Molecular and structural characterization of oxidized ribonucleotide insertion into DNA by human DNA polymerase β

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During oxidative stress, inflammation, or environmental exposure, ribo- and deoxyribonucleotides are oxidatively modified. 8-Oxo-7,8-dihydro-2′-guanosine (8-oxo-G) is a common oxidized nucleobase whose deoxyribonucleotide form, 8-oxo-dGTP, has been widely studied and demonstrated to be a mutagenic substrate for DNA polymerases. Guanine ribonucleotides are analogously oxidized to r8-oxo-GTP, which can constitute up to 5% of the rGTP pool. Because ribonucleotides are commonly misinserted into DNA, and 8-oxo-G causes replication errors, we were motivated to investigate how the oxidized ribonucleotide is utilized by DNA polymerases. To do this, here we employed human DNA polymerase β (pol β) and characterized r8-oxo-GTP insertion with DNA substrates containing either a templating cytosine (nonmutagenic) or adenine (mutagenic). Our results show that pol β has a diminished catalytic efficiency for r8-oxo-GTP compared with canonical deoxyribonucleotides but that r8-oxo-GTP is inserted mutagenically at a rate similar to those of other common DNA replication errors (i.e. ribonucleotide and mismatch insertions). Using FRET assays to monitor conformational changes of pol β with r8-oxo-GTP, we demonstrate impaired pol β closure that correlates with a reduced insertion efficiency. X-ray crystallographic analyses revealed that, similar to 8-oxo-dGTP, r8-oxo-GTP adopts an anti conformation opposite a templating cytosine and a syn conformation opposite adenine. However, unlike 8-oxo-dGTP, r8-oxo-GTP did not form a planar base pair with either templating base. These results suggest that r8-oxo-GTP is a potential mutagenic substrate for DNA polymerases and provide structural insights into how r8-oxo-GTP is processed by DNA polymerases.

Oxygen radicals produced during oxidative stress can damage bases within both duplex DNA and the nucleotide pool, causing cytotoxicity and mutagenesis (1, 2). Specifically, oxidation of the nucleotide pool has been shown to be a significant contributor to DNA damage (3–6). Guanine is particularly susceptible to oxidation and readily forms 8-oxo-7,8-dihydro-2′-guanosine (8-oxo-G)² (7). The deoxynucleotide form of 8-oxo-G is a highly mutagenic nucleobase substrate for DNA polymerases and, consequently, has been implicated in several human diseases (8–11). In contrast, the ribonucleotide form of 8-oxo-G (r8-oxo-G) remains to be fully characterized. The r8-oxo-G lesion has two deviations from the canonical nucleotide: an adducted oxygen on the nucleotide base (O8) and an additional oxygen on the ribose sugar (O2′) (Fig. 1). Importantly, the combined effect of both noncanonical oxygens during nucleotide discrimination is not well-understood.

During DNA replication, polymerases must select the correct base and sugar combination to ensure high fidelity. DNA polymerase β (pol β) is an established mammalian model for studying nucleotide discrimination because of its amenability to structure–function studies and conformational nucleotide selection mechanism akin to replicative polymerases (12, 13). The conformational change occurs upon correct nucleotide binding, where the polymerase N-subdomain closes to facilitate key nucleic acid–protein interactions for efficient catalysis (12–18). Following closure, catalysis is carried out by nucleophilic attack of the primer terminus oxyanion (O3′) on the α-phosphate (αP) of the incoming nucleotide, resulting in deoxyribose monophosphate incorporation and generation of a pyrophosphate (19).

There are several key steps involved in pol β closure and nucleotide insertion that discriminate between correct and incorrect nucleotides (20). To discriminate against ribonucleotides, Tyr-271 on α-helix M acts as a steric gate to prevent insertion of incoming ribonucleotides. Specifically, the backbone carbonyl of Tyr-271 clashes with ribose O2′ of the incoming nucleotide (Fig. 2) (21, 22). Although most DNA polymerases have an equivalent steric gate, misincorporation of ribonucleotides is an extremely common replication error that is estimated to occur millions of times per cell division in normal cells (23–25). Moreover, mildly oxidative conditions in human cells results in oxidation of 0.2%–5% of free guanine

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1 To whom correspondence should be addressed: E-mail: bfreudenthal@kumc.edu.
2 The abbreviations used are: 8-oxo-G, 8-oxo-7,8-dihydro-2′-guanosine; pol β, DNA polymerase β; AEDANS, N-(acetylaminoethyl)-5-naphthylamine-1-sulfonic acid; IAEDANS, 5(((2-iodoacetyl)amino)(ethyl)amino)-naphthalene-1-sulfonic acid.

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ribonucleotides (rGTP) into r8-oxo-GTP (4, 24, 26). Recent studies in yeast, bacteria, and humans have shown that r8-oxo-GTP is incorporated into DNA, indicating that r8-oxo-GTP is a potential polymerase substrate (27–29). Furthermore, human DNA polymerase \( \beta \) is unable to proofread lesions containing either a O2' or O8, suggesting reduced proofreading of r8-oxo-GMP during replication (24, 30).

To gain mechanistic insight into r8-oxo-GTP insertion, we utilized pol β for structure–function studies of the nucleotidyl transferase reaction with an incoming r8-oxo-GTP. Here we report precatalytic ternary (pol β:DNA:rNTP) substrate X-ray crystallographic structures of r8-oxo-GTP opposite templating adenine (mutagenic) or cytosine (nonmutagenic) FRET measurements of pol β closure, and kinetic characterization of r8-oxo-GTP insertion efficiencies. Our results provide molecular insight into how DNA polymerases incorporate r8-oxo-GTP into DNA.

**Results**

**Kinetic characterization of r8-oxo-GTP insertion**

The efficiency of r8-oxo-GTP and rGTP insertion into a single-nucleotide gap by pol β was measured with both a templating cytosine (dC) and adenine (dA) (Fig. 3). To contextualize r8-oxo-GTP insertion, these insertion efficiencies were compared with undamaged deoxyribonucleotide (dGTP) and oxidized deoxyribonucleotide (8-oxo-dGTP) insertion (11, 12). Relative to correct dGTP insertion, r8-oxo-GTP insertion efficiency opposite dC and dA is reduced more than 250,000-fold and 4,000-fold, respectively. The 60-fold preference for insertion of r8-oxo-GTP opposite dA, compared with dC, demonstrates the mutagenicity of r8-oxo-GTP. This is consistent with 8-oxo-dGTP, which is also preferentially inserted opposite dA. However, insertion of r8-oxo-GTP is less efficient than 8-oxo-dGTP by 700- and 4,300-fold opposite dA and dC, respectively. This demonstrates that the ribose O2' hinders insertion of the 8-oxo-G base, but the effect is not strictly additive. For comparison, the O2' of the undamaged base (rGTP) reduces insertion efficiency by 3,500-fold opposite the correct base, dC. Notably, rGTP is extremely difficult to misinsert opposite the wrong base; rGTP insertion opposite dA could not to be measured in our assay or previously (31). Despite the reduced efficiencies imparted by the O2', ribonucleotides are still frequently inserted into DNA (25), and we show that mutagenic r8-oxo-GTP insertion has a similar efficiency as ribonucleotide insertion. To provide molecular insight into the basis of these observations, we turned to a FRET polymerase closure assay and X-ray crystallography.

**Polymerase closure is impaired for ribonucleotide and mismatched substrates**

To evaluate how polymerase subdomain closure might contribute to the decrease in kinetic efficiency for r8-oxo-GTP insertion, we utilized a steady-state FRET-based assay with pol β. Closure of the N-subdomain, triggered by binding of the nucleotide, was measured using FRET between the fluorescent AEDANS label, located at position V303C of the N-subdomain, and the quencher (Dabcyl) located at position –8 relative to the templating base of the DNA substrate (Fig. S2). As expected, the correct pairing of dGTP opposite dC results in closure of pol β,
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Figure 3. Discrimination plot evaluating r8-oxo-GTP insertion. The catalytic efficiencies ($k_{cat}/K_m$) for insertion of r8-oxo-GTP opposite dC (yellow) or dA (green) for pol β are shown. The distance between the respective catalytic efficiencies is a measure of discrimination/fidelity. Each short horizontal bar represents the standard deviation of the mean of triplicate independent determinations. The insertion efficiencies for dGTP were reported in Refs. 11, 12 and 8-oxo-dGTP in Ref. 11. The catalytic efficiency values are reported in Fig. S1 and are as follows: $2.4 \times 10^{-3} \pm 3.0 \times 10^{-4} \mu M^{-1} s^{-1}$ (dGTP:dc), $3.0 \times 10^{-3} \pm 1.3 \times 10^{-4} \mu M^{-1} s^{-1}$ (rGTP:dc), $3.9 \times 10^{-3} \pm 2.0 \times 10^{-4} \mu M^{-1} s^{-1}$ (8-oxo-dGTP:dc), $6.0 \times 10^{-3} \pm 4.0 \times 10^{-4} \mu M^{-1} s^{-1}$ (r8-oxo-GTP:dc), $1.3 \times 10^{-3} \pm 0.4 \times 10^{-4} \mu M^{-1} s^{-1}$ (dGTP:dc), $3.5 \times 10^{-3} \pm 7.0 \times 10^{-4} \mu M^{-1} s^{-1}$ (rGTP:dc), $1.2 \times 10^{-3} \pm 0.4 \times 10^{-4} \mu M^{-1} s^{-1}$ (8-oxo-dGTP:dc), $1.3 \times 10^{-3} \pm 0.4 \times 10^{-4} \mu M^{-1} s^{-1}$ (r8-oxo-GTP:dc), and $1.0 \times 10^{-3} \pm 0.4 \times 10^{-4} \mu M^{-1} s^{-1}$ (Y271Gr8-oxo-GTP:dc).

Figure 4. Calculated interprobe distances from FRET efficiencies during steady-state FRET experiments. Efficiencies from triplicate measurements are plotted for the following complexes opposite either a templating cytosine (dark blue) or templating adenine (red); binary; ternary with dGTP, rGTP, or r8-oxo-GTP; with error bars (black) calculated using standard deviation of the mean. Distance values are reported in Fig. S3 and are as follows: $42.6 \pm 1.3$ Å (binary:dc), $38.0 \pm 0.3$ Å (binary:da), $30.7 \pm 0.7$ Å (dGTP:dc), $36.0 \pm 0.5$ Å (dGTP:da), $38.1 \pm 0.8$ Å (rGTP:dc), $35.8 \pm 0.4$ Å (rGTP:da), $38.1 \pm 0.7$ Å (8-oxo-dGTP:dc), $31.8 \pm 0.5$ Å (8-oxo-dGTP:da), $37.7 \pm 0.9$ Å (r8-oxo-GTP:dc), and $35.2 \pm 0.5$ Å (r8-oxo-GTP:da).

indicated by a shift of the N-subdomain ~12 Å closer to the DNA compared with the binary open complex (Fig. 4). Of note, our calculated distances for the open binary and closed correct ternary associate closely with previous measurements using this assay and those expected from crystallographic data (15). Performing the same experiment with either 8-oxo-dGTP, rGTP, or r8-oxo-GTP insertion opposite dC resulted in a lack of significant N-subdomain closure (shifting only 2.9–4.5 Å). These results indicate that modifications on either the sugar (O2’) or the base (O8) inhibit the enzyme from closing and are consistent with the observed reduction in their insertion efficiency opposite dC (Fig. 3).

The mismatched insertion of dGTP opposite dA results in only a 2.0 Å shift of the N-subdomain, from 38.0 Å in the binary complex to 36.0 Å in the ternary, and is associated with a significant loss in catalytic efficiency (Fig. 3). In contrast, insertion of 8-oxo-dGTP, the oxidized deoxyribonucleotide, opposite dA leads to a 6.2 Å shift in the N-subdomain, from 38.0 Å to 31.8 Å, which is similar to the distance observed in the presence of the correct dTTP:da pair (30.9 Å) (Fig. S3). The ability of pol β to undergo subdomain closure during 8-oxo-dGTP insertion opposite dA correlates with the high catalytic efficiency of this mutagenic deoxyribonucleotide. The lack of closure measured by FRET during rGTP insertion opposite dA provides a potential explanation for the unmeasurable catalytic activity (Fig. 4). Last, insertion of r8-oxo-GTP opposite dA resulted in only a 2.8 Å shift in the N-subdomain, which indicates an intermediate or partially closed state and correlates with its intermediate insertion efficiency.

Structural snapshots of r8-oxo-GTP insertion opposite dA and dC

To investigate the active-site geometries and base-pairing properties of r8-oxo-GTP, we sought to collect X-ray crystallographic ternary complex structures with WT pol β. Unfortunately, we were unable to obtain a WT pol β:DNA:r8-oxo-GTP ternary complex, likely as a result of the poor binding between r8-oxo-GTP and pol β. A previous study determined that removal of the Tyr-271 side chain did not appreciably alter insertion of dCTP opposite dG and increased nondamaged ribonucleotide insertion efficiency by 12-fold compared with WT pol β (31). Therefore, we utilized a Y271G pol β variant to obtain ternary structures of pol β bound to r8-oxo-GTP. Consistent with this, Y271G insertion of r8-oxo-GTP showed increased catalytic efficiency opposite dA (2,700-fold) but not opposite dC when compared with the WT (Fig. 3). We proceeded to use the Y271G pol β variant to obtain ternary crystal structures of r8-oxo-GTP insertion opposite both dA and dC with MnCl2 and a deoxyterminated primer, which allows for binding of r8-oxo-GTP while preventing catalysis (12, 22, 32).

The ternary complex, showing Y271G pol β bound to single-nucleotide gapped DNA with a templating dC and an incoming r8-oxo-GTP, diffracted to 2.55 Å (Table S1). The resulting structure contained two pol β complexes in the asymmetric unit, with one complex in the open conformation and the other in the closed conformation. The density for r8-oxo-GTP in the
open active site of pol β was relatively poor, with only clearly defined density for the triphosphate moiety (Fig. S4). In contrast, the density for r8-oxo-GTP in the closed pol β conformation was more defined and is the focus of our analysis (Fig. 5A).

In this structure, the r8-oxo-GTP is bound to the pol β active site with a single Mn\(^{2+}\) in the nucleotide metal-binding site, and the catalytic metal-binding site contains a sodium ion. The lack of divalent catalytic metal binding has been observed previously for dideoxy-terminated ternary complexes (33). The incoming r8-oxo-GTP resides in the anti conformation opposite a dC template in a closed complex, but the base-pairing is not planar. Instead, the angle between base planes in the r8-oxo-GTP:dC nascent pair is 27°, positioning the O8 oxygen in the direction of the N-helix (34). This twist results in the adducted O8 of r8-oxo-GTP sitting 0.5 Å farther away from the O5′ compared with the O8 in a previously solved 8-oxo-dGTP:dC complex (35). In the 8-oxo-dGTP:dC complex, a Ca\(^{2+}\) ion interacts with the α-phosphate of the incoming nucleotide to neutralize the close proximity of the O8 and O5′. This metal is not present in the r8-oxo-GTP:dC complex, likely because of the propeller twist accommodating any potential clash between O8 and O5′. The ribose sugar of the incoming nucleotide resides in an O4′-endo conformation, with the O5′ oriented toward the backbone carbonyl of Gly-271 (Fig. 5B). This results in the backbone carbonyl of Gly-271 shifting 0.9 Å, relative to its position in the dGTP:dC complex, to avoid a clash with the O2′ (Fig. 5B). A stabilizing hydrogen bond between the 3′-OH of the incoming nucleotide and the nonbridging β-phosphate oxygen, observed in the rGTP:dC complex, is not present with r8-oxo-GTP because the 3′-OH is 5 Å from the nonbridging β-phosphate oxygen of the nucleotide. In a previously obtained Y271A pol β rCTP:dG complex, a rotameric shift of Phe-272 (Fig. S5) and a shift in the primer terminus toward the major groove were observed (22). These structural features are also present in our r8-oxo-GTP:dC complex as a result of removing the Tyr-271 side chain. In the r8-oxo-GTP:dC complex, we also observed a shift of the templating dC along with the 5′-end of the templating strand upstream compared with an rGTP:dC complex control (Fig. 5C). Ternary mismatches also shift the templating strand upstream; however, the r8-oxo-GTP:dC complex does not shift to the same extent (Fig. 5D) (12, 36). This is likely due to weak interactions between the Watson-Crick faces of r8-oxo-GTP and the templating dC.
8-oxo-dGTP prefers to insert opposite adenine via a rotation of the glycosidic bond and formation of a Hoogsteen base pair with dA. In our solution studies, r8-oxo-GTP similarly prefers to insert opposite adenine (Fig. 3). To evaluate whether r8-oxo-GTP uses Hoogsteen base-pairing, we obtained a ternary Y271G pol_H9252 structure with an incoming r8-oxo-GTP opposite a templating dA. This complex diffracted to 2.05 Å, with only one pol_H9252 molecule per asymmetric unit (Table S1). In the resulting structure, pol_H9252 assumed a closed conformation with clear density for r8-oxo-GTP in the nucleotide binding pocket (Fig. 6A). r8-oxo-GTP was bound with Mn2_H11001 in both the nucleotide and catalytic metal binding sites, indicating a more optimal active-site geometry compared with r8-oxo-GTP opposite dC (Fig. 5A). Similar to 8-oxo-dGTP, r8-oxo-GTP Hoogsteen base-pairs opposite dA using the syn conformation. This places the adducted O8 oxygen into the minor groove, where it is stabilized by Asn-279, which was also observed with 8-oxo-dGTP (Fig. 6B). However, one clear difference between structures with 8-oxo-dGTP and r8-oxo-GTP is that the nucleoside breaks from planarity by 17° with the ribo form, pointing the N2 away from the α-phosphate and toward the N-helix (Fig. 6B) (37). Despite being nonplanar, there was no significant change to the distance of the hydrogen bond between the N2 and the α-phosphate (2.9 Å), which has been proposed to stabilize the Hoogsteen conformation during 8-oxo-dGTP insertion opposite dA (3.0 Å) (Fig. 6B) (35). The overall organization of the active site during r8-oxo-GTP insertion opposite dA aligns well with our rGTP:dC structure and is consistent with respect to their similar kinetic efficiencies (Fig. 6C). The ribose O2’ was positioned 2.8 Å away from the Gly-271 backbone carbonyl, similar to rGTP:dC (2.6 Å) (Fig. 6D). However, the ribose sugar adopts a C4’-exo conformation as opposed to the more competent 3’-endo in our rGTP:dC complex and other pol_H9252:rNTP structures (Fig. 6D). Additionally, the r8-oxo-GTP 3’-OH and its nonbridging β-phosphate oxygen are within hydrogen bonding distance at 3.2 Å.

Discussion

In this study, we characterized r8-oxo-GTP insertion with DNA polymerase β using single-turnover kinetics, a steady-state FRET assay, and X-ray crystallography. Our results show that polymerase subdomain closure opposite both dA and dC is reduced, but differences in active site geometries upon closure lead to the preference for mutagenic (dA) insertion of r8-oxo-GTP (Fig. 7). We also showed that mutagenic insertion of r8-oxo-GTP opposite dA occurs at a similar catalytic efficiency as that of a mismatched deoxyribonucleotide or correctly matched undamaged ribonucleotide. Based on the error rate of...
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The adducted O8 promotes mutagenic r8-oxo-GTP insertion by pol β

It is well characterized that 8-oxo-dGTP is inserted by polymerases across from dA more efficiently than dC, and 8-oxo-GTP continues this trend. 8-Oxo-dGTP and 8-oxo-GTP, respectively, have a 10-fold and 60-fold increase in insertion efficiency opposite dA compared with dC (Fig. 3). Moreover, when the steric gate residue Tyr-271 is mutated, r8-oxo-GTP:dA insertion increases 40-fold, which is only 17-fold less than r8-oxo-GTP:dC. Furthermore, we observed an open r8-oxo-GTP:dA and dGTP:dA complexes (35.2 Å and 36.0 Å, respectively) that corroborates the similar catalytic efficiency opposite dA and dC, and pol β is only 1.7-fold less than its nondamaged mismatch insertion (dGTP:dA). Mutagenic r8-oxo-GTP insertion by WT pol β is only 1.7-fold less than its nondamaged mismatch insertion (dGTP:dA), demonstrating the potential frequency of r8-oxo-GTP, given that mismatches are a common replication error. In our FRET experiments, we observed a similar distance between the r8-oxo-GTP:dA and dGTP:dA complexes (35.2 Å and 36.0 Å, respectively) that corroborates the similar catalytic efficiencies (Fig. 3). Conversely, ribonucleotide insertion (rGTP:dC), another frequent replication error, is also within 1.3-fold of r8-oxo-GTP:dA but has a more open conformation (38.1 Å), indicating that other steps in the enzymatic pathway account for the observed differences in catalytic efficiency.

In our X-ray crystal structures, we see similarities between 8-oxo-dGTP and r8-oxo-GTP insertion. Particularly, the adducted O8 oxygen imparts the same general base-pairing properties in the deoxyribo- and ribonucleotide forms. When base-pairing opposite cytosine, there is a retention of the anti conformation that forms Watson–Crick base pairs. Opposite adenine, both r8-oxo-GTP and 8-oxo-dGTP rotate about their glycosidic bond to a syn conformation and Hoogsteen base-pair with dA. Retention of the base pairing properties between 8-oxo-dGTP and r8-oxo-GTP supports the retained mutagenic behavior of the adducted O8.

Mechanistic insight into r8-oxo-GTP discrimination by pol β

The induced-fit model posits that, after initial correct dNTP binding, conformational changes of the ternary complex induce catalytic residues to properly align for catalysis (38). The induced-fit model would also assert that mismatch complexes do not properly align the active site for catalysis and, instead, promote dissociation (38). In a mismatched complex (dG: dAP-CPP; PDB code 3C2M), the templating strand is shifted out of coding position, and pol β is positioned in an open conformation (12, 33, 39–41). Similarly, we observed these features in our r8-oxo-GTP:dC complex (Figs. 5D and 4), demonstrating a similar method of discrimination between mismatches and r8-oxo-GTP:dC. Furthermore, we observed an open pol β complex within the asymmetric unit with poorly defined density for the nucleoside and only moderately defined triphosphate density (Fig. S4). Retention of triphosphate density without nucleobase density is consistent with previous findings demonstrating that triphosphate binding occurs prior to Watson–Crick sampling (42, 43). The observance of poor density and reduced occupany of the r8-oxo-GTP:dC open complex structurally supports our solution experiments showing both a decrease in catalytic efficiency and reduction in pol β closure for this complex. Another feature of the mismatch complex is the absence of Watson–Crick hydrogen bonds in the nascent pair, which led to the hypothesis that optimal base-pairing interactions during nucleotide binding facilitate proper active-site assembly (12). However, we found that r8-oxo-GTP does come within hydrogen-bonding distance with the templating dC despite the template strand shift and propeller twist of the nascent pair. Of note, a similar degree of propeller shift was observed during rCTP:dG insertion with polymerase η (27°) compared with our r8-oxo-GTP:dC twist (27°) (44). Overall, pol β strongly deters r8-oxo-GTP insertion opposite dC via poor r8-oxo-GTP binding, impaired subdomain closure, and significant active-site geometry perturbations should closure occur.

The r8-oxo-GTP lesion is distinctly nonplanar opposite both dC and dA. However, the O8 and O2’ by themselves do not significantly alter base-pair planarity, as observed during 8-oxo-dGTP and rNTP insertion (22, 35). This suggests that the combined steric burden of O2’ and O8 prevent formation of a planar base pair with either the templating dA or dC. In a pre-
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Consequences of r8-oxo-GTP insertion

Our results show that the ribose O2’ of r8-oxo-GTP heavily diminishes the rate of insertion opposite adenine and cytosine. Despite this, r8-oxo-GTP can still be mutagenically inserted by pol β at an efficiency similar to undamaged ribonucleotides. If replicative polymerases were to have a similar discrimination for r8-oxo-GTP as pol β, then we can speculate on the amount of mutagenic r8-oxo-GTP insertion events during DNA replication. Using replicative DNA polymerase ε as an example, the discrimination between rGTP and dGTP is 12 times worse than pol β, indicating that r8-oxo-GTP may be inserted more efficiently by pol ε than by pol β (46). Replicative polymerases are estimated to misinsert more than 1 million ribonucleotides (~250,000 rGTP) per round of replication (26), and levels of r8-oxo-GTP have been measured to be between 0.2%–5% of the rGTP pool during mild oxidative stress (4, 47). If 0.2% of the ~250,000 inserted rGTP are oxidized to r8-oxo-GTP, then ~500 r8-oxo-GTP mutagenic insertion events could potentially occur per cell division under mildly oxidative conditions. The subsequent cellular ramifications of these lesions in duplex DNA are still unclear. Many DNA damage-processing enzymes have been investigated, but most are unable to process r8-oxo-GTP or r8-oxo-G, including 8-oxoguanine glycosylase (OOG1), RNase H2, and ribonucleotide reductase (29, 48–50). MutT-homolog 1 (MTH1) and AP endonuclease (APE1) only have weak activity on r8-oxo-GTP and r8-oxo-G processing, making it uncertain whether the reduced activities are sufficient to protect the cell from r8-oxo-G(TP) (50–52). In cellular extracts subjected to mildly oxidized conditions, r8-oxo-GTP does accumulate (0.3 nmol/10⁶ cells), whereas 8-oxo-dGTP was not detected (4). This suggests that MTH1 is not able to sufficiently remove r8-oxo-GTP from the nucleotide pool, in contrast to its efficient removal of 8-oxo-dGTP. MutY DNA Glycosylase (MUTYH) was found to have r8-oxo-GMP activity and remove the opposing mutagenic dA. However, to our knowledge, the efficiency for inserting the correct base opposite r8-oxo-GTP currently remains unknown.

Experimental procedures

DNA sequences

The DNA sequences used in crystallization studies (16-mer), kinetic studies (34-mer), and FRET studies (45-mer) are provided in Table S2. Each oligonucleotide was suspended in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA, and the concentration was determined from their UV absorbance at 260 nm. DNA substrates were prepared by annealing three purified oligonucleotides (1:1.2:1.2 molar ratio, respectively) at 95 °C for 5 min, followed by 65 °C for 30 min, and finally cooling 1 °C min⁻¹ to 10 °C in a PCR thermocycler.

Pol β expression, purification, and crystallization

Human WT pol β was subjected to site-directed mutagenesis to generate the Y271G mutant enzyme using the QuikChange II site-directed mutagenesis protocol and kit. The Y271G mutation was confirmed via sequencing and was overexpressed in BL21-CodonPlus (DE3)-RP Escherichia coli and purified as described previously (53). This mutant was rationally designed to provide flexibility to the steric gate backbone carbonyl of Tyr-271 in pol β. Y271G pol β was incubated with 1-nt gapped/dideoxy-terminated DNA (1:1.2) containing either a templating dA or dC for 30 min. Binary complex crystals were grown via sitting drop vapor diffusion using 2 μl of protein/DNA mixture combined with 2 μl of mother liquor (50 mM imidazole (pH 7.5), 16%–20% PEG3350, and 350 mM sodium acetate) as described previously (33). Binary Y271G pol β:DNA complex crystals were then soaked in a cryosolution containing 20% ethylene glycol, 50 mM imidazole (pH 7.5), 16%–19% PEG3350, 70 mM sodium acetate, 5 mM r8-oxo-GTP, and 50 mM MnCl2 for 1–3 h. This resulted in ternary pol β:DNA:r8-oxo-GTP crystals for X-ray crystallography.

Data collection and refinement

Data were collected at 100 K on a MicroMax-007HF rotating anode generator at a wavelength of 1.54 Å. Diffraction images were collected using a Dectris Pilatus3R 200K-A detector, and the HKL3000 software package was used for processing and scaling the data (54). Initial models were determined using molecular replacement with the previously determined closed ternary (PDB code 2FMS) structure of pol β, and Rfree flags were taken from the starting model, except for PDB code 6UOK, which had two pol β in the asymmetric unit. The metal–ligand coordination restraints were generated by ReadYset (PHENIX). Refinement was performed using PHENIX and model building using Coot (55, 56). Density maps in structure figures (green mesh) were generated using Polder Maps in PHENIX. Polder OMIT maps are reduced-bias σ-A weighted difference density maps (mFo-DFc) that exclude bulk solvent within a 5 Å radius when calculating OMIT maps to improve visualization of weak densities (57). All polder maps were scaled to 3.0 σ, except for Fig. S4, where it was scaled to 4.0σ. Ramachandran analysis...
determined that 100% of nonglycine residues lie in the allowed regions and at least 96% in favored regions. Local base-pair parameters were calculated using 3DNA, and local interbase angles were corrected for both buckle and propeller contributions (37). The figures were prepared in PyMOL (Schrödinger LLC).

**Single-nucleotide gap-filling DNA synthesis**

Catalytic efficiencies ($k_{pol}/K_d$) for single-nucleotide gap-filling reactions were determined by single-turnover analysis (i.e. enzyme $\gg$ DNA). At least seven time points were gathered for each single-exponential time course determined with three subsaturating concentrations of r8-oxo-GTP. The standard reaction mixture contained 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 100 µg/ml BSA, 10% glycerol, 100 mM single-nucleotide gapped DNA, and 1 µM pol β. Concentrations of r8-oxo-GTP varied depending on the identity of the templating base to achieve a wide range of product concentration while avoiding multiple insertions and/or product inhibition. Templating dA kinetics were performed with 10 µM, 25 µM, and 50 µM r8-oxo-GTP during a 4-h time course, with samples taken at 5, 15, 30, 45, 60, 90, 132, 170, 230, and 330 min. Templating dC kinetics were performed with 100 µM, 250 µM, and 500 µM r8-oxo-GTP during a time course of 6.5 h, with samples taken at 15, 30, 60, 95, 132, 180, 230, 275, and 400 min. For rGTP insertion kinetics, 10 µM, 25 µM, and 50 µM rGTP were used in our reactions over a time course of 2.5 h, with samples taken at 2, 5, 15, 30, 45, 60, 90, 120, and 150 min. With the Y271G pol β mutant, kinetic experiments with a templating adenine used 50 nM, 100 nM, and 250 nM r8-oxo-GTP over a 4-h time course, with samples taken at 1, 3, 10, 30, 60, 90, 120, 180, and 240 min. Y271G pol β mutant experiments with a templating cytosine used 200 µM, 400 µM, and 600 µM r8-oxo-GTP over a 6-h time course, with samples taken at 5, 15, 30, 45, 60, 90, 132, 170, 230, and 330 min. Each reaction time course was performed at 37 °C. Reactions were stopped with addition of a quench solution containing 100 mM EDTA, 10 mM urea, and 6-Carboxyfluorescein 5'-labeled primer was used in these assays, the gels were imaged using the GE Typhoon Phosphorimager in fluorescence mode. Substrate and product bands were quantified using ImageJ (58) and then plotted using Kaleidagraph (45). For each concentration of r8-oxo-GTP, the data were fit to a single exponential curve (Fig. S1A). Because subsaturating concentrations of r8-oxo-GTP ($<K_d$) were used, the data were fit to an alternate form of the Michaelis equation to extract apparent catalytic efficiencies ($k_{pol}/K_d$): $k_{obs} = ((k_{pol}/K_d) \times S)/(1 + S/K_d)$, where S refers to the concentration of r8-oxo-GTP (Fig. S1B). Catalytic efficiencies were calculated as an average of three independent experiments ± S.D. (Fig. S1C).

**Steady-state FRET**

Human pol β containing mutations C239S, C267S, and V303C was used to fluorescently label the N-subdomain with the thiol-reactive IAEDANS, as described previously (15). Fluorescence emission of IAEDANS-labeled pol β was measured in either the apoenzyme form (400 nM IAEDANS-labeled pol β in 50 mM Tris (pH 8.0), 20 mM NaCl, and 10 mM MgCl₂), the binary complex (400 nM IAEDANS-labeled pol β mixed with 400 nM single-nucleotide gapped DNA substrate with a diode-terminated primer terminus containing Dabcyl dT at the −8 position relative the templating base), or the ternary complex (formed by adding 1 mM dGTP, 8-oxo-dGTP, rGTP, or r8-oxo-GTP to the binary complex) (Fig. S2). IAEDANS was excited at 336 nm, and emission was collected at 400–550 nm using a QuantaMaster 800 fluorometer at room temperature (Fig. S3, A and B) (15). Distances were estimated from the FRET efficiency between IAEDANS and Dabcyl and by using a Förster radius of 37.7 Å. Experiments were performed in triplicate, and the error was calculated as a standard deviation of the mean. A table with the calculated distances is provided (Fig. S3C).

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