Kinetics of Oxidized Cytosine Repair by Endonuclease III of *Escherichia coli*†

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ABSTRACT: Endonuclease III of *Escherichia coli* excises a broad range of oxidized, hydrated and ring-fragmented pyrimidines from DNA. The kinetic parameters were compared for repair of three potentially mutagenic oxidized cytosine lesions: 5,6-dihydroxy-5,6-dihydro-2′-deoxyuridine (uracil glycol or Ug), 5-hydroxy-2′-deoxycytidine (5-ohC), and 5-hydroxy-2′-deoxyuridine (5-ohU). Site-specifically modified 40-mer oligonucleotides containing each of the three lesions in the same sequence context were synthesized chemically or by a combination of chemical and enzymatic methods. Appropriately protected phosphoramidites of 5-ohC and 5-ohU were synthesized and incorporated into oligonucleotides by standard solid-phase synthetic methods. The lability of Ug made it necessary to use an alternative approach to prepare the analogous 40-mers containing Ug. An uracil containing pentamer oligonucleotide was oxidized with OsO_4 to generate the corresponding Ug containing product, which was then ligated into an oligonucleotide scaffold to generate 40 base pair duplexes. Using ^{32}P-labeled substrates and a gel electrophoresis based assay, the values of K_m and V_{max} for excision of 5-ohC, 5-ohU, and Ug were determined. In this experimental system, the order of repair efficiency is Ug > 5-ohC > 5-ohU based on ratios of V_{max}/K_m. Modest effects were observed when the base paired opposite the lesion was changed from G to A.

Oxidative DNA damage is implicated in the process of mutagenesis and is suggested to be involved in carcinogenesis and aging (*1*). These effects are likely to be mediated by potent oxidizing agents, including O_2^-, H_2O_2, and ·OH, generated from either endogenous or exogenous sources such as aerobic metabolism or ionizing radiation, respectively. Reaction of these oxidants with the bases or deoxyribose residues in DNA leads to the formation of numerous lesions, some of which may miscode or block replication (*2*). It is estimated that DNA in a human cell may suffer as many as 10^4 oxidative "hits" per day (*3*). In order to mitigate the effects of these and other deleterious lesions and to preserve the integrity of the genome, organisms have evolved multiple defense mechanisms to repair DNA damage (*4*). Not surprisingly, several human disease syndromes and cancers have been correlated with deficiencies in DNA repair systems.

Among the many oxidized lesions formed are three potentially mutagenic deoxycytidine derivatives: 5,6-dihydroxy-5,6-dihydro-2′-deoxyuridine (uracil glycol or Ug),[1] 5-hydroxy-2′-deoxycytidine (5-ohC), and 5-hydroxy-2′-deoxyuridine (5-ohU) (Figure 1). Oxidation of cytosine can result in formation of the unstable cytosine glycol, which can subsequently deaminate, dehydrate, or undergo both reactions to give rise to Ug, 5-ohC, or 5-ohU (*5*). Interest in these lesions stems from their relative abundance in mammalian tissues where they have been detected at levels comparable to 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoG), the most prevalent oxidized base observed to date (*6*). Moreover, C → T transition mutations dominate the mutational spectra of both oxidizing agents and ionizing radiation but have not yet been attributed to any specific cytosine lesion (*7*−*9*). Speculation that these lesions may be partially or completely responsible for the C → T transition is supported by data from recent experiments. DNA polymerase extension assays in vitro have determined that both 5-ohC and 5-ohU are capable of base pairing with A and that the corresponding triphosphates are accepted by polymerases, suggesting that they may be mutagenic in vivo (*10*,*11*). Indeed, one report indicates that 5-ohC induces a mutation frequency of 2.5% in a reporter construct in *Escherichia coli* (*12*). Additionally, removal of these lesions from DNA by the repair enzymes endonuclease III and uracil glycosylase has been reported. (*13*−*16*).

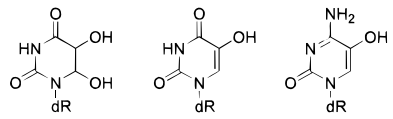

FIGURE 1: Structures of the three oxidized lesions with dR = deoxyribose. 5,6-Dihydroxy-5,6-dihydro-2′-deoxyuridine (Ug); 5-hydroxy-2′-deoxycytidine (5-ohC); 5-hydroxy-2′-deoxyuridine (5-ohU).

Endonuclease III from *E. coli* is a 24 kDa protein possessing both *N*-glycosylase and apurinic/apyrimidinic (AP) endonuclease activity. The enzyme has a broad substrate specificity, acting upon a host of hydrated, oxidized, or ring-fragmented pyrimidines (*15*,*17*). Release of the damaged base is followed by a β-elimination that cleaves the phosphodiester bond. Interestingly, endonuclease III

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[1] Abbreviations: 5-ohC, 5-hydroxy-2′-deoxycytidine; 5-ohU, 5-hydroxy-2′-deoxyuridine; A, 2′-deoxyadenosine; A_{260}, UV absorbance at 260 nm; bp, base pair; BSA, bovine serum albumin; dR, deoxyribose; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FAB, fast atom bombardment; GC, gas chromatography; HPLC, high-pressure liquid chromotography; MALDI-TOF, matrix assisted laser desorption time-of-flight; MS, mass spectrometry; O^6-MeG, O^6-methyl-2′-deoxyguanosine; OAc, acetate; U, 2′-deoxyuridine; Ug, 5,6-dihydroxy-5,6-dihydro-2′-deoxyuridine.

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possesses an Fe-S cluster that has been hypothesized to serve primarily a structural rather than catalytic role (*18*). Crystal structure data combined with modeling studies suggest that catalysis involves an intermediate in which the damaged base is rotated out of the helix (*19*). The *n*th gene of *E. coli*, which encodes endonuclease III, has been cloned and sequenced, and homologs have been identified in yeast and mammals (*20*−*23*). A significant observation is that yeast strains deficient in this activity are hypersensitive to oxidative damage (*22*).

In this paper, we present experiments comparing the kinetics of base excision repair in various base-pairing schemes by endonuclease III using site-specifically modified substrates. Toward that end, we have developed the first synthesis of the labile *cis*-Ug lesion in the context of an oligonucleotide. This report is also the first to describe the kinetic parameters of endonuclease III mediated excision of oxidized cytosines.

EXPERIMENTAL PROCEDURES

Repair Enzymes. Purified endonuclease III from *E. coli* was the gift of Dr. Richard Cunningham, State University of New York, Albany. Purified endonuclease IV from *E. coli* was the gift of Dr. Bruce Demple, Harvard School of Public Health. Protein concentration was determined by the method of Bradford, using bovine serum albumin as standard (*24*).

5′-End-Labeling. T4 polynucleotide kinase (PNK) (New England Biolabs) and [γ-^{32}P]ATP (6000Ci/mmol) (New England Nuclear) were incubated in 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 5 mM DTT at 37 °C for 15 min. Exhaustive phosphorylation was achieved by addition of 1 mM ATP to the reaction and incubating for an additional 30 min at 37 °C. PNK was heat inactivated by treating at 65 °C for 15 min.

Oligonucleotides Containing 5-ohC and 5-ohU. All oligonucleotides were synthesized on an Applied Biosystems 391 automated DNA synthesizer. For oligonucleotides containing 5-ohC and 5-ohU, phosphoramidites were prepared (Morningstar, M., and Essigmann, J. M., unpublished results) and the 40-mers 5′- AATTGCGATCTAGCTCGCCAG**X**AGCGACCTTATCTGATGA-3′, where X= 5-ohC or 5-ohU, were synthesized. All oligonucleotides were purified by 20% polyacrylamide gel electrophoresis. Concentrations were determined by measuring the A_{260} and calculating the extinction coefficient (ϵ) of the single-stranded oligonucleotide as described (*25*). Duplexes containing 5-ohC or 5-ohU were prepared by mixing ^{32}P-labeled oligonucleotides with 1.1 equiv of the complementary strand, which contained either G or A at the site opposite the lesion, in 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, and 0.1 mg/mL BSA, and heating for 2 min at 75 °C. The samples were allowed to cool for 1 h at room temperature and then 1 h at 4 °C.

Synthesis and Characterization of Oligonucleotides Containing Ug. Typically, 375 μg of the 5-mer oligonucleotide GUAGC in NH_4Cl-NH_4OH (pH 9.0) was treated with 2.4% OsO_4 for 30 min at 55 °C to generate the Ug containing pentamer GUgAGC. The modified oligonucleotide was purified by reversed-phase HPLC with UV detection on a Beckman ODS column using a gradient 0−30% B over 60 min (A= 0.1 M NH_4OAc in H_2O; B= 0.1 NH_4OAc in 50% H_2O/acetonitrile) followed by 23% denaturing polyacrylamide gel electrophoresis. The oligonucleotide was characterized by MALDI-TOF MS as well as enzymatic digestion to the nucleosides and comparison to standards. The DNA was digested with 0.05 unit of nuclease P1 (ICN) in 66 mM NaOAc (pH 5) and 0.13 mM $ZnCl_2$ for 1 h at 37 °C followed by incubation with 25 units of calf intestinal phosphatase (Sigma) in 100 mM Tris HCl (pH 8.8). HPLC analysis of the nucleosides utilized a linear gradient 0 to 20% B over 40 min (A = H_2O; B = 50% H_2O/acetonitrile). Authentic Ug was synthesized by treating U with OsO_4; the product was purified by HPLC and identified by FAB-MS. Ug was detected by monitoring UV absorbance at 214 nm. To determine the stability of Ug, the oligonucleotide GUgAGC was subjected to the conditions of duplex construction and examined for formation of degradation products by reversed-phase HPLC.

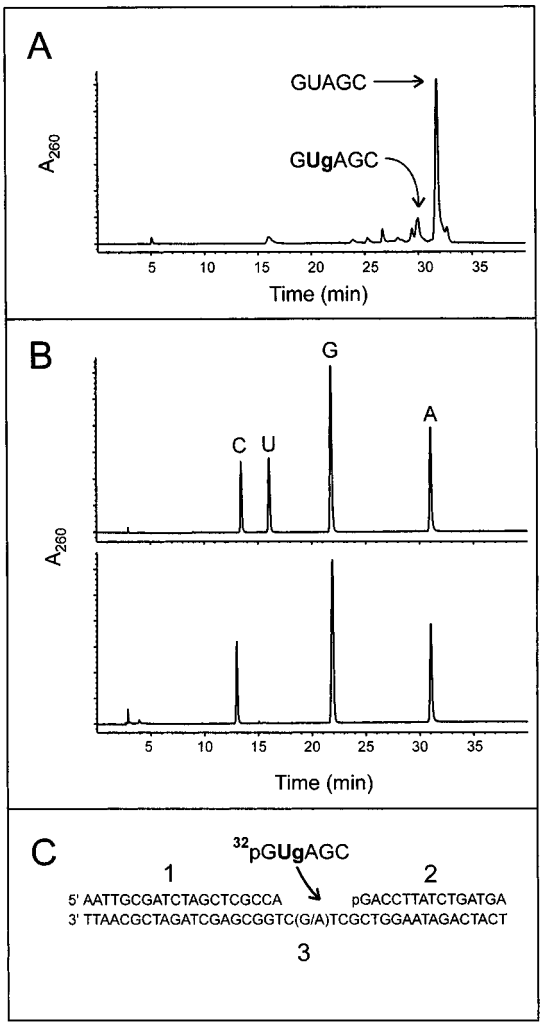

FIGURE 2: (Panel A) HPLC trace of reaction of GUAGC with OsO_4. (Panel B) 260 nm HPLC trace of enzymatic digest of GUAGC (upper) and GUgAGC (lower). The order of elution of the nucleosides is C, U, G, A. (Panel C) Oligonucleotides used in ligation of GUgAGC to form a 40 bp duplex.

Construction of Duplexes Containing Ug. Duplex oligonucleotide substrates were prepared as follows. Oligonucleotide 1 (Figure 2C) was exhaustively phosphorylated with ATP, and GUgAGC was ^{32}P-end labeled as described above. Typically 1800 pmol of each of the three oligonucleotides 1, 2, and 3 (Figure 2C) were annealed by heating to 75 °C in 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT,

and 1 mM ATP, followed by cooling to room temperature over 45 min. An equimolar amount of phosphorylated GUgAGC was added along with 2000 units of T4 DNA ligase (New England Biolabs) and incubated at 16 °C for 3−4 h. Five units of endonuclease IV were added, and the sample was incubated at 37 °C for 30 min to cleave any abasic sites formed during the construction process. The ligation mixture was concentrated using Microcon 10 filters and then subjected to 20% polyacrylamide gel electrophoresis containing 8 M urea for 3 h at 15 V/cm. Bands were visualized by autoradiography and the band corresponding to full length duplex was excised and recovered by the method of crush and soak. The samples were desalted and transferred to 1× TE buffer using Centricon 10 membranes. To confirm that the recovered DNA was in duplex form, a sample was electrophoresed on a 12% native acrylamide gel. The purified duplexes were quantitated by measuring the A_{260} in a 1 cm path length cell and using an ϵ value of 661 L/mmol estimated by summing the individual ϵ values of the single strands and subtracting 2.0 L/mmol per G:C bp and 3.3 L/mmol per A:T bp to account for hypochromicity (*26*). Finally, the samples were ethanol precipitated and resuspended in 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, and 0.1 mg/mL BSA.

Enzymatic Reactions. Reactions using endonuclease III were performed in 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, and 0.1 mg/mL BSA. Reactions were terminated by addition of an equal volume of formamide gel-loading dye. For initial determinations of enzyme activity, samples were incubated for 30 min at 37 °C at endonuclease III concentration between 6.25 and 625 nM. For time course experiments, 6.25 nM endonuclease III was incubated with 1000 nM DNA in 20 μL, and 2 or 3 μL aliqots were removed and quenched at appropriate time points.

Determinations of K_m and V_{max} Values. Enzymatic reactions (10 or 20 μL) containing varying amounts of ^{32}P-labeled substrate (100−3000 nM) were incubated with 6.25 nM endonuclease III for 10 min at 37 °C. The samples were electrophoresed on 20% denaturing polyacrylamide gels, and the bands corresponding to substrate and product were quantitated using a Molecular Dynamics PhosphorImager. Reaction velocity (V) was calculated as picomoles of product formed per minute per nanogram of protein. Substrate concentration [S] was calculated as nanomolarity. The apparent K_m and V_{max} values were determined by performing nonlinear least-squares fitting of the averaged data of at least three determinations to the Michaelis−Menton equation using Microcal Origin.

RESULTS

The objective of this work was to study the relative efficiency of removal of three oxidized cytosine derivatives by endonuclease III. A series of site-specifically modified oligonucleotide duplexes was used to determine the kinetics of base excision using phosphodiester bond cleavage as an end point. The substrates were generated by either total chemical synthesis or a combination of chemical and enzymatic methods.

Synthesis and Construction of Duplexes Containing Ug. The lability of Ug made it necessary to invoke a combination of chemical and enzymatic methods to prepare a duplex containing a single Ug lesion. A small pentamer oligonucleotide was chemically oxidized with OsO_4 to generate a singly modified oligonucleotide. The oxidation proceeded in low yield (5−10%) but gave rise to a single major product peak as determined by HPLC (Figure 2A). Isolation of this material yielded a product possessing physical properties consistent with those expected of an oligonucleotide containing an Ug residue; the purified oligonucleotide eluted earlier on reversed-phase HPLC suggesting that it was more polar than the starting material, and it had retarded mobility relative to the unmodified oligonucleotide during PAGE (data not shown). MALDI-TOF MS confirmed that the isolated peak had a mass 34 amu greater than the unmodified ($M\text{-}H_{calc}$ 1522.0, $M\text{-}H_{obs}$ 1522.5), which is the expected mass increase upon dihydroxylation of uridine. Enzymatic digestion of the product peak to the nucleoside level revealed the absence of uridine in the sample, indicating that the chemical modification was specific for uridine (Figure 2B). Moreover, the uridine derivative, like authentic Ug, lacked UV absorbance at 260 nm (*27*). Monitoring of the chromatogram at 214 nm revealed two peaks eluting at 4 and 5 min (data not shown); the later peak corresponds to authentic Ug; it is believed that the earlier peak is residual Ug 5′-monophosphate resulting from incomplete reaction of the calf intestinal phosphatase.

The stability of the Ug containing pentamer was examined under the conditions necessary for preparation of the 40-mer duplex. Treatment at 37 °C for 30 min followed by 65 °C for 15 min (end labeling) and 16 °C for 3 h (ligation) revealed between 2 and 4.5% degradation to the AP site containing oligonucleotide (data not shown), which was subsequently removed after the ligation step by endonuclease IV treatment.

Upon phosphorylation, the modified oligonucleotide was ligated into a scaffold construct containing a single-stranded gap complementary in sequence to the modified oligonucleotide (Figure 2C). Using equimolar quantities of each of the four oligonucleotide components, ligation efficiencies ranging between 30 and 60% were regularly achieved. There was no significant difference in the ligation efficiency of incorporating Ug opposite A or G. The fully ligated material was purified from an 8 M urea polyacrylamide gel. In order to preserve the integrity of the Ug lesion, the gel was electrophoresed at 15 V/cm to minimize heat generation; additionally, the sample was not heated prior to loading on the gel. A consequence of these steps was that a significant fraction of the DNA remained duplex under these conditions. This duplex DNA band could be recovered, and subsequent electrophoresis under standard denaturing conditions resulted in the appearance of a band corresponding to a 40-mer. Electrophoresis of the duplex under native conditions revealed >90% double-stranded material (data not shown).

Endonuclease III Removes Ug, 5-ohC, and 5-ohU from DNA. As seen in Figure 3, Ug, 5-ohC, and 5-ohU were excised from DNA and the subsequent AP site cleaved regardless of whether the lesions were paired with G or A. The band that appears in lanes treated with endonuclease III migrated as expected as approximately a 21-mer. Endonuclease III had no effect on the control duplexes containing either C:G or C:A. Time course experiments reveal that enzymatic activity was linear for the first 20−30 min (Figure 4) and that the extent of cleavage was greatest for Ug, followed by 5-ohC and then 5-ohU.

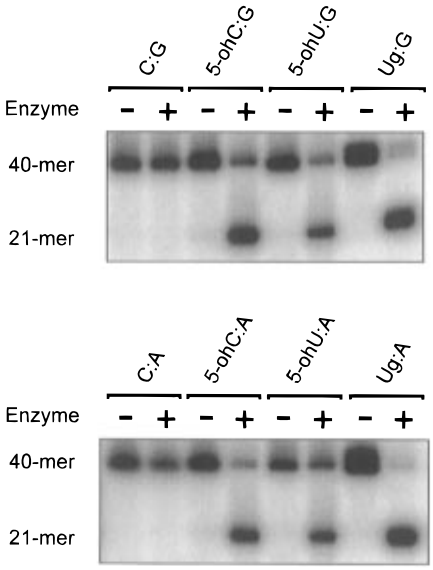

FIGURE 3: Duplexes of 40 bp containing C, 5-ohC, 5-ohU, and Ug incubated with 62.5 nM endonuclease III. (Upper panel) Bases paired opposite G; (Lower panel) Bases paired opposite A.

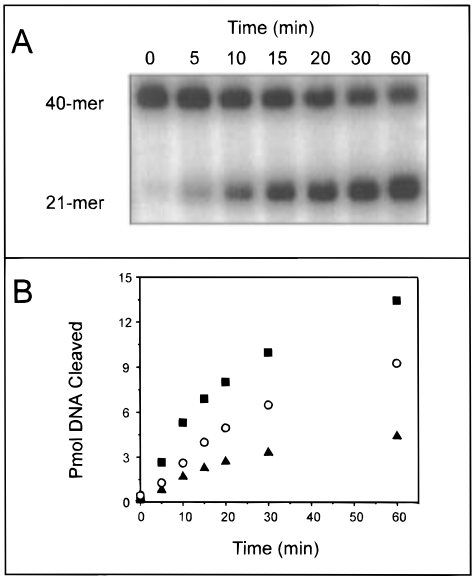

FIGURE 4: (Panel A) Representative time course for endonuclease III cleavage of 5-ohC:G. (Panel B) Extent of cleavage vs time for Ug (■), 5-ohC (○), 5-ohU (▲).

Comparative Kinetics of 5-ohC, 5-ohU, and Ug Removal. Assays in which the concentration of substrate was varied were carried out and values of K_m and V_{max} were determined for each lesion in two different base-pairing contexts: paired with G or paired with A (Figure 5). As shown in Table 1, based on values of V_{max}/K_m, Ug was repaired more efficiently than 5-ohC, which was in turn repaired more efficiently than 5-ohU. For each lesion, positioning of an A residue in the opposite strand resulted in both higher K_m values as well as higher V_{max} values. V_{max}/K_m, however, was always greater for the pairing with G. The values of V_{max} for the three lesions varied rather modestly over a 2.6-fold range, whereas the K_m values spanned a slightly greater 6-fold range.

DISCUSSION

We have determined the relative kinetics of removal of the oxidized lesions Ug, 5-ohU, and 5-ohC from site-specifically modified DNA substrates and found that endonuclease III processes the lesions in the order Ug > 5-ohC > 5-ohU. In order to prepare the substrates used for this direct comparison, it was necessary to develop several new synthetic methodologies. We shall report elsewhere novel solid phase methods to synthesize oligonucleotides containing 5-ohC and 5-ohU, thus, enabling the study of these lesions in any desired DNA sequence (Morningstar, M., and Essigmann, J. M., unpublished results). In addition, in this work, we report the first synthesis of the thermally labile Ug lesion in an oligonucleotide. The use of these site-specifically modified substates also enabled us to examine the effects of changing the base positioned opposite each lesion to reflect potential biologically important pairings.

Although the solid phase incorporation of a labile saturated pyrimidine, (5*R*)-5,6-dihydro-5-hydroxythymidine into an oligonucleotide has been reported, no analogous synthesis for the structurally similar Ug has been advanced to date (*28*). In the absence of such technology, we resorted to postsynthetic oxidation of a uridine containing oligonucle-

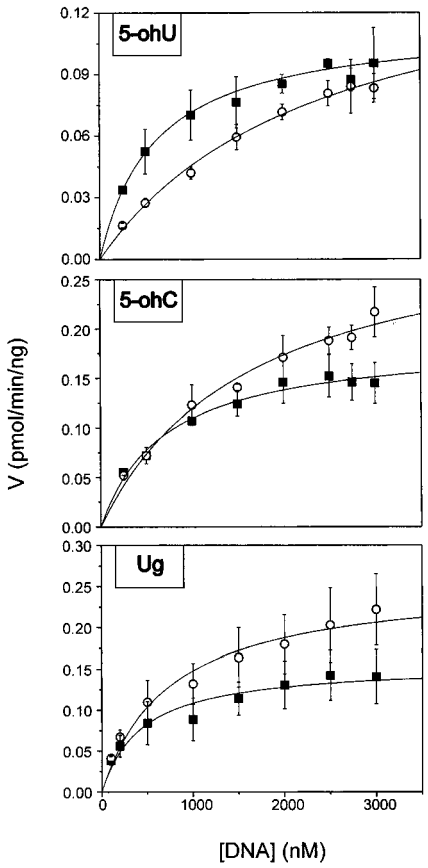

FIGURE 5: Plots of reaction velocity (V) vs substrate concentration. Values are mean and standard deviation of at least three experiments. Curves shown are the best fit to the Michaelis−Menten equation of the averaged data. (■) Lesion paired with G; (○) lesion paired with A.

Table 1: Kinetic Analysis of Oxidized Cytosines[a]

substrate	K_m (nM)	V_{max} (pmol/min/ng)	relative V_{max}/K_m
Ug:G	440 (120)	0.16 (0.01)	1.0
Ug:A	740 (170)	0.26 (0.02)	0.98
5-ohC:G	710 (110)	0.19 (0.01)	0.74
5-ohC:A	1660 (320)	0.32 (0.03)	0.54
5-ohU:G	610 (130)	0.12 (0.01)	0.53
5-ohU:A	2440 (340)	0.16 (0.01)	0.18

[a] Parameters derived from graphs in Figure 5. Numbers in parenthesis indicate standard errors.

otide. OsO_4 is widely used as a probe of DNA structure owing to its specific reactivity with the 5,6-double bond of T and has been used to synthesize site-specifically modified oligonucleotides containing thymidine glycol (Tg) (*29*). The reaction of OsO_4 with uridine proceeds analogously, albeit less vigorously, to the reaction with T (*30*). Moreover, OsO_4 has been used to synthesize deoxyuridine glycol nucleoside (Ug) (*16*, *27*). A logical extension of this approach was to treat an oligonucleotide containing a single U with OsO_4. We oxidized a very short oligonucleotide, GUAGC, which could be readily purified and characterized using HPLC, gel electrophoresis, MALDI-TOF MS, and enzymatic digestion. Subsequent enzymatic manipulations introduced the pentamer into 40 bp duplexes. Similar strategies have been used by others to prepare large duplexes for in vitro DNA repair studies (*31*).

Oxidation with OsO_4 is known to give exclusively *cis*-glycols (*32*). It is likely that oxidation of the oligonucleotide GUAGC affords a mixture of the two diastereomers of *cis*-Ug (5*S*,6*R* and 5*R*,6*S*), as has been reported for the $KMnO_4$ oxidation of T to Tg (*33*). Although Douki et al. report that the two diastereomeric nucleosides are separable by reversed-phase HPLC (*27*), under our conditions, Ug synthesized by treatment of deoxyuridine with OsO_4 eluted as a single peak during reversed-phase HPLC (data not shown). Similarly, Luo and co-workers observe only a single peak corresponding to Ug of unknown stereochemistry upon subjecting C to the Fenton reaction (*34*). Thus, the kinetic parameters we have determined may reflect enzymatic activity directed toward a mixture of the two diastereomers. Of relevance are studies by Wagner et al., who observe comparable endonuclease III activity against the *cis*- and *trans*-Ug isomers as measured by gas chromatography of the TMS derivatives, suggesting that the relative configuration of the hydroxyl groups may not dramatically perturb the reaction kinetics (*16*).

The results of the kinetic experiments are summarized in Table 1 and indicate that the relative ability of endonuclease III to process these three lesions varies over a modest 6-fold range. We have used phosphodiester bond cleavage as an end point to measure the reaction kinetics. Other studies have demonstrated that the glycosylase activity of endonuclease III varies widely depending upon the lesion, suggesting that phosphodiester bond cleavage is not rate limiting (*35*−*37*). Comparison of the values of V_{max}/K_m, or the specificity constant, revealed that at low substrate concentrations, the order of preference for repair was Ug:G ≈ Ug:A > 5-ohC:G > 5-ohC:A ≈ 5-ohU:G > 5-ohU:A. Direct comparison of V_{max} values indicates that, under saturating conditions, Ug and 5-ohC are repaired at roughly equal rates while 5-ohU is repaired somewhat more slowly. Examination of K_m values reveals that for each lesion, base pairing with A led to slightly higher values of the K_m than when the lesion was paired with G. The V_{max} values we observed, 0.12−0.32 pmol/min/ng, fall well within the range of specific activities previously reported for endonuclease III toward various pyrimidine hydrates in homopolymeric DNA. These values vary from 2.8×10^{-5} pmol/min/ng of protein for thymine hydrate to 0.044 for cytosine hydrate and 0.35 for uracil hydrate (*36*, *37*). Although other laboratories have reported that endonuclease III acts upon 5-ohC, 5-ohU, and Ug, no absolute kinetic parameters have been previously determined. In addition to placing the repairability of these lesions on an absolute scale, our findings are in good agreement with the observation that 5-ohC is repaired about twice as well as 5-ohU (*13*, *16*) as well as with the observation that *cis*-Ug is a slightly better substrate than 5-ohC (*16*). Moreover, it has been reported that 5-ohU:G is repaired slightly better than 5-ohU:A, which is also consistent with our data (*13*). The only other kinetic parameter reported for any of these lesions is a recent GC-MS study of 5-ohU removal by human uracil glycosylase, which acts with a similar apparent K_m of 450 nM (*14*).

The identity of the base opposite a lesion when it is excised from DNA can have critical consequences for the welfare of a cell since removal of a lesion when it is mispaired will result in fixation of the mutation rather than productive repair. Enzymes have been ascribed physiological roles in reducing mutation frequency based on their observed ability to discriminate between appropriate and inappropriate base pairings. For example, hypoxanthine glycosylase removes inosine, the deamination product of adenine, 20-fold more rapidly from I:T as compared to I:C (*38*). Similarly, the MutM protein has an approximately 13-fold lower K_m value for removing 8-oxoG from 8-oxoG:C as compared to 8-oxoG:A (*39*). In both of these cases, nonproductive repair is disfavored. If one of the physiological roles of endonuclease III is to protect the cell from oxidative damage to C, it might be expected that repair of lesions paired with G would be favored. On the basis of values of V_{max}/K_m, we indeed observe that excision of 5-ohC and 5-ohU when paired opposite G is favored over removal when paired with A, albeit only by 2−3-fold. Interestingly, comparable levels of discrimination are observed for uracil glycosylase, which demonstrates a slight but consistent preference for removing uridine when paired with G versus A (*40*).

The recent structural characterization of endonuclease III indicates that an extrahelical base can be accommodated in the enzyme crystal structure (*19*). This motif is becoming increasingly common as more structures of DNA-modifying enzymes are solved (*41*). Interestingly, NMR studies have indicated that a site-specifically located Tg residue in duplex DNA displays extrahelicity in the absence of protein (*42*), highlighting an unresolved issue of much current interest; does this class of proteins recognize and bind to an extrahelical base, or does the protein actively participate in the eversion process (*43*)? In either case, the observed differences in the kinetic parameters when the opposing base is varied could reflect differential interactions between protein side chains and the orphan base upon binding. Another possibility is that the stability of the Watson−Crick hydrogen bonding between the two bases correlates with the propensity of the base to "flip-out"; a stable interaction is more resistant to the extrusion process. Structural and thermodynamic comparisons of the lesions in different base-pairing schemes might shed further light on the mechanisms of protein-DNA recognition, binding, and catalysis.

The physiological relevance of these observations is difficult to assess with certainty. Steady state levels of Ug, 5-ohC, and 5-ohU have been measured, indicating a balance between the rate of oxidative damage and the repair capacity of the cell. The high steady state levels in mammalian tissues, on the order of 1 in 10^6 nucleotides, indicate that between 10^3−10^4 lesions could be present in each genome, posing a serious threat to genomic integrity (*6*). Unfortunately, direct identification of repair systems responsible for

correcting oxidative damage has proven elusive. Although our studies demonstrate that endonuclease III is clearly capable of processing all three of these oxidative lesions in vitro, *E. coli* cells with *n*th mutations, which lack endonuclease III, are only weak mutators and are not sensitized toward killing by oxidants (*44*), suggesting the existence of alternative repair pathways. The existence of multiple repair systems to repair similar types of damage is well precedented. For example, alkylation damage in the form of O^6-MeG can be directly reversed by the the repair proteins Ada and Ogt, and similarly, the base excision repair enzymes AlkA and Tag act in concert to remove N-alkylated lesions (*4*). The finding that the UVR (A)BC excision repair complex, in addition to endonuclease III, removes Tg lesions supports the notion that there may be functional redundancy in the repair of oxidized pyrimidines (*45*, *46*), as does the identification of endonuclease VIII, which also appears to repair some modified pyrimidines (*47*). Interestingly, the recent cloning of an endonuclease III homolog in yeast and the observed sensitivity of the corresponding knockout strain to oxidizing agents indicates that this base excision repair pathway is physiologically important, at least in yeast (*22*). A human homolog of endonuclease III has also been cloned (*23*), and examination of the kinetics and substrate specificity of this enzyme may shed more light as to the true physiological role of this enzyme family. It will be of great interest to determine whether additional enzymes are involved in the processing of these oxidized lesions.

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