The critical active-site amine of the human 8-oxoguanine DNA glycosylase, hOgg1: direct identification, ablation and chemical reconstitution

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Background: Base-excision DNA repair (BER) is the principal pathway responsible for the removal of aberrant, genotoxic bases from the genome and restoration of the original sequence. Key components of the BER pathway are DNA glycosylases, enzymes that recognize aberrant bases in the genome and catalyze their expulsion. One major class of such enzymes, glycosylase/lyases, also catalyze scission of the DNA backbone following base expulsion. Recent studies indicate that the glycosylase and lyase functions of these enzymes are mechanistically unified through a common amine-bearing residue on the enzyme, which acts as both the electrophile that displaces the aberrant base and an electron sink that facilitates DNA strand scission through imine (Schiff base)/conjugate elimination chemistry. The identity of this critical amine-bearing residue has not been rigorously established for any member of a superfamily of BER glycosylase/lyases.

Results: Here, we report the identification of the active-site amine of the human 8-oxoguanine DNA glycosylase (hOgg1), a human BER superfamily protein that repairs the mutagenic 8-oxoguanine lesion in DNA. We employed Edman sequencing of an active-site peptide irreversibly linked to substrate DNA to identify directly the active-site amine of hOgg1 as the ε-NH_2 group of Lys249. In addition, we observed that the repair-inactive but recognition-competent Cys249 mutant (Lys249→Cys) of hOgg1 can be functionally rescued by alkylation with 2-bromoethylamine, which functionally replaces the lysine residue by generating a γ-thia-lysine.

Conclusions: This study provides the first direct identification of the active-site amine for any DNA glycosylase/lyase belonging to the BER superfamily, members of which are characterized by the presence of a helix–hairpin–helix–Gly/Pro–Asp active-site motif. The critical lysine residue identified here is conserved in all members of the BER superfamily that exhibit robust glycosylase/lyase activity. The ability to trigger the catalytic activity of the Lys249→Cys mutant of hOgg1 by treatment with the chemical inducer 2-bromoethylamine may permit snapshots to be taken of the enzyme acting on its substrate and could represent a novel strategy for conditional activation of catalysis by hOgg1 in cells.

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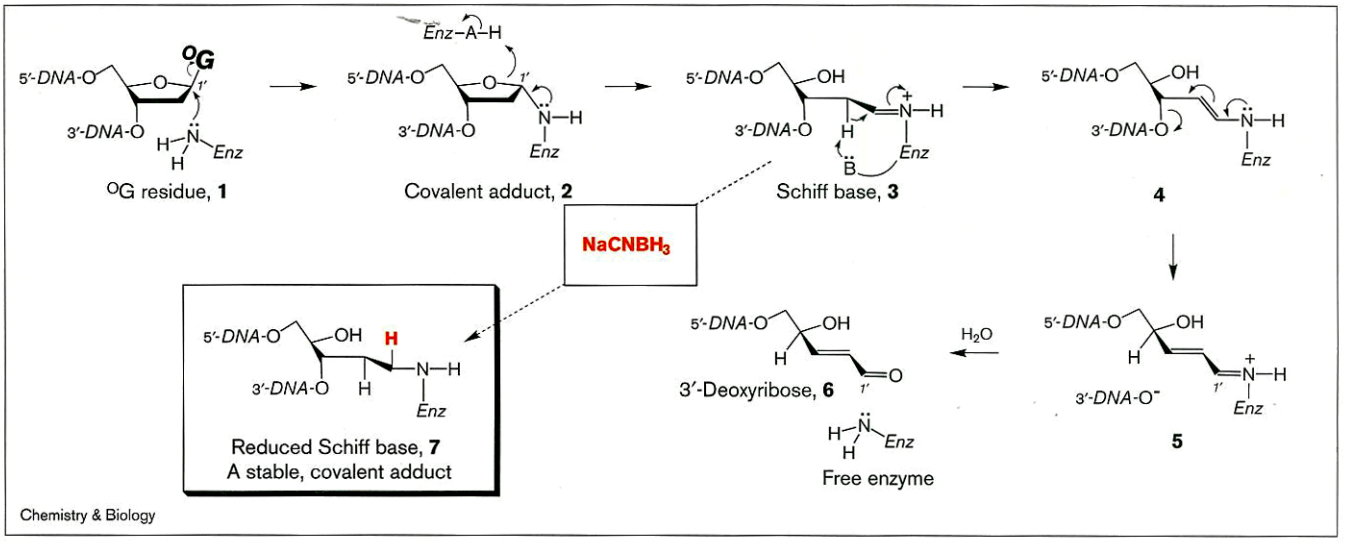
Introduction

Base-excision DNA repair (BER) is a cellular pathway dedicated to the removal of damaged nucleobases from the genome [1]. Lesions generated as the result of diverse DNA-damaging events, such as alkylation, oxidation, hydrolysis, ultra-violet photolysis, γ-irradiation and mis-replication are all subject to eradication via this pathway. BER is initiated by DNA glycosylases, enzymes that recognize aberrant bases in DNA and catalyze their expulsion through glycosidic-bond cleavage. The abasic DNA product thus generated is further processed through a multienzyme cascade, ultimately resulting in restoration of the original DNA sequence [2]. Consistent with the key genoprotective role served by DNA glycosylases, inactivation of the genes encoding these enzymes can render cells profoundly sensitive toward particular DNA-damaging agents and unusually susceptible to spontaneous mutagenesis [3–7].

Because each DNA glycosylase recognizes a particular set of lesions, all free-standing organisms express multiple enzymes in order to protect themselves broadly against DNA-base damage. Known DNA glycosylases fall into two distinct mechanistic classes: *monofunctional DNA glycosylases*, which catalyze only hydrolysis of the glycosidic bond; and *DNA glycosylase/lyases*, bifunctional enzymes that catalyze

Figure 1



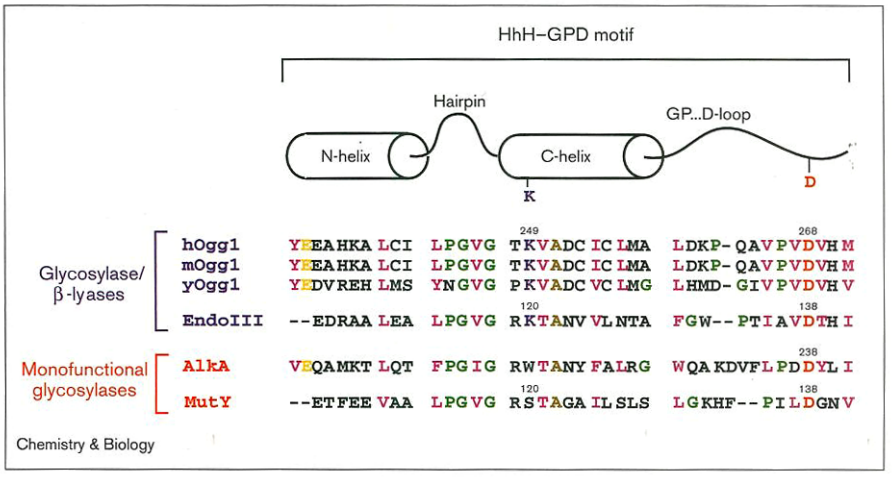
Unified mechanism for the glycosylase and β-lyase activities of the Ogg1 proteins, and mechanism for the borohydride-dependent formation of a stable, covalent protein–DNA complex (adapted from [18]). ^{O}G refers to 8-oxoguanine, *Enz* denotes the Ogg1 enzyme, B refers to a basic residue and A refers to an acidic residue on the Ogg1 enzyme. Attack of an amine nucleophile (*Enz*–NH_2) of the Ogg1 enzyme on the glycosidic bond of **1** expels the base and forms the covalently linked enzyme–substrate aminal intermediate, **2**. Facile rearrangement of **2** generates the Schiff base, **3**, which bears an acidified 2′-H. Enzyme-assisted abstraction of the 2′-H yields the enamine, **4**, which then undergoes conjugate elimination to cleave the 3′-C–O bond, thereby breaking the DNA backbone on the 3′-side of the original lesion. The α,β-unsaturated imine thus generated, **5**, undergoes hydrolysis to liberate the free enzyme and the α,β-unsaturated aldehyde, **6** (or a hydrated form thereof). $NaCNBH_3$ [17] can intercept the imine intermediate, **3**, resulting in the formation of an irreversible enzyme–DNA cross-link, **7**.

cleavage of the glycosidic bond, followed by scission of the DNA backbone; it is unknown whether this mechanistic distinction *per se* impacts upon the biology of DNA repair. The simple reaction chemistry catalyzed by monofunctional glycosylases — delivery of an activated water molecule to the glycosidic bond of the substrate — has been relatively straightforward to reconcile with structural [8–12] and biochemical information; by contrast, the complex, multistep reaction catalyzed by DNA glycosylase/lyases has presented a difficult mechanistic challenge, despite the availability of X-ray crystallographic structures [13–16].

One of the most critical issues concerns the extent to which the glycosylase and lyase functions of DNA glycosylase/lyases are mechanistically coupled. A breakthrough on this front was the proposal of Lloyd and coworkers [17,18], which draws a fundamental connection between these two catalytic functions: in the mechanism, an amine nucleophile on the enzyme (represented as a lysine ε-NH_2 group in Figure 1) attacks the glycosidic bond, displacing the aberrant base and forming an intermediate in which the enzyme is covalently attached to the 2′-deoxyribose moiety of the DNA substrate. Isomerization of the aminal intermediate thus produced generates an imine (Schiff base), which promotes abstraction of the 2′-H and cleavage of the 3′-C–O bond through a conjugate elimination mechanism [19–21]. Of the numerous lines of evidence supporting this mechanism, the strongest and most pertinent to the present study is the observation that glycosylase/lyases become irreversibly cross-linked to their substrates when the DNA repair reaction is carried out in the presence of borohydride [17], which effects reduction to a stable amine (Figure 1). This is consistent with the interception of a transient imine intermediate by a hydride ion. Efficient borohydride-dependent trapping of DNA glycosylases to their substrates has now been demonstrated in numerous instances [22–26] and, as such, it amounts to a diagnostic test for distinguishing glycosylase/lyases from monofunctional glycosylases. Borohydride trapping has also been of tremendous practical value in facilitating the isolation and characterization of eukaryotic DNA glycosylase/lyases [24–27].

A central question in DNA glycosylase/lyase action, then, is the identity of the enzymic amine that acts both as catalytic nucleophile and Schiff-base donor. For two structurally unrelated glycosylase/lyases, bacteriophage T4 endonuclease V and *Escherichia coli* MutM/Fpg, the catalytic amine has been rigorously determined as the amino terminus of the polypeptide mainchain [17,23]; the catalytic amine has not been determined unambiguously for any other glycosylase/lyase. Although these findings might be taken as suggesting that all glycosylase/lyases use their amino terminus as the catalytic amine, this appears not

Figure 2



Schematic representation of the helix–hairpin–helix–Gly/Pro–Asp (HhH–GPD) motif and amino-acid sequence alignment of the Ogg1 proteins with the bacterial DNA glycosylase/lyase endonuclease III (EndoIII) and the bacterial DNA glycosylases AlkA and MutY. Note the conserved lysine residue (purple) which is believed to act as the nucleophile that participates in the cleavage of the glycosidic bond by the bifunctional DNA glycosylase/lyases (Ogg1 and EndoIII). The conserved aspartate residue (red) is believed to activate the catalytic nucleophile. We have proposed that all six proteins shown share a common fold as members of a larger base-excision DNA repair (BER) superfamily of proteins [25]. The high-resolution X-ray crystal structures of EndoIII and AlkA have been solved and they are, therefore, structural prototypes of the BER protein superfamily [11,16]. N-helix, amino-terminal α-helix; C-helix, carboxy-terminal α-helix. Purple, conserved lysine; red, conserved aspartic acid; green, glycine or proline; pink, conserved hydrophobic residue; gold, conserved alanine; yellow, conserved glutamic acid.

to be the case, as radical amino terminal modifications of other glycosylase/lyases have little or no effect on their activity (H.M.N. and G.L.V., unpublished observations; [7,28–31]). Instead, recent evidence suggests that most DNA glycosylase/lyases use the ε-NH_2 group of an as yet undetermined internal lysine as the catalytic nucleophile. The X-ray crystallographic structure of the monofunctional glycosylase AlkA [11,12] unexpectedly revealed a remarkably similar overall fold to that of the glycosylase/lyase endonuclease III (EndoIII) [15]; the similarity between the two proteins was found to be especially striking within a region that in both had been independently pinpointed as the enzyme active site [11,12,16]. This region comprises an α-helix–hairpin–α-helix (HhH) motif, a supersecondary element that may represent a general module for recognition of non-canonical DNA structures [16,32]. Upon cloning of the yeast 8-oxoguanine (^{O}G) DNA glycosylase, yOgg1, it was further recognized that DNA glycosylases related in structure to EndoIII and AlkA contain, in addition to the HhH motif, a glycine/proline-rich loop terminated by an invariant, catalytically essential aspartate residue. The presence of this HhH–GPD motif (Figure 2) in numerous such enzymes strongly suggested the existence of a superfamily of DNA glycosylase enzymes, members of which share a common overall fold, active-site location and invariant catalytic aspartate residue [25]. Despite the fact that the majority of known DNA glycosylases, including most notably a number of eukaryotic proteins cloned in the past year [7,25,26,28–31,33–36], belong to this BER superfamily, relatively little is known about how these proteins recognize or process their substrates. It has been noted that a lysine residue within the HhH–GPD motif is conserved in all members of the BER superfamily known to possess efficient lyase activity (Lys249 in hOgg1; Figure 2) and to be absent in all members that lack this activity. Mutation of this or the corresponding lysine in other BER superfamily glycosylase/lyases abrogates catalysis [16,25,26]. Such loss-of-function alterations do not, however, constitute a conclusive test of involvement in catalysis.

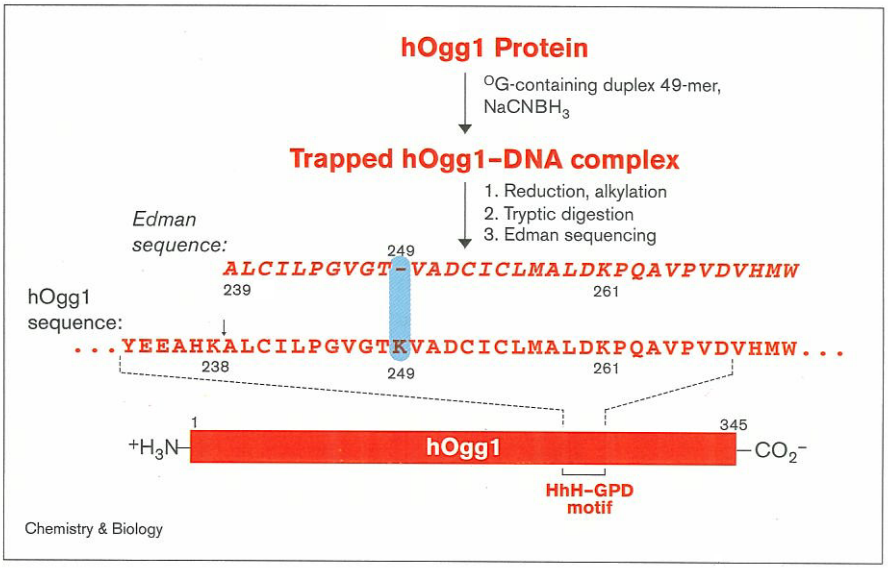
Here, we report the direct identification of Lys249 as the residue that becomes irreversibly attached to DNA upon borohydride trapping of hOgg1 to an ^{O}G-containing substrate. Mutants of hOgg1 having cysteine or glutamine at position 249 (Lys249→Cys or Lys249→Gln, respectively) lack detectable glycosylase or lyase activity. The Lys249→Cys mutant can be functionally rescued by treatment with 2-bromoethylamine, which presumably generates γ-thia-lysine *in situ* at position 249. Together, these data provide definitive evidence that the residue corresponding to Lys249 is the catalytic amine that acts as a nucleophile/Schiff-base N-donor in all BER superfamily glycosylase/lyases. The ability to induce chemically a gain-of-function in Lys249→Cys hOgg1 may be of substantial value in structural and functional studies of base-excision DNA repair.

Results

Direct identification of the ε-NH_2 group of Lys249 as the active-site amine of hOgg1

Our strategy for direct identification of the hOgg1 active-site amine (shown schematically in Figure 3) relies upon the premise that this amine becomes irreversibly linked to DNA upon borohydride trapping. Protease digestion of the trapped hOgg1–DNA complex followed by amino-acid sequencing of the resulting DNA-cross-linked hOgg1 peptide is, therefore, expected to reveal a single unidentifiable residue, which corresponds to the DNA-linked amino-acid residue. hOgg1 was incubated with a 49 base-pair oligonucleotide duplex containing a central ^{O}G:C base pair in

Figure 3



The active-site amine of hOgg1 is the ε-NH_2 group of the evolutionally conserved Lys249. The borohydride-trapped protein–DNA complex (**7**, Figure 1) was purified by anion exchange chromatography before reduction, alkylation, and exhaustive tryptic digestion (see Materials and methods section). Anion exchange chromatography was then used to separate the peptide–DNA complex from all other hOgg1 tryptic peptides and trypsin. Edman sequencing was performed for a total of 40 cycles, and no phenylthiohydantoin (PTH) amino acid derivative was observed at cycle 11 (Lys249). PTH identities were read with high confidence to cycle 34 (Trp272), after which no PTH-amino acid derivatives were detected. We presume that the apparent proteolytic cleavage at Trp272 resulted from chymotryptic activity under the aggressive tryptic digestion conditions used. For exact conditions, see Materials and methods section.

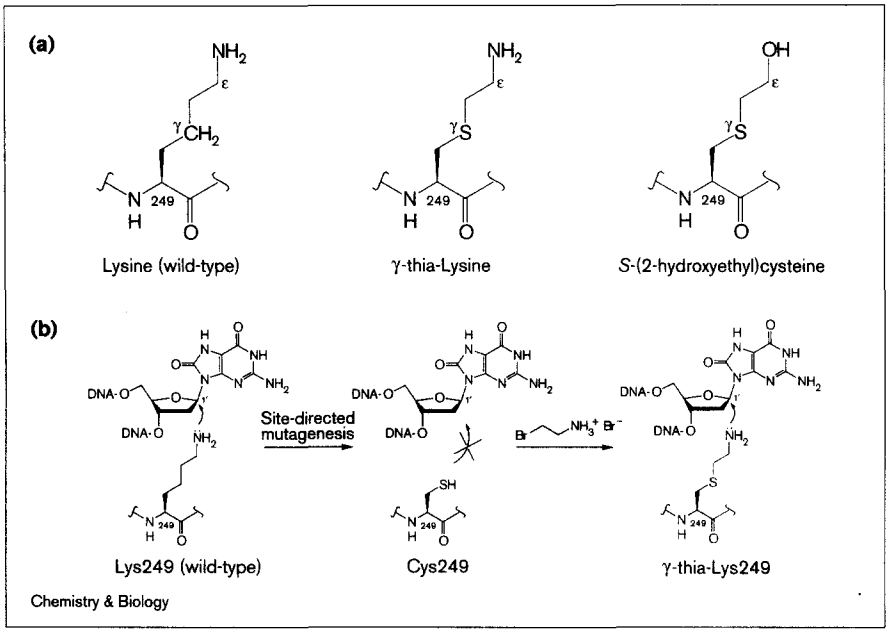
the presence of $NaCNBH_3$. The reaction mixture was then purified by anion exchange chromatography, which separated the unreacted protein and DNA from the trapped hOgg1–DNA complex. As this complex was found to be rather resistant to protease digestion, we exposed it to high concentrations of trypsin under partially denaturing conditions, and followed the course of the digestion by SDS-PAGE. After the reaction products largely coalesced into a single low-molecular-weight band on SDS-PAGE, we again subjected the reaction mixture to anion exchange chromatography, which separated the peptide covalently linked to DNA from other hOgg1-derived peptides and trypsin. Edman sequencing of the peptide–DNA complex yielded a readily assignable sequence except at one position, at which a phenylthiohydantoin (PTH) derivative was absent. Comparison of the Edman sequence with the cDNA-derived amino-acid sequence of hOgg1 (Figure 3) revealed that the active-site peptide comprised almost the complete HhH–GPD motif of hOgg1, and that Lys249 was missing from the Edman sequence. The failure to observe a PTH-Lys derivative at position 249 was not due to a general inability to detect PTH-Lys, as this derivative was clearly observed at position 261 in the sequence (the Edman sequence continues past Lys261, even though this is a potential tryptic site; trypsin is known to cleave inefficiently at Lys–Pro sequences). Observation of a normal PTH-Lys at position 261 unambiguously rules out Lys261 as the site of linkage to DNA. We thus conclude that the ε-NH_2 group of Lys249 is covalently cross-linked to DNA in the trapped hOgg1–substrate complex.

Reengineering the active-site amine of hOgg1

In a previous study of the mammalian Ogg1 proteins, we reported that replacement of Lys249 in murine Ogg1 (mOgg1) by Gln (Lys249→Gln) yields a mutant protein that lacks DNA glycosylase/lyase activity but retains ^{O}G-specific DNA-binding activity (electrophoretic mobility shift assay; EMSA [26]); similar observations have been reported in the case of EndoIII [16]. Because these studies involve an alteration of the protein that results in a loss-of-function, they cannot establish unambiguously that Lys249 participates directly in covalent catalysis. A more convincing case could be made if there existed a means to restore the activity of a catalytically inactive Lys249 mutant. An attractive possibility for accomplishing this is suggested by the work of Smith and Hartman [37] and others [38–40], which showed that lysine residues in proteins could be functionally replaced by γ-thia-lysine (Figure 4a). The γ-thia-lysine residue can be produced by alkylation of a cysteine residue using 2-bromoethylamine [41]. Substitution of the γ-CH_2 of lysine with a sulfur atom is nearly isosteric, although the thioether-containing sidechain is slightly longer and more conformationally mobile than the corresponding sidechain of lysine, and the ε-NH_2 group of γ-thia-lysine is slightly less basic (~1 pK_a unit lower) than that of lysine [42]. We thus reasoned that it might be possible to carry out a loss-of-function/restoration-of-function sequence in hOgg1 through mutation of Lys249 to Cys, followed by treatment of the resulting Lys249→Cys mutant protein with 2-bromoethylamine (Figure 4b). Two major concerns at the outset of these experiments were that the 2-bromoethylamine might not react efficiently with Cys249, or might react with the other eight cysteine residues in the protein and thereby inactivate it. To test this strategy, we generated the Lys249→Cys mutant of hOgg1 by site-directed mutagenesis. As a stringent test for the regiochemical effects of alkylation, we also generated a control loss-of-function mutant having

Figure 4

Sidechain derivatives and their use in re-engineering the active-site Lys249 of hOgg1. **(a)** Re-engineered sidechain derivatives of amino acid 249 studied in this work. The γ-thia-Lys249 sidechain is obtained by alkylation of Cys249 with 2-bromoethylamine hydrobromide, and the *S*-(2-hydroxyethyl)Cys249 sidechain is obtained by alkylation of Cys249 with 2-bromoethanol. **(b)** Strategy for reengineering the active-site Lys249 of hOgg1. Site-directed mutagenesis of Lys249 to Cys yields a repair-inactive Lys249→Cys mutant protein. Alkylation of Cys249 generates the unnatural lysine analog γ-thia-Lys249, which should lead to a gain of repair function.



a lysine to glutamine change at position 249 (Lys249→Gln); this protein should be incapable of rescue upon treatment with 2-bromoethylamine. We first confirmed by EMSA that both the Lys249→Cys and Lys249→Gln mutant proteins possessed affinities for ^{O}G-containing DNA that were comparable to that of the wild-type protein (Figure 5). We then incubated the Lys249 (wild-type), Lys249→Cys, and Lys249→Gln hOgg1 proteins with 2-bromoethylamine and compared the borohydride trapping (Figure 6) and DNA cleavage activities (Figure 7) of the untreated and treated proteins. As a further test for the requirement of the ε-NH_2 group for catalysis, we reacted the Lys249→Cys hOgg1 protein with 2-bromoethanol, which produces the corresponding sidechain bearing an ε-hydroxyl group (refer to Figure 4a).

Treatment of Lys249 hOgg1 with 2-bromoethylamine had no detectable effect on the borohydride-trapping activity (Figure 6, compare lanes 1 and 2), or β-lyase activity (Figure 7, compare lanes 3 and 4) of wild-type hOgg1, indicating that exposure of the protein to 2-bromoethylamine does not result in an impairment of protein function. On the other hand, whereas the Lys249→Cys mutant of hOgg1 lacked detectable borohydride trapping or β-lyase activity, both of these activities were clearly evident when the protein was treated with 2-bromoethylamine (compare lane 5 with 6 in Figure 6 and lane 7 with 8 in Figure 7). Functional rescue must result from alkylation of Cys249 to generate γ-thia-Lys249, as no gain in activity was observed upon 2-bromoethylamine treatment of the Lys249→Gln mutant protein (Figure 6, lanes 3 and 4; Figure 7, lanes 5

Figure 5

Electrophoretic mobility shift assay (EMSA) of the wild-type Lys249, Lys249→Cys and Lys249→Gln hOgg1 proteins confirmed that all three hOgg1 proteins possessed comparable affinities for ^{O}G-containing DNA. Mutation of Lys249 did not significantly affect the non-covalent binding of hOgg1 to substrate DNA. For exact reaction conditions, see Materials and methods section.

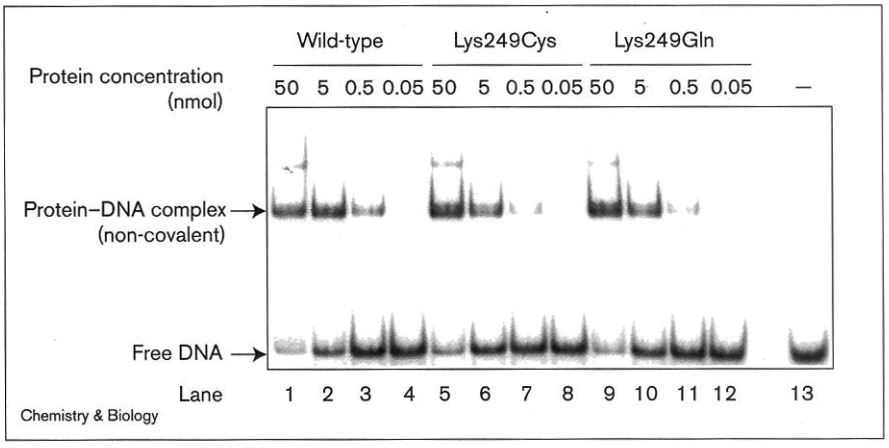
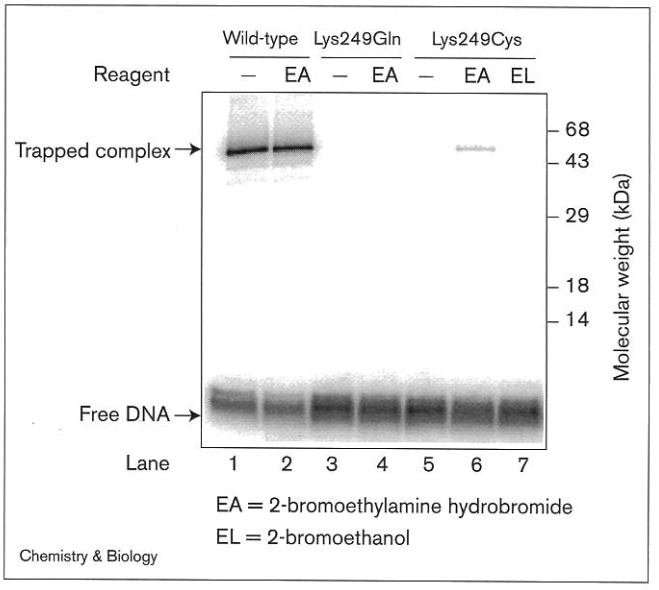


Figure 6



Borohydride trapping analysis of wild-type and mutant hOgg1 proteins revealed a modest, but significant, gain of repair function with the Lys249→Cys mutant protein only after alkylation with 2-bromoethylamine (compare lanes 5 and 6). Treatment of the wild-type hOgg1 protein did not lead to a decrease in activity (compare lanes 1 and 2). No gain of repair function was observed upon pretreatment of the Lys249→Cys protein with 2-bromoethanol (lane 7), and no repair function was observed for the Lys249→Gln mutant protein either before or after alkylation with 2-bromoethylamine (lanes 3 and 4, respectively). –, no alkylation; EA, alkylation with 25 mM 2-bromoethylamine hydrobromide (pH 8, room temperature, 1 h); EL, alkylation with 25 mM 2-bromoethanol (pH 8, room temperature, 1 h). For exact reaction conditions, see Materials and methods section. Molecular weights were assigned by comparison with ^{14}C-labeled protein standards.

and 6). It is apparent from Figures 6 and 7 that treatment of Lys249→Cys hOgg1 with 2-bromoethylamine did not restore the enzyme activity to wild-type levels. In principle, this partial restoration could result from incomplete alkylation or a decrease in catalytic efficiency of the γ-thia-Lys249 hOgg1 relative to wild-type, or both; our data do not permit us to distinguish these possibilities. A determined attempt to optimize the alkylation reaction conditions at this stage has not yet been made.

Finally, the amine group of γ-thia-lysine is clearly required for functional rescue to occur, as treatment of Lys249→Cys hOgg1 with 2-bromoethanol resulted in no detectable gain of trapping (Figure 6, lane 7) or β-lyase activity (Figure 7, lane 9). It is possible that the *S*-(2-hydroxyethyl)Cys249 protein carries out base excision to form an enzyme–substrate acetal adduct, but then is incapable of undergoing the sugar-ring opening rearrangement (Figure 1). This possibility was ruled out on the basis that no protein–DNA cross-link was observed when the 2-bromoethylamine-treated Lys249→Cys protein was incubated with ^{O}G substrate and the mixture was analyzed under mild denaturing conditions (data not shown).

Discussion

The catalytic machinery of BER superfamily glycosylases

The BER superfamily [25] makes up a large and important class of proteins that serve a key role as DNA damage sensors and excisors, initiating the overall BER process [2]. All members of this superfamily contain a conserved aspartate residue, located at the carboxy-terminal end of the HhH–GPD motif (Asp268 in hOgg1), which has been proposed to deprotonate and thereby activate the nucleophile that attacks the glycosidic bond [11]. In the case of monofunctional glycosylases of the BER superfamily, the attacking nucleophile is most likely to be a tightly bound water molecule [11,18]. Although the reaction is certainly more complicated for the glycosylase/lyase class of BER superfamily enzymes, the nucleophilic activation step has been proposed to be similar to that of monofunctional glycosylases, except that instead of deprotonating water, the invariant aspartate residue of these enzymes deprotonates an amino group of the enzyme [11,25]. This same amino group has been proposed to promote the lyase reaction by acting as an electron sink for imine/enamine chemistry (Figure 1). Thus there has been significant interest in the identity of this amine, a key catalytic component that differentiates the two major classes of BER superfamily enzymes. The present studies resolve this issue.

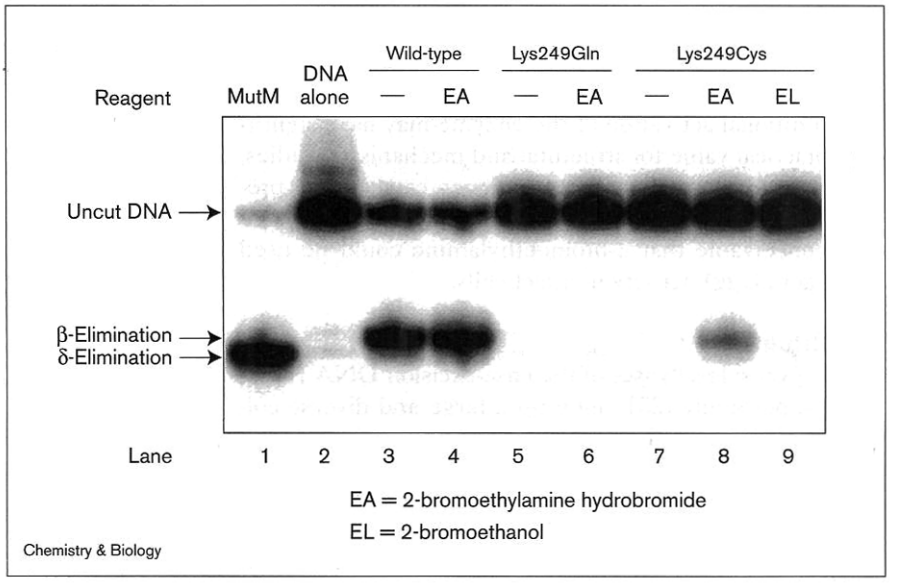
The ε-NH_2 group of Lys249 is the active-site amine of hOgg1

Here, we have taken two complementary approaches to identify conclusively the active-site amine of hOgg1, a glycosylase/lyase of the BER superfamily. In the first approach, we isolated an active-site peptide that had become directly and irreversibly bound to substrate DNA through borohydride trapping of hOgg1 to an ^{O}G-containing duplex. Edman sequencing of this peptide revealed the disappearance of a single amino acid, which was located at the position corresponding to Lys249. We thus conclude that Lys249 of this peptide is irreversibly linked to DNA. The second approach involved a chemically induced restoration of enzymatic activity by introducing a close structural analog of the active-site amine. Thus we showed that whereas mutation of Lys249 to cysteine resulted in complete loss of catalytic activity, treatment of the mutant protein with 2-bromoethylamine restored enzyme activity to a significant extent, by inducing the formation of γ-thia-lysine at position 249. Together, these data provide a compelling case for the sidechain ε-amino group of Lys249 being the catalytic amine of hOgg1.

The active-site amine of BER superfamily glycosylase/lyases

The sequence similarity between hOgg1 and other proteins of the BER superfamily strongly suggest that these proteins contain a similar overall fold [26]. Moreover, because all BER superfamily members possess an active-site motif

Figure 7



DNA-cleavage analysis of wild-type and mutant hOgg1 proteins revealed a modest, but significant, gain of DNA glycosylase/β-lyase activity with the Lys249→Cys mutant protein only after alkylation with 2-bromoethylamine (compare lanes 7 and 8). Treatment of the wild-type hOgg1 protein did not lead to a decrease in cleavage activity (compare lanes 3 and 4). No gain of cleavage activity was observed upon pretreatment of the Lys249→Cys protein with 2-bromoethanol (lane 9), and no cleavage activity was observed for the Lys249→Gln mutant protein either before or after alkylation with 2-bromoethylamine (lanes 5 and 6, respectively). Note that the DNA-cleavage efficiencies paralleled the borohydride-trapping efficiencies observed in Figure 6; lanes 1–7 of Figure 6 correspond to lanes 3–9 of Figure 7. Comparison of the cleavage products obtained by reaction of ^{O}G·C DNA with either wild-type hOgg1 or the bacterial ^{O}G DNA glycosylase/β,δ-lyase MutM indicated that hOgg1 possesses an efficient β-lyase activity, but lacks a detectable δ-lyase activity. –, no alkylation; EA, alkylation with 25 mM 2-bromoethylamine hydrobromide (pH 8, room temperature, 1 h); EL, alkylation with 25 mM 2-bromoethanol (pH 8, room temperature, 1 h). For exact reaction conditions, see Materials and methods section.

including an invariant, catalytically essential aspartate residue at a similar position in their sequence, it seems likely that these enzymes will possess active sites located similarly within a conserved core fold [11]. Indeed, this core fold has already been observed in at least three proteins that bear little in common apart from having DNA-repair activity and an HhH–GPD motif: *E. coli* EndoIII [15], *E. coli* AlkA [11,12], and the DNA repair domain of human DNA polymerase β [43]. Furthermore, all BER superfamily members known to possess potent glycosylase/lyase activity have a lysine residue at the same position within their HhH–GPD motif as Lys249 of hOgg1 and, conversely, all BER superfamily members that act as monofunctional glycosylases possess a residue other than lysine at this position [25]. This being the case, it seems almost certain that the residue corresponding to Lys249 in hOgg1 is the catalytic amine of all BER superfamily glycosylase/lyases. Thus, prior speculations of the identity of the catalytic amine of BER superfamily glycosylase/lyases are substantiated by the present experimental results [11,16, 22,25,28,29]. In the X-ray crystallographic structure of EndoIII, the postulated catalytic lysine residue (Lys120) projects into a cavity that also contains at its floor the invariant aspartate residue [16]; simple rotations of side-chain dihedral angles allow the aspartate to be positioned close enough to enable it to deprotonate the lysine, as proposed to precede attack on the glycosidic bond [11]. An intriguing mechanistic question is whether it will be possible to reengineer glycosylases into glycosylase/lyases through simple introduction of the active-site lysine or *vice versa*, or whether additional residues will be required (see below). If successful, such an engineering exercise could have a significant impact on our understanding of the biology of base-excision DNA repair, because a major unanswered question in this field concerns the biological relevance of having two mechanistically distinct ways of repairing DNA-base damage.

We mention parenthetically that the MutY protein of *E. coli* has been reported to possess lyase activity and to undergo borohydride trapping to its substrate [44]. This issue is controversial, however, because others have failed to observe such activities under seemingly similar conditions [22] and because the lyase and trapping activities of MutY appear to be inefficient, relative to other confirmed glycosylase/lyases. We note that MutY lacks the conserved lysine residue, which we have identified here as a critical feature of BER superfamily glycosylase/lyases (Figure 2), and instead possesses a serine at this position (Ser120). It is possible that the trapping and perhaps even lyase activities of wild-type MutY are epiphenomena that result from the presence of a high concentration of lysine residues on the DNA-binding surface of the protein, where the ε-NH_2 group of lysine could experience a chance encounter with the abasic site produced by the *bona fide* catalytic activity of the enzyme; small lysine-containing peptides have been shown to possess lyase activity on abasic sites in DNA [45].

Conditional activation of a mutant BER glycosylase/lyase

We have demonstrated the ability to switch a mutant glycosylase/lyase from an inactive state to an active state by

treatment with a small molecule reagent, 2-bromoethylamine. Importantly, similar treatment of the wild-type protein has no discernible effect on its catalytic activity, thus indicating that the reagent does not cause deleterious non-specific alkylation of the enzyme. This strategy for conditional activation of the enzyme may have significant practical value for structural and mechanistic studies, allowing, for example, one to trigger catalysis in preformed co-crystals of the mutant enzyme bound to DNA. It is conceivable that 2-bromoethylamine could be used to trigger hOgg1 activity in intact cells.

Significance

DNA glycosylase/lyases of the base-excision DNA repair BER superfamily [25] make up a large and diverse collection of enzymes that expel aberrant bases from DNA and catalyze scission of the DNA backbone. These two seemingly unrelated activities are apparently unified through a mechanism involving covalent catalysis, in which an amine-bearing residue on the enzyme acts both as a nucleophile to displace the aberrant base from its 2′-deoxyribose moiety and as an electron sink to facilitate conjugate elimination through imine (Schiff base) chemistry [18]. The identity of the critical amine residue on the enzyme has been the subject of much speculation, but to date it has not been identified directly.

In this study, we have determined through two independent assays that a lysine residue located within the HhH–GPD (helix–hairpin–helix–Gly/Pro–Asp) active-site motif of BER superfamily glycosylase/lyases is the catalytic amine of these proteins. Specifically, we found that Lys249 of the human 8-oxoguanine DNA glycosylase (hOgg1), a BER superfamily glycosylase/lyase, becomes irreversibly linked to DNA upon borohydride trapping of a Schiff base intermediate formed during normal enzymatic turnover. A lysine residue at the position corresponding to Lys249 is found in all BER superfamily glycosylases that exhibit robust lyase activity [25,26]. We also report that the glycosylase and lyase activities of hOgg1 can be inactivated by mutation of Lys249 to Cys, but, remarkably, both activities can be restored by treatment of the Lys249→Cys mutant with the alkylating reagent 2-bromoethylamine. We propose that this chemical inducer of hOgg1 function acts by alkylating the active-site cysteine to generate γ-thia-lysine, an isosteric analog of lysine [37,41]. This ability to chemically trigger the catalytic function of Lys249→Cys hOgg1, a mutant glycosylase/lyase that retains the ability to bind 8-oxoguanine lesions in DNA with high affinity, may permit high-resolution structural analysis of intermediates that lie along the hOgg1-catalyzed reaction pathway. Thus, in addition to providing fundamental insight into the catalytic machinery of a large class of important DNA-repair proteins, the present work suggests a way for snapshots to be taken of these enzymes in action.

Materials and methods

Oligonucleotide duplex substrates

All oligonucleotides were synthesized by β-cyanoethyl solid-phase chemistry on a 1 mmol scale using an ABI model 392 DNA synthesizer, and were purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE). Full-length DNA was eluted from the acrylamide gel by crushing and soaking in 1 M triethylammonium bicarbonate, pH 7, followed by C_{18} Sep-Pak concentration/desalting. Concentrations were determined by A_{260} quantitation, and radiolabeling reactions were performed with T4 polynucleotide kinase (New England Biolabs) and γ^{32}P-labeled ATP. Duplexes were prepared by annealing with a 50-fold molar excess of the complementary strand in 1 × TE/100 mM NaCl. The top, ^{O}G-containing strand of the 25-mer duplex (**9**; see below) was radiolabeled for the borohydride-trapping and DNA cleavage assays.

8-oxo-dG·dC 49-mer (**8**):

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5′-GGC AAG TCT GAT GGA TAG TGT CCA OG-3′→
3′-CCG TTC AGA CTA CCT ATC ACA GGT C-5′→
→5′-GTT ACT CGA AGC AGT TCG AAC TGG-3′
→3′-CAA TGA GCT TCG TCA AGC TTG ACC-5′
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8-oxo-dG·dC 25-mer (**9**):

```
5′-GGA TAG TGT CCA OG GTT ACT CGA AGC-3′
3′-CCT ATC ACA GGT C CAA TGA GCT TCG-5′
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The phosphoramidite of 8-oxo-dG was synthesized according to published procedures.

Formation and purification of the hOgg1 protein–DNA complex

An estimated 40 nmol of purified, recombinant hOgg1 protein was incubated with 30 nmol of ^{O}G·C 49-mer duplex **8** in the presence of 50 mM $NaCNBH_3$, 35 mM Tris pH 7.5, 70 mM KCl, 7 mM 2-mercaptoethanol, and 0.7 mM EDTA at 37°C for 1 h in siliconized microcentrifuge tubes. The reaction was centrifuged at 14,000 rpm at 4°C for 30 min and applied to a 1 ml MonoQ anion-exchange FPLC column (Pharmacia; pre-equilibrated with 100 mM KCl in 20 mM Tris pH 7.5). After washing the column with 10 ml 100 mM KCl in Tris pH 7.5, the trapped protein–DNA complex was eluted with a 30 ml gradient of 100 mM to 2 M KCl in 20 mM Tris pH 7.5, and 1 ml fractions were collected in siliconized microcentrifuge tubes. Fractions were assayed for the presence of the protein–DNA complex by 5′ end-labeling 2 μl aliquots of the column fractions and analyzing by 20% 200:1 SDS-PAGE. The protein DNA complex was observed to elute at 700 mM to 850 mM KCl, and complete separation of the protein–DNA complex from the free DNA was not obtained.

Tryptic digestion and Edman sequencing of the hOgg1 peptide–DNA complex

MonoQ fractions containing the majority of the protein–DNA complex (fractions 20–22, 3 ml total) were lyophilized by Speed Vac (Savant) and redissolved in a total of 500 μl CRA buffer (8 M urea, 400 mM ammonium bicarbonate). DTT was added to a final concentration of 2.5 mM and the mixture was heated at 60°C for 45 min. After cooling to room temperature, the cysteines of the reduced complex were alkylated with 5 mM iodoacetamide at room temperature for 30 min. The reaction mixture was diluted fourfold with double-distilled water to yield a final urea concentration of 2 M in a total volume of 2 ml, and $CaCl_2$ was added to a final concentration of 1 mM. 40 μg of trypsin (Promega, sequencing grade) was added and the complex was digested at 37°C for 84 h. At this time, SDS-PAGE analysis indicated that the tryptic digestion was still incomplete, and the partially digested peptide–DNA complexes were purified by MonoQ chromatography (30 ml gradient of 100 mM to 2 M KCl in 20 mM Tris pH 7.5, 1 M urea). The majority of the peptide–DNA complexes were observed to elute at 600–850 mM KCl, and these fractions were pooled and redigested with 80 μg trypsin. After 72 h at 37°C, the tryptic digest was judged by SDS-PAGE to be complete, and the active-site peptide–DNA complex was purified by MonoQ chromatography, using an elution gradient of 100 mM to 1 M

KCl in 20 mM Tris pH 7.5, 10% CH_3CN. The completely digested peptide–DNA complex was observed to elute at 700 mM KCl, and this 1 ml fraction was partially concentrated by Speed Vac lyophilization to a final volume of 200 µl. The final yield of purified peptide–DNA complex was 2 nmol, as determined by quantitative amino acid analysis. 200 pmols of purified peptide–DNA complex was applied to a Hewlett Packard G1000A Protein Sequencer equipped with an on-line 1090 HPLC. The peptide was submitted to standard Edman degradation using the manufacturer's ROUTINE 3.5 protocol. A sequence of ALCILPGVGT(–)VADCICLMALDKPQAVPVDVHMW was read with confidence, with the (–) denoting the absence of a PTH-Lys249 or any other uncharacterized amino acid derivative at cycle 11. We presume that termination at Trp272 resulted from chymotryptic activity under the aggressive tryptic digestion conditions used. We note that a reduced yield of the PTH-Lys261 was observed, with the difference corresponding to an uncharacterized derivative with a retention time similar, but not identical, to PTH-His. High-resolution mass spectroscopic analysis of peptides obtained by subdigest of the original peptide–DNA complex with aspartyl endopeptidase revealed that a fraction of the Lys261 residues possessed a modification of +43 Da (not shown).

Site-directed mutagenesis of hOgg1

The coding sequence of *hOgg1* was subcloned into the bacterial T7 expression vector pET-30a (Novagen) as a *Bam*HI/*Hin*dIII cassette, so as to express the protein as a fusion with an amino-terminal 6XHis tag. The resulting plasmid was transformed into CJ236 cells, and single-stranded uracil-containing DNA (coding strand) was obtained by transfection of these cells with M13-K07 helper phage. Site-directed mutagenesis reactions were performed by the Kunkel method with unmodified T7 DNA polymerase, T4 DNA ligase, and the 5′ phosphorylated primers listed below (the mutations are italicized):

Lys249Cys: 5′-GCA GTC AGC CAC *GCA* GGT GCC CAC TCC-3′

Lys249Gln: 5′-GTC AGC CAC *CTG* GGT GCC CAC-3′

The presence of the Lys249→Cys or Lys249→Gln mutation and the absence of any other undesired mutations was confirmed by DNA sequencing.

Overexpression and purification of wild-type and mutant hOgg1 proteins

Plasmids containing the wild-type and mutant *hOgg1* genes subcloned into pET-30a were transformed into BL21(DE3)pLysS MutM::Tn10 cells (H.M.N. and G.L.V., unpublished observations), and the cells were grown to $OD_{600} = 0.5$ under antibiotic selection (kanamycin, chloramphenicol, tetracycline) before protein expression was induced with 1 mM (final concentration) IPTG. Induced growth was continued for 3.5 h at 30°C, and the cells were pelleted at 4°C. All subsequent steps were performed at 4°C. The cells were lysed by French Press disruption in 4–5 cell volumes of lysis buffer (20 mM Tris, pH 7.5, 20% glycerol, 500 mM KCl, 10 mM 2-mercaptoethanol) and the lysates were clarified by centrifugation at 23,000 × g. Nucleic acids were removed by batchwise treatment of the crude lysates with 1/6 volume (preswollen) of DEAE cellulose (rotary mixing, 1 h). Imidazole was added to the DEAE supernatants to a final concentration of 5 mM, and 1/12 volume of preequilibrated TALON metal affinity resin (Clontech) was added. Binding to the TALON resin was performed in batch for 1 h with rotary mixing, and the resin was washed batchwise with 3 × 10 resin volumes of 5 mM imidazole lysis buffer before transfer to a disposable gravity-flow column. The TALON resin was washed in the column with 10 resin volumes of 5 mM imidazole lysis buffer, and protein was eluted with 10 resin volumes of 500 mM imidazole lysis buffer, collecting 1 ml fractions in siliconized microcentrifuge tubes. The majority of hOgg1 protein was observed to elute in the first four fractions. Proteins were dialyzed for 20 h at 4°C against three changes of storage buffer (20 mM Tris, 7.5, 100 mM KCl, 10 mM 2-mercaptoethanol, 50% glycerol), and were stored at −20°C in siliconized microcentrifuge tubes. Protein concentrations were calculated by Bradford assay, with BSA as the standard. Working dilutions of 0.04 mg/ml were made in storage buffer for each protein.

Electrophoretic mobility shift assay

Wild-type (Lys249), Lys249→Cys, and Lys249→Gln hOgg1 proteins were diluted to final concentrations of 50 nM, 5 nM, 0.5 nM and 0.05 nM in 20 µl of 1 × binding buffer (50 mM Tris 7.5, 2 mM EDTA, 100 mM NaCl, 1 mM 2-mercaptoethanol, 7.5% glycerol), and radiolabeled ^{O}G·C duplex **9** was added to a final concentration of 10 pM. The binding reactions were allowed to proceed at room temperature for 10 min, at which time the reactions were placed on ice and immediately loaded onto a pre-run 10% 0.5 × TBE non-denaturing polyacrylamide gel. The gel was run at 250 V for 2.5 h at 4°C, then dried and exposed to a phosphorimaging plate (Fuji BAS 1000). The image was imported into Adobe Photoshop 3.0 for captioning.

$NaCNBH_3$ trapping analysis

Wild-type and mutant hOgg1 proteins (0.04 µg/reaction) were alkylated with 25 mM 2-bromoethylamine hydrobromide or 25 mM 2-bromoethanol in 100 mM HEPES, pH 8 for 1 h at room temperature (total volume 20 µl). Control reactions were performed in parallel without alkylating reagent. After 1 h, $NaCNBH_3$ was added to a 50 mM final concentration, non-radiolabeled ^{O}G·C duplex **9** was added to a 33 nM final concentration, and radiolabeled ^{O}G·C duplex **9** was added to a 0.7 nM final concentration. Trapping reactions were allowed to proceed for 1 h at 37°C before termination with 1 × SDS loading dye (final concentration) and heating at 95°C for 5 min. Terminated reactions were analyzed by 20% 200:1 SDS-PAGE. The gel was imaged using a phosphorimaging plate (Fuji BAS 1000) and imported into Adobe Photoshop 3.0 for captioning.

DNA-cleavage analysis

Wild-type and mutant hOgg1 proteins (0.04 µg/reaction) were alkylated with 25 mM 2-bromoethylamine hydrobromide or 25 mM 2-bromoethanol in 100 mM HEPES, pH 8 for 1 h at room temperature (total volume 20 µl). Control reactions were performed in parallel without alkylating reagent. After 1 h, NaCl was added to a 50 mM final concentration, non-radiolabeled ^{O}G·C duplex **9** was added to a 33 nM final concentration , and radiolabeled ^{O}G·C duplex **9** was added to a 0.7 nM final concentration. Cleavage reactions were allowed to proceed for 1 h at 37°C, and were terminated by the addition of 1/10 volume 1 mg/ml salmon sperm DNA in 2 M sodium acetate plus 3 volumes absolute ethanol. DNA was precipitated by incubation on dry ice ($CO_{2(s)}$) for 30 min, followed by centrifugation at 14,000 rpm at 4°C. DNA pellets were washed with cold (−20°C) 70% ethanol and allowed to air dry before resuspension in 20 µl 80% formamide/1 × TBE loading dye. Samples were heated at 75°C for 5 min, and 5 µl of each sample was loaded on a prerun, 0.75 mm thick, 20% 19:1 denaturing polyacrylamide gel. The gel was run at 300 V for 3 h, imaged without drying using a phosphorimaging plate (Fuji BAS 1000) and imported into Adobe Photoshop 3.0 for captioning.

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