of Gln-38 and N2 of 8-oxoG by hydrogen bonds. This bridging water hydrogen-bonds with the Νο-mimicking water as well as N2 of 8-oxoG in the Q38A/R61A-G8-oxoG-dCTP complex (Fig. 7).

**X-ray Crystallography of Mutant hpol η Inserting dATP Opposite 8-oxoG**—To explore the role of Arg-61 in the fidelity of hpol η, we crystallized the hpol η R61M mutant with dATP opposite 8-oxoG. Similar to the wild-type hpol η8-oxoG-dCTP complex, 8-oxoG adopted the syn conformation and formed a Hoogsteen pair with the incoming dATP when Arg-61 was mutated to Met. The distance from the 3'-OH of the primer end to Pφ of dATP was 3.7 Å, com-
Figure 5. Electron density maps for the active sites of mutant ternary complexes. The Fourier (2Fo-HFc) sum electron density is at the 1/σ threshold.

A, R61M/GdCTP, Q38A/GdCTP, G61M/WgGdCTP; B, Q38A/R61M/GdCTP; C, R61M/8-oxoGdCTP; D, Q38A/R61A/8-oxoGdCTP; E, R61M/8-oxoGdATP. The metal ions (Ca2+) are shown in red in each panel.

Table 5

| Complex | R-merge | R-free | Resolution (Å) | Data collection | Space group |
|---------|---------|--------|----------------|----------------|-------------|
| R61M/GdCTP, Q38A/GdCTP | 0.172 (0.282) | 0.152 (0.272) | 50.00-1.78 (1.81-1.78) | 0.9785 | P61 |
| Q38A/R61A/GdCTP | 0.176 (0.295) | 0.155 (0.284) | 50.00-1.78 (1.81-1.78) | 0.9785 | P61 |
| R61M/8-oxoGdCTP | 0.179 (0.295) | 0.152 (0.272) | 50.00-1.78 (1.81-1.78) | 0.9785 | P61 |
| Q38A/R61A/8-oxoGdCTP | 0.174 (0.295) | 0.155 (0.284) | 50.00-1.78 (1.81-1.78) | 0.9785 | P61 |
| R61M/8-oxoGdATP | 0.170 (0.295) | 0.150 (0.272) | 50.00-1.78 (1.81-1.78) | 0.9785 | P61 |

Data shown in parentheses are from the highest resolution shell.

$^a$ R-merge: R-linear/SUM(ABS(I(H2O855/I(H20856)))/SUM(I(H20856)).

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pared with 3.1 or 3.3 Å in the complex with wild-type hpol η (30) (Fig. 8).

The methionine side chain in the point mutant R61M exhibited two conformations in the ternary structure. One of the conformations partially superimposed with the side chain of Arg-61 in the wild-type hpol η-8-oxoG-dATP complex. However, due to the hydrophobic nature of the Met side chain, the electrostatic interaction seen in the wild-type complex with the incoming dATP was lost. The N\(^{\#}\) of Gln-38 from either wild-type or R61M mutant hpol η donated a hydrogen bond to the O8 atom of 8-oxoG in the minor groove (Fig. 8).

**Multimeric Forms of hpol η**—Wild-type hpol η and the mutants R61M and Q38A/R61A were purified by gel filtration chromatography (Superdex-75 10/300 GL) prior to crystallization. Two distinct peaks, eluting with \(t_r\) of ~8.1 and 11.3 ml, were observed in the chromatogram in each case, suggesting the presence of proteins with different molecular masses (Fig. 9A). The second peak was eluted with the molecular mass of monomeric hpol η, as established using the standards BSA (67 kDa) and ovalbumin (43 kDa). The proteins from the two peaks had the same mobility in SDS-PAGE (data not shown), indicating that the protein in the first peak was a multimeric form of hpol η. The R61M mutant had the largest ratio of peak 1 (multimer) over peak 2 (monomer) (Fig. 9A), and kinetic assays with minimal protein dilution following the chromatography (final dilution ratio 1:4 (v/v) for the reaction) were used to measure their catalytic activities (burst phase, pre-steady state). The protein from peak 2 (monomer) showed a 1.5-fold higher burst rate compared with that from peak 1 (multimer) for dCTP insertion opposite template G. The burst amplitudes were 65 and 202 nM (compared with the input concentrations of 566 and 772 nM for the peak 1 and 2 proteins), respectively. In addition, both peaks of the R61M mutant were crystallized with DNA and incoming dCTP with no dramatic structural differences (data not shown). To characterize the multimeric composition, the mutant R61M, as well as two markers, thyroglobulin (670 kDa) and BSA (67 kDa), were used to calibrate a Superdex-200 10/300 GL gel filtration column. The mutant enzyme was eluted in a peak corresponding to monomeric hpol η, a broad peak with molecular mass in the range of 400–600
kDa, and the void peak, indicating the presence of an even higher fold ensemble (Fig. 9B).

**Discussion**

The fidelity of some DNA polymerases is determined largely by a single key residue. For example, Arg-332 in the archbacterial *Sulfolobus solfataricus* Y-family DNA polymerase Dpo4 directly interacts with the O8 atom of 8-oxoG and determines its fidelity in the bypass of 8-oxoG (50). Structures of hpol h9257 show that two highly conserved residues, Arg-61 and Gln-38, are important in the catalytic reaction (10, 11, 21, 28, 30, 34–39). In order to delineate their roles for the activity and fidelity of hpol h9257, we mutated the two residues to amino acids with different side chain properties. The unmodified G and a common DNA lesion, 8-oxoG, were used in DNA templates in our study.

The misincorporation frequencies of wild-type and hpol h9257 mutants for dATP insertion opposite unmodified G were very small, all <4%, as judged from the steady-state kinetic assay results. However, the misincorporation frequency was increased 36-fold for mutant Q38L, 13-fold for R61K, 8-fold for Q38A, and 7-fold for Q38A/R61A compared with that for wild-type hpol h9257, whereas mutants R61A and R61M had misincorporation frequencies similar to that of the wild-type protein in incorporating dATP opposite G (Fig. 2A and Table 1). On the other hand, because 8-oxoG can readily form a syn conformation due to a steric effect and pair with A, the dATP insertion frequency was increased 280-fold for wild-type hpol h9257 compared with that opposite unmodified G (30). Interestingly, mutant R61K had the highest misincorporation frequency (0.60) for dATP insertion opposite 8-oxoG, whereas R61M had the lowest value (0.079) compared with wild-type hpol h9257 (0.28). The other mutants had slightly reduced misincorporation frequency (Fig. 2B and Table 2). In addition, previous reports also showed that the template base/lesion as well as the flanking sequence also affected the misincorporation frequency for wild-type or mutant hpol h9257 (21, 51). Altogether, we conclude that active site residues, the template base/lesion, and the flank-
ing DNA sequence synergistically determine the misincorporation frequency of hpol η. It was unexpected that the R61A mutant would be more active than R61M; however, x-ray crystal structures of the corresponding complexes provide a potential explanation. Polarized water molecules mimic the side chain of Arg-61 and partially compensate for its functions in the R61A mutant. In order to understand how these water molecules participate in the nucleotidyl transfer reaction, the Q38A/R61A 8-oxoG·dCTP complex was compared with the other reported wild-type hpol η structures. A third metal ion (Mg²⁺) has been reported to be involved in the catalytic process, whereas the side chain of Arg-61 of wild-type hpol η adopted the conformation facing toward the major groove (Fig. 10A) (34). After superimposition, the water interacting with the –phosphate in the Q38A/R61A 8-oxoG·dCTP complex partially overlapped with a water molecule coordinating with the third Mg²⁺ (Fig. 10, B and C). Therefore, the water in the mutant hpol η may not only play a role in mimicking the missing side chain of Arg-61 in the ground state but may also interact with the third metal ion during the catalytic process.

Because H₂O and monovalent Na⁺ may look very similar in electron density maps (52), close investigation of the coordination, occupancy, and B factor was conducted (for those water molecules). In particular, the water molecule on top of the base ring of the incoming dCTP was of interest. This water is 2.9 Å from the centroid of the aromatic ring in the Q38A/R61A·dCTP complex, and the corresponding distance in the Q38A/R61A·8-oxoG·dCTP complex is 3.1 Å, indicating the possibility of the formation of a lone pair–π or H–π interaction between H₂O and the base ring (49). To distinguish H₂O from a monovalent metal ion, the former was replaced by a Na⁺ ion with half-occupancy in the Q38A/R61A·8-oxoG·dCTP model.
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structure. After refinement using the program Phenix, the occupancy of Na\(^+\) was 0.38, and the B factor dropped from 35.6 to 21 Å\(^2\) compared with a fully occupied water (data not presented). Thus, we cannot completely exclude the possibility that the electron density peak represents a partially occupied Na\(^+\) instead of H\(_2\)O on top of the base ring. Soaking crystals with K\(^+\) or Rb\(^+\) and collecting anomalous data may help to further resolve the issue, although K\(^+\) and Rb\(^+\) have larger ionic radii than Na\(^+\) (53).

On the other hand, the electrostatic interaction with the incoming dNTP was lost when Arg-61 was mutated to the hydrophobic Met. In addition, the side chain of R61M was too big to allow room for Arg-61-mimicking water molecules, as seen in the ternary structures with the mutation R61A. As a result, hpol η with the single substitution R61A was more active than with R61M. Similar results were observed with the Q38A and Q38L mutants (Tables 1 and 2 and Figs. 6 and 7).

A second unexpected result was observed from the pre-steady-state kinetic data; the burst amplitudes for wild type hpol η inserting dCTP opposite unmodified G or 8-oxoG were ~100 or 70% of the input, respectively, whereas the amplitude was <21% for either the R61M or Q38A/R61A mutant. The result indicated the presence of either an inactive form(s) of mutant protein or an equilibrium of reactive and nonreactive ternary complexes (1). Interestingly, gel filtration chromatography showed the presence of both multimeric and monomeric forms of wild type or mutants of hpol η. Both forms of the proteins were active and formed single crystals (data not shown; however, the extent of the multimeric state is not known at the high concentration used for crystal formation). These results suggest that hpol η may assemble to multimers in vivo, and the two mutants showed an increased tendency to multimeric assembly compared with wild-type hpol η (Fig. 9), although extensive cellular studies would be needed for further conclusions about physiological relevance. The reason for this change in behavior is unknown, considering that the mutations are in the interior of the protein. Interestingly, multimeric forms of the other polymerases have been reported previously. For example, S. solfataricus Dpo4 can form a dimer on DNA in vitro (54). Small angle x-ray scattering and ultracentrifugation results reveal that mammalian DNA polymerase β binds with DNA in ratios of both 2:1 and 1:1 in solution (55, 56). Recently, an A-family DNA polymerase (polymerase θ) has been reported to be able to form a dimer, which may be important for polymerase θ to participate in microhomology-mediated end joining (57). Considering those examples, it is possible that the multimeric form(s) of hpol η plays a specific role in certain biological processes, although further investigation will be required.

hpol η has been shown to have active site prealignment, dNTP-metal ion binding, and third metal ion binding steps during its catalytic cycle (34, 38). In our study, the biochemical and structural results provide direct evidence that the conserved active site residues Arg-61 and Gln-38 synergistically contribute to enzyme efficiency and fidelity, further delineating the catalytic mechanism of the nucleotidyl transfer reaction catalyzed by hpol η.

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References
1. Guengerich, F. P. (2006) Interactions of carcinogen-bound DNA with individual DNA polymerases. Chem. Rev. 106, 420–452
2. Ohmori, H., Friedberg, E. C., Fuchs, R. P. P., Goodman, M. F., Hanaoka, F., Hinkle, D., Kunkel, T. A., Lawrence, C. W., Livneh, Z., Nohmi, T., Prakash, L., Prakash, S., Todo, T., Walker, G. C., Wang, Z., and Woodgate, R. (2001) The Y-family of DNA polymerases. Mol. Cell 8, 7–8
3. Yang, W., and Woodgate, R. (2007) What a difference a decade makes: insights into translesion DNA synthesis. Proc. Natl. Acad. Sci. U.S.A. 104, 15591–15598
4. Sale, J. E., Lehmann, A. R., and Woodgate, R. (2012) Y-family DNA polymerases and their role in tolerance of cellular DNA damage. Nat. Rev. Mol. Cell. Biol. 13, 731–744
Arg-61 and Gln-38 Roles in Human Polymerase η

Cell Biol. 13, 141–152

1. Yang, W. (2014) An overview of Y-family DNA polymerases and a case study of human DNA polymerase η. Biochemistry 53, 2793–2803

5. Reha-Krantz, L. J. (2010) DNA polymerase proofreading: multiple roles maintain genome stability. Biochim. Biophys. Acta 1804, 1094–1063

2. Inui, H., Oh, K. S., Nadem, C., Ueda, T., Khan, S. G., Metin, A., Gozukara, E., Emmert, S., Svor, H., Busch, D. B., Baker, C. C., DiGiovanna, J. I., Tamura, D., Seitz, C. S., Gorbatchev, A., Wu, W. H., Chung, K. Y., Chung, H. I., Azizi, E., Woodgate, R., Schneider, T. D., and Kraemer, K. H. (2008) Xeroderma pigmentosum-variant patients from America, Europe, and Asia. J. Invest. Dermatol. 128, 2055–2068

8. Masutani, C., Kusumoto, R., Yamada, A., Dohmae, N., Yokoi, M., Yuasa, M., Araki, M., Iwai, S., Takio, K., and Hanaoka, F. (1999) The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase η. Nature 399, 700–704

10. Zhao, Y., Biertümpfel, C., Gregory, M. T., Hua, Y. J., Hanaoka, F., and Yang, W. (2007) Rad54 dissociates from strand invasion intermediates of homologous recombination. Proc. Natl. Acad. Sci. U.S.A. 104, 7269–7274

12. Vaisman, A., Biertümpfel, C., Gregory, M. T., Hua, Y. J., Hanaoka, F., and Masutani, C. (2000) Efficient translesion replication past oxaliplatin and cisplatin GpG adducts by human DNA polymerase η. Biochemistry 39, 4575–4580

14. Chen, Y. W., Cleaver, J. E., Hanaoka, F., Chang, C. F., and Chou, K. M. (2005) Contribution of DNA polymerase η to immunoglobulin gene diversity to chemotherapeutic agents. Mol. Cancer Res. 537–541

16. Johnson, R. E., Kondratick, C. M., Prakash, S., and Prakash, L. (1999) hRAD30 mutations in the variant form of xeroderma pigmentosum. Science 285, 263–266

18. Hick, J. K., Chute, C. L., Paulsen, M. T., Ragland, R. L., Howelett, N. G., Güéranger, Q., Glover, T. W., and Canman, C. E. (2010) Differential roles for DNA polymerases η, zeta, and REV1 in lesion bypass of intrastrand crosslinks. Proc. Natl. Acad. Sci. U.S.A. 107, 9790–9805

20. Zeng, X., Winter, D. B., Kasmer, C., Kraemer, K. H., Lehmann, A. R., and Gearhart, P. J. (2001) DNA polymerase η is an A-T mutator in somatic hypermutation of immunoglobulin variable genes. Nat. Immunol. 2, 537–541

24. Rada, C., and Kunkel, T. A. (2002) Correlation of somatic hypermutation specificity and A-T base pair substitution errors by DNA polymerase η during copying of a mouse immunoglobulin kappa light chain transgene. Proc. Natl. Acad. Sci. U.S.A. 99, 9594–9599

26. Wilson, T. M., Vaisman, A., Martomo, S. A., Sullivan, P., Lan, L., Hanaoka, F., Yasui, A., Woodgate, R., and Gearhart, P. J. (2005) MSH2-MSH6 stimulates DNA polymerase η suggesting a role for A-T mutations in antibody genes. J. Exp. Med. 201, 637–645

28. Maga, G., Villani, G., Crespan, E., Wimmer, U., Ferrari, E., Bertocci, B., and Hübscher, U. (2007) 8-Oxo-guanine bypass by human DNA polymerase η. Structure 15, 1463–1470

30. Schorr, S., Schneider, S., Lammens, K., Hopfner, K. P., and Carell, T. (2010) Mechanism of replication blocking and bypass of Y-family polymerase η in the presence of auxiliary proteins. Nature 474, 606–608

32. Maga, G., Villani, G., Crespan, E., Wimmer, U., Ferrari, E., Bertocci, B., and Hübscher, U. (2007) 8-Oxo-guanine bypass by human DNA polymerases in the presence of auxiliary proteins. Nature 447, 606–608

34. Zahn, K. E., Wallace, S. S., and Doublé, S. (2011) DNA polymerases provide a canon of strategies for translesion synthesis past oxidatively generated lesions. Curr. Opin. Struct. Biol. 21, 358–369

36. Zhang, L., Qu, Y., Zeng, X., Wu, J., and Yang, W. (2012) DNA polymerase η promotes translesion DNA synthesis past oxidatively damaged DNA by yeast DNA polymerase η. Structure 20, 196–201

38. Solovey, L., Zhao, Y., Kondor, Y., Ramón-Maqués, S., Gregory, M., Lee, J. Y., Masutani, C., Lehmann, A. R., Hanaoka, F., and Yang, W. (2010) Structure and mechanism of human DNA polymerase η. Nature 465, 1044–1048

40. Slonim, E., Solovey, L., and Yang, W. (2012) Structural basis for the suppression of skin cancers by DNA polymerase η. Nature 459, 257–265

42.STRUCTURAL BASIS FOR ERROR-FREE SYNTHESIS OF DNA BY Yeast DNA POLYMERASE η. Nature 459, 257–265
comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* 66, 213–221
44. Langer, G. G., Hazledine, S., Wiegels, T., Carolan, C., and Lamzin, V. S. (2013) Visual automated macromolecular model building. *Acta Crystallogr. D Biol. Crystallogr.* 69, 635–641
45. Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F., and Vagin, A. A. (2011) REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr. D Biol. Crystallogr.* 67, 355–367
46. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* 66, 486–501
47. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera: a visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612
48. Furge, L. L., and Guengerich, F. P. (1999) Explanation of pre-steady-state kinetics and decreased burst amplitude of HIV-1 reverse transcriptase at sites of modified DNA bases with an additional, nonproductive enzyme-DNA-nucleotide complex. *Biochemistry* 38, 4818–4825
49. Egli, M., and Sarkhel, S. (2007) Lone pair-aromatic interactions: to stabilize or not to stabilize. *Acc. Chem. Res.* 40, 197–205
50. Eoff, R. L., Irimia, A., Angel, K. C., Egli, M., and Guengerich, F. P. (2007) Hydrogen bonding of 7,8-dihydro-8-oxodeoxyguanosine with a charged residue in the little finger domain determines miscoding events in *Sulfolobus solfataricus* DNA polymerase Dpo4. *J. Biol. Chem.* 282, 19831–19843
51. Suarez, S. C., Beardslee, R. A., Toftton, S. M., and McCulloch, S. D. (2013) Biochemical analysis of active site mutations of human polymerase δ. *Mutat. Res.* 745, 46–54
52. Wouters, J. (1998) Cation-π (Na⁺-Trp) interactions in the crystal structure of tetragonal lysozyme. *Protein Sci.* 7, 2472–2475
53. Tereshko, V., Wilds, C. J., Minasov, G., Prakash, T. P., Maier, M. A., Howard, A., Wawrzak, Z., Manoharan, M., and Egli, M. (2001) Detection of alkali metal ions in DNA crystals using state-of-the-art x-ray diffraction experiments. *Nucleic Acids Res.* 29, 1208–1215
54. Lin, H. K., Chase, S. F., Laue, T. M., Jen-Jacobson, L., and Traksels, M. A. (2012) Differential temperature-dependent multimeric assemblies of replication and repair polymerases on DNA increase processivity. *Biochemistry* 51, 7367–7382
55. Tang, K. H., Niebuhr, M., Aulabaugh, A., and Tsai, M. D. (2008) Solution structures of 2:1 and 1:1 DNA polymerase-DNA complexes probed by ultracentrifugation and small-angle x-ray scattering. *Nucleic Acids Res.* 36, 849–860
56. Tang, K. H., and Tsai, M. D. (2008) Structure and function of 2:1 DNA polymerase. DNA complexes. *J. Cell Physiol.* 216, 315–320
57. Kent, T., Chandramouly, G., McDevitt, S. M., Ozdemir, A. Y., and Pomerantz, R. T. (2015) Mechanism of microhomology-mediated end-joining promoted by human DNA polymerase η. *Nat. Struct. Mol. Biol.* 22, 230–237