In Vitro Replication and Repair of DNA Containing a C2′-Oxidized Abasic Site†

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ABSTRACT: Abasic lesions are unable to form Watson−Crick hydrogen bonds with nucleotides. Nonetheless, polymerase and repair enzymes distinguish between various oxidized abasic lesions, as well as from nonoxidized abasic sites (AP). The C2-AP lesion is produced when DNA is exposed to γ-radiolysis. Its effects on polymerases and repair enzymes are unknown. A recently reported method for the chemical synthesis of oligonucleotides containing C2-AP at a defined site was utilized for studying the activity of Klenow exo$^-$ and repair enzymes on templates containing the lesion. The C2-AP lesion has a similar effect on Klenow exo$^-$ as do AP and C4-AP sites. Deoxyadenosine is preferentially incorporated opposite C2-AP, but extension of the primer past the lesion is strongly blocked. C2-AP is incised less efficiently by exonuclease III and endonuclease IV than are other abasic lesions. Furthermore, although a Schiff base between C2-AP and endonuclease III can be chemically trapped, the location of the 3′-phosphate α with respect to the aldehyde prevents β-elimination associated with the lyase activity of type I base excision repair enzymes. The interactions of the C2′-oxidized abasic site with Klenow exo$^-$ and repair enzymes suggest that the lesion will be mutagenic and that it will be removed by strand displacement synthesis and flap endonuclease processing via a long patch repair mechanism.

Abasic lesions (AP)[1] are often referred to as noninstructional because of their inability to form Watson−Crick hydrogen bonds with opposing nucleotides. However, recent in vitro and in vivo studies showing that oxidized abasic lesions interact differently with polymerases and DNA repair enzymes than AP sites, which result from hydrolysis of a nucleotide's glycosidic bond, suggest that this is a misnomer. Unique behavior is particularly evident when considering the C1′-oxidized abasic lesion 2-deoxyribonolactone (L). Replication of L in *E. coli* does not follow the A-rule, and the lesion forms DNA−protein cross-links with enzymes that repair AP sites (*1*−*4*). We also recently reported that a C4-AP lesion induces pol II exo$^-$ to incorporate thymidine preferentially, whereas the A-rule is followed when a template contains an AP lesion (*5*). The individuality exhibited by abasic lesions led us to investigate the properties of the abasic site that results from formal C2′-oxidation (C2-AP). The activity of Klenow exo$^-$ and repair enzymes in the presence of DNA templates containing C2-AP is described below. These experiments, which were facilitated by a method for chemically synthesizing DNA containing the lesion, suggest that C2-AP will be mutagenic in *Escherichia coli* (*6*).

The C2′-oxidized abasic site (C2-AP) is produced as a result of exposing DNA to γ-radiolysis and is also formed when nucleic acids containing the 5-halopyrimidine radiosensitizing agents (5-bromodeoxyuridine, 5-iododeoxyuridine) are irradiated (*7*−*12*). In addition to being smaller than the three other abasic lesions, which may affect translesional synthesis (*13*), C2-AP contains a β-hydroxyaldehyde. This arrangement of functional groups potentially enables the C2-AP lesion to undergo a retroaldol reaction under alkaline conditions, which produces fragmented DNA containing electrophilic phosphoglycoaldehyde at one terminus of each fragment (Scheme 1) (*10*). The phosphoglycoaldehyde component may produce premutagenic exocyclic adducts via reaction with nucleobases within the biopolymer (*14*). The structural relationship between the aldehyde group and the

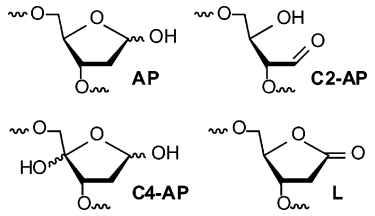
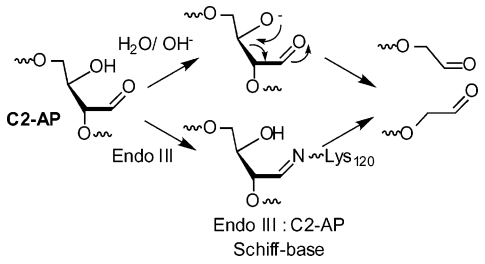


FIGURE 1: Structures of abasic lesions: "regular" abasic lesions (AP), C2′-oxidized abasic site (C2-AP), C4′-oxidized abasic site (C4-AP), and 2-deoxyribonolactone (L).

Scheme 1: Cleavage of C2′-Oxidized Abasic Lesion To Produce Phosphoglycoaldehyde Termini



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[1] Abbreviations: C2-AP, C2′-oxidized abasic site; C4-AP, C4′-oxidized abasic site; AP, abasic site; L, 2-deoxyribonolactone; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; Endo III, endonuclease III; Endo IV, endonuclease IV; Exo III, exonuclease III.

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Table 1: Oligonucleotides and Duplexes Employed in Experiments

5′-d-GTC ACG TGC TGC A**X**A CGA CGT GCT GAG CCT

a X = **1**

b X = C2-AP

5′-d-AGG CTC AGC ACG TCG TAT GCA GCA CGT GAC

c

5′-d-AGG CTC AGC ACG TCG T

d

5′-d-AGG CTC AGC ACG TC

e

5′-d-AGG CTC AGC ACG TC
3′-d-TCC GAG TCG TGC AGC A**X**A CGT CGT GCA CTG

2a X = C2-AP
2b X = T

5′-d-AGG CTC AGC ACG TCG T
3′-d-TCC GAG TCG TGC AGC A**X**A CGT CGT GCA CTG

3 X = C2-AP

5′-d-GTC ACG TGC TGC A**X**A CGA CGT GCT GAG CCT
3′-d-CAG TGC ACG ACG TAT GCT GCA CGA CTC GGA

4 X = C2-AP

α-phosphate and β-hydroxy groups derived from the 3′- and 4′-positions, respectively, of the original nucleotides raises interesting possibilities regarding base excision repair (BER) of the C2-AP lesion. Type I BER enzymes excise lesions by catalyzing a β-elimination reaction. The Schiff base formed between the lesion and a type I BER enzyme (e.g., Lys120 of Endo III) is analogous to the product initially formed between phosphoglycoaldehyde and the enamine produced from lysine and another molecule of the aldehyde (Scheme 1). Application of the principle of microscopic reversibility raises the specter that the enzyme could catalyze conversion of C2-AP into other toxic lesions. If the retrocondensation reaction is not catalyzed by the BER enzyme(s), situation of the phosphate group α to the Schiff base (instead of β as in an AP site) should prevent type I enzymes from excising the lesion. This would place an even greater emphasis on the need for type II repair enzymes, which act as 5′-phosphodiesterases, to initiate repair via efficient C2-AP incision.

MATERIALS AND METHODS

General Procedures. Oligonucleotide synthesis was carried out on an Applied Biosystems Incorporated 394 DNA synthesizer using standard protocols. Oligonucleotides containing C2-AP were synthesized as described (*6*). Commercially available oligonucleotide synthesis reagents were obtained from Glen Research. DNA manipulation, including enzymatic labeling, was carried out using standard procedures (*15*). Oligonucleotides and duplexes used in these experiments are presented in Table 1. Preparative and analytical oligonucleotide separations were carried out on 20% polyacrylamide denaturing gel electrophoresis [5% cross-link, 45% urea (by weight)]. T4 polynucleotide kinase, uracil DNA glycosylase (UDG), Klenow, and Klenow exo$^-$ were obtained from New England Biolabs. Endonuclease IV was from Trevigen. Exonuclease III was from Promega. [γ-^{32}P]-ATP was purchased from Amersham Pharmacia Biotech. Radioactive samples were quantitated by Cerenkov counting using a Beckman LS6500 liquid scintillation counter. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Storm 840 phosphorimager equipped with ImageQuant Version 5.1 software.

General Preparation of Primer-Template and Template-Complement Duplexes. Primers or complements were hybridized at 55 °C to template oligonucleotides for 5 min and cooled to room temperature over 2 h. Samples were hybridized immediately before their use in experiments. For polymerase reactions, 5 pmol of 5′-^{32}P-labeled primer and 34.1 pmol of unlabeled primer were annealed to 58.5 pmol of template in 20 mM Tris-HCl (pH 7.5) and 100 mM NaCl to produce a DNA working solution (3.13 μM). For cleavage reactions with Endo IV and Exo III, 25 pmol of 5′-^{32}P-labeled template was hybridized to 50 pmol of complement in aqueous NaCl (100 mM) to produce a 500 nM working DNA solution.

Qualitative Full-Length Primer Extension by Klenow exo$^-$. Full-length primer extension of **2a** (75 nM) was carried out using Klenow exo$^-$ (1 nM). Reactions were carried out in 0.02% (w/v) BSA, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 15 mM DTT. A (2×) solution (30 μL) of **2a** (150 nM) and Klenow exo$^-$ (2 nM) in buffer was added to a solution (30 μL) containing all four dNTP's (0.2 mM each). The reaction mixture was incubated at room temperature for 180 min. Aliquots (5 μL) were collected at 1, 5, 10, 30, 60, 120, and 180 min and quenched immediately in 95% formamide loading buffer containing 10 mM EDTA (20 μL). Samples were denatured and loaded on a 20% denaturing PAGE. Marker lanes were loaded with independently synthesized 5′-^{32}P-16mer and -17mer. A control experiment using **2b** as substrate was carried out under the same conditions.

Insertion Kinetics Opposite a C2′-Oxidized Abasic Site (C2-AP). A (2×) DNA−enzyme solution (100 μL) was prepared containing **3** (150 nM) and Klenow exo$^-$ (2 nM) in 0.02% (w/v) BSA, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 15 mM DTT. The DNA−enzyme solution (5 μL) was added to the appropriate 2× dNTP solution (5 μL). The reaction was allowed to run for a fixed period of time at room temperature and then quenched with 95% formamide loading buffer containing 10 mM EDTA (20 μL). The samples were denatured by heating at 90 °C for 3 min and immediately placed on ice before the sample (4 μL) was loaded on a 20% PAGE gel. The concentration range and reaction time were as follows: dATP, 10−300 μM, 3 min; dGTP, 10−300 μM, 30 min; dCTP, 25−400 μM, 45 min; dTTP, 25−400 μM, 45 min.

Action of Endo IV on a C2′-Oxidized Abasic Site (C2-AP). The ability of Endo IV to recognize the C2-AP site was investigated using duplex **4**. A (2×) enzyme solution was prepared containing 100 mM Hepes−KOH buffer (pH 7.6), 100 mM KCl, 2 mM DTT, 20% glycerol (w/v), and Endo IV (6 nM). The enzyme solution (5 μL) was added to 5 μL of a 2× solution of **4** (20−500 nM), prepared as described above. The reaction was allowed to run for 3 min at room temperature and then quenched with 95% formamide loading buffer containing 10 mM EDTA (20 μL). The samples were denatured by heating at 55 °C for 3 min and immediately placed on ice before the sample (5 μL) was loaded on a 20% PAGE gel.

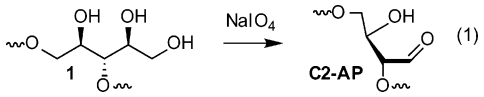
Action of Exo III on a C2-AP Oxidized Abasic Site. The ability of Exo III to recognize the C2-AP site was investigated using duplex **4**. A (2×) enzyme solution was prepared containing 132 mM Tris-HCl (pH 8.0), 1.32 mM $MgCl_2$,

and Exo III (25 pM). The enzyme solution (5 μL) was added to 5 μL of a 2× solution of **4** (20−500 nM), and the reaction was allowed to run for 15 min at room temperature. Reactions were quenched with 95% formamide loading buffer containing 10 mM EDTA (20 μL). The samples were denatured by heating at 55 °C for 3 min and immediately placed on ice before the sample (5 μL) was loaded on a 20% PAGE gel.

Chemical Trapping of Schiff Base Formation between Endo III and C2-AP. 5′-^{32}P-**4** (10 nM) in Tris-HCl (100 mM), pH 8.0, NaCl (50 mM), EDTA (1 mM), and DTT (0.1 mM) was incubated with Endo III (1 μM) for 10 min at 37 °C. $NaCNBH_3$ (1 μL, 100 mM) was added to the reaction, making a final reaction volume of 10 μL, and the mixture was incubated for 30 min at 37 °C. The reaction was quenched with 10 μL of loading buffer (100 mM Tris-HCl, pH = 6.8, 4% SDS, 150 mM DTT, 20% glycerol, 0.05% bromophenol blue). The entire sample was loaded onto a 12% SDS−PAGE and run using TG buffer (25 mM Tris-HCl, 192 mM glycine, and 0.1% SDS). Reactions containing **4** (1 μM) in excess were carried out in the same manner with the exception that $NaCNBH_3$ was present at 50 mM. Endo III concentrations utilized in these experiments were 100, 200, or 500 nM.

RESULTS

Oligonucleotide Synthesis and Hybridization. A 30 nucleotide long oligonucleotide template (a, Table 1) containing the precursor (**1**, eq 1) of C2-AP was prepared and purified



by gel electrophoresis, as previously described (*6*). The precursor-containing oligonucleotide was converted to the aldehyde by treatment with sodium periodate, and the product containing C2-AP (b, Table 1) was characterized by ESI-MS (*6*). The C2-AP lesion is considerably more stable toward cleavage under alkaline conditions than are oligonucleotides containing other abasic lesions. Hence, stock solutions of b could be used for several weeks with no evidence for degradation. The greater stability of the C2-AP lesion also made hybridization conditions less restrictive than those required when working with other abasic lesions (*16*). Nonetheless, we chose to err on the side of caution and carried out hybridizations at 55 °C.

Primer Extension by Klenow exo$^-$. Qualitative analysis of Klenow exo$^-$'s ability to read through a C2-AP site was carried out using duplex **2a** in which the enzyme was given a running start. Two pause sites corresponding to inhibition of translesion incorporation (accumulation of a 16 nucleotide long product) and single nucleotide extension past the lesion (accumulation of a 17 nucleotide long product) were observed (Figure 2). Although translesion synthesis was achieved, Klenow exo$^-$ was unable to extend a primer past C2-AP after 3 h. Complete extension of a comparable duplex (**2b**) containing thymidine in place of the lesion was observed within 60 min under identical conditions (data not shown). Carrying out the reaction using higher concentrations of enzyme did not result in extension past the lesion (data not shown).

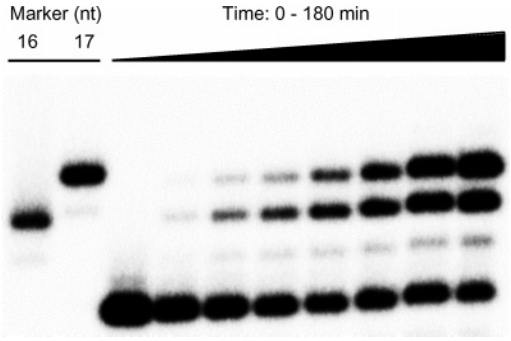


FIGURE 2: Qualitative Klenow exo$^-$ replication of a template-primer complex containing C2-AP (**2a**).

Table 2: Steady-State Analysis of Translesion Synthesis Opposite C2-AP in **3** by Klenow exo$^-$

dNTP	V_{max} (%·min^{-1})	K_m (μM)	V_{max}/K_m (%·min^{-1}·M^{-1})
A	5.1 ± 0.8	55.4 ± 5.5	(9.3 ± 1.0) × 10^4
G	0.6 ± 0.1	39.0 ± 1.4	(1.5 ± 0.2) × 10^4

Table 3: Steady-State Analysis of C2-AP Incision in **4** by Exo III and Endo IV

enzyme	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}·M^{-1})
Exo III	23.5 ± 4.9	18.9 ± 4.1	(8.2 ± 1.8) × 10^8
Endo IV	85.6 ± 6.7	2.4 ± 0.6	(2.8 ± 0.6) × 10^7

Steady-state experiments were carried out using **3** in order to measure the efficiency of individual nucleotide incorporation opposite (translesion synthesis) the C2-AP lesion (Table 2) by Klenow exo$^-$. The kinetic constants reported (Table 2) represent the average of at least three individual experiments. Each experiment consists of three replicates. To facilitate comparisons, measurements were made using a template-primer complex identical in sequence (with the exception of the identity of the DNA lesion) and in the presence of the same enzyme and DNA concentration as was used in studies on AP, L, and C4-AP lesions (*17*).

Klenow exo$^-$ showed an ∼6-fold preference for incorporating dA over dG opposite the lesion. Although the apparent K_m of dG translesion synthesis was approximately one-third lower than that of dA, the bulk of the selectivity was attributable to an almost 10-fold greater V_{max} for translesion incorporation of dA. Incorporation of dT or dC opposite the C2-AP site was extremely inefficient. Despite increasing the reaction time to 45 min in the presence of as much as 400 μM dTTP or dCTP, only ∼1% extension of the primer was detected. Consequently, kinetic analysis of translesion incorporation of dCTP or dTTP by Klenow exo$^-$ was not investigated further.

Incision of C2-AP by Endo IV and Exo III. To facilitate comparisons with other lesions, the incision of C2-AP was examined using a duplex (**4**) consisting of the identical surrounding sequence that was used previously to investigate the repair of AP, L, and C4-AP sites (*17*). The kinetic constants reported (Table 3) represent the average of at least three individual experiments. Each experiment consists of three replicates. When measuring the reaction velocity, background cleavage of the alkali-labile lesion was accounted for as described previously (*17*). However, C2-AP is significantly more stable than other AP lesions, and the

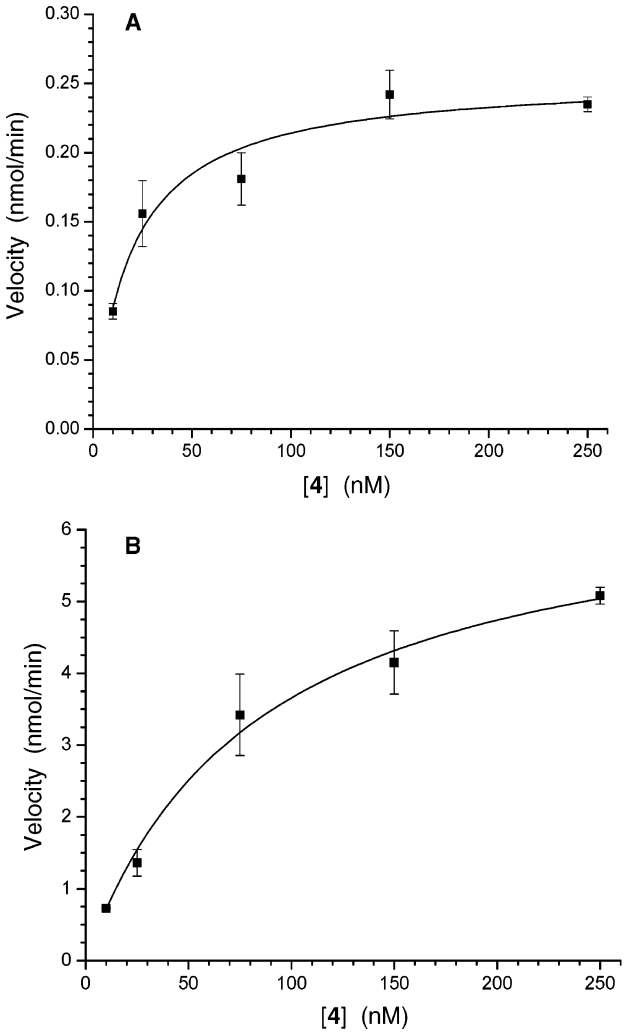


FIGURE 3: Representative plots (velocity versus [**4**]) for incision of the C2′-oxidized abasic site (C2-AP) by (A) exonuclease III and (B) endonuclease IV.

correction was negligible (*6*). Consequently, the kinetic plots (velocity versus [**4**]) obtained for C2-AP kinetics (Figure 3) exhibited less scatter at higher DNA concentrations and better overall fits than did those for L and C4-AP lesions. Exo III was approximately 30-fold more proficient at incising the C2-AP lesion than was Endo IV. The greater ability of Exo III to incise C2-AP was attributable to both a higher k_{cat} and more favorable K_m. The more favorable kinetic parameters for incision of C2-AP by Exo III are consistent with the relative abilities of these enzymes to incise other abasic lesions (e.g., AP, L, and C4-AP) (*17*).

Covalent Trapping of the Schiff Base Formed between Endonuclease III and the C2′-Oxidized Abasic Lesion. AP and C4-AP lesions are excised by bifunctional type I BER enzymes, such as endonuclease III (Endo III). Endo III exhibits high lyase activity relative to other BER enzymes, which is reflected in the nucleophilicity of the lysine residue involved in Schiff base formation (*18*−*21*). Hence, we investigated the interaction between the C2-AP lesion in **4** and Endo III. No strand scission was observed when **4** was incubated with Endo III, indicating that the lesion is not excised by the enzyme. However, trapping studies using sodium cyanoborohydride to reduce the possible Schiff base formed between Endo III and C2-AP indicated that the lesion

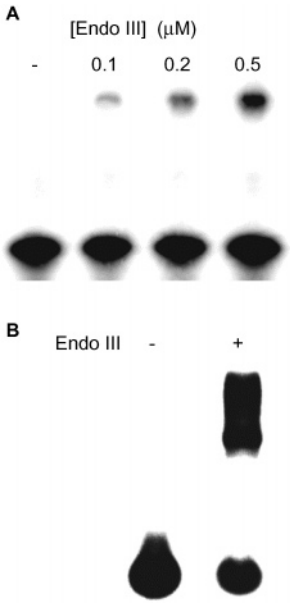


FIGURE 4: Chemical trapping of the Schiff base formed between C2-AP (**4**) and endonuclease III in the presence of (A) excess DNA (**4**, 1 μM) or (B) excess enzyme (Endo III, 1 μM; **4**, 10 nM).

was strongly recognized by the enzyme (Figure 4). The amount of cross-linked product was proportional to enzyme concentration in the presence of excess **4** (1 μM) and corresponded to ∼20% of the amount of Endo III (0.1−0.5 μM) present (Figure 4A). More than 70% of C2-AP in **4** (10 nM) was cross-linked when the enzyme (1 μM) was in large excess (Figure 4B).

DISCUSSION

The overall efficiency of translesion incorporation by Klenow exo$^-$ in the presence of a template containing the C2-AP lesion is comparable to that observed with templates containing AP or C4-AP lesions in otherwise identical templates (*5*, *22*). Pyrimidines are incorporated so inefficiently opposite C2-AP that their kinetic parameters were not determined. This too is consistent with the effects of other abasic lesions (*5*, *22*). There is an approximately 6-fold preference overall for translesion incorporation of dA versus dG, with the differences in V_{max} (∼10×) being the dominant effect. This too is similar to what is observed in experiments with templates containing AP or C4-AP, as is the inability of the polymerase to extend the primer past the lesion. However, the selectivity for dA incorporation over dG is slightly greater than that reported for 2-deoxyribonolactone (*23*). If purines are incorporated preferably opposite AP (and its tetrahydrofuran analogue, F) because of their more extensive π-systems, it is not surprising that the even smaller C2-AP lesion would induce Klenow exo$^-$ to behave similarly (*13*). In all previously reported studies concerning Klenow activity on templates containing AP, L, or C4-AP, the enzyme's poor ability to read through the lesion has served as a good prognosticator for in vivo replication (*5*, *22*, *23*). In each instance SOS polymerases were required to bypass the abasic lesions in *E. coli* (*3*). Using these studies on other abasic lesions as a guide, the effect of C2-AP on Klenow exo$^-$ suggests that SOS-inducible polymerases will be responsible for bypassing this lesion in *E. coli* (*3*, *24*). Furthermore, although Klenow is not the enzyme responsible

for bypassing other abasic lesions in vivo, the modest selectivity for translesion nucleotide incorporation in vitro has served as a good indicator of what happens in *E. coli* (*3*, *24*). Considering that C2-AP is produced randomly in a nucleotide-independent manner, if the correlation between in vitro and in vivo experiments continues, we expect that bypass of this lesion in *E. coli* will be accompanied by a high mutation frequency.

The anticipated high mutation frequency due to bypass of C2-AP underscores the need for its efficient repair. However, C2-AP incision by Exo III and Endo IV, the primary defense in *E. coli* against AP, L, and C4-AP, reveals that this lesion is incised less efficiently than other abasic sites (*25*). Endo IV incises C2-AP from **4** between 2 and 3 times more slowly than either L or C4-AP (*17*). The difference in specificity constants for Endo IV incision of AP and C2-AP lesions is even greater (*17*, *26*). The approximately 10-fold less efficient incision of C2-AP is attributed to higher K_m and lower k_{cat}. The 13−64-fold higher specificity constants for incision of C4-AP, L, or AP lesions compared to C2-AP in an otherwise identical template by Exo III is also an aggregate effect of both higher K_m and lower k_{cat} (*17*). Of these molecules C2-AP is the only acyclic abasic lesion. The efficiency of its incision by Exo III and Endo IV is between that of two acyclic models of abasic lesions (*26*). It is possible that the greater conformational freedom of C2-AP contributes to making it a poorer substrate for these type II repair enzymes.

Although C2-AP is repaired less efficiently than other abasic lesions by the two nucleases present in largest amounts in *E. coli*, it is a substrate nonetheless. In contrast, C2-AP is not a substrate for Endo III, a prototypical type I endonuclease that possesses a relatively high lyase activity. Hydride trapping experiments (Figure 4) suggest that Endo III recognizes the lesion. The fact that C2-AP is not excised by Endo III is readily understood on chemical grounds. Lyase reactions require a β-relationship between the carbonyl carbon, which participates in Schiff base formation, and the phosphate leaving group. The potential phosphate leaving group is α to the carbonyl in C2-AP, thwarting elimination.

Comparison of the results with type I and II repair enzymes and Klenow exo$^-$ suggests that C2-AP will provide a more difficult challenge to *E. coli* than AP lesions. The inability of Endo III to excise C2-AP could have ramifications regarding its repair in cells. One possibility is that Endo III:C2-AP Schiff base formation could impede repair of the lesion by type II enzymes in *E. coli*. Alternatively, in mammalian cells AP lesions are removed by a sequence involving Ape1 incision, followed by removal of the 5′-deoxyribose phosphate by DNA polymerase β. On the basis of comparisons to other abasic lesions, we expect that C2-AP will be incised by Ape1 (*17*, *27*). However, DNA polymerase β utilizes a Schiff base lyase mechanism to remove 5′-deoxyribose phosphates produced by Ape1 (*28*). Removal of a 5′-C2-AP phosphate group will be incompatible with this mechanism. Hence, we propose that C2-AP will have to be excised by strand displacement synthesis and flap endonuclease processing via a long patch repair mechanism.

In summary, in vitro studies on the interaction between the C2′-oxidized abasic site (C2-AP) and *E. coli* enzymes reveal similarities between this lesion and other abasic sites, but significant differences as well. One similarity between C2-AP and other abasic lesions (e.g., AP, C4-AP) is the preferential incorporation of purine nucleotides opposite it. In contrast, incision of the C2-AP lesion by Exo III and Endo IV is less efficient than repair of other abasic sites by these enzymes. The greatest difference between C2-AP and other abasic lesions is evident in its interaction with Endo III. C2-AP is not a substrate, as AP and C4-AP are, or an irreversible inhibitor, as is L. Overall, these studies suggest that there is no reason to expect that the C2-AP lesion will be any less toxic to cells than are other abasic lesions.

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