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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
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Surprising Repair Activities of Nonpolar Analogs of 8-oxoG Expose Features of Recognition and Catalysis by Base Excision Repair Glycosylases

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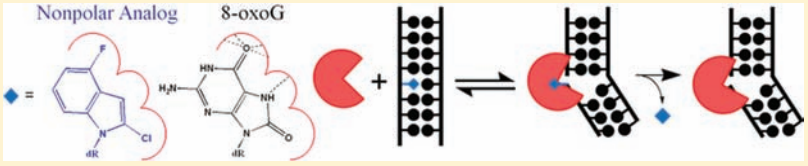
Supporting Information



ABSTRACT: Repair glycosylases locate and excise damaged bases from DNA, playing central roles in preservation of the genome and prevention of disease. Two key glycosylases, Fpg and hOGG1, function to remove the mutagenic oxidized base 8-oxoG (OG) from DNA. To investigate the relative contributions of conformational preferences, leaving group ability, enzyme-base hydrogen bonding, and nucleobase shape on damage recognition by these glycosylases, a series of four substituted indole nucleosides, based on the parent OG nonpolar isostere 2Cl-4F-indole, were tested as possible direct substrates of these enzymes in the context of 30 base pair duplexes paired with C. Surprisingly, single-turnover experiments revealed that Fpg-catalyzed base removal activity of two of the nonpolar analogs was superior to the native OG substrate. The hOGG1 glycosylase was also found to catalyze removal of three of the nonpolar analogs, albeit considerably less efficiently than removal of OG. Of note, the analog that was completely resistant to hOGG1-catalyzed excision has a chloro-substituent at the position of NH7 of OG, implicating the importance of recognition of this position in catalysis. Both hOGG1 and Fpg retained high affinity for the duplexes containing the nonpolar isosteres. These studies show that hydrogen bonds between base and enzyme are not needed for efficient damage recognition and repair by Fpg and underscore the importance of facile extrusion from the helix in its damaged base selection. In contrast, damage removal by hOGG1 is sensitive to both hydrogen bonding groups and nucleobase shape. The relative rates of excision of the analogs with the two glycosylases highlight key differences in their mechanisms of damaged base recognition and removal.

INTRODUCTION

DNA damage can threaten the integrity of the genome by increasing the frequency of mutations and exacerbating replication errors.[1] Organisms possess a cornucopia of damage-specific repair pathways that prevent the deleterious consequences of DNA damage. One of these, the base excision repair (BER) pathway, is primarily responsible for the recognition and initiation of repair of modified nucleobases arising from oxidation, alkylation, methylation, and deamination reactions. The low redox potential of guanine makes it particularly vulnerable to oxidative damage, a major product of which is 8-oxo-7,8-dihydroguanine (8-OxoG or OG). The incorporation of the oxo-substituent at the C-8 position of the purine ring causes the nucleotide to preferentially adopt the syn orientation (Figure 1A).[2] In this conformation in the template strand during DNA replication, OG codes like thymine, causing adenine to be preferentially incorporated over cytosine. The stable OG:A base pair, if allowed to persist in DNA, will eventually produce a G→T transversion mutation (Figure 1A). Base excision repair glycosylases, Fpg in *Escherichia coli* and its human homologue, hOGG1, prevent mutations associated with OG by catalyzing removal of OG bases from OG:C base pairs within DNA.[3,4] Subsequent trimming of the sugar fragment, nucleotide insertion, and ligation serve to restore the original G:C base pair.[5]

The mechanisms by which BER glycosylases locate subtle alterations in DNA bases and catalyze *N*-glycosidic bond cleavage with high efficiency and accuracy within the context of a large excess of normal DNA bases are questions central to their roles in the prevention of mutations and disease.[1] Synthetically derived nucleotide analogs are particularly useful in measuring how specific modifications to an enzyme substrate impact recognition, excision, and overall repair. Nonpolar isosteres have been used to investigate the importance of hydrogen bonding within varied enzyme DNA complexes while preserving the nucleobase shape.[2,6−11] Previous work established that such nonpolar nucleotide analogs can effectively mimic the natural nucleotides when used to evaluate DNA polymerase activity.[8−10,12] The hydrophobic adenine analogs, 4-

Received: September 8, 2011
Published: December 14, 2011

dx.doi.org/10.1021/ja208510m | J. Am. Chem.Soc. 2012, 134, 1653−1661

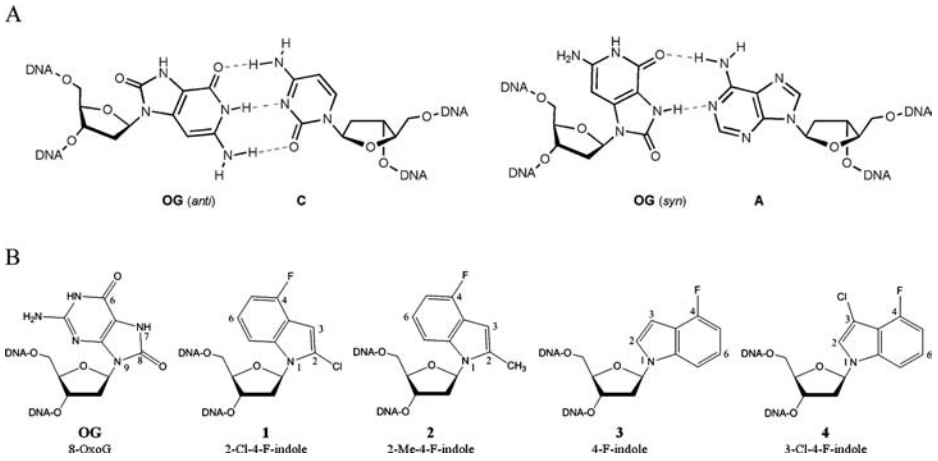


Figure 1. Structures of OG(anti):C(anti) pair and OG(syn):A(anti) mispair in DNA (A). Structures of OG deoxynucleoside and of OG analogs 1−4 (B). Nucleosides are drawn with their preferred glycosidic orientation (i.e., **OG**,**1**,**2** are syn; **3**,**4** are anti). The standard numbering scheme for purines and indoles is shown for key positions analyzed in this work.

methylbenzimidazole (B) and 9-methyl-1*H*-imidazo-[4,5-*b*]-pyridine (Q) were also shown in previous studies with Fpg to be useful at revealing features of base excision and recognition.[7] Fpg was found to cleave OG faster when paired opposite these A analogs, which suggested that the lack of hydrogen bonds between the base pair facilitates OG flipping into the Fpg active site.[7]

Although considerable structural and mechanistic study has focused on these enzymes, the salient features allowing for efficient damaged base recognition and excision by Fpg and hOGG1 remain unclear. A structure of Fpg bound to DNA shows the OG base oriented in the syn conformation, with hydrogen bonds to NH7 and to amino and imino groups along the Watson−Crick pairing edge.[13] In addition to OG, Fpg cleaves a wide variety of oxidized guanine and thymine bases with a common feature being the presence of a carbonyl group adjacent to the C−N glycosidic bond. This common substrate feature has prompted the hypothesis that this carbonyl group acts as an electron acceptor, allowing the base to leave the sugar.[14] In contrast, structures of hOGG1 bound to DNA show the OG base in the anti conformation, with hydrogen bonds from the enzyme using amino acid residues as well as tightly bound water molecules to the amino and imino groups and the N7H of OG.[15] Despite the structural studies with these two enzymes, it is not known how the syn preference of OG relates to the efficiency of cleavage by either of these enzymes, and it is not known to what degree the ability to form hydrogen bonds affects the recognition of the damaged base. Similarly, it is not clear how critical the presence of the 8-oxo carbonyl group of the substrate base is in promoting leaving of the base, nor is it known the extent and site(s) at which the bases are protonated during the excision process.

To address these questions, we have designed a set of nonpolar OG analogs with varied conformational and leaving group abilities. Recently, a synthetic nonpolar isostere of OG, 2-chloro-4-fluoroindole (**1**), was shown to adopt the syn glycosidic bond conformation and code like T in polymerization reactions with Klenow fragment of *E. coli* DNA polymerase Pol 1 (KF exo-) (Figure 1B).[2,16] Because it was shown to effectively behave like OG in polymerase reactions, we sought to determine if this nonpolar isostere would mimic OG with the BER glycosylases that are involved in repair of OG, specifically Fpg and hOGG1. Although the compound mirrors the structure of OG closely, it lacks basic atoms and canonical hydrogen bonding groups, and so is ideal for testing the importance of such groups in *N*-glycosidic bond hydrolysis. To further test mechanisms of recognition, we synthesized and studied a series of three additional related nonpolar analogs (**2**−**4**) that alter the electron withdrawing abilities and/or the positioning of indole substituents. This would also allow for discernment of the relative importance of base shape, hydrogen bonding capacity and glycosidic bond conformation in substrate recognition and catalysis by Fpg and hOGG1.

Here we report the surprising finding that these two BER glycosylases can recognize and cleave nonpolar OG mimics, despite their lack of hydrogen bonding groups and basic atoms. Most remarkably, we observed for Fpg an approximate 2-fold *increase* in the rate of excision of analogs **2** and **3** relative to OG. In contrast, hOGG1 was able to recognize and initiate cleavage of three of the nonpolar analogs, but at levels that are significantly reduced compared to those for OG. The results suggest that Fpg does not require activation of the leaving group via hydrogen-bonding or base protonation in catalysis of base excision, whereas hOGG1 does gain an advantage from polar/hydrogen bonding atoms. Neither enzyme was strongly influenced by the syn/anti base conformational preference nor by the leaving group ability of the base. Finally, one analog (**4**) was resistant to hOGG1 enzymatic cleavage but was still found to bind tightly to DNA containing it paired opposite C. The results provide useful new insights into the mechanisms of damage repair by these two central enzymes.

RESULTS

Design, Synthesis, and Structure of OG Analogs 1−4.

The nonpolar OG analog nucleotides **1**−**4** (Figure 1B) were designed to test a number of questions regarding the mechanisms by which the glycosylases Fpg and hOGG1 recognize the damaged OG base and effectively release it from the C-1′ of deoxyribose in DNA. First is the question of the contribution of hydrogen bonding to enzyme recognition and base excision catalysis. The four analogs lack all the imino, amino, and carbonyl groups of OG; thus if hydrogen bonds are mechanistically important, the compounds would be expected to be very poor substrates. Second is the issue of whether the inherent syn preference of OG affects its ability to be processed by the enzymes; thus we included syn- (**1**, **2**) and anti-oriented

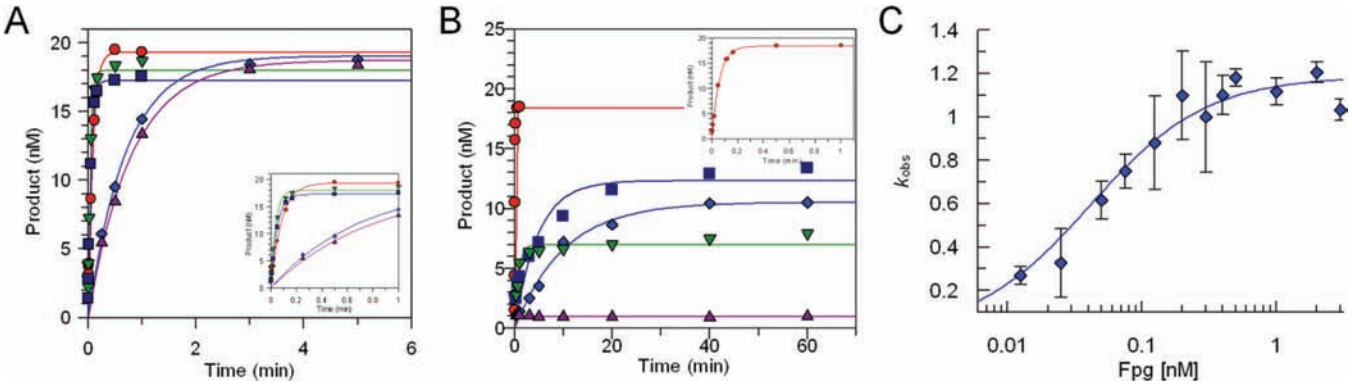


Figure 2. (A) Representative plot of extent of Fpg catalyzed removal of OG and analogs **1**−**4** as a function of time under conditions of single-turnover from 30 base pair duplexes containing OG:C (●), **1**:C (◆), **2**:C (■), **3**:C (▼), and **4**:C (▲). The inset shows region of Fpg experiments expanded to 1 min time scale. Experiments were performed using 200 nM Fpg and 20 nM duplex DNA at 37 °C. (B) Representative plot for extent of hOGG1 catalyzed removal of OG and nonpolar OG analogs **1**−**4** as a function of time under conditions of single-turnover from 30 base pair duplexes containing OG:C (●), **1**:C (◆), **2**:C (■), **3**:C (▼), and **4**:C (▲). The inset shows region of OG:C (●) fit expanded to 1 min time scale. Experiments were performed using 200 nM hOGG1 and 20 nM duplex DNA at 37 °C. (C) Measurement of Fpg affinity for a DNA duplex containing compound **1**:C (◆) pair. The dissociation constant K_d was determined from k_{obs} measurements as a function of [Fpg] performed under STO conditions with 5 pM duplex DNA.

(**3**, **4**) analogs to test this. The third question being addressed is how the base leaves with negative charge; 8-oxoG and related damaged bases (e.g Fapy and hydantoin lesions) might require the oxo group to accept electrons and stabilize charge buildup. Because the analogs **1**−**4** lack any basic atoms to accept the developing charge, they would be expected to be resistant to enzyme-mediated cleavage if such an acceptor were required. In a related issue, compound **1** has an electronegative chlorine adjacent to the nitrogen leaving atom of the indole, which might enhance leaving group ability by decreasing basicity of the anion; to control for this effect we can compare **1** and **4** (which also has a chlorine in the ring) to **2** (which has a similarly sized, but nonelectronegative, methyl group replacing chlorine).

Compounds **1** and **3** were known previously but neither has been studied as a BER glycosylase substrate. These were synthesized following published methods.[16,17] Compounds **2** and **4** are unknown in the literature; we developed synthetic approaches to them from commercial 4-fluoroindole. Schemes and methods are given in detail in the Supporting Information file. We established the glycosidic conformations of all four nucleosides by 2-D NOESY methods; strong nuclear Overhauser enhancements from the indole C-6 proton to the 5′ protons on deoxyribose signaled syn conformation, as expected from the 2-substitution, whereas similarly strong enhancements to the 1′ proton were consistent with a normal anti orientation of **3** and **4**, which lack this substitution. Details of the conformational studies are given in the Supporting Information.

Glycosylase Activity of Fpg with DNA Substrates Containing OG or Analogs 1−4. The glycosylase activity of Fpg was evaluated in vitro using a 30 base pair duplex containing a central X:C base pair (X = OG, **1**−**4**) (see sequence in the Experimental Section). The general method involved 5′-end-labeling the X-containing strand with [γ-^{32}P]-ATP and monitoring by denaturing PAGE the extent of strand cleavage at the X-nucleotide upon quenching with 0.5 M NaOH. The base treatment ensured that cleavage occurred at all abasic sites and that the ability of Fpg to remove the modified bases was evaluated, rather than an alteration in the associated β or δ-lyase activities. Under conditions of single-turnover (STO) where [Fpg] > [DNA], the reaction profiles exhibited first-order kinetics and proceeded to completion. The data were fit to a single-exponential equation to determine k_{obs}, which under these pseudo-first-order conditions allows for k_{obs} to be simplified to the rate constant, k_g, which includes all steps involving base excision (see Scheme 3 in the Supporting Information). Representative reaction progress curves of the reaction of Fpg with DNA duplexes containing OG:C, **1**:C, **2**:C, **3**:C, and **4**:C base pairs are shown in Figure 2A, and rate constants are listed in Table 1. Surprisingly, the rate constants for Fpg-catalyzed removal of **2** and **3** are approximately 2-fold greater (k_g = 23 ± 1 and 31 ± 3 min^{-1}, respectively) than the corresponding rate constant measured for OG removal (14 ± 3 min^{-1}), establishing these analogs as *better* substrates for Fpg than the natural substrate OG. The rate constants measured for the duplexes containing **1**:C and **4**:C (k_g = 1.6 ± 0.2 and k_g = 1.3 ± 0.1 min^{-1}) indicate that both chloro-containing analogs are good substrates for Fpg, albeit approximately 10-fold slower in this duplex context than OG (Table 1).

Table 1. Summary of Rate Constants for Base Excision (k_g) and Equilibrium Dissociation Constants (K_d) Determined for Fpg and hOGGl with Nonpolar Analogs

	rate constants (k_g) (min^{-1})		equilibrium dissociation constants (K_d) (nM)	
substrate	Fpg[a]	hOGG1[a]	Fpg[d]	hOGGl[e]
OG	14 ± 3	20 ± 3	0.46 ± 0.03	ND[f]
1	16 ± 0.2	0.09 ± 0.01[b]	0.04 ± 0.01	<0.003[g]
2	23 ± 1	0.20 ± 0.02[b]	0.56 ± 0.04	<0.003[g]
3	31 ± 3	1.0 ± 0.2[b]	0.8 ± 0.2	<0.003[g]
4	1.3 ± 0.1	NC[c]	0.05 ± 0.01	0.04 ± 0.02

[a]Rate constants determined at 37 °C under single-turnover conditions with OG and nonpolar analogs base paired to cytosine in 30 bp duplex. [b]These reactions did not go to completion, and these rates are based on the fits; see Figure 2B. [c]NC = no detectable cleavage (<5% after background correction at all time points). [d]K_d values determined from glycosylase reaction at 37 °C (see methods). [e]K_d values determined using EMSA at 25 °C (see methods). [f]ND = not determined. [g]Upper limit estimation: too tight to measure accurately, K_d is estimated to be lower than the [enzyme] used. Errors reported are the standard deviations of the average of at least three independent trials.

dx.doi.org/10.1021/ja208510m | J. Am. Chem.Soc. 2012, 134, 1653−1661

Glycosylase Activity of hOGG1 with DNA Substrates Containing OG or Analogs 1−4. The glycosylase activity of hOGG1 with the nonpolar OG analogs was carried out in a manner similar to that with Fpg. The hOGG1-catalyzed OG removal from an OG:C base pair in this duplex context is efficient (k_g = 20 ± 3 min^{-1}) and is similar to previous reports of the reaction of hOGG1 with this base pair in different duplex sequence contexts.[18,19] However, the reactions in this duplex under the analogous conditions with the nonpolar isosteres paired with C were considerably less efficient (Figure 2B and Table 1) and did not go to completion. The incomplete reactions were unanticipated because the same duplexes were converted completely to product by Fpg (Figure 1A). Rate constants listed in Table 1 were obtained from fitting of the observed progress curves. Clearly all of the nonpolar isosteres are removed less efficiently than OG. In the case of the duplex containing analog **3**, the measured rate constant (k_g = 1.0 ± 0.2 min^{-1}) is approximately 20-fold reduced compared to the corresponding rate constant with the OG:C substrate. The rates of excision of the corresponding duplexes with analogs **1** and **2** when base paired to C by hOGG1 were found to be approximately 200-fold reduced compared to those for OG (k_g = 0.09 ± 0.01 min^{-1} and k_g = 0.20 ± 0.02 min^{-1}, respectively). Albeit reduced compared to that for OG, the ability to observe *any* cleavage of these nonpolar isosteres was surprising due to the narrow substrate preference of hOGG1 (e.g., primarily OG and FapyG).[18−20] This suggests that the correct size and conformation of **1**, **2**, and **3** is enough to at least partially fulfill the OG-like lesion requirements in the active site to allow for observation of glycosidic bond cleavage. In contrast, no detectable cleavage was measured with the duplex containing analog **4**. This shows that 3-chloro-substitution on the indole ring is particularly deleterious to hOGG1-catalyzed cleavage.

Equilibrium Dissociation Constants of Fpg with Nonpolar Analog-Containing DNA Measured with Glycosylase Activity. Under single turnover (STO) conditions the dissociation constant (K_d) is equal to the enzyme concentration when the k_{obs} equals half of the k_g.[21] Therefore, it is possible to evaluate k_{obs} as a function of varied enzyme concentration under these conditions and determine the substrate K_d. We used this approach to determine the K_d of Fpg for DNA duplexes containing **1**−**4** or OG paired opposite C, at varied [Fpg] ranging from 12.5 pM to 20 nM with 5 pM duplex. Low DNA concentrations were used to ensure a [DNA] below the K_d value. Concentrations of Fpg greater than 20 nM did not result in significant increases in the k_{obs}. A representative plot of k_{obs} for the **1**:C duplex as a function of Fpg enzyme concentration is shown in Figure 2C, and binding data are given in Table 1. The data show that Fpg binds tightly to all four nonpolar analogs in this study. The K_d of Fpg for OG:C, **2**:C, and **3**:C is respectively 460 ± 30, 560 ± 40, and 800 ± 200 pM, with the enzyme showing similar affinity to DNA containing the analogs or OG. Significantly, the K_d with chlorinated analogs **1**:C and **4**:C was 41 ± 8 and 50 ± 10 pM (Table 1); thus Fpg binds over 10-fold more tightly to the DNA containing these latter analogs than to the natural substrate in this context. It is noteworthy that the two nonpolar isosteres that exhibit the highest affinity for Fpg are not the ones that are removed with highest efficiency. These results show that interactions within the Fpg base binding site are extremely sensitive to the electronic characteristics of the nonpolar base analog, and presumably also the natural damaged bases. Moreover, these results illustrate that characteristics that lead to high affinity enzyme recognition are not necessarily the same as those that lead to facile glycosidic bond cleavage, such as inherent lability of the base or base analog (vide infra).

Determination of Equilibrium Dissociation Constants (K_d) for hOGG1 with Nonpolar Analog-Containing Duplexes. Due to the poor or absent activity of hOGG1 with the nonpolar isostere duplexes, we were unable to measure dissociation constants using the glycosylase activity. However, the lack of activity hOGG1 with the duplex containing the nonpolar analog **4**, permitted determination of the relative equilibrium dissociation constants (K_d) using electrophoretic mobility shift assays (EMSA). Previously, we have shown that hOGG1 has a strong affinity for duplexes containing the noncleavable nucleotide FOG opposite C and therefore this duplex provides an excellent benchmark for high affinity hOGG1 binding.[22] EMSA studies were performed under conditions where [DNA-duplex] < K_d, to detect the [hOGG1-DNA complex] as a function of [hOGG1] and the resulting data were fit to a one-site binding isotherm. The K_d values for duplexes containing FOG:C (2 ± 1 nM) relative to **4**:C (0.04 ± 0.02 nM) indicate a higher affinity for the nonpolar analog **4** than the 2′F version of the natural substrate (Table 1). Indeed, the extremely high affinity of the **4**:C-containing duplex clearly shows that reduced binding is not the origin of the low activity with this particular analog. In the case of duplexes containing **1**:C, **2**:C, and **3**:C, the extent of conversion to product during the incubation period for the K_d experiments is relatively small (15−25%) such that we used EMSA to estimate the relative dissociation constants for these analog-containing duplexes as well. In these experiments, the duplexes containing analogs **1**:C, **2**:C, and **3**:C were completely bound to hOGG1, even at the lowest enzyme concentration tested (3 pM). This suggests an extremely high affinity for hOGG1 with K_d value that is lower than 3 pM. The observation of complete and tight binding of hOGG1 to these duplexes also shows that incomplete processing of these duplexes cannot be due to lack of binding to the analog duplex. An experiment with the OG:C substrate under the same conditions showed similar behavior; however, in this case the high affinity would be due to the binding to the product (∼85% product under these conditions). Importantly, these results reveal that reduced excision for analogs **1**, **2**, and **3** and lack of excision for **4** is not due to an inability of hOGG1 to recognize the nonpolar base isosteres within DNA.

Acid-Catalyzed Depurination of Nonpolar Analog 1−4 Containing DNA. The base excision mechanism for DNA glycosylases has been suggested to be a highly dissociative S_N1 mechanism involving the formation of an oxacarbenium ion intermediate based on kinetic isotope studies.[23,24] The ability of the base to be a leaving group, either in the protonated state or as an anion, would be an important factor in the efficiency of the reaction. To examine the susceptibility of the nonpolar analogs to acid-catalyzed depurination, the 5′-^{32}P-end-labeled ss DNA containing the analogs **1**−**4** were subjected to a modified Maxam−Gilbert G+A sequencing reaction utilizing piperidine-formate to initiate depurination, followed by standard piperidine treatment to reveal abasic sites.[25] The extent of depurination along the DNA was resolved with gel electrophoresis and quantified using storage phosphor autoradiography (see the Supporting Information). The relative extents of depurination of **1**−**4** as well as G and OG located at position 16 of the oligonucleotide sequence were normalized to the depurination of guanine at position 12 in each sequence

(Figure 3). The results reveal that OG is the most easily depurinated, yielding 2-fold more depurination than G under

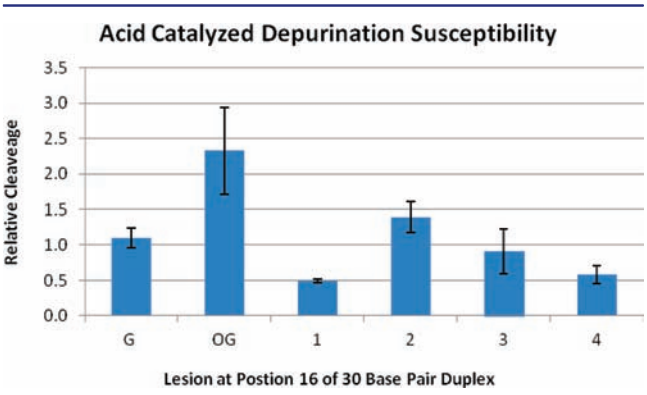


Figure 3. Histogram illustrating the extent of depurination of G, OG, and analogs **1**−**4** at position 16 of a 30 base pair duplex normalized to extent of cleavage of G at position 12. Quantitation of data from several experiments provided the relative % extents of cleavage: G, 1.1 ± 0.1; OG, 2.3 ± 0.6; **1**, 0.5 ± 0.1; **2**, 1.4 ± 0.2; **3**, 0.9 ± 0.3; **4**, 0.6 ± 0.1.

these conditions (Figure 3). The extents of acid-catalyzed depurination of analogs **2** and **3** were within error of that of G, suggesting that **2** and **3** have comparable sensitivity to G despite the lack of basic proton acceptors. Lastly, the chlorinated analogs **1** and **4** exhibited approximately half the susceptibility of G to acid-catalyzed depurination, suggesting that they are relatively resistant to depurination (Figure 3) despite containing electronegative substituents.

DISCUSSION

The study of the "GO" repair glycosylases hOGG1 and Fpg through the use of synthetically derived nonpolar nucleoside analogs of the OG damaged base provides a new approach to examine the recognition and catalytic properties of these two enzymes.[9,10] Previously, nonpolar adenine analogs were used only as a partner of OG to study the adenine glycosylase, MutY, revealing that the lack of possible hydrogen bonding interactions reduced the kinetic rate of base excision but did not obliterate substrate recognition.[7] In addition, Fpg was found to cleave OG faster when paired with nonpolar adenine analogs than from an OG:A base pair. This was attributed to the lack of hydrogen bonds between the bases in the target base pair leading to more facile flipping of the OG base into the Fpg active site.[7] Indeed, disruption of proper base pairing leading to increased base excision activity has been observed with several glycosylases.[26−28] The present studies are distinct in that the designed nonpolar analogs are mimics of the damaged base. The nonpolar isostere of OG, analog **1**, was earlier shown to be an effective mimic of OG in its ability both to adopt the syn orientation in DNA and to miscode as thymine in polymerase studies.[2] The preferred syn orientation of OG has long been suggested to be an important recognition feature utilized by glycosylases in lesion detection.[14] Here we find for the first time that a number of nonpolar OG analogs act as surprisingly good substrates for enzymatic deglycosylation, despite their lack of polar and basic atoms.

The activity of hOGG1 on this series of nonpolar analogs provides insight into how this enzyme accurately selects OG for excision. The enzyme has been shown to be highly selective for OG and formamidopyrimidine lesions within specific base pair contexts, preferably excising the lesions when base paired to cytosine.[19] Here we find that the nonpolar analogs **1**, **2**, and **3** can in fact be excised by hOGG1, but at a reduced efficiency compared to that of OG. Notably, the 3-chlorinated analog **4** was not cleaved by hOGG1 in DNA. The substantial reduction in cleavage rate that results with these analogs lacking polar groups suggests a substantial requirement of the enzyme for hydrogen-bonded interactions in its cleavage mechanism. A wealth of crystallographic studies of hOGG1 bound to an OG:C containing DNA duplex have revealed many of the intricacies of OG-specific recognition mediated by this enzyme (Figure 4A).[1] There is a critical OG-specific contact between the Gly42 carbonyl oxygen of hOGG1 and the NH7 of OG.[29,30] With G, this results in an unfavorable interaction between the lone pair on N7G and the backbone carbonyl O of Gly42. In the case of the nonpolar analogs **1**−**3**, a hydrophobic C−H group replaces NH7 of OG and therefore lacks the

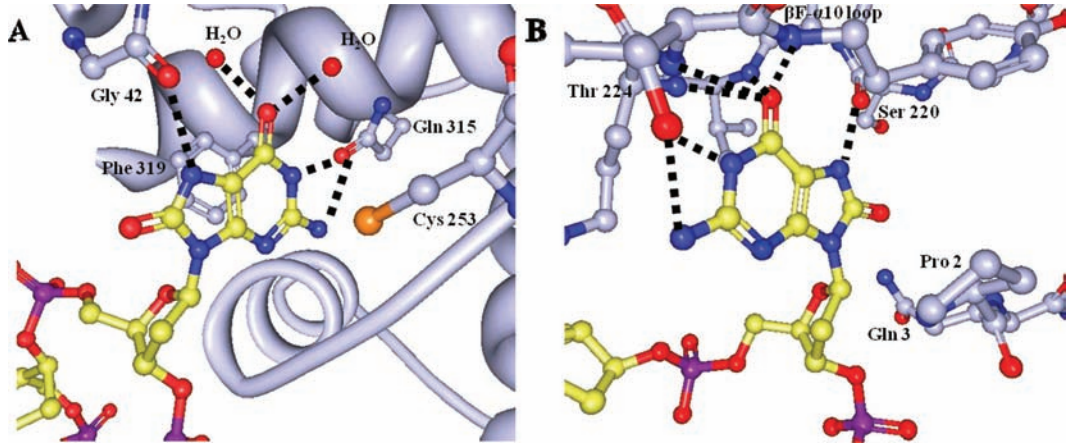


Figure 4. Hydrogen bonding interactions of OG observed in X-ray crystal structure of K249Q hOGG1 and E3Q Fpg bound to an OG:C containing duplex. DNA is shown in yellow with oxygen (red), nitrogen (blue), and phosphorus (purple). Important residues are labeled and shown in gray with oxygen (red), nitrogen (blue), and sulfur (orange). Hydrogen bonds are shown as dotted lines. (A) The view of the base-specific pocket of hOGG1, showing residues involved in hydrogen bonding interactions in recognition of OG. (B) The view of the base-specific pocket of Fpg, showing residues involved in hydrogen bonding interactions in recognition of OG. Images generated from the pdb files 1EBM (hOGG1) and 1R2Y (Fpg) from the Worldwide Protein Data Bank based on data from refs 13 and 15.

dx.doi.org/10.1021/ja208510m | J. Am. Chem.Soc. 2012, 134, 1653−1661

favorable hydrogen-bonding interaction; however, it also lacks the unfavorable interaction that would be present for G, thus providing a rationale for the higher activity relative to G. Another feature of hOGG1 leading to OG specific recognition is a complementary dipole−dipole interaction for OG created by Lys-249-NH_3^+ and Cys-253-S^- within the lesion-binding site.[30] This dipole−dipole interaction would be lacking in the active site with the nonpolar analogs, contributing to less efficient cleavage activity. Several other OG interactions observed in hOGG1 structures include stacking interactions with Phe319 and hydrogen-bonding to the Watson−Crick face (N1, 2-NH_2) by Gln315 (Figure 4A).[15] Consistent with the importance of Gln315-mediated hydrogen bonding, Q315A hOGG1 exhibits no OG glycosylase activity;[31] however, this apparent critical interaction is absent using the nonpolar analogs, establishing that this contact is not required to support base cleavage and may be more critical for base recognition (e.g., damaged G over A) and proper orientation within the active site pocket. In addition, our analog studies suggest that syn versus anti geometry does not have a discernible influence on OG recognition or catalysis by hOGG1, because syn and anti analogs were cleaved at a similar rate.

A particularly revealing result with hOGG1 was that one analog (**4**) was completely resistant to base cleavage by the enzyme. On the basis of the structural studies, the presence of a 3-chloro substituent on the indole at the position equivalent to purine NH7 likely provides an unfavorable interaction. Surprisingly, however, the measured dissociation constant with the **4**:C-containing DNA, showing considerably greater affinity than with substrate analog FOG, indicates that this steric clash, if present, does not prevent high-affinity binding to hOGG1. We surmise that the lack of cleavage may be due to incomplete or inappropriate engagement of the base within the OG-specific pocket compared to the case of OG and the other OG nonpolar isosteres (e.g., **1**, which is shaped very similarly to OG). Indeed, duplexes containing **1**:C, **2**:C, and **3**:C exhibited higher affinity for hOGG1 than **4**:C (Table 1). In addition, the incomplete processing of these three duplexes, despite high affinity, suggests considerable nonproductive binding of hOGG1 to these nonpolar analogs as well. These results are reminiscent of the structure of Q315F hOGG1 bound to an OG:C duplex, where the OG residue was found to be almost but not completely inserted in the active site pocket.[32] Notably, Q315F hOGG1 was unable to cleave OG from OG:C base pairs.[32] Similarly, structures of hOGG1 bound to a product analog show that in the absence of OG, key catalytic residues are improperly positioned, indicating a strong coupling between substrate recognition and catalysis.[33] Furthermore, recent studies from our laboratory have shown that hOGG1 exhibits a preference for a pyrrolidine transition state analog harboring a benzyl substituent to mimic the base over the analogous analog lacking the substituent.[34] The results herein further illustrate the high level of quality control utilized by hOGG1 to ensure proper base excision by requiring proper alignment of the target base within the active site to attain the transition state needed for cleavage. The presence of the bulky chloro substituent in **4** at the key recognition site may alter the base docking within the base specific pocket and positioning of active site residues, thereby thwarting catalysis. Alternatively, the nonpolar analogs may be tightly bound in an alternative "exo" site that has been shown in structural studies of hOGG1 with a nonspecific duplex to bind G.[30] Binding at this site would be nonproductive and provides an additional checkpoint to prevent inappropriate bases from accessing the OG binding site.

In contrast to hOGG1, Fpg was found to efficiently remove all of the analogs examined in this study when paired opposite C. Remarkably, Fpg cleaved the analogs **2** and **3** with an approximately 2-fold *increase* relative to OG. Analogs **1** and **4** were also good substrates for Fpg with cleavage rates only ∼10-fold reduced relative to OG:C. The ability of Fpg to catalyze the removal of all of the nonpolar analogs suggests that neither the presence of the OG polar groups nor any syn/anti conformational preference are critical for activity. We find that Fpg functions extremely well without making hydrogen bonds to the base and can flip base conformation with little cost. Indeed, Fpg seems to be more sensitive to the types of substituents on the indole ring rather than their orientation, because the chlorinated compounds were slower substrates than the nonchlorinated ones. In addition to efficient catalysis, the glycosylase was also able to capably bind to all four nonpolar analogs in DNA, again confirming that aberrant base recognition by this enzyme can occur regardless of hydrogen bonding groups or base conformation. Again the types of substituents rather than their positition influenced Fpg binding the most with the chlorinated analogs binding more tightly than the nonchlorinated version. However, high affinity did not translate to the most efficient removal, likely due to the additional complication of altering base lability by the addition of chloro substituents. The lack of influence of the nonpolar isostere conformation on activity with Fpg is also consistent with the observation in structural studies of OG and a carbacyclic-FapyG lesion located within the same binding pocket in syn versus anti conformations, respectively.[13,35]

The presence of atoms corresponding to the purine NH7 and O6 of OG are common features of many Fpg substrates, such as the formamidopyrimidines, and the hydantoin lesions, guanidinohydantoin and spiroiminodihydantoin.[5,18,36] In the structure of Fpg with OG, there is an OG-specific hydrogen bond between the main chain amide of Ser220 and the NH7 whereas G and OG-specific contacts are provided by several hydrogen bonds to both the 2-amino and 1-imino positions of OG (Figure 4B). Moreover, an important feature of lesion-containing structures of Fpg is ordering of the βFα10 peptide loop to position the main chain amides for hydrogen-bonding to the O6 atom of OG (Figure 4B). Despite this network of hydrogen-bonding and polar interactions, the current results show that they apparently add little, if anything, to stabilization of the transition state needed for facile base cleavage. Indeed, such interactions may be more important for OG base recognition rather than glycosidic bond cleavage and therefore may only be needed for processing OG and similar lesions.

The overall process leading to lesion excision mediated by DNA glycosylases requires multiple steps, including initial nonspecific binding to DNA, encounter with the lesion base pair, nucleotide flipping, and glycosidic bond cleavage.[1,37] Under single-turnover conditions, the rate constant k_g describes the process of ES to E-abasic site product, including all steps subsequent to the initial encounter of the enzyme with the lesion-containing base pair. The overall efficiency of excision of the nonpolar isosteres by Fpg will be related to the consequences of the modified substrate on all of these various steps in the process. Because nonpolar base analogs generally exhibit low pairing affinity for polar natural bases,[2,38] the current nonpolar analogs are expected to be more easily extruded from the helix than OG, and this would provide for an

increased excision rate compared to that for OG if all other steps in the base excision process remained unaltered. In addition, the facile extrusion of the nonpolar analogs **1**−**4** may explain the lack of observable dependence of the rates of base excision due to a substituent at the position corresponding to the 8-oxo group in OG. Indeed, with Fpg, analog **3**, which lacks a substituent that would favor the syn orientation, is the best substrate in this series, and is superior to OG. The presence of steric bulk at C8 (e.g., oxo or halogen) in purines destabilizes the duplex relative to a normal base,[39,40] suggesting that the 8-oxo substituent may aid in lesion recognition by facilitating lesion base-pair opening. Moreover, recent structural studies of a mutated version of Fpg lacking a portion of βFα10 peptide loop covalently trapped interrogating an OG:C base pair provided compelling evidence that the 8-oxo-substituent of OG provides the means for its interhelical detection.[41] Specifically, these studies illustrated that upon Fpg interrogation of OG:C base pairs, a steric clash of the 8-oxo group with the DNA backbone occurs that helps propel the OG out the helix into the base specific pocket. In the case of the nonpolar analogs, the lack of base pair hydrogen bonding likely removes the reliance on this enzyme-mediated steric destabilization provided by the 8-substituent of the syn nucleotide in the initial recognition event. Once lodged within the base specific pocket, there are no direct contacts of Fpg (or hOGG1) to the 8-oxo group of OG, which is also consistent with a lack of an effect of substituents in the indole at this position on glycosidic bond cleavage.

Another important aspect of base excision is the ability of the enzyme to stabilize the departing base, in either anionic or neutral protonated form. Indeed, kinetic isotope effect studies on glycosylases to date indicate highly dissociative transition states consistent with an S_N1-type mechanism.[24] In such a mechanism, the nature and stability of the leaving nucleobase anion would be expected to be an important factor. Indeed, such a direct correlation has been documented for thymine−DNA glycosylase.[42] Our data measuring the leaving group ability of the nonpolar analogs under acid-catalyzed depurination conditions revealed that the nonpolar isosteres are less easily depurinated than OG, suggesting that the analogs are not highly labile. However, they are surprisingly more easily removed than might be expected; for example, analogs **2** and **3** exhibit susceptibility to depurination that is comparable to that of G. Moreover, the nonpolar compounds are remarkably well excised by Fpg. Most notably, analogs **1** and **4** are the least susceptible to acid catalyzed depurination and are also the least efficiently removed of the nonpolar analogs by Fpg. However, the removal of these nonpolar analogs to any extent is surprising because at first glance an indole is a much poorer leaving group than OG. The carbonyl (at position 8 or 6) that can accept negative charge in OG is missing in indole. Indeed, the positioning of an array of amides in the βFα10 peptide loop of Fpg toward the O6 of OG suggests a role in stabilizing the developing negative charge at this position as the base is excised.[13] How, then, does indole leave in an S_N1-like mechanism? Clearly it cannot leave with full negative charge, as the indole anion is highly basic. We propose that, instead, the indoles are protonated on carbon, at C3, analogous to the well-known nucleophilicity of indoles at this position. If an acidic proton from an amino acid side chain or from water were nearby, this protonation could occur simultaneously with leaving of the base, thus stabilizing the transition state. Also consistent with this are our acid-catalyzed depurination data, which show that all the indoles can be depurinated, but that the less basic chlorinated cases are less well depurinated. In the enzyme active site several different sources for the protonation of OG have been previously proposed including the backbone amides of the βFα10 loop, Glu 3, Glu 174, Lys 57, and Lys 155.[13,43,44] Analysis of the consequences of mutations of the βFα10 loop and other amino acids within the active site on cleavage of OG versus the nonpolar analogs would be enlightening. Our expectation is that mutations of amino acids that participate in intrinsic aspects of catalysis of bond hydrolysis will reduce excision of both OG and the nonpolar analogs, whereas alterations of residues that participate in lesion recognition and extrusion will only reduce activity with OG.

■ CONCLUSION

The superior activity of Fpg relative to hOGG1 with the nonpolar substrates reveals distinct differences in mechanisms that are intimately related to their distinct biological functions requiring balance between accuracy and efficiency.[20] Clearly, the presence of polar groups, involved either in hydrogen bonding or in stabilizing the anion, are much more important for hOGG1 than for Fpg. This suggests that hOGG1 relies on hydrogen bonds within the active site to aid in leaving group departure and to couple correct base recognition with proper positioning of catalytic residues. In contrast, Fpg utilizes a flexible lesion recognition loop and active site to accommodate a wide variety of different types of damaged bases while still attaining the proper transition state for catalysis. To ensure accuracy of base excision, a glycosylase may use an initial recognition step to only allow specific bases to be extruded from the helix and additional catalytic checkpoints to prevent cleavage of inappropriate *N*-glycosidic linkages. The distinct differences revealed herein and taken together with the wealth of structural and biochemical information on these two enzymes suggest that hOGG1 uses both extrusion and catalytic checkpoints to ensure proper substrate base excision. In contrast, Fpg appears to rely more on the extrusion step to select the appropriate bases for excision. This illustrates that fine-tuning of overall similar catalytic strategies allows for broad versus specific substrate processing and the exquisite control needed to preserve the genome.

■ EXPERIMENTAL SECTION

General Synthetic Methods. 2′-Deoxynucleoside compounds **1** and **3** were synthesized following published methods.[16,17] New nucleoside analogs **2** and **4** were synthesized from 4-fluoroindole (see the Supporting Information for detailed methods and characterization data). 5′-DMT and 3′ phosphoramidite derivatives of all four analogs were prepared using standard methods (see the Supporting Information for details). 8-Oxo-7,8-dihydro-2′-deoxyguanosine (OG) phosphoramidite was purchased from Glen Research whereas 8-oxo-7,8-dihydro-9-(2′-deoxy-2′-fluoro-β-D-ribofuranosyl) guanine (FOG) phosphoramidite was synthesized as reported previously.[22] All compounds were characterized by ^{1}H and ^{13}C NMR and by high-resolution mass spectrometry. Conformations were measured by ^{1}H NOESY methods; details are given in the Supporting Information file. Oligodeoxynucleotides containing analogs **1**−**4** were synthesized on CPG solid support by standard automated methods on an ABI 392 synthesizer. They were purified by preparative polyacrylamide gel electrophoresis and characterized by MALDI-mass spectrometry (see the Supporting Information for details and data). DNA oligonucleotides containing standard phosphoramidites were purchased from Integrated DNA technologies. Oligodeoxynucleotides containing the OG and FOG phosphoramidites were synthesized at the University of Utah core facility. The oligonucleotides containing natural bases, FOG,

and OG were purified by ion-exchange HPLC. The 5′ radiolabeling was done using [γ-^{32}P]-ATP and T4 polynucleotide kinase following standard protocols. Gels were imaged using a Typhoon Trio scanner.

Substrate Preparation for Glycosylase Assays. The following duplex sequence was used in all glycosylase assays: 5′-CGA TCA TGG AGG CTA **X**CG CTC CCG TTA CAG-3′:3′-GCT AGT ACC TCC GAT **Y**GC GAG GGC AAT GTC-5′ where X = **1**−**4**, G, OG, or FOG, and Y = C. Glycosylase assays were conducted using this duplex with the 5′-hydroxyl of the X-strand labeled with γ-^{32}P-[ATP] (see the Supporting Information for details). For glycosylase assays, an additional nonlabeled X-containing strand was added to the labeled strand to produce a 5% labeled X-oligonucleotide. The was was then annealed with a 20% excess of the complement by heating to 90 °C for 10 min in annealing buffer (20 mM Tris−HCl, pH 7.6, 10 mM EDTA, and 150 mM NaCl) and then allowed to cool slowly overnight.

Enzyme Purification and Glycosylase Assays. Recombinant Fpg and hOGG1 were purified and active enzyme concentrations determined as described previously.[19,36] All enzyme concentrations listed are active enzyme concentrations. Single-turnover experiments, where [enzyme] > [DNA], were performed using the 30 base pair duplex sequence (vide supra) to evaluate the glycosylase activity of Fpg and hOGG1.[19,36,45] For reactions under single-turnover conditions in which the glycosylase reaction was too rapid to measure manually, a Rapid Quench Flow instrument (RQF-3) from Kintek was used. The enzyme was mixed with 20 nM final DNA duplex for time points ranging from 0.2 s to 1 min and quenched with 0.5 M NaOH. Denaturing polyacrylamide gel analysis provided separation of the 15-nucleotide DNA fragment arising from the product and the 30-nucleotide fragment originating from the substrate. Gels were imaged using storage phosphor autoradiography and band intensities quantitated to provide binding plots. For kinetic determination of the K_d values for Fpg with the lesion and analog containing DNA the substrate concentration was 5 pM of 100% label X-strand and the [Fpg] was varied between 20 nM and 12.5 pM. Values for K_d were determined by fitting the observed rate of glycosylase activity (k_{obs}) versus log [Fpg] to the one-site binding isotherm (GraFit 5.0).

Equilibrium Dissociation Constant (K_d) Measurements. Electrophoretic mobility shift assays (EMSA) were performed to determine the K_d values of hOGG1 for lesion and analog containing DNA similarly to previously reported.[7] Reaction volumes of 30 μL contained duplex DNA concentrations estimated at 1 pM. The reaction contained DNA ^{32}P 5′-end-labeled X-strand, 20 mM Tris−HCl pH 7.6, 10 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1 mg/mL BSA, and enzyme concentrations ranging from 2.3 μM to 3 pM.

Acid-Catalyzed Depurination Assays. A modified Maxam−Gilbert G+A sequencing reaction was performed as previously described.[7,25] Details are given in the Supporting Information.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

General synthetic methods and materials, synthetic methods for synthesis of nonpolar analogs (**2** and **4**), mass spectrometry characterization of oligonucleotides, enzyme purification and assay methods, PAGE analysis of acid-catalyzed depurinations, and NMR spectra. This information is available free of charge via the Internet at http://pubs.acs.org/.

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■ ACKNOWLEDGMENTS

We thank the National Institutes of Health (CA090689 to S.S.D. and GM072705 to E.T.K.) for support. A.K. acknowledges the KIT Faculty Research Abroad Fellowship Program.

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