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DNA Polymerase λ Inactivation by Oxidized Abasic Sites

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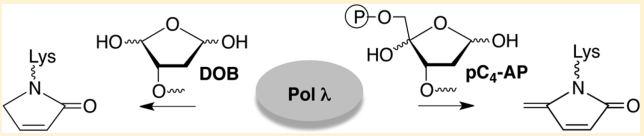
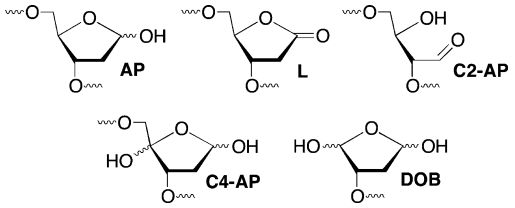
Supporting Information

ABSTRACT: Base excision repair (BER) plays a vital role in maintaining genomic integrity in mammalian cells. DNA polymerase λ (Pol λ) is believed to play a backup role to DNA polymerase β (Pol β) in base excision repair. Two oxidized abasic lesions that are produced by a variety of DNA-damaging agents, including several antitumor antibiotics, the C4′-oxidized abasic site following Ape1 incision (pC4-AP), and 5′-(2-phosphoryl-1,4-dioxobutane) (DOB), irreversibly inactivate Pol β and Pol λ. The interactions of DOB and pC4-AP with Pol λ are examined in detail using DNA substrates containing these lesions at defined sites. Single-turnover kinetic experiments show that Pol λ excises DOB almost 13 times more slowly than a 5′-phosphorylated 2-deoxyribose (dRP). pC4-AP is excised approximately twice as fast as DOB. The absolute rate constants are considerably slower than those reported for Pol β for the respective reactions, suggesting that Pol λ may be an inefficient backup in BER. DOB inactivates Pol λ approximately 3-fold less efficiently than it does Pol β, and the difference can be attributed to a higher K_I (33 ± 7 nM). Inactivation of Pol λ's lyase activity by DOB also prevents the enzyme from conducting polymerization following preincubation of the protein and DNA. Mass spectral analysis of GluC-digested Pol λ inactivated by DOB shows that Lys324 is modified. There is inferential support for the idea that Lys312 may also be modified. Both residues are within the Pol λ lyase active site. When acting on pC4-AP, Pol λ achieves approximately four turnovers on average before being inactivated. Lyase inactivation by pC4-AP is also accompanied by loss of polymerase activity, and mass spectrometry indicates that Lys312 and Lys324 are modified by the lesion. The ability of DOB and pC4-AP to inactivate Pol λ provides additional evidence that these lesions are significant sources of the cytotoxicity of DNA-damaging agents that produce them.



An apurinic/apyrimidinic (AP) site is the prototypical member of the abasic lesion family. AP sites arise from hydrolysis of a nucleotide's glycosidic bond and are produced via adventitious DNA hydrolysis, as products of glycosylases involved in DNA repair, and following alkylation.[1−3] Oxidized abasic sites (e.g., C2-AP, C4-AP, DOB, and L) are derived from radicals that are produced by agents that abstract hydrogen atoms from the deoxyribose ring in DNA.[4−6] Abasic sites exhibit a rich and diverse chemistry. They are mutagenic, and despite lacking a nucleobase capable of forming Watson−Crick base pairs, only AP adheres to the "A-rule".[7−10] The C2-AP, C4-AP, and L oxidized abasic sites leave distinctive signatures of replication in *Escherichia coli*.[11−14] Recently, other aspects of abasic site chemistry have been uncovered. For instance, AP, DOB, and C4-AP form interstrand DNA cross-links.[15−18] Cross-links involving C4-AP were detected in cellular DNA.[19] In addition, AP, L, and C4-AP are alkali-labile lesions prone to strand breaks, and cleavage of DNA containing them is significantly accelerated in nucleosome core particles.[20−23] These examples illustrate how abasic lesions are spontaneously converted to forms of DNA damage (e.g., interstrand cross-links) that are viewed as more deleterious to cells and highlight the importance of their efficient repair. Consequently, reports that L, DOB, and C4-AP irreversibly inhibit enzymes involved in base excision repair (BER) are potentially important.[24−26] Herein, we report on the irreversible inactivation of DNA polymerase λ by DOB and C4-AP.



C4-AP and DOB are formed following abstraction of a hydrogen atom from positions C4′ and C5′, respectively (Scheme 1).[6] These hydrogen atoms are highly accessible to diffusible species compared to those at other positions of the 2′-deoxyribose ring.[27] Positions C4′ and C5′ are also common sites of hydrogen atom abstraction by minor groove binding molecules. C4-AP is produced by a variety of oxidizing agents, some of which have therapeutic potential, such as bleomycin.[28] DOB is formed less frequently than C4-AP but is a product of some of the most potent antitumor antibiotics.[29] The reactivity and biochemical behavior of DOB and C4-AP suggest that

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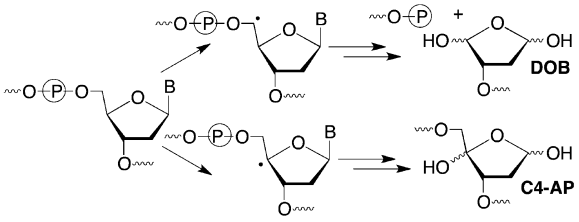
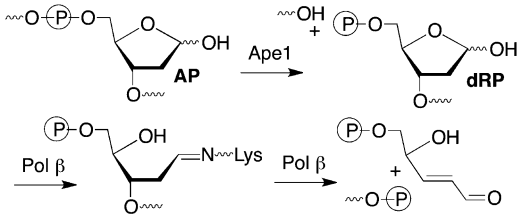
Scheme 1. Formation of C4-AP and DOB



these lesions provide the chemical bases for the cytotoxicity of the agents that produce them. DOB and C4-AP are among a growing number of examples of DNA modifications that spontaneously form potentially more deleterious lesions and/or inhibit BER. For instance, DOB undergoes elimination, and the butenedial released forms mutagenic adducts with dA, dC, and dG.[30,31] In addition, DOB and C4-AP form interstrand cross-links with dA and dC.[16−18] Cross-links involving C4-AP are converted to double-strand breaks ∼15% of the time because of misrepair by bacterial nucleotide excision repair (UvrABC).[32] C4-AP is a substrate for 5′-endonucleases, including Xth and Ape1.[26,33,34] However, it is the next step in BER (Scheme 2) that C4-AP and DOB inhibit.

Scheme 2. Role of Pol β in BER



Pol β is responsible for excising the 5′-phosphorylated 2′-deoxyribose fragment (dRP) remaining at the 5′-terminus of the 3′-fragment after the Ape1 reaction.[35] The lyase activity of Pol β lies in an 8 kDa domain of the protein.[36] Lys72 is accepted as being the primary residue responsible for Schiff base formation. A second lysine (Lys84) present in the binding pocket was also implicated in the lyase activity. Direct and indirect evidence of modification of Lys84 and Lys72 by DOB and pC4-AP were reported. Cellular experiments suggest that Pol λ, whose primary biological function is in repair of DSBs by nonhomologous end joining (NHEJ), also serves as a backup to Pol β in BER.[37,38] Pol λ contains a homologous 8 kDa domain, which also catalyzes elimination of dRP.[39−41] Lys312 in Pol λ is homologous to Lys72 in Pol β, and mutagenesis and trapping studies support its involvement in Schiff base formation. Lys324 is homologous to Lys84, but its role in Pol λ's lyase activity is uncertain.

MATERIALS AND METHODS

Materials and General Methods. Oligonucleotides were prepared on an Applied Biosystems Inc. 394 DNA synthesizer. Commercially available DNA synthesis reagents were obtained from Glen Research Inc. Oligonucleotides containing the photolabile DOB and C4-AP precursors used to prepare **1**−**3**, including [^{3}H]DOB, were synthesized as previously described.[24,42,43] 5′-^{32}P-**4** was prepared as previously described by incubating 5′-^{32}P-**5** with uracil-DNA glycosylase (UDG).[26] All others were synthesized and deprotected using standard protocols. DNA substrates used in this study are presented in Chart 1. T4 polynucleotide kinase, terminal deoxynucleotide

Chart 1. DNA Substrates Used in This Study

5′-d(TAA TGG CTA ACG CAA **X**TC GTA ATG CAG TCT)
3′-d(ATT ACC GAT TGC GTT AAG CAT TAC GTC AGA)

1 **X** = DOB

5′-d(TAA TGG CTA ACG CAA **X**AC GTA ATG CAG TCT)
3′-d(ATT ACC GAT TGC GTT ATG CAT TAC GTC AGA)

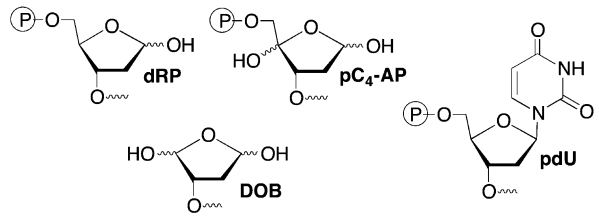
2 **X** = dRP

5′-d(CGA CCG GCT CGT ATG **X** TGT GTG GAC CTG TGG)
3′-d(GCT GGC CGA GCA TAC T ACA CAC CTC GAC ACC)

3 **X** = pC4-AP

5′-d(CCC CGA CCG GCT CGT ATG **X**TG TGT GGA GCT GTG GCG G)
3′-d(GGG GCT GGC CGA GCA TAC AAC ACA CCT CGA CAC CGC C)

4 **X** = dRP
5 **X** = pdU



transferase, UDG, Glu C, and trypsin were obtained from New England Biolabs. DNA Pol β was obtained from Trevigen. DNA Pol λ was expressed and purified as previously described.[44] ^{32}P-labeled nucleotides were obtained from Perkin-Elmer. ZipTips were from Millipore. Analysis of radiolabeled oligonucleotides was conducted using a Storm 840 Phosphorimager and ImageQuant TL. UPLC analysis was conducted on an Agilent Infinity 1290 system as previously described.[26] MALDI-TOF MS data were obtained on a Bruker AutoFlex spectrometer. MALDI-TOF mass spectrometry was performed in reflectron positive mode. The laser power was varied, starting at lower values and increasing until the signal was ∼10^3−10^4 units for 10^3 shots (at 100 Hz). The detection range was varied but was commonly set at m/z 440−2000, and the instrument was programmed to perform a "partial sample" random walk to gain appropriate signal coverage. Single-turnover kinetic experiments for the Pol λ lyase reaction on dRP (3′-^{32}P-**2**) and pC4-AP (3′-^{32}P-**3**) were conducted using a KinTek rapid quench system. Single-turnover kinetic experiments for the Pol λ lyase reaction on DOB (3′-^{32}P-**1**) were conducted manually. Please note that either the 3′-terminus of the strand containing the modified nucleotide or the 5′-terminus of the flanking oligonucleotide is radiolabeled in all of the experiments described below.

Single-Turnover Kinetics with Pol λ. The ternary complex 3′-^{32}P-**2** or 3′-^{32}P-**3** (20 nM) in HEPES buffer (460 μL, 50 mM, pH 7.4, containing 5 mM $MgCl_2$) was prepared. A solution of Pol λ (200 nM) in HEPES buffer (460 μL, 50 mM, pH 7.4, containing 5 mM $MgCl_2$) was prepared and stored on ice. Both solutions were loaded in 1 mL sterile disposable syringes and the syringes attached to the KinTek rapid quench instrument. An experiment consisted of eight time points (1, 3, 5, 10, 15, 20, 30, and 60 s for 3′-^{32}P-**2** or 2, 5, 10, 20, 30, 40, 60, and 180 s for 3′-^{32}P-**3**) conducted in triplicate. Prior to each

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measurement, the reaction loop was rinsed with water, followed by methanol, and evacuated for 30 s with a vacuum pump. A solution of DNA complex (15 μL) and enzyme (15 μL) were mixed, and the reaction was performed for the indicated times before being quenched by 0.1% TFA. The reaction solution (300 μL) from the rapid quench was immediately mixed with $NaBH_4$ (20 μL, 1 M) and incubated at room temperature for 30 min. The sample was then mixed with NaOAc (20 μL, 3 M, containing 200 μg/mL calf thymus DNA). The DNA was precipitated from ethanol (700 μL) and resuspended in formamide loading buffer (10 μL, 90%, 10 mM EDTA). The products were separated via a 20% denaturing polyacrylamide gel and analyzed using a phosphorimager. The percent cleaved was plotted versus time. The resulting graph was fit to the exponential equation percent cleaved = (maximal percent cleaved)$(1 - e^{-k_{obs}t})$.

Single-turnover kinetic experiments with DOB (3′-^{32}P-**1**) were conducted under the same buffer conditions and concentrations of reactants described above, except the reaction mixtures (50 μL) were mixed manually and aliquots (5 μL) were removed and quenched (1 μL, 1 M $NaBH_4$) at 10, 30, 60, 120, 180, 240, 300, and 600 s. After incubation for 30 min at room temperature, the quenched aliquot was mixed with 90% formamide loading buffer (10 μL) and separated via a 20% denaturing polyacrylamide gel.

Pol λ Extension Reaction Assay. The ternary complex of 5′-^{32}P-**1**, -**3**, or -**4** (200 nM) was reacted with Pol λ (10 nM) in HEPES buffer (60 μL, 50 mM, pH 7.4, containing 5 mM $MgCl_2$, 50 mM KCl, and 0.2 mM EDTA) and dNTPs (50 μM, dATP, dGTP, dCTP, and dTTP) at 37 °C. Aliquots (5 μL) were removed at various times between 0 and 60 min, and reactions were quenched with 90% formamide loading buffer (10 μL). An aliquot of the mixture (5 μL) was loaded onto a 20% denaturing polyacrylamide gel, and the product was analyzed using a phosphorimager. Upon preincubation of the Pol λ with DNA, the dNTPs were added after the prescribed time as a 10× solution. Otherwise, the reactions were conducted as described above.

Phenol Extraction Assay. ^{3}H-**1** or 3′-^{32}P-**1** (125 nM) was incubated with Pol β (1.25 μM) or Pol λ (1.25 μM) in HEPES buffer (200 μL, 50 mM, pH 7.4, containing 5 mM $MgCl_2$) at 37 °C for 2 h (4 h for Pol β). Calf thymus DNA (1 μL, 10 μg) was then added, and the resulting solution was incubated at 37 °C for 15 min. The solution was extracted with buffer-saturated (Tris-HCl, pH 7.5) phenol (200 μL) three times. The mixtures were vortexed and the layers separated by being spun for 5 min at 16000*g*. The phenol layers were combined. The phenol layer and aqueous layer were mixed separately with liquid scintillation cocktail (16 mL), and the ^{3}H or ^{32}P in each sample was counted using a liquid scintillation counter after equilibration for 2 h. Upon examination of the effect of incubation time on the extractions, the reaction mixtures were set up as described above. However, aliquots were removed at 0.5, 1, and 2 h (Pol λ) and 1, 2, and 4 h (Pol β).

Kinetics of Inhibition of Pol λ by DOB. Various concentrations of **1** (0, 15, 30, 60, 90, and 120 nM) were incubated with 3′-^{32}P-**4** (500 nM) and Pol λ (7.5 nM) in HEPES buffer (100 μL total volume, 50 mM, pH 7.4, containing 5 mM $MgCl_2$) at 37 °C. Aliquots (10 μL) were removed at the indicated times up to 1 h and reactions quenched with $NaBH_4$ (2 μL, 500 mM) on ice for 1 h. The samples were mixed with formamide loading buffer (20 μL, 90%, 10 mM EDTA). An aliquot of the mixture (5 μL) was loaded onto a 20% denaturing polyacrylamide gel, and the product was analyzed using a phosphorimager.

Stepwise Inhibition of the Pol λ Lyase Reaction by pC4-AP. 3′-^{32}P-**3** (200 nM) was incubated with Pol λ (10 nM) in HEPES buffer (500 μL total volume, 50 mM, pH 7.4, containing 5 mM $MgCl_2$) at 37 °C. Aliquots (5 μL) were removed at the indicated times (1, 5, 10, 30, 60, 90, and 120 min) and reactions quenched with $NaBH_4$ (1 μL, 500 mM) at 37 °C for 30 min. After 120 min, another portion of Pol λ (10 μL, 500 nM, 5 pmol) was added to the solution. Aliquots (5 μL) were removed after the following additional times (1, 5, 10, 30, 60, 90, and 120 min) and reactions quenched with $NaBH_4$ as described above. After an additional 120 min, a final aliquot of Pol λ (10 μL, 500 nM, 5 pmol) was added to the solution. Aliquots (5 μL) were removed after the following additional times (1, 5, 10, 30, 60, 90, and 120 min) and reactions quenched with $NaBH_4$ (1 μL, 1 M $NaBH_4$). The samples were mixed with formamide loading buffer (15 μL, 90%, 10 mM EDTA). An aliquot of each mixture (5 μL) was loaded onto a 20% denaturing polyacrylamide gel, and the product was analyzed using a phosphorimager.

Protease Digestions and MALDI-TOF Analysis of Pol λ Reactions. A solution (100 μL) of pol λ (10 μM) was incubated with or without ternary complex **1** or **3** (50 μM) in HEPES buffer (50 mM, pH 7.4, containing 5 mM $MgCl_2$) at 37 °C for 2 h. Ammonium bicarbonate (5.5 μL, pH 8.0, 1 M) and GluC or trypsin (5 μL, 0.4 μg/μL in water) were added, and the mixture was incubated for 4 h at 37 °C, at which time the reaction mixtures were acidified with 10% TFA. An aliquot (2 μL) of the reaction mixture was removed and analyzed by MALDI-TOF MS. The sample was desalted using a ZipTip as follows. The ZipTip was washed with CH_3CN (3 × 10 μL) and then with 0.1% TFA (3 × 10 μL). The sample was bound by pipetting up and down 10 times, then washed with 0.1% TFA (3 × 10 μL), and eluted and spotted directly on the MALDI-TOF plate with 2 μL of CH_3CN and 0.1% TFA containing α-cyano-4-hydroxycinnamic acid (10 mg/mL).

RESULTS

Single-Turnover Kinetics with Pol λ. To the best of our knowledge, the kinetics of Pol λ's dRPase activity have not been reported. Consequently, to provide a benchmark for DOB and pC4-AP incision, Pol λ incision of dRP was examined under single-turnover conditions (Figure 1A and Table 1). Of the three lesions examined, dRP was incised most readily by Pol λ. dRP was incised almost 13 times more efficiently than DOB, which lacks a 5′-phosphate. However, the absence of a 5′-phosphate could not be the sole source of the greater reactivity with dRP because this terminal lesion is incised ∼7-fold more rapidly than pC4-AP.

Pol λ Inactivation by DOB. Previous experiments qualitatively established that DOB irreversibly inactivates Pol λ.[25] A more quantitative analysis examined the ability of DOB (**1**) at concentrations ranging from 15 to 120 nM to inhibit Pol λ's dRPase activity on 3′-^{32}P-**4** (Figure 2). The activity of Pol λ declined as a function of time (Figure 2A). The period of time that it took for the amount of product to reach one-half of that when no DOB-containing substrate was present was inversely proportional to inhibitor concentration (Figure 2B) and yielded an approximate K_I of 33 ± 7 nM and a k_{inact} of (5.2 ± 0.7) × 10^{-4} s^{-1}. Inactivation of Pol λ's lyase activity also eliminates the enzyme's ability to polymerize DNA but only if the DOB-

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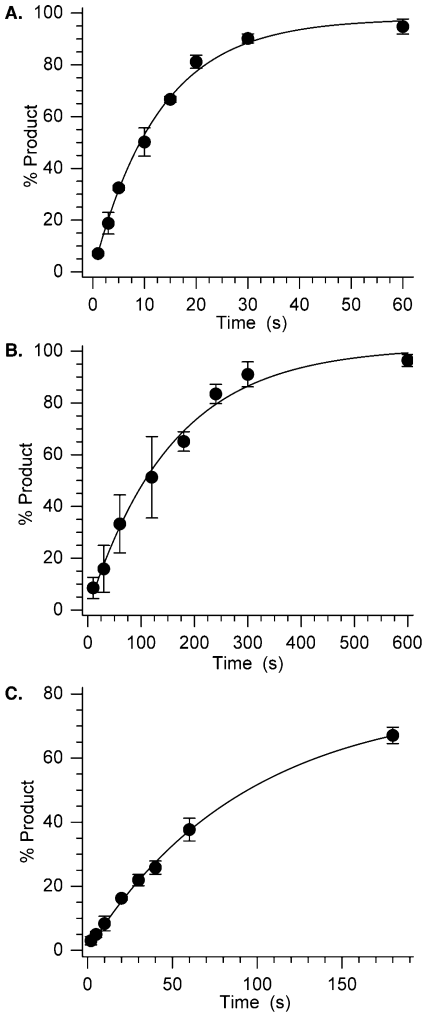

Figure 1. DNA lesion excision by Pol λ under single-turnover conditions: (A) dRP (3′-^{32}P-**2**), (B) DOB (3′-^{32}P-**1**), and (C) pC4-AP (3′-^{32}P-**3**).

Table 1. Single-Turnover Kinetics for Pol λ Incision

substrate	observed rate constant (s^{-1})[a]
dRP	$(7.4 \pm 0.6) \times 10^{-2}$ (2)
DOB	$(5.8 \pm 0.7) \times 10^{-3}$ (2)
pC4-AP	$(1.1 \pm 0.2) \times 10^{-2}$ (3)

[a]Rate constants are the average of the number of experiments noted in parentheses ± the standard error (for two measurements) or the standard deviation (for three measurements). Each experiment consisted of three replicates.

containing substrate and enzyme are incubated prior to adding dNTP substrates (Figure 3).

On the basis of DOB inactivation of Pol β, we envisioned that Pol λ may form covalent bonds to the DNA substrate containing the lesion or the enzyme could serve as a trap for released but-2-ene-1,4-dial [**6** (Scheme 3)]. These pathways were distinguished from one another using appropriately radiolabeled substrates[24] under single-turnover conditions. 3′-^{32}P-**1** allows detection of the DOB-mediated DNA−protein cross-link. Incorporation of tritium at a nonexchangeable position of DOB gives rise to radiolabeled enzyme via both pathways. Inactivation attributable to but-2-ene-1,4-dial (**6**) is estimated by determining the difference in the percent of

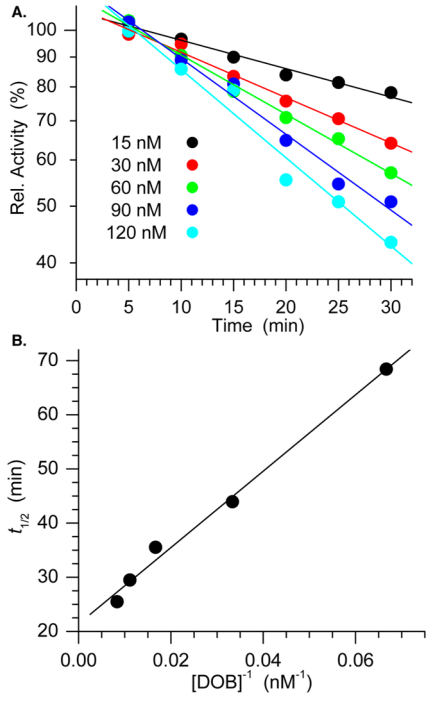

Figure 2. Kinetic analysis of irreversible inhibition of Pol λ by DOB (**1**, 15−120 nM). (A) Effect of increasing DOB concentration (**1**) on Pol λ (7.5 nM) lyase reaction of AP (3′-^{32}P-**4**, 500 nM). (B) Half-life of Pol λ inactivation as a function of DOB concentration.

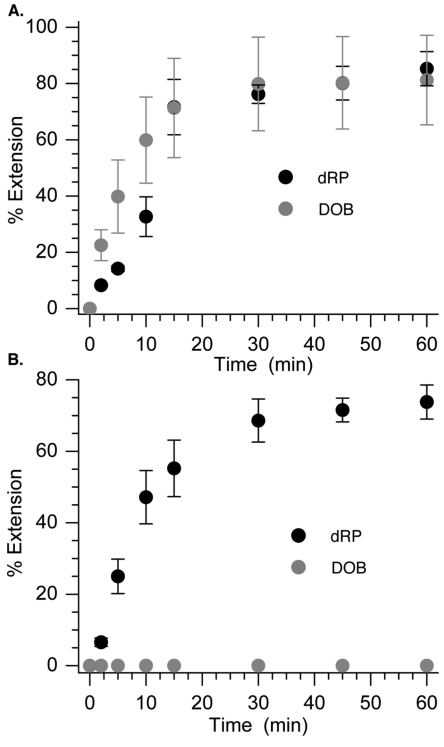

Figure 3. Pol λ (10 nM) extension of 200 nM dRP (5′-^{32}P-**4**) and DOB (5′-^{32}P-**1**) in the presence of dNTPs (50 μM). (A) Preincubation time of 0 min. (B) Preincubation time of 40 min.

radiation bound by the protein using the ^{3}H- and ^{32}P-labeled DOB substrates. The amount of radiation transferred to the protein is determined using liquid scintillation counting after phenol extraction. When DOB inactivates Pol β, the percentage

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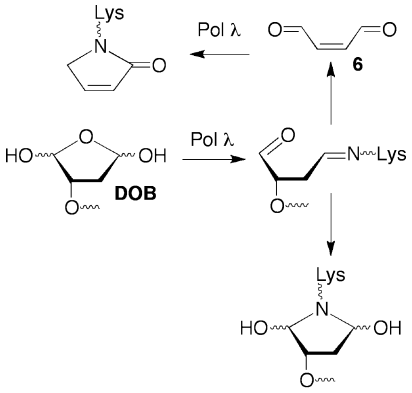
Scheme 3. Hypothetical Covalent Modification of Pol λ by DOB



of ^{3}H transferred to the phenol layer is slightly higher than the amount of ^{32}P from ^{32}P-**1**, confirming that covalent trapping of lesion-containing DNA was the major pathway for inactivation (Figure 4). In contrast, the level of transfer of ^{32}P to the phenol

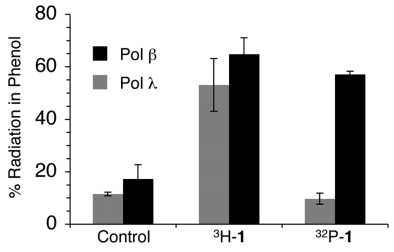


Figure 4. Determination of covalent modification of Pol β (1.25 μM) and Pol λ (1.25 μM) via phenol extraction of reaction mixtures containing radiolabeled 3′-^{32}P-**1** (125 nM) or ^{3}H-**1** (125 nM). Control corresponds to 3′-^{32}P-**1** extracted in the absence of enzyme.

layer when incubating Pol λ with ^{32}P-**1** is within experimental error of that in the absence of any enzyme. Decreasing the time that the DNA and protein are incubated together does not alter the percentage of ^{32}P transferred to the phenol layer (data not shown), indicating that if a covalent DNA−protein cross-link is formed it must decompose more rapidly than the analogous adduct formed from Pol β. However, more than 60% of the radiation from ^{3}H-**1** is transferred to the phenol layer (Figure 1). These results suggest that Pol λ inactivation is due to reaction with released but-2-ene-1,4-dial [**6** (Scheme 3)]. For both Pol β and λ, the net signal of ^{3}H above background is approximately 50% and provides an estimate of the fraction of reactions that result in protein modification.

The identity of the amino acid(s) modified by the released dialdehyde was probed using MALDI-TOF MS and liquid chromatography of the digested enzyme. The lyase region of Pol λ is homologous to the respective 8 kDa region of Pol β.[40] Lys312 is the primary amino acid believed to be responsible for Schiff base formation. A peptide corresponding to amino acids Ile313−Arg323 (m/z 1279) was observed following tryptic digestion of unmodified Pol λ, which cleaves on the carboxyl side of Lys and Arg. This peptide is absent in the trypsin digest of Pol λ that was incubated with 5 equiv of DOB-containing DNA (**1**). This is presumably due to cleavage inhibition at ε-amino-modified Lys312, although a corresponding longer peptide containing a modification was not detected. The peptide containing Lys312 (Ala299−Glu315) with an ion at m/z 1816 is detected in the GluC digest of modified Pol λ but not from protein incubated with DOB-containing DNA (**1**). However, a modified peptide corresponding to this segment is not observed in its place. A modified peptide is detected by MALDI-TOF MS following GluC digestion of Pol λ modified with **1**. The peptide containing Ser319−Glu330 (m/z 1391.7) is replaced by one 66 Da greater (m/z 1457.7) when the digested Pol λ is first incubated with DOB-containing DNA. Previously, the 66 Da increase upon incubation of Pol β with DOB was ascribed to reaction with **6** (Scheme 3), which results from dehydration and tautomerization of the condensation product between the ε-amino group and the 1,4-dialdehyde. The corresponding peptide obtained from Pol λ contains a single lysine residue at position 324, and MS/MS analysis indicates that this amino acid is modified (see the Supporting Information). Analysis of the digested material by UPLC reveals the total disappearance of all unmodified fragments containing Lys312 and Lys324, confirming the complete modification of Lys312 and Lys324 indicated by the MS data. In addition, a modified peptide encompassing residues 266−275 (m/z 1209.5 in unmodified Pol λ) was detected in the tryptic digest (see the Supporting Information). Lys273 in Pol λ may have access to the nucleotide binding pocket and is not conserved in Pol β. The modified peptide (m/z 1275.5) was consistent with the same 66 Da species proposed above [**6** (Scheme 3)]. MS/MS fragmentation shows that the 66 Da modification is directly on Lys273 (see the Supporting Information).

Pol λ Inactivation by pC4-AP. The similarities between Pol β and Pol λ inactivation by DOB led us to examine the effect of pC4-AP, which inactivates the former, on Pol λ.[26] Treating 3′-^{32}P-**3** with substoichiometric amounts of Pol λ yielded a burst of activity, followed by a plateau after approximately four turnovers (Figure 5). Although this was a

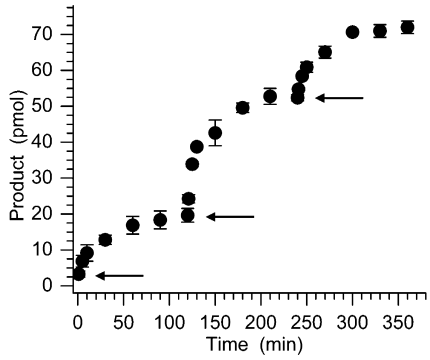


Figure 5. Inhibition of Pol λ lyase reaction by pC4-AP (3′-^{32}P-**3**, 200 nM). Each arrow indicates the addition of 5 pmol of Pol λ.

comparable number of turnovers that Pol β and Pol λ undergo before being inactivated by DOB, Pol β reacts on average with seven turnovers of pC4-AP before losing activity.[26] Preincubation of pC4-AP (5′-^{32}P-**3**) with Pol λ for varying amounts of time prior to dNTP addition also inhibits extension (Figure 6). The inhibition level is proportional to the preincubation time, and the amount of time required to completely inactivate Pol λ is comparable to that required by DOB (Figure 3).

Protease digestions and mass spectrometric analysis of Pol λ incubated with pC4-AP (**3**) demonstrated that Lys312 and Lys324 were modified following reaction with DNA containing pC4-AP (**3**). Positive evidence of Lys324 modification was

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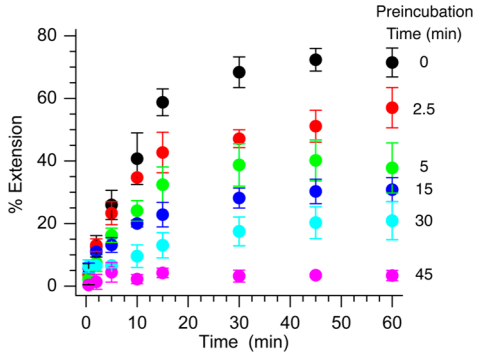
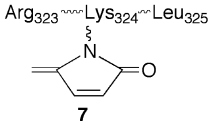


Figure 6. Inhibition of pC4-AP (5′-^{32}P-**3**, 200 nM) extension by Pol λ (20 nM) in the presence of dNTPs (50 μM) as a function of preincubation time.

obtained following GluC digestion. The ion corresponding to Ser319−Glu330 (m/z 1391.8) was replaced by a 78 Da larger one (m/z 1469.9). This is consistent with a Lys324 adduct of the C4-AP lesion (e.g., **7**), as was previously observed with Pol β and the reaction of this lesion with simple primary amines.[26,45] Once again, we observe a peak corresponding to residues Ala299−Glu315 (m/z 1816) in the unmodified digest that is absent in the digest of material enzyme reacted with DNA containing pC4-AP (**3**).



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DISCUSSION

A variety of molecules that oxidatively damage DNA are chemotherapeutic candidates. Understanding the biochemical effects of the damaged DNA created by these molecules helps to provide chemical bases for their biological activity. It is difficult to decipher the effects of specific DNA lesions by using the oxidizing agents themselves as a source of the damage because these molecules typically show poor sequence selectivity and sometimes produce multiple forms of damage. Independent generation of DNA lesions in synthetic oligonucleotides greatly facilitates such investigations. For instance, this approach recently revealed that electrophilic lesions can produce DNA interstrand cross-links (ICLs) and that these ICLs are converted to double-strand breaks during nucleotide excision repair.[32] Two lesions containing 1,4-dicarbonyls, DOB and C4-AP, have been shown to irreversibly inactivate Pol β, an enzyme that is critical in BER.[24−26] Preliminary studies also showed that DOB inactivates the structurally and functionally homologous protein Pol λ.[26]

Quantitative data on the lyase activity of Pol λ were lacking. Hence, to calibrate our results, the enzyme's ability to conduct the excision of dRP, DOB, and pC4-AP was measured under single-turnover conditions. Previous studies established that Ape1 converts C4-AP to pC4-AP and that Pol β excises the remaining fragment of the lesion approximately one-half as fast as it removes dRP.[26] Pol λ exhibits greater selectivity in its preference for removing the remnants of abasic lesions from the 5′-termini of the ternary complexes produced upon incision by Ape1. dRP is cleaved ∼7 times more rapidly than pC4-AP and more than 12 times faster than DOB. However, Pol λ reacts with dRP and pC4-AP 2 orders of magnitude more slowly than Pol β does. This significant difference raises the question of how important Pol λ is as a backup for Pol β in the repair of some lesions.[37,38]

Under steady-state conditions, Pol λ inactivation by DOB is comparable to the lesion's effect on Pol β (Figure 2). The apparent K_I for Pol λ inactivation by DOB is ∼3 times higher than that observed for Pol β, but both enzymes conduct approximately four turnovers prior to inactivation.[24] Pol λ contains separate binding sites to catalyze lyase and polymerase reactions. We were unsure whether inactivation of the lyase activity would also prevent Pol λ from conducting DNA polymerization. Indeed, extension experiments employing 5′-^{32}P-**1** or 5′-^{32}P-**3** indicate that DOB and pC4-AP knock out Pol λ polymerase activity as well. To prevent polymerization, it is necessary to incubate the lesion-containing DNA and Pol λ before adding the dNTPs. We attribute this to the greater efficiency of the enzyme's polymerase site compared to its lyase activity, which correlates with the belief that the rate-limiting step in BER is the dRP lyase reaction by Pol β.[46] These results indicate that this may also be the case for Pol λ.

The effects of DOB and pC4-AP on Pol λ activity are very similar to those on Pol β. However, how DOB modifies the two proteins is very different. The major pathway (∼90%) for Pol β inactivation involves covalent bond formation with the modified oligonucleotide, whereas none of this respective product is detected when ^{32}P-**1**, which contains DOB, inactivates Pol λ (Scheme 3).[18] Tritium labeling experiments indicate that Pol λ inactivation by DOB results entirely in protein modified by but-2-ene-1,4-dial (**6**). We were unsuccessful in detecting a DNA−protein cross-link by conducting the incubation for shorter times. However, we cannot distinguish between rapid decomposition of such a cross-link and direct reaction of **6** released in the active site following the lyase reaction.

Mass spectral examination of protease digests of inactivated Pol λ reveals similarities with the homologous residues of Pol β. Indirect and direct evidence of modification of Lys312 and Lys324, respectively, which occupy the 8 kDa lyase domain (Figure 7), was obtained following reaction between Pol λ and substrates containing DOB and pC4-AP. $NaBH_4$ trapping experiments establish the functional analogy between Lys312 in Pol λ and the primary lysine residue (Lys72) believed to be responsible for Schiff base formation in Pol β.[39] Sequence homology suggests that Lys324 is analogous to Lys84 in Pol β,

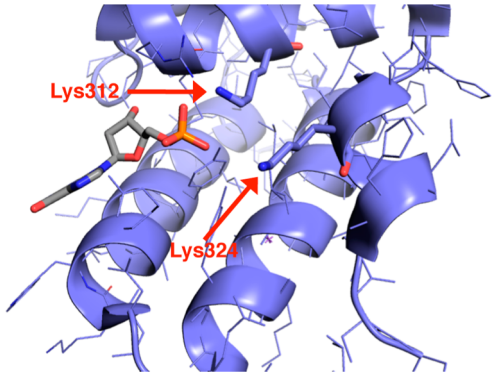

Figure 7. Portion of the Pol λ 8 kDa lyase domain showing the proximity of Lys312 and Lys324 to the terminus of bound DNA. Structure taken from Protein Data Bank entry 1RZT.

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which is also modified following inactivation by DOB- and pC4-AP-containing DNA. Lys84 is believed to be a minor contributor to lyase activity in Pol β.[36] Chromatographic separation of the digested fragments revealed that Lys312 and Lys324 are completely modified. When considered in the context of finding approximately half of the first turnover modified in the ^{3}H experiment described above, and the observation that Pol λ conducts approximately four turnovers prior to inactivation, these data suggest that more than one modification is required to inactivate the enzyme. Lys312 has previously been shown to be the key residue for Schiff base formation with dRP, but our results show Lys324 also has strong reactivity with but-2-ene-1,4-dial released in the active site. The complete modification of both residues suggests that Lys324 also contributes to the lyase activity of Pol λ. A third modification, Lys273, was observed in Pol λ, albeit less frequently, when DOB (but not pC4-AP) inactivates the enzyme. Lys273 is also within the lyase domain, and a homologous residue is absent in Pol β.

CONCLUSIONS

Molecules that damage DNA are often cytotoxic and useful as anticancer agents. How the DNA damage translates into cell death is not always clear. Understanding the effects of specific forms of DNA damage on biochemical processes provides insight into how drugs that produce these lesions kill cells. Pol β and Pol λ possess 5′-dRPase activity. Although Pol λ conducts this reaction more slowly, under some circumstances it may act as a backup of Pol β in BER. Recent in vitro studies using DNA substrates containing DOB or pC4-AP at defined sites revealed that these lesions inactivate Pol β, an enzyme that is vital in BER, by reacting with lysine residues in the protein's lyase domain. We have now shown that DOB and pC4-AP inactivate Pol λ in a mechanistically analogous manner. In this regard, Pol λ joins the ranks of other proteins, such as AlkB, Ku, and Pol γ, that also exhibit lyase activities.[47−49] Pol λ has been shown to play a role in the repair of 8-oxo-7,8-dihydro-2′-deoxyguanosine,[50,51] and studies in cells suggest it serves as a backup for Pol β in BER.[37,38] Our results indicate that DOB and pC4-AP inactivate both enzymes, providing an obstacle to repair in the cell.

Pol λ inactivation by these lesions also may have bearing on NHEJ in which this polymerase plays an important role.[41] Antitumor agents form bistranded lesions containing DOB or C4-AP, which may serve as precursors to double-strand breaks that would be repaired by NHEJ.[52−54] Ku has been found to act as a dRPase via Schiff base formation in this process.[48,55] Although it has not yet been explicitly shown, it is possible that DOB and pC4-AP (and maybe even C4-AP) might also inhibit Ku, thus impacting the efficiency of NHEJ.

ASSOCIATED CONTENT

Supporting Information

Mass spectra and UPLC chromatograms of enzyme digests. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AP, apurinic/apyrimidinic site; L, 2-deoxyribonolactone; C2-AP, C2′-oxidized abasic site; C4-AP, C4′-oxidized abasic site; BER, base excision repair; DOB, 5′-(2-phosphoryl-1,4-dioxobutane); dsb, double-strand break; dRP, 5′-deoxyribose phosphate; Pol β, DNA polymerase β; Pol λ, DNA polymerase λ; ssb, single-strand break; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; UPLC, ultraperformance liquid chromatography.

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