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The Ogg1 protein of *Saccharomyces cerevisiae*: a 7,8-dihydro-8-oxoguanine DNA glycosylase/AP lyase whose lysine 241 is a critical residue for catalytic activity

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ABSTRACT

The *OGG1* gene of *Saccharomyces cerevisiae* codes for a DNA glycosylase that excises 7,8-dihydro-8-oxoguanine (8-OxoG) and 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (Fapy) from damaged DNA. In this paper, we have analysed the substrate specificity and the catalytic mechanism of the Ogg1 protein acting on DNA subtrates containing 8-OxoG residues or apurinic/apyrimidinic (AP) sites. The Ogg1 protein displays a marked preference for DNA duplexes containing 8-OxoG placed opposite a cytosine, the rank order for excision of 8-OxoG and cleavage efficiencies being 8-OxoG/C > 8-OxoG/T >> 8-OxoG/G and 8-OxoG/A. The cleavage of the DNA strand implies the excision of 8-OxoG followed by a β-elimination reaction at the 3′-side of the resulting AP site. The Ogg1 protein efficiently cleaves a DNA duplex where a preformed AP site is placed opposite a cytosine (AP/C). In contrast, AP/T, AP/A or AP/G substrates are incised with a very low efficiency. Furthermore, cleavage of 8-OxoG/C or AP/C substrates implies the formation of a reaction intermediate that is converted into a stable covalent adduct in the presence of sodium borohydre ($NaBH_4$). Therefore, the Ogg1 protein is a eukaryotic DNA glycosylase/AP lyase. Sequence homology searches reveal that Ogg1 probably shares a common ancestor gene with the endonuclease III of *Escherichia coli*. A consensus sequence indicates a highly conserved lysine residue, K120 of endonuclease III or K241 of Ogg1, respectively. Mutations of K241 to Gln (K241Q) and Arg (K241R) have been obtained after site directed mutagenesis of *OGG1*. Mutation K241Q completely abolishes DNA glycosylase activity and covalent complex formation in the presence of $NaBH_4$. However, the K241Q mutant still binds DNA duplexes containing 8-OxoG/C. In contrast, K241R mutation results in a catalytically active form of Ogg1. These results strongly suggest that the free amino group of Lys241 is involved in the catalytic mechanism of the Ogg1 protein.

INTRODUCTION

Free radicals and reactive oxygen species (ROS) can attack DNA causing base and sugar damage (1). Oxidative DNA damage has been suggested to play a role in the etiologies of degenerative pathologies in man such as cancer and aging (2–4). Several lines of evidence suggest that an oxidatively damaged form of guanine, 7,8-dihydro-8-oxoguanine (8-OxoG), is a highly mutagenic DNA lesion (5,6). *Escherichia coli* possesses two DNA glycosylases that prevent mutagenesis by 8-OxoG: the Fpg protein which excises 8-OxoG in damaged DNA and the MutY protein which excises the adenine residues incorporated by DNA polymerases opposite 8-OxoG (5–7). Inactivation of both the *fpg* (*mut*M) and *mut*Y (*mic*A) genes of *E.coli* results in a strong GC→TA mutator phenotype (8–10). In *Saccharomyces cerevisiae*, the *OGG1* gene encodes a protein of 376 amino acids, the Ogg1 protein, which possesses a DNA glycosylase activity that catalyses the removal of 8-OxoG and Fapy residues from damaged DNA (11). Recently, we have demonstrated that Ogg1-deficient strains of *S.cerevisiae* exhibit a mutator phenotype and specifically accumulate GC→TA transversions (12). These results show that base excision repair of 8-OxoG, by proteins like Fpg or Ogg1, protects genomes from the deleterious action of ROS in prokaryotes or in eukaryotes (13).

The Fpg protein of *E.coli* was initially isolated as a DNA glycosylase which excises Fapy residues in alkylated DNA (14,15). The Fpg protein also releases other damaged purines such as 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-G), 4,6-diamino-5-formamidopyrimidine (Fapy-A) and 8-oxoG (16–19). Besides its DNA glycosylase activity, the Fpg protein has a physically associated activity which incises DNA at apurinic/apyrimidinic (AP) sites and another activity which removes 5′-terminal deoxyribose-phosphate from DNA (20–22). The AP nicking mechanism implies successive β- and δ-elimination reactions leaving a single nucleoside gap in DNA (20,23).

The Ogg1 protein of *S.cerevisiae* which excises Fapy and 8-OxoG is also endowed with an AP nicking activity (11). It was proposed that the strand cleavage at the 3′ side of an AP site occurs via a β-elimination reaction (11,24). Several studies have led to the emergence of a unified catalytic mechanism for DNA glycosylases/AP lyases such as Fpg protein or endonuclease III of *E.coli* (25,26). These enzymes proceed by a nucleophilic attack

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at the sugar of the damaged base nucleotide using an imino group as the attacking nucleophile. The consequence is the formation of an imino enzyme–DNA intermediate as the product of the glycosylase step. Sodium borohydride has been used to trap this intermediate in reactions catalysed by DNA glycosylases/AP lyases (24–27).

In this study, we report an analysis of the substrate specificity and of the catalytic mechanism of the Ogg1 protein of *S.cerevisiae*. The results show that the Ogg1 protein has a marked preference for lesions, either 8-OxoG or AP sites, placed opposite a cytosine in DNA. The mechanisms of strand cleavage for both 8-OxoG/C and AP/C duplexes imply the formation of a Schiff base intermediate which can be converted into a stable adduct in the presence of $NaBH_4$. The results also show that lysine 241 is a crucial residue for the catalytic activities of the Ogg1 protein but not for DNA binding.

MATERIALS AND METHODS

Bacterial strains, plasmids and enzymes

Escherichia coli strains BH410 (*fpg*::Kan^r) and PR195 (*fpg*::Kan^r *mutY*::Kan^r) and plasmid pYSB10 containing the *OGG1* gene of *S.cerevisiae* were from our laboratory (11,28,29). Endonuclease III (Nth), endonuclease IV (Nfo), uracil DNA glycosylase and Fpg protein from *E.coli* were purified from overproducing strains. Restriction endonucleases, DNA polymerases, T4 polynucleotide kinase and T4 DNA ligase were from commercial sources.

Recombinant DNA methods

Plasmid pYSB10 was used as a template in a PCR reaction to amplify *OGG1*. Primer 1 was used to engineer an *Eco*RI restriction site at the beginning of *OGG1* (start codon is in bold). Primer 2 was used to introduce a *Hin*dIII restriction site at the end of *OGG1* (stop codon is in bold).

Primer 1: 5′-CCGGAATTC**ATG**TCTTATAAATTCGG-3′

Primer 2: 5′-GCCCAAGCTT**CTA**ATCTATTTTTGCTTC-3′

The amplified fragment containing the *OGG1* gene was cloned into plasmid pKK223-3 (Pharmacia) after digestion by *Eco*RI and *Hin*dIII restriction enzymes yielding the pYSB160 plasmid. The sequence of the *OGG1* gene in pYSB160 does not reveal mutations when compared with the published sequence of the *OGG1* gene (11).

Site-directed mutagenesis

The Altered site II Systems (Promega) was used to introduce single base pair mutations in the *OGG1* gene. For this purpose, *OGG1* from pYSB160 was cloned in the pALTER-1 vector between the *Eco*RI and *Hin*dIII sites. The following oligonucleotides were used to generate the mutant Ogg1 proteins where the base resulting in the Lys (K)→Gln (Q) or Lys→Arg (R) substitutions are indicated in bold type and underlined: K241Q protein, GGTGTAGGCCCC**C**AAGTTGCTGATTGC; K241R protein, GGTGTAGGCCCCA**G**AGTTGCTGATTGC. Following mutagenesis, the sequences were verified and the *Eco*RI/*Hin*dIII fragments were inserted into the pKK223-3 vector yielding the pYSB161(K241Q) and pYSB162(K241R) plasmids, respectively.

Purification of the Ogg1 protein

The Ogg1 protein was purified from isopropyl β-D-thiogalactopyranoside (IPTG)-induced *E.coli* BH410 (*fpg*$^-$) hosting pYSB160. The cells (29 g) were resuspended in 200 ml of Buffer A (25 mM Tris–HCl pH 7.6, 2 mM Na_2EDTA and 5% glycerol) containing 250 mM NaCl. The cell suspension was supplemented with lysozyme (1 mg/ml) and incubated at 0°C for 20 min, followed by 15 min at 37°C and 15 min at –80°C. The resulting lysate was centrifuged at 130 000 *g* for 45 min at 4°C. The supernatant fraction was taken as crude extract (fraction I: 200 ml). Fraction I was loaded onto a QMA-Acell anion exchange column (Waters) equilibrated with Buffer A containing 250 mM NaCl. The Fapy DNA glycosylase activity was not retained under these conditions (fraction II: 380 ml). Fraction II was dialysed against Buffer A containing 50 mM NaCl and applied to a Phospho-Ultrogel A6 column (IBF-LKB). Proteins were eluted with a linear salt gradient (50–600 mM NaCl). Fractions containing the activity eluted at 350 mM NaCl (fraction III: 130 ml). Fraction III was precipitated using ammonium sulfate, 500 g/l (w/v). The precipitate was collected by centrifugation and resuspended in Buffer A containing 1 M NaCl and loaded onto an AcA54 gel filtration column (IBF-LKB). Fractions containing the activity (fraction IV: 43 ml) were dialysed against Buffer A containing 100 mM NaCl and loaded onto a double-stranded DNA cellulose column (Sigma). Proteins were eluted with a linear salt gradient (100–800 mM NaCl). The active fractions eluted at 500 mM NaCl (fraction V: 12 ml). Fraction V was dialysed against Buffer A containing 100 mM NaCl and loaded onto a MonoS HR5/5 (FPLC, Pharmacia). Proteins were eluted with a salt gradient (100–800 mM NaCl). Fractions containing the activity eluted at 450 mM NaCl (fraction VI: 4 ml). Protein concentration was measured according to Bradford (30).

Fapy DNA glycosylase activity assay

The [^{3}H]Fapy–poly(dG-dC) was prepared as previously described (31). The assay mixture (100 µl) was composed of 25 mM Tris–HCl pH 7.6, 100 mM KCl, [^{3}H]Fapy–poly(dG-dC) and Ogg1 protein. The reaction was carried out at 37°C for 15 min. Ethanol-soluble radioactive material was quantitated and the chemical nature of this material was monitored by HPLC (31).

8-OxoG DNA glycosylase assay

The assay mixture (50 µl) contained 25 mM Tris–HCl pH 7.6, 100 mM KCl, 10 pmol 8-OxoG/N duplex (34mer DNA duplexes containing a single 8-OxoG placed opposite each of the four DNA bases in the complementary strand) and Ogg1 protein. The reaction was carried out at 37°C for 15 min. The products of the reaction were analysed by HPLC with electrochemical detection as previously described (11).

Assay for nicking activity at 8-OxoG

The 34mer oligonucleotide containing a single 8-oxoG was synthesized as previously described (32): 5′-GGCTTCATCGTT-ATT(**8-OxoG**)ATGACCTGGTGGATACCG-5′*. Complementary sequences with a C, T, G or A placed opposite 8-OxoG after hybridization were also synthesized. The nucleotide at the 3′-end was inverted yielding a 5′-$(N)_n$-3′-P-3′-N-5′* sequence with two 5′-ends (33). Thus, the 34mer containing 8-OxoG was labelled at both ends using [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase (32,33). The labeled strand was hybridized with a

complementary sequence yielding 8-OxoG/N duplexes. The assay mixture (20 µl) contained 25 mM Tris–HCl pH 7.6, 2 mM Na_2EDTA, 100 mM KCl, 50 fmol of ^{32}P-labeled 8-OxoG/N duplex and Ogg1 protein. The reactions were performed at 37°C for 15 min. The products of the reactions were separated by 20% PAGE containing 7 M urea. Cleavage was quantitated after scanning of the autoradiographs using the NIH V1.59 software.

Assay for nicking activity at AP sites

The 30mer oligonucleotide containing a single uracil residue and the four complementary sequences with C, T, G or A placed opposite the uracil after hybridization were of commercial origin: 5′-TACGGATCGCAG(**U**)TGGGTTAGGGAAGTTGG-3′. This 30mer oligonucleotide was ^{32}P-labeled at the 5′-end and annealed with one of the four complementary sequences yielding U/N duplexes. To generate AP sites, 500 fmol of each U/N duplex was incubated with 20 ng of purified uracil DNA glycosylase for 15 min at 37°C yielding the AP/N duplexes (30mer DNA duplexes containing a single AP site placed opposite each of the four DNA bases in the complementary strand). Assay conditions are as described for 8-OxoG/C nicking assay.

Formation of enzyme–DNA covalent complexes (trapping assay)

The trapping assays were performed using either 8-OxoG/N (Reaction 1) or AP/N (Reaction 2) duplexes as substrates. Reaction 1 (20 µl) contained 25 mM Tris–HCl pH 7.6, 2 mM Na_2EDTA, 100 fmol labelled 8-OxoG/N duplex and 50 mM of either $NaBH_4$, $NaCNBH_3$ or NaCl. Finally, 50 ng of either Ogg1 or Fpg protein was added to the mixture. The reaction was carried out at 37°C for 20 min. Reaction 2 (20 µl) contained 25 mM Tris–HCl buffer pH 7.6, 2 mM Na_2EDTA and 100 fmol labeled AP/N duplex, 50 mM $NaBH_4$ and 50 ng of Ogg1 protein. Immediately before the reaction, the Ogg1 protein was premixed with $NaBH_4$ and added to the rest of the assay mixture. The reaction was carried out at 4°C for 20 min followed by 5 min at 37°C. The reactions were stopped by addition of 10 µl SDS–PAGE loading buffer/formamide blue dye and heating at 90°C for 3 min. The products of the reactions were separated onto 10–20% gradient SDS–PAGE and analysed as previously described.

Gel retardation assay with bacterial crude extracts

Binding reactions were performed at 4°C for 20 min. The reaction mixture (20 µl) contained 50 mM Tris–HCl pH 7.6, 100 mM KCl, 2 mM Na_2EDTA, 5% glycerol, 0.2 mg/ml poly(dI-dC), 100 pM (8000 c.p.m.) of ^{32}P-labelled 8-OxoG/C oligonucleotide duplex and 10 µg of proteins from bacterial crude lysate. The samples were analysed using non-denaturing 10% PAGE as described by Castaing *et al.* (34).

Antibody preparation and immunoblotting

Polyclonal antibodies against Ogg1 were obtained after immunisation of female New Zealand rabbits. Antibodies were purified from the antiserum and the resulting preparation was referred to as BCE-1691. Total proteins from bacterial extracts were separated using 12.5% SDS–PAGE and transferred to Hybond-C membranes (Amersham). Western blots were performed using BCE-1691 at a dilution of 1:10 000 in phosphate buffered saline–0.1% Tween 20 (pH 7.4). Western blots were then developed using an anti-rabbit horseradish peroxidase-conjugated (Amersham) and detected with the BM chemiluminescence blotting substrate (POD) system (Boehringer).

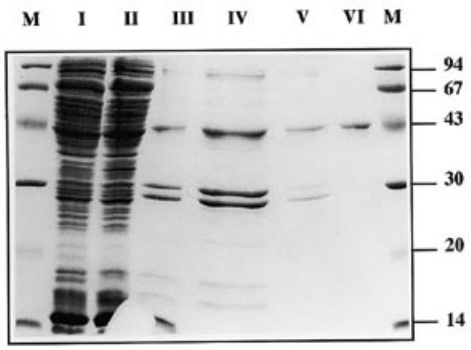

Figure 1. SDS–PAGE analysis of the Ogg1 protein purification fractions. Lane M, molecular weight markers (Pharmacia); lanes I–VI, purification steps of the Ogg1 protein (Fig. 1). The amounts of protein loaded on the gel are: lanes I and II, 50 µg; lanes III and IV, 10 µg; lanes V and VI, 2 µg. The gel was 15% acrylamide, 0.4% bis-acrylamide and stained with Coomassie brilliant blue.

Determination of mutation frequencies in *E.coli*

PR195 cells harbouring either the pKK223-3, pYSB160, pYSB161 or pYSB162 vectors were grown in LB broth containing 100 µg/ml ampicillin and 0.5 mM IPTG. To determine the spontaneous frequencies of rifampicin-resistant mutants and lactose revertants, appropriate dilutions of at least 10 overnight cultures, originally inoculated with 10^3 cells, were plated on either LB agar, LB agar with rifampicin (100 µg/ml), or minimal lactose (0.2%) agar. Colonies were counted the following day in the case of the LB plates and 48 h after plating for the minimal lactose plates.

RESULTS

Purification of the Ogg1 protein

To overproduce the Ogg1 protein, the coding sequence of the *OGG1* gene of *S.cerevisiae* was PCR-amplified and cloned in the expression vector pKK223-3 yielding the pYSB160 plasmid. The Ogg1 protein was purified from IPTG-induced *E.coli* BH410 (*fpg::*Kan^r) cells harbouring pYSB160 (Table 1). The release of Fapy residues from [^3H]Fapy–poly(dG-dC) was used as an activity assay during the course of the purification. The purity of the final fraction VI was assessed by the observation of a single protein band on a SDS–PAGE with a molecular weight of 43 kDa (Fig. 1). This 43 kDa polypeptide was transfered to a membrane and the N-terminal sequence was determined. The unique N-terminal sequence for the 10 first amino acids was (Ser-Tyr-Lys-Phe-Gly-Lys-Leu-Ala-Ile-Asn). This sequence is identical to that deduced from the nucleotide sequence of *OGG1* which is confirmed as the structural gene coding for the Ogg1 protein.

Repair of 8-OxoG/N substrates by the Ogg1 protein and probing for imino enzyme–DNA intermediate

The substrate specificity of the Ogg1 protein was investigated using as substrates 34mer oligonucleotides containing a single 8-OxoG placed opposite each of the four DNA bases. The release of 8-OxoG as a free base was determined using HPLC with electrochemical detection (11). Figure 2a shows that the Ogg1 protein preferentially excises 8-OxoG placed opposite a cytosine

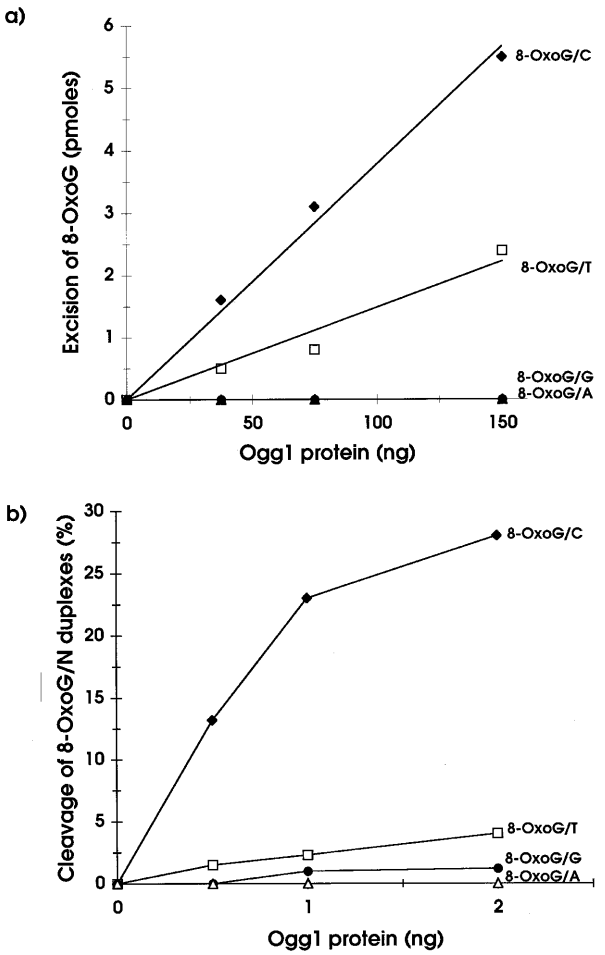

Figure 2. Repair by the Ogg1 protein of 34mer DNA duplexes containing a single 8-OxoG residue placed opposite each of the four DNA bases. Each 8-OxoG/N duplex was incubated with purified Ogg1 protein for 15 min at 37°C and the products of the reaction were separated (**a**) by HPLC and analysed with electrochemical detection (**b**) on denaturing 20% PAGE containing 7 M urea.

as compared to the three other mismatches, the efficiency order being 8-OxoG/C > 8-OxoG/T >> 8-OxoG/G and 8-OxoG/A. We have also analysed the cleavage of 8-OxoG/N duplexes by the purified Ogg1 protein. Figure 2b shows that Ogg1 cleaves efficiently 8-OxoG/C duplex as compared to 8-OxoG/T. Furthermore, the incision of 8-OxoG/G and 8-OxoG/A duplexes occurs at a very slow rate (Fig. 2b) as previously described (11,24).

Table 1. Purification of the Ogg1 protein

Step	Protein (mg)	Specific activity (U/mg)	Yield %	Purification (fold)
I. Crude extract	2080	23	100	1
II. QMA-Acell	1976	30	100	1
III. Phospho-Ultrogel	248	159	82	7
IV. Ultrogel AcA54	48	645	65	28
V. DNA–cellulose	10	1260	26	55
VI. MonoS–FPLC	2.6	2365	13	103

The Ogg1 protein was purified from 29 g of IPTG-induced *E.coli* BH410 (*fpg*⁻) harboring the pYSB160 plasmid. The Fapy DNA glycosylase activity was measured using [^{3}H]Fapy–poly(dG-dC) as substrate. One unit releases 1 pmol of Fapy in 15 min at 37°C. Purification steps are as described in Materials and Methods.

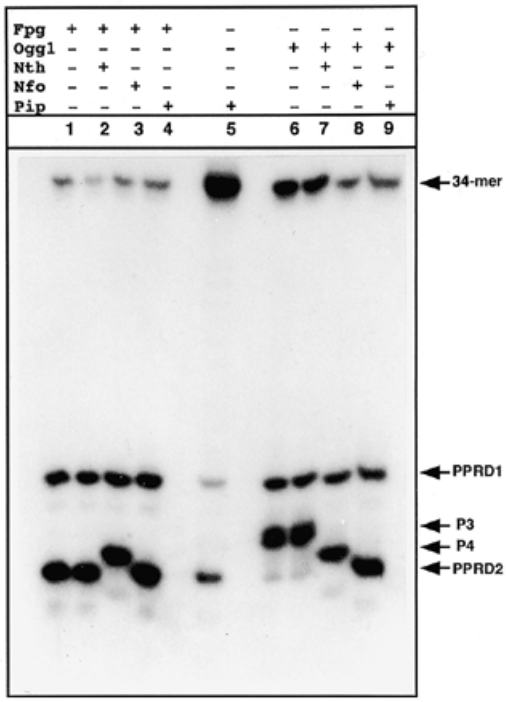

Figure 3. Analysis of the reaction products of the incision by the Fpg or the Ogg1 proteins of a 34mer oligonucleotide substrate containing a single 8-OxoG placed opposite a cytosine. The 8-OxoG containing strand was labeled at both ends and annealed with a complementary sequence with a cytosine opposite the lesion. The 8-OxoG/C duplex was incubated for 15 min at 37°C in the presence of 1 ng of Ogg1 or Fpg proteins. The enzymes were inactivated by heating for 5 min at 50°C. Reaction mixtures were then incubated with 10 ng of either endonuclease III (Nth) or endonuclease IV (Nfo). Hot piperidine (Pip) treatment was with 1 M of piperidine for 90 min at 90°C. The products were separated by denaturing 20% PAGE containing 7 M urea. PPRD1, PPRD2, P3 and P4 bands are described in the Results section.

Our understanding of the mechanism of strand cleavage by the Ogg1 protein required the identification of the 3′- and 5′-ends of the DNA fragments resulting from the incision of the 8-OxoG/C duplex. Figure 3 shows an analysis of the 8-OxoG/C substrate reacted with either Ogg1 or Fpg protein. The Fpg protein (lane 1) generates two fragments which are identical to those generated by hot piperidine treatment (lane 5), namely PPRD1 (5′-P-[N_{17}]-3′-P-3′-N-[^{32}P]-5′) and PPRD2 (5′-[^{32}P]-[N_{15}]-3′-P) (32,35). These two fragments are the products of successive β- and δ-elimination reactions at the AP site resulting from the excision of the 8-OxoG lesion, leaving a single nucleoside gap limited by 3′- and 5′-phosphate ends in DNA. (7,20,23). As expected, the products generated by the Fpg protein are not modified by endonuclease III (Fig. 3, lane 2). On the other hand, the PPRD2 product generated by Fpg is converted into the P4 product (5′-[^{32}P]-[N_{15}]-3′-OH) after removal of phosphorus at the 3′-end of PPRD2 by the Nfo protein (Fig. 3, lane 3). The Ogg1 protein generates a DNA fragment which comigrates with PPRD1 and another product which migrates as a doublet, P3 (5′-[^{32}P]-[N_{15}]-3′-P-dR) (Fig. 3, lane 6). Similarly, a doublet has been observed after nicking at the 3′-side of an AP site by the endonuclease III of *E.coli* leaving an unsaturated sugar at the 3′-end of the 5′-DNA fragment (20). These results imply that the Ogg1 protein, as in the case of endonuclease III, catalyses a

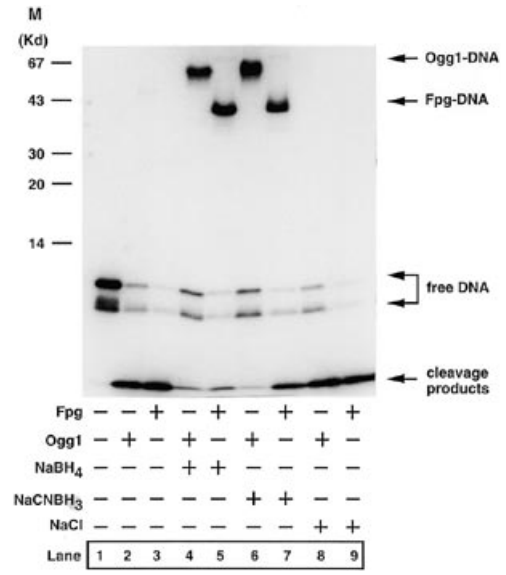

Figure 4. Formation by $NaBH_4$ or $NaCNBH_3$ reduction of a covalent complex between Ogg1 or Fpg protein and a 34mer DNA duplex containing a single 8-OxoG. Fifty nanograms of Ogg1 (1.16 pmol) or Fpg (1.66 pmol) proteins were allowed to react with 0.1 pmol of a labeled 34mer duplex containing a single 8-OxoG paired with a cytosine in the presence of 50 mM NaCl, $NaBH_4$ or $NaCNBH_3$ as described in Materials and Methods. The products of the reactions were analysed using a 10–20% polyacrylamide gradient SDS–PAGE allowing the identification of intact DNA, cleavage products and DNA–protein complexes (trapped complex). The molecular weight marker is as in the legend of Figure 1.

β-elimination reaction. To reinforce this conclusion, the cleavage products generated by the Ogg1 protein were incubated in the presence of other repair enzymes or hot piperidine. The addition of the endonuclease III (Nth) after Ogg1 does not result in alteration of cleavage products nor additional cleavage of the 34mer (Fig. 3, lane 7). This observation suggests that the Ogg1 protein possesses an AP nicking activity which is not limiting in our assay conditions. The addition of endonuclease IV (Nfo) after cleavage by the Ogg1 protein results in a quantitative convertion of the P3 product yielding the P4 product (5′-[^{32}P]-[N_{15}]-3′-OH) (Fig. 3, lane 8). The P4 product is probably generated by the release of the deoxyribose-phosphate at the 3′-end of the P3 fragment by the Nfo protein (Fig. 3, lane 8). Finally, treatment of the DNA products generated by the Ogg1 protein with 1 M piperidine provokes the modification of the P3 product to yield PPRD2 (Fig. 3; lane 9). It can be seen that the PPRD1 fragment is not modified by any treatment (Fig. 3). These results strongly suggest that the mechanism of strand cleavage by the Ogg1 protein implicates the release of the 8-OxoG residue followed by a β-elimination reaction at the 3′-side of the resulting AP site.

The mechanism of DNA strand cleavage after excision of base damage has been established for T4 endonuclease V, *Micrococcus luteus* UV-endonuclease, endonuclease III and Fpg proteins from *E.coli*. These proteins use an amino group as a nucleophile to attack at the C-1′ of the sugar moiety leading to the formation of an enzyme–DNA Schiff base intermediate (26,27,36,37). To probe for such an intermediate, the Ogg1 protein was allowed to react with a labeled 8-OxoG/C substrate in the presence of reducing agents such as $NaCNBH_3$ or $NaBH_4$ and the products of the reactions were analysed by SDS–PAGE. Figure 4 shows that $NaBH_4$ or $NaCNBH_3$ in the assay mixture results both in the inhibition of the cleavage activity of the Ogg1 protein and in the formation of a shifted band (trapped complex) with an apparent molecular weight of between 55 and 60 kDa (Fig. 4, lanes 4 and 6). The Ogg1–DNA complex can be digested with proteinase K to yield a product which co-migrates with free DNA (data not shown). Control experiments show the cleavage of the 8-OxoG/C substrate by the Ogg1 protein in the presence of NaCl (Fig. 4, lane 8). Figure 4 also shows the formation of an Fpg–DNA complex with an apparent molecular weight of between 40 and 45 kDa (lanes 5 and 7) confirming other studies (26,27). These results suggest that the Ogg1 protein forms a transient Schiff base intermediate which is converted into a covalent protein–DNA adduct in the presence of $NaBH_4$. Furthermore, we used the four 8-OxoG/N substrates for Ogg1–DNA trapping assay. Indeed, the rank order for Ogg1 mediated DNA trapping efficiency was as follows, 8-OxoG/C >> 8-OxoG/T >> 8-OxoG/G or 8-OxoG/A (data not shown), paralleling the cleavage efficiency. These conclusions are fully in accord with a recent independent study (24).

Cleavage of AP/N substrates by the Ogg1 protein and probing for an imino enzyme–DNA intermediate

Our study shows that the Ogg1 protein is a DNA glycosylase/AP lyase that cleaves DNA after the removal of a damaged base. However, we have not demonstrated that the Ogg1 protein possesses an activity that nicks DNA at preformed AP sites. Therefore, we have prepared 30mer DNA substrates, AP/N, in which a single AP site was placed opposite each of the four DNA bases. Figure 5 shows that the Ogg1 protein very efficiently cleaves the AP/C duplex where a cytosine was placed opposite the AP site. Indeed the cleavage efficiency of the AP/C substrate is similar to that of the 8-OxoG/C in a parallel reaction (Fig. 5, insert). In contrast, the AP/T duplex is a very poor substrate for the Ogg1 protein and AP/G and AP/A duplexes are not incised at all (Fig. 5). Furthermore, the reduction of the AP site with $NaBH_4$ before the reaction with Ogg1 makes the AP/C duplex resistant to cleavage by the Ogg1 protein (data not shown). These results strongly suggest that the Ogg1 protein possesses an intrinsic AP lyase activity.

To analyse the mechanism of strand cleavage at AP sites, the Ogg1 protein was allowed to react with AP/N duplexes in the presence of $NaBH_4$. The reaction was performed at 4°C and the reaction products were analysed by SDS–PAGE. Figure 6 shows that the Ogg1 protein forms a stable covalent complex with a duplex DNA containing an AP site. The complex formation is strictly dependent upon the presence of the Ogg1 protein and of $NaBH_4$. Figure 6 also shows that the Ogg1 protein reacts very efficiently with an AP/C substrate as compared with the other AP/N duplexes. The rank order for trapping efficiency is AP/C >> AP/T >> AP/G or AP/A paralleling the cleavage efficiency order at AP sites or 8-OxoG (Figs 2, 5 and 6). These results show that the catalytic mechanism for strand cleavage at preformed AP sites by the Ogg1 protein also involves the formation of a transient imino enzyme–DNA intermediate.

Functional analysis of mutant Ogg1 proteins: Lys241→Gln241 (K241Q) and Lys241→Arg241 (K241R)

The Ogg1 protein belongs to a family of base-excision repair proteins related to the endonuclease III of *E.coli* (24). The sequence similarity between these two proteins is limited to a

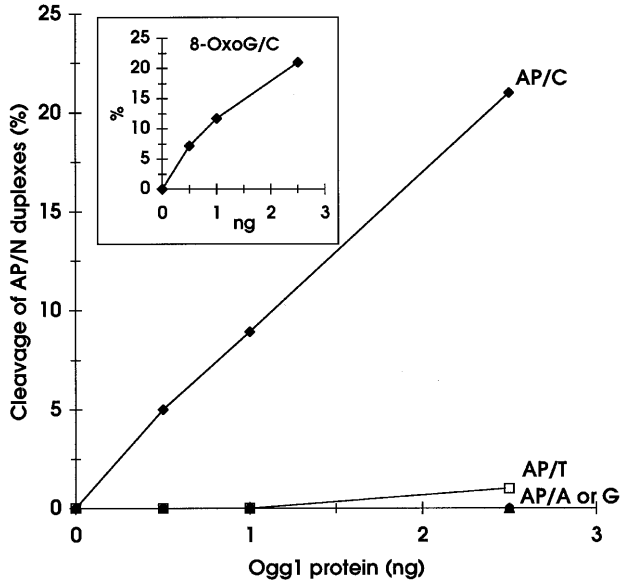

Figure 5. Cleavage of 30mer DNA duplexes containing a single AP site placed opposite each of the four DNA bases by the Ogg1 protein. The 30mer sequence used is reported in Materials and Methods. The AP site results from the excision of U_{13} by the uracil DNA glycosylase from *E.coli*. The AP site containing strand was ^{32}P-labeled at the 5′-end and annealed with each of the four possible complementary sequences. Each AP/N duplex was incubated with purified Ogg1 protein for 15 min at 37°C and the products of the reaction were separated on denaturing 20% PAGE containing 7 M urea. The insert shows the cleavage of 8-OxoG/C duplex in a parallel experiment.

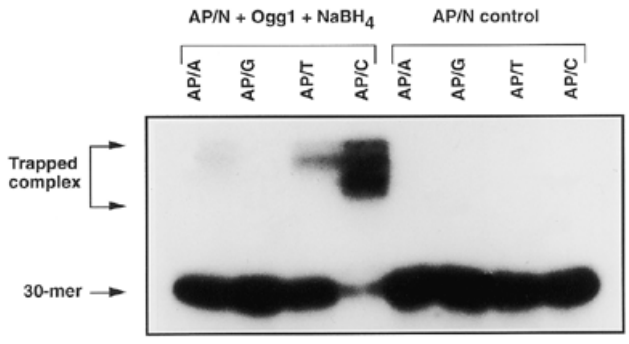

Figure 6. Formation by $NaBH_4$ reduction of a covalent complex between the Ogg1 protein and a 30mer DNA duplex containing a single AP site placed opposite each of the four DNA bases. Fifty nanograms of Ogg1 protein (1.16 pmol) was allowed to react with each of the four 30mer AP/N duplexes in the presence of 50 mM $NaBH_4$ as described in Materials and Methods. The products of the reactions were analysed using a 10–20% polyacrylamide gradient gel SDS–PAGE.

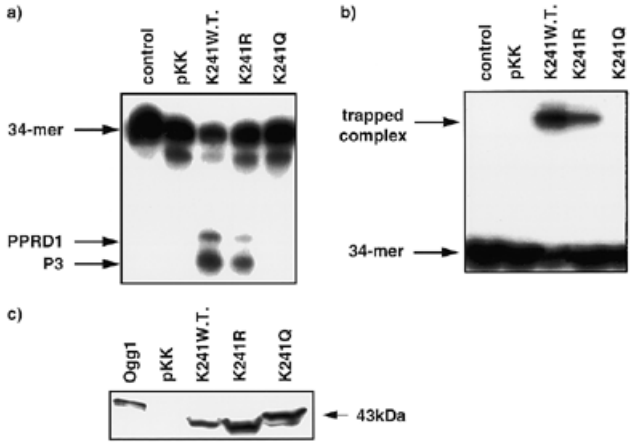

Figure 7. Comparison of the properties of the wild-type and mutant forms, K241R or K241Q, of the Ogg1 protein. Cell free extracts (10 µg-total protein) of *E.coli* BH410 harboring the various plasmids were used in the different reactions. pKK: plasmid pKK223-3 does not express Ogg1. K241W.T., wild-type Ogg1; K241R and K241Q, mutant forms of Ogg1 at Lys241. (**a**) Cleavage of 8-OxoG/C duplex (see Fig. 3). (**b**) Trapping assay in the presence of $NaBH_4$ (see Fig. 4). (**c**) Western blot analysis. Lane Ogg1 contained 100 ng of purified Ogg1 protein.

region spanning amino acid residues 232–265 and 111–143 of Ogg1 and endonuclease III, respectively. This region corresponds to the helix–hairpin–helix (HhH) structural motif and to the catalytic active site of the *E.coli* endonuclease III (38). Sequence alignment suggests that lysine 241 of Ogg1 corresponds to lysine 120 of endonuclease III (24). On these basis, lysine 241 of Ogg1 was used as a target for substitution to yield the K241Q (lysine→glutamine) and K241R (lysine→arginine) mutants. The various Ogg1 proteins were expressed in *E.coli* BH410 from pYSB160 (K241W.T.), pYSB161(K241R) or pYSB162 (K241Q) plasmids, respectively. Figure 7 shows that cell free extracts containing the wild-type Ogg1 or the K241R mutant cleave the 8-OxoG/C duplex and form a high molecular weight covalent complex in the presence of $NaBH_4$ (Fig. 7a and b). In contrast, cell free extracts containing K241Q mutants do not incise nor form retardation complexes with 8-OxoG/C (Fig. 7a and b). Western-blot analysis using BCE-1691 antibodies shows that the wild-type and the two mutant Ogg1 proteins are present in similar amounts in cell free extracts (Fig. 7c). These results strongly suggest that the replacement of Lys241 by Arg241 generates a catalytically functional protein, perhaps less active. In contrast, replacement of Lys241 by Gln241 results in a protein without catalytic activity. The expression vectors pKK223-3, pYSB160 (K241W.T.), pYSB161 (K241Q) and pYSB162 (K241R) were transformed in *E.coli* PR195 which displays a strong spontaneous mutator phenotype. The frequencies of either mutation to rifampicine resistance or reversion to *lac*$^+$ were determined. The results shown in Table 2 demonstrate that expression of wild-type Ogg1 protein (K241W.T.) and K241R mutant in PR95 *E.coli* strain reduces the mutation frequencies for both markers analysed whereas expression of K241Q mutant does not. Thus, the cleavage and trapping efficiency of K241W.T., K241R and K241Q proteins parallels the antimutator effect of these proteins. Nevertheless, the lack of activity of the K241Q mutant could be due either to a defect in the catalytic mechanism or to an incapacity to bind modified DNA. Figure 8 shows that the wild-type as well as the mutant K241R or K241Q Ogg1 proteins bind the 34mer 8-OxoG/C duplex. The shifted bands were found to migrate at the same position either with purified Ogg1 protein or with the various cell extracts containing wild-type or mutant Ogg1 proteins (Fig. 8). These latter observations suggest that lysine 241 has a functional role rather than a structural role in the catalytic activities of Ogg1 protein.

Table 2. Spontaneous mutagenesis of the mutant strain *E.coli fpg mutY* (PR195) harboring the pKK223-3, pYSB160, pYSB161 or pYSB162 vectors

Vector	Cloned gene	$Rif^r/10^8$	$Lac^+/10^8$
pKK223-3	none	118 ± 18	2027 ± 263
pYSB160	*OGG1*	13 ± 1	83 ± 7
pYSB161	*OGG1*K241Q	185 ± 61	1698 ± 300
pYSB162	*OGG1*K241R	12 ± 3	620 ± 132

DISCUSSION

In this study, we have purified the Ogg1 protein of *S.cerevisiae* from *E.coli* (*fpg*⁻) cells harboring the overproducing plasmid pYSB160. The Ogg1 protein is a monomer of 43 kDa which is endowed with two enzymatic activities, (i) a DNA glycosylase activity which excises Fapy and 8-OxoG, and (ii) an AP lyase activity which incises DNA at AP sites either preformed or resulting from the excision of a damaged base. The catalytic mechanism of the Ogg1 protein for the cleavage of DNA substrates containing either 8-OxoG or an AP site implies the formation of a transient enzyme–DNA intermediate which can be converted into a stable covalent adduct in the presence of $NaBH_4$. This is the first demonstration of a covalent intermediate between a DNA glycosylase and a preformed AP site. Therefore, these results suggest a unique mechanism for cleavage of DNA by the Ogg1 protein, (i) after excision of a damaged base and (ii) at a preformed AP site. Our data also show that the Ogg1 protein reacts preferentially with 8-OxoG and AP sites placed opposite a cytosine. This strong bias in favor of lesions placed opposite a cytosine is a hallmark of the Ogg1 protein and could reflect the biological properties of the Ogg1 protein that is required to repair endogenous mutagenic DNA damage thus preventing G.C→T.A transversions (12). It is worth noting that the Fpg protein, which is the functional homologue of the Ogg1 protein in bacteria, incises AP/N duplexes at approximatively the same rate (unpublished results). On the other hand, the rank order for cleavage efficiency by the Fpg protein is as follows, 8-Oxo/C = 8-OxoG/T > 8-OxoG/G >> 8-OxoG/A (32). Furthermore, the mechanism of strand cleavage at AP sites is also different since Ogg1 proceeds via a β-elimination reaction whereas Fpg proceeds via β- and δ-elimination reactions, respectively. To conclude, the substrate specificities and the catalytic mechanism of Fpg and Ogg1 are significantly different which, in turn, may suggest that these two proteins are not closely related.

The fact that Ogg1 is a DNA glycosylase/AP lyase prompted us to search for sequence homology between the Ogg1 protein and other proteins catalysing similar reactions. Indeed, sequence alignment amongst DNA glycosylases/AP lyases reveals sequence similarity between the Ogg1 protein and proteins related to the endonuclease III of *E.coli* rather than to the Fpg protein. The homology between the Ogg1 protein and the endonuclease III is limited to a region that includes a structural HhH motif found in the crystal structure of endonuclease III (38). Using this sequence, we have been able to align Ogg1 with other proteins suh as the UV-endonuclease of *M.luteus* (37), the Ntg1 protein of *S.cerevisiae* (39) and the Spo-Nth protein of *Schizosaccharomyces pombe* (40). A consensus sequence emerged from these comparison. It spans two blocks of conserved residues [(L/Y)(P/N)**GVG**(P/R)**K**] and [(V/I)X**VD(**V/T)**H**] separated by 14 or 15 amino acids. Two amino acids, K120 and D138 of *E.coli* endonuclease III, are conserved in all sequences (24,38). These residues correspond to K241 and D260 of the Ogg1 protein. The replacement of Lys241 of Ogg1 by an Arg (K241R) results in a catalytically active protein. In contrast, the K241Q mutation completely abolish the catalytic activity. However, both K241R and K241Q mutants bind to damaged DNA. These results are identical to those obtained with the K120Q mutant of the endonuclease III of *E.coli* (38) and confirm that sequence homologies between Ogg1 and endonuclease III reflect functional and/or structural similarities. Therefore, our results reinforce the concept that these proteins may have a common ancestor gene which may be the ancestor of most if not all eukaryotic DNA glycosylases/AP lyases. It is important to notice that neither the Fpg protein nor the T4 UV-endonuclease are included in this list of proteins. Indeed, Fpg proteins are highly conserved amongst eubacteria but no eukaryotic homologue has been so far identified.

Finally, studies of proteins like Ogg1 in the simple eukaryote *S.cerevisiae* are important to understand the biological impact of base excision repair in mammalian cells. Indeed, based on sequence similarity, the human homolog (*hOGG1*) of the *OGG1* gene of *S.cerevisiae* has been cloned recently (29,41).

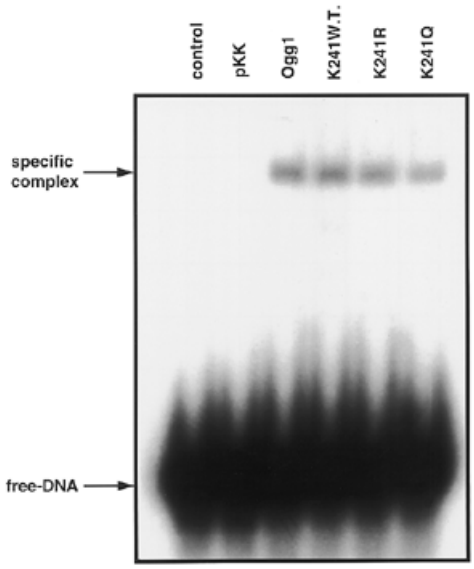

Figure 8. Binding of the 8-OxoG/C duplex by bacterial crude extracts containing either the wild type, K241R or K241Q mutant Ogg1 proteins. Cell free extracts were prepared from BH410 hosting various plasmids (see Fig. 7). Control, ^{32}P-labelled 8-OxoG/C duplex alone; Ogg1, 8-OxoG/C plus 50 ng of purified Ogg1. Other reactions contained 10 µg of total proteins from BH410 hosting pKK223-3 (pKK), pYSB160 (K241W.T.) or pYSB161 (K241R) or pYSB162 (K241Q). The products of the reactions were loaded onto non-denaturing PAGE for retardation analysis.

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