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Expanded Substrate Scope of DNA Polymerase Theta and DNA Polymerase Beta: Lyase Activity on 5'-Overhangs and Clustered Lesions

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Abstract

DNA polymerase θ (Pol θ) is a multifunctional enzyme with double strand break (DSB) repair, translesion synthesis, and lyase activities. Pol θ lyase activity on ternary substrates containing a 5'-dRP that are produced during base excision repair of abasic sites (AP) is weak compared to DNA polymerase β (Pol β), a polymerase integrally involved in base excision repair (BER). This led us to explore whether Pol θ utilizes its lyase activity to remove 5'-dRP and incise abasic sites from alternative substrates that might be produced during DNA damage and repair. We found that Pol θ exhibited lyase activity on abasic lesions near DSB termini and on clustered lesions. To calibrate the Pol θ activity, Pol β reactivity was examined with the same substrates. Pol β excised 5'-dRP from within a 5'-overhang 80-times faster than did Pol θ . Pol θ and Pol β also incised AP within clustered lesions but showed opposite preferences with respect to the polarity of the lesions. AP lesions in 5'-overhangs were typically excised by Pol β 35–50-times faster than those in a duplex substrate but 15- to 20-fold less than 5'-dRP in a ternary complex. This is the first report of Pol θ exhibiting lyase activity within an unincised strand. These results suggest that bifunctional polymerases may exhibit lyase activity on a greater variety of substrates than previously recognized. A role in DSB repair could potentially be beneficial, while the aberrant activity exhibited on clustered lesions may be deleterious due to their conversion to DSBs.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

Representative kinetic plots, fluorescence anisotropy data, mass spectra of oligonucleotides containing abasic site precursors
Supporting Information is available free of charge on the ACS Publications website.

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Introduction

Base excision repair (BER) removes damaged nucleobases and sugars from DNA, replaces them with nucleotides in a template-dependent manner, and completes the process by ligating the 3'-hydroxyl and 5'-phosphate in an ATP dependent reaction (Scheme 1A).¹ BER of a damaged base is initiated by a glycosylase which hydrolyzes the N-glycosidic bond to create an apurinic/aprimidinic site (AP, Chart 1). In humans, AP endonuclease 1 (Ape1) incises the 5'-phosphate of AP, generating 5'-dRP within a ternary complex. Repair proceeds by short-patch BER (SP-BER) involving the lyase activity of a BER bifunctional polymerase or by long-patch BER (LP-BER) involving strand displacement synthesis by Pol β, Pol δ, or Pol ε.^{1,2} Pol β is the major BER polymerase in human cells.^{3,4} Other polymerases, including Pol θ, possess lyase activity and function in BER *in vitro*.⁵⁻⁷ However, the 5'-dRPase activity of Pol θ in a ternary complex is approximately 1000-fold weaker than that of Pol β, calling into question the importance of this activity in cells.^{5,8,9} Because Pol θ is unequivocally associated with DSB repair, we postulated that a ternary complex containing a 5'-dRP may not be the primary substrate for its lyase activity. Consequently, we examined Pol θ lyase activity on atypical substrates and compared this with Pol β.

Pol β, the major BER polymerase in humans, is a small (39 kDa) polymerase belonging to the X family.¹⁰ It possesses an 8 kDa N-terminal lyase domain and a C-terminal polymerase domain. The former catalyzes the removal of 5'-dRP from DNA via Schiff base formation and elimination of an unsaturated sugar fragment (Scheme 1B), while the latter conducts gap-filling DNA synthesis.¹¹ The lyase activity of Pol β promotes cellular resistance to alkylating agents, indicating that 5'-dRP removal by Pol β is important for efficient BER of alkylation damage.¹² The lyase activity of Pol β appears to largely be restricted to abasic lesions in duplex DNA containing a nick immediately 5'-to the lesion. For instance, under single turnover conditions, Pol β excises 5'-dRP, pC4-AP, and DOB (Chart 1) from nicked duplexes, although the oxidized abasic lesions irreversibly inhibit the enzyme.^{9,11-13} In contrast, Pol β lyase activity on unincised AP is extremely low, and a 5'-dRP present in the 5'-overhang of a DSB is excised more slowly than from a ternary complex.^{9,14,15} Whether Pol β has proficient lyase activity on abasic lesions present in other structural contexts is unknown.

In addition to Pol β , human cells possess several other polymerases which may contribute to BER, including Pol θ .^{5,16–18} Pol θ is a large (290 kDa), error-prone polymerase belonging to the A-family.^{19,20} It possesses a unique domain organization with an N-terminal helicase domain, a large central domain which is predicted to be unstructured, and a C-terminal polymerase domain (98 kDa).²⁰ The polymerase domain contains an associated lyase activity, distinguishing Pol θ from X-family polymerases such as Pol β and Pol λ which have a separate lyase domain.^{5,21} The lyase activity of Pol θ on 5'-dRP has been directly demonstrated *in vitro* and in cell extracts from chicken DT40 cells.^{5,6} This activity on a 5'-dRP in a ternary complex is weak compared to Pol β (~1000-fold slower) but comparable to other repair polymerases.^{5,8,9,22} It also excises DOB and pC4-AP, although turnover in the latter is limited due to irreversible inhibition.⁷ Pol θ is unique in that a single residue, Lys₂₃₈₃, located in the O-helix of the fingers subdomain, is critical for lyase and polymerase activities.²³ Although the identification of the catalytic residue for Pol θ lyase sheds light on this reaction, additional questions remain. For instance, it is unclear whether Pol θ utilizes its lyase activity in cells or upon which substrates it uses this activity. Because Pol θ plays important roles in DSB repair,^{20,24,25} we considered whether its lyase activity is used to remove abasic lesions from DSBs or from other substrates.

BER acts upon damaged bases, apurinic/apyrimidinic sites (AP), and oxidized abasic sites such as C4-AP and DOB, which are generated by ionizing radiation and some chemotherapeutics.^{9,13,26–29} BER of various lesions within duplex DNA is well characterized; however, many lesions repaired by BER are formed within different structural contexts. For instance, ionizing radiation generates clusters of DNA damage, where two or more lesions are present within two helical turns.³⁰ Oxidized abasic sites (e.g. DOB, C4-AP) are produced as part of bistranded lesions by antitumor antibiotics, such as bleomycin and the enediynes.^{31,32} In some cases, BER of clustered lesions is inhibited.^{33–35} In others, BER of clustered damage generates DSBs, which are more cytotoxic than the original damage.^{30,33,36} The contribution of different BER enzymes to clustered lesion repair may therefore be important to understanding cellular response to ionizing radiation. Additionally, DSBs generated by abortive BER of clustered lesions can possess associated base damage or abasic lesions.³³ Such DSBs are refractory to repair by end-joining pathways unless the abasic damage is excised by a 5'-dRP/AP lyase.¹⁵ We speculated that Pol θ may utilize its lyase activity to excise abasic damage from DSB termini, as this polymerase plays important roles in DSB repair.²⁰ Pol θ exhibited lyase activity on abasic lesions present at the 5'-terminus within the 5'-overhang of a DSB, as well as on some clustered lesions containing a single nucleotide gap on the opposite strand. Pol β showed even greater lyase activity on abasic lesions at a DSB terminus and show an opposite preference with respect to polarity for abasic lesion incision within the intact strands of clustered lesions compared to those processed by Pol θ . These results suggest that repair polymerases may act upon abasic lesions present in structural contexts other than those typically produced during BER.

Methods

General Methods.

Oligonucleotides were synthesized on an Applied Biosystems Inc. 394 DNA synthesizer using reagents from Glen Research (Sterling, VA). C4-AP, DOB, and AP photochemical precursor phosphoramidites were synthesized as described previously and incorporated into DNA by solid phase oligonucleotide synthesis.^{13,37,38} γ -³²P-dATP and α -³²P-ATP (cordycepin) were obtained from PerkinElmer. Protein purification was conducted using an AKTA FPLC using columns from GE Healthcare. C₁₈ Sep Pak cartridges were from Millipore. Terminal deoxynucleotidyl transferase, uracil DNA glycosylase (UDG) and T4 polynucleotide kinase were obtained from New England Biolabs. Analysis of radiolabeled oligonucleotides was carried out using a Storm 860 Phosphorimager and ImageQuant 7.0 TL software. Fluorescence anisotropy measurements were conducted using an AVIV Biomedical Model ATF 107 spectrofluorometer at the Center for Molecular Biophysics at Johns Hopkins University. Photolyses were carried out in a Rayonet photoreactor fitted with 16 lamps having a maximum output at 350 nm. Pol θ catalytic core (residues 1792–2590) was expressed and purified as previously described.^{39,40}

All oligonucleotides prepared solely using commercially available phosphoramidites were deprotected according to the manufacturer's instructions. Those containing photolabile C4-AP, DOB, and AP precursors were deprotected with a solution (1 mL) of concentrated ammonium hydroxide (500 μ L) and 40% methylamine in water (500 μ L) at 65 °C for 1.5 h. All oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE) and desalted by C₁₈ Sep Pak. Modified oligonucleotides were characterized by ESI-MS, UPLC-MS, or MALDI-MS. Oligonucleotides were 3'-³²P-labeled using α -³²P-cordycepin (for **1–3**) or 5'-³²P-labeled (all other substrates) using γ -³²PATP. Oligonucleotide duplexes were prepared by hybridizing the ³²P-labeled strand with the template in a 1:1.5 ratio in phosphate buffered saline (10 mM sodium phosphate, 100 mM NaCl, pH 7.2), heating to 95 °C, and slowly cooling to 25 °C. Ternary complexes were prepared in the same fashion except the labeled strand was annealed to two complementary strands in a 1:1.5:1.5 ratio.

Analysis of Pol θ or Pol β lyase activity under single turnover conditions.

Oligonucleotide duplexes (3'-³²P-**1–3**, 50 nM) or ternary complexes (5'-³²P-**10–13**, 50 nM) were incubated with Pol θ (250 nM) or Pol β (250 nM) at 37 °C in a reaction buffer consisting of 50 mM HEPES pH 7.5, 20 mM KCl, 1 mM EDTA, 1 mM β -mercaptoethanol. In a typical experiment, a 10 $\times$ solution of DNA (500 nM) in 1 $\times$ phosphate buffered saline (10 mM sodium phosphate 100 mM NaCl, pH 7.2) was prepared. Abasic lesions were generated by photolysis (350 nm, 10 min) for **1–3** or UDG treatment (0.5 μ L, 5 units, 37 °C, 15 min) for **10–13** in phosphate buffered saline (10 mM sodium phosphate, 100 mM NaCl, pH 7.2). The 10 $\times$ DNA solution (3 μ L) was added to a solution of H₂O (21 μ L) and 10 $\times$ reaction buffer (3 μ L). A 10 $\times$ solution of Pol θ or Pol β (3 μ L) in storage buffer (20 mM Tris HCl pH 7, 300 mM NaCl, 10% glycerol, 5 mM BME) was added and the reaction was incubated at 37 °C. Aliquots (4 μ L) were removed from the reaction at the indicated times (below) and frozen on dry ice. At the end of the experiment, each aliquot was quenched by

addition of a freshly prepared solution of NaBH₄ (1 μ L, 500 mM). The reactions were incubated at room temperature for 1.5 h with occasional centrifugation on a bench-top centrifuge to allow for complete reaction of residual NaBH₄. Samples were mixed with an equal volume (5 μ L) of 95% formamide containing 10 mM EDTA, bromophenol blue and xylene cyanol. A portion (4 μ L) was subjected to 20% denaturing polyacrylamide gel electrophoresis at 55 W. For **1–3**, electrophoresis was conducted for approximately 4 h, while electrophoresis was 1 h for **10–13**. The gel was exposed to a phosphor storage cassette and imaged using phosphorimaging. The fraction of product formed by enzymatic reaction was plotted as a function of time. Control reactions were conducted in the same fashion except enzyme was omitted from the reaction. Subtraction of the uncatalyzed reaction was unnecessary, because the background reaction was essentially negligible over the time scale of these experiments. Reactions were conducted in triplicate for each experiment, and each experiment was conducted at least twice. Aliquots in Pol θ reactions were removed as follows: **1**: 2.5, 5, 7.5, 10, 20, 30 min; **2**: 1, 2, 3, 4, 6, 10 min; **3**: 0.5, 1, 2, 4, 6, 10 min; **6–9**: 5, 10, 20, 30, 60 min; **10a** and **13a**: 2, 5, 10, 20, 30, 60 min; **10b–13b**: 1, 2.5, 5, 10, 20, 30 min.

Pol β single turnover kinetic experiments were conducted in the same fashion as those using Pol θ . Aliquots were removed as follows: **1**: 5, 10, 20, 30, 60, 300 s; **2**: 1, 2, 4, 6, 10, 20 min; **3**: 10, 20, 40, 60, 120, 300 s; **6**: 10, 20, 30, 45, 60 min; **10a**, **11a**, **13a**: 1, 2.5, 5, 10, 20, 30 min; **12a**: 0.5, 1, 2.5, 5, 10, 30 min; **10b** and **13b**: 2.5, 5, 10, 20, 30, 60 min; **11b**: 1, 2.5, 5, 10, 20, 30 min; **12b**: 0.5, 1, 2.5, 5, 10, 30 min.

Fluorescence anisotropy measurements.

Anisotropy measurements were conducted as described previously.²³ Dichloro-diphenyl-fluorescein-labeled DNA (**4**, **5**, **10–13c**) (250 pM) was incubated with Pol θ or Pol β (varying concentrations) in reaction buffer (50 mM HEPES pH 7.5, 20 mM KCl, 1 mM EDTA, and 1 mM β -mercaptoethanol). Samples also contained 10 % by volume enzyme storage buffer (20 mM Tris HCl pH 7, 300 mM NaCl, 10% glycerol, 5 mM BME). In a typical experiment, a sample (300 μ L) was prepared by mixing polymerase (30 μ L, 2 μ M) in storage buffer with 10 $\times$ reaction buffer (30 μ L), DNA substrate (30 μ L, 2.5 nM), and H₂O (210 μ L). The concentration of polymerase in this solution, termed solution 1, is 100 nM. Samples containing varying concentrations of polymerase were prepared by serial dilution with solution 2. Solution 2 (10 mL) was prepared by mixing H₂O (7.95 mL) with 10 $\times$ reaction buffer (1 mL), enzyme storage buffer (1 mL), and DNA (50 nM, 50 μ L). Mixing solution 1 (150 μ L) and solution 2 (150 μ L) halved the polymerase concentration (from 100 nM to 50 nM). The concentration of DNA, reaction buffer, and storage buffer remained unchanged. An aliquot (150 μ L) of this new solution was mixed with solution 2 (150 μ L) to prepare a solution containing 25 nM polymerase. Serial dilutions were repeated in this fashion to prepare samples containing 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39 nM polymerase.

Samples were incubated at 25 $^{\circ}$ C for 1 h, and fluorescence anisotropy (A) was measured using a portion (125 μ L) of each sample with PMT voltage of 800 mV, 8 nm slit width, and 535 nm excitation and 556 nm emission. Fluorescence anisotropy was measured for each

DNA complex in the absence of polymerase (A_0), and the change in anisotropy (ΔA_0) was calculated for each sample and plotted against the concentration of Pol θ . The data were fit to the single binding site Hill equation $A = A_{\max}([\text{enzyme}]/K_d)$ using Origin 7.0.

Results and Discussion.

Substrate design and general experimental approach.

The motivation for this study was to explore the possibility that Pol θ , an enzyme whose role in DSB repair is well established, utilizes its lyase activity on abasic sites in substrates (Chart 2, 3) other than those that arise during BER of an isolated lesion (Scheme 1A).^{36,41–44,31} For comparison, the interaction of Pol β with these same alternative substrates was examined. Lyase activity by a polymerase on uncleaved abasic site-containing DNA strands is unusual and weak for Pol β .¹⁴ Consequently, we also examined the ability of Pol β and Pol θ to cleave at abasic lesions that are components of clustered lesions (Chart 3), an important type of DNA damage. Substrates were designed in which the abasic site is present in the intact strand of a clustered lesion, with the complementary strand containing a 1-nt gap between 1 and 5 nucleotides away. The effects of orientation and spacing between damaged sites in clustered lesions on other steps in BER have been examined.^{30,33–35,44} However, to our knowledge neither Pol β or Pol θ lyase activity has been reported on such substrates.

The types of lesions examined are frequently produced in cancer cells treated with DNA damaging agents, including γ -radiolysis and anti-tumor antibiotics.^{30,31,41–43,45} Pol β and Pol θ expression is often upregulated in cancer cells.^{20,46–48} We anticipate that the relative polymerase and DNA lesion levels will be relatively high in these environments, indicating that the enzymes will not need to carry out many turnovers. In addition, because the rate-limiting step for bypass polymerases is often product release, steady-state rate constants are heavily influenced by this step.^{49,50} Therefore, lyase activity was measured under single turnover conditions, which enabled us to more directly probe the chemical step under conditions that might more aptly describe the concentration conditions of the enzymes and substrate encounters.

Lyase activity of Pol θ and Pol β on 5'-dRP, DOB, and pC4-AP at a DSB terminus.

Substrates **1–3** were prepared, where either 5'-dRP, DOB, or pC4-AP was present at the 5'-terminus of the duplex (Chart 2). Single turnover Pol β kinetics were previously reported on similar substrates containing 5'-dRP or pC4-AP.⁹ Substrates of this general type could be generated by BER of clustered abasic lesions in the -1 polarity, an orientation where Ape1 shows robust activity.⁵¹ Pol θ excised all three lesions, following the trend pC4-AP > DOB > 5'-dRP (Table 1). The single-turnover Pol θ rate constants (k_{obs}) were very similar to those observed for the ternary substrates representative of BER containing the same lesions and followed the same order of preference.²³ Pol β was reported to exhibit negligible lyase activity on 5'-dRP in the 5'-overhang of a DSB terminus.¹⁵ In contrast, Jacobs reported that k_{obs} for dRP excision by Pol β from a 5'-overhang was $\sim 0.4 \text{ s}^{-1}$. The rate constants reported here (Table 1) are ~ 8 -fold slower than that determined by Jacobs, and 5'-dRP excision by

Pol β is far slower when the lesion is part of a 5'-overhang than in a ternary complex but it is appreciable.

Pol θ and Pol β exhibit differences in their relative preferences for 5'-overhang substrates (Table 1) and ternary complexes typically formed as intermediates during BER (Scheme 1A). Pol θ excises lesions from BER intermediates and DSBs with similar efficiency, but Pol β exhibits a preference for BER intermediates (Table 1).^{9,23,52} Comparison with previous reports suggests that Pol β lyase activity is at least 60-fold more active on 5'-dRP in a ternary substrate than within a 5'-overhang.^{9,52} The enzymes also exhibit different preferences in 5'-overhang substrates (Table 1). Pol β excises 5'-dRP more rapidly than oxidized abasic sites, following the trend 5'-dRP > pC4-AP > DOB; whereas Pol θ exhibits a preference for oxidized abasic sites, following the trend pC4-AP > DOB > 5'-dRP. Fluorescence anisotropy measurements (Table 2) on 5'-overhang (4) and ternary (5) complexes containing a stabilized abasic site (F) suggest that although Pol θ binds the former more strongly, both enzymes bind tightly to the substrates. Therefore, Pol β 's preferred activity on BER intermediate substrates over those corresponding to DSBs cannot be explained by poor binding to the latter.

Exploring the Substrate Scope of Pol θ Lyase Activity.

The relatively high Pol θ lyase activity on substrates containing abasic lesions with the 5'-overhangs produced upon double-strand cleavage led us to examine other atypical substrates (Chart 2). In our hands, Pol θ had no detectable lyase activity (<1% reaction in 1 h) within a duplex substrate (6). Lyase activity was analyzed on AP recessed within either a 5'-overhang (7) or a 3'-overhang (8) of a DSB. Such complex DSBs could be generated by high LET radiation or by abortive BER of clustered lesions.^{30,53} Pol θ had minimal activity (estimated k_{obs} : $8 \times 10^{-6} \text{ s}^{-1}$) on AP recessed within the 5'-overhang (7). Lyase activity was slightly greater when AP was recessed in a 3'-overhang (estimated k_{obs} : $\sim 4 \times 10^{-5} \text{ s}^{-1}$), although this was at least 40-fold slower than Pol θ excision of 5'-dRP in a 5'-overhang (1, Table 1).

Pol β prefers to act on substrates containing 5'-phosphates. A similar preference for 5'-phosphate containing substrates may explain the weak AP excision by Pol θ in 6–8. However, one atypical, albeit possibly biologically irrelevant substrate was an outlier. Pol θ cleaved an intact strand containing AP (k_{obs} : $2.3 \pm 0.2 \times 10^{-4} \text{ s}^{-1}$) when the lesion was opposite a two-nucleotide gap on the other strand (9). The only difference between 9 and 6 (a substrate upon which Pol θ has no activity, Table 3) is the presence of a two-nucleotide gap on the opposing strand. This raised an intriguing possibility that Pol θ may have lyase activity on clustered lesions, specifically on those containing an abasic lesion on one strand and a nearby gap on the other. The potential for human enzymes to act upon clustered lesions is of interest because faulty repair of a clustered lesion can convert it into a more deleterious DSB.^{36,54}

Lyase Activity of Pol θ and Pol β on Clustered Lesions

Several substrates (10–13) were prepared containing a single nucleotide gap on the strand base paired to that containing an AP or C4-AP lesion (Chart 3). Briefly, 10–13 could be generated by BER of a clustered lesion, producing a single nucleotide gap on the top strand.

(Scheme 1). Glycosylase activity on a damaged nucleobase on the bottom strand would generate AP (**10a–13a**), while hydrogen abstraction from the deoxyribose sugar, induced by ionizing radiation or radiomimetic agents, would generate C4-AP directly. The position of the single nucleotide gap was chosen so that the substrate scope could be analyzed as a function of clustered lesion polarity (commonly denoted by the distance between two lesions in the plus (5') or minus (3') direction).

Pol θ incised bistranded lesions containing AP when the single nucleotide gap was located in the negative polarity (–1 (**10a**); –4; (**12a**), Table 3). The observed rate constant for incision in these substrates was comparable to that for 5'-dRP excision from a 5'-overhang (**1**, Table 1). In contrast, no activity was detected on positive polarity substrates (+1 (**11a**); +5 (**12a**), Table 3). Pol β showed the opposite polarity preference, incising AP in positive polarity substrates **11a** (+1) and **12a** (+5) more than 20 times as rapidly as the negative polarity substrates (**10a** and **13a**, Table 3). However, its incision of AP in the positive polarity clustered lesions (**11a**, **12a**) was still more than 10-fold less efficient than 5'-dRP excision in a 5'-overhang (Table 1). Pol β was no more active on negative polarity substrates than an intact duplex (**6**, Table 3). Overall, these results indicate that the presence of a single nucleotide gap on the opposite strand only increases activity of Pol θ and Pol β (relative to an intact duplex, **6**) when that gap is present in a specific and complementary polarity.

Qualitatively, the same trend was observed when examining Pol θ and Pol β activity on bistranded lesions containing C4-AP (**10b–13b**, Table 4, Chart 3). Pol θ is more active on negative polarity clustered lesions (**10b**, **13b**) than positive polarity ones (**11b**, **12b**). The observed rate constants for incising C4-AP in negative polarity clustered lesions are modestly faster than those measured for AP (Table 3). However, C4-AP is incised more rapidly by Pol θ within **11b** and **12b** than AP was in the corresponding positive polarity substrates (**11a**, **12a**, Table 3), resulting in only an approximately 2-fold preference for C4-AP incision in negative polarity substrates. Pol β retained its preference for positive polarity bistranded substrates to a greater extent when incising C4-AP than did Pol θ (Table 4). However, the preference was also diminished compared to substrates containing AP. The decrease in selectivity for types of clustered lesions may be due partially to the greater chemical reactivity of C4-AP than AP. C4-AP undergoes strand scission in free DNA under physiologically relevant buffer conditions considerably more rapidly than AP.^{38,55–57}

Role of Lys₂₃₈₃ in Pol θ Lyase Activity on Clustered Lesions

The only crystal structures of Pol θ show it bound to a primer-template in the closed conformation.⁵⁸ In these structures, the O-helix of the fingers subdomain occupies the position which would likely be occupied by the flanking DNA strand present in structures **10–13**. Therefore, it is difficult to envision how Pol θ binds to **10–13** and how this would affect the positioning of the O-helix. The positioning of the O-helix is likely important for lyase activity, because the major nucleophile responsible for Schiff base formation, Lys₂₃₈₃, is located in this helix.²³ To investigate the role of Lys₂₃₈₃ in cleavage of negative polarity AP substrates (**10a** and **13a**), the activity of the K2383R variant of Pol θ was measured on each substrate. K2383R Pol θ showed a 60% reduction in activity on the –1-polarity substrate (**10a**, $k_{\text{obs}} = 4.7 \pm 1.2 \times 10^{-4} \text{ s}^{-1}$) and a 78% reduction in activity on the –4-

polarity substrate (**13a**, $k_{\text{obs}} = 5.5 \pm 0.2 \times 10^{-4} \text{ s}^{-1}$) compared to wild type enzyme. The percent reductions in rate constants were similar to that observed involving a ternary 5'-dRP substrate (Scheme 1).²³ This suggests that clustered lesions are accommodated in the Pol θ lyase active site, allowing for attack and strand cleavage catalyzed by the major nucleophile, Lys₂₃₈₃, in a similar manner as the previously reported BER substrate. However, the basis for the polarity preference which Pol θ exhibits upon **10–13** is unclear.

Possible Structural Basis for Pol β Lyase Activity on Clustered Lesions.

To our knowledge, the opposite clustered lesion polarity preferences exhibited by Pol θ and Pol β were unprecedented. The possibility that they resulted from more favorable enzyme binding to the preferred substrates was probed by measuring the dissociation constants for model clustered lesions containing the chemically stable AP analogue, F (**10c–13c**, Table 2) in place of AP or C4-AP. Dissociation constants for Pol θ binding to **10c–13c** were within experimental error of each other, indicating that Pol θ does not show a polarity preference for binding to clustered lesions (Table 2). Although Pol β binding to **10c–13c** was approximately two- to three-fold weaker than that of Pol θ , there also was no discernible pattern based upon the polarity of the lesions.

Since macroscopic binding did not provide insight into the polarity preferences during lyase activity on **10–13** by Pol β and Pol θ , we turned to structural data in the literature for a possible explanation. The co-crystal structure of Pol β with gapped DNA (PDB ID: 1BPX)⁵⁹ may provide insight into the binding of **10–13** by this polymerase. The DNA substrate in this structure is a good approximation of clustered lesions **10–13**, because it is a ternary complex with a single nucleotide gap on one strand. In this structure, the DNA is kinked 90° so that the 5'-phosphate adjacent to the gap is positioned within the lyase active site (Figure 1). If Pol β binds the single nucleotide gap in **10–13** in a similar fashion, positive polarity clustered lesions **11a,b** and **12a,b** would appear to position AP (or C4-AP) close to the lyase active (Figure 1). Meanwhile negative polarity clusters (**10a,b** and **13a,b**) would have AP (or C4-AP) far removed from the lyase active site, potentially explaining why positive polarity clusters are better substrates for Pol β than those of negative polarity. However, the +1 and +5 positions are still quite far (16.3 Å and 21.3 Å, respectively) from Lys₇₂ in this snapshot. Protein conformational change and/or DNA substrate flexibility would be required to enable Lys₇₂ to catalyze lyase activity on **11a,b** and **12a,b**. Alternatively, residues other than Lys₇₂ may act as nucleophiles. For instance, multiple lysine residues in Ku appear to contribute to dRPase reactions,⁶⁰ so it is possible that other residues contribute to incision of **11a,b** and **12a,b** by Pol β . For instance, Lys₃₅ is closer to the +1 position (10.3 Å) than Lys₇₂, while Lys₆₀ is closer than Lys₇₂ to the +5 position (17 Å), but these distances are still quite large.

Although the –1 and –4 positions lie well outside of the Pol β lyase active site, strand scission is still observed on **10a,b** and **13a,b**, albeit at a slower rate than **11a,b** and **12a,b**. Weak lyase activity on negative polarity clusters could be catalyzed by lysine residues within the polymerase domain. For instance, Lys₂₃₄ is 6.4 Å from the nucleotide at the –4 position of gapped DNA. Carboxylate residues, such as Glu₂₆ in the lyase domain of Pol β are often proposed to deprotonate the Schiff base intermediate,¹⁰ although lysine residues and/or histidines may also contribute to lyase reactions.^{38,61} His₁₃₅ is 9.9 Å from the C2' carbon of

the -4 position, while Glu₂₃₂ is 9.2 Å from this position, suggesting that either of these residues may function in strand scission by β -elimination. There is no obvious candidate for nucleophilic attack at the -1 position, although Glu₂₉₅ is 6.4 Å from the C2' carbon of the -1 position, suggesting that this residue may catalyze β -elimination. Strand scission catalyzed by residues outside of the lyase domain could also explain the greater than 20-fold preference for positive polarity substrates (positioned within the lyase active site) over negative polarity substrates.

Conclusions

Pol θ and Pol β exhibit lyase activity on several alternative substrates that are not typically associated with BER. Notably, both polymerases exhibit lyase activity on 5'-dRP, DOB, and pC4-AP in a 5'-overhang of a DSB terminus. Abasic site excision from the terminus of a 5'-overhang could be a useful housekeeping role in double strand break repair as it provides a 5'-phosphate that can ultimately be ligated. Pol β removes 5'-dRP from a DSB terminus with a single turnover rate constant (0.14 s^{-1}) that is 33-fold greater than the value reported for Ku ($4.3 \times 10^{-3} \text{ s}^{-1}$), another protein involved in double strand break repair.¹⁵ Extrapolation of kinetic results in the test tube to cells may be complicated because Ku is recruited to laser-induced DSBs within seconds of their formation, and appears to remove the majority of abasic lesions present at DSB termini in some cell extracts.¹⁵ The rapid recruitment of Ku to DSBs suggests that Ku could restrict access of these polymerases to such damage. However, a role for Pol θ in DSB repair is well-established,^{20,24,25} and Pol β is also proposed to function in repair of DSBs by end-joining.⁶² Therefore, it is possible that the lyase activity on abasic lesions of one or both of these polymerases contributes to double strand break repair.

Pol θ and Pol β also exhibit lyase activity on AP and C4-AP within an intact strand of DNA when a single nucleotide gap is present in the other strand. The enzymes exhibit complementary preferences with respect to the polarity of the clustered lesions. This is the first report of lyase activity of either polymerase on these types of clustered lesions and suggests that Pol θ and Pol β can convert some clustered lesions to DSBs. This activity was relatively surprising, especially for Pol β , which has minimal activity on DNA strands containing unincised, isolated AP.¹⁴ Furthermore, although the incisions were considerably slower than that for Pol β acting on a 5'-dRP within the type of ternary substrate that it normally encounters during BER (Scheme 1), the observed rate constants under single turnover conditions were comparable to the 5'-dRPase activity of other polymerases or AP incision by other atypical lyase repair enzymes.^{16,63-65}

Other types of repair enzymes can convert similar clustered lesions to DSBs. For instance, bifunctional glycosylases can cleave AP, potentially converting clustered lesions in which one strand is cleaved into DSBs.⁶⁶ Ape1 may also convert these clustered lesions to DSBs, as it is known to convert bistranded lesions containing AP or F in both strands into DSBs.^{33,51} Although rate constants were not reported, Ape1 conducts hundreds of turnovers within 1 h on negative polarity substrates, generating DSBs at rates that would be faster than those determined here involving Pol β and Pol θ .³³ However, Ape1 activity is strongly inhibited *in vitro* when two AP sites are present in the +1 orientation (e.g. **11a,b**).^{33,51} The observation

that Pol β efficiently cleaves **11a** to generate a DSB suggests that this clustered lesion can be converted to a double strand break without the activity of Ape1. These results expand the scope of enzymatic reactions by which clustered lesions may be converted to double strand breaks by aberrant BER.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Superimposition of clustered lesions (**10–13**) on top of Pol β with gapped DNA crystal structure (PDB ID: 1BPX). The locations of relevant nucleotides (relative to the single nucleotide gap) are indicated (-4 , -1 , $+1$, $+5$). 8-kDa lyase domain cyan; polymerase domain, green. Relevant lysines in the 8-kDa lyase domain are colored by element (C: green, N: blue).

Scheme 1.
Base excision repair.

Chart 1.
Abasic site structures

5'-d(**X**G GAA ATC AAA TGT AAG TAG AGG TCA)
 3'-d(CTT TAG TTT ACA TTC ATC TCC AGT)
X: **1**, 5'-dRP; **2**, DOB; **3**, pC4-AP

5'-d(**P**FG GAA ATC AAA TGT AAG TAG AGG TCA)
 3'-d(CTT TAG TTT ACA TTC ATC TCC AGT)-**FI**
4 FI = dichloro-diphenyl-fluorescein (SIMA-HEX)

5'-d(TAA TGG CTA ACG CTT **P**FCC GTA ATG CAG TCT)
 3'-d(ATT ACC GAT TGC GAA AGG CAT TAC GTC AGA)-**FI**
5 FI = dichloro-diphenyl-fluorescein (SIMA-HEX)

5'-d(CGA CCG GCT CGT ATG **X**TG TGT GGA GCT GTG G)
 3'-d(GGT GGC CGA GCA TAC AAC ACA CCT CGA CAC C)
6 X = AP

5'-d(TAT G**X**T GAC CTA CAC GCT AGA AC)
 3'-d(A CTG GAT GTG CGA TCT TG)
7 X = AP

5'-d(AGC TGT CTA CGA CCA ATT GAG **C**XG CCT CGG)
 3'-d(TCG ACA GTA GCT GGT TAA CTC)
8 X = AP

5'-d(CGA CCG GCT CGT ATG **X**TG TGT GGA GCT GTG G)
 3'-d(GGT GGC CGA GCA TAC C ACA CCT CGA CAC C)
9 X = AP

Chart 2.

Lyase and binding substrates

		Clustered Lesion Polarity
5'-d(CCA CAG CAC CAC AC	P _{GAA} TAC GAG CCG GTC G)	-1
3'-d(GGT GTC GAG GTG TGT <u>X</u> TT ATG CTC GGC CAG C)		
10a X = AP; 10b X = C4-AP		
5'-d(CCA CAG CAC CAC AC	P _{GAA} TAC GAG CCG GTC G)	-1
3'-d(GGT GTC GAG GTG TGT <u>X</u> TT ATG CTC GGC CAG C)-FI		
10c X = F; FI = dichloro-diphenyl-fluorescein (SIMA-HEX)		
5'-d(CCA CAG CAC CAC ACA C	P _{ATA} CGA GCC GGT CG)	+1
3'-d(GGT GTC GAG GTG TGT <u>XT</u> TAT GCT CGG CCA GC)		
11a X = AP; 11b X = C4-AP		
5'-d(CCA CAG CAC CAC ACA C	P _{ATA} CGA GCC GGT CG)	+1
3'-d(GGT GTC GAG GTG TGT <u>XT</u> TAT GCT CGG CCA GC)-FI		
11c X = F; FI = dichloro-diphenyl-fluorescein (SIMA-HEX)		
5'-d(CCA CAG CAC CAC ACA CAA TA	P _{GAG} CCG GTC G)	+5
3'-d(GGT GTC GAG GTG TGT <u>XTT</u> ATG CTC GGC CAG C)		
12a X = AP; 12b X = C4-AP		
5'-d(CCA CAG CAC CAC ACA CAA TA	P _{GAG} CCG GTC G)	+5
3'-d(GGT GTC GAG GTG TGT <u>XTT</u> ATG CTC GGC CAG C)-FI		
12c X = F; FI = dichloro-diphenyl-fluorescein (SIMA-HEX)		
5'-d(CCA CAG CAC CA	P _{ACA} GAA TAC GAG CCG GTC G)	-4
3'-d(GGT GTC GAG GTG <u>TGT</u> XTT ATG CTC GGC CAG C)		
13a X = AP; 13b X = C4-AP		
5'-d(CCA CAG CAC CA	P _{ACA} GAA TAC GAG CCG GTC G)	-4
3'-d(GGT GTC GAG GTG <u>TGT</u> XTT ATG CTC GGC CAG C)-FI		
13c X = F; FI = dichloro-diphenyl-fluorescein (SIMA-HEX)		

Chart 3.

Clustered lesion lyase and binding substrates

Table 1.

Single turnover rate constants for Pol θ and Pol β lyase activity on abasic sites in 5'-overhangs.

Lesion	K_{obs} (s ⁻¹) Pol θ^I	K_{obs} (s ⁻¹) Pol β^I
5'-dRP (1)	$(1.7 \pm 0.1) \times 10^{-3}$	$(1.4 \pm 0.3) \times 10^{-1}$
DOB (2)	$(5.3 \pm 0.3) \times 10^{-3}$	$(5.3 \pm 0.1) \times 10^{-3}$
pC4-AP (3)	$(1.5 \pm 0.6) \times 10^{-2}$	$(6.7 \pm 0.1) \times 10^{-2}$

^IData are the average $\pm$ std. dev. of two experiments, each consisting of 3 replicates.

Table 2.DNA dissociation constants for Pol θ and Pol β .

Substrate	$K_d(\text{nM})\text{Pol } \theta^I$	$K_d(\text{nM})\text{Pol } \beta^I$
5'-overhang (4)	0.4 ± 0.1	1.3 ± 0.1
ternary complex (5)	6.7 ± 0.5	2.7 ± 1.2
-4 cluster (10c)	0.9 ± 0.03	2.9 ± 1.2
-1 cluster (11c)	1.1 ± 0.3	3.6 ± 1.1
+ 5 cluster (12c)	0.7 ± 0.2	2.6 ± 1.4
+ 1 cluster (13c)	0.9 ± 0.4	2.0 ± 0.5

^IData are the average $\pm$ std. dev. of three experiments.

Table 3.Lyase activity of Pol θ and Pol β on substrates containing AP.

Substrate	K_{obs} (s^{-1}) Pol θ^I	K_{obs} (s^{-1}) Pol β^I
duplex (6)	N.D. ²	$(2.0 \pm 1.1) \times 10^{-4}$
cluster- 1 (10a)	$(1.2 \pm 0.2) \times 10^{-3}$	$(3.3 \pm 0.2) \times 10^{-4}$
cluster + 1 (11a)	N.D. ²	$(7.0 \pm 0.1) \times 10^{-3}$
cluster+ 5 (12a)	N.D. ²	$(1.0 \pm 0.1) \times 10^{-2}$
cluster-4 (13a)	$(2.0 \pm 0.5) \times 10^{-3}$	$(2.2 \pm 0.1) \times 10^{-4}$

^IData are the average $\pm$ std. dev. of two experiments, each consisting of 3 replicates.²N.D., not detected.

Table 4.Lyase activity of Pol θ and Pol β on substrates containing C4-AP.

Substrate	K_{obs} (s^{-1}) Pol θ^I	K_{obs} (s^{-1}) Pol β^I
– 1 cluster (10b)	$(3.2 \pm 0.1) \times 10^{-3}$	$(6.7 \pm 0.2) \times 10^{-4}$
+ 1 cluster (11b)	$(1.7 \pm 0.1) \times 10^{-3}$	$(1.1 \pm 0.1) \times 10^{-2}$
+ 5 cluster (12b)	$(1.5 \pm 0.1) \times 10^{-3}$	$(1.6 \pm 0.1) \times 10^{-2}$
–4 cluster (13b)	$(4.3 \pm 0.3) \times 10^{-3}$	$(1.5 \pm 0.1) \times 10^{-3}$

^IData are the average $\pm$ std. dev. of two experiments, each consisting of 3 replicates.