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Long Patch Base Excision Repair Compensates for DNA Polymerase β Inactivation by the C4′-Oxidized Abasic Site[†]

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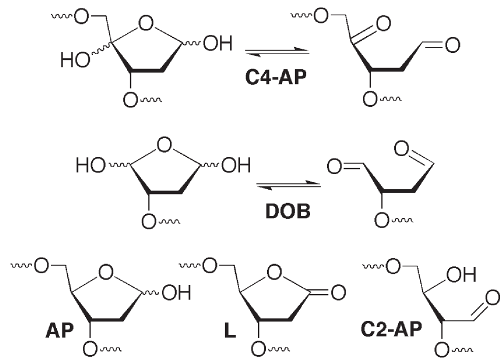
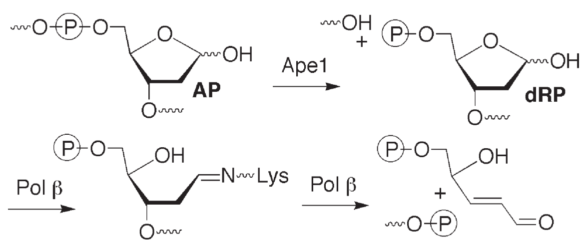
ABSTRACT: The C4′-oxidized abasic site (C4-AP), which is produced by a variety of damaging agents, has significant consequences for DNA. The lesion is highly mutagenic and reactive, resulting in interstrand cross-links. The base excision repair of DNA containing independently generated C4-AP was examined. C4-AP is incised by Ape1 ∼12-fold less efficiently than an apurinic/apyrimidinic lesion. DNA polymerase β induces the β-elimination of incised C4-AP in ternary complexes, duplexes, and single-stranded substrate. However, excision from a ternary complex is most rapid. In addition, the lesion inactivates the enzyme after approximately seven turnovers on average by reacting with one or more lysine residues in the lyase active site. Unlike 5′-(2-phosphoryl-1,4-dioxobutane), which very efficiently irreversibly inhibits DNA polymerase β, the lesion is readily removed by strand displacement synthesis conducted by the polymerase in conjunction with flap endonuclease 1. DNA repair inhibition by C4-AP may be a partial cause of the cytotoxicity of drugs that produce this lesion.

Abstraction of a hydrogen atom from the 2′-deoxyribose rings of nucleotides in DNA gives rise to several oxidized abasic lesions (*1*, *2*). One of these, the C4′-oxidized abasic site [C4-AP[1] (Chart 1)], is produced by a variety of DNA-damaging agents, including γ-radiolysis and antitumor antibiotics (*3*−*5*). Its frequent occurrence is attributed to the high accessibility of the C4′ hydrogen atom to diffusible species, and the relatively low bond dissociation energy of the respective carbon−hydrogen bond (*6*, *7*). C4-AP is efficiently incised by the endonucleases in *Escherichia coli* that are responsible for AP incision (*8*). In addition, previous studies using C4-AP produced by bleomycin indicated that the lesion is a substrate for mammalian BER enzymes, including Ape1 and Pol β (*9*). Ape1 incision and subsequent Pol β excision are the first two steps in BER of AP lesions (Scheme 1). However, this pathway does not efficiently excise all abasic lesions. DOB is an oxidized abasic lesion that is produced by a variety of DNA-damaging agents. Recent experiments revealed that DOB very efficiently inhibits DNA polymerase β irreversibly (*10*). C4-AP contains the 1,4-dicarbonyl functional group that is crucial for irreversible Pol β inhibition by DOB. The structural similarity between these two lesions, the central role played by Pol β in BER, and its increased expression level in some cancer cells motivated us to investigate C4-AP repair using synthetic oligonucleotides in which the lesion was independently generated at a defined position (*11*−*15*).

Chart 1: DNA Lesions



Scheme 1: Role of Pol β in AP Site Repair



Efficient C4-AP repair is likely important for protecting cells against oxidative stress. The lesion is highly mutagenic in *E. coli* where it exhibits the distinctive property of giving rise to three-nucleotide deletions (*16*). The 1,4-dicarbonyl moiety in C4-AP is also highly reactive with nucleophiles, including those in DNA. Interstrand cross-links that can be ascribed to C4-AP have been detected in cellular DNA, and their formation was verified using synthetic oligonucleotides (*17*−*19*). The significance of these interstrand cross-links was demonstrated by their misrepair by the bacterial nucleotide excision repair proteins, UvrABC, which resulted in their transformation into double strand breaks (*20*).

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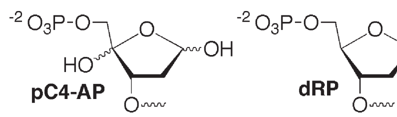
[1]Abbreviations: AP, apurinic/apyrimidinic site; L, 2-deoxyribonolactone; C2-AP, C2′-oxidized abasic site; C4-AP, C4′-oxidized abasic site; pC4-AP, 5′-phosphorylated C4′-oxidized abasic site; BER, base excision repair; DOB, 5′-(2-phosphoryl-1,4-dioxobutane); dsb, double strand break; dRP, 5′-deoxyribose phosphate; Pol β, DNA polymerase β; ssb, single strand break; FEN1, flap endonuclease 1; Ape1, apurinic endonuclease 1.

In addition, model studies and inferential experiments suggest that C4-AP reacts with the amines in proteins (*21–23*). This reactivity with amines could present challenges to BER proteins such as Pol β that utilize Schiff base formation to carry out their designated function. In view of this possibility, we characterized C4-AP's repair by Ape1, Pol β, and FEN1 because if left unrepaired the lesion could impart such deleterious consequences on cellular DNA.

MATERIALS AND METHODS

Materials and General Methods. Oligonucleotides were prepared on an Applied Biosystems Inc. 394 DNA synthesizer. Commercially available DNA synthesis reagents, including 5′-phosphorylation agent, were obtained from Glen Research Inc. Oligonucleotides containing the photolabile C4-AP precursor were synthesized as previously described (*18*, *24*). All others were synthesized and deprotected using standard protocols. Oligonucleotides containing AP or dRP were obtained via UDG treatment of the corresponding DNA complex containing dU. T4 polynucleotide kinase, UDG, terminal deoxytransferase, trypsin, and GluC were obtained from New England Biolabs. DNA Pol β and FEN1 were obtained from Trevigen. Radionuclides were obtained from Perkin-Elmer. ZipTips were from Millipore. Single-turnover experiments were conducted using a KinTek rapid quench instrument. Quantitative analysis of radiolabeled oligonucleotides was conducted using a Storm 840 Phosphorimager and ImageQuant version 5.1. UPLC was conducted using an Agilent Infinity 1290 system. MALDI-TOF MS data were obtained on a Bruker AutoFlex spectrometer. MALDI-TOF mass spectrometry was performed in reflective positive mode. Laser power was varied, starting at lower values and increasing until the signal was $\sim 10^3-10^4$ 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 obtain appropriate signal coverage. Please note that when radiolabeling was used in the experiments described below either the 3′-terminus of the strand containing the modified nucleotide or the 5′-terminus of the flanking oligonucleotide (when appropriate) was labeled.



Preparation of Nucleic Acids Containing C4-AP, pC4-AP, and dRP Lesions. The lesions were unmasked after hybridizing (if appropriate) with the corresponding oligonucleotide(s). Hybridization was conducted at 90 °C (5 min), followed by slow cooling to room temperature in the appropriate buffer. The radiolabeled oligonucleotide was hybridized with 2 equiv of the template strand and the flanking oligonucleotide (when appropriate). The corresponding oligonucleotides or oligonucleotide complexes containing the C4-AP precursor were photolyzed in clear Eppendorf tubes using a Rayonet photoreactor (RPR-100) equipped with 16 lamps having an output maximum at 350 nm. The tubes were placed ~10 cm from the lamps and rotated, maintaining the samples at room temperature with ventilation. The volume of the solutions varied but was never more than 100 μL (in a 600 μL tube). For most reactions, the concentration of labeled oligonucleotide was 1 μM during photolysis. For intact C4-AP precursors, the samples were photolyzed for 20 min. For pC4-AP precursors, a 5 min photolysis was sufficient. Complexes containing dU were treated with UDG (1 unit/pmol of dU) in UDG reaction buffer [100 μL; 20 mM Tris-HCl (pH 8.0), 1 mM DTT, and 1 mM EDTA] for 10 min at 37 °C, cooled in an ice bath, and used immediately (*25*).

Ape1 Incision Kinetics on C4-AP. We treated the radiolabeled duplex [5′-^{32}P]**17** (3–60 nM) with Ape1 (150 pM) in a total volume of 10 μL at room temperature for 3 min by mixing a 2× solution of substrate (5 μL) containing 100 mM KCl and 20 mM $MgCl_2$ with a 2× enzyme solution (5 μL) in 50 mM Hepes-KOH (pH 7.5), 200 μg/mL BSA, 10% glycerol, and 0.05% Triton X-100. The reactions were quenched with an equal volume of 90% formamide loading buffer containing EDTA (1 mM) and analyzed by 20% denaturing PAGE. All experiments were conducted in triplicate. Control experiments without enzyme were conducted under identical conditions to establish the amount of background cleavage. The percent cleavage in the reaction was determined by subtracting the amount of cleavage in the control from that in the reaction.

Steady-State Kinetics of the Lyase Activity of Polymerase β. Ternary complexes ([3′-^{32}P]**14** or [3′-^{32}P]**16**) were used in reaction mixtures (30 μL) containing Pol β (10 nM) and varying concentrations of substrate (100–1000 nM) in HM buffer [50 mM Hepes (pH 7.4) and 5 mM $MgCl_2$]. Reactions were initiated via addition of a 2× enzyme solution (15 μL) to a 2× DNA solution (15 μL) and mixtures incubated at room temperature. At time points of 3, 6, 10, 30, and 60 min, an aliquot (3 μL) was removed and quenched with 1 μL of $NaBH_4$ (400 mM). The quenching reaction was nearly instantaneous, but letting the $NaBH_4$ sit for 1 h prevented further reaction with the polyacrylamide gel. Aliquots were removed from a control reaction mixture containing all of the ingredients described above except enzyme and reactions quenched at the same time points. Products were separated by denaturing 20% PAGE (prerun for at least 3 h, xylene cyanol run ~30 cm at 3000 V for best separation). The percent cleavage in the reaction was determined by subtracting the amount of cleavage in the control from that in the reaction at each time point.

Preincubation Assay. The unlabeled pC4-AP ternary complex (4 pmol, **16**) was incubated with Pol β (0.4 pmol) in a 24 μL reaction mixture containing HM buffer for 15 min at room temperature. Ternary dRP complex ([3′-^{32}P]**14**, 20 pmol) in HM buffer was added to dilute the reaction mixture to 40 μL, yielding final concentrations of 100 nM **16**, 5 nM Pol β, and 500 nM [3′-^{32}P]**14**. The mixture was incubated at room temperature. Aliquots (5 μL) were removed at 5, 30, and 60 min, and quenched with 1 μL $NaBH_4$ (500 mM). Products were separated by 20% denaturing PAGE. Control reactions with initial incubations of unlabeled dRP ternary complex (**14**) or single-stranded C4-AP oligo (**18**) were conducted in the same manner using the same concentrations of reactants.

Single-Turnover Kinetics of Polymerase β. The respec tive substrates ([3′-^{32}P]**2**, **-4**, **-6**, **-8**, **-10**, **-12**, **-14**, and **-16**) were prepared as described above. Working solutions (2×) of 200 nM Pol β (460 μL) and 40 nM DNA substrate (400 μL) in HM buffer were prepared and stored on ice. A portion of the solution (~120 μL) was loaded in a sterile disposable 1 mL syringe, and the syringe was attached to the rapid quench instrument. The buffer syringes of the instrument were loaded with HM buffer, and the quench syringe was loaded with methanol. During the experimental cycle, the reaction loop was first rinsed with methanol and evacuated for 30 s by vacuum pump. Aliquots of substrate and enzyme (~15 μL each) were loaded from their respective inlets and reacted for a defined period before methanol quench. Products released from the rapid quench (~300 μL) were immediately stabilized with 1 M $NaBH_4$ (20 μL pre-added in a 1.6 mL Eppendorf tube) and cooled to 0 °C. To each sample was added a solution (20 μL) containing NaOAc (3 M) and calf thymus DNA (200 μg/mL), and the sample was incubated for at least 10 min at room temperature (to quench the $NaBH_4$). The precipitation was completed with ethanol (1 mL). The sample was resuspended in formamide loading buffer (10 μL) and the products were separated by 20% denaturing PAGE (with the xylene cyanol running ~30 cm, products migrated ~33 cm at 3000 V to achieve optimal resolution).

The cycle was repeated for seven time points (usually 0.02, 0.05, 0.1, 0.2, 0.5, 1, and 2 s for ternary substrates **14** and **16** or 0.1, 0.2, 0.5, 1, 2, 5, and 10 s for other substrates). After a control reaction (no enzyme) had been quenched, the loading syringes were replaced with new syringes containing fresh solutions. The seven time points were assessed in triplicate, and the fraction cleaved was plotted versus time. The resulting graph was fit to the exponential equation fraction cleaved = (maximal fraction cleaved)$(1 - e^{-k_{obs}t})$.

Identification of the pC4-AP DNA Polymerase β Adduct. For the GluC digestion, a solution (100 μL) of Pol β (5 μM) was incubated with or without ternary complex **16** (25 μM) in HM buffer at room temperature for 20 min. The solution was diluted with 47.5 μL of GluC buffer [50 mM Tris-HCl (pH 8.0) and 0.5 mM Glu-Glu] and 2.5 μL of resuspended GluC (10 mM) and then incubated at 37 °C for 4 h. For the trypsin digestion, a solution (100 μL) of Pol β (5 μM) was incubated with or without ternary complex **16** (25 μM) in HM buffer at room temperature for 20 min. The solution was diluted with 47.5 μL of trypsin buffer [50 mM Tris-HCl (pH 8.0) and 20 mM $CaCl_2$] and 2.5 μL of resuspended TPCK-treated trypsin (10 mM) and then incubated at 37 °C for 4 h. Crude digestions were either separated by UPLC or directly spotted on a MALDI plate.

MALDI-MS Preparation for Crude Pol β Digests. The crude samples were concentrated and desalted with a ZipTip (Milipore). The tip was wetted with 50% MeCN (10 μL) and equilibrated with 0.1% TFA (3 × 10 μL). The sample (100 μL) was adjusted to 0.1% TFA (with a 2.5% stock solution) and bound to the column by pipeting up and down ~10 times, allowing the full solution to pass through the tip. The tip was then washed with 0.1% TFA (3 × 10 μL) and eluted with 2 μL of CHCA matrix (10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% MeCN and 0.1% TFA) directly onto the MALDI plate.

UPLC Analysis of Pol β Digests. Digested samples (100 μL) were diluted with 250 μL of water and passed by syringe (1 mL) through a filter (0.22 μm, 4 mm diameter). The samples were further purified with a 10K microcon (Milipore), washing with 200 μL of water and keeping the filtrate, which was concentrated to a dry residue. The products were resuspended in 20 μL of water and injected onto a UPLC system as 2 μL (analytical) or 6 μL (collection) samples. The column (Zorbax RRHD SB-C18, 2.1 mm × 100 mm, 1.8 μm) was heated to 40 °C and equilibrated with 97% A (0.1% TFA in water) and 3% B (0.09% TFA in acetonitrile). The total flow rate was 0.5 mL/min. Both solvents were 0.22 μm filtered and made fresh every 2−3 days. The 3% B solution was maintained for 2 min after injection, after which its level was increased to 6% at 10 min, followed by a faster gradient that reached 50% B at 25 min. The column was purged with 90% B from 26 to 30 min and then re-equilibrated at 3% B. The 214 nm wavelength was monitored for products. A background run consisting of a water injection was subtracted to remove the gradual increase in the magnitude of the signal associated with increasing solvent B content. Individual peaks were collected from a short splint of tubing off the detector. These peaks were collected in Eppendorf tubes and concentrated to dryness in a speedvac before being resuspended in CHCA matrix (2 μL). The products were spotted on a MALDI plate for further characterization.

Chart 2: Modified DNA Molecules Used in This Study

5'-d(**X**TG TGT GGA GCT GTG GCG G)
1 X = pdU **3 X** = pNV
2 X = dRP **4 X** = pC4-AP

(**X** TGT GTG GAG CTG TGG CGG)d-3'
(TAC A ACA CAC CTC GAC ACC GCC)d-5'
5 X = pdU **7 X** = pNV
6 X = dRP **8 X** = pC4-AP

(**X** TGT GTG GAG CTG TGG CGG)d-3'
(A CAC CTC GAC ACC GCC)d-5'
9 X = pdU **11 X** = pNV
10 X = dRP **12 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)
13 X = pdU **15 X** = pNV
14 X = dRP **16 X** = pC4-AP

5'-d(GTC ACG TGC TGC A**X**A CGA CGT GCT GAG CCT)
3'-d(CAG TGC ACG ACG TTT GCT GCA CGA CTC GGA)
17 X = C4-AP

5'-d(CGA CCG GCT CGT ATG **X**TG TGT GGA GCT GTG G)
18 X = C4-AP

Strand Displacement Synthesis of pC4-AP with and without FEN1. Primer-template ternary complex [5′-^{32}P]**16** was assembled as described above. Reactions (20 μL) including Pol β (2 nM) and [5′-^{32}P]**16** (200 nM) with HM buffer, dTTP (50 μM), dGTP (50 μM), and 0.1 mg/mL BSA were initiated by mixing a 2× solution of DNA and dNTPs (10 μL) with a 2× solution of enzyme (10 μL) and mixtures incubated at 37 °C in the presence or absence of FEN1 (10 nM). Aliquots (3 μL) were removed at 2, 5, 10, and 15 min, and reactions were quenched with 5 μL of formamide loading buffer. Ternary complex [3′-^{32}P]**16** (200 nM) was reacted (20 μL) with Pol β (2 nM) in HM buffer, along with dTTP (50 μM), dGTP (50 μM), and 0.1 mg/mL BSA at 37 °C only in the presence of FEN1 (10 nM). Aliquots (3 μL) were removed at 2, 5, 10, and 15 min, and quenched with 1 μL $NaBH_4$ (400 μM). After sitting for 1 h, 10 μL formamide loading buffer was added and the products were separated by 20% denaturing PAGE. A no-enzyme control time course was determined side by side.

RESULTS

Preparation of DNA Substrates Containing C4-AP, pC4-AP, or dRP. Oligonucleotides containing 5′-phosphorylated lesions at their termini (e.g., dRP or pC4-AP) were prepared via solid phase synthesis using commercially available 5′-phosphorylation reagent (Chart 2). dRP sites were generated by reaction with UDG in 5′-phosphorylated oligonucleotides containing dU (*25*). Oligonucleotides containing dU were synthesized via standard oligonucleotide synthesis conditions and reagents. Oligonucleotides containing C4-AP were obtained via photolysis of

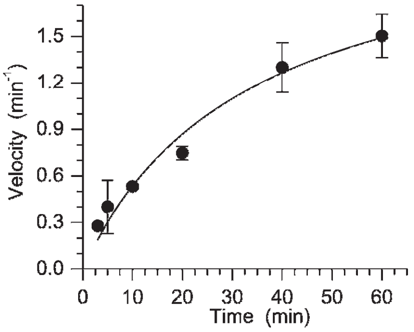
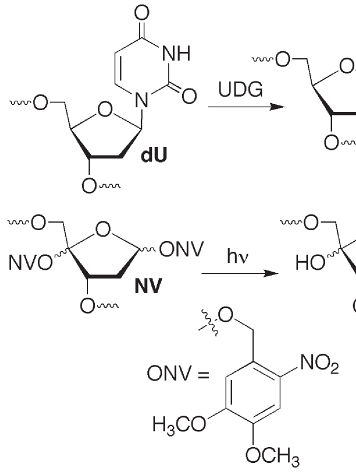


Figure 1: Representative plot of the incision of C4-AP by Ape1 in [5′-^{32}P]**17** (K_m = 33.4 ± 10.0 nM, and V_{max} = 2.3 ± 0.3 min^{-1}).

Scheme 2: Generation of Abasic Lesions from Synthetic Precursors



the respective biopolymer containing NV on an as needed basis (Scheme 2) (*18*, *24*). Duplexes and ternary complexes containing C4-AP were prepared by hybridizing the oligonucleotide containing NV prior to photolysis as previously described (*18*).

Incision of C4-AP by Ape1. Incision of C4-AP ([5′-^{32}P]**17**) by Ape1 (representative data in Figure 1) was studied under steady-state conditions as previously described for the related oxidized abasic site, C2-AP (*26*). The kinetic parameters (K_m = 28.0 ± 9.0 nM, V_{max} = 2.4 ± 0.9 nM/min at 150 pM Ape1, and k_{cat} = 16.0 ± 6.0 min^{-1}) are the average of three experiments, each conducted using three replicates. The kinetic parameters are very similar to those measured for the incision of C2-AP by Ape1 but are less efficient than the incision of C4-AP by Xth in a duplex with a similar sequence (*8*, *26*). Oligonucleotides and complexes containing 5′-phosphates (e.g., pC4-AP and dRP) were obtained by incorporating the phosphate group using commercially available 5′-phosphorylation reagent.

Single-Turnover Kinetic Analysis of the Pol β Lyase Reaction with dRP and pC4-AP. The lyase reaction of dRP is known to be the rate-determining step in BER of AP lesions (*25*, *27*). Although qualitative experiments show that pC4-AP is a substrate for Pol β lyase activity, we took a more comprehensive, quantitative approach because of the lesion's structural similarity to DOB (*9*, *10*). pC4-AP and DOB are both 1,4-dicarbonyl-containing molecules that readily react with amines. Given that DOB efficiently irreversibly inhibits DNA Pol β, we investigated whether pC4-AP also inactivates Pol β. Irreversible inhibition of Pol β by C4-AP would bias steady-state measurements (vide infra). Hence, pC4-AP excision in various structural situations was assessed under single-turnover conditions (Table 1). For comparison, the rate constants for dRP excision were measured in otherwise identical substrates. dRP was excised from a ternary complex (**14**) less than twice as fast as pC4-AP [**16** (Figure 2)]. In addition, both lesions were excised by Pol β more rapidly from a ternary complex than when incorporated in single-stranded (**2** and **4**) or duplex substrates. The double-stranded substrates included those in which the 5′-phosphorylated lesions were present in a recessed region (**6** and **8**) or an overhang (**10** and **12**).

Steady-State Analysis of the Pol β Lyase Reaction with pC4-AP and dRP. Kinetic constants were extracted by measuring initial velocities of reactions including various concentrations of the ternary complex containing dRP ([3′-^{32}P]**14**) with Pol β (10 nM) (see the Supporting Information). The kinetic parameters measured (K_m = 82 nM, and k_{cat} = 2.1 min^{-1}) were similar to those reported previously for excision of dRP in a different sequence (*25*). The observed K_m is ~5 times smaller, and the k_{cat} is

Table 1: Rate Constants for Excision of pC4-AP and dRP by Pol β under Single-Turnover Conditions

k_{Obs} (s^{-1}) X:	pC4-AP[a]	dRP[a]
——X——3′ / ————5′ **14**, **16**	3.6 ± 0.9 (6)	6.2 ± 2.2 (5)
X——3′ / ————5′ **6**, **8**	1.8 ± 0.9 (3)	0.9 ± 0.6 (4)
X——3′ / ——5′ **10**, **12**	0.4 ± 0.1 (3)	0.4 ± 0.2 (2)
X——3′ **2**, **4**	0.8 ± 0.2 (3)	2.8 ± 1.2 (3)

[a]Number of replicates in parentheses. Each replicate consisted of three independent samples.

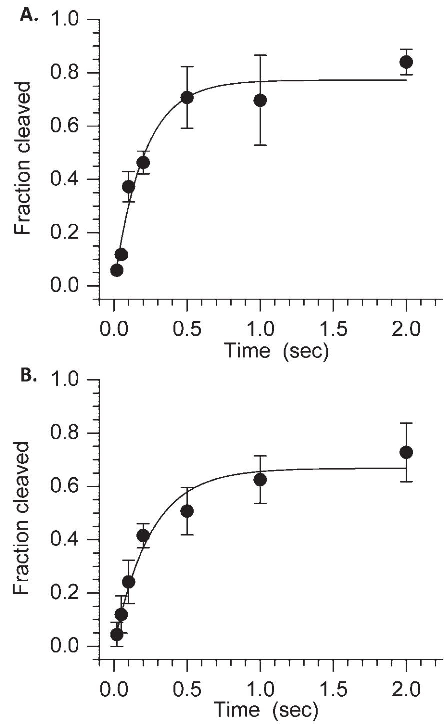


Figure 2: Representative excision of ternary complexes containing (A) dRP ([3′-^{32}P]**14**) and (B) pC4-AP ([3′-^{32}P]**16**) by Pol β under single-turnover conditions.

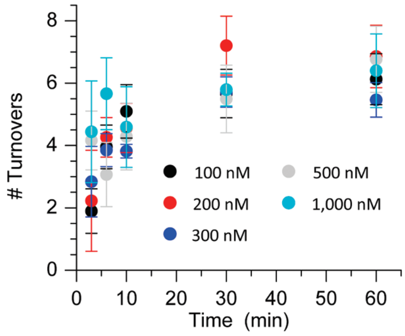


FIGURE 3: Number of Pol β (10 nM) turnovers during excision of various concentrations (100−1000 nM) of pC4-AP ([3′-^{32}P]**16**) as a function of time.

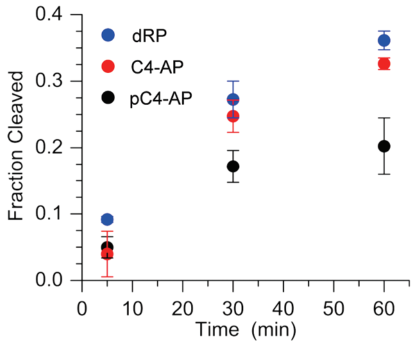


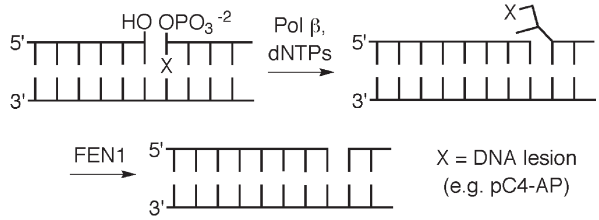
FIGURE 4: Pol β (10 nM) excision of dRP in [3′-^{32}P]**14** (500 nM) after preincubation of the enzyme with 100 nM dRP (**14**), pC4-AP (**16**), or single-stranded DNA containing C4-AP (**18**).

approximately half as fast previously reported in a different sequence. Attempts to determine Michaelis−Menten parameters for excision of pC4-AP ([3′-^{32}P]**16**) were unsuccessful (data not shown). Unlike time course studies of dRP excision that showed reaction progress over the course of 1 h, reactions between pC4-AP and Pol β ceased before 30 min regardless of the ratio of substrate to enzyme. The number of turnovers as a function of time at [3′-^{32}P]**16** concentrations from 0.1 to 1.0 μM converged between six and seven even when the substrate was in 100-fold excess of enzyme (Figure 3). In contrast, more than 40 turnovers were observed when dRP was present in a sufficiently large excess (see the Supporting Information).

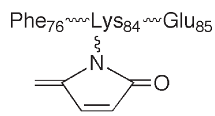
Inhibition of Pol β by pC4-AP. The premature ceasing of Pol β lyase activity and the lack of dependence of turnover number on pC4-AP concentration (Figure 3) suggested that the oxidized abasic lesion irreversibly inhibited the enzyme. Further support for pC4-AP inactivation of Pol β was garnered by preincubating the enzyme (10 nM) with **16** (100 nM) for 15 min prior to measuring the excision of dRP from [3′-^{32}P]**14** (500 nM) (Figure 4). The extent of dRP excision was significantly reduced compared to that in samples in which the enzyme was preincubated with either unlabeled **14** (100 nM) or a single-stranded oligonucleotide containing C4-AP (**18**, 100 nM). However, unlike experiments with DOB, we were unable to kinetically characterize irreversible inhibition of Pol β by pC4-AP. This was attributed to the high concentration of pC4-AP required to observe this effect. When the concentration of pC4-AP was less than 10-fold higher than that of Pol β, the moderate pC4-AP turnover masked its inhibition of the dRPase activity.

Despite the absence of kinetic data for the irreversible Pol β inhibition by pC4-AP, qualitative support for this process

Scheme 3: Long Patch BER by Pol β and FEN1



provided the impetus to examine the enzyme for modification. We anticipated that one or more of the nucleophilic lysine residues in the Pol β lyase active site would be modified. Direct evidence of Pol β modification was obtained by comparing GluC protein digests with or without prior incubation with pC4-AP (**16**). GluC hydrolyzes peptides at the C-terminus of glutamic acid residues. Several peptide fragments, including an ion that corresponds to amino acids 76−85 (m/z 1275.9), were identified via MALDI-TOF MS analysis of the crude Pol β digest. This ion was not detected in the GluC digestion conducted following incubation of Pol β with 5 equiv of **16**. Instead, a fragment corresponding to the amino acid peptide fragment of residues 76−85+78 amu (m/z 1353.8) was observed (see the Supporting Information). This fragment contains two lysine residues, but only Lys84 is accessible to the lyase binding site (*28*). The adduct (**19**) is consistent with Pol β structure and the type of product previously observed in reactions between C4-AP and primary amines (*22*, *23*). More inferential evidence of protein modification was obtained by comparing trypsin digests of Pol β to trypsin digests of the enzyme after pC4-AP incubation (**16**) (see the Supporting Information). Trypsin hydrolyzes the C-termini of lysine residues. The digestion of Pol β in the absence of pC4AP yielded a peak in the UPLC chromatogram whose molecular ion (m/z 993.4) corresponded to residues 73−81. This peptide results from cleavage at Lys72, the residue shown to form a Schiff base with dRP, and Lys81 (*29*). The intensity of this peptide was greatly reduced in the digest of enzyme that was incubated with pC4-AP (**16**). This is consistent with modification of Lys72, which prevents hydrolysis by trypsin at this site. However, a corresponding longer peptide containing a sugar fragment was not observed.



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Removal of pC4-AP by Long Patch BER. Pol β contributes to long patch BER by conducting strand displacement synthesis. During this process, Pol β extends the 3′-terminus at the point of incision in the DNA and in the process displaces the incised lesion and one or more of the 3′-adjacent nucleotides (Scheme 3). The displaced segment of DNA ("flap") is cleaved by FEN1, producing a nicked duplex that is poised for completion of repair by DNA ligase. Extension of [5′-^{32}P]**16** by Pol β (2 nM) was monitored in the presence of dTTP (50 μM) and dGTP (50 μM) in the absence and presence of FEN1 (10 nM) (Figure 5). One and two nucleotide extension products were observed. Although dGTP was present to allow addition of a third nucleotide, none of this product was observed. In addition, the amount of product with a one-nucleotide extension reaches a maximum in ∼5 min and then begins to decrease, whereas the

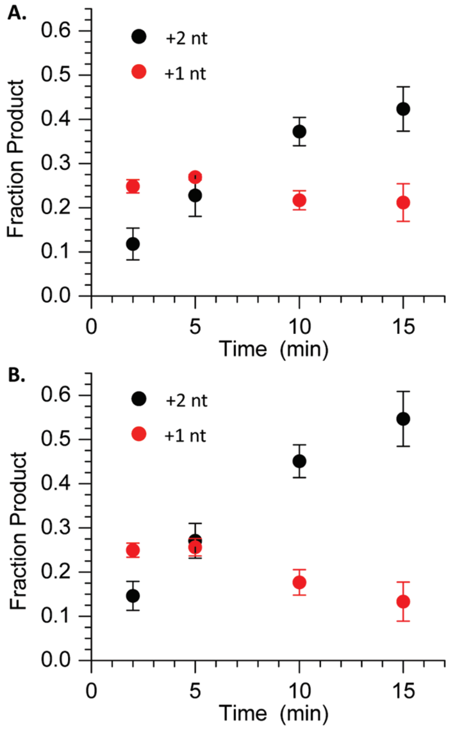

FIGURE 5: Extension of [5′-^{32}P]**16** (200 nM) by Pol β (2 nM) in the presence of dTTP (50 μM) and dGTP (50 μM) in the (A) absence or (B) presence of FEN1 (10 nM).

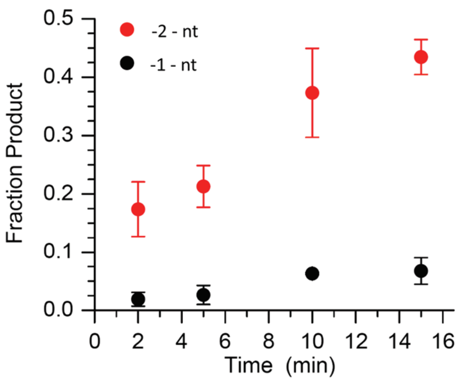

FIGURE 6: Cleavage of [3′-^{32}P]**16** (200 nM) by FEN1 (10 nM) in the presence of Pol β (2 nM), dTTP (50 μM), and dGTP (50 μM).

amount of product with a two-nucleotide extension continues to increase throughout the course of the reaction. The overall amount of extension was insignificantly affected by FEN1, but the fraction of two-nucleotide extension product increased modestly at the expense of single-nucleotide addition in the presence of the endonuclease. The amount of two-nucleotide cleavage product observed from [3′-^{32}P]**16**, which results from FEN1 cleavage of the flap produced as a result of strand displacement, subjected to the same reaction conditions was comparable to the yield of two-nucleotide extension product (Figure 6).

DISCUSSION

AP sites are produced spontaneously via depurination, as a result of oxidative stress, and as intermediates in BER of damaged nucleotides. They are efficiently repaired by the BER pathway in which the first two steps involve Ape1 incision, followed by removal of the 5′-(2′-deoxyribose)phosphate (dRP) fragment by the lyase activity of Pol β. Oxidized abasic sites such as L and C2-AP are substrates for Ape1 but pose obstacles to Pol β that require alternative repair processes (*26*, *30*, *31*). Previous qualitative studies showed that the oxidized abasic lesion, C4-AP, was a substrate for Ape1 and Pol β, two principle enzymes responsible for the BER of AP lesions (*9*). However, kinetic parameters were not reported. Given the structural similarity (Chart 1) between C4-AP and the potent irreversible inhibitor of Pol β, DOB, we took advantage of our ability to chemically synthesize oligonucleotides containing C4-AP or pC4-AP to reinvestigate its repair (*10*, *18*).

C4-AP is incised ~12-fold less efficiently than AP by Ape1 and is a slightly poorer substrate than 2-deoxyribonolactone (L) (*31*). However, because of an ~2-fold lower K_m and 2-fold higher k_{cat}, Ape1 incises C4-AP ~4-fold more efficiently than C2-AP (*26*). Incision by Ape1 is a necessary first step for BER of an abasic site. These data indicate that C4-AP is a viable candidate for this pathway. Excision of dRP or pC4-AP by Pol β is the next step in short patch BER. Single-turnover kinetic experiments (Table 1) indicate that the polymerase's lyase activity removes pC4-AP almost as rapidly as it excises dRP. Excision of either lesion when it is part of a single strand or at the 5′-terminus of a recessed or overhanging duplex was considerably less efficient (Table 1). From these data alone, it is unclear whether Pol β plays a significant role as a housekeeping enzyme to clean up DNA termini of single and double strand breaks in preparation for religation (*32*, *33*). However, steady-state experiments reveal that excision of pC4-AP by Pol β is different from that of dRP in a ternary complex of the type formed upon Ape1 hydrolysis. Excision of pC4-AP under multiple-turnover conditions showed that this lesion irreversibly inhibits Pol β. Previous studies, which were conducted with relatively large concentrations of Pol β, did not uncover this mode of action (*9*). Interestingly, our data show that on average each Pol β molecule turns over six or seven times before being inactivated. The earlier study was conducted using <6-fold excess substrate. Hence, previous investigators would not have detected this inhibition under their reaction conditions. Pol β turns over a greater number of times when excising pC4-AP than when removing DOB (two to four turnovers) from the 5′-terminus of DNA. A previous study of DOB indicated that the 1,4-dicarbonyl component of the lesion was required for efficient irreversible inhibition (*34*). We attribute the less efficient inhibition of Pol β by pC4-AP than by DOB to the reduced electrophilicity of the former. pC4-AP contains the requisite 1,4-dicarbonyl functionality, but the ketone group at the C4 position is less reactive than the aldehyde in DOB at the comparable position.

Although quantitative analysis of irreversible inhibition of Pol β by pC4-AP was not conducted, GluC digestion and subsequent MALDI-TOF MS analysis provided direct evidence of Lys modification in the peptide fragment containing residues 76−86. Two Lys residues are present in this fragment, but the X-ray crystal structure of Pol β in a complex with a stable dRP analogue indicates that only Lys84 is present in the lyase active site (*28*). The modified fragment could result from liberated sugar fragment and/or trapping by pC4-AP and subsequent decomposition. Although Lys84 may play an active role in the lyase reaction, Lys72 is believed to be responsible for the majority of Schiff base formation en route to dRP excision (*35*−*37*). We did not detect a modified peptide fragment by MS containing this residue, but inferential support for the Lys72 modification during inactivation of Pol β by pC4-AP was evident from analysis of the trypsin digest. Disappearance of the amino acid fragment of residues 73−81 is consistent with modification of Lys72 (*29*).

The less efficient inactivation of Pol β by pC4-AP compared to that by DOB is also evident in long patch repair of the incised lesion (Scheme 3). Whereas Pol β achieved insignificant amounts of strand displacement synthesis when acting on DNA containing DOB, Pol β readily extended the strand flanking pC4-AP by two nucleotides (*34*). Pol β-mediated displacement of pC4-AP and the 3′-adjacent nucleotide was accompanied by comparable amounts of 2-nucleotoide excision by FEN1 in the strand containing the lesion. The level of long patch BER of pC4-AP was similar to that observed for C2-AP (*26*). We attribute the more effective long patch BER of pC4-AP compared to that of DOB to the less efficient Pol β inactivation by the former, which allows this process to proceed.

CONCLUSIONS

The C4′-oxidized abasic site is a commonly produced lesion, which exhibits effects on base excision repair that are intermediate between those of structurally related AP and DOB lesions. The lesion is not repaired as efficiently as an AP lesion. Moreover, like the DOB lesion, it irreversibly inhibits Pol β, a critical enzyme involved in BER, albeit less efficiently. The less efficient inactivation of Pol β also allows this enzyme in conjunction with FEN1 to excise C4-AP through long patch BER. These data suggest that C4-AP is not as toxic as DOB but that its ability to inhibit BER may contribute to the cytotoxicity of drugs that produce it.

SUPPORTING INFORMATION AVAILABLE

Plots of excision of dRP by Pol β and GluC and trypsin digests of Pol β following reaction with pC4-AP. This material is available free of charge via the Internet at http://pubs.acs.org.

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