Structural Characterization of Human 8-Oxoguanine DNA Glycosylase Variants Bearing Active Site Mutations*

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The human 8-oxoG DNA glycosylase (hOGG1) protein is responsible for initiating base excision DNA repair of the endogenous mutagen 8-oxoG. Like nearly all DNA glycosylases, hOGG1 extrudes its substrate from the DNA helix and inserts it into an extrahelical enzyme active site pocket lined with residues that participate in lesion recognition and catalysis. Structural analysis has been performed on mutant versions of hOGG1 having changes in catalytic residues but not on variants having altered 7,8-dihydro-8-oxoGuanine (oxoG) contact residues. Here we report high resolution structural analysis of such recognition variants. We found that Ala substitution at residues that contact the phosphate 5′ to the lesion (H270A mutation) and its Watson-Crick face (Q315A mutation) simply removed key functionality from the contact interface but otherwise had no effect on structure. Ala substitution at the only residue making an oxoG-specific contact (G42A mutation) introduced torsional stress into the DNA contact surface of hOGG1, but this was overcome by local interactions within the folded protein, indicating that this oxoG recognition motif is “hardwired.”

The internal components of aerobic cells are under chronic bombardment by electrophilic agents known collectively as reactive oxygen species (1). These ferocious oxidants typically arise endogenously as by-products of aerobic respiration but can also be produced via exposure to external sources such as ionizing radiation and exogenous carcinogens (2). Attack of reactive oxygen species on the genome is particularly deleterious because the resulting strand breaks and nucleobase lesions seriously compromise the informational integrity of DNA. The most troublesome site of reactive oxygen species attack on the genome is the C8 position of G because oxidation at this position gives rise to a genotoxic product, 7,8-dihydro-8-oxoguanine (oxoG)(3) (1, 3), which mispairs with A during processes of DNA replication, leading to G:C to T:A transversion mutations (4, 5).

Cells defend themselves against nucleobase lesions such as oxoG through the operation of the evolutionarily conserved base excision DNA repair pathway (6–8), the key components of which are DNA glycosylases, lesion-specific enzymes that scan the genome for aberrant nucleobases and catalyze their excision (9, 10). The resulting abasic DNA product is subsequently processed by downstream components of the repair pathway, ultimately resulting in restoration of the original DNA sequence (11–13). The DNA glycosylase responsible for recognition and removal of oxoG in bacteria is known as MutM (also Fpg) (14, 15), whereas that in eukaryotes is Ogg1 (16–19). Although MutM and Ogg1 perform essentially the same reaction, the two enzymes are completely unrelated in overall fold. Notwithstanding their distinct structures, MutM and Ogg1, like all known DNA glycosylases that act on single base lesions in DNA, recognize and cleave their cognate damaged nucleoside substrates by extruding them completely from the DNA helix and inserting them into an extrahelical active site pocket on the enzyme (20–23). DNA glycosylases belonging to each of the four known structural superfamilies of DNA glycosylases, two of which are represented by MutM and Ogg1, contain in their active site pocket conserved residues that serve critical roles in catalysis plus variable residues that are responsible for conferring the unique lesion recognition preferences of individual enzymes. Elucidating the roles that each of these residues serves in lesion recognition, discrimination, and excision is a key goal in the structural biology of base excision DNA repair.

Human Ogg1 (hOGG1), like all other members of the HhH-GPD (helix-hairpin-helix glycine/proline-rich loop terminated

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† The on-line version of this article (available at http://www.jbc.org) contains a supplemental table and Figs. 1–9.

‡ The atomic coordinates and structure factors (code 2NOB (H270A)), 2NOE (G42A), 2NOI (G42A), 2NOH (Q315A), 2NOF (Q315F*149), 2NOL (LRC*292), and 2NOZ (Q315F*292) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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The abbreviations used are: oxoG, 7,8-dihydro-8-oxoguanine; Ogg1, 8-oxoguanine glycosylase; hOGG1, human 8-oxoguanine glycosylase; LRC, lesion recognition complex; DXL, disulfide cross-linking; Kd, equilibrium binding constant; MOPS, 4-morpholinepropanesulfonic acid.
by an aspartic acid) superfamily (24, 25) to which it belongs, contains an invariant active site Asp residue (Asp-268 in human Ogg1; mutated to Asn in Fig. 1A) that is absolutely required for catalysis of base excision (26). The catalytic nucleophile, Lys-249, is essential both to initiate excision of oxoG and to catalyze a subsequent cascade of reactions leading to strand scission by conjugate elimination on the 3’-side of the lesion (lyase cascade). Mutation of Asp-268 and Lys-249 to Asn and Gln, respectively, generate variant forms (D268N and K249Q) that lack base excision activity but retain the ability to bind oxoG-containing DNA tightly and specifically (26, 27); x-ray crystal structures of lesion recognition complexes (LRCs) comprising K249Q and D268N hOGG1 bound to oxoG-containing DNA have yielded valuable insights into the particular features of oxoG recognition and discrimination in this system (22, 26). Of particular interest in this regard are residues that hydrogen bond directly to the oxoG nucleobase, Gly-42 and Gln-315, and His-270, which hydrogen bonds to the 5’-phosphate of the oxoG lesion (Fig. 1A). Crystallographic evidence indicates that the His-270/5’-phosphate contact is formed late in the process of extruding the target nucleobase from DNA (28) (see also below). Ala point substitutions at Gln-315 and His-270 have a deleterious effect on binding and hence catalysis (29), whereas Gly-42 has not been subjected to mutagenic analysis.

Here we report the characterization of variant forms of hOGG1 having point mutations at Gly-42, His-270, and Gln-315. These studies support the notion that the reported H270A mutation merely diminishes binding by removing functionality that stabilizes the lesion recognition complex. This work also revealed that the positioning of Gly-42 to contact N7-H of oxoG was retained despite the mutational introduction of steric repulsion, indicating that the Gly-42 amide carbonyl is “hard-wired” to provide maximal discrimination of oxoG from its normal relative, G. Finally we report the identification of a point substitution at Gln-315 that slightly mispositioned oxoG in the active site but completely ablated catalysis of base excision, suggesting that the transition state for this S\textsubscript{1,1}-like displacement is geometrically demanding.

**EXPERIMENTAL PROCEDURES**

**Cloning and Protein Preparation**—The fragment of hOGG1 used for crystallographic characterization (22) was overproduced in bacteria from a vector encoding residues 12–327 of the human Ogg1 cDNA cloned into the pET30a vector (Novagen) using the restriction sites EcoRI and HindIII. Fragments used for electrophoretic mobility gel shift assays were overproduced in *Escherichia coli* by cloning a PCR fragment of hOGG1 containing residues 12–345 into the pET30a vector using the BamHI and XhoI restriction sites. Mutagenesis was performed via the megaprimer method (30). All new constructs were sequenced throughout the hOGG1-coding sequence. All proteins were overexpressed and purified by the published method (26).

**DNA Preparation, Disulfide Cross-linking, and Crystallization**—Oligomers used for electrophoretic mobility shift assays, 5’-GGATAGTGCTCAAXGTTACTCGAAGC-3’, where the X indicates either G or oxoG, and its complement 5’-GCTTC-GAGTAACCTGGACACTATCC-3’, were synthesized on an ABI Expedite 8909 DNA synthesizer using standard methods and purified by 15% urea-PAGE. For crystallization, DNA oligomers 5’-AGCGTCCA(xoxoG)GTCTACC-3’, 5’-AGCGTCCAGGTCTACC-3’, and 5’-TGGTAGACCTGGAGGC-3’ were also synthesized on an ABI Expedite 8909 DNA synthesizer using standard reagents. The DNA oligomers 5’-TGGTAGACCTGGAGGC-3’ (proximal cross-link to Cys-149) and 5’-TGGTAGACCTGGACGC-3’ (distal cross-link to Cys-292), where the underlined position denotes the site of modification with the thiol-bearing tether, were synthesized and functionalized with a two-carbon linker and a four-carbon linker, respectively, as described previously (31, 32). Phosphoramidite derivatives of oxoG, O\textsuperscript{4}-triazolyl-dU, and O\textsuperscript{6}-phenyl-dl were purchased from Glen Research. DNA was purified by 20% urea-PAGE and dissolved in 10 mM Tris, pH 8.0, 1 mM EDTA. Non-cross-linked and cross-linked protein-DNA complexes were formed and crystallized as described previously (22, 28). Briefly the cross-linked complexes were formed by mixing duplex DNA with 2-fold molar excess protein and incubating at 4 °C for several days. The cross-linked complexes were purified away from the unreacted DNA and protein by Mono Q chromatography (GE Healthcare), buffer-exchanged and concentrated to 280 μM, and crystallized by the hanging droplet vapor diffusion method at 4 °C. Crystals appeared within a few days in well solution containing 100 mM sodium cacodylate, pH 6.0 or 6.5, 50–200 mM CaCl\textsubscript{2}, and 12–15% polyethylene glycol 8000. Crystals were allowed to grow for several days, transferred to a cryoprotectant solution containing mother liquor supplemented with 25% glycerol, and then frozen in liquid nitrogen for data collection.

**Data Collection and Refinement**—X-ray data were collected at 100 K at the X25 beamline of the National Synchrotron Light Source and 19-ID beamline of the Advanced Photon Source. All diffraction data were processed using HKL2000 (33). Data collection statistics are summarized in the supplemental table. Co-crystal and cross-linked structures were determined by molecular replacement using the coordinates of the protein from the structure of the K249Q hOGG1 and cross-linked recognition complex, respectively, as the initial search model during refinement using Crystallography & NMR System (CNS) (34). Residues involved in catalysis and DNA interaction were omitted from the initial search model. A partial model was generated by rigid body fit, energy minimization, and simulated annealing. Electron density for the omitted residues was clearly observed in an σA-weighted (35) *F\textsubscript{o} - F\textsubscript{c}* map. Iterative rounds of energy minimization, simulated annealing, and grouped B-factor refinement were performed. Subsequent rounds of energy minimization, simulated annealing, and individual B-factor refinement were performed. Simulated annealing omit maps were used to reduce model bias. Manual adjustments to the model were made using Quanta2000 (Accelrys). Water molecules were added to the model using automated (CNS) and manual inspection of the difference maps. Density for some amino acid side chains was occasionally incomplete, and in these cases, only the ordered portion of the side chain was built into the model. Renderings of the structures were generated using PyMOL (37).
**hOGG1 Active Site Mutations**

**Electrophoretic Mobility Shift Assay**—A 25-mer oligonucleotide containing a single centrally located oxoG residue was radioactively labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (PerkinElmer Life Sciences) and then annealed to a complementary strand with C opposite oxoG. A normal duplex, whose labeled strand contained a G instead of oxoG, was prepared in an identical manner. The duplex was then incubated with serial dilutions of protein (residues 12–345) in binding buffer containing 50 mM Tris, pH 7.4, 100 mM NaCl, 0.5 mM EDTA, 0.5 mM β-mercaptoethanol, and 5% glycerol. In the case of wild-type and G42A hOGG1, the protein contained the K249Q mutation to prevent base excision from taking place. The protein-DNA mixture was incubated for 30 min at room temperature. Samples were loaded on a prerun 10% nondenaturing polyacrylamide gel in 0.5× Tris borate-EDTA buffer and electrophoresed for several hours at room temperature. Bands were quantified using ImageQuant TL (GE Healthcare) and plotted in Kaleidagraph (Synergy Software) to determine $K_d$ values.

**Circular Dichroism**—CD spectra were obtained on a Jasco J-715 spectropolarimeter in buffer containing 10 mM potassium phosphate, pH 7.5, and 100 mM KCl in a 0.1-cm cell at 13 °C. The temperature of the sample cell was regulated by a Jasco PTC-348 Peltier temperature control. Thermal denaturation experiments were performed by raising the cell temperature from 13 to 70 °C at 1 °C/min and monitoring the CD signal at 222 nm. Melting points were determined by taking the maximum of the first derivative plot.

**Cross-link Time Course Assay**—Proteins were mixed in a 2:1 ratio at 4 °C with 10 mM DNA containing oxoG on one strand and equipped for proximal cross-linking (C2 tether) on the opposite strand in buffer containing 20 mM Tris, pH 7.4, 100 mM NaCl, and 1 mM EDTA. Aliquots were removed at various time points and quenched by addition of methyl methanethiosulfonate to 5 mM. The samples were analyzed on 4–12% SDS-PAGE gels in 1× MOPS buffer. Gels were stained with Coomassie Blue, and band intensities were quantified with ImageQuant TL (GE Healthcare), then plotted in Igor Pro (Wavemetrics), and fit to exponential curves.

**RESULTS**

**H270A**—In all structures of hOGG1 LRCs, His-270 is hydrogen-bonded to the phosphate on the 5'-oxygen of oxoG; this interaction is reinforced by further hydrogen bonding of His-270 to Asp-322 (Fig. 1A and supplemental Fig. 1A). On the other hand, in the structure of hOGG1 bound to DNA containing an abasic site analog (tetrahydrofuranyl; supplemental Fig. 1B), the His-270 side-chain imidazolium group is disengaged from the 5'-phosphate and instead is engaged in an aryl/aryl interaction with Phe-319 while maintaining contact with Asp-322 (38); this interaction appears to be mutually exclusive with oxoG insertion into the lesion recognition pocket as the two His-270 orientations require different side-chain rotamers of Phe-319 (compare supplemental Fig. 1, A and B). The His-270/Phe-319 interaction is also observed in the structure of hOGG1 not bound to DNA (unliganded) (39). His-270 is likewise diverted from the 5'-phosphate through interaction with Phe-319 in the structure of hOGG1 bound to DNA containing an extrahelical G residue bound to an exosite flanking, but outside, the active site pocket (28). Together these data have led to the suggestion (28) that the disruption of the His-270/Phe-319 interaction and the subsequent establishment of Phe-319/oxoG and His-270/5'-phosphate contacts are a late event in the process of base extrusion and insertion into the active site pocket. We were thus interested in probing the structural effects of removing the His-270 side-chain imidazolium group (by mutation to Ala) on substrate recognition by hOGG1. It has been
shown previously that the H270A mutation substantially decreases the binding affinity of hOGG1 for oxoG-containing DNA and slows glycosylase activity 160-fold under conditions of substrate saturation (29). On the other hand, the introduction of the H270A mutation diminishes the affinity of hOGG1 for tetrahydrofuranyl-containing DNA by less than 2-fold, consistent with the structural findings that His-270 does not contact DNA in this complex.

To overcome the low affinity of H270A hOGG1 for DNA, we used the intermolecular disulfide cross-linking (DXL) strategy shown previously to stabilize otherwise unstable hOGG1-DNA complexes (28), thereby enabling detailed structural characterization. In one implementation of this strategy (Fig. 1B, proximal cross-linking), a Cys residue engineered into hOGG1 by point mutation of Asn-149 engages in disulfide bond formation with a thiol-bearing tether introduced at the N\textsuperscript{4} position of the cytosine opposite oxoG. The protein constructs used throughout this work, unless otherwise noted, contained an additional mutation (K249Q) known to abrogate catalysis while preserving specific oxoG recognition. We first examined the effect of mutating His-270 to Ala on the rate of DXL. Under conditions in which both hOGG1 and the oxoG-containing DNA duplex were present well above $K_{d}$, Ala substitution at position 270 was found to slow the rate of DXL by ~5-fold (supplemental Fig. 2).

Because cross-linking in this system is dependent upon extrusion of the target oxoG from DNA, the modest effect of the mutation on DXL suggests that His-270 does not play a significant role in promoting base extrusion.

H270A/K249Q/N149C hOGG1 (hereafter designated H270A hOGG1), disulfide-cross-linked to the same oxoG-containing duplex as was used to solve the hOGG1 LRC structure (22), readily yielded crystals isomorphous to those of the corresponding LRC\textsuperscript{*149} (asterisk denotes cross-linking to the Cys at the indicated position). Using phases from the LRC\textsuperscript{*149} as initial phases for H270A’ followed by rounds of refinement, we solved the structure of the cross-linked H270A’ complex to 2.10 Å (supplemental table). In this structure (Fig. 2, A and B), the oxoG nucleobase is fully inserted in the lesion recognition pocket just as in the LRC\textsuperscript{*149} structure. Indeed the C\textsubscript{\gamma} atoms in the H270A’ structure superimpose on those of the LRC\textsuperscript{*149} with a root mean square deviation of 0.15 Å (Fig. 2B), indicative of close similarity between the two; the DNA conformation is also essentially identical (not shown).

Furthermore there is excellent correspondence between the positions of side chains in the active site region with the exception of Asp-322 (Fig. 2B). In LRC\textsuperscript{*149}, Asp-322 hydrogen bonds to His-270; when His-270 is mutated to Ala, the side chain of Asp-322 retracts slightly from the active site and participates in a network of hydrogen bonding interactions with a cluster of four ordered water molecules that are also in contact with Asp-268 and the 5’-phosphate and 5’-oxygen of oxoG (Fig. 2B). Notwithstanding this one accommodation in the structure, the marked overall similarity between the H270A’ and LRC\textsuperscript{*149} complex structures indicate that the deficiency in binding to, and base excision of, oxoG-containing DNA results from removal of an important phosphate contact between His-270 and the 5’-phosphate and not from some structural reorganization of the protein or protein-DNA complex. A binding deficit of at least 22.5-fold observed in gel shift assays corresponds to an energetic contribution of at least 1.9 kcal/mol for this contact.

$G42A$—Gly-42 is positioned at the base of a loop that sits directly above the active site pocket, which positions the Gly-42 main-chain carbonyl to accept a hydrogen bond from N\textsuperscript{7}-H of 8-oxoG (Fig. 1A). Gly-42 is the only residue in hOGG1 that directly reads out the structural difference between G and oxoG, both through its hydrogen bonding to N\textsuperscript{7}-H and through a dipole/dipole interaction between the Gly-42 C-terminal amide and the oxoG C8=O/N7-H ureide system (28). With G, the Gly-42 carbonyl would suffer a repulsive interaction with the lone pair of electrons on N\textsuperscript{7} of G, and the dipole/dipole interaction would likewise be repulsive rather than attractive. In all DNA-bound structures of hOGG1 solved to date and most importantly in the structure of the unliganded protein, Gly-42 and the amino acids flanking it adopt nearly identical conformations even though other residues in the active site undergo substantial ligand-dependent structural changes (supplemental Fig. 3).

The values of the backbone torsion angles at Gly-42 lie outside the permissible region for any amino acid residue other than Gly (supplemental Fig. 4), suggesting that Gly may be uniquely capable of mediating the interaction with oxoG at position 42. To test this notion, we mutated Gly-42 to Ala. Replacement of a hydrogen atom at C\textsubscript{\gamma} (Gly) with the much larger methyl group (Ala) would introduce a substantial steric clash with the carbonyl of residue 42, and this repulsive interaction would have to be alleviated through some sort of backbone conformational reorganization; it was unclear, however, whether these structural accommodations would compromise the stability of hOGG1 or its hydrogen bonding interaction with oxoG. The wild-type and G42A forms of hOGG1 forms gave identical CD spectra at room temperature, signifying that both were folded (data not shown). We then used CD to determine the thermal denaturation temperatures of wild-type ver-
sus G42A hOGG1 and found that the mutation diminished the thermal stability of hOGG1 by the relatively modest amount of 4.6 °C (wild-type hOGG1 melts at 42.4 °C; supplemental Fig. 5).

Next we measured the affinity of G42A hOGG1 for oxoG-containing DNA using electrophoretic mobility shift assays. For the G42A/K249Q mutant form of hOGG1, we determined an equilibrium binding constant ($K_d$) of $15 \pm 2 \text{ nM}$, whereas that for the K249Q hOGG1 (wild type at position 42) was $16 \pm 3 \text{ nM}$ under the same conditions (data not shown). Taken together, these biochemical results demonstrate that the introduction of a local steric clash at position 42 diminishes the thermal stability of hOGG1 but has no effect on the affinity of its interaction with lesion-containing DNA.

To elucidate the structural impact of the G42A mutation in hOGG1, we determined the structure of the corresponding DNA complex by x-ray crystallography. G42A/K249Q hOGG1 was co-crystallized with the oxoG-containing duplex, and the structure was refined to 2.2 Å (supplemental table); in this case, disulfide cross-linking was not necessary to obtain crystals. The co-crystal structure revealed the Ala mutant complex to be nearly identical with its wild-type counterpart (22) (Fig. 3). Strikingly the main-chain carbonyl of Ala-42 remains in the eclipsed conformation observed in the complex with a bound oxoG, although the eclipsing interaction is not as severe ($C\alpha - C\beta$ bond of Ala-42 is nearly eclipsed with its own carbonyl ($C\beta C\alpha C = 0$ torsion angle $=-11.3^\circ$), an unfavorable situation that places the Ala-42 conformation slightly outside the allowed region for a non-Gly residue in the left-handed $\alpha$-helix conformation (supplemental Fig. 4).

We next considered the possibility that the energetically unfavorable conformation of Ala-42 might be stabilized by the interaction of Ala-42 with oxoG and might therefore be alleviated by structural adjustment in the absence of oxoG. This possibility was of particular interest to us because such local structural changes might even enable the protein to accept G into the lesion recognition pocket, something wild-type hOGG1 does not do (28). To test this, we introduced the G42A mutation into a previously described complex, the so-called G-interrogation complex, staged to present an extrahelical G to the lesion recognition pocket of hOGG1; the structure of the G-interrogation complex revealed that the extrahelical target G residue is not bound in the active site but instead lies against a nearby exosite (28). We therefore introduced the N149C mutation into G42A/K249Q hOGG1 to give G42A’ hOGG1, and we cross-linked this hOGG1 variant to a DNA duplex containing a target G (Fig. 1B). This G42A’ G-interrogation complex was crystallized, and its structure was refined to 2.35 Å (supplemental table). In the structure of the G-interrogation complex of G42A’ hOGG1 (supplemental Fig. 6), G resides in the same exosite flanking the active site first identified with the corresponding Gly-42 complex, although the backbone conformation at the extruded G may be slightly different in the two. Importantly the main-chain carbonyl of Ala-42 remains in the eclipsed conformation observed in the complex with a bound oxoG, although the eclipsing interaction is not as severe ($C\beta C\alpha C = 0$ torsion angle $=-22.6^\circ$).

The propensity of residue 42 to retain its conformation despite the introduction of a repulsive steric interaction suggests that the polypeptide chain is buttressed in this region. To gain insight into this issue, we analyzed the interactions of the loop containing residue 42 with the surrounding elements of protein structure. Indeed the loop engages in extensive hydrogen bonding contacts involving both main-chain and side-chain functionality (Fig. 3, A and B) plus ordered water molecules, which together rigidify residues 35–43 into compact organization. Consequently conformational adjustment at position 42 to place the residue in a more favorable region of Ramachandran space would require disruption of this network of interactions.

These results establish that the protein conformation surrounding Gly-42 is hardwired to present the Gly-42 carbonyl to the oxoG recognition pocket in a manner that is predisposed toward recognition of oxoG and rejection of G. Whereas other
side chains that make up the lesion recognition pocket, notably Gln-315 and Phe-319, change their conformation upon insertion of an oxoG into the pocket (supplemental Fig. 3). Gly-42 does not. These other residues are not involved in discriminating oxoG from G, whereas Gly-42 is. We speculate that this hardwiring of Gly-42 is evolved to maximize the ability of hOGG1 to interact productively with an oxoG lesion while rejecting the normal nucleobase G from admission to the active site pocket.

Q315A—Besides Gly-42, Gln-315 is the only other residue in the lesion recognition pocket that directly hydrogen bonds with the oxoG nucleobase. The side-chain amide of Gln-315 projects out from the deepest recess of the lesion recognition pocket and contacts the Watson-Crick face of oxoG; the carbonyl oxygen directly contacts N1 and N2, and the amide-NH$_2$ contacts O$_6$ through the intermediacy of a bound water molecule (Fig. 4A). As noted above, these contacts provide no discrimination of oxoG versus G, because both nucleobases contain the same functionality on their Watson-Crick face, but the contacts do enable discrimination of oxoG versus A, C, and T. Previous biochemical studies have established that replacement of Gln with Ala reduced the affinity of the enzyme for oxoG-containing DNA by 7-fold and diminished glycosylase activity to 62% of wild-type levels (29).

We determined the x-ray co-crystal structure of the catalytically inactivated Q315A mutant version of hOGG1 (Q315A/K249Q hOGG1) in complex with oxoG/C-containing DNA. In this case, DXL was not necessary to obtain crystals. The structure, refined to 2.0-Å resolution (supplemental table), revealed two highly ordered waters in the active site occupying the positions vacated by the oxygen and nitrogen heteroatoms on the Gln side chain (Fig. 4A, Wat A and B). Indeed these two water molecules appear to make the same contacts to the oxoG and surrounding protein as were made by the heteroatoms of the molecules. We speculate that this hardwiring of Gly-42 is evolved to maximize the ability of hOGG1 to interact productively with an oxoG lesion while rejecting the normal nucleobase G from admission to the active site pocket.

Q315F Proximal Cross-link—In the case of MutM, we have found that destabilization of oxoG binding to the lesion-recognition pocket enables the formation and structural characterization of early intermediates in the search/extrusion/base-excision pathway. The lack of structural similarity between hOGG1 and MutM raises the question of whether there exists any commonality to the search/extrusion/base excision pathway used by the two enzymes. The strategy used to destabilize extrahelical oxoG in the case of MutM is not applicable to hOGG1 because the structure of hOGG1 is different from MutM. We reasoned that introduction of a bulky side chain into the hOGG1 lesion recognition pocket might sterically interfere with insertion of oxoG into the pocket. Of the several candidate positions, we chose Gln-315 because (i) it is in direct contact with the Watson-Crick face of oxoG and is therefore of the correct size to interact productively with oxoG; (ii) it projects directly toward the edge of the nucleobase, a disposition that accentuates the effect of an increase in size; and (iii) it is farther away from the remainder of the DNA than any other residue in the pocket (Fig. 1A). We therefore constructed and expressed a mutant version of hOGG1 in which Gln-315 was changed to Phe (Q315F hOGG1) and measured the affinity of the mutant protein for non-lesion-containing and oxoG-containing DNA duplexes (data not shown). The $K_D$ of Q315F hOGG1 for the oxoG-containing 25-mer duplex was 2.4 ± 0.6 μM as compared with 3.0 ± 0.7 μM for the normal 25-mer having G in place of oxoG. Thus, the Q315F mutation essentially abrogates specific recognition of oxoG.

The lack of specific binding of Q315F hOGG1 to DNA necessitated the use of DXL to crystallize and structurally characterize this complex. We therefore introduced the N149C mutation into Q315F hOGG1, cross-linked the double mutant protein (Q315F*149 hOGG1) to oxoG-containing DNA, and purified the resulting complex. Because this complex retains all of the amino acid residues required for catalysis and is therefore in principle capable of processing oxoG-containing DNA, we assayed it for oxoG cleavage activity. Electrophoretic analysis of the DNA disulfide-cross-linked to Q315F*149 hOGG1 in solution at 4 °C revealed a modest but clearly detectable extent of time-dependent cleavage at the position of the oxoG; ~4% of the cleavage was observed after 24 h (data not shown). Because our MutM-DNA complexes tended to crystallize rapidly, we thought it should be possible to crystallize the complex of Q315F*149 hOGG1 disulfide-cross-linked to oxoG-containing DNA before cleavage had proceeded to a significant extent and then cryogenically protect the crystals from further cleavage. Crystals of the Q315F*149 hOGG1-oxoG DNA complex grew rapidly and were cryogenically protected as soon as they

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5 A. Banerjee, S. Jiralerspong, and G. L. Verdine, unpublished results.
The Q315F proximal cross-linked complex. A, active site view of the Q315F\textsuperscript{+49} complex (Phe-315 in magenta and other protein side chains in teal, DNA in gold, and oxoG in red). The hydrogen bonding interaction between His-270 and the 5′-phosphate of oxoG is denoted by dashed lines. Note that although Phe-315 is rotated away from the active site, it is not stacked with Phe-319. B, superposition showing the active site of the Q315F\textsuperscript{+49} complex (colored as in A) and the structure of hOGG1 bound to DNA containing an extrahelical G in the exosite (G-complex; Protein Data Bank code 1YQK (28)) (protein and DNA in white). C, superposition showing the active site of the Q315F\textsuperscript{+49} complex represented as in A and B with the proximally cross-linked lesion recognition complex (LRC\textsuperscript{+49}, protein, and DNA in white).

Other features of interest in this structure include the engagement of His-270 in a hydrogen bond with the 5′-phosphate (Fig. 5A). Neither His-270 nor the 5′-phosphate are in the positions they occupy when an oxoG is fully inserted into the active site (Fig. 5C), but they nonetheless contact each other. This particular contact is disengaged in the structure of the G-complex, which has an extrahelical G in the exosite, suggesting that the contact is established after the nucleobase exits the exosite but before it is fully inserted into the lesion recognition pocket. Also of interest is that fact that the catalytic nucleophile, Lys-249, is not engaged in an interaction with the Cys-253 (28). A close interaction between these two active site residues has been observed in the x-ray structure of a hOGG1 LRC containing Lys-249 but having another catalytically essential residue, Asp-268, mutated to Asn (Fig. 1A and Ref. 26). High level computational studies (28) have indicated that this interaction takes place between Lys-249 in the protonated form (NH\textsubscript{3}+) and Cys-253 in the deprotonated (S−) form. The computational results indicate that the dipole resulting from this solvent-shielded ion pair makes an important contribution to specific recognition of oxoG.

Finally, the Asp-268 carboxyl, which is required to stabilize the incipient oxocarbonium ion during the base excision step, is rotated in a way that results in its being drawn away from the sugar moiety of the substrate. This conformation is different from that observed in any hOGG1 lesion recognition complex.

Q315F Distal Cross-link—The particular cross-linking site used in our previous work and here to study the effects of the H270A, Q315F, and G42A mutations favors extrusion of the target nucleobase from DNA because the cross-link itself occupies the space vacated by the extruded nucleoside and therefore enforces the extrahelical state by sterically obstructing reannealing. To remove this bias, we sought an alternative cross-linking site that would lie in the duplex at some distance from the site of the lesion, thereby eliminating any direct influence of the cross-link on the extra- versus intrahelical disposition of the target oxoG nucleoside. Through inspection of the hOGG1 LRC, we identified what appeared to be a suitable distal cross-linking site. An adenine residue located on the non-lesion-containing DNA 4 base pairs away from the target oxoG/C pair possesses a tether attachment point (exocyclic N6 amine nitrogen) that lies within 10 Å of Ser-292 on hOGG1 (distal cross-linking site, Fig. 1B). Starting with the catalytically inactive K249Q mutant of hOGG1, we introduced the S292C mutation required for cross-linking and analyzed the covalent coupling of this double mutant protein to DNA containing a thiol-tethered adenine at the relevant position in DNA. K249Q/S292C hOGG1 was found to undergo efficient disulfide cross-linking to an oligonucleotide containing a four-methylene tether at the selected adenine residue; cross-linking was considerably less efficient with oligonucleotides having three- or two-methylene tethers (data not shown). We crystallized this control, cross-linked lesion recognition complex, designated LRC\textsuperscript{+292}, and refined the structure to 2.57 Å (supplemental table). Inspection of the structure revealed that it is virtually identical in all important respects to LRC and LRC\textsuperscript{+49}; no substantial cross-link-induced structural perturbation was evident at the site of cross-linking.

Having validated the S292C cross-linking strategy, we set out to apply it to the investigation of the influence of the Q315F mutation in hOGG1 on its extrusion preference toward a target oxoG lesion. In this instance, we decided to preserve all active site residues required for catalysis, introducing only the Q315F point mutation and the S292C mutation required for cross-linking (Q315F/S292C hOGG1) (Q315F\textsuperscript{+49} hOGG1). As had been done with the Q315F\textsuperscript{+49} oxoG DNA complex, we ana-
lyzed DNA cleavage in the cross-linked Q315F*292-oxoG DNA complex and found that the latter also underwent slow time-dependent DNA cleavage at the oxoG position in solution with 4.6% cleaved after 24 h incubation in solution at 4 °C (data not shown). Crystals of this complex were grown and cryoprotected, and the structure was solved to 2.43 Å (supplemental table). In this structure, density for the oxoG is considerably better than in the corresponding complex cross-linked at the proximal site (see section above) (supplemental Fig. 9). Not only is the oxoG extrahelical in the distal complex, but indeed the oxoG nucleobase is almost fully inserted into the active site pocket (Fig. 6, A and B). The striking similarity of the Q315F*292 complex to the corresponding LRC*292 is plainly evident from their superposition (Fig. 6B). Even the one oxoG-specific hydrogen bond, the aforementioned one between the Gly-42 carbonyl oxygen and N7-H of oxoG, appears to be formed in the Q315F*292 complex, and His-270 is engaged in the hydrogen bond to the 5'-phosphate characteristic of late extrusion complexes (see above). Slotting of the oxoG into the lesion recognition pocket is dependent upon some accommodation to the presence of the bulky phenyl ring of Phe-315; this consists of the Phe-319 side chain’s rotating slightly downward and away from the active site, occupying the space normally filled by the Met-271 side chain, which in turn becomes disordered (Fig. 6B). The oxoG nucleobase base is angled slightly upward in the distal structure, relative to its position in LRC and LRC*292, but the remainder of the DNA deviates in no substantial way from the LRCs. Furthermore the structure revealed a close interaction between the side chains of Lys-249 and Cys-253 (nitrogen-to-sulfur distance = 2.69 Å) (Fig. 6A) as also seen in a structure of D268N hOGG1 complexed with oxoG/C DNA (Fig. 1A). As mentioned above, computational studies have indicated that this interaction consists of a Lys-249(NH3+)/Cys-253(S−) salt bridge, which makes a substantial contribution to both recognition of oxoG and discrimination of oxoG from G (28).

**DISCUSSION**

In this study, we characterized the structural effects of mutations in active site residues of hOGG1 that directly interact with the extrahelical oxoG nucleotide. These effects, which range from substantial to nearly negligible, shed light on aspects of recognition and catalysis by hOGG1.

The most straightforward mutations to interpret are H270A and Q315A. Both of these mutations remove contact functionality from the hOGG1/DNA interface, and that is precisely what we observed in the corresponding structures. Namely these structures are virtually identical to those of the hOGG1 LRCs outside the immediate vicinity of the mutation site. At the position of the mutation, the space vacated by truncation of the side chain (His or Gln to Ala) has become filled by ordered water molecules. This replacement is chemically literal in the case of the Q315A mutation in which the exact positions formerly occupied by the heteroatoms of the Gln side chain become occupied by the oxygen heteroatoms of two ordered water molecules (Fig. 4B). Such a precise one-to-one atomic correspondence was not observed at the H270A mutation site where local adjustments of the Asp-322 side chain and the nearby polypeptide backbone were also evident. We interpret these changes as being driven by electrostatic repulsion between the Asp-322 carboxylate and the phosphate 5' to oxoG; this repulsion would be dissipated in the wild-type complex through interposition of the positively charged His-270 side-chain imidazolium group. Notwithstanding these relatively minor changes, the structures of the H270A and Q315A mutant hOGG1 proteins clearly point to their biochemical defects resulting simply from removal of critical contacts in the protein/DNA interface. The deleterious effect of the H270A mutation is more pronounced than that of Q315A; the structures suggest that this difference may result from the fact that Q315A merely removes favorable contacts, whereas H270A also introduces a repulsive carboxylate/phosphate interaction.

Gly-42 is the only residue of hOGG1 that makes a direct contribution to both recognition of oxoG and discrimination of G from oxoG. Here we carried out the first mutational analysis of position 42, changing Gly to Ala. We found that this mutation had no measurable effect on recognition of oxoG, had a minor impact on the thermal stability of the protein, and was nearly imperceptible in terms of structure apart from the obvious constitutional difference of introducing a methyl substituent at Cα of residue 42. Such a modest impact is not expected given the important role served by this residue and the fact that it is conserved in all hOGG1 orthologs sequenced to date. It is also unexpected in structural terms because Gly-42 adopts a conformation that is permissible only for Gly residues in proteins, and introduction of a methyl substituent at Cα would be expected to engender an energetically punishing steric clash. Inspection of the structure revealed that the protein structure does in fact adjust to alleviate the clash, bringing Ala-42 within the “generously allowed” region of Ramachandran space. This particular conformation is relatively rare in protein structures, being
formed typically by residues in short stretches of left-handed α-helical structure, and is higher in energy than the right-handed α-helical or β-sheet conformations (40). We considered the possibility that this high energy conformer was being stabilized through its interaction with oxoG, which remains intact despite the Ala mutation, but discounted this when we found that Ala-42 remains in the same conformation even when oxoG is not bound in the active site pocket. Thus, we solved the structure of the proximally cross-linked base to the enzyme active site. Thus it was not unexpected was rigged (by DXL) to present an extrahelical oxoG nucleobase.

completed abrogation of catalytic activity even when the system bind oxoG-containing DNA over normal DNA and a nearly naturally. First we found that the Q315F mutation caused a complete abrogation of catalytic activity of Ala-42. If these interactions stabilize Ala-42, then they also must stabilize Gly-42; in other words, the backbone conformation at Gly-42 is reorganized as opposed to being acquired through induced fit. We propose that this “hardwiring” of Gly-42 is critical to its essential function in discrimination of oxoG from G. As we have shown previously, this discrimination arises mainly from repulsive contacts between the Gly-42 amide and G (electron/electron and dipole/dipole interactions) that become attractive with oxoG. Both of these discriminatory modes require a precise positioning of the Gly-42 amide with respect to the 7-position of the bound nucleobase and also are dependent upon cutting off any simple conformational route of escape from the unfavorable interactions experienced with G. Both these objectives are served by having the backbone conformation at position 42 be rigid and predisposed for both recognition of oxoG and rejection of G.

One of the major goals of the field of base excision repair is to understand the basis of lesion recognition and discrimination. The present work on the H270A, K249Q, and G42A mutations, along with a major body of previously published work, has illuminated the structural and energetic basis for lesion recognition and discrimination by the extrahelical active site of hOGG1 and MutM (21, 22, 26). What remains poorly understood is how hOGG1 and MutM recognize oxoG lesions upon first encounter, i.e. when the lesions still reside in the DNA helix. Insight into this question can be gained by identifying variants of hOGG1 that are able to perform intrahelical lesion recognition and discrimination but have a significantly diminished ability to stabilize an extrahelical lesion. As a first step toward achieving this objective, we reasoned that oxoG might be sterically blocked from binding in the lesion recognition pocket if a relatively small residue at the base of the pocket were mutated into a larger one. We therefore mutated Gln-315 to Phe and analyzed the mutant protein biochemically and structurally. First we found that the Q315F mutation caused a complete loss of the ordinarily substantial preference of hOGG1 to bind oxoG-containing DNA over normal DNA and a nearly complete abrogation of catalytic activity even when the system was rigor (by DXL) to present an extrahelical oxoG nucleobase-base to the enzyme active site. Thus it was not unexpected when we solved the structure of the proximally cross-linked complex (Q315F<sup>+</sup>oxoG) containing the Q315F mutation and found that the oxoG was not inserted into the lesion recognition pocket. However, it was completely unexpected to observe that the distally cross-linked complex (Q315F<sup>+</sup>oxoG) bearing the same mutation exhibited the oxoG inserted almost fully into the lesion recognition pocket, such that the distinctive hydrogen bond between Gly-42 and the oxoG N7-H was apparently formed. It is important to note that whereas the proximal cross-link (Cys-149) enforces an extrahelical oxoG by blocking renaturation of the target nucleobase, the distal cross-link is remote from the site of base extrusion and consequently exerts no direct bias on the helical status of the target nucleobase. These results have several implications.

What force stabilizes oxoG in the lesion recognition pocket in the distal complex when the protein exhibits no thermodynamic preference to bind DNA containing oxoG? Of course, crystal structures offer no direct information with respect to energetics, but they can offer clues into energetics. In the distal complex, for example, we noted that the amino residues that make up the active site had undergone many adjustments relative to their usual positions in lesion recognition complexes (Fig. 6B); the most significant of these is the loss of order in the Met-271 side chain, which is forced to move from its usual position to avoid a steric clash with Phe-315. These changes most likely come at an energetic cost that is balanced against the favorable interactions gained by base insertion. If the oxoG nucleobase has destabilizing interactions with the active site, then what overcome these interactions in the distal complex? We believe that crystal packing forces might provide the missing energy. In the particular crystal form obtained here, the DNA of neighboring complexes is coaxially stacked, and crystal formation is critically dependent upon these DNA/DNA interactions. The conformation of DNA that exists in this crystal form is one in which the DNA is drastically bent and contains an extrahelical target nucleobase. Changes in the crystal form that modulate the DNA conformation, for example replacing the target oxoG with its normal counterpart G, lead to a loss of crystallization when cross-linked at the distal site<sup>6</sup> but not when cross-linked at the proximal site. As discussed previously, the target G is forced to be extrahelical by proximal cross-linking (28) but has no such bias and is probably intrahelical when distally cross-linked. In summary, we believe that crystal packing forces favor a conformation of DNA containing an extrahelical target base, and in the case of the distally cross-linked Q315F mutant of hOGG1, this factor stabilizes the relatively weak interaction of the oxoG nucleobase with the lesion recognition pocket. A corollary of the crystal packing argument is that it may be possible to capture the elusive hOGG1-DNA complex containing an intrahelical oxoG using the Q315F mutant version, but this will require the discovery of a new crystal form free of bias toward a DNA conformation having an extruded target nucleobase.

Despite the fact that the oxoG is nearly fully inserted into the active site in the distal cross-linked complex, this crystalline complex is inefficient at promoting base excision. The deficiency is not due to the presence of the cross-link, as we have shown, using photocaged versions of oxoG and hOGG1 having a wild-type active site, that base excision proceeds in distally cross-linked crystals within minutes of photodeprotection.<sup>7</sup> It thus appears that the slight displacement of oxoG from the active site is sufficient to render inefficient the catalysis of base

<sup>6</sup> A. Banerjee, C. M. Crenshaw, and G. L. Verdine, unpublished results.

<sup>7</sup> C. Radom, S. Lee, and G. L. Verdine, unpublished results.
FIGURE 7. Superpositions of the DNA component of various hOGG1/DNA complexes. Shown are least squares superpositions of the DNA duplexes bound to hOGG1 with the protein component removed. A–F, complexes as indicated with the legend color-coded to the structure. G, histogram showing the extent of rotation about the indicated bonds flanking the lesion. Inset, a schematic depicting the relevant bonds. Torsional changes of greater than ±60° we consider noteworthy (dashed lines).
excision. This reaction is believed to proceed through an S_{n,1}-like pathway, initiated by cleavage of the glycosidic bond and formation of an oxocarbenium ion intermediate. This intermediate is then trapped by the active site Lys-249. Although we cannot rule out the possibility that the defect is in the trapping step, this seems unlikely as the oxocarbenium ion is highly reactive and could readily be trapped by water if it were being formed. We did not observe the product of water trapping, an abasic site, so the most likely explanation is that the protein has difficulty stabilizing the oxocarbenium ion and hence cannot readily catalyze its formation. It is reasonable to expect that the transition state leading to such a high energy intermediate as an oxocarbenium ion would be demanding with respect to the precise positioning of reactants and products within the catalytic apparatus of the protein, but to our knowledge these studies provide the first experimental evidence of just how demanding it is.

An unexpected benefit of this study is the rich trove of information it provides on alternative DNA backbone conformations at the site of the extruded target base. Superposition of the DNA component of the hOGG1-DNA complexes revealed in exquisite detail the conformational changes that distinguish one structure from the other (Fig. 7). We have argued previously that the process of base extrusion is sufficiently complex that it must involve multiple steps that together define a pathway, much as the steps in folding of a protein define a folding pathway (28). Just as with protein folding, the oxoG extrusion pathway could in principle always proceed through the same set of discrete, structurally defined intermediates, or it might proceed through ensembles of intermediates having roughly equivalent energies. Whether discrete or ensembles, the intermediates can be characterized as early, middle, or late, depending on where they lie along the kinetic pathway of base extrusion. Thus, for example, we have characterized the G-complex (Fig. 7, A and E, green) as representing a late intermediate because it is fully extrahelical, but its nucleobase is not yet inserted into the lesion recognition pocket (28). Yet another complex (the oxoG/G complex) we have described as being early because the target nucleobase is extruded from the helical stack but is still disposed inward toward the major groove rather than outward as in extrahelical complexes (Fig. 7F) (41). Remarkably large (>60°) rotations about only three bonds were necessary to convert the backbone conformation in the G-complex into that of an LRC (Fig. 7, A and G, magenta bars); that is, for the lesion to move from the exosite to the active site. How do the new structures reported here relate to this emerging picture of an extrusion pathway, and what do they reveal about the nature of the overall pathway?

Superposition of the Q315F*292 DNA structure on that of the corresponding LRC (LRC*292) revealed how closely related are and the final extrahelical state (D). Hydrogen bonds are indicated by dashed lines, and a Ca^{2+} ion (probably Mg^{2+} under physiologic conditions) is shown as a green sphere. Note that progression from A and B, with movement about the 3'-pivot (curved arrow), to C results in acquisition of hydrogen bonds and Ca^{2+} coordination by the 3'-phosphate (also denoted by dashed lines), contacts that are necessary to form a lesion recognition complex (D). Note also that the catalytically essential Lys-249 is hydrogen-bonded to the 3'-phosphate in the late complexes (A and B) but has swung back into the active site during progression along the base extrusion pathway (mutated to Gln in D).
that Q315F*149 is obliged to undergo a greater number of large directional rotations about two different bonds on the 3'-side of the lesion are necessary to interconvert the oxoG/G complex and the G-complex (Fig. 7, F and G, gray bars), that is, to proceed from an early to a late complex along the base extrusion pathway.

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