Catalytic and DNA Binding Properties of the Ogg1 Protein of *Saccharomyces cerevisiae*: Comparison between the Wild Type and the K241R and K241Q Active-Site Mutant Proteins†

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ABSTRACT: The Ogg1 protein of *Saccharomyces cerevisiae* belongs to a family of DNA glycosylases and apurinic/apyrimidinic site (AP) lyases, the signature of which is the α-helix−hairpin−α-helix−Gly/Pro−Asp (HhH-GPD) active site motif together with a conserved catalytic lysine residue, to which we refer as the HhH-GPD/K family. In the yeast Ogg1 protein, yOgg1, the HhH-GPD/K motif spans residues 225−260 and the conserved lysine is K241. In this study, we have purified the K241R and K241Q mutant proteins and compared their catalytic and DNA binding properties to that of the wild-type yOgg1. The results show that the K241R mutation greatly impairs both the DNA glycosylase and the AP lyase activities of yOgg1. Specificity constants for cleavage of a 34mer oligodeoxyribonucleotide containing a 7,8-dihydro-8-oxoguanine (8-OxoG) paired with a cytosine, [8-OxoG·C], are 56×10^{-3} and 5×10^{-3} min^{-1} nM^{-1} for the wild-type and the K241R protein, respectively. On the other hand, the K241Q mutation abolishes the DNA glycosylase and AP lyase activities of yOgg1. In contrast, the K241R and K241Q proteins have conserved wild-type DNA binding properties. K_{dapp} values for binding of [8-OxoG·C] are 6.9, 7.4, and 4.8 nM for the wild-type, K241R, and K241Q proteins, respectively. The results also show that AP site analogues such as 1,3-propanediol (Pr), tetrahydrofuran (F), or cyclopentanol (Cy) are not substrates but constitute good inhibitors of the wild-type yOgg1. Therefore, we have used a 59mer [Pr·C] duplex to further analyze the DNA binding properties of the wild-type, K241R, and K241Q proteins. Hydroxyl radical footprints of the wild-type yOgg1 show strong protection of six nucleotides centered around the Pr lesion in the damaged strand. On the complementary strand, only the cytosine placed opposite Pr was strongly protected. The same footprints were observed with the K241R and K241Q proteins, confirming their wild-type DNA binding properties. These results indicate that the K241Q mutant protein can be used to study interactions between yOgg1 and DNA containing metabolizable substrates such as 8-OxoG or an AP site.

Reactive oxygen species (ROS)[1] formed in cells as byproducts of aerobic metabolism or during oxidative stress have been suggested to play a role in biological processes such as carcinogenesis or aging (*1*−*4*). ROS can attack DNA, producing a multiplicity of lesions such as oxidized bases, apurinic/apyrimidinic (AP) sites, and strand breaks (*5*, *6*). In the case of cancer, unrepaired oxidative DNA damage presumably causes mutations that can activate oncogenes or inactivate tumor suppressor genes that control cell proliferation (*7*). An oxidatively damaged form of guanine, 7,8-dihydro-8-oxoguanine (8-OxoG), is critical in terms of mutagenesis by endogenous ROS (*8*, *9*). In most organisms, the repair of 8-OxoG is primarily mediated by the base excision repair (BER) pathway (*10*−*12*). The first step in the course of BER is the excision of the damaged base by a DNA glycosylase activity, followed by the incision of the resulting AP site and successive actions of a phosphodiesterase, a DNA polymerase, and a DNA ligase (*10*−*12*). In *Escherichia coli*, two DNA glycosylases cooperate to prevent mutagenesis by 8-OxoG: the Fpg protein, which excises 8-OxoG paired with a cytosine in the damaged DNA, and the MutY protein, which excises the adenine residues incorporated by DNA polymerases opposite to 8-OxoG (*8*, *9*). Inactivation of both the *fpg* (*mut*M) and *mut*Y(*mic*A) genes of *E. coli* results in a strong GC to TA mutator phenotype (*13*, *14*). In *Saccharomyces cerevisiae*, the *OGG1* gene codes for a DNA glycosylase that catalyzes the removal

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[1] Abbreviations: ROS, reactive oxygen species; 8-OxoA, 7,8-dihydro-8-oxoadenine; 8-OxoG, 7,8-dihydro-8-oxoguanine; FapyG, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; Me-FapyG, 2,6-diamino-4-hydroxy-5-(*N*-methylformamido)pyrimidine; AP site, apurinic/apyrimidinic site; EMSA, electrophoretic mobility shift assay; Pr, 1,3-propanediol; Cy, cyclopentanol; F, tetrahydrofuran.

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Table 1: Sequences of Oligodeoxyribonucleotides Used as DNA Probes in This Study[a]

name	sequence
34mer G	5′-GGCTTCATCGTTGTC**G**CAGACCTGGTGGATACCG- 3′
34mer A	5′-GGCTTCATCGTTGTC**A**CAGACCTGGTGGATACCG-3′
34mer U/AP	5′-GGCTTCATCGTTGTC**U**CAGACCTGGTGGATACCG-3′
34mer 8-OxoG[b]	5′-GGCTTCATCGTTGTC**8-OxoG**CAGACCTGGTGGATACCG-3′
34mer 8-OxoA[b]	5′-GGCTTCATCGTTGTC**8-OxoA**CAGACCTGGTGGATACCG-3′
34mer C	5′-CGGTATCCACCAGGTCTG**C**GACAACGATGAAGCC-3′
34mer A	5′-CGGTATCCACCAGGTCTG**A**GACAACGATGAAGCC-3′
13mer G[c]	5′-CTCTTT**G**TTTCTC-3′
13mer Pr[c]	5′-CTCTTT**Pr**TTTCTC-3′
13mer F[c]	5′-CTCTTT**F**TTTCTC-3′
13mer Cy[c]	5′-CTCTTT**Cy**TTTCTC-3′
13mer C	5′-GAGAAA**C**AAAGAG-3′
59mer G	5′-AGCTTACTCTAGAAGAATTCTCACTCTTT**G**TTTCTCACTGGATCCACAGATATCACACA-3′
59mer Pr[c]	5′-AGCTTACTCTAGAAGAATTCTCACTCTTT**Pr**TTTCTCACTGGATCCACAGATATCACACA-3′
59mer C	5′-ATGAGATCTTCTTAAGAGTGAGAAA**C**AAAGAGTGACCTAGGTGTCTATAGTGTGTTCGA-3′

[a] Pr, 1,3-propanediol; F, tetrahydrofuran; Cy, cyclopentanol; G, guanine; A, adenine; C, cytosine; U, uracil; AP, AP site. If not specified, oligodeoxyribonucleotides are of commercial origin (OLIGO-Express, Paris). [b] These oligodeoxyribonucleotides are a gift of Drs. A. Guy and J. Cadet (CEA, Grenoble) (*51*). [c] These oligodeoxyribonucleotides are a gift of Dr. J. L. Fourrey (CNRS, Gif sur Yvette) (*40*).

of 8-OxoG from damaged DNA (*15*, *16*). Furthermore, Ogg1-deficient strains of *S. cerevisiae* exhibit a spontaneous GC to TA mutator phenotype (*17*, *18*). Using the yOgg1 sequence, groups from several laboratories were able to retrieve human and mouse cDNAs coding for proteins showing a strong sequence similarity with the yeast Ogg1 (*19*−*25*).

The *OGG1* gene of *S. cerevisiae* is localized on chromosome XIII and codes for a 43-kDa protein, yOgg1, which excises 8-OxoG, 2,6-diamino-4-hydroxy-5-(*N*-methylformamido)pyrimidine (Me-FapyG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) from damaged DNA (*15*, *16*, *26*, *27*). Furthermore, the yOgg1 protein efficiently removes 7,8-dihydro-8-oxoadenine (8-OxoA) placed opposite a cytosine or a 5-methylcytosine in oligodeoxyribonucleotides (*28*). Moreover, the yOgg1 protein is also endowed with an AP lyase activity that incises DNA at preformed AP sites in DNA via a β-elimination reaction (*15*, *16*). However, cleavage at an AP site requires the presence of a cytosine in the complementary strand opposite to the lesion (*27*). The yOgg1 protein also possesses a deoxyribophosphodiesterase (dRPase) activity that releases 5′-deoxyribose phosphate residues after incision of AP sites by the Nfo protein of *E. coli* (*29*). The catalytic mechanism of the yOgg1 protein involves the formation of a transient covalent imino enzyme−DNA intermediate, presumably between the ϵ-NH_2 group of lysine 241 and the C1′ of the abasic sugar moiety (*16*, *27*). The critical role of lysine 241 is suggested by its location at position two of the second α-helix of the highly conserved HhH-GPD (α-helix−hairpin−α-helix−Gly/Pro−Asp) motif (*16*, *30*). Indeed, the yOgg1 protein belongs to a class of DNA glycosylases/AP lyases sharing an ancestor gene with endonuclease III of *E. coli*, to which we refer as the HhH-GPD/K class. On this basis, lysine 241 of yOgg1 was used as a target for site-directed mutagenesis to generate K241Q and K241R mutant proteins (*16*, *27*). Preliminary studies using cell-free protein extracts of *E. coli* overexpressing K241R and K241Q mutants indicated an essential role of K241 for cleavage but not for binding of 8-OxoG-containing substrates (*27*).

In this study, we have purified the wild-type yOgg1 and two mutant proteins, K241R and K241Q, to perform a quantitative analysis of their catalytic and DNA binding properties. These purified proteins were required to determine specificity constants (k_{cat}/K_M) and apparent dissociation constants (K_{dapp}) for substrates containing or not containing oxidative DNA damage. Furthermore, hydroxyl radical footprint analysis was performed for the first time with the wild-type, K241R, and K241Q yOgg1 proteins bound to a 59mer [Pr·C] duplex. Taken together, the results reported in this study show that the K to Q mutation at position 241 completely abolishes the catalytic properties of yOgg1, whereas the DNA binding properties are preserved. Since the K241Q protein binds to damaged DNA as does the wild-type protein, it may be used as a new approach to dissect the interactions between HhH-GPD/K repair enzymes and DNA containing metabolizable base damage such as 8-OxoG or an AP site.

MATERIALS AND METHODS

DNA and Other Materials. Sequences of the oligodeoxyribonucleotides used in this study are listed in Table 1.

Bacterial Strains, Plasmids, and Enzymes. Escherichia coli strain BH410 (*fpg*$^-$) and plasmids pYSB160, pYSB161, and pYSB162 expressing wild-type, K241R, and K241Q proteins were from our laboratory stock (*27*). Uracil DNA glycosylase from *E. coli* was purified from an overproducing strain (our laboratory stock).

Purification of yOgg1 Proteins. Wild-type and the two mutant yOgg1 proteins were purified from IPTG-induced *E. coli* cells BH410 (*fpg*$^-$) harboring plasmid pYSB160, pYSB161, or pYSB162, respectively. For each preparation, about 10 g of cells (w/w) were collected, lysed, and purified as previously described (*27*). Wild-type and K241R proteins were purified by using the release of Me-FapyG from [^{3}H]-Me-FapyG-poly(dG-dC)·poly(dG-dC) as an activity assay (*31*). The K241Q protein purification was followed by SDS−PAGE analysis and immunoblotting with purified anti-yOgg1 antibodies BCE-1691 (*27*). The protein solutions were adjusted to 1 mg/mL in 25 mM Tris-HCl, pH 7.6, 2 mM Na_2EDTA, 250 mM NaCl, and 50% glycerol (v/v) and stored at −20 °C. Protein concentration was measured according to the Bradford method (*32*).

DNA Glycosylase Activity Assays. 8-OxoG DNA glycosylase activity: Reaction mixture (25 μL) contained 25 mM

Tris-HCl, pH 7.6, 100 mM KCl, 30 pmol of 34mer [8-OxoG·C] duplex, and yOgg1 protein. The reaction was carried out at 37 °C for 30 min. The products of the reaction were separated by HPLC and detected with electrochemical detection (ECD) as described (*15*). Me-FapyG DNA glycosylase activity: Reaction mixture (50 μL) contained 25 mM Tris-HCl, pH 7.6, 100 mM KCl, [^{3}H]-Me-FapyG-poly(dG-dC)·poly(dG-dC) (1.5 pmol of [^{3}H]-Me-FapyG), and yOgg1 protein. The reaction was carried out at 37 °C for 30 min. Ethanol-soluble radioactive material was quantitated and the chemical nature of this material was monitored by HPLC (*31*). One unit releases 1 pmol of Me-FapyG or 8-OxoG in 15 min at 37 °C.

Cleavage of [8-OxoG·C], [8-OxoA·C], or [AP·C] Duplexes. Oligodeoxyribonucleotides (34 mers) containing 8-OxoG or 8-OxoA were a kind gift of Drs. A. Guy and J. Cadet (CEA, Grenoble) (Table 1). The DNA strand containing 8-OxoG, 8-OxoA, or uracil was labeled at the 5′-end with [γ-^{32}P]ATP (3000 Ci/mmol, ICN) and T4 polynucleotide kinase (New England Biolabs) (*33*). The [^{32}P]-labeled strand was hybridized with a complementary sequence yielding [8-OxoG·C], [8-OxoA·C], or [U·C] duplexes (Table 1). To generate an AP site, [U·C] duplex was incubated with uracil DNA glycosylase, yielding the [AP·C] duplex (*27*). The assay mixture (20 μL) contained 25 mM Tris-HCl, pH 7.6, 100 mM KCl, 50 fmol of [^{32}P]-labeled [8-OxoG·C], [8-OxoA·C], or [AP·C] duplex, and yOgg1 protein. The reactions were performed at 37 °C for 15 min and stopped by adding 6 μL of formamide dye (90% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, and 5 mM Na_2-EDTA) and heating for 5 min at 95 °C. For [AP·C] cleavage reaction, the stop buffer was supplemented with 50 mM $NaBH_4$ to stabilize unreacted AP sites. The products of the reactions were separated by 20% PAGE containing 7 M urea. Cleavage was quantified with a Molecular Dynamics PhosphorImager. For kinetic studies, the yOgg1 protein was added to the reaction mixture and allowed to proceed at 37 °C for 2 and 4 min. The initial reaction rate was plotted versus substrate concentration. K_M and V_{max} values were derived from a computer-fitted curve, by use of the GraphPad Prism 2.0a program (*27*).

Inhibition by AP Site Analogues. Cleavage of the [8-OxoG·C] duplex was carried out at 4 °C for 12 h in the presence of 25 ng of wild-type yOgg1 protein. Competition was performed as follows: Unlabeled 13mer duplexes either unmodified [G·C] or modified [Cy·C], [F·C], or [Pr·C], were diluted in TE buffer containing 0.5 M NaCl and 10 mM $MgCl_2$ and incubated for 10 min at 4 °C with 25 ng of wild-type yOgg1. These mixtures were supplemented with 50 fmol of [^{32}P]-labeled 34mer [8-OxoG·C] and incubated for 12 h at 4 °C. The reaction products were separated by electrophoresis on a denaturing 20% polyacrylamide gel containing 7 M urea, and cleavage was quantified as described.

Electrophoretic Mobility Shift Assay and Determination of the Apparent Equilibrium Dissociation Constants (K_{dapp}). The assay mixture (10 μL) contained 25 mM Tris-HCl, pH 7.6, 175 mM NaCl, 1 mM Na_2EDTA, 1 mM dithiothreitol, 300 μg/mL BSA, 12% glycerol (v/v), 2.5 fmol of 5′-end [^{32}P]-labeled oligodeoxyribonucleotide duplex and yOgg1 protein. The binding reaction was performed at 4 °C for 30 min and loaded onto a nondenaturing 10% polyacrylamide gel for electrophoresis as described (*34*). Gels were run at 4 °C for 3 h. The gel was fixed and dried on 3MM Whatman paper and exposed for autoradiography on X-ray film (Fuji) at −80 °C. The percentage of free and bound DNA was quantified after scanning of the autoradiographs by use of the NIH V1.59 software (*33, 34*). Assuming a 1:1 stoichiometry between the monomeric yOgg1 protein and the DNA probe, an apparent dissociation constant, K_{dapp}, can be calculated from the concentrations of free yOgg1 = [yOgg1], free DNA = (f), and bound [DNA−yOgg1] complex = (1 − f) at equilibrium: K_{dapp} = [yOgg1](f)/(1 − f).

If the total concentration of protein $[yOgg1]_0$ is close to that of the free [yOgg1] at equilibrium, then the K_{dapp} = $[yOgg1]_0$ when 50% of the DNA is bound by the protein.

Hydroxyl Radical Footprinting. Binding assays were carried out as follows: 40 nM of purified 5′-end [^{32}P]-labeled 59mer [G·C] or [Pr·C] duplexes were incubated for 30 min at 4 °C with 0, 0.2, 0.4, or 0.8 μM yOgg1 protein in 25 mM Tris-HCl, pH 7.6, 175 mM NaCl, 1 mM Na_2EDTA, 1 mM dithiothreitol, and 300 μg/mL BSA. Hydroxyl radical footprinting was performed as follows: 10 μL of binding reaction was incubated for 2 min at room temperature with 3 μL of a fresh and cooled solution containing 0.1% H_2O_2, 6.7 mM ascorbate, and 0.1 mM $[Fe(EDTA)]^{2-}$ [0.2 mM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ and 0.4 mM Na_2EDTA] in order to produce a Fenton reaction (*35, 36*). Reactions were quenched by addition of 1.8 μL of a stop solution containing 80 mM thiourea and 13 mM Na_2EDTA. These experimental conditions were determined to obtain less than one strand break per DNA molecule. Samples were precipitated with cold ethanol, washed with 70% ethanol, treated for 30 min at 90 °C in 1 M piperidine, dried, and analyzed by electrophoresis on a denaturing polyacrylamide sequencing gel [8% acrylamide:bisacrylamide (19:1), 7 M urea]. Gels were fixed, dried, and scanned with a Molecular Dynamics PhosphorImager. Densitometer scans of each lane were done with ImageQuant software and the normalized densitometer scans obtained were plotted with Microsoft Excel software (*36*).

RESULTS

Purification of the Wild-Type, K241R, and K241Q yOgg1 Proteins. The yOgg1 proteins were overexpressed and purified from *E. coli* BH410 (*fpg*$^-$) hosting recombinant plasmids as previously described (*27*). The same purification steps were used for the wild-type and the mutant proteins. The release of [^{3}H]-Me-FapyG was used as an activity assay during the purification of the wild-type and the K241R proteins (*31*). The K241Q protein was detected by Coomassie blue staining and immunoblotting during purification steps (*27*). The purity of the yOgg1 proteins was assessed by SDS−PAGE analysis (Figure 1) and determination of N-terminal sequences. The presence of two or three close bands on SDS−PAGE was systematically observed in our preparations, independent of the nature of the expressed protein (Figure 1). This reflects short deletions at the C-terminus, since sequencing of these polypeptides only reveals the N-terminal sequence of yOgg1. Most probably these deletions do not affect enzyme activity, since deletions of up to 44 amino acids from the C-terminus result in a fully active yOgg1 protein (data not shown).

8-OxoG and Me-FapyG DNA Glycosylase Activity of the Wild-Type, K241R, and K241Q yOgg1 Proteins. The ability

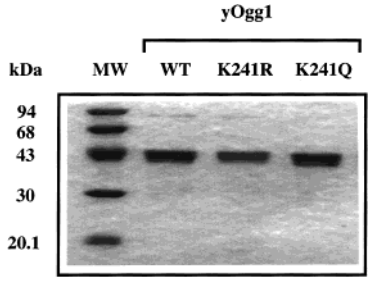

FIGURE 1: Purification of the wild-type, K241R and K241Q yOgg1 proteins of *S. cerevisiae*. The purification of the yOgg1 proteins was performed as described under Materials and Methods. The final purification fractions were analyzed by SDS−PAGE and stained by Coomassie brillant blue. Lane MW: molecular weight markers (Pharmacia-Biotech). The amounts of protein loaded onto the gel were 1 μg for each lane.

of the wild-type and the mutant proteins to release 8-OxoG and Me-FapyG was investigated. Figure 2A shows the release of 8-OxoG by the yOgg1 proteins from 34mer oligodeoxyribonucleotides containing a single 8-OxoG paired with a cytosine, [8-OxoG·C]. The wild-type yOgg1 excises 8-OxoG 7-fold faster than the K241R mutant (Figure 2A). Both the wild-type and the K241R proteins preferentially excise 8-OxoG placed opposite a cytosine, the efficiency order being [8-OxoG·C] > [8-OxoG·T] ≫ [8-OxoG·G] and [8-OxoG·A] (data not shown). Figure 2B shows that the wild-type yOgg1 excises Me-FapyG 10-fold faster than the K241R protein. In contrast, the K241Q protein does not release 8-OxoG nor Me-FapyG at a detectable rate (Figure 2).

Cleavage of [8-OxoG·C], [8-oxoA·C], or [AP·C] Duplexes by the Wild-Type, K241R, and K241Q yOgg1 Proteins. The results show that the wild-type yOgg1 efficiently cleaves the following 34mer oligodeoxyribonucleotide duplexes: [8-OxoG·C], [8-OxoA·C], and [AP·C] (Figure 3, panels A−C, respectively). They also show that [8-OxoG·C] and [AP·C] duplexes are cleaved at slower rates by the K241R protein (Figure 3A,C), whereas [8-OxoA·C] is not incised at all (Figure 3B). Furthermore, the K241Q protein does not cleave any of the three duplexes tested (Figure 3). Specificity constants, k_{cat}/K_M, for cleavage of [8-OxoG·C] by the wild-type and K241R proteins are 56×10^{-3} and 5×10^{-3} nM^{-1} min^{-1}, respectively (Table 2). The higher specificity constant of the wild-type yOgg1 is primarily due to its k_{cat} value, which is 6-fold higher than that of the K241R protein, suggesting a defect in the catalytic mechanism. The ability of the yOgg1 proteins to form a covalent complex with [8-OxoG·C] and [AP·C] in the presence of $NaBH_4$ was also analyzed. The results indicate that the trapping efficiencies for the various yOgg1 proteins reflect the cleavage efficiencies for the same substrates (Figure 3 and data not shown). It should be noticed that the K241Q protein is not able to form a covalent imino enzyme−DNA intermediate with any of the three substrates tested.

DNA Binding Activity of the Wild-Type, K241R, and K241Q yOgg1 Proteins and Determination of Apparent Dissociation Constants (K_{dapp}) by EMSA. For these studies, [^{32}P]-labeled 34mer oligodeoxyribonucleotide duplexes containing or not containing an oxidized DNA base were incubated with the wild-type or mutant yOgg1 proteins and the products of the reactions were analyzed by the electrophoretic mobility shift assay (EMSA). To minimize cleavage of DNA duplexes by the wild-type yOgg1, the binding

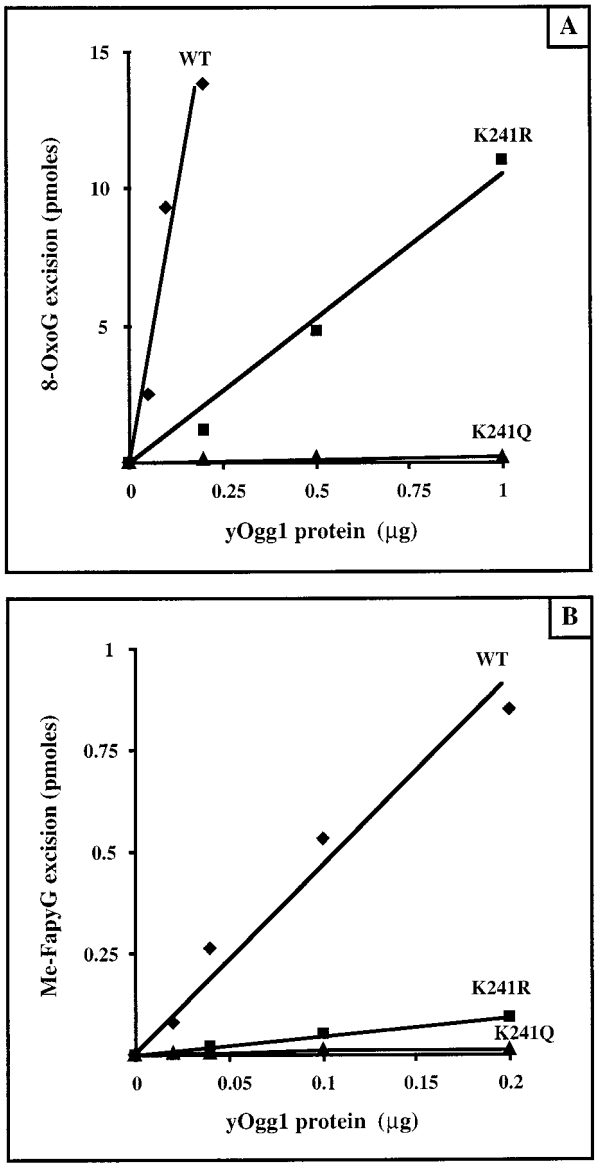

FIGURE 2: Excision of 8-oxoG and Me-FapyG by the wild-type, K241R, and K241Q yOgg1 proteins. (A) 8-OxoG DNA glycosylase activity: 34mer [8-OxoG·C] duplex was incubated with yOgg1 proteins for 30 min at 37 °C. The products of the reaction were analyzed by HPLC-ECD (*15*). (B) Fapy DNA glycosylase activity: [^{3}H]-Me-FapyG(polydG-dC)·poly(dG-dC) was incubated with yOgg1 proteins for 30 min at 37 °C. The products of the reaction were analyzed by HPLC as described (*31*).

reactions and gel electrophoresis were performed at 4 °C. Figure 4 shows that incubation of the [8-OxoG·C] duplex with increasing concentration of the wild-type or K241Q proteins produced increasing amounts of a band with reduced electrophoretic mobility, presumably a 1:1 [yOgg1−DNA] complex. A control experiment shows that 14.5 nM wild-type yOgg1 binds more than 80% of the [8-OxoG·C] duplex, whereas it cleaves less than 10% of the same duplex, under the assay conditions used (Figure 3 and data not shown). At high protein concentration another complex is observed, due to the binding of a second protein molecule to DNA. The results also show that both the wild-type and K241Q proteins bind [8-OxoG·C] duplex with similar efficiencies (Figure 4). The apparent dissociation constants (K_{dapp}) for the binding of yOgg1 proteins to 34mer oligodeoxyribonucleotide duplexes were determined by EMSA (*33*, *34*). Under the assay

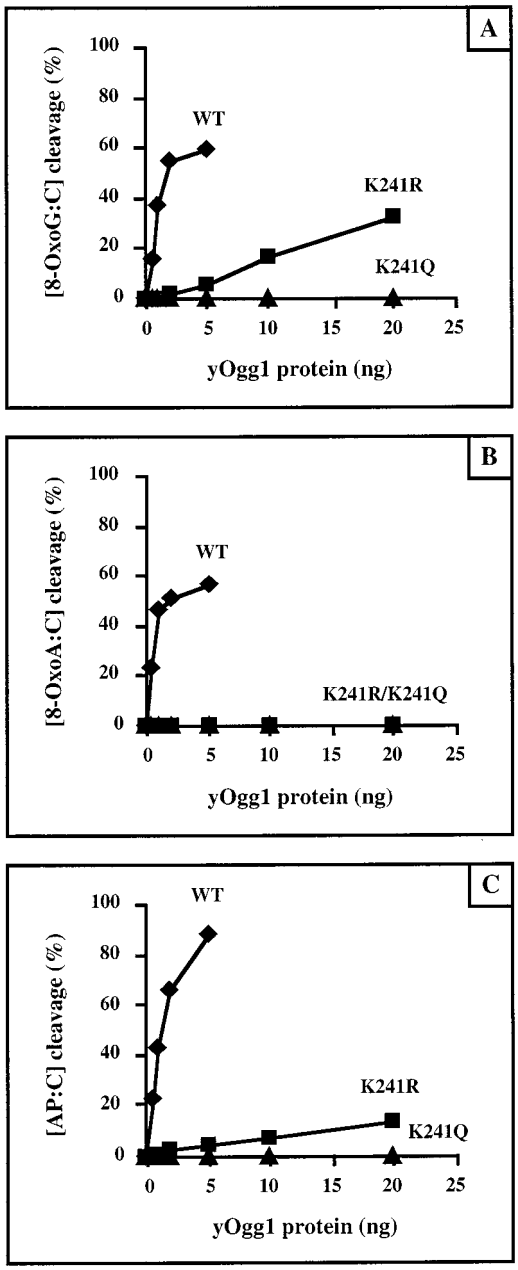

FIGURE 3: Cleavage of [8-OxoG·C], [8-OxoA·C], and [AP·C] duplexes by the wild-type, K241R, and K241Q yOgg1 proteins. Oligodeoxyribonucleotide duplexes (34 mers) were [^{32}P]-labeled at the 5′-end of the lesion-containing strand. Each reaction was carried out for 15 min at 37 °C. Products were separated on a 20% polyacrylamide gel containing 7 M urea. Cleavage was quantified with a Molecular Dynamics PhosphorImager.

Table 2: Kinetic Constants for Cleavage of the [8-OxoG·C] Duplex by Wild-Type and K241R yOgg1 Proteins[a]

protein	K_M (nM)	k_{cat} (min^{-1})	K_{cat}/K_M (min^{-1} nM^{-1})
wild type	7.3	0.41	56×10^{-3}
K241R	14	0.071	5×10^{-3}

[a] The sequence of the 34mer oligodeoxyribonucleotide used is reported in Table 1. The kinetic parameters were determined from Lineweaver−Burk plots.

conditions used, the total concentration of yOgg1 protein required to bind 50% of the input DNA is equal to K_{dapp}. The results show that the wild-type yOgg1 protein efficiently binds [8-OxoG·C] and [8-OxoA·C] duplexes with K_{dapp}

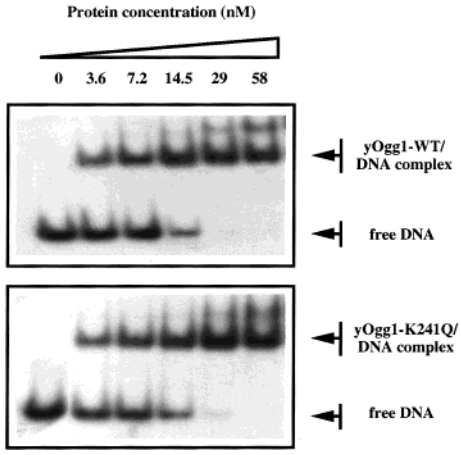

FIGURE 4: Binding of the wild-type and K241Q yOgg1 proteins to [8-OxoG·C] duplex. Electrophoretic mobility shift assay (EMSA) was performed with increasing concentrations of the wild-type and K241Q proteins and [^{32}P]-labeled 34mer [8-OxoG·C] duplex. Since DNA concentration in each reaction mixture (0.25 nM) is low compared to the total $[yOgg1]_0$ protein concentration (3.6−116 nM), the free [yOgg1] at equilibrium is very close to $[yOgg1]_0$. Therefore, K_{dapp} values corresponded to $[yOgg1]_0$ required to bind 50% of the DNA probe, determined by EMSA.

Table 3: Apparent Dissociation Constants (K_{dapp}) of the Wild-Type, K241R, and K241Q yOgg1 Proteins for Modified or Unmodified DNA Duplexes[a]

	apparent dissociation constant, K_{dapp} (nM)		
DNA probe	wild type	K241R	K241Q
[8-OxoG·C]	6.9 ± 0.8	7.4 ± 0.3	4.8 ± 0.7
[8-OxoG·A]	19.8 ± 4.1	19.1 ± 1.3	42.8 ± 8.8
[8-OxoA·C]	5.9 ± 1.2	7.9 ± 0.3	7.1 ± 0.1
[G·C]	37.5 ± 2.2	nd	76.3 ± 3.1
[A·C]	36.2 ± 4.9	nd	78.4 ± 0.2

[a] DNA probes are 34-mer oligodeoxyribonucleotide duplexes whose sequences are reported in Table 1. K_{dapp} values are determined by EMSA as described under Materials and Methods. DNA concentration was 0.25 nM and the protein concentration range was 0.9−116 nM. Values are average of 3−6 independent experiments. nd, not done.

values of 6.9 and 5.9 nM, respectively (Table 3). Other DNA tested are less tightly bound by yOgg1, the rank order for binding being as follows: [8-OxoG·C] = [8-OxoA·C] > [8-OxoG·A] > [G·C] = [A·C] (Table 3). These results are well fitting with the cleavage efficiency of the same duplexes by the yOgg1 protein (*15*, *16*, *27*). Furthermore, K_{dapp} values for the K241R or K241Q mutant proteins are not significantly different from that of the wild type for the same duplex (Table 3). As an example, K_{dapp} values for binding of [8-OxoG·C] are 6.9, 7.4, and 4.8 nM for the wild-type, K241R, and K241Q proteins, respectively (Table 3). In addition, the rank order for binding of the different duplexes is identical for the three yOgg1 proteins tested (Table 3). These results strongly suggest that the K241R and K241Q mutant proteins, which have partly or totally impaired catalytic properties, have retained wild-type DNA binding properties.

Cyclic and Noncyclic AP Site Analogues Are Inhibitors of the Wild-Type yOgg1 Protein. To investigate the mechanisms of interaction between the yOgg1 protein and damaged DNA, it is important to generate specific and stable abortive [protein−DNA] complexes. Such complexes have been described between several DNA glycosylases and DNA

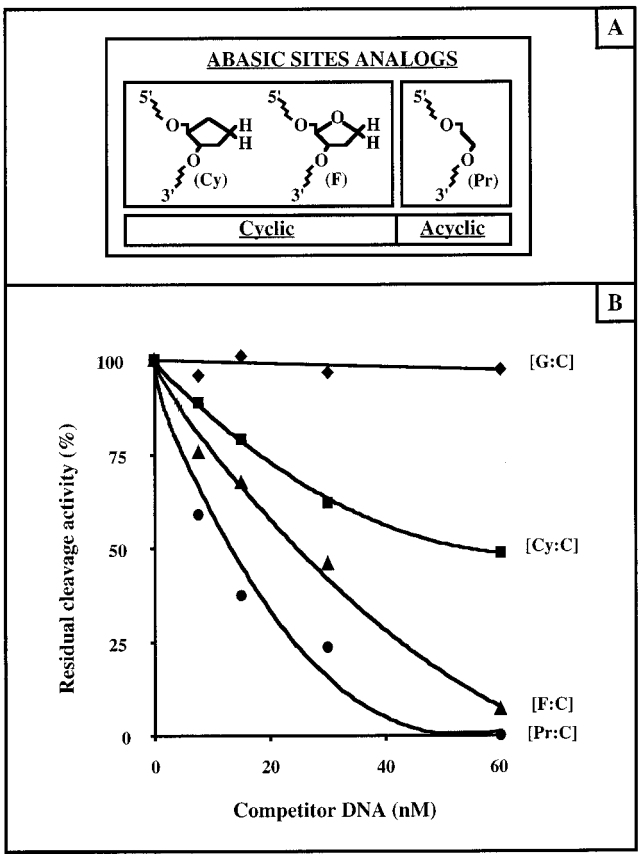

FIGURE 5: Inhibition of the wild-type yOgg1 protein by AP site analogues in 13mer duplexes. (A) Chemical structure of cyclic and acyclic AP site analogues used. Sequences of the 13mer duplexes containing Cy, F, Pr, or G are reported in Table 1. (B) Cleavage of the [8-OxoG·C] duplex was carried out at 4 °C for 12 h in the presence of 25 ng of wild-type yOgg1 protein. Under these conditions approximately 60% of the [8-OxoG·C] duplex was cleaved. Competitors are the following 13mer duplexes: [G·C], [Cy·C], [F·C], and [Pr·C].

duplexes containing AP site analogues such as reduced AP site, 1,3-propanediol (Pr), tetrahydrofuran (F), or pyrrolidine (*34*, *36*−*39*). In this study, we have tested the ability of the wild-type yOgg1 protein to specifically bind 13mer duplexes containing AP site analogues that are known to be efficiently bound by the Fpg protein (*36*). Figure 5A shows the chemical structures of the three analogues tested: 1,3-propanediol (Pr), tetrahydrofuran (F), and the newly synthesized cyclopentanol (Cy) (*40*). Figure 5B shows that 13mer DNA duplexes containing Pr, F, or Cy inhibit cleavage of [8-OxoG·C] by the yOgg1 protein. The most potent inhibitor is the 13mer containing the acyclic Pr, followed by the 13mer containing cyclic F or Cy residues (Figure 5B). In contrast, unmodified [G·C] duplex does not significantly inhibit yOgg1 demonstrating the specificity of the binding to the AP site analogues containing duplexes (Figure 5B). In addition, yOgg1 does not incise [Pr·C] duplex as expected for a DNA glycosylase/AP lyase of the HhH-GPD/K family (data not shown). Furthermore, binding of the yOgg1 protein to these 13mer duplexes was also confirmed by EMSA (data not shown). Therefore, the yOgg1 protein can form a stable abortive [protein−DNA] complex with [Pr·C] that can be used to study interactions between yOgg1 and modified DNA.

Hydroxyl Radical Footprints of the Wild-Type, K241R, and K241Q yOgg1 Proteins on a 59mer [Pr·C] Duplex. High-resolution hydroxyl radical footprinting analysis was performed to determine more precisely how the yOgg1 protein interacts with lesions in damaged DNA (*35*). For these experiments, the wild-type yOgg1 was incubated with 59mer oligodeoxyribonucleotide duplexes containing either [G·C] or [Pr·C]. The Pr lesion was placed in the same sequence environment as in the 13mer duplexes used in the competition assays (Table 1 and Figure 5). The formation of a specific complex between yOgg1 and the [Pr·C] duplex was assessed by EMSA. Under the assay conditions used, only one specific complex was observed and about 80% of the [Pr·C] probe was titrated in this complex, whereas less than 20% of the control [G·C] duplex was bound (data not shown). The preformed [yOgg1−DNA] complexes were exposed to the hydroxyl radical generating system [$(Fe^{II}/Na_2EDTA)^{2-}/H_2O_2$/ascorbate], and the products of the reaction were analyzed by sequencing PAGE (*36*). Figure 6A shows footprints of the wild-type yOgg1 at a 5:1 protein:DNA ratio, on 59mer [G·C] and [Pr·C] duplexes that are [^{32}P]-labeled at the 5′-end on either the G- or Pr-containing strand (upper strand) or the complementary strand containing a cytosine opposite to the lesion (lower strand). The densitometer scans for the upper strand show that the yOgg1 protein strongly protects six nucleotides including the Pr residue as well as 2 and 3 nucleotides at the 3′ and 5′ side of the lesion, respectively (Figure 6B, panel a). On the complementary strand, yOgg1 footprint indicates a strong protection of the cytosine residue paired with Pr in the [Pr·C] duplex (Figure 6B, panel b). Footprinting analysis was also performed at protein:DNA ratios of 10:1 and 20:1. In these conditions the footprint of yOgg1 covers 10 nucleotides on the Pr-containing strand (4 and 5 nucleotides at the 3′ and 5′ side of the lesion) and 7 nucleotides on the complementary strand (3 and 3 nucleotides at the 3′ and 5′ side of the cytosine opposite Pr) (Figure 7 and data not shown). Furthermore, footprints of the wild-type, K241R, and K241Q proteins bound to [G·C] and [Pr·C] duplexes have been compared (Figure 7). After scanning and quantitative analysis, we conclude that the wild type, K241R, and K241Q footprints are identical (Figure 7). Taken together, these results demonstrate that K241R and K241Q mutant proteins have conserved wild-type DNA binding properties and they can be used to analyze interactions between yOgg1 and damaged DNA.

DISCUSSION

An important objective in the field of DNA repair is to understand how DNA repair enzymes locate and remove aberrant bases among a vast excess of normal bases in DNA. One approach relies on the careful investigation of the structural and biochemical properties of DNA glycosylases catalyzing the first step in the course of the BER process (*10*−*12*). The Ogg1 protein is one of the three DNA glycosylases/AP lyases involved in the removal of oxidative DNA base lesions in *S. cerevisiae* (*41*). These yeast proteins belong to a family of DNA glycosylases/AP lyases, to which we refer as the HhH-GPD/K family. The critical lysine in yOgg1 is K241, whose importance was suggested by a preliminary study with cell-free protein extracts (*27*). In the present study, we purified to apparent homogeneity the wild-type and the K241R and K241Q yOgg1 proteins for a quantitative analysis of their catalytic and DNA binding

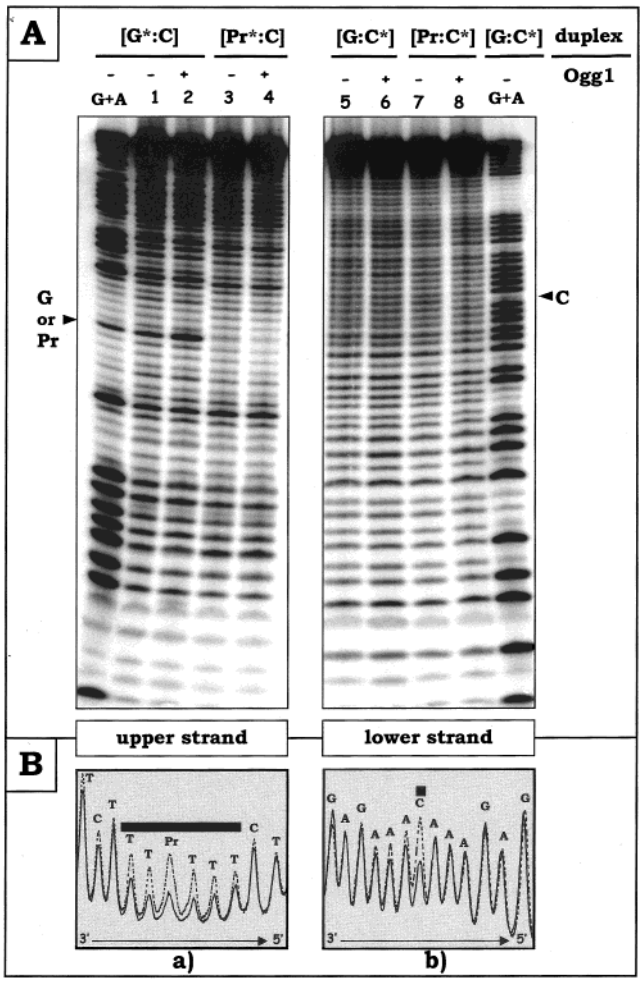

FIGURE 6: Hydroxyl radical footprints of the wild-type yOgg1 protein bound to a 59mer oligodeoxyribonucleotide duplex containing the 1,3-propanediol AP site analogue [Pr·C]. (A) Gel autoradiography: Before exposure to the hydroxyl radical generating system, 5′-[^{32}P]-labeled 59mer [G·C] or [Pr·C] duplexes were incubated in the absence (lanes 1, 3, 5, and 7) or in the presence of yOgg1 at a protein:DNA ratio of 5:1 (lanes 2, 4, 6, and 8). The products of the reactions were separated by sequencing PAGE. An asterisk indicates 5′-end [^{32}P] radioactive labeling. "Upper strand" contained a G or Pr (Table 1). "Lower strand" contained a cytosine (C) opposite G or Pr. G + A lanes are Maxam−Gilbert sequencing reactions. (B) Quantification: Sequencing gel were dried and normalized densitometer scans obtained were plotted. a) Scans of lanes 3 and 4 and b) scans of lanes 7 and 8 were obtained from two independent experiments. The broken line represents the hydroxyl radical cleavage pattern for naked DNA (lanes 3 or 7), whereas the continuous line represents the cleavage pattern obtained for the yOgg1−DNA complex (lanes 4 or 8). The black boxes indicate the strongly protected nucleotides.

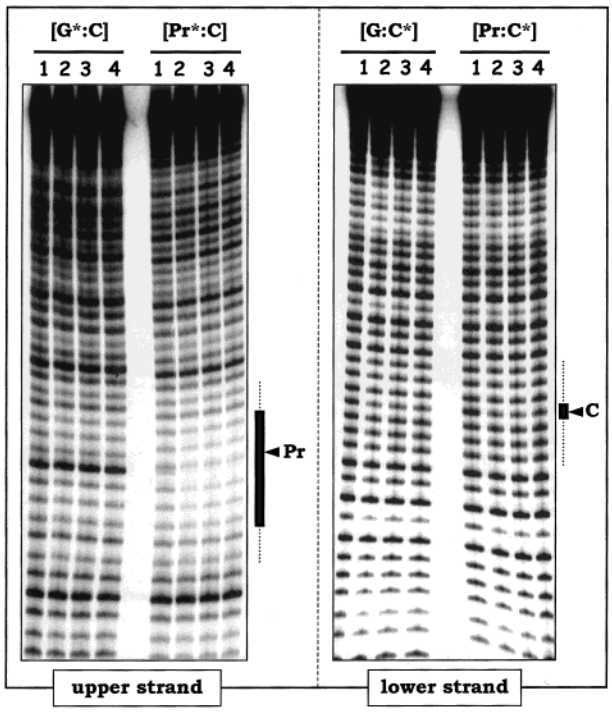

FIGURE 7: Hydroxyl radical footprints for the wild-type, K241R, and K241Q yOgg1 proteins bound to 59mer [Pr·C] duplexes. These experiments were performed as described under Materials and Methods and in the caption to of Figure 6. Hydroxyl radical patterns of 5′-[^{32}P]-labeled [Pr·C] and [G·C] in the absence (lane 1) or in the presence of yOgg1 protein at a protein:DNA ratio of 10:1 are shown: lane 2, wild-type yOgg1; Lane 3, K241R; lane 4, K241Q. Black bars on the right side of each panel indicate the protein footprint determined from densitometer scans as in Figure 6. The dashed lines indicate additional nucleotides protected at higher protein concentrations (20:1).

properties. In the K241R mutant protein, a basic residue (arginine) was conserved, whereas in the other, a neutral residue (glutamine) was substituted for K241. The results show that the K241R protein exhibits reduced DNA glycosylase and AP lyase activities, compared to those of the wild-type yOgg1 protein. All reactions are not altered to the same extent; thus cleavage of [8-OxoG·C] and [AP·C] duplexes occurs at slow rates, whereas cleavage of [8-OxoA·C] is completely abolished. The reduced cleavage activity of the K241R mutant is mostly due to a 6-fold lower k_{cat} value compared to that of the wild-type yOgg1. Similar alteration of the catalytic functions was observed for the K212R mutant of hNth1 (*42*). It is also important to notice that the excision of 8-OxoG, the formation of a covalent imino enzyme−DNA intermediate, and the cleavage of the [8-OxoG·C] duplex are similarly altered by the K to R mutation of yOgg1 at position 241. On the other hand, the K to Q mutation in yOgg1 completely abolishes all tested catalytic activities. This also holds true for *E. coli* endonuclease III (K120Q) (*30*), human hNth1 (K212Q) (*31*), and human α-hOgg1 (K249Q) (*43*). These results strongly suggest a single active site for all the reactions catalyzed by yOgg1 leading to excision of the lesion and cleavage at the resulting AP site. These results also show that the free ϵ-amino group of lysine 241 is required for fully catalytically active yOgg1 protein. Although basic, arginine cannot efficiently substitute for lysine at the active site. This is in agreement with the fact that among more than 20 endonuclease III homologues found in sequence databases, none possesses an arginine instead of a lysine at position 2 of the second α-helix of the HhH motif. This observation confirms that the lysine of the HhH-GPD/K motif is essential for the catalytic activity.

In this study, we also report on the role of the K241 residue of yOgg1 on its DNA binding properties. Comparison of K_{dapp} values shows that the wild-type, K241R, and K241Q proteins bind to [8-OxoG·C] or [8-OxoA·C] with the same efficacy. The strong binding of yOgg1 to [8-OxoA·C] duplex compared to that of [A·C] duplex indicates that the lesion rather than the mismatch is targeted by yOgg1 proteins. These results indicate that the K241 residue does not play a major role in the recognition of the modified base in DNA.

Furthermore, we compared hydroxyl radical footprints of the wild-type, K241R, and K241Q yOgg1 proteins bound to a 59mer [Pr•C] duplex. Indeed, the three proteins exhibit identical footprints with a strong protection of a region of 6 nucleotides centered on the Pr residue in the damage-containing strand and a single nucleotide carrying the cytosine placed opposite Pr in the complementary strand. Comparable hydroxyl radical footprints were observed for the Fpg protein bound to [F•C] or [Pr•C] duplexes (*36*, *37*). These results strongly suggest that yOgg1 as well as Fpg tightly contact the damaged strand. The strong protection of the cytosine is well fitting with the fact that yOgg1 preferentially repairs [8-OxoG•C], [8-OxoA•C], and [AP•C] duplexes (*27*, *28*). These results suggest that the cytosine opposite 8-OxoG, 8-OxoA, AP site, or Pr residues is specifically recognized by the yOgg1 protein. The recognition of the base opposite the lesion is not a general mechanism selected by DNA glycosylases. Nevertheless, in at least one other case, the Mug protein, the evidence for opposite-base recognition was demonstrated by EMSA and footprinting analysis (*44*). The visualization of this interaction has been done by the atomic structure of the Mug protein complexed with a DNA containing an AP site (*45*). The Mug protein makes three hydrogen bonds with the guanine localized opposite the AP site (*45*). Details of the Mug structure explain the enzyme specificity for [U•G] or [T•G] mispairs, which derives from direct recognition of the widowed guanine (*45*). On the other hand, such a specific interaction was not identified in the crystallographic structure of the human Aag protein (*46*). In this case, it was shown that the Aag protein does not specify a preference for the base placed opposite the lesion (*47*, *48*) and no specific contacts are made with the thymine opposite pyrrolidine as it could be seen in the cocrystal structure of Aag (*46*). Thus in the case of yOgg1, we propose that one or more protein residues interacts with the cytosine opposite the lesion. Only an atomic model of yOgg1 complexed to its target DNA will demonstrate this hypothesis.

The structural features required for the lesion recognition by DNA glycosylases are often examined by use of nonproductive protein−DNA complexes between the wild-type protein and substrate analogues such as AP site analogues or 2′-fluoro-2′-deoxyuridine or 2′-fluoro-2′-deoxyadenine (*36*, *39*, *49*, *50*). In this study, we show that the K241Q mutant of yOgg1 has no detectable catalytic activities but has conserved wild-type DNA binding properties. Therefore, the K241Q protein can be used to form stable, nonproductive complexes with DNA fragment containing metabolizable substrates. This type of approach might be applicable to all DNA glycosylases/AP lyases harboring the HhH-GPD/K motif by generating a K to Q mutation at the critical lysine.

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