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Identification of *Escherichia coli* 8-oxoguanine endonuclease

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Abbreviations: ROS, reactive oxygen species; oh^{8}Gua, 7,8-dihydro-8-oxoguanine; Fapy, 2,6-diamino-4-hydroxyformamidopyrimidine; Fpg, 2,6-diamino-4-hydroxyformamidopyrimidine-DNA glycosylase; OGG, 7,8-dihydro-8-oxoguanine-DNA glycosylase; DTT, dithiothreitol

Abstract

7,8-Dihydro-8-oxoguanine (oh^{8}Gua) endonuclease is a DNA repair enzyme in *Escherichia coli* to remove oh^{8}Gua, a promutagenic DNA adduct. Due to the unique mode of enzyme action and substrate specificity, this DNA repair enzyme has been suggested to be identical to 2,6-diamino-4-hydroxyformamidopyrimidine (Fapy)-DNA glycosylase (Fpg). However, oh^{8}Gua endonuclease had not been definitely identified because it had not been homogenously purified. In this study, we attempted to purify and identify the enzyme. Through several purification procedures, we obtained two proteins (32 kD and 29 kD). The larger protein co-migrated with Fpg in 12% SDS-PAGE gel. Sequences of N-terminal amino acids of these two proteins were identical to that of Fpg; the smaller one is a degraded product of oh^{8}Gua endonuclease during purification steps. These results indicate that oh^{8}Gua endonuclease is identical to Fpg, implying that oh^{8}Gua in oxidatively damaged DNA rather than Fapy is more physiologically relevant substrate for Fpg.

Keywords: 7,8-dihydro-8-oxoguanine, reactive oxygen species, DNA repair, glycosylase, 2,6-diamino-4-hydroxyformamidopyrimidine, mutagenesis

Introduction

Reactive oxygen species (ROS) is produced from endogenous metabolism or environmental oxidative stresses, and these noxious stimuli have been reported to cause damages to cellular components including DNA by its reactivity (Marnett, 2000; Gracy *et al*., 1999; Beckman and Ames, 1997). Oxidative damage to cellular DNA is implicated in mutagenesis and carcinogenesis (Emerit, 1994). 7,8-Dihydro-8-oxoguanine (oh^{8}Gua) is one of promutagenic adducts in DNA formed by ROS (Cunningham, 1997).

oh^{8}Gua is formed by hydroxylation at C8 of guanine in DNA by ROS (Kasai and Nishimura, 1983). By tautomerism, oh^{8}Gua in DNA is in 8-keto-6-enolic form or 6, 8-diketo form (Oda *et al*., 1991). The content of oh^{8}Gua in DNA is increased by oxidative stresses *in vitro* as well as *in vivo*. Irradiation increased oh^{8}Gua contents in isolated DNA as well as cellular DNA in mice or HeLa cells (Kasai *et al*., 1986). Furthermore, increase of oh^{8}Gua in DNA was reported in several human diseases in which oxidative stresses are suggested to play important roles in pathogenesis (Liu *et al*., 1996; Lyras *et al*., 1998). From these findings, oh^{8}Gua is currently considered as an index of oxidative DNA damage (Helbock *et al*., 1999).

By structural preference in DNA helix (Oda *et al*., 1991), dATP tends to be incoporated opposite to oh^{8}Gua in DNA (Shibutani *et al*., 1991), which leads to GC to TA transversion (Wood *et al*., 1990). Living organisms from prokaryotes to eukaryotes have DNA repair enzymes to remove this promutagenic adduct from DNA (Chung *et al*., 1991; van der Kemp *et al*., 1996; Radicella *et al*., 1997; Rosenquist *et al*., 1997; Arai *et al*., 1997). In *E. coli*, Chung *et al.* (1991) reported the presence of oh^{8}Gua endonuclease activity to remove oh^{8}Gua. The enzyme cuts oh^{8}Gua-containing strand at the sites 5′ and 3′ to oh^{8}Gua, producing a single nucleotide gap. In addition, this enzyme has other activities: oh^{8}Gua glycosylase, apurinic/apyrimidinic (AP) lyase (Tchou *et al*., 1991). Based on this unique mode of enzyme action, substrate specificity of oh^{8}Gua endonuclease was compared to that of formamidopyrimidine (Fapy)-DNA glycosylase (Fpg) with the assumption that two enzymes might have been identical (Tchou *et al*., 1991).

Fapy is a DNA adduct formed by opening of purine ring by ROS (Chetsanga and Lindahl, 1979). Fpg removes Fapy from DNA and nicks DNA strand at AP sites (Boiteux, 1990). By comparison of substrate specificity of oh^{8}Gua endonuclease and Fpg, it was suggested that oh^{8}Gua endonuclease is identical to Fpg (Tchou *et al*., 1991). However, its identity was not determined definitely because partially purified oh^{8}Gua endonuclease was used in that report. In this study, we tried to determine its identity by peptide sequencing with homogenously purified protein.

Materials and Methods

Culture of *E. coli*

E. coli (BW9053, exonuclease III-deficient strain) was cultured in 500 l fermentor (Korea Fermentor Inc., Korea) in LB broth at 37°C. At mid-logarithmic phase, *E. coli* was harvested by centrifugation (10,000 *g*, 5 min) and frozen at -70°C until use.

Preparation of crude extract

All procedures were done at 4°C as previously described (Chung *et al*., 1991). Frozen *E. coli* (2.4 kg) was thawed and suspended in 6.0 l of buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.2 mM dithiothreitol (DTT), 10% glycerol). *E. coli* was ruptured by sonication (Model W-385, Heatsystems-Ultrasonics Inc, USA) and centrifuged at 25,000 *g* for 30 min. Nucleic acids were removed by addition of 5% streptomycin solution to a final concentration of 0.8% with stirring and centrifugation at 25,000 *g* for 30 min.

Purification procedures

Except gel filtration chromatography, all the purification procedures were done using HPLC (Model 302 pump, Model 811B gradient mixer, Model 115 UV detector; Gilson, France). Active fractions obtained from each steps were concentrated with pressurized ultrafiltration cell with YM10 membrane (Amicon, USA), extensively dialyzed against buffers with which columns were equilibrated, and centrifuged at 100,000 *g* for 30 min to remove particulates. For purification, the following columns were used; TSK gel Phenyl-5PW (21.5 × 150 mm, Toyo Soda, Japan) for phenyl HPLC, TSK gel DEAE-5PW (21.5 × 150 mm) for DEAE HPLC, a column (2.6 × 100 cm) packed with Sephacryl S-200 (Pharmacia, Sweden) for gel filtration column chromatography, TSK gel Heparin-5PW (7.5 × 75 mm) for heparin affinity HPLC and TSK gel HA 1000 (21.5 × 150 mm) for hydroxylapatite HPLC. Fractions from each HPLC and gel filtration chromatography were collected for 2 min and 12 min, respectively.

Assay of oh^{8}Gua endonuclease activity

Assay of oh^{8}Gua endonuclease activity was done as described by Chung *et al.* (1991). In brief, oligonucleotide-containing oh^{8}Gua (***G***^{OH}) at the specified position (5′ CAG CCA ATC AGT ***G***^{OH}CA CCA TCC 3′, a generous gift from Dr. Kasai in University of Occupational and Environmental Health, Japan) was 5′ end labeled with [γ-^{32}P] ATP and annealed with complementary strand. Chromatographic fractions (5 or 10 μl) were mixed with 0.4 pmole double stranded DNA in total volume of 50 μl reaction mixture (50 mM Tris-HCl, pH 7.5, 2 mM EDTA) and incubated at room temperature for 1 hr. After extraction with phenol-chloroform and precipitation with yeast tRNA and sodium acetate, nicked double-stranded oligonucleotide was visualized with 8 M urea-sequencing gel electrophoresis and autoradiography. To determine the collection of active fractions, % of DNA breakage was estimated by densitometry (Model 620, BioRad, USA). Yield of purification was determined as one unit that was defined as activity to nick 1 fmol of substrate DNA for 60 min.

SDS-PAGE and determination of N-terminal amino acid sequences

Purified proteins were dialyzed in a buffer (25 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.2 mM DTT and 35 mM NaCl) and separated in 12% SDS-PAGE gel. Fpg was a generous gift from Dr. Larval (Institut Gustav-Roussy, France). Separated proteins in gels were stained with silver staining kit (BioRad, USA).

For determination of N-terminal sequences, purified proteins were transferred to PVDF membrane (Applied Biosystems) in tank blotter at 2 mA/cm^2 for 1 hr. The membrane was dried and proteins were stained with Coomassie Blue. Protein bands were excised and their N-terminal sequences were determined with a gas phase sequencer connected to phenylthiohydantoin analyzer (model 473A, Applied Biosystems, USA).

Results

First Phenyl HPLC

Upon removal of nucleic acids with streptomycin, KCl at a final concentration of 2.0 M was added to crude extract. Supernatant after centrifugation at 100,000 *g* for 30 min was separated with phenyl HPLC with decreasing gradient of KCl (Figure 1A) at 4.0 ml/min. During loading 3 g of crude extract (80 ml) and washing the column with 2 M KCl, most of proteins were eluted without binding to the column. Active fractions were eluted in the range of 1 to 0.5 M of KCl.

DEAE HPLC

Active fractions collected from repeated phenyl HPLC were dialyzed against a buffer (25 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.2 mM DTT, 35 mM NaCl) and separated with DEAE HPLC at 4.0 ml/min (Figure 1B). Activities of oh^{8}Gua endonuclease were detected in fractions eluted from 50 to 200 mM NaCl. From these steps, no other DNA nicking activities were observed making it possible to calculate the yield of purification (Table 1).

Gel filtration chromatography

Concentrated active fractions from DEAE HPLC were dialyzed against a buffer (25 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.2 mM DTT, 0.2 M NaCl) and separated with a column packed with Sephacryl S-200 at a flow

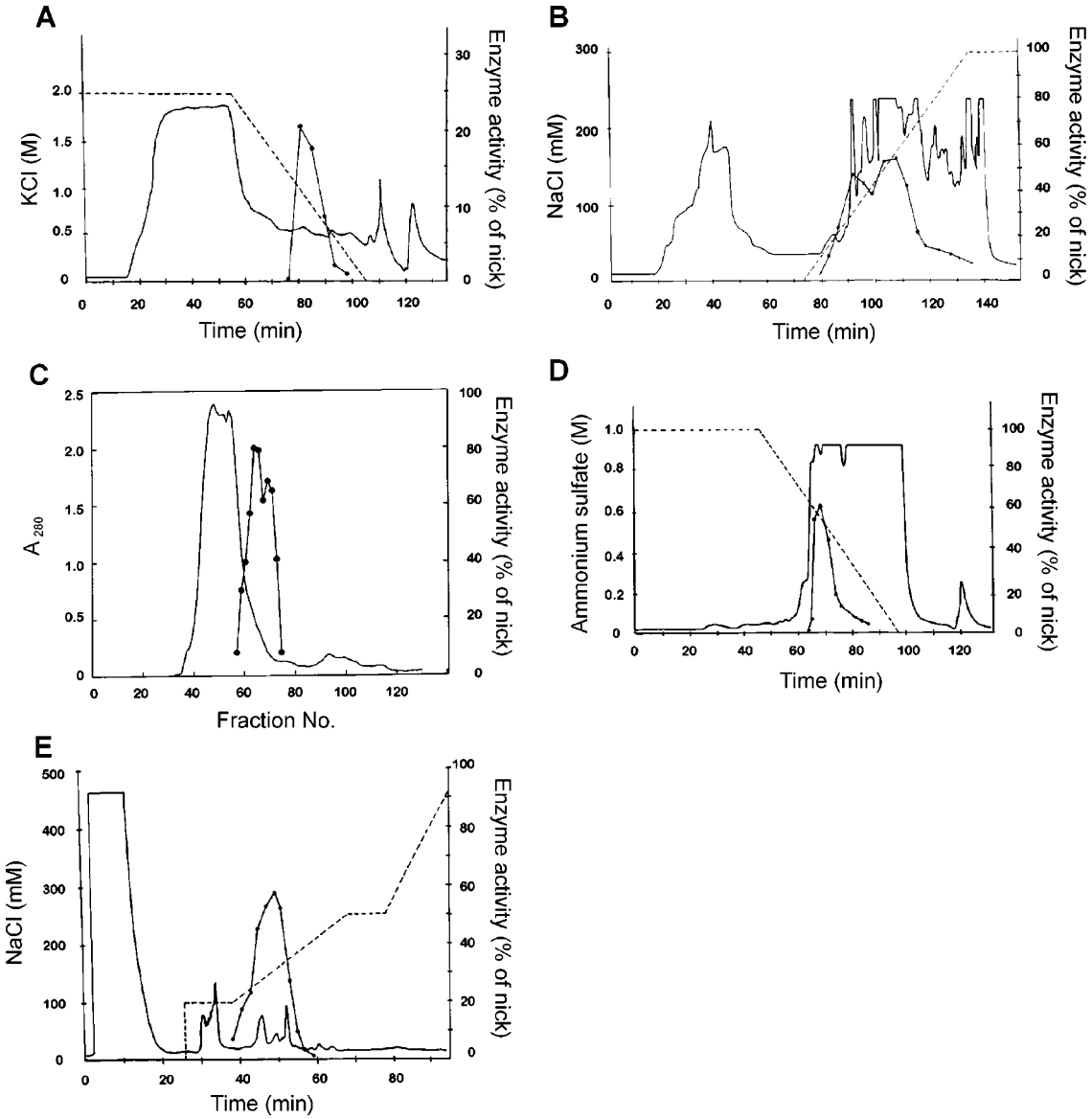


Figure 1. Chromatographic purification of *E. coli* oh^{8}Gua endonuclease. After removal of nucleic acids with streptomycin, crude extract was separated by first phenyl HPLC (**A**), DEAE HPLC (**B**), gel filtration chromatography (**C**), second phenyl HPLC (**D**) and heparin affinity HPLC (**E**). For activity assay, 5 or 10 μl of each fraction was incubated with 5' end-labeled substrate DNA containing oh^{8}Gua. DNA nick at the site of oh^{8}Gua was visualized with sequencing gel electrophoresis and autoradiography. For collection of active fractions, percentage of cleavage was determined by densitometry. Solid line, dashed line and dotted solid line indicate absorbance at 280 nm (protein profile; arbitrary unit except gel filtration chromatography), salt concentration and activity (% of nick), respectively.

rate of 0.5 ml/min (Figure 1C). In this step, about 50 % of proteins were excluded, but 5 % of total activity was lost (Table 1).

Second phenyl HPLC

After addition of ammonium sulfate to a final concentration of 1 M and centrifugation, supernatant was separated by phenyl HPLC again with a gradient of ammonium sulfate (Figure 1D). By addition of ammonium sulfate, few proteins precipitated. Active fractions were eluted around 0.6 M ammonium sulfate, while most of proteins bound tightly to the phenyl column.

Heparin affinity HPLC

After removal of ammonium sulfate with dialysis against a buffer (20 mM potassium phosphate, pH 7.7, 0.5 mM EDTA, 0.2 mM DTT), active fractions from second phenyl HPLC were fractionated with heparin affinity HPLC (Figure 1E) at 1.0 ml/min. While most of proteins (95%) were removed by washing with 0 and 100 mM

Table 1. Purification of oh^{8}Gua endonuclease from 2.4 kg of E. coli

Fraction	Protein amount (mg)	Total activity ($\times 10^3$ units)	Specific activity ($\times 10^3$ units/mg)	Fold of purification	Yield (%)
Crude extract	157,000	N.D.	N.D.	N.D.	N.D.
First phenyl HPLC	15,500	N.D.	N.D.	N.D.	N.D.
DEAE HPLC	2,400	52,000	22	1	100
Gel filtration chromatography	1,400	49,000	35	1.6	94
Second phenyl HPLC	220	45,000	205	9.4	86
Heparin HPLC	9.1	8,500	934	43	16
Hydroxylapatite HPLC	0.036	270	7,500	346	0.5

One unit is defined as activity to nick 1 fmol of substrate DNA for 60 min. Total activity, specific activity, fold of purification and yield were determined after the purification steps of DEAE HPLC because other DNA nicking activities were observed in crude extract and 1st phenyl HPLC (N.D., *not determined*).

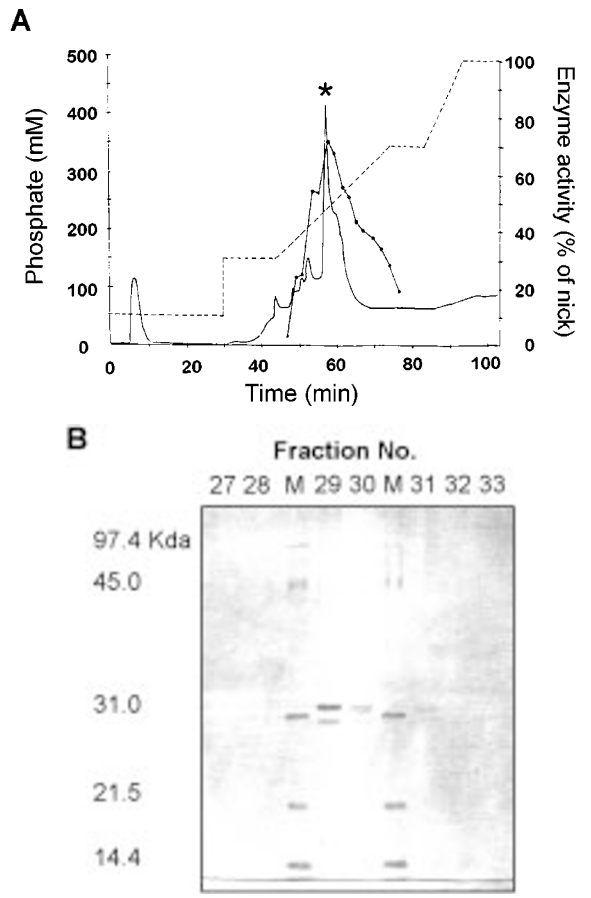


Figure 2. Purification of oh^{8}Gua endonuclease by hydroxylapatite HPLC. (**A**) Oh^{8}Gua endonuclease was purified by hydroxylapatite HPLC. After washing the column with 50 mM and 150 mM phosphate, gradient elution from 150 to 300 mM phosphate was done at 0.6 ml/min. Asterisk (·) indicates 29th fraction. Solid line, dashed line and dotted solid line indicate absorbance at 280 nm (protein profile; arbitrary unit except gel filtration chromatography), salt concentration and activity (% of nick), respectively. (**B**) 30 μl of each fraction (1.2 ml) was electrophoresed in 12% SDS-PAGE gel with molecular weight markers (M). Proteins in the gel were stained with silver.

NaCl, oh^{8}Gua endonuclease activity was detected in the narrow range around 150 mM. By this purification, 80% of total activity was lost (Table 1).

Hydroxylapatite HPLC

After dialysis against a buffer (50 mM potassium phosphate, pH 7.0), oh^{8}Gua endonuclease in the active fractions from heparin affinity HPLC was purified by hydroxylapatite HPLC (Figure 2A) with phosphate gradient at 0.6 ml/min. Protein peak (optical density at 280 nm) was observed at 220 mM phosphate and coincided with activity peak (29th fraction). In 12% SDS-PAGE gel, two protein bands (32 kD and 29 kD) were observed in the 29th fraction (Figure 2B). Through this last purification step, we obtained 36 μg of protein and the purification yield was about 3% after DEAE HPLC (Table 1).

Determination of N-terminal amino acid sequences of purified oh^{8}Gua endonuclease and comparison of electrophoretic mobility with Fpg in SDS-PAGE gel

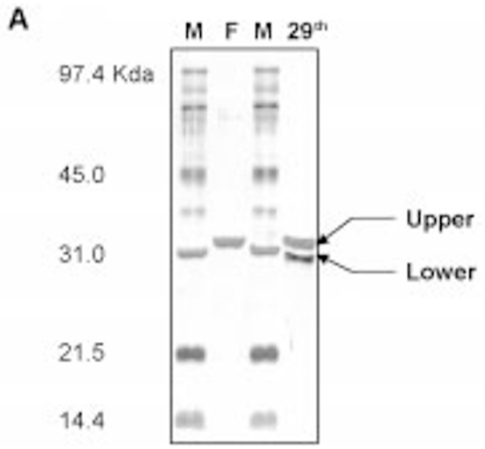
The purified proteins in 29th fraction was electrophoresed together with Fpg protein in 12% SDS PAGE gel. The upper band co-migrated with Fpg.

By peptide sequencing, N-terminal sequences of two proteins of 29th fraction were identical to that of Fpg (Figure 3C). So, the protein of the lower band was to be a degraded product of oh^{8}Gua endonuclease during purification.

Discussion

oh^{8}Gua is found in DNA following oxidative damage by ROS *in vitro* as well as *in vivo* (Kasai *et al*., 1986). In the absence of conformational restraints, it prefers to pair with adenine during DNA synthesis (Oda *et al*., 1991; Shibutani *et al*., 1991). Thus, without DNA repair, this mismatch leads GC to TA transversion (Wood *et al*., 1990).

Against this promutagenic DNA adduct, living organisms have several kinds of defense systems. In *E. coli*, several DNA repair enzymes known as the "GO system" prevents mutagenesis via oh^{8}Gua (Michaels and Miller, 1992). This "GO system" consists of three components.



B

Fpg

N' Pro-Glu-Leu-Pro-Glu-Val-Glu-The-Ser-Arg--- C'

Upper

N' Pro-Glu-Leu-Pro-Glu-Val-Glu C'

Lower

N' Pro-Glu-Leu-Pro-Glu-Val-Glu-The-Ser C'

Figure 3. Comparison of oh[8]Gua endonuclease and Fpg. (**A**) Fpg (F, 100 ng) and 29th fraction (29th, 100 μl) were separated with molecular weight markers (M) in 12% SDS-PAGE gel. (**B**) Sequences of N-terminal amino acids of Fpg (F), the upper (Upper) and the lower band (Lower) of 29th fraction.

First, MutT, oh[8]dGTPase, is a triphosphatase to prevent incorporation of oh[8]dGTP from nucleotide pool to DNA, thus preventing TA to GC mutation (Maki and Sekiguchi, 1992). Second, Fpg or MutM, identical to oh[8]Gua endonuclease removes preferentially oh[8]Gua paired with C (Chung *et al*., 1991; Tchou *et al*., 1991). Third, MutY, adenine-DNA glycosylase preferentially excises adenine paired with oh[8]Gua (Michaels *et al*., 1992). Human homologues to MutT, MutM and MutY have been also discovered and cloned (MutT, Sakumi *et al*., 1993; MutM, van der Kemp *et al*., 1996; MutY, Slupska *et al*., 1996; Arai *et al*., 1997; Radicella *et al*., 1997; Rosenquist *et al*., 1997).

In this experiment, we revealed that oh[8]Gua endonuclease is identical to Fpg by comparison of N-terminal amino acid sequences and electrophoretic mobility. Fpg was originally found as activity to release Fapy from DNA (Chetsanga and Lindahl T, 1979). By this study, it is confirmed that oh8Gua endonuclease is identical to Fpg. However, oh[8]Gua rather than Fapy in DNA seems to be more natural substrate for this enzyme for the following reasons.

Oh[8]Gua has been reported as a major DNA adduct by ROS. In isolated DNA, much lower dose of irradiation was required to induce an increase of oh[8]Gua than any other DNA adducts including Fapy (Dizdaroglu and Bergtold, 1986). Among DNA adducts caused by hypoxanthine/xanthine oxidase/Fe^{3+}, oh[8]Gua was a major product (Aruoma *et al*., 1989). Meaningfully, it is also a major adduct to be removed *in vivo*. Oh[8]Gua was not only detected in biological fluid in rats fed with nucleic acid-free diet, but also in culture medium of *E. coli* (Park *et al*., 1992). In human urine samples, oh[8]Gua was detected as a major oxidized DNA base while Fapy could not be observed by HPLC-GC/MS which is currently one of the most sensitive assay methods (Ravanat *et al*., 1999). This implies that oh[8]Gua rather than Fapy is a major modified base to be repaired *in vivo*.

In addition, other reports support the possibility that the enzyme may repair preferentially oh[8]Gua-containing DNA rather than Fapy-containing DNA. Fpg has greater *Km* value for Fapy- than oh[8]Gua-conatining DNA (Tchou *et al*., 1991). While Fapy in DNA blocks DNA synthesis (O'Connor *et al*., 1988), oh[8]Gua induces mutagenesis. In *mutM* strain of *E. coli* deficient in Fpg, the frequency of GC to TA transversion was increased and this increase was inhibited by overexpression of Fpg (Michaels *et al*., 1991). These findings indicate that Fpg prevents mutagenesis caused by oh[8]Gua in *E. coli*. Furthermore, it was recently reported that *nth* protein (endonuclease III) excised Fapy from DNA damaged by ROS with the same preference to thymine glycol or uracil glycol, which were known as major substrates for *nth* protein. However, they did not detect the removal of oh[8]Gua by this enzyme (Dizdaroglu *et al*., 2000). This finding suggests the possibility that Fapy might be removed primarily by endonuclease III rather than Fpg. In this context, we suggest that oh[8]Gua endonuclease identified as Fpg in this study, plays a primary physiological role in removing oh[8]Gua from DNA rather than Fapy.

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