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Incidence and persistence of 8-oxo-7,8-dihydroguanine within a hairpin intermediate exacerbates a toxic oxidation cycle associated with trinucleotide repeat expansion

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Abstract

The repair protein 8-oxo-7,8-dihydroguanine glycosylase (OGG1) initiates base excision repair (BER) in mammalian cells by removing the oxidized base 8-oxo-7,8-dihydroguanine (8-oxoG) from DNA. Interestingly, OGG1 has been implicated in somatic expansion of the trinucleotide repeat (TNR) sequence CAG/CTG. Furthermore, a 'toxic oxidation cycle' has been proposed for age-dependent expansion in somatic cells. In this cycle, duplex TNR DNA is (1) oxidized by endogenous species; (2) BER is initiated by OGG1 and the DNA is further processed by AP endonuclease 1 (APE1); (3) a stem-loop hairpin forms during strand-displacement synthesis by polymerase β (pol β); (4) the hairpin is ligated and (5) incorporated into duplex DNA to generate an expanded CAG/CTG region. This expanded region is again subject to oxidation and the cycle continues. We reported previously that the hairpin adopted by TNR repeats contains a hot spot for oxidation. This finding prompted us to examine the possibility that the generation of a hairpin during a BER event exacerbates the toxic oxidation cycle due to accumulation of damage. Therefore, in this work we used mixed-sequence and TNR substrates containing a site-specific 8-oxoG lesion to define the kinetic parameters of human OGG1 (hOGG1) activity on duplex and hairpin substrates. We report that hOGG1 activity on TNR duplexes is indistinguishable from a mixed-sequence control. Thus, BER is initiated on TNR sequences as readily as non-repetitive DNA in order to start the toxic oxidation cycle. However, we find that for hairpin substrates hOGG1 has reduced affinity and excises 8-oxoG at a significantly slower rate as compared to duplexes. Therefore, 8-oxoG is expected to accumulate in the hairpin intermediate. This damage-containing hairpin can then be incorporated into duplex, resulting in an expanded TNR tract that now contains an oxidative lesion. Thus, the cycle restarts and the DNA can incrementally expand.

Keywords

OGG1; trinucleotide repeat expansion; toxic oxidation cycle

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Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A: Supplementary data

Supplementary data associated with this article can be found in the online version; stability of 8-oxoG-containing oligonucleotide, hOGG1 active site titration, EMSA of K249Q hOGG1 and wild-type hOGG1 binding to Stem-HP, autoradiogram for multiple-turnover experiments on Mixed-DUP with and without APE1, multiple-turnover experiments with Loop-HP.

1. Introduction

The expansion of a CAG/CTG TNR sequence has been identified as the pathogenic signature of several neurodegenerative disorders [1–3], with one such disorder being Huntington's disease (HD). In healthy individuals, exon 1 of the HD gene contains 5–35 CAG/CTG repeats; repeat tracts of this length are not prone to expansion [4]. Sequences containing 36–39 CAG/CTG repeats are described as the pre-mutation allele and are known to be prone to expansion. The disease state is characterized by greater than 40 repeats.

Following expansion of the CAG/CTG sequence, the HD gene is transcribed and translated and results in a protein product with an expanded glutamine tract [5]. Although the function of the normal HD protein remains an active area of research, it is known that the mutant HD protein, which has a tract of more than 40 glutamine residues, has aberrant properties that cause the death of brain cells [6]. Indeed, it is the death of these cells that leads to both the mental decline and uncoordinated body movements associated with HD.

Significant insight into the molecular mechanism of the expansion of CAG/CTG sequences was gained from work using an R6/1 mouse model for HD [7]. In these mice, which harbor a transgene containing exon 1 of the human HD gene, TNR expansion correlated with accumulation of the oxidatively damaged base 8-oxoG. Intriguingly, when the R6/1 mice were crossed with mice lacking the DNA repair protein OGG1, the CAG/CTG repeat expansion was abrogated in somatic cells [7]. These results indicate that the presence of the OGG1 repair protein is implicated in TNR expansion.

OGG1 is a glycosylase that initiates the base excision repair (BER) process in mammalian cells by excising oxidized bases from DNA, with 8-oxoG being the prototypic and most well-studied substrate [8]. Like most glycosylases, OGG1 is a base-flipping enzyme that cleaves the *N*-glycosidic bond to excise 8-oxoG following extrusion of the modified base from the DNA helix. Cleavage of the glycosidic bond generates an apurinic (AP) site. The remaining steps in the BER pathway are believed to occur in a manner that has been compared to the 'passing of a baton' [9, 10]. AP endonuclease 1 (APE1) converts the AP site to a single nucleotide gap by cleaving the sugar-phosphate backbone 5' to the AP site. This cleavage event produces a gap with 3'-OH and 5'-deoxyribose phosphate (5'-dRP) termini [11]. Polymerase β (Pol β) catalyzes the addition of dGTP to the 3'-OH and also removes the 5'-dRP using its dRPase activity [12]. Finally, DNA ligase III (LigIII) seals the nick to complete the repair event. Since a single nucleotide is incorporated by pol β , the process is referred to as short-patch BER (SP-BER).

It is of note that, in addition to its glycosylase activity, OGG1 possesses AP lyase activity that is able to cleave at the AP site to generate a gap with 3'-phospho α,β -unsaturated aldehyde (3'-dRP) and 5'-phosphate termini [13]. APE1 can remove the 3'-dRP to generate the 3'-OH terminus necessary for DNA synthesis by pol β [11]. Although OGG1 possesses AP lyase activity it has been shown to be ~500-fold slower than the associated glycosylase step [14]. Furthermore, it has been shown *in vitro* that APE1 improves the efficiency of repair by stimulating the product release rate of OGG1 and cleaving at the AP site, which bypasses the AP lyase activity of OGG1 [15, 16]. Thus, it is likely that APE1 and not OGG1, cleaves at the AP site *in vivo*.

It has also been demonstrated that, in addition to SP-BER, BER can occur via a pathway that generates a repair patch of two or more nucleotides and is referred to as long-patch BER (LP-BER). The LP-BER pathway is utilized when a chemically modified 5'-dRP group is present. For example, alkylation [17] or oxidation [18] of an AP site, followed by processing by APE1, generates a modified 5'-dRP group that is refractory to the dRPase activity of pol β [17, 18]. Pol β -dependent strand displacement synthesis then generates a repair patch, as

well as a displaced single-stranded DNA flap containing the modified 5'-dRP. The flap is subsequently removed by flap endonuclease 1 (FEN1). LigIII seals the nick and completes the repair.

In an effort to elucidate the role of OGG1 in CAG/CTG repeat expansion the OGG1 repair event has been reconstituted *in vitro* [7]. Two 100 base pair (bp) DNA duplexes were used as substrates. The first duplex contained 8-oxoG flanked on both sides by mixed sequence. In the second duplex the 8-oxoG was flanked on the 5' side by mixed sequence and on the 3' side by (CAG)₁₉/(CTG)₁₉. Using purified human OGG1 (hOGG1), which shares 83% sequence homology with murine OGG1 [19], 8-oxoG was excised from both duplexes [7]. Furthermore, APE1 also had activity on both duplexes. In contrast, while pol β incorporated a single nucleotide in the mixed-sequence duplex, more than one nucleotide was incorporated in the duplex containing the TNR sequence [7]. Following ligation by bacteriophage T4 DNA ligase, a 100 bp product was restored for the mixed-sequence duplex, but expanded products were obtained for the duplex containing the repeat sequence (the length of the expanded products was not reported). In other work, when the same 100 bp duplex substrates were incubated with cell extracts from mouse embryonic fibroblasts, instead of purified BER proteins, similar results were obtained [20]. The mixed-sequence duplex was faithfully repaired, but expansion of the repeat-containing duplex resulted in products containing up to 130 bp. It was identified *in vitro* that the expansion occurred via an LP-BER pathway. With TNR sequences, LP-BER occurred when the 5'-dRP contained an analog of the deoxyribose sugar ring that is known to be refractory to the dRPase activity of pol β and, interestingly, also when there was an authentic 5'-dRP group present. In contrast, for the mixed-sequence duplex pol β processed the authentic 5'-dRP group and repair occurred via SP-BER. Therefore, it was suggested that the TNR region facilitates LP-BER. Furthermore, it was proposed that the formation of a hairpin by the displaced single strand of TNR DNA generates a flap that is refractory to FEN1 and leads to incorporation of the hairpin into the DNA.

Indeed, in addition to the duplex conformation, CAG/CTG repeat sequences have been shown to adopt non-B conformations. *In vitro* studies using NMR, optical melting analysis, native gel electrophoresis, differential scanning calorimetry, circular dichroism, and chemical probes have shown that single-stranded (CAG)_n and (CTG)_n sequences can fold to form intramolecular hairpins and other slipped-structure intermediates [21–26]. There is also evidence for these TNR sequences to adopt hairpins *in vivo*. Two zinc finger nucleases were designed to cleave the stem of CAG or CTG hairpins but not CAG/CTG duplex [27]. Expression of either of these nucleases in isogenic HeLa cell lines containing 45 or 102 CAG/CTG repeats resulted in the accumulation of products due to hairpin cleavage.

In previous work we have shown that, relative to (CAG)₁₀/(CTG)₁₀ duplex, the hairpin adopted by (CAG)₁₀ is hyper-susceptible to modification by peroxynitrite [28]. Notably, peroxynitrite is known to convert G to several damage products, one of which is 8-oxoG [29–31]. Furthermore, it is the solution-accessible loop of the hairpin that contains a hotspot for DNA damage. Given the demonstrated role of OGG1 in initiating CAG/CTG repeat expansion in both *in vitro* and *in vivo* systems [7, 20, 32], in this work we characterized fully the kinetic parameters of hOGG1 acting on TNR sequences. We determined the binding affinity, the rate of cleavage of the *N*-glycosidic bond of 8-oxoG, and the rate of product release, in the absence and presence of APE1, for hOGG1 acting on DNA substrates with different structural contexts, namely duplex and hairpin, and substrates in which the location of the 8-oxoG was varied. Furthermore, comparison of the results obtained for the TNR sequences to those obtained for a mixed-sequence duplex allowed us to define the contribution of sequence context of the damage to enzyme activity. We demonstrate that hOGG1 binding, catalytic activity and rate of product release for TNR duplexes is

indistinguishable from a comparable mixed-sequence control, indicating that BER can be initiated by hOGG1 just as efficiently in TNR regions as elsewhere in the genome. We also establish that APE1 can stimulate turnover for both the mixed and TNR duplex substrates, albeit to a lesser extent for repeat sequences. Additionally, we demonstrate that the activity of hOGG1 is modulated by the structure of the DNA substrate. With hairpin structures, hOGG1 has a reduced affinity for both substrate and product and excises 8-oxoG at a slower rate as compared to the corresponding TNR duplex substrates.

These data contribute to our understanding of the proposed toxic oxidation cycle [7] in which somatic TNR expansion contributes to the onset and progression of HD. In this model of TNR expansion the repetitive duplex DNA is oxidized, BER is initiated by OGG1, formation and ligation of a TNR hairpin into the DNA occurs during a LP-BER event, and the hairpin is incorporated into duplex to generate an expanded CAG/CTG sequence. Several models have been proposed for hairpin incorporation into duplex and involve a subsequent round of replication or nick-induced gap-filling synthesis [3]. The expanded CAG/CTG duplex is then oxidatively damaged to restart the cycle. Here we show that hOGG1 would effectively initiate this cycle by removing 8-oxoG from duplex TNR DNA. However, the hairpin intermediate is hyper-susceptible to oxidative damage and 8-oxoG is not efficiently processed at the hotspot location. Incorporation of the 8-oxoG containing hairpin results in expanded duplex which now contains oxidative damage. Therefore, we describe a system in which the final step of the cycle regenerates a substrate for the toxic process and works to incrementally expand the TNR DNA.

2. Materials and methods

2.1. Oligonucleotide synthesis and purification

Oligonucleotides were synthesized by standard phosphoramidite chemistry using a BioAutomation DNA/RNA synthesizer [33]. The 8-oxoG-CE phosphoramidite was purchased from Glen Research and used according to the manufacturers specifications. For sequences containing 8-oxoG, the 5'-dimethoxytrityl (DMT) group was removed by the synthesizer and two rounds of HPLC purification were performed using a Dionex DNAPac PA100 anion-exchange column (4 × 250 mm); 10% acetonitrile (aq) (solvent A) and 0.8 M ammonium chloride in 10% acetonitrile (aq) (solvent B) were used as mobile phases (gradient: solvent B was increased from 30% to 55% over 10 min, 55% to 75% over 5 min, 75% to 90% over 15 min, and 90% to 100% over 5 min; 1 mL/min). Oligonucleotides were desalted using Sephadex G-25 fine resin after each HPLC purification. Stability of the oligonucleotides containing 8-oxoG to the experimental conditions was confirmed by HPLC and mass spectral analysis (Supplementary Fig. 1).

For sequences lacking 8-oxoG, the 5'-DMT group was retained to aid in purification. HPLC purification was performed using a Dynamax Microsorb C18 column (10 × 250 mm) with acetonitrile (solvent A) and 30 mM ammonium acetate (solvent B) as mobile phases (gradient: solvent A was increased from 5% to 25% over 25 min; 3.5 mL/min). After the removal of the DMT group by incubation in 80% glacial acetic acid for 12 min at room temperature, a second round of purification was performed (gradient: solvent A was increased from 0% to 15% over 35 min; 3.5 mL/min). Quantification of oligonucleotides was performed at 90 °C using the ϵ_{260} estimated for single stranded DNA [34] and a Beckman Coulter DU800 UV-Vis spectrophotometer equipped with a Peltier thermoelectric device.

2.2. Electrophoretic mobility shift assay to determine K_D

Mutant K249Q hOGG1 was expressed and purified as described previously [35] and total protein concentration was determined using the Bradford assay with bovine γ -globulin as a standard. The oligonucleotides containing 8-oxoG were 5'-³²P end-labeled with T4 polynucleotide kinase following the manufacturer's protocol. 5'-³²P-labeled single-stranded DNA (15 nM) alone (for hairpin samples) or with a 1.25-fold excess of the unlabeled complementary oligonucleotide (for duplex samples) in 20 mM Tris-HCl, 10 mM Na₂EDTA, 140 mM NaCl, pH 7.6 was incubated for 5 min at 90 °C, followed by cooling to room temperature over ~2.5 h. Using a dilution buffer (20 mM Tris-HCl, 10 mM Na₂EDTA, pH 7.6, 7.5% glycerol, 111 μ g/mL bovine serum albumin (BSA)) the DNA was then diluted before adding K249Q hOGG1 (in 20 mM Tris-HCl, 10 mM Na₂EDTA, pH 7.6, 7.5% glycerol, 111 μ g/mL BSA) to yield a final sample of 1.5 nM DNA, 0–500 nM K249Q hOGG1 in 20 mM Tris-HCl, 10 mM Na₂EDTA, 14 mM NaCl, 6.75% glycerol, 100 μ g/mL BSA (20 μ L total sample volume) and incubated for 10 min at room temperature. Following incubation with K249Q hOGG1, samples were placed on ice until loading onto a 10% non-denaturing polyacrylamide gel. Electrophoresis was performed at 4°C and 100 V. The products were visualized by phosphorimager and the amount of K249Q hOGG1•DNA complex and unbound DNA were quantified. Percent of K249Q hOGG1•DNA complex was plotted versus log[K249Q hOGG1], the data were fitted with a sigmoidal curve, and the K_D was taken as the concentration of K249Q hOGG1 at which 50% of the DNA was bound.

2.3. Determination of the glycosylase activity of hOGG1 (k_2)

Wild-type hOGG1 was expressed and purified as described previously [35] and total protein concentration was determined using the Bradford assay with bovine γ -globulin as a standard. The concentration of active hOGG1 was determined using an active site titration as described previously [36] using the 8-oxoG-containing duplex Mixed-DUP (Supplementary Fig. 2). The hOGG1 preparation was found to be 39% active. All hOGG1 concentrations given below or in figure captions are active enzyme concentrations.

The oligonucleotides containing 8-oxoG were 5'-³²P end-labeled with T4 polynucleotide kinase following the manufacturer's protocol. 5'-³²P-labeled single-stranded DNA (80 nM) with a 1.25-fold excess of the unlabeled complementary oligonucleotide in 20 mM Tris-HCl, 10 mM Na₂EDTA, 140 mM NaCl, pH 7.6 was incubated for 5 min at 90 °C, followed by cooling to room temperature over ~2.5 h. The DNA was diluted to a final concentration of 40 nM in 20 mM Tris-HCl, 10 mM Na₂EDTA, 70 mM NaCl, 100 μ g/mL BSA, pH 7.6. This diluted DNA sample and 200 nM hOGG1 in 20 mM Tris-HCl, 10 mM Na₂EDTA, 210 μ g/mL BSA, pH 7.6 were loaded into separate syringes of a Rapid Quench Flow (RQF) instrument (Kintek) and equilibrated for 5 min at 37 °C. The DNA and hOGG1 were then combined in the reaction loop to yield a sample containing 20 nM DNA, 100 nM hOGG1 in 20 mM Tris-HCl, 10 mM Na₂EDTA, 35 mM NaCl, 155 μ g/mL BSA, pH 7.6. Using the RQF instrument, after 0.5–100 sec the reactions were quenched with NaOH (0.5 M final concentration) and incubated at 90 °C for 2 min. After incubation at 90 °C, 15 μ L of denaturing loading buffer (80% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) were added, and the samples were stored on dry ice until electrophoresis. Prior to loading onto an 18% denaturing polyacrylamide gel, all samples were incubated at 90 °C for 3 min. The products were visualized by phosphorimager and the amount of product was plotted versus time. The data were then fitted as described previously [37] in order to obtain values for the rate of *N*-glycosidic bond cleavage of 8-oxoG (k_2).

2.4. Determining the rate of product release of hOGG1 (k_3)

Oligonucleotides containing 8-oxoG were 5'-³²P end-labeled with T4 polynucleotide kinase following the manufacturer's protocol. 5'-³²P-labeled single-stranded DNA (100 nM) alone

(for hairpin samples) or with a 1.25-fold excess of the unlabeled complementary oligonucleotide (for duplex samples) in 20 mM Tris-HCl, 140 mM NaCl, pH 7.6 was incubated for 5 min at 90 °C, followed by cooling to room temperature over ~2.5 h. Using a dilution buffer (20 mM Tris-HCl, 4 mM MgCl₂, 200 µg/mL BSA, pH 7.6) the DNA was then diluted and equilibrated at 37 °C for 5 min. After equilibration at 37 °C, hOGG1 (in 20 mM Tris-HCl, 4 mM MgCl₂, 200 µg/mL BSA, pH 7.6) and APE1 (NEB; in 20 mM Tris-HCl, 4 mM MgCl₂, 200 µg/mL BSA, pH 7.6) were added to yield a final sample of 50 nM DNA, 5 nM hOGG1 or 5 nM hOGG1/50 nM APE1 in 20 mM Tris-HCl, 70 mM NaCl, 2 mM MgCl₂, 100 µg/mL BSA, pH 7.6 (80 µL total sample volume). After addition of hOGG1 or hOGG1/APE1, the sample was incubated at 37 °C and aliquots of 5 µL were removed as a function of time (0.25–20 min), quenched by the addition of 5 µL of 0.5 M NaOH, and incubated at 90 °C for 2 min. Following quenching by NaOH/heat treatment, 5 µL of denaturing loading buffer were added and the samples were placed on dry ice until electrophoresis through an 18% polyacrylamide gel. The products were visualized by phosphorimager and the concentration of product was plotted versus time. Both a burst phase and a linear phase are observed with the slope of the linear phase (k_{ss}) equal to $k_3 \times [\text{active enzyme}]$ [37].

2.5. Trapping by sodium borohydride

Oligonucleotides containing 8-oxoG were 5'-³²P end-labeled with T4 polynucleotide kinase following the manufacturer's protocol. 5'-³²P-labeled single-stranded DNA (80 nM) alone (for hairpin samples) or with a 1.25-fold excess of the unlabeled complementary oligonucleotide (for duplex samples) in 20 mM Tris-HCl, 10 mM Na₂EDTA, 140 mM NaCl, pH 7.6 was incubated for 5 min at 90 °C, followed by cooling to room temperature over ~2.5 h. Using a dilution buffer (20 mM Tris-HCl, 10 mM Na₂EDTA, pH 7.6) the DNA was diluted before adding sodium borohydride (NaBH₄) and hOGG1 (in 20 mM Tris-HCl, 10 mM Na₂EDTA, pH 7.6) to yield a final sample of 20 nM DNA, 500 nM hOGG1, 50 mM NaBH₄ in 20 mM Tris-HCl, 10 mM Na₂EDTA, 35 mM NaCl, pH 7.6 (10 µL total sample volume). Samples were incubated at 37 °C for 1 h and the reaction was quenched by addition of 10 µL of SDS loading buffer (0.5 M Tris-HCl, 20% glycerol, 10% SDS (w/v), 10% β-mercaptoethanol (v/v), 0.1% bromophenol blue) and incubation at 90 °C for 5 min. Samples were electrophoresed through a 10% SDS-PAGE at 200 V for 45 min at room temperature. The products were visualized by phosphorimager.

3. Results and discussion

3.1. DNA substrates

Five DNA substrates were utilized in these studies (Fig. 1). All the substrates are 30 nucleotides in length and each contains a single, site-specifically incorporated 8-oxoG (Fig. 1A). The substrates differ based on their structure, sequence composition, and/or positioning of the 8-oxoG. The first substrate is a 30 bp duplex of mixed sequence with 8-oxoG in the 16th bp (Mixed-DUP; Fig. 1B). This mixed-sequence duplex has been used previously to establish the rate of *N*-glycosidic bond cleavage of 8-oxoG in duplex DNA by hOGG1 and serves here as a control [14]. Two substrates are comprised of CAG trinucleotide repeats that adopt an intramolecular fold to form stem-loop hairpins. These hairpins have a loop with 4 bases and a stem comprised of G-C base pairs and A•A mismatches. In one CAG hairpin, the 8-oxoG is located in the 2nd repeat, positioning the damage in the stem of the hairpin (Stem-HP; Fig. 1C); in the other CAG hairpin, the 8-oxoG is in the 5th repeat, positioning the damage in the loop of the hairpin (Loop-HP; Fig. 1E). In order to generate CAG/CTG duplexes, the (CTG)₁₀ sequence was hybridized with Stem-HP or Loop-HP to yield Stem-DUP (Fig. 1D) and Loop-DUP (Fig. 1F), respectively; these substrates can be considered the duplex counterparts of Stem-HP and Loop-HP. Notably, in Loop-DUP the 8-

oxoG is in the center of the 30 bp duplex whereas in Stem-DUP the damage is closer to one end of the duplex.

3.2. Minimal kinetic scheme of hOGG1

The minimal kinetic scheme used for the analysis of hOGG1 activity is provided in Fig. 2. The figure shows the three steps of the enzymatic cycle: binding to the DNA substrate (DNA)_S, which is described by the rate constants k_1 and k_{-1} , cleavage of the *N*-glycosidic bond to excise 8-oxoG and subsequent cleavage of the AP site, which are described by the rate constant k_2 , and release of the DNA product (DNA)_P, which is described by the rate constant k_3 . In this work, using the five DNA substrates shown in Fig. 1, we characterized the three steps of the hOGG1 enzymatic cycle and report values for the dissociation constant (K_D ; where $K_D = k_{-1}/k_1$), k_2 , and k_3 .

3.3. Dissociation constant (K_D) for hOGG1 binding DNA substrates

The K_D describes the affinity of hOGG1 for a particular 8-oxoG-containing DNA substrate. This affinity is governed by non-covalent interactions such as hydrogen bonding and electrostatic interactions, as well as hydrophobic and Van der Waals forces. Values for K_D are reported in molar units and represent the concentration of hOGG1 enzyme at which half of the substrate is bound [38]. An important point to consider when determining a K_D is that, in addition to binding to the substrate, an enzyme can be catalytically active over the course of the experiment. For example, if turnover of hOGG1 were to occur during these experiments, the reported K_D could reflect binding to the DNA substrate and/or the DNA product. Thus, in order to prevent cleavage of the *N*-glycosidic bond, we performed an electrophoretic mobility shift assay (EMSA) for each DNA substrate using a K249Q mutant of hOGG1 (Fig. 3). It has previously been shown that K249Q hOGG1 contains an active site mutation such that the enzyme retains its ability to bind duplex DNA, but is catalytically inactive [35].

Within the error of our experimental methods the K_D values obtained for the three duplex substrates, Stem-DUP, Loop-DUP, and Mixed-DUP, are the same (Table 1) and are comparable to values reported for hOGG1 binding to mixed-sequence duplex substrates [15]. Thus, for the duplex substrates used in this work neither sequence context nor position of 8-oxoG seem to influence the affinity of hOGG1 for the substrate. These results indicate that hOGG1 would bind equally well to mixed sequence and TNR regions of DNA. However, it should be noted that comparing previously reported data [28] and data reported in this work (section 3.4), we have shown that a centrally-located 8-oxoG, as is found in Mixed-DUP or Loop-DUP, is excised ~17-fold faster than the 8-oxoG in Stem-DUP, for which the damage is closer to one end of the duplex. One possible interpretation of the K_D data is that for the duplex substrates used in this work neither sequence context nor position of 8-oxoG influence the affinity of hOGG1 for the substrate but proximity of 8-oxoG to the end of the duplex does decrease the rate of removal of 8-oxoG. A second possibility is that proximity of 8-oxoG to the end of the duplex (i.e., for Stem-DUP) does influence binding affinity of hOGG1, likely resulting in a larger K_D and the slower rate of excision of 8-oxoG, but the EMSA technique is not sufficient to reveal this distinction.

In contrast to the duplex substrates in which a shift in electrophoretic mobility of the DNA was observed upon addition of K249Q hOGG1, a stable hOGG1•DNA complex was not observed for either of the hairpin substrates. Furthermore, addition of a large excess of K249Q hOGG1 (1.5 nM DNA:1,000 nM enzyme) also yielded no detectable enzyme•DNA complex (Supplementary Fig. 3). In order to investigate the possibility that mutation of the active site lysine to glutamine affects binding of hOGG1 to non-B DNA conformations such as Stem-HP and Loop-HP, EMSA experiments were also performed using wild-type

hOGG1. Indeed, using wild-type hOGG1, a small amount (4%) of Stem-HP was observed as a hOGG1•Stem-HP complex (Supplementary Fig. 3). This complex was observed at hOGG1 concentrations as low as 250 nM. Notably, as the concentration of wild-type hOGG1 was increased up to 2,000 nM the amount of Stem-HP observed as a complex with hOGG1 did not change. In fact, only the amount of cleaved substrate increased (from 9% to 22%) as the amount of hOGG1 was increased over the range of 250 to 2,000 nM. In an attempt to minimize cleavage of the DNA substrate over the course of the experiment the incubation time was shortened and the temperature was lowered. These changes were sufficient to reduce the amount of cleavage of the substrate, but also yielded less hOGG1•Stem-HP complex (data not shown).

EMSA experiments using wild-type hOGG1 and Stem-HP revealed that some substrate is converted to product. Therefore, it is possible that the small amount of Stem-HP observed in complex with the enzyme is due to a covalent interaction formed during the processing of substrate to product (section 3.7) [35]. Because no complex was observed between Stem-HP and K249Q hOGG1, and EMSA experiments performed with the wild-type enzyme yielded only a small amount of enzyme•DNA complex that is further complicated by substrate turnover, we do not report a K_D for the Stem-HP substrate.

For the Loop-HP substrate a complex with hOGG1 was observed neither with the K249Q mutant nor wild-type enzyme (data not shown). Therefore, a value for K_D could not be determined for the CAG hairpin with 8-oxoG in the loop.

Although a K_D could not be obtained for either of the CAG hairpin substrates we have shown previously that hOGG1 can excise 8-oxoG from both the stem and loop of the CAG hairpin, although significantly less efficiently than from duplex substrates (section 3.4) [28]. When taken together, our inability to determine a K_D for Stem-HP or Loop-HP and the slower rate of excision of 8-oxoG from these substrates, indicate that this non-B DNA conformation is a less ideal substrate for hOGG1 than duplex. Therefore, it is likely that the interactions of hOGG1 with the hairpins are transient and cannot be monitored by EMSA.

In addition to the role of hOGG1 in triggering TNR expansion via a BER-dependent pathway, we proposed previously that hOGG1 may also contribute to replication-dependent TNR expansion [28]. hOGG1 could bind to 8-oxoG-containing hairpins and this binding event could further stabilize the stem-loop structure and prolong its lifetime. DNA polymerase could then replicate the stabilized hairpin with the full length of the hairpin being incorporated into the nascent strand [39, 40]. Furthermore, others have proposed that as part of the toxic oxidation cycle, the hairpin formed by the TNR sequence is stabilized by the mismatch repair proteins Msh2 and Msh3 [7]. Thus, we considered that hOGG1 could also serve in this role and stabilize a hairpin intermediate. However, the transient nature of the hOGG1•hairpin interactions revealed here suggests that hOGG1 does not contribute to the stability of the hairpin structure and subsequently does not facilitate replication-dependent expansion or the toxic oxidation cycle in this manner.

3.4. Glycosylase activity of hOGG1 is not influenced by sequence context

With most glycosylases product release is rate limiting [41]. Therefore, k_{cat} measured under steady-state conditions is a reflection of the rate of product release rather than the glycosylase step in which the *N*-glycosidic bond is cleaved. Indeed, for this reason hOGG1 does not follow Michaelis-Menten kinetics [15]. By carrying out experiments under single-turnover conditions ($[hOGG1] \gg [DNA]$), the enzyme is not required to process more than one substrate and the rate of product release does not contribute to k_{obs} ; therefore the rate of excision of 8-oxoG is revealed. As described in work from our lab [28] and other labs [14, 37] single-turnover conditions can be established and k_2 can be determined by incubating the

8-oxoG-containing substrates with a 5-fold excess of hOGG1. Furthermore, quenching the reactions by addition of NaOH and incubation at 90 °C converts AP sites to strand breaks, which can be visualized by polyacrylamide gel electrophoresis (PAGE). Thus, quenching with NaOH/heat ensures that the AP lyase activity of hOGG1 does not limit the observed rate [14]. Therefore, k_2 will reflect the rate constant for cleavage of the *N*-glycosidic bond [36].

In our previous work with TNR substrates we reported a k_2 for hOGG1 excising 8-oxoG from Stem-HP, Loop-HP, and Stem-DUP [28]. However, quantitative rate constants describing hOGG1 removal of 8-oxoG from Loop-DUP and Mixed-DUP could not be obtained using manual methods. After only 15 sec at 37 °C, hOGG1 had fully converted these two substrates to product; therefore, in our previous work only lower limits for the k_2 could be determined. To address this issue, here we used a RQF instrument to determine k_2 for Loop-DUP and Mixed-DUP (Fig. 4). hOGG1 was found to remove 8-oxoG from Loop-DUP and Mixed-DUP at a rate of $56 \pm 2.2 \text{ min}^{-1}$ and $54 \pm 11 \text{ min}^{-1}$, respectively (Table 1). This rate constant for hOGG1 removing 8-oxoG from Mixed-DUP is in agreement with the value reported previously for this duplex substrate [14]. Furthermore, hOGG1 removes 8-oxoG from Loop-DUP at the same rate as from Mixed-DUP. While the lesion is centrally-located in both duplexes (at the 15th and 16th bp for Loop-DUP and Mixed-DUP, respectively) the sequence context of the lesion is different in these substrates. This result indicates that hOGG1 removes 8-oxoG with the same efficiency regardless of sequence context and that non-repetitive and TNR sequences have equal potential to serve as substrates for the initiation of the BER pathway.

Furthermore, comparison of the results obtained here for Loop-DUP with the k_2 results reported previously for Stem-DUP [28] reveal that whereas sequence context does not affect k_2 , location of the lesion in the duplex does influence efficiency of hOGG1. The k_2 for Stem-DUP is 17-fold slower than Loop-DUP. Although the sequence context of the 8-oxoG is identical in these two substrates, positioning the 8-oxoG closer to the end of the duplex affects the ability of hOGG1 to process the substrate. Indeed, it has been shown previously that position of 8-oxoG in a duplex influences the k_2 of hOGG1. Literature reports show a 2-fold slower k_2 for an 18 bp duplex with 8-oxoG positioned 9 bp from the end than for a 30 bp duplex with 8-oxoG 15 bp from the end [14, 37].

3.5. Rate of product release (k_3) by hOGG1 is modulated by DNA structure

In the minimal kinetic scheme in Fig. 2, k_3 is the rate constant associated with the release of product by hOGG1. For most glycosylases, including hOGG1, the rate of product release is the rate-determining step [41]. Therefore, by performing experiments under multiple-turnover conditions ($[\text{hOGG1}] \ll [\text{DNA}]$) the k_{obs} will be k_3 . This rate constant provides a measure of the turnover efficiency, or k_{cat} , of hOGG1 acting on a particular DNA substrate. Shown in Supplementary Fig. 4A are representative PAGE results for Mixed-DUP. Fig. 5 displays product concentration as a function of time for experiments performed with Mixed-DUP, Loop-DUP, Stem-DUP, and Stem-HP. In all cases there is an initial burst of product formation, followed by a linear phase, which represents hOGG1 operating under steady-state conditions in which the accumulation of product is governed by the rate of product release.

The duplex substrates Mixed-DUP and Loop-DUP have k_3 values that are the same within error (Table 1) and are comparable to a previously reported rate of product release when 8-oxoG is in a duplex and is paired to C [37]. These rates for k_3 are ~1,300-fold slower than those reported for k_2 in this and previous work [28]. Thus, these data support the notion that the rate-limiting step in the enzymatic cycle for the removal of 8-oxoG by hOGG1 from both non-repetitive and TNR duplex DNA substrates is product release. It has been proposed

that this lingering of glycosylases with their product ensures the proper cascade of events in BER and prevents the exposure of an AP site [9, 10].

When compared to Mixed-DUP and Loop-DUP, where the 8-oxoG lesion is found in the center of the duplex, the k_3 obtained for both Stem-DUP and Stem-HP is ~2-fold faster. This result indicates that hOGG1 releases the Stem-DUP and Stem-HP products more quickly than the Mixed-DUP and Loop-DUP products and reveals that the enzyme has a lower affinity for the product of the reaction obtained with Stem-HP and Stem-DUP. In both of these substrates the 8-oxoG is positioned 5 base pairs from one end of the substrate. It has previously been shown for duplex substrates [14, 37] that the distance of an 8-oxoG from the end of a duplex can influence the catalytic activity of hOGG1 and, therefore, it is likely that the affinity of the enzyme for both the substrates and products is also affected.

Using the same concentrations of hOGG1 (5 nM) and DNA (100 nM) as for the other substrates, a value for k_3 could not be obtained for Loop-HP even after incubation for 8 h. Only a small amount of product accumulation was observed and no burst or linear phase could be identified (Supplementary Fig. 5). Given the transient nature of the hOGG1•Loop-HP interaction, experiments were also performed in which the concentration of Loop-HP was increased to 500 nM. Again, after 24 h only a small amount of product was observed and no discernable burst or linear phase was identified (Supplementary Fig. 6). Notably, although a k_3 could not be determined, a k_2 for the Loop-HP substrate was obtained, albeit 800 times slower than for Loop-DUP. It has been shown that when 8-oxoG is paired with G in a duplex, a value for k_2 could be obtained for hOGG1, but due to very slow turnover k_3 could not be attained [37]. Therefore, 8-oxoG in a loop of a hairpin or 8-oxoG paired to a G in a duplex is a poor substrate for hOGG1. When present in excess over hOGG1 these substrates reduce the rate of turnover to such an extent that a minimal amount of substrate is converted to product.

3.6. Rate of product release (k_3) by hOGG1 in the presence of APE1

APE1 is the enzyme that follows hOGG1 in the BER cascade. APE1 has been shown to improve the efficiency of repair by stimulating the product release rate of OGG1 [15, 16, 42, 43]. For a mixed-sequence duplex containing 8-oxoG, the rate of product release of hOGG1 was stimulated 5 to 8-fold in the presence of 10 molar equivalents of APE1 [15, 43]. Given that APE1 is known to influence the product release rate of hOGG1 we also determined the k_3 for hOGG1 in the presence of APE1.

We found that the k_3 of hOGG1 acting on Mixed-DUP is ~9-fold faster in the presence of 10 molar equivalents of APE1 (Table 1, Fig. 5A). The k_3 for the TNR duplex Loop-DUP was stimulated ~3.5-fold in the presence of APE1 (Fig. 5B). This difference in amount of stimulation for the two duplex substrates suggests that while sequence context does not influence k_2 or k_3 of hOGG1, the ability of APE1 to stimulate product release of hOGG1 may be modulated by sequence. Furthermore, consistent with the observation that proximity of 8-oxoG to the end of a duplex influences the catalytic activity of hOGG1, we find that APE1 is similarly influenced and for Stem-DUP the k_3 of hOGG1 is stimulated ~2-fold in the presence of APE1 (Fig. 5C).

For the hairpin substrate Stem-HP, the addition of APE1 does not stimulate product release of hOGG1 (Fig. 5D); the k_3 is the same in the absence and presence of APE1. This lack of stimulation may be a result of the pseudo-duplex nature of Stem-HP. The presence of G-C base pairs and A•A mismatches in Stem-HP result in a stem that is more dynamic than a well-matched duplex. Indeed, we have shown through structural characterizations of CAG repeat hairpins [28] that adenines in the A•A mismatches are more reactive towards the chemical probe diethyl pyrocarbonate than adenines in A-T bp, indicating increased

dynamics. This increased dynamics may contribute to the lack of stimulation of hOGG1 by APE1 observed for Stem-HP. Lastly, even with the addition of APE1, only a small amount of product accumulated for Loop-HP. No burst or linear phase could be identified and, therefore, a value for k_3 could not be obtained.

3.7. A stable hOGG1•DNA complex can be trapped for all hairpin and duplex substrates

During the excision of 8-oxoG by hOGG1 a Schiff base intermediate is formed between the active site lysine and C1' of the nucleoside. This intermediate can be intercepted by the reducing agent NaBH₄ to generate a stable covalent hOGG1•DNA complex [35]. The complex can be separated from unbound DNA substrate by SDS-PAGE. Two key aspects of this covalent trapping experiment are (1) a covalent hOGG1•DNA complex can only be formed if hOGG1 progresses down the catalytic pathway and utilizes a nucleophilic residue to initiate the *N*-glycosidic bond cleavage and (2) this covalent trapping can reveal hOGG1•DNA interactions that are too weak and/or transient to be observed by a standard EMSA.

We performed NaBH₄ trapping experiments with wild-type hOGG1 and each of the DNA substrates. Indeed, all five DNA substrates could be trapped in a complex with the enzyme (Fig. 6). Approximately 45–75% of Stem-DUP, Stem-HP, Loop-DUP, or Mixed-DUP can be trapped in a complex with hOGG1. Loop-HP is trapped least efficiently with ~5% of the substrate observed in a complex with hOGG1. These results are consistent with the K_D , k_2 , and k_3 results obtained for Loop-HP and support our conclusion that a hairpin substrate with 8-oxoG in the loop is a poor substrate for hOGG1.

3.8. Implications for TNR expansion and a toxic oxidation cycle

DNA glycosylases initiate the BER pathway by recognition and excision of a damaged base, and are followed in action by downstream proteins and particular polymerases which incorporate unmodified bases in place of the damaged DNA [8, 11, 12]. Such repair events typically help to maintain genetic stability by minimizing mutations that occur due to errors in DNA replication. However, the presence of OGG1 has been implicated in the disease-initiating CAG/CTG repeat expansion [7]. Indeed, it has been demonstrated that if BER is initiated by hOGG1 on TNR duplex DNA, pol β ultimately catalyzes the addition of excess TNR repeats [7, 20]. Therefore, hOGG1 contributes to TNR expansion by initiating BER on damage-containing duplex DNA.

Here, by determining the dissociation constant, the rate of *N*-glycosidic bond cleavage, and the rate of product release of hOGG1 for TNR duplex DNA, we provide a comprehensive description of the kinetic parameters for the initiating event in TNR expansion. With respect to duplex substrates, we found that the kinetic parameters describing hOGG1 activity on CAG/CTG sequences and mixed-sequences are indistinguishable when the 8-oxoG is located the same distance from the end of the substrate. These results indicate that hOGG1 is not more or less likely to initiate BER on TNR duplexes than on non-repetitive sequences, but rather processes these substrates with the same efficiency. While the ability of hOGG1 to excise 8-oxoG was found to be influenced by the proximity of the damage to the end of the duplex, the processing of genomic DNA is likely not affected by this property of hOGG1 since most damage will not be located near a double-stranded end. It is of note that although product release by hOGG1 is stimulated by APE1 on both TNR and mixed-sequence duplexes the effect is larger for mixed-sequence duplexes.

Due to our previous studies that identified hairpin TNR structures as containing hot spots for oxidative damage, we were also motivated to examine the ability of hOGG1 to initiate BER on such DNA substrates. We describe here that these non-B conformations are poor

substrates for hOGG1. It is noteworthy that although hOGG1 can process these hairpin substrates to a small extent, the enzyme-DNA interaction is transient in nature. This transient interaction provides insight into the differential catalytic activity of hOGG1 observed for hairpin substrates relative to duplex substrates and suggests that hairpins are not biologically relevant substrates for hOGG1. Additionally, this data suggests it is unlikely that hOGG1 contributes to stabilization of a hairpin during a repair or replication-dependent expansion mechanism.

Taken together, the data presented in this work provide further insight into the mechanistic pathway by which hOGG1 and BER contribute to a toxic oxidation cycle (Fig. 7). We have shown here that the first step of this pathway, initiation of BER by hOGG1 on duplex DNA, occurs with high efficiency regardless of sequence context. More simply, the TNR repeats do not decrease the likelihood for this process to occur. We determined previously that hairpins contain hot spots for oxidative damage, particularly in the loop region that is solvent exposed. Importantly, it is these same hairpin conformations that are proposed to form during LP-BER mediated TNR expansion. *Therefore the likelihood of DNA oxidation within a TNR region is greatly increased during the process that is intended to remove oxidative damage.* Here, we provide a full kinetic analysis of hOGG1 activity on hairpin substrates containing 8-oxoG and demonstrate that the probability that oxidative damage is removed from hairpins is dramatically reduced relative to duplex. In fact, we were unable to measure K_D or k_3 for hOGG1 in the presence of a hairpin containing an 8-oxoG lesion in the loop due to the transient interaction of hOGG1 with the substrate and product of the glycosylase reaction, respectively. Thus, we proposed the addition of a step to the toxic oxidation cycle in which 8-oxoG forms and accumulates in the hairpin intermediate. This hairpin DNA is ligated and incorporated into duplex resulting in an expanded duplex, which now contains oxidative damage, poisoning the system to begin the toxic oxidation cycle again.

4. Conclusions

In this study we performed a full kinetic analysis of hOGG1 acting on TNR duplex and hairpin substrates and report that the formation of a hairpin intermediate during LP-BER exacerbates a toxic oxidation cycle. This hairpin intermediate is hyper-susceptible to oxidative damage and is a poor substrate for hOGG1. Thus, damage is expected to accumulate in the hairpin. As part of the toxic cycle the hairpin intermediate is incorporated into duplex and therefore, a damage-containing expanded TNR tract is generated. Lastly, we demonstrated that the TNR repeats do not affect hOGG1 binding, catalytic activity, or product release rate for duplex substrates and only minimally effect the ability of APE1 to stimulate the turnover of hOGG1. Therefore, the cycle can be readily initiated again by hOGG1 to further expand the repeat region.

Highlights

> OGG1 has been implicated in trinucleotide repeat expansion. > The activity of OGG1 is not influenced by DNA sequence context. > DNA structure does modulate activity of OGG1. > This structure-dependent activity can lead to an accumulation of DNA damage in trinucleotide repeat sequences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

8-oxoG	8-oxo-7,8-dihydroguanine
AP	apurinic
APE1	apurinic endonuclease 1
BER	base excision repair
BSA	bovine serum albumin
DMT	dimethoxytrityl
EDTA	ethylenediaminetetraacetic acid
FEN1	flap endonuclease 1
HD	Huntington's disease
hOGG1	human 8-oxo-7,8-dihydroguanine glycosylase
LigIII	DNA ligase III
OGG1	8-oxo-7,8-dihydroguanine glycosylase
PAGE	polyacrylamide gel electrophoresis
pol β	polymerase β
T_m	melting temperature
TNR	trinucleotide repeat
Tris	tris(hydroxymethyl)aminomethane

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Fig. 1. Schematic illustrations of lesion-containing DNA substrates used in this work. Shown first is (A) guanine and 8-oxo-7,8-dihydroguanine (8-oxoG) followed by (B) mixed-sequence duplex with a centrally located 8-oxoG (Mixed-DUP), (C) (CAG)₁₀ hairpin with 8-oxoG in the stem (Stem-HP), (D) duplex obtained by base pairing Stem-HP to (CTG)₁₀ (Stem-DUP), (E) (CAG)₁₀ hairpin with 8-oxoG in the loop (Loop-HP) and (F) duplex obtained by base pairing Loop-HP to (CTG)₁₀ (Loop-DUP). The asterisk represents the location of the ³²P-radiolabel.

Fig. 2.

Minimal kinetic scheme used for analysis of hOGG1 activity. (DNA)_S and (DNA)_P indicate DNA substrate and product, respectively.

Fig. 3.

Representative EMSA data for determination of K_D . (A) Autoradiogram revealing binding of K249Q hOGG1 to Loop-DUP and (B) a graph of percent DNA bound versus $\log[K249Q \text{ hOGG1}]$ with sigmoidal fit. Conditions were 1.5 nM DNA, 20 mM Tris-HCl, 10 mM Na_2EDTA , 14 mM NaCl, 6.7% glycerol, 100 $\mu\text{g/mL}$ BSA, pH 7.6 with 0–500 nM K249Q hOGG1.

Fig. 4.

Graph of concentration of product as a function of time obtained under single-turnover conditions. Results for (A) Mixed-DUP and (B) Loop-DUP are shown and were obtained using a RQF instrument. Conditions were 20 nM DNA, 100 nM hOGG1, 20 mM Tris-HCl, 10 mM Na₂EDTA, 35 mM NaCl, 155 μ g/mL BSA, pH 7.6.

Fig. 5. Data obtained under multiple-turnover conditions for determination of k_3 . Shown is a graph of concentration of product as a function of time for (A) Mixed-DUP, (B) Loop-DUP, (C) Stem-DUP, and (D) Stem-HP. Conditions were 50 nM DNA, 5 nM hOGG1 or 5 nM hOGG1/50 nM APE1 in 20 mM Tris-HCl, 70 mM NaCl, 2 mM MgCl₂, 100 µg/mL BSA, pH 7.6.

Fig. 6.

Trapping of a hOGG1•DNA complex by NaBH₄. Shown is the autoradiogram for Stem-DUP, Stem-HP, Loop-DUP, Loop-HP, and Mixed-DUP. Conditions were 20 nM DNA, 500 nM hOGG1, 50 mM NaBH₄, 20 mM Tris-HCl, 10 mM Na₂EDTA, 35 mM NaCl, pH 7.6. Samples were incubated at 37 °C for 1 h.

Fig. 7.

Toxic oxidation cycle. Schematic representation of the 'toxic oxidation cycle' proposed previously [7] and characterized further in this work. (1) Genomic DNA is oxidized to generate 8-oxoG and (2) OGG1 and APE1 initiate BER by removing the oxidative lesion from duplex TNR DNA and cleaving the backbone at the AP site. (3) The resulting gap is repaired via LP-BER, producing a TNR flap that (4) is refractory to FEN1 and ligation occurs, trapping a hairpin in the duplex. (5) The resulting hairpin is highly susceptible to DNA damage in the loop region and the lack of efficient repair of this damage results in (6) it being incorporated into the expanded duplex. The final product of the cycle now contains DNA damage that will be recognized by OGG1. Therefore, the cycle is repeated and the DNA can be processed again, incrementally expanding the TNR tract of DNA.

Table 1**K_D, *k*₂ and *k*₃ Values Determined for hOGG1 with DNA Substrates**

Substrate	K _D (nM) ^a	<i>k</i> ₂ (min ⁻¹) ^a	<i>k</i> ₃ (min ⁻¹) ^a	
			- APE1	+ APE1 ^d
Mixed-DUP	17.1 ± 4.7	54 ± 11	0.040 ± .009	0.348 ± .033
Stem-DUP	21.7 ± 6.8	2.8 ± 0.17 ^c	0.093 ± .016	0.156 ± .027
Stem-HP	N.D. ^b	1.3 ± 0.11 ^c	0.077 ± .008	0.070 ± .012
Loop-DUP	10.0 ± 4.4	56 ± 2.2	0.046 ± .004	0.163 ± .038
Loop-HP	N.D. ^b	0.07 ± 0.004 ^c	N.D. ^e	N.D. ^e

^aError represents standard deviation obtained from a minimum of three experiments.^bNo K249Q hOGG1•DNA complex observed.^cValues from reference [28].^dAPE1 was present at 10 molar equivalents with respect to hOGG1.^eNo significant turnover observed over 24 h.