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Critical amino acids in human DNA polymerases η and κ involved in erroneous incorporation of oxidized nucleotides

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ABSTRACT

Oxidized DNA precursors can cause mutagenesis and carcinogenesis when they are incorporated into the genome. Some human Y-family DNA polymerases (Pols) can effectively incorporate 8-oxo-dGTP, an oxidized form of dGTP, into a position opposite a template dA. This inappropriate G:A pairing may lead to transversions of A to C. To gain insight into the mechanisms underlying erroneous nucleotide incorporation, we changed amino acids in human Polη and Polκ proteins that might modulate their specificity for incorporating 8-oxo-dGTP into DNA. We found that Arg61 in Polη was crucial for erroneous nucleotide incorporation. When Arg61 was substituted with lysine (R61K), the ratio of pairing of dA to 8-oxo-dGTP compared to pairing of dC was reduced from 660:1 (wild-type Polη) to 7:1 (R61K). Similarly, Tyr112 in Polκ was crucial for erroneous nucleotide incorporation. When Tyr112 was substituted with alanine (Y112A), the ratio of pairing was reduced from 11:1 (wild-type Polκ) to almost 1:1 (Y112A). Interestingly, substitution at the corresponding position in Polη, i.e. Phe18 to alanine, did not alter the specificity. These results suggested that amino acids at distinct positions in the active sites of Polη and Polκ might enhance 8-oxo-dGTP to favor the *syn* conformation, and thus direct its misincorporation into DNA.

INTRODUCTION

Reactive oxygen species (ROS) are constantly generated in cells during normal aerobic metabolism. The intracellular levels of ROS are further enhanced by exposure of cells to redox agents or ionizing radiation (1–3). To counteract the potential genotoxicity and cytotoxicity of ROS, cells possess a number of defense systems, e.g. low-molecular-weight scavengers, ROS-degrading enzymes and DNA repair. Nevertheless, some ROS molecules escape the defense systems and eventually damage nearby bio-molecules including DNA, proteins and membrane lipids. Therefore, ROS has been implicated in the etiology of human degenerative diseases, aging and cancer (4,5).

DNA precursors (dNTPs) in the cellular nucleotide pool are subject to oxidation by ROS (6,7). Oxidized forms of DNA precursors include 7,8-dihydro-8-oxo-dGTP (8-oxo-dGTP), 7,8-dihydro-8-oxo-dATP (8-oxo-dATP) and 1,2-dihydro-2-oxo-dATP (2-OH-dATP). These oxidized dNTPs cause various deleterious effects in cells. For example, 8-oxo-dGTP can be incorporated opposite a dA residue in the template strand during DNA replication; this can result in an A to C transversion (8). *Escherichia coli* mutants deficient in the *mutT* gene, whose gene product hydrolyzes 8-oxo-dGTP, display spontaneous A to C transversion rates that are over 1000 times higher than those in wild-type strains (9,10). Similarly, 2-OH-dATP can be incorporated opposite template dG, and this induces G to T transversions. *Escherichia coli* mutants deficient in the *orf135* gene, whose gene product hydrolyzes 2-OH-dATP, display higher spontaneous G to T transversion rates than the

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wild-type strains (11,12). In higher-order organisms, the human MTH1 gene product, a functional counterpart of the *E. coli* MutT protein, hydrolyzes 8-oxo-dGTP, 8-oxo-dATP and also 2-OH-dATP; in contrast, MutT does not hydrolyze 2-OH-dATP (13,14). Overexpression of hMTH1 reduced total cellular 8-oxo-dG levels in human cells and transgenic mice. This overexpression also suppressed genome instability in cells with defective mismatch repair mechanisms; in addition, it caused delayed cellular senescence, and ameliorated neuropathological and behavioral symptoms in mice that resembled those of Huntington's disease (15,16). Alternatively, suppression of hMTH1 expression induced genomic DNA damage and caused accelerated cellular senescence in human skin fibroblasts (17). Mice deficient in the *Mth1* gene exhibited increased tumorigenicity in the lung, liver and stomach compared to wild-type mice (18). Thus, the nucleotide pool is a critical target of intracellular ROS, and oxidized nucleotides, unless continuously eliminated, can induce a variety of cellular abnormalities.

To exert these adverse effects, oxidized dNTPs must be incorporated into the genome DNA. Actually, in culture medium, 8-oxo-dG is readily incorporated into the genome DNA upon phosphorylation in human cells (19). Interestingly, Y-family DNA polymerases (Pols), a novel family of Pols involved in translesion DNA synthesis (20), efficiently and almost exclusively incorporated 8-oxo-dGTP into the DNA chain opposite a template dA (21). This specificity for erroneous pairing appears to be conserved in all Y-family Pols from bacteria, Archea and humans that have been examined. In *E. coli*, two Y-family Pols, i.e. Pol IV (DinB) and Pol V (UmuD′C), were involved in the erroneous incorporation of 8-oxo-dGTP and 2-OH-dATP into DNA in the *sod fur* mutants. In these mutants, intracellular ROS levels were elevated and, hence, the rates of spontaneous A to C and G to T transversions were elevated (22). The human Y-family Polη efficiently paired 8-oxo-dGTP with template dA (23). The incorporation of 8-oxo-dGTP into the genome of phage M13 by human Polη *in vitro* induced A to C transversions and deletions (24). In human cells, 8-oxo-dGTP induced an increase in the frequency of A to C mutations in the *supF* gene; this mutation frequency was reduced with the suppression of REV1, Polη and Polζ expression (25).

It has been shown that 8-oxo-dG assumes the *anti* conformation when it pairs with dC, but it assumes the *syn* conformation when pairing with dA (26,27). Therefore, we hypothesized that certain amino acids in the active sites of the Y-family Pols might force 8-oxo-dGTP to assume the *syn* conformation. In this study, we tested this hypothesis by amino acid substitutions of two Y-family human polymerases, Polη and Polκ. We changed three amino acids that might affect the specificity for pairing 8-oxo-dGTP with a template dA (28). The first candidates for amino acid alterations were the 'steric gate' amino acids, i.e. phenylalanine 18 (F18) of Polη and tyrosine 112 (Y112) in Polκ. These amino acids distinguish dNTPs and rNTPs by sensing their 2′-OH-groups (29). We reasoned that the proximity of these amino acids to the incoming dNTPs might play a critical role in determining the conformation of 8-oxo-dGTP. The second candidate for an amino acid alteration was arginine 61 (R61) in human Polη. This is the counterpart of yeast R73, which is located adjacent to the base of the incoming dNTP and stabilize dNTPs during bypass DNA synthesis across cisplatin adducts and cyclobutane pyrimidine dimers (30). The arginine residue is conserved in yeast and humans, but not in other Y-family Pols, including Polκ. The third candidate for an amino acid alteration was isoleucine 48 (I48) in Polη, which is the counterpart of yeast I59. This residue is also located close to the base of the dNTPs in the active site (30). Our results suggested that R61 in Polη and Y112 in Polκ were critical for the preferential pairing of 8-oxo-dGTP with template dA. This implies that distinctly positioned amino acids in the active sites of two Y-family Pols direct the conformation of the incoming 8-oxo-dGTP to the *syn* conformation. We discuss how R61 modulates the conformation of the incoming 8-oxo-dGTP in the active site of Polη.

MATERIALS AND METHODS

Substrates and enzymes

All oligonucleotides were purchased from BEX Corp. (Tokyo). Unaltered dNTPs were purchased from GE-Healthcare and oxidized dNTPs, i.e. 8-oxo-dGTP and 8-oxo-dATP, were purchased from TriLink BioTechnologies. 2-OH-dATP was kindly provided by Dr H. Kamiya (Hokkaido University). Human Polκ and the mutant Polκ with an alanine substitution at Y112 (Y112A) were prepared as described previously (31). The human Polη mutant enzymes included substitutions at F18 with alanine (F18A), at R61 with alanine, methionine, asparagine, glutamine, histidine or lysine (R61A, R61M, R61N, R61Q, R61H or R61K), and at I48 with serine, methionine, phenylalanine, asparagine or glutamine (I48S, I48M, I48F, I48N or I48Q). Polη and its mutant proteins were prepared with the pET21bXPV(1-511) plasmid that carried the human Polη cDNA sequence. Site-directed mutagenesis protocols were used to exchange the DNA sequences that encoded the targeted amino acids (QuickChange Lightening Site-Directed Mutagenesis Kit; STRATAGENE). The resulting plasmids were transformed into Rosetta (DE3) plysS cells (Novagen). The cells were grown in 1 l of Luria–Bertani (LB) medium until the cell density reached an $OD_{600} = 0.6$. The expression of Polη and the mutant proteins were induced with the addition of 0.2 mM IPTG at 15°C for 10 h (32). The resulting cell pastes were resuspended in lysis buffer comprised of 50 mM potassium phosphate buffer pH 7.0, 500 mM NaCl, 10% sucrose, 20% glycerol, 1× BugBuster (Novagen), 5 mM imidazole, 5 mM β-mercaptoethanol, benzonase nuclease (Novagen) and complete EDTA-free, which is a protease inhibitor cocktail (Roche). The resuspended mixtures were incubated on ice for 30 min. The lysates were clarified by centrifugation at 20 000*g* for 20 min at 4°C. TALON super flow metal affinity resin (Clontech) was washed twice with

wash buffer (50 mM potassium phosphate buffer pH 7.0, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol, complete EDTA-free), and the washed resin was gently mixed with the supernatant containing the Polη or the mutant proteins for 30 min. After three subsequent washings, the resins were placed on the column, and washed twice. The Polη and the mutant proteins were eluted with 5 ml of elution buffer (the wash buffer plus 350 mM imidazole). For kinetics analyses, the proteins were further purified with HiLoad 16/60 Superdex 200 pg and HiTrap Heparin HP columns (both from GE Healthcare) with an FPLC system (AKTAexplorer 10 s, GE Healthcare). The pooled fractions containing wild-type hPolη, R61A and R61K were dialyzed against 25 mM Tris–HCl pH 7.5, 2.5 mM β-mercaptoethanol and 50% glycerol. All purified proteins were stored at −80°C.

Incorporation of oxidized dNTPs

DNA extension assays were performed to test the behavior of the native and mutant Pols. The DNA primers used in the DNA extension assays were annealed with the template DNA sequence (5′-GAAGG GATCCTTAAGACNGTAACCGGTCTTCGCGCG-3′, where N represents A, C, G or T) at a molar ratio of 1 : 1.2. The template/primer (100 nM) was combined with appropriate concentrations of Polη (5 nM), Polκ (10 nM) and mutant proteins (5, 50 or 100 nM), and incubated in a reaction buffer [40 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM dithiothreitol, 100 μg/ml BSA, 60 mM KCl, 2.5% glycerol] with 50 μM of oxidized dNTPs for 10 min at 37°C. The exact concentrations of proteins are described in legends of figures and Supplementary Figures. Reactions were terminated by addition of the termination buffer (98% formamide, 10 mM EDTA, 10 mg/ml Blue dextran). After heat denaturation, the mixtures were loaded onto a denaturing 15% polyacrylamide gel for electrophoresis (PAGE), and run with a buffer containing 8 M urea. The products were visualized with a Molecular Imager FX Pro System (Bio-Rad Laboratories) and quantified with Quantity One software (Bio-Rad Laboratories). For steady-state kinetic analyses of incorporation of oxidized dNTP, the reactions were carried out with 1 nM Polη and 5 nM Polκ. Both the concentrations of dNTP and the incubation times were varied according to the activity of a given enzyme. The products were analyzed as described in the primer extension assay. The rate of incorporation was plotted against dNTP concentrations, and the apparent Michaelis–Menten constant, K_m and V_{max} values were determined by Enzyme Kinetics Module 1.1 of SigmaPlot 2001 software (SPSS Inc., IL). The k_{cat} was calculated by dividing the V_{max} by the enzyme concentration. All values represent means plus standard errors from three independent experiments.

Translesion DNA synthesis assay

Primer DNA (5′-CTTCCCTAGGAATTCTGC-3′) labeled with Cy3 at the 5′-end was annealed to template DNA (5′-GCGCGCTTCTGGCCAATXGCAGAATTC CTAGGGAAG-3′, where X represents 8-oxo-dG or 8-oxo-dA) at a molar ratio of 1: 1.2. Similarly, primer DNA (5′-CTTCCCTAGGAATT-3′) labeled with Cy3 at the 5′-end was annealed with template DNA (5′-GCGCG CTTCTGGCCAATTGCAGAATTCCTAGGGAAG-3′, where TT represents a *cis-syn* thymine dimer) at a ratio of 1 : 1.2. The template/primer containing 8-oxo-dG or 8-oxo-dA were incubated with 5 nM of Polη and R61K, or 10 nM of wild-type Polκ and 100 nM of Y112A. The reaction buffer contained 5 μM of unaltered dNTPs, and the reactions were incubated for 10 min at 37°C. The template/primer that contained the thymine dimer was incubated with 1, 5 and 20 nM of either wild-type Polη or R61K in the reaction buffer. This reaction buffer contained 250 μM dNTPs, and the reactions were incubated for 5 min at 37°C. The products were analyzed as described earlier.

Modeling 8-oxo-dGTP in the active site of Polη

Models were created of 8-oxo-dGTP in the *syn* conformation opposite a dA residue and in the *anti* conformation opposite a dC residue in the active site of yeast Polη by 2007.09 version of the Molecular Operating Environment (MOE: Chemical Computing Group Inc., Montreal, Canada). These models were based on the reported crystallographic structures [PDB#: 2R8J (21)]. The active site of yeast Polη was displayed with Gaussian surfaces. The template and primer sequences were 3′-CACCTACT CX-5′ and 5′-GTGGATGAG-3′, respectively, where X represents the dA or dC that was paired with 8-oxo-dGTP.

RESULTS

The steric gate of Polκ is involved in the orientation of incoming 8-oxo-dGTP

The steric gate amino acids, which distinguish between dNTP and rNTP, are highly conserved. In most Pols, including those in the Y-family, phenylalanine or tyrosine forms the steric gate (29). In human Polκ, Y112 acts as the steric gate and also plays important roles in both the insertion of dCTP opposite a benzo[a]pyrene diolepoxide-N^2-dG adduct in the template DNA and the extension of mismatched termini (31). In Polη, F18 is assumed to be the steric gate, based on the amino acid sequence alignment and the crystallographic structure comparison with yeast Polη (Supplementary Figure S1) (30,33,34). We found that the mutant of Polη with the alanine substitution of F18 (F18A) was able to incorporate both rNTPs and dNTPs into DNA. However, its ability to incorporate dNTPs was substantially attenuated compared to the native Pol (data not shown). As the steric gates lie adjacent to the incoming dNTPs, they may affect the specificity incorporating oxidized dNTPs into DNA. To examine the possibility, we compared the specificities of Polκ versus Y112A and Polη versus F18A for preferentially pairing 8-oxo-dGTP with template dA. We found that Polκ showed a preference for pairing 8-oxo-dGTP with template dA (Figure 1A). However, this preferential pairing was absent in Y112A, which

paired 8-oxo-dGTP with both dA and dC with nearly equal frequency. The kinetic analyses indicated that the substitution of Y112 with alanine reduced the efficiency (k_{cat}/K_m) of pairing 8-oxo-dGTP with template dA by more than 200-fold. This resulted in nearly equal frequencies of pairing 8-oxo-dGTP with template dA and dC (Table 1). In contrast, both the wild-type Polκ and the Y112A mutant showed similar specificities for mainly incorporating 8-oxo-dATP opposite dT and 2-OH-dATP with dT and dG (Figure 1B and C).

We also examined the specificity of wild-type Polκ and Y112A for translesion DNA synthesis across 8-oxo-dG and 8-oxo-dA lesions in template strands. We found that both Pols had similar specificity for inserting dATP and, less frequently dCTP, opposite a template 8-oxo-dG; both also preferentially inserted dTTP opposite to a template 8-oxo-dA (Supplementary Figure S2). These data indicated that the Y112 residue in Polκ was involved in the orientation of incoming 8-oxo-dGTP opposite dA, but not other specificity, i.e. incorporating 8-oxo-dATP or 2-OH-dATP into DNA or for a translesion bypass across an oxidized dG or dA in template strands.

In Polη, the corresponding substitution of F18 by alanine did not alter the specificity for incorporating oxidized dNTPs, including 8-oxo-dGTP, into DNA (Supplementary Figure S3). Therefore, we concluded that the steric gate of Polκ, but not Polη, was critical for inducing incoming 8-oxo-dGTP into the *syn* conformation in the active site, thereby facilitating its pairing with dA.

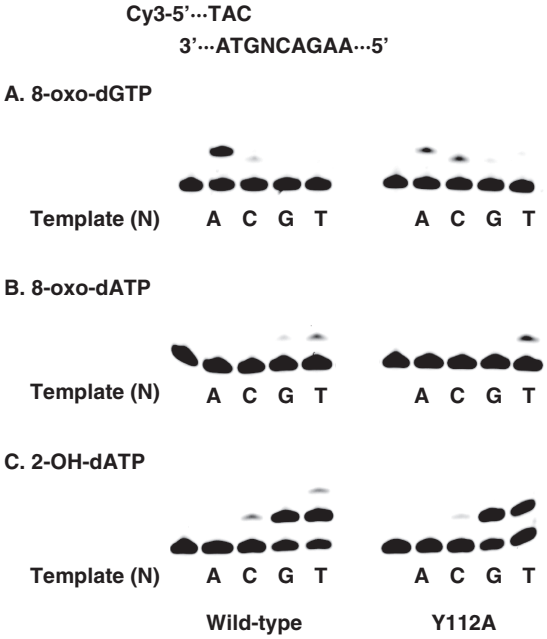

Figure 1. Incorporation of oxidized dNTPs into DNA by Polκ and its Y112A mutant. The primer/template DNA (100 nM, N in the template strand represents A, C, G or T) was incubated with wild-type Polκ (10 nM) or Y112A mutant (100 nM) in the presence of 50 μM 8-oxo-dGTP (**A**), 8-oxo-dATP (**B**) or 2-OH-dATP (**C**) for 10 min at 37°C. Extended primers were separated by denaturing PAGE. The first lanes represent the results of control experiments where no dNTPs were added to the reaction mixtures.

R61 of Polη determines specificity for pairing 8-oxo-dGTP with template dA

As F18 did not appear to be involved in the orientation of 8-oxo-dGTP in the active site of Polη, we investigated other amino acids that might influence its specificity for incorporating 8-oxo-dGTP into DNA. We reasoned that R61 was the best candidate, because it was predicted to lie adjacent to the base of the incoming dNTP. Thus, we substituted R61 with alanine, methionine, asparagine, glutamine, histidine or lysine. We also changed I48 to alanine, serine, methionine, phenylalanine, asparagine, glutamine, aspartic acid or glutamic acid because it was also predicted to lie close to the base of the incoming dNTP. We found that R61A, R61M, R61N, R61Q, R61H and all the I48 mutants displayed similar specificity to the wild-type Polη and/or exhibited extremely low abilities to incorporate 8-oxo-dGTP into DNA (Supplementary Figure S4). However, we found that R61K was able to pair 8-oxo-dGTP with dC or dA with nearly equal specificity (Figure 2A). In contrast, the wild-type Polη preferentially paired 8-oxo-dGTP with dA (Figure 2A). In addition to changing the preference for incorrect pairing of 8-oxo-dGTP, R61K paired 8-oxo-dATP more efficiently with dT than with dG. In contrast, the wild-type Polη showed almost equal specificity for pairing 8-oxo-dATP with dT and dG (Figure 2B). However, R61K exhibited equal specificity for pairing 2-OH-dATP with dG, dT and dC, and less efficiency for pairing with dA; this specificity was similar to that observed for the wild-type Polη (Figure 2C). The wild-type Polη and R61K showed similar translesion activities for DNA synthesis across 8-oxo-dG, 8-oxo-dA, or a thymine dimer in the template strands

Table 1. Steady-state kinetic parameters for 8-oxo-dGTP by wild-type Polκ and Y112A mutant

Template/dNTP	k_{cat}, min^{-1}		K_m, μM		k_{cat}/K_m, μM^{-1} min^{-1}		Y112A/WT
	WT[a]	Y112A	WT	Y112A	WT	Y112A	
dA/dTTP	14 ± 3.0	9.2 ± 3.5	4.3 ± 2.0	72 ± 52	3.3	0.13	1/26
dA/8-oxo-dGTP	2.1 ± 0.38	0.029 ± 0.0052	83 ± 34	300 ± 140	0.026	0.000096	1/270
dC/dGTP	4.4 ± 0.64	5.4 ± 0.33	2.1 ± 0.84	20 ± 3.4	2.1	0.28	1/7.7
dC/8-oxo-dGTP	0.70 ± 0.12	0.11 ± 0.030	290 ± 120	660 ± 360	0.0024	0.00016	1/15

[a]WT: wild-type Polκ

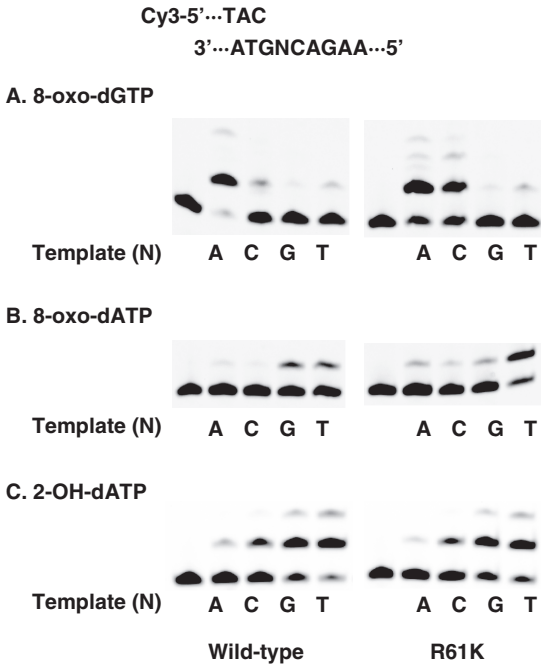

Figure 2. Incorporation of oxidized dNTPs into DNA by Polη and its R61K mutant. The primer/template DNA (100 nM, N in the template strand represents A, C, G or T) was incubated with wild-type Polη and R61K mutant (5 nM) in the presence of 50 μM 8-oxo-dGTP (**A**), 8-oxo-dATP (**B**) or 2-OH-dATP (**C**) for 10 min at 37°C. Extended primers were separated by denaturing PAGE. The first lanes represent the results of control experiments where no dNTPs were added to the reaction mixtures.

(Supplementary Figure S5). Thus, the effect of the R61K mutation appeared to be specific for conferring specificity for the incorporation of 8-oxo-dGTP and 8-oxo-dATP into DNA.

To quantitatively compare the efficiencies (k_{cat}/K_m) of incorporating 8-oxo-dGTP and 8-oxo-dATP into DNA among the wild-type Polη, R61K and R61A, we determined the kinetic parameters of each Pol for incorporating unaltered and oxidized dNTPs into DNA (Table 2). We found that the kinetic parameters for pairing unaltered dNTPs with the correct template bases were almost identical between the wild-type Polη and R61K. Likewise, the kinetics were not substantially different between the two Pols for pairing 8-oxo-dGTP and 8-oxo-dATP with incorrect template bases (8-oxo-dGTP with dA and 8-oxo-dATP with dG). However, R61K exhibited efficiencies that were 24-fold and 14-fold higher than wild type for correctly pairing 8-oxo-dGTP with dC and pairing 8-oxo-dATP with dT. Similarly, R61A exhibited efficiencies that were 1.7-fold higher than wild type for pairing both 8-oxo-dGTP and 8-oxo-dATP with dC and dT, respectively. These results suggested that R61 in Polη specifically inhibited pairing the C8-oxidized deoxypurine triphosphates with the correct template bases; i.e. 8-oxo-dGTP with dC and 8-oxo-dATP with dT. We concluded, therefore, that R61 was critical for orienting the incoming 8-oxo-dGTP into the *syn* conformation in the active site of Polη.

DISCUSSION

Oxidation of the nucleotide pool can be a source of DNA damage when oxidized dNTPs are incorporated into DNA by Pols during chromosome replication (35). However, oxidized dNTPs are generally poor substrates for Pols. For example, calf thymus Polδ incorporated 8-oxo-dGTP into DNA with only about 10^{-4} times the efficiency showed for incorporating unaltered dGTP; moreover, the Pol exhibited a preference for correctly pairing 8-oxo-dGTP with template dC (36). Several other Pols exhibited poor efficiencies for 8-oxo-dGTP incorporation into DNA, including human Polγ, T7 Pol exo$^-$, HIV reverse transcriptase, *E. coli* Pol II, ϕ29 Pol and Klenow exo$^-$ (37,38). An exception may be human Polβ, which incorporated 8-oxo-dGTP into DNA with 10–20% of the efficiency it showed for unaltered dGTP incorporation, and it showed a preference for incorrect pairing with template dA (39). Calf thymus Polα incorporated 2-OH-dATP with <1% and 0.1% of the efficiencies it showed for incorporating unaltered dATP and dGTP, respectively; moreover, the Pol showed a similar preference for pairing 2-OH-dATP with template T and C (40). In this respect, it is interesting that Y-family Pols from bacteria, Archaea and humans exhibit a preference for incorrectly pairing 8-oxo-dGTP with template dA (21,22,24) and human Polη incorporates it with a relatively high efficiency (23,41).

To examine the mechanisms underlying the conserved specificity of Y-family Pols for incorrectly pairing 8-oxo-dGTP with template dA, we substituted amino acids of human Polη and Polκ, which might modulate the specificity. The results indicated that R61 of Polη and Y112 of Polκ played important roles in the preferential pairing of 8-oxo-dGTP with template dA. In Polη, when R61 was substituted with alanine, the ratio of pairing of dA to 8-oxo-dGTP compared to pairing of dC was reduced from 660 : 1 in wild type to 65 : 1 in R61A (0.79 : 0.0012 in wild type and 0.13 : 0.0020 in R61A, Table 2). Moreover, the substitution of R61 with lysine (R61K) resulted in the ratio of pairing of dA to 8-oxo-dGTP compared to pairing of dC of 7 : 1 (0.19 : 0.028 in R61K, Table 2, Figure 2). In Polκ, the Y112A mutant had less preference for incorrectly pairing 8-oxo-dGTP with template dA (Figure 1). This was primarily because the replacement of Y112 with A more severely reduced its efficiency for pairing 8-oxo-dGTP with template dA (270-fold reduction) compared to the reduction in its efficiency for pairing with template dC (15-fold reduction) (Table 1). Thus, the ratio of pairing of dA to 8-oxo-dGTP compared to pairing of dC was reduced from 11 : 1 to 0.6 : 1 by the Y112A amino acid substitution (0.026 : 0.0024 in wild type and 0.000096 : 0.00016 in Y112A, Table 1). In addition to its role as the steric gate, Y112 was previously shown to be involved in bypass DNA synthesis; e.g. pairing dCTP with template a benzo[a]pyrene diol epoxide-N^2-dG adduct and the extension of primers with terminal mismatches (31). Thus, the amino acid at the 112 position may interact with both the base and the sugar moiety of the incoming dNTPs. These data suggest that

Table 2. Steady-state kinetic parameters for C8-oxidized dNTP by wild-type Polη and R61 mutants

Template/dNTP	hPolη	k_{cat}, min^{-1}	K_m, μM^{-1}	k_{cat}/K_m, μM^{-1} min^{-1}	Related efficiency
dA/dTTP					
	WT[a]	28 ± 2.7	5.1 ± 1.4	5.4	1.0
	R61K	40 ± 5.9	17 ± 4.5	2.3	0.43
	R61A	8.0 ± 1.2	12 ± 3.5	0.7	0.13
dA/8-oxo-dGTP					
	WT	18 ± 1.8	22 ± 6.2	0.79	1.0
	R61K	21 ± 4.8	110 ± 41	0.19	0.24
	R61A	5.4 ± 1.3	42 ± 23	0.13	0.16
dC/dGTP					
	WT	11 ± 1.8	3.1 ± 1.2	3.6	1.0
	R61K	17 ± 1.2	4.6 ± 0.71	3.7	1.0
	R61A	6.3 ± 1.8	6.3 ± 3.5	1.0	0.27
dC/8-oxo-dGTP					
	WT	0.87 ± 0.21	720 ± 320	0.0012	1.0
	R61K	4.6 ± 0.58	160 ± 49	0.028	23
	R61A	0.37 ± 0.050	180 ± 56	0.0020	1.7
dG/dCTP					
	WT	33 ± 2.9	3.2 ± 87	10	1.0
	R61K	38 ± 3.3	7.4 ± 1.5	5.1	0.49
	R61A	14 ± 2.1	3.7 ± 1.7	3.7	0.35
dG/8-oxo-dATP					
	WT	0.90 ± 0.16	370 ± 150	0.0024	1.0
	R61K	0.78 ± 0.I4	510 ± 190	0.0015	0.63
	R61A	0.33 ± 0.063	180 ± 100	0.0018	0.74
dT/dATP					
	WT	18 ± 2.4	5.7 ± 1.5	3.1	1.0
	R61K	22 ± 2.8	6.8 ± 1.7	3.2	1.0
	R61A	14 ± 4.6	11 ± 5.7	1.3	0.43
dT/8-oxo-dATP					
	WT	0.56 ± 0.070	150 ± 46	0.0036	1.0
	R61K	3.2 ± 0.33	61 ± 16	0.052	14
	R61A	0.26 ± 0.016	43 ± 9.2	0.0061	1.7

[a]WT: wild-type hPolη

Y112 may stabilize the pairing of 8-oxo-dGTP with template dA in the active site of the Pol. In contrast, the F18 position of Polη, which corresponds to Y112 in Polκ, did not appear to play a role in the specificity for pairing 8-oxo-dGTP with template dA. Therefore, amino acids involved in the specificity of incorporating 8-oxo-dGTP into DNA are not conserved between Polη and Polκ, despite the fact that they belong to the same Y-family.

In Polη, the substitution of R61 with lysine elevated its preferences for pairing 8-oxo-dGTP with dC and 8-oxo-dATP with dT without affecting other activities; e.g. incorporating unaltered dNTP into DNA or bypassing oxidized dG or dA or thymine dimers in template DNA (Figure 2, Table 2, Supplementary Figure S5). These results suggested that R61 specifically inhibited the ability to correctly pair C8-oxidized dNTPs with template DNA. To investigate how R61 modulated the conformation of incoming 8-oxo-dGTP in the active site, we modeled the predicted *syn* and *anti* structures of 8-oxo-dGTP paired with dA or dC in the active site of yeast Polη. In this model, arginine 73, which corresponds to R61 of human Polη, was substituted with lysine or alanine (Figure 3). The model suggested that the side chain of arginine may constitute a steric constraint, because it would not accommodate the O^8 of an 8-oxo-dGTP in the *anti* conformation, but could accommodate it in the *syn* conformation (Figure 3A). This may be the basis for the preference shown by Polη for pairing 8-oxo-dGTP with template dA. Interestingly, when the arginine was substituted with lysine, the model indicated that the ε-amino group and O^8 of 8-oxo-dGTP in the *anti* conformation may be interacted electrostatically (Figure 3B). These results suggested that the lysine, but not arginine, might stabilize 8-oxo-dGTP in the *anti* formation; this would enhance the preference for correctly pairing 8-oxo-dGTP with template dC. In the case of alanine, the side chain did not interact with 8-oxo-dGTP (Figure 3C). Thus, the slight enhancement in the preference of R61A for pairing 8-oxo-dGTP with template dA may be due to the lack of a steric constraint of R61 in the active site of Polη. This prediction, based on model structure, could also be applicable to the preference of Polη for correctly pairing 8-oxo-dATP with template dT; the arginine may not accommodate the 8-oxo-dATP in the *anti* conformation, and the substituted lysine may induce 8-oxo-dATP to assume the *anti* conformation with the electrostatic interaction, and thus, would result in pairing with template dT. These predictions are in accordance with our biochemical findings that R61A and R61K exhibited increased preferences for pairing 8-oxo-dGTP with dC and 8-oxo-dATP with dT compared to the wild-type Polη (Table 2). Therefore, R61 appeared to play a critical role in modulating the *anti* or *syn* conformation of 8-oxo-dGTP in the active site of Polη. In human

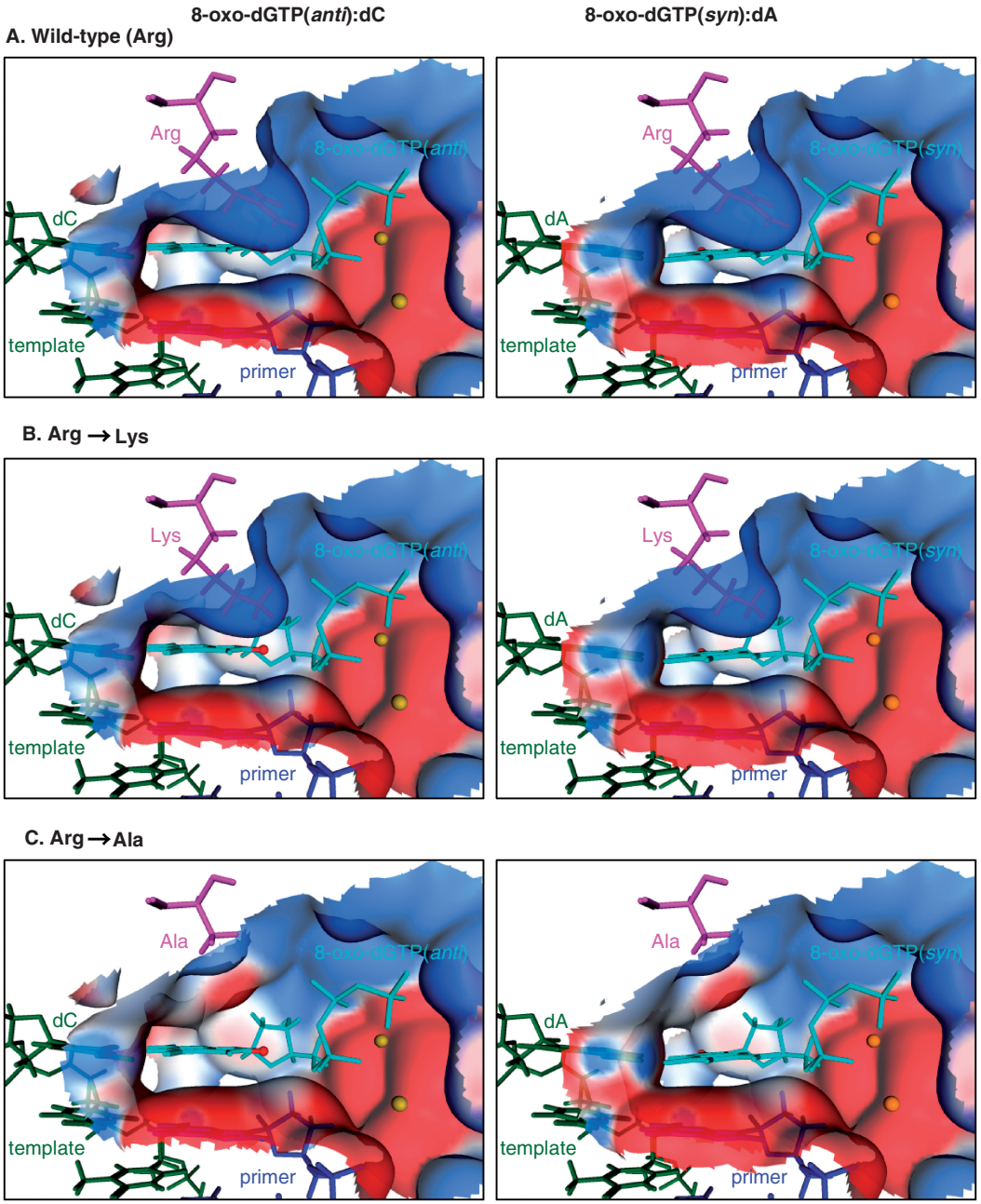

Figure 3. Molecular models of the incoming 8-oxo-dGTP in the active site of Polη. When the incoming 8-oxo-dGTP (cyan stick) forms the *anti* conformation (left panels), the side chain of arginine (displayed as a purple stick) may sterically clash with O^8 (red ball) of the incoming 8-oxo-dGTP, which may be the reason for the poor incorporation of 8-oxo-dGTP opposite template dC (green stick) (**A**). Proper pairing with template dC can be achieved, however, when arginine is substituted with lysine (**B**) or alanine (**C**) because of no steric hindrance. An electrostatic interaction between the lysine residue and O^8 of 8-oxo-dGTP may facilitate the incorporation of 8-oxo-dGTP opposite template dC (B). When the incoming 8-oxo-dGTP forms the *syn* conformation (right panels), there appear no steric or electrostatic interactions between the amino acids and O^8 of 8-oxo-dGTP (A–C). All models were constructed based on the crystal structure of yeast Polη [PDB#2R8J (21)]. The active site of Polη is displayed as Gaussian surface (colored according to its electric charge).

Polι, there is a lysine residue at the position corresponding to the R61 of Polη (Supplementary Figure S1). However, Polι always paired 8-oxo-dGTP with dA. Thus, the mechanism underlying the preference of Polη for pairing 8-oxo-dGTP with template dA may be unique to this enzyme.

Human Polβ has an asparagine in its active site, i.e. N279, that was shown to be involved in its specificity for the incorporation of oxidized dNTPs (39). Polβ was shown to preferentially pair 8-oxo-dGTP with template dA. The substitution of N279 with alanine (N279A) reduced the activity of Polβ incorporating 8-oxo-dGTP with template dA by almost 1000-fold, but it decreased the activity incorporating it with template dC by about 3-fold. Thus, the ratio of pairing of dA to 8-oxo-dGTP compared to pairing of dC was altered from 24 : 1

(wild-type Polβ) to 1 : 14 (N279A). It was proposed that the side chain of N279 might favorably interact with 8-oxo-dGTP in the *syn* conformation. For instance, there may be a hydrogen bond between the O^8 of 8-oxo-dGTP in the *syn* conformation and the asparagine side chain of the Pol; this would stabilize the *syn* conformation. This proposed mechanism is contrary to the mechanism we proposed here for Polη, where the side chain of R61 appeared to disturb the formation of the *anti* conformation and thus enhance the formation of the *syn* conformation. Bacteriophage ϕ29 Pol, which belongs to the B-family, has shown a preference for pairing 8-oxo-dGTP with dC over dA; thus the ratio of pairing of dA to 8-oxo-dGTP compared to pairing of dC was 3 : 1 (38). It has been shown that the lysine residue (K560) of ϕ29 Pol was involved with this specificity. Contrary to Polη, the K560 residue appeared to collide with the N^1, N^2 and O^6 atoms of 8-oxo-dGTP in the *syn* conformation, thus inducing the *anti* conformation and correct pairing activity. These results show that the amino acids that govern the specificity for the incorporation of 8-oxo-dGTP into DNA are distinct among various Pols.

Why do various Pols have different mechanisms to govern the specificity for the incorporation of oxidized dGTP? In general, replicative Pols incorporate 8-oxo-dGTP poorly and prefer to incorporate it opposite template dC as described earlier. This seems reasonable because the poor incorporation and correct specificity contribute to the genome stability. In this regard, it is understandable that the replicative Pols have effective mechanisms to exclude 8-oxo-dGTP pairing with template dA such as collision of K560 of bacteriophage ϕ29 pol with the N^1, N^2 and O^6 atoms of 8-oxo-dGTP in the *syn* conformation (38). In contrary, Polβ (X-family) and Y-family Pols incorporate 8-oxo-dGTP into DNA in relatively high efficiency and their incorporation specificity is opposite template dA rather than template dC (41). Because of their roles in DNA repair and translesion bypass, DNA synthesis may be more important (higher priority) than the fidelity. Therefore, they may incorporate dNTPs, even oxidized ones, into DNA in relatively high yield. Incorporation of 8-oxo-dGTP opposite dA may be beneficial for the cells exposed to oxidative stress. For example, pairing of 8-oxo-dGMP:dA may induce genetic diversity, which may be helpful to survive in stressful environment for Archaea and bacteria (42). The pairing may be recognized by mismatch repair proteins (43) and the recognition may induce apoptosis in mammalian cells, which eliminates cells damaged by ROS (44). Each Y-family Pol may have created its own mechanism to incorporate it opposite template dA during evolution.

In summary, human Polη and Polκ showed a preference for incorrectly pairing 8-oxo-dGTP with template dA, and this specificity appeared to be caused by the presence of distinct amino acids. The Y112 residue in Polκ may interact with the sugar and/or base of the incoming 8-oxo-dGTP, thereby forcing it to assume the *syn* conformation in the active site. The R61 residue of Polη appeared to inhibit the *anti* conformation of 8-oxo-dGTP by steric and/or electrostatic hindrance between the O^8 of 8-oxo-dGTP and the side chain of R61. These key amino acids had distinct positions in the active sites, but both were involved in inducing the *anti* conformation of 8-oxo-dGTP. Thus, we proposed that two Y-family Pols, human Polη and Polκ, employed different mechanisms for achieving the same specificity for incorporating oxidized dNTPs into DNA. The efficiency and specificity of Polη and Polκ for pairing 8-oxo-dGTP with template dA during DNA synthesis may lead to transversions of A to C. We are currently investigating this hypothesis with human cells in our laboratory.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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