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Catalytic and DNA-binding properties of the human Ogg1 DNA *N*-glycosylase/AP lyase: biochemical exploration of H270, Q315 and F319, three amino acids of the 8-oxoguanine-binding pocket

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ABSTRACT

The human Ogg1 protein (hOgg1) is an antimutator DNA glycosylase/AP lyase that catalyzes the excision of 8-oxo-7,8-dihydroguanine (8-oxoG) and the incision of apurinic and apyrimidinic (AP) sites in DNA. In this study, we have investigated the functional role of H270, Q315 and F319, three amino acids that are located in the 8-oxoG-binding pocket of hOgg1. Wild-type and mutant hOgg1 proteins (H270A, H270R, H270L, Q315A and F319A) were purified to apparent homogeneity. The catalytic activities and the DNA-binding properties of the various hOgg1 mutants were compared to those of the wild-type. The results show that hOgg1 mutated at H270 (H270A and H270L) or F319 (F319A) exhibits greatly reduced (50- to 1000-fold) DNA glycosylase activity, whereas the AP lyase activity is only moderately affected (<4-fold). The affinity of the hOgg1 mutants (H270A, H270L and F319A) for 8-oxoG.C-containing DNA is also greatly reduced (>30-fold), whereas their affinity for THF.C-containing DNA is only moderately reduced (<7-fold). The results also show that hOgg1 mutated at Q315 (Q315A) exhibits catalytic and DNA-binding properties similar to those of the wild-type. Therefore, H270 and F319 are essential to form the functional 8-oxoG-binding pocket, whereas Q315 is less crucial. In contrast, H270, Q315 and F319 are not required for efficient binding of THF.C and cleavage of AP sites. Finally, hOgg1 mutant proteins with a substitution of H270A or F319A are members of a new type of hOgg1 that is deficient in DNA glycosylase but proficient in AP lyase.

INTRODUCTION

The integrity of DNA in the cell is under constant threat from physical and chemical damaging agents of endogenous and exogenous origin (1–3). Reactive oxygen species (ROS) that escape cellular metabolism are an important source of formation of DNA damage, which is involved in pathological processes such as carcinogenesis and aging (1–7). ROS can attack base and sugar moieties in DNA yielding a variety of lesions, such as damaged bases, apurinic and apyrimidinic (AP) sites and DNA strand breaks (1–8). An oxidized guanine, 8-oxo-7,8-dihydroguanine (8-oxoG), is an abundant and arguably mutagenic lesion in DNA exposed to ROS (9–13). To counteract the deleterious effects of 8-oxoG, living organisms have evolved robust DNA repair mechanisms (9–13). In *Escherichia coli*, the repair of 8-oxoG mostly relies on two DNA glycosylases, Fpg and MutY, whose simultaneous inactivation results in a strong and specific (G.C→T.A) spontaneous mutator phenotype (9–13). In eukaryotic cells, the main defense against the mutagenic effect of 8-oxoG is the base excision repair (BER) pathway, which is initiated by the Ogg1 proteins (11,12). In *Saccharomyces cerevisiae*, Ogg1 is a DNA glycosylase/AP lyase that excises 8-oxoG and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) from γ-irradiated DNA (12–15). Inactivation of Ogg1 in yeast also results in a G.C→T.A spontaneous mutator phenotype (12,16,17). Indeed, yeast Ogg1 is the functional homolog of bacterial Fpg, although they do not belong to the same structural family (18).

In human cells, the *OGG1* gene is located on chromosome 3p25, and expresses two main forms of mRNAs with open reading frames coding for peptides of 345 and 424 amino acids, α-hOgg1 and β-hOgg1, respectively (19–23). The genomic structure of the human *OGG1* gene indicates alternative splicing of a primary transcript at the origin of α-hOgg1 and β-hOgg1 (24). The two forms of hOgg1 have identical first 316 amino acids but completely different C-terminal ends (19–24). Analysis of the cellular localization of Ogg1 proteins in human cells reveals that α-hOgg1 is localized in the nucleus whereas β-hOgg1 is localized in

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mitochondria (24). Both α-hOgg1 and β-hOgg1 are DNA glycosylases/AP lyases, which are able to excise 8-oxoG in damaged DNA (19–23). The nuclear α-hOgg1 is highly conserved in the course of evolution and has been characterized in *S.cerevisiae*, *Arabidopsis thaliana*, *Drosophila melanogaster* and mammals (11). The homology between yeast and human α-hOgg1 proteins spans the whole sequence (38% identity) (11). So far the presence of β-type Ogg1 has only been reported in human cells (11). The biological functions of Ogg1 in higher eukaryotes have been investigated using $ogg1^{-/-}$ knockout mice. The $ogg1^{-/-}$ animals are viable and after 12 months do not show gross pathological changes (25,26). However, $ogg1^{-/-}$ mice accumulate 8-oxoG in DNA and exhibit moderately elevated mutation rates in liver (25,26). Furthermore, an excess of lung adenoma and carcinoma has been reported in 18 months old $ogg1^{-/-}$ mice, compared with control animals (27). In human, somatic mutations and several polymorphisms of hOgg1 have been found in lung and kidney cancer (28–30). Furthermore, two recent studies suggest that low hOgg1 activity in peripheral blood mononuclear cells of smokers correlates with an increased risk of lung cancer (31,32).

The catalytic mechanisms and structural properties of human Ogg1 have been exclusively investigated using the nuclear α-hOgg1 protein (11). The present study also deals with α-hOgg1, which we will refer to as hOgg1 for simplicity. The hOgg1 protein belongs to a family of DNA glycosylases the signature of which is an active site HhH-GPD motif composed of a helix–hairpin–helix (HhH) element followed by a Gly/Pro-rich loop and terminating in an invariant aspartic acid residue (D268) (18,33). This family includes monofunctional DNA glycosylases such as AlkA and MutY from *E.coli* and bifunctional DNA glycosylases/AP lyases such as Nth from *E.coli* and Ntg1, Ntg2 and Ogg1 from *S.cerevisiae* and human hNth1 and hOgg1 (18,33). So far, all DNA glycosylases/AP lyases of the HhH-GPD family use a lysine residue on the enzyme as the catalytic nucleophile (K249 of hOgg1 or K241 of yeast Ogg1) (11,12,33–36). hOgg1 protein catalyzes the excision of purine lesions such as 8-oxoG, FapyG, 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (N^7-meFapyG) and 8-oxo-7,8-dihydroadenine (8-oxoA) and the incision of DNA at the 3′-side of regular AP sites via a β-elimination reaction (37–39). Importantly, removal of the damaged base and cleavage of an AP site by hOgg1 require the presence of a cytosine in the complementary strand opposite the lesion (37–40). The catalytic mechanism of hOgg1 involves an attack on C-1′of the 8-oxoG-containing nucleotide by the ε-NH_2 group of K249, thereby expelling the 8-oxoG base (DNA glycosylase activity) (17,33). Afterwards, hOgg1 can form a covalent enzyme–DNA intermediate that can undergo rearrangements leading to the formation of a ring-opened Schiff base and scission of the sugar–phosphate backbone on the 3′-side of the AP site (AP lyase activity) (33). Finally, hydrolysis liberates hOgg1 from the product DNA that contains a single-strand break with a 3′-unsaturated aldehydic end (33). Mutation of the catalytic lysine of hOgg1 (K249Q) or yeast Ogg1 (K241Q) abolishes all the catalytic activities, whereas it does not affect the specific recognition of 8-oxoG or tetrahydrofuranyl (THF) residues (34,36).

The X-ray structure of hOgg1 (K249Q) bound to 8-oxoG.C-containing DNA was first reported and revealed that 8-oxoG is extruded from the DNA helix and deeply buried in an enzyme pocket (41) (Fig. 1A). The X-ray structures of hOgg1 either free or bound to an AP site analog THF-containing DNA as well as the borohydride-trapped complex were also reported (42–45). Overall, the free and 8-oxoG.C-bound structures of hOgg1 are very similar, except for amino acids involved in formation of the 8-oxoG-binding pocket, such as H270, Q315 and F319 (41,42). The side chains of these three amino acids of hOgg1 define two main conformations of the 8-oxoG-binding pocket: an 'open state' in hOgg1 bound to 8-oxoG and a 'closed state' in hOgg1 either free or bound to THF (41–43). Switching between the 'open' and 'closed' states results from a combined rearrangement of the side chains of H270, Q315 and F319, which could act as a single functional entity (Fig. 1B).

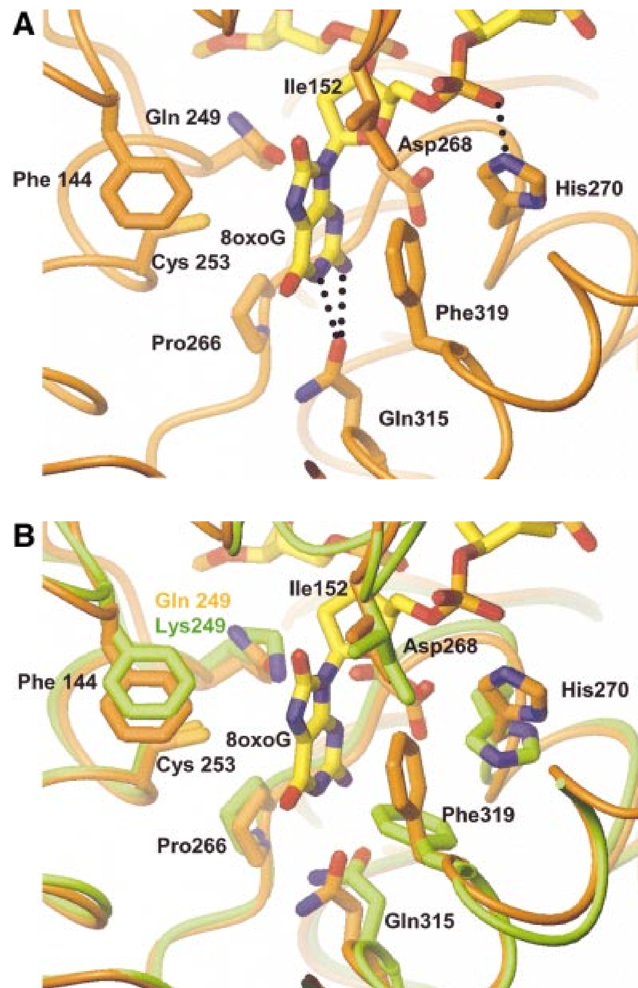

Figure 1. The 8-oxoG-binding pocket of hOgg1. (**A**) Active site of hOgg1 (K249Q) bound to an 8-oxoG.C-containing DNA (41) (pdb code 1EBM). Hydrogen bonds made by the H270 and Q315 side chains are represented by dotted lines. The human Ogg1 protein is in orange and the DNA in yellow. (**B**) Superimposition of the active site of the free hOgg1 enzyme (green) (42) (pdb code 1KO9) with the active site of the complex hOgg1–8-oxoG.C (orange). The view is in the same orientation as (A). The amino acids that form the 8-oxoG-binding pocket superimpose well except for the triad H270–F319–Q315 that switch from an 'open state' (8-oxoG, orange) to a 'closed state' (free enzyme, green). Theses figures were produced by the program MOLMOL (51).

Table 1. Sequences of oligodeoxyribonucleotides used as DNA probes in this study

Oligo	Sequence
34mer G	5′-GGCTTCATCGTTGTC[**G**]CAGACCTGGTGGATACCG-3′
34mer U	5′-GGCTTCATCGTTGTC[**U**]CAGACCTGGTGGATACCG-3′
34mer THF	5′-GGCTTCATCGTTGTC[**THF**]CAGACCTGGTGGATACCG-3′
34mer 8-oxoG[a]	5′-GGCTTCATCGTTGTC[**8-oxoG**]CAGACCTGGTGGATACCG-3′
34mer 8-oxoA[a]	5′-GGCTTCATCGTTGTC[**8-oxoA**]CAGACCTGGTGGATACCG-3′
34mer C[b]	5′-CGGTATCCACCAGGTCTG[**C**]GACAACGATGAAGCC-3′

If not specified, oligodeoxyribonucleotides used are of commercial origin (Genosys or Eurogentec).
[a]Oligodeoxyribonucleotides containing 8-oxoG or 8-oxoA are kind gifts from Drs A. Guy and J. Cadet (CEA, Grenoble).
[b]Complementary sequence harboring a cytosine opposite the lesion in duplexes. G, guanine; U, uracil; THF, tetrahydrofuranyl; 8-oxoG, 8-oxoguanine; 8-oxoA, 8-oxoadenine; C, cytosine.

In this study, we have investigated the role of H270, Q315 and F319 of hOgg1 using biochemical approaches. The mutant versions of hOgg1 (H270A, H270R, H270L, Q315A and F319A) have been purified to homogeneity, like the wild-type and the K249Q mutant. The catalytic properties of the wild-type and mutant hOgg1 proteins were analyzed using 8-oxoG.C-, 8-oxoA.C-, N^7-meFapyG.C- or AP.C-containing DNA. The binding properties were also analyzed using 8-oxoG.C- or THF.C-containing DNA. The results show that mutation of H270 and F319 residues greatly impair recognition and excision of 8-oxoG residues by hOgg1, whereas they moderately affect recognition of THF and AP lyase activity. On the other hand, mutation of Q315 causes only a minor alteration of hOgg1 activities. We propose a model where H270 and F319 are critical residues and act as a single entity to form the 'open' 8-oxoG-binding pocket of hOgg1.

MATERIALS AND METHODS

DNA probes and other materials

Sequences of the oligodeoxyribonucleotides used in this study are listed in Table 1. Uracil DNA glycosylase from *E.coli* was purified from an overproducing strain (our laboratory stock).

Preparation of wild-type and mutant hOgg1 proteins

The open reading frame coding for the nuclear version of hOgg1 (α-hOgg1, 345 amino acids) was cloned into pGEX-4T1 (Pharmacia Biotech) yielding plasmid pPR71 (19). This plasmid allows the expression of a fusion protein, GST–hOgg1, where GST is fused to the N-terminus of hOgg1. The hOgg1 coding sequence in pPR71 was mutagenized using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol to generate plasmids pVDK1 (hOgg1-K249Q), pVDK2 (hOgg1-H270A), pVDK3 (hOgg1-H270R), pVDK4 (hOgg1-H270L), pVDK5 (hOgg1-Q315A) and pVDK6 (hOgg1-F319A), respectively. The oligodeoxyribonucleotides used for mutagenesis and their complementary sequences were purchased from Genosys. Mutations, as well as the integrity of the rest of the hOgg1 coding sequence, were confirmed by DNA sequencing. Sequences of oligodeoxyribonucleotide primers used and plasmid sequences are available upon request.

The plasmids expressing GST–hOgg1, either wild-type (pPR71) or mutant (pVDK1–pVDK6), were transformed into *E.coli* strain BH410 (*fpg*⁻). Expression and purification of hOgg1 proteins were performed as previously described (30). Briefly, the GST–hOgg1 proteins were purified by gluthatione–Sepharose 4B (Pharmacia Biotech) chromatography. Afterwards, the GST tag was removed by thrombin (ICN) and the hOgg1-containing solution was dialyzed overnight against a buffer containing 20 mM Tris–HCl pH 8.0, 2 mM Na_2EDTA, 50 mM NaCl and 2% glycerol and applied to a MonoS column (FPLC System; Pharmacia Biotech). Proteins were eluted with a linear salt gradient (50–800 mM NaCl). The fractions containing hOgg1 were determined after SDS–PAGE analysis and Coomassie blue staining. The protein solution was adjusted to 1 mg/ml in 25 mM Tris–HCl pH 7.6, 2 mM Na_2EDTA, 250 mM NaCl and to 50% glycerol (v/v) and stored at –20°C. Protein concentration was measured according to the Bradford method.

Circular dichroism

Circular dichroism (CD) spectra were recorded using a Jobin Yvon CD6 spectropolarimeter in a 0.1 cm cell. Solutions of wild-type hOgg1 and the three mutants (H270A, Q315A and F319A) were prepared at 10 μM and dialyzed against a buffer containing 20 mM Tris–HCl pH 7.6 and 150 mM NaCl. Each CD signal is the average of four scans between 200 and 250 nm. The spectra were measured at 20, 30, 37 and 44°C.

DNA glycosylase activity assays

N^7-meFapyG DNA glycosylase assay. Reaction mixture (50 μl final volume) contained 25 mM Tris–HCl pH 7.6, 100 mM NaCl, [^{3}H]N7-meFapyG–poly(dG·dC) (1.5 pmol [^{3}H]N^7-meFapyG) and hOgg1. The reactions were performed at 37°C for 15 min. Ethanol-soluble radioactive material was quantified by scintillation counting and the chemical nature of this material was monitored by HPLC as described (46). One unit releases 1 pmol N^7-meFapyG in 15 min at 37°C.

8-oxoG or 8-oxoA DNA glycosylase assay. 34mer oligodeoxyribonucleotides containing a single 8-oxoG or 8-oxoA (Table 1) were labeled at the 5′-end using [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) and T4 polynucleotide kinase (New England Biolabs) (35,38). The ^{32}P-labeled strand was purified and hybridized with a complementary sequence containing a cytosine opposite the lesion, yielding an 8-oxoG.C or 8-oxoA.C duplex, respectively. The assay mixture

(15 µl final volume) contained 25 mM Tris–HCl pH 7.6, 100 mM NaCl, 50 fmol ^{32}P-labeled 8-oxoG.C or 8-oxoA.C and hOgg1. The reactions were performed at 37°C for 15 min. Afterwards, the reactions were supplemented with 2 µl of 2N NaOH and heated at 60°C for 5 min to cleave all residual AP sites. Then, 6 µl of formamide dye (90% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol and 5 mM Na_2EDTA) was added before heating at 95°C for 5 min. The products of the reaction were separated by 20% PAGE containing 7 M urea and quantified using a Molecular Dynamics PhosphorImager. One unit cleaves 1 pmol 8-oxoG.C or 8-oxoA.C duplex in 15 min at 37°C.

AP lyase activity assay

A 34mer oligodeoxyribonucleotide containing a single uracil (Table 1) was ^{32}P-labeled at the 5′-end and hybridized with a complementary sequence yielding a U.C duplex. To generate an AP site, the U.C duplex was incubated with uracil DNA glycosylase yielding an AP.C duplex (35). The assay mixture (15 µl final volume) contained 25 mM Tris–HCl pH 7.6, 100 mM KCl, 50 fmol ^{32}P-labeled AP.C duplex and hOgg1. The reactions were performed at 37°C for 15 min. Afterwards, they were supplemented with $NaBH_4$ (50 mM final concentration at 4°C for 5 min) to stabilize intact AP sites. The reaction products were analyzed and quantified as described for DNA glycosylase assays. One unit cleaves 1 pmol AP.C duplex in 15 min at 37°C.

Determination of the apparent dissociation constant (K_d)

34mer oligodeoxyribonucleotides containing a single 8-oxoG or THF residue (Table 1) were ^{32}P-labeled at the 5′-end and hybridized with a complementary sequence yielding 8-oxoG.C or THF.C duplex, respectively.

Electrophoretic mobility shift assay (EMSA). The assay mixture (10 µl final volume) contained 25 mM Tris–HCl pH 7.6, 175 mM NaCl, 1 mM Na_2EDTA, 1 mM dithiothreitol, 300 µg/ml bovine serum albumin, 12% (v/v) glycerol, 2.5 fmol ^{32}P-labeled 8-oxoG.C or THF.C duplex and hOgg1. The reactions were performed at 4°C for 30 min and loaded onto non-denaturing 10% PAGE gels as described (47,48). Gel electrophoresis was performed at 4°C for 3 h. Free DNA and hOgg1-bound DNA were quantified using a Molecular Dynamics PhosphorImager.

Determination of K_d. Assuming a 1:1 stoichiometry between hOgg1 and the DNA probe, the apparent dissociation constant (K_d) can be calculated from the concentrations of: free hOgg1 ([hOgg1]), free DNA (f) and bound DNA–hOgg1 complex ($1 - f$) at equilibrium: $K_d = [\text{hOgg1}] \times f/(1 - f)$. If the total concentration of protein $[\text{hOgg1}]_0$ is close to that of the free hOgg1 at equilibrium: $K_d = [\text{hOgg1}]_0$ when 50% of the DNA is bound by the protein (36,47,48).

Mutagenesis experiments

Complementation of the spontaneous mutator phenotype of an *E.coli* (*fpg*$^-$ *mutY*$^-$) double mutant (BH990) by expression of different GST–hOgg1 fusion proteins was analyzed by determining the frequency of rifampicin-resistant cells in 20 independent cultures (19).

RESULTS

Purification of wild-type and mutant hOgg1 proteins

In order to evaluate the role of H270, Q315 and F319 in the catalytic and DNA-binding activities of hOgg1, human *OGG1* cDNA was cloned into a bacterial expression vector and mutants were generated. The hOgg1 proteins were expressed as GST fusions, with the GST tag at the N-terminus. Afterwards, the GST tag was cleaved off and native hOgg1 was purified to apparent homogeneity. The purity of each hOgg1 protein was assessed by SDS–PAGE and determination of the N-terminal sequence, which indicates deletion of the first four amino acids. The molecular mass of hOgg1 (wild-type) was determined by MALDI-TOF MS, with a major peak at m/z 38 327 ± 5, corresponding to the expected mass of hOgg1 (amino acids 5–345) (49). This deletion does not affect the enzyme properties, since hOgg1 (amino acids 12–327) has *in vitro* activities very similar to those of full-length hOgg1 (amino acids 1–345) (41). Figure 2 shows the hOgg1 proteins used in this study: wild-type, H270A, Q315A, F319A and K249Q. To further investigate the role of H270, two additional mutants were prepared (H270R and H270L) (data not shown). Secondary structures of wild-type hOgg1 and the H270A, Q315A and F319A mutants were followed by CD at 20, 30, 37 and 44°C. The CD spectra of the hOgg1 mutants at temperatures up to 37°C are similar to that observed with the wild-type (Supplementary Material, Fig. S1). Therefore, the alanine replacement does not significantly alter the α-helix and β-sheet contents of hOgg1 up to 37°C, however, minor local rearrangements cannot be excluded. It should be noted that a slight decrease in CD signal is observed at 37°C for mutant H270A, with 80% of the signal still present. Thus, the hOgg1 proteins used in this study are correctly folded and, therefore, can be used to gain insight into whether H270, Q315 and F319 are critical for the catalytic activities or lesion recognition or both steps.

DNA glycosylase activity of wild-type and mutant hOgg1 proteins

The ability of purified wild-type hOgg1 and the mutants to excise N^7-meFapyG, 8-oxoG and 8-oxoA was investigated using specific substrates. A representative experiment and a quantitative analysis are reported (Fig. 3A,B and Table 2). The results show that hOgg1(wild-type) and hOgg1(Q315A) efficiently release N^7-meFapyG, 8-oxoG and 8-oxoA from damaged DNA (Table 2 and Fig. 3A,B). In contrast, the hOgg1 mutants H270A, H270L and F319A exhibit greatly impaired DNA glycosylase activities towards N^7-meFapyG, 8-oxoG and 8-oxoA (Table 2 and Fig. 3A,B). The 8-oxoG DNA glycosylase activities of the hOgg1 mutants H270A, H270L and F319A are 160-, 1000- and 200-fold lower than that of the wild-type, respectively (Table 2). However, the 8-oxoG DNA glycosylase activities of hOgg1(H270A) and hOgg1(F319A) are still detectable compared to that of hOgg1(K249Q) (Table 2).

On the other hand, the H270A, H270L and F319A mutants have detectable N^7-meFapyG DNA glycosylase activity, with loss of activity ranging from 45- to 71-fold compared to the wild-type (Table 2). The results also show that hOgg1(H270R) presents an intermediate situation with DNA glycosylase

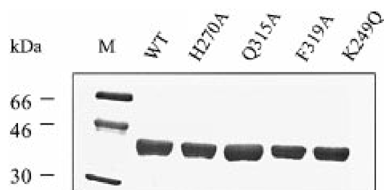

Figure 2. Purification of wild-type and mutant hOgg1 proteins. Purified fractions were analyzed by 12.5% SDS–PAGE and stained with Coomassie brillant blue. Lane M, molecular weight markers (Amersham Bioscience). The amounts of protein loaded onto the gel were 2 µg for each lane.

activities that are only 3- to 4-fold lower than those of the wild-type (Table 2). It should be noted that identical results are obtained when reactions are performed at 25 or 30°C (data not shown). Therefore, inactivation of the DNA glycosylase activities of the hOgg1 mutants is probably not due to protein instability at 37°C. Taken together, these results show that mutation of F319 or H270 in hOgg1 can greatly affect the excision of damaged bases, whereas mutation of Q315 has more limited consequences.

AP lyase activity of wild-type and mutant hOgg1 proteins

The AP lyase activity of wild-type hOgg1 and the mutants was investigated using an AP.C-containing duplex as substrate. A representative experiment and a quantitative analysis are reported (Fig. 3C and Table 2). The results show that the hOgg1 mutants H270R and Q315A possess an efficient AP lyase activity like the wild-type (Table 2 and Fig. 3C). Furthermore, the hOgg1 mutants H270A, H270L and F319A exhibit only moderately reduced AP lyase activities (1.5- to 3.7-fold) compared to the wild-type (Table 2 and Fig. 3C). It should be noted that the AP lyase activities of the hOgg1 mutants H270A, H270L and F319A are highly significant when compared with that of the K249Q mutant (Table 2). The cleavage products generated by hOgg1(wild-type) and mutant proteins are identical and correspond to the expected β-elimination products (data not shown). These results show that the amino acids of hOgg1 explored in this study, H270, Q315 and F319, are not essential for AP lyase activity.

Binding properties of wild-type and mutant hOgg1 proteins for 8-oxoG.C-containing DNA

The possibility of dissociating the DNA glycosylase and AP lyase activities of hOgg1 by mutation at H270 or F319 could rely on specific alteration of the recognition of damaged bases, whereas recognition of AP sites is conserved. The DNA-binding properties of wild-type hOgg1 and the mutants were investigated using an 8-oxoG.C-containing DNA, which was previously used in the cleavage assay. The K_d values of hOgg1 proteins for 8-oxoG.C-containing DNA were determined using EMSA (47,48). In order to minimize DNA strand cleavage by hOgg1, the binding reactions and gel electrophoresis were performed at 4°C. A representative experiment and a quantitative analysis are reported (Fig. 4A and Table 3). Figure 4A shows that incubation of the 8-oxoG.C duplex with increasing concentrations of hOgg1(wild-type) produces increased amounts of a band with reduced electrophoretic mobility, presumably a 1:1 hOgg1–DNA complex. Such experiments were used to determine the K_d values (47,48). Table 3 shows that all hOgg1 mutants tested are significantly affected for 8-oxoG.C binding compared to the wild-type and K249Q. Two mutant proteins, H270R and Q315A, present an intermediate behavior with K_d values that are 4.4- and 7-fold higher than that of the wild-type (Table 3). On the other hand, mutants H270A, H270L and F319A are so affected that K_d values cannot be determined experimentally (Table 3 and Fig. 4A). Indeed, these three mutants exhibit the same affinity for 8-oxoG.C- and unmodified G.C-containing DNA (data not shown). These results strongly suggest that H270 and F319 are critical for the recognition of 8-oxoG, whereas the contribution of Q315 is weaker. Therefore, the weakest catalysts (H270A, H270L and F319A) are also the weakest binders, which could suggest that these mutations affect the excision of 8-oxoG by reducing the recognition step.

Binding properties of wild-type and mutant hOgg1 proteins for THF.C-containing DNA

The ability of wild-type hOgg1 and the mutants to bind an AP site analog-containing (THF.C) DNA was also investigated. Although the THF.C-containing duplex cannot be cleaved by the AP lyase activity of hOgg1, binding assays and gel electrophoresis were performed at 4°C. A representative experiment and a quantitative analysis are reported (Fig. 4B and Table 3). The results show that all hOgg1 mutants tested, except hOgg1(H270L), can efficiently bind THF.C-containing DNA like the wild-type or the K249Q mutant (Fig. 4B and Table 3). The H270L mutation significantly affects binding of THF.C, with a 6.2-fold increase in K_d value (Table 3). Taken together, these results indicate that H270, Q315 and F319 are not critical for the recognition of an AP site analog such as THF in DNA. In addition, efficient binding of the THF.C-containing DNA confirms that all tested hOgg1 proteins, with the possible exception of hOgg1(H270L), are correctly folded, as suggested by the CD spectra. The effects observed on AP lyase activity can be analyzed in the light of the THF.C binding experiments. As observed for 8-oxoG, effects on binding and catalysis are well correlated, since hOgg1 mutants that bind THF.C with high affinity are also endowed with efficient AP lyase activity.

Expression of wild-type and mutant GST–hOgg1 proteins in *E.coli fpg mutY*

Plasmids expressing wild-type or mutant GST–hOgg1 fusion proteins were transformed into an *E.coli* strain in which the *fpg* and *mutY* genes are disrupted. This strain displays a strong spontaneous mutator phenotype, which is thought to be due to the accumulation of 8-oxoG in DNA (14,19). The ability of a GST–hOgg1 protein to suppress mutagenesis was previously reported (19). Table 4 shows that GST–hOgg1(wild-type) and GST–hOgg1(Q315A) efficiently suppress the mutator phenotype of the *fpg mutY* double mutant, whereas GST–hOgg1(H270A) and GST–hOgg1(F319A) do not. These results reveal a strong correlation between efficient DNA glycosylase activity and suppression of the mutator phenotype. These results also confirm that the Q315A mutant possesses a functional 8-oxoG DNA glycosylase activity. Finally, they strongly suggest that the antimutator potency of hOgg1 relies on its DNA glycosylase activity.

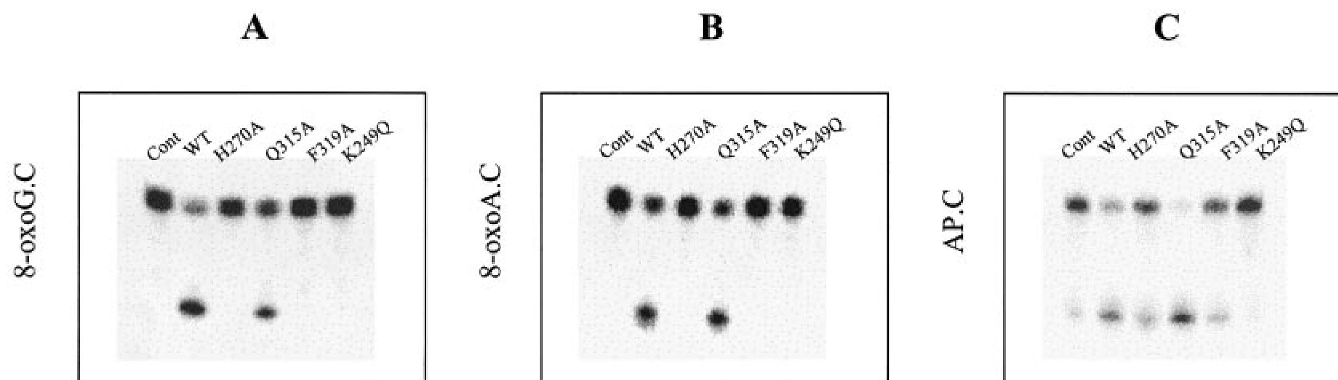

Figure 3. Catalytic activities of wild-type and mutant hOgg1 proteins. The ^{32}P-labeled 8-oxoG.C-, 8-oxoA.C- or AP.C-containing duplexes were incubated with hOgg1 (2 ng) for 15 min at 37°C. Reactions with 8-oxoG.C and 8-oxoA.C were terminated by addition of 0.2 N NaOH to cleave all AP sites. Under these assay conditions, cleavage of the 8-oxoG- or 8-oxoA-containing strand quantitatively translates into DNA glycosylase activity.

Table 2. DNA glycosylase and AP lyase activities of wild-type and mutant hOgg1 proteins

hOgg1 protein	N^7-meFapyG units/mg P[a] (%)	8oxoG units/mg P[a,b] (%)	AP sites units/mg P[a] (%)
Wild-type	18 500 (100)[c]	27 000 (100)[c]	6230 (100)[c]
H270A	405 (2.2)	162 (0.6)	2679 (43)
H270R	4750 (25.7)	7850 (29.1)	6410 (103)
H270L	250 (1.4)	≤20 (≤0.1)	1674 (27)
Q315A	11 563 (62.5)	16 740 (62.0)	7351 (118)
F319A	345 (1.9)	147 (0.5)	4203 (67)
K249Q	≤50 (≤0.3)	≤20 (≤0.1)	≤20 (≤0.3)

[a]Specific activities were determined from curves where excision or cleavage is a function of enzyme amount: 2–200 ng for excision of N^7-meFapyG or 0.1–50 ng for excision of 8-oxoG and cleavage of AP sites. All reaction were performed at 37°C for 15 min.
[b]Reactions with 8-oxoG.C were terminated by addition of 0.2 N NaOH to cleave all AP sites formed. Under these assay conditions, cleavage of the 8-oxoG-containing strand quantitatively translates into DNA glycosylase activity.
[c]Numbers in parentheses are the residual percent activity (hOgg1 mutant/wild-type hOgg1). The values are the average of three independent experiments.

DISCUSSION

An important objective in the field of DNA repair is to understand how repair enzymes locate and remove aberrant bases amongst a vast excess of normal bases in double-stranded DNA. One approach relies on the investigation of the structural and biochemical properties of DNA repair proteins. DNA glycosylases constitute an important class of repair enzymes that initiate the BER process in prokaryotes and eukaryotes. Furthermore, DNA glycosylases are small, monomeric enzymes that can act without cofactors (33). Amongst DNA glycosylases, hOgg1 is an important member of a subfamily characterized by the HhH-GPD motif (18,33). The catalytic mechanisms of hOgg1 have been investigated in detail and X-ray structures of free hOgg1 and hOgg1 bound to DNA are available (41–45). Overall, the X-ray structures of hOgg1 free or bound to 8-oxoG-containing DNA superimpose well, however, significant conformational changes are observed (41,42). The rearrangements are mostly localized in the 8-oxoG-binding pocket and involve positions H270, F319 and Q315 (Fig. 1B). This triad of amino acids appears to behave as a single functional unit switching from a 'closed' conformation in the free and THF.C-bound structures (42,43) to an 'open' conformation in the 8-oxoG.C-bound structure and in the structure of hOgg1 with a borohydride-trapped intermediate and a 8-oxoG (41,45). The objective of this biochemical study was to analyze the individual role of H270, Q315 and F319 on hOgg1 functions.

H270 is a key residue to define the 8-oxoG binding pocket of hOgg1

In the structure of hOgg1(K249Q) bound to an 8-oxoG.C-containing DNA, the H270 side chain makes a hydrogen bond with the phosphate on the 5′-side of 8-oxoG (41) and a second hydrogen bond with Asp322. The imidazole ring of H270 makes van der Waals contacts with F319 (Fig. 1A). Replacement of H270 with an alanine (H270A) results in a greatly impaired capacity to excise 8-oxoG, 8-oxoA and N^7-meFapyG and to bind 8-oxoG.C-containing DNA. We propose that this decrease in affinity for 8-oxoG.C (>32-fold) may correspond to both disruption of the hydrogen bond between H270 and the phosphate at the 5′-side of 8-oxoG and to destabilization of the F319 side chain conformation. Therefore, the decreased 8-oxoG DNA glycosylase activity (160-fold) may be due mainly to a decrease in substrate recognition. To further investigate the role of H270, two other mutants were constructed, H270R and H270L. Replacement of H270 with an arginine results in a moderate decrease in both 8-oxoG excision (3-fold) and 8-oxoG.C binding (4.5-fold). Modeling of the H270R mutant suggests that the guanidinium group of R270 could be accommodated in the active site and that it could be within hydrogen bond distance and angles with the phosphate group on the 5′-side of 8-oxoG and could maintain the F319 position (data not shown). It suggests that this basic residue though not isosteric might mimic the histidine role. Replacement of H270 with a leucine

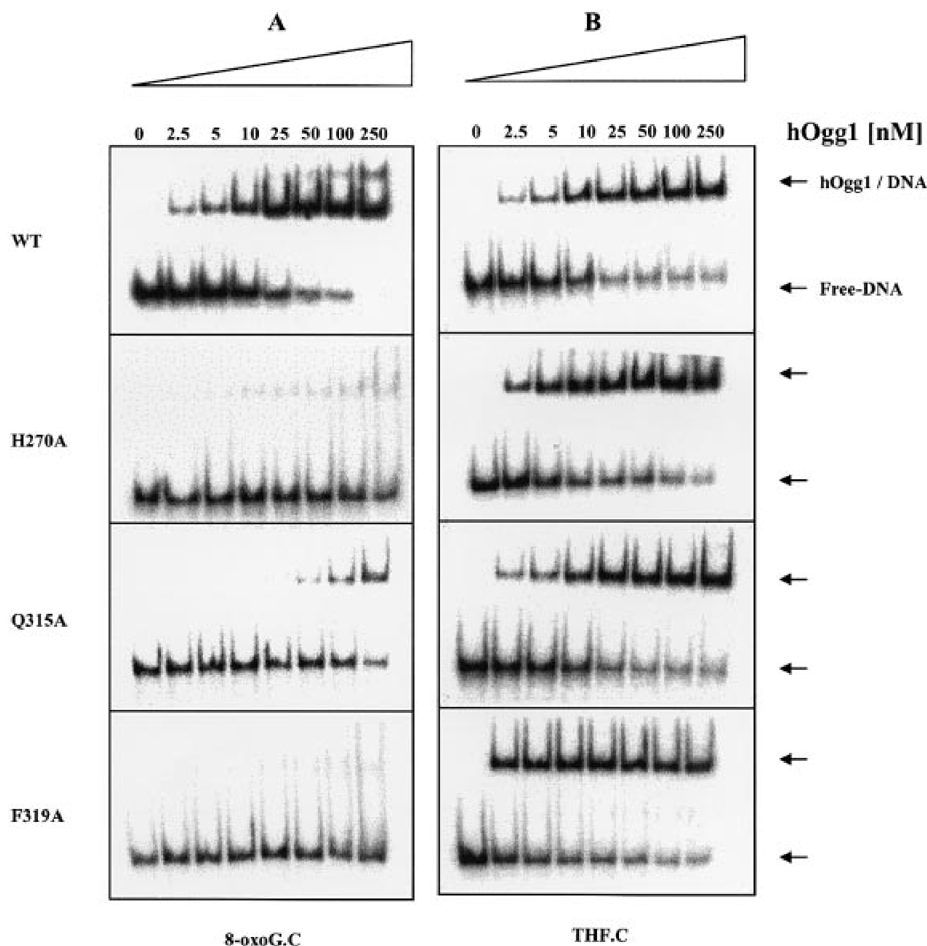

Figure 4. Binding of the wild-type and mutant hOgg1 proteins to 8-oxoG.C- and THF.C-containing DNA. EMSA was performed using increasing concentrations of wild-type and mutant hOgg1 and ^{32}P-labeled 8-oxoG.C- or THF.C-containing DNA. Such gels were used for the determination of K_d values, which correspond to the [hOgg1] required to bind 50% of the DNA probe.

Table 3. Binding of 8-oxoG.C- and THF.C-containing DNA by wild-type and mutant hOgg1 proteins

hOgg1 protein	Binding of 8-oxoG.C [K_d (nM)]	Binding of THF.C [K_d (nM)]
Wild-type	8.2 ± 0.9	2.4 ± 0.3
H270A	>266[a]	4.6 ± 0.7
H270R	36.3 ± 4.9	4.2 ± 1.1
H270L	>266[a]	14.8 ± 3.3
Q315A	57.3 ± 10.9	5.5 ± 0.8
F319A	>266[a]	2.2 ± 0.4
K249Q	11.8 ± 4.3	4.8 ± 1.6

DNA probes were ^{32}P-labeled 34mer oligodeoxyribonucleotide duplexes (Table 1). K_d values were determined by EMSA as described in Materials and Methods. DNA concentration was 0.25 nM and the hOgg1 concentration range was 1.25–250 nM. Values are averages of three independent experiments ± SD.
[a]The amount of DNA bound at the highest concentration of hOgg1 was less than 50% of the total DNA. Therefore, the K_d values cannot be determined. It should be noted that the binding curves of hOgg1 (H270A, H270L and F319A) with the 8-oxoG.C-containing DNA were similar to those obtained with control DNA where the 8-oxoG.C pair was replaced with a G.C pair within the range of concentrations used.

Table 4. Frequencies of spontaneous mutation to rifampicin resistance in *E.coli* BH990 (*fpg mutY*) expressing GST or GST–hOgg1 fusion proteins

Protein expressed	Rifampicin resistant cells/10^9 cells
GST	3350
GST–hOgg1	460
GST–hOgg1(H270A)	3054
GST–hOgg1(Q315A)	675
GST–hOgg1(F319A)	2945

abolishes excision of 8-oxoG and 8-oxoG.C-binding. Modeling of the H270L mutant suggests steric hindrance in addition to disruption of the hydrogen bond made with the phosphate backbone that could explain why this mutation has more severe effects than H270A (data not shown).

The role of H270 must also be discussed in the light of the wild-type affinity of the H270A mutant for THF.C-containing DNA and of its efficient AP lyase activity. In the structures of hOgg1 either free or bound to THF.C, H270 makes a π stacking interaction with the aryl group of F319, and it does not make any hydrogen bond with the DNA backbone phosphate (42,43). Therefore, the X-ray structure of hOgg1 bound to THF and the high affinity of the H270A mutant for THF.C-containing DNA strongly suggests that the hydrogen

bond between H270 and the phosphate on the 5′-side of the THF is not required for binding and curving DNA. Taken together, these results suggest that H270 plays a critical and specific role in formation of the 8-oxoG-binding pocket. As a speculation, we propose that the hydrogen bond between H270 and DNA favors an orientation of F319 to π stack with 8-oxoG, suggesting a system involving 8-oxoG, F319, H270 and the DNA backbone (Fig. 1).

F319 is another key residue defining the 8-oxoG binding pocket of hOgg1

In the structure of hOgg1 bound to 8-oxoG.C-containing DNA, F319 stacks against the 8-oxoG base, contributing significantly to the delimitation of the 8-oxoG-binding pocket (41). It is in van der Waals contact with Asp268, His270 and Leu323 (Fig. 1). Here we show that the F319A mutant presents a severe decrease in both 8-oxoG excision (200-fold) and 8-oxoG.C binding (>32-fold). Therefore, the H270A and F319A mutants exhibit very similar properties. The decrease in affinity for 8-oxoG.C may correspond to disruption of the π stacking interaction with the 8-oxoG. In the structures of hOgg1 either free or bound to THF.C, F319 has its aromatic ring almost perpendicular to its orientation in the 8-oxoG.C-bound structure (41–43). These structural data suggest that the orientation of F319 is critical for formation of the 8-oxoG-binding pocket. The F319A mutant presents AP lyase activity and THF.C binding similar to the wild-type, as for H270A. These results are coherent with our previous speculation about cooperation between H270 and F319 to form the 8-oxoG-binding pocket (Fig. 1). Chen *et al*. proposed, from direct visualization of the hOgg1–DNA interaction by atomic force microscopy, that hOgg1 is able to bend DNA at non-specific sites in searching for 8-oxoG (50). From our data, we propose that in this model H270 and F319 may change conformation upon hOgg1 binding at 8-oxoG sites and thus stabilize the extra-helical position of the lesion.

An unexpected minor contribution of Q315 in 8-oxoG recognition and excision

Q315 is located at the beginning of the last helix of hOgg1, helix O. It is in contact with L256, P266 and F319 (Fig. 1). In the structure of hOgg1 bound to 8-oxoG.C, Q315 interacts with the Watson–Crick face of the 8-oxoG and makes two hydrogen bonds with the N^1-imino and N^2-amino groups of the 8-oxoG (41). The Q315A mutant presents a small decrease in 8-oxoG excision (1.6-fold) and a more significant decrease in 8-oxoG.C binding (7-fold). The decrease in affinity may correspond to disruption of the hydrogen bonds made by Q315 with 8-oxoG. Although significant, these hydrogen bonds are not crucial for the DNA glycosylase activity of hOgg1. The results also show that hOgg1 efficiently excises 8-oxoA, like the yeast and mouse enzymes (38,39). The efficient removal of 8-oxoA confirms that the hydrogen bonds are not essential and that they are not used to exclude adenine from the hOgg1-binding site as previously suggested (41). Finally, the ability of hOgg1(Q315A) to suppress the mutator phenotype of a *fpg*$^-$ *mutY*$^-$ double mutant of *E.coli* demonstrates it ability to repair 8-oxoG *in vivo*. Furthermore, the Q315A mutant of hOgg1 presents an intact AP lyase activity coherent with the absence of an interaction between Q315 and THF (43). Therefore, Q315 presumably stabilizes 8-oxoG in the active site but is not crucial for the catalytic and binding properties of hOgg1.

A new type of mutant hOgg1 proteins

Active site mutants of hOgg1(K249Q) or yeast Ogg1(K241Q) have no detectable DNA glycosylase and AP lyases activities, whereas they can bind 8-oxoG.C- and THF.C-containing DNA like the wild type (34,36). The hOgg1(H270A) and hOgg1(F319A) mutants described in the present study are deficient in recognition and excision of 8-oxoG but proficient in binding and cleavage of AP sites. Therefore, H270A and F319A define a new type of hOgg1 proteins that can help in our understanding of the biochemical and biological properties of this important family of DNA repair proteins.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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