Base excision repair synthesis of DNA containing 8-oxoguanine in *Escherichia coli*

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Accepted 29 March 2003

Abbreviations: 8-oxo-G, 8-oxo-7,8-dihydroguanine; Fapy, 2,6-dihydroxy-5N-formamidopyrimidine; FPG, Fapy-DNA glycosylase; BER, base excision repair; AP, apurinic/apyrimidinic; dRPase, deoxyribose phosphatase

Abstract

8-oxo-7,8-dihydroguanine (8-oxo-G) in DNA is a mutagenic adduct formed by reactive oxygen species. In *Escherichia coli*, 2,6-dihydroxy-5N-formamidopyrimidine (Fapy)-DNA glycosylase (Fpg) removes this mutagenic adduct from DNA. In this report, we demonstrate base excision repair (BER) synthesis of DNA containing 8-oxo-G with Fpg *in vitro*. Fpg cut the oligonucleotide at the site of 8-oxo-G, producing one nucleotide gap with 3' and 5' phosphate termini. Next, 3' phosphatase(s) in the supernatant obtained by precipitating a crude extract of *E. coli* with 40% ammonium sulfate, removed the 3' phosphate group at the gap, thus exposing the 3' hydroxyl group to prime DNA synthesis. DNA polymerase and DNA ligase then completed the repair. These results indicate the biological significance of the glycosylase and apurinic/apyrimidinic (AP) lyase activities of Fpg, in concert with 3' phosphatase(s) to create an appropriately gapped substrate for efficient BER synthesis of DNA containing 8-oxo-G.

Keywords: DNA repair; *Escherichia coli*; mutagenesis; oxidative stress; reactive oxygen species; phosphatases

Introduction

8-oxo-7,8-dihydroguanine (8-oxo-G) in DNA is a mutagenic adduct formed by reactive oxygen species (Kasai and Nishimura, 1984). As a structural preference, adenine is frequently incorporated into opposite template 8-oxo-G (Shibutani *et al.*, 1991), and 8-oxo-dGTP is incorporated into opposite template dA during DNA synthesis (Cheng *et al.*, 1992). Thus, unrepaired, these mismatches lead to GT and AC transversions, respectively (Grollman and Morya, 1993).

In *Escherichia coli*, several DNA repair enzymes, preventing mutagenesis by 8-oxo-G, are known as the GO system (Michaels *et al.*, 1992). The GO system consists of MutT (8-oxo-dGTPase), MutM (2,6-dihydroxy-5N-formamidopyrimidine (Fapy)-DNA glycosylase, Fpg) and MutY (adenine-DNA glycosylase). 8-oxo-dGTPase prevents incorporation of 8-oxo-dGTP into DNA by degrading 8-oxo-dGTP. Adenine-DNA glycosylase preferentially excises A paired with 8-oxo-G, restoring the 8-oxo-G:C pair, a substrate for Fpg.

Fpg has multiple enzymatic activities. Through its glycosylase activity, it removes Fapy and 8-oxo-G from oxidatively damaged DNA (Tchou *et al.*, 1991), and it has apurinic/apyrimidinic (AP) lyase activity to remove the AP site by successive β - and δ -elimination mechanisms, which results in a single nucleotide gap with 3' and 5' phosphate termini at the gap (Tchou *et al.*, 1991; Bhagwat and Gerlt, 1996). In addition, it has 5' deoxyribophosphatase (dRPase) activity to remove 5' deoxyribophosphate from DNA strands (Graves *et al.*, 1992).

Various kinds of DNA glycosylases specific to each of the modified bases have been known to initiate base excision repair (BER) DNA synthesis by removing the modified DNA bases. In the case of the DNA glycosylases with AP lyase activity to induce β - and δ -elimination, short patch BER synthesis has been proposed (Memisoglu and Samson, 2000). Initially, the glycosylases produce one nucleotide gap with 3' and 5' phosphate termini at the gap through glycosylase and AP lyase activities. Then, 3' phosphatase removes the 3' terminal phosphate at the gap, providing a 3' prime end for the insertion of a single nucleotide. Thereafter, DNA polymerase inserts one nucleotide and DNA ligase seals the nick to complete short patch BER DNA synthesis. Here, we demonstrate *in vitro* short patch BER synthesis of DNA containing 8-oxo-G by Fpg based on this proposed repair model.

Material and Methods

Materials

Exonuclease III-deficient *E. coli* (BW 9053) was used for the purification of Fpg and the AS 40/100 fraction. Purification procedures were performed using HPLCs (Model 302 pump, Model 811B gradient mixer, Model 115 UV detector; Gilson, France), and a conventional gel filtration chromatography (2.6×100 cm column packed with Sephacryl S-200; Amersham Pharmacia, UK). Columns for phenyl HPLC (Phenyl-5PW, 21.5×150 mm), DEAE HPLC (DEAE-5PW, 21.5×150 mm), heparin affinity HPLC (Heparin-5PW, 7.5×75 mm) and hydroxylapatite HPLC (HA 1000, 7.5×75 mm) were from Toyo Soda (Japan). Radioactive nucleotides were obtained from Amersham Pharmacia (UK) or NEN (Boston, MA). T4 polynucleotide kinase and 3' end-labeling kits and nucleotides were from Roche (Germany). T4 DNA polymerase, T4 DNA ligase and gene 32 product were from BioRad (Hercules, CA). All other chemicals were from Sigma (St. Louis, MO).

Preparation of enzymes

Fpg was purified chromatographically. Briefly, from the crude extract of *E. coli* deprived of nucleic acids by 0.8% streptomycin, homogenous Fpg was obtained by fractionation with ammonium sulfate, phenyl HPLC, DEAE HPLC, gel filtration, phenyl HPLC, heparin affinity HPLC and hydroxylapatite HPLC applied sequentially. One unit of enzyme activity was defined as the activity required to cleave 1 fmol of double stranded DNA containing 8-oxo-G at 37°C for 60 min (Chung *et al.*, 1991).

AS 40/100 fraction: ammonium sulfate was added to a final concentration of 40% to the crude extract devoid of nucleic acids with stirring at 4°C for 1 h. After centrifugation at 25,000 g for 30 min, the supernatant was dialyzed against a buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.2 mM dithiothreitol and 10% glycerol). The dialysate was centrifuged and the supernatant (AS 40/100 fraction) was used in the experiment.

Labeling of substrate double-stranded DNA and other oligonucleotides

For BER synthesis, 21-mer (5' CAGCCAATCAGT- $\underline{\text{G}}^{\text{oxo}}$ CACCATCC 3'; $\underline{\text{G}}^{\text{oxo}}$ =8-oxo-G) was used (a kind gift from Dr. Kasai at the University of Occupational and Environmental Health, Japan). The 21-mer oligonucleotide was labeled at the 5' end with [γ -³²P] ATP and T4 polynucleotide kinase, and then annealed to its complementary strand as described previously (Chung *et al.*, 1991). To confirm incision site 3' to 8-oxo-G by Fpg, 46-mer oligonucleotide (5' CAGCCA-

ATCAGT $\underline{\text{G}}^{\text{oxo}}$ CACCATCCCGGGTCGTTTTACAACGTCGTGACT 3') was labeled at the 3' end with a 3' end-labeling kit and [α -³²P]cordycepin ATP, and annealed to its complementary oligonucleotide as described previously (Chung *et al.*, 1991). For identification of the 3' or 5' termini at the incision site by Fpg or the AS 40/100 fraction, single-stranded 5' end-labeled oligonucleotide (21-mer) or single-stranded 3' end-labeled oligonucleotide (46-mer) was incubated with piperidine at 90°C for 30 min (Chung *et al.*, 1992). To confirm removal of 3' terminal phosphate, synthetic 12-mer oligonucleotide without 3' terminal phosphate was labeled at 5' end.

Enzyme reactions

Formation of a single nucleotide gap by Fpg

Fpg or the AS 40/100 fraction was incubated with 0.4 pmol of 5' end-labeled or 3' end-labeled substrate DNA in 50 μ l of a reaction mixture containing 50 μ M Tris-HCl, pH 7.4, 50 mM KCl and 2 mM EDTA at 25°C.

Removal of 3' terminal phosphate by the AS40/100 fraction

To a reaction mixture containing 0.4 pmol of substrate 21-mer DNA treated with 400 units of Fpg at 25°C for 90 min, 200 μ g of AS 40/100 fraction was added, and the mixture was incubated at 25°C for 90 min. 50 μ l of distilled water and 100 μ l of phenol:chloroform (1:1) were then added, vigorously mixed and centrifuged at 12,000 g for 5 min, and 50 μ l of the supernatant was then collected and dried using a vacuum concentrator (Savant, NY).

Insertion of dG by DNA polymerase and sealing of the nick by DNA ligase

The above 21 mer substrate DNA gapped by successive treatment with Fpg and the AS 40/100 fraction was incubated with 1 unit of T4 DNA polymerase, 3 units of T4 DNA ligase, 50 mg of gene 32 product, 5 mM of dNTPs and 10 mM ATP in 100 μ l of a reaction buffer containing 5 mM MgCl₂, 2 mM dithiothreitol and 100 mM Tris-HCl, at 4°C for 5 min, 25°C for 5 min and 36°C for 60 min.

Visualization of enzyme products

To visualize products at each step, reaction mixtures were dried in a vacuum concentrator after phenol-chloroform extraction. The dried reaction mixtures were dissolved in loading buffer (80% formamide, 0.1% xylene cyanol and 0.1% bromophenol blue) and the products were separated in 8 M urea-denaturing 20% polyacrylamide gels at 2,000 V for 3 h. Gels were

autoradiographed with X-Omat film (Kodak).

Results

Trimming of 3' terminal phosphate at the gap

In the early purification step of Fpg, we observed two cleavage products of the substrate DNA. By treatment of the substrate DNA with the AS 40/100 fraction, two cleavage products, the lower and the upper band were observed (Figure 1, lane 4). The lower band co-migrated with the nicked fragment of substrate DNA treated with purified Fpg (lane 3) or hot piperidine (lane 1), while the upper band was located at the same position as synthetic 12 mer without the 3' terminal phosphate group (lane 5). Thus, we hypothesized that certain kinds of 3' phosphatases in the AS 40/100 fraction might remove the 3' terminal phosphate group from the gap site after the action of Fpg.

To confirm this idea, the AS 40/100 fraction was incubated after the substrate DNA was completely cleaved at the 8-oxo-G site by Fpg. After complete cleavage by Fpg (lane 6), the lower band was shifted to the upper position by the AS 40/100 fraction (lane 7, 8 and 9). After 90 min, the lower band almost completely shifted to the upper position (lane 9), which indicates that 3' phosphatases in the AS 40/100 fraction remove 3' terminal phosphate after the removal of 8-oxo-G and the resulting AP site by Fpg.

Cleavage site 3' to 8-oxo-G

For determination of cleavage site 3' to 8-oxo-G by Fpg or the AS 40/100 fraction, 3' end-labeled substrate

Figure 1. Trimming of 3' terminal phosphate by the AS 40/100 fraction after the removal of the 8-oxo-G and AP sites from 5' end-labeled 21-mer substrate DNA by Fpg. Lane 1, treated with piperidine at 90°C for 30 min; lane 2, incubated without enzymes; lane 3, incubated with 400 units of Fpg at 25°C, for 5 min; lane 4, incubated with 400 mg of AS 40/100 fraction for 60 min; lane 5, synthetic 12 mer; lane 6, incubated with 400 units of Fpg for 90 min; lane 7, 8 and 9 treated with 200 µg of AS 40/100 fraction for 10, 60 and 90 min, respectively, after incubation with 400 units of Fpg for 90 min.

DNA was incubated with either Fpg or the AS 40/100 fraction. The nicked product by Fpg (Figure 2, lane 2) co-migrated with the fragment produced by hot piperidine (lane 4), which was known to result in the 5' terminal phosphate at the gap (Chung *et al.*, 1992). While two products of 5' end-labeled DNA were formed by the AS 40/100 fraction, only one cleaved fragment of 3' end-labeled DNA by the AS 40/100 fraction was observed at the same position as the fragments produced by Fpg or hot piperidine (lane 3). Thus, Fpg or the AS 40/100 fraction did not remove 5' terminal phosphate at the gap.

Filling the gap produced by Fpg

The above results indicated that Fpg formed one nucleotide gap with 3' and 5' terminal phosphate at the site of 8-oxo-G, and that 3' phosphatases in the AS 40/100 fraction then removed the 3' terminal phosphate, thus resulting in one nucleotide gap adequate for the insertion of dGTP. We then tried to complete the BER synthesis by filling the gap (Figure 3). After complete cleavage of substrate DNA by Fpg (lane 1) and successive removal of 3' terminal phosphate by the AS 40/100 fraction (lane 2), the gap was completely filled and the nick was sealed by T4 DNA polymerase, 4 dNTPs, and T4 DNA ligase (lane 4). However, no gap filling was observed without the removal of 3' phosphate by the AS 40/100 fraction (lane 3) or without 4 dNTPs (lane 5).

When only one nucleotide was used, dGTP was selectively inserted into the gap (Figure 4, lane 4). In the case of dATP, dCTP or dTTP, the gap was not filled and the DNA was degraded (lane 1, 2 and 3, respectively). With dATP, dCTP and dTTP, some

Figure 2. Cleavage of 3' end-labeled 46-mer substrate DNA by Fpg or the AS 40/100 fraction. Lane 1, incubated without enzymes; lane 2, incubated with 200 units of Fpg at 25°C, for 60 min; lane 3, 200 µg of AS 40/100 fraction for 60 min; lane 4, treated with piperidine at 90°C for 30 min.

Figure 3. Essential requirements for the trimming of 3' terminal phosphate for filling the gap in 5' end-labeled 21-mer substrate DNA generated by Fpg. Lane 1, incubated with 400 units of Fpg for 90 min; lane 2, incubated with 400 units of Fpg for 90 min and 200 μ g of AS 40/100 fraction for 90 min; lane 3, gap filling in the presence of T4 DNA polymerase, 4 dNTPs and T4 DNA ligase without treatment of AS 40/100 fraction after incubation with Fpg; lane 4, gap filling after treatment of Fpg followed by incubation with AS 40/100 fraction; lane 5, gap filling without 4 dNTPs; lane 21-mer, no treatment; lane 12-mer, synthetic 12-mer having no 3' terminal phosphate; lane PPRD, single-stranded 21-mer treated with hot piperidine.

misincorporation was observed (lane 5). Laddering in lane 4 might be due to the 3' to 5' exonuclease activity of T4 DNA polymerase in the presence of only a subset of 4 dNTPs (Tabor *et al.*, 1987).

Discussion

In the present study, the BER synthesis of DNA containing 8-oxo-G is demonstrated *in vitro*, according to the scheme (Figure 5). Initially, Fpg initiates BER synthesis by generating a single nucleotide gap through its 8-oxo-G glycosylase/AP lyase activity. Then, 3'-phosphatases in the AS 40/100 fraction trim the 3' terminal phosphate off to prime DNA synthesis. Finally, DNA ligase and DNA polymerase complete the repair synthesis.

Fpg is a DNA glycosylase to remove Fapy as well as 8-oxo-G from DNA (Tchou *et al.*, 1991). In addition to glycosylase activity, Fpg has other DNA repair activities; AP lyase (Tchou *et al.*, 1991) and 5' dRPase (Graves *et al.*, 1992). 8-oxo-G glycosylases (OGG1), functionally homologous to Fpg, have been found and cloned in eukaryotic cells including *Saccharomyces* (van der Kemp *et al.*, 1996), mouse (Rosenquist *et al.*, 1997) and human (Aburatani *et al.*, 1997; Radicella *et al.*, 1997). Fpg and the eukaryotic DNA repair

Figure 4. Selective insertion of dGTP to the gap. After treatment of 5' end-labeled 21-mer substrate DNA with Fpg and AS 40/100 fraction, the gapped DNA was incubated with dATP (lane 1), dCTP (lane 2), dTTP (lane 3), dGTP (lane 4) or dNTPs except dGTP (lane 5) in the presence of T4 DNA polymerase, 4 dNTPs and T4 DNA ligase. Lane 6 indicates substrate DNA treated with hot piperidine.

Figure 5. Schematic base excision repair synthesis of DNA containing 8-oxo-G in *Escherichia coli*.

enzymes are known to possess AP lyase activity (N-glycosylase/AP lyase) and these enzymes are suggested to remove 8-oxo-G through the formation of Schiff base with 8-oxo-G (McCullough *et al.*, 1999; Fedorova *et al.*, 2002; Fromme *et al.*, 2003). Fpg encounters 8-oxo-G in DNA by processive search (DNA sliding) rather than distributive search (random-diffusion-mediated) (Francis and David, 2003). Fpg form a specific complex, a Schiff base intermediate, with 8-oxo-G in DNA through interaction between a lysine residue (Lys249) of Fpg and 8-oxo-G. 8-oxo-G is removed from the DNA strand, and the one nucleotide gap is formed through β -elimination followed by δ -elimination.

While Fpg removes the AP site through β , δ -elimination mechanisms after removal of 8-oxo-G (Bhagwat and Gerlt, 1996), yeast OGG1 was suggested to remove the sugar moiety as trans-4-hydroxy-2-pentyl-5-phosphate via possible hydrolysis in the presence of Mg^{2+} (Sandigursky *et al.*, 1997). In contrast, mouse OGG1 elicits only β -elimination resulting in 3'-terminal α , β -unsaturated sugar that must be processed for gap filling (Rosenquist *et al.*, 1997; Zharkov *et al.*, 2000). Therefore, 3' diesterase may be necessary to trim the 3' terminus for DNA polb, XRCC1 and DNA ligase to complete the short patch BER repair synthesis (Memisoglu and Samson, 2000). Recently, Pascucci *et al.* (2002) showed reconstitution of the BER repair synthesis for 8-oxo-G with purified human OGG1. In the experiment, 3'-terminal sugar phosphate was removed by human AP endonuclease1 (HAP1) containing 3' diesterase activity, and Pol β and DNA ligase I completed repair synthesis.

Our results showed that 3' phosphatase activity in the AS 40/100 fraction trimmed 3' terminal phosphate at the gap. In DNA repair synthesis, exonuclease III (Xth, Henner *et al.*, 1983) or endonuclease IV (Nfo, Bailly and Verly, 1989) might remove 3' terminal phosphate to prime the DNA synthesis. In this study, endonuclease IV might remove 3' terminal phosphate, because we used an exonuclease III-deficient strain of *E. coli*. With *in vitro* reconstitution system, endonuclease IV in concert with Fpg generated one nucleotide gap to prime DNA synthesis (Harrison *et al.*, 1999). However, involvement of exonuclease III cannot be excluded in BER synthesis because both *xth* and *nfo* mutants, but not *xth/nfo* double mutant, could carry out complete repair of DNA strand breaks mediated by Fpg when treated with hydrogen peroxide (Galhardo *et al.*, 2000).

Even though BER DNA synthesis by Fpg is suggested to be a major mechanism of 8-oxo-G repair, other mechanisms including nucleotide excision repair have been proposed to backup BER in *E. coli*, because of the strong miscoding property of 8-oxo-G. Nucleotide excision repair (NER) by UvrABC endonu-

lease was suggested to be a candidate for the repair of 8-oxo-G (Czeczot *et al.*, 1991). However, it was recently reported that the frequency of GC to TA mutation induced by γ -radiation is greater in the *fpg*⁻ strain than the *fpg*⁻*uvrA*⁻ double mutant of *E. coli*. This implies that NER may not act as a correct backup system, but is responsible for making mistakes leading to mutation (Kuipers *et al.*, 2000). Another possible candidate is a newly found 8-oxo-G glycosylase (Nei) that is identical to endonuclease VIII and is considered as *E. coli* homologue of mammalian OGG2 (Hazra *et al.*, 2000). While Fpg did not excise 8-oxo-G efficiently when paired with a Nei excised 8-oxo-G in 8-oxo-G:A and in 8-oxo-G:C pair to a similar degree. Thus, Nei can backup Fpg by repairing 8-oxo-G:C in addition to 8-oxo-G:A. Nei shares the N-terminal amino acids of Fpg, especially, the N-terminal Pro, which is considered as an active site of Fpg in terms of 8-oxo-G glycosylase/AP lyase activity (McCullough *et al.*, 1999). Recently, MutH, one member of methyl-directed mismatch repair system of *E. coli* was reported to be involved in repair of 8-oxo-G in DNA, even though it was not demonstrated whether it remove directly 8-oxo-G, adenine misincorporated opposite 8-oxo-G or both (Wyrzykowski and Volkert, 2003).

Like *E. coli*, several repair pathways have been proposed in humans. Involvement of human NER and long patch (3 to 4 nucleotides) BER in the repair of 8-oxo-G has been demonstrated (Jaiswal *et al.*, 1998). Contrastingly, the preferential repair of 8-oxo-G by short patch BER was demonstrated with HeLa cell extracts and circular DNA containing a single 8-oxo-G (Fortini *et al.*, 1999). In addition to core components of 8-oxo-G BER synthesis including OGG1, HAP, DNA polymerase and DNA ligase, xeroderma pigmentosum G (XPG), Cockayne syndrome B (CSB) and TFIIH (Le Page *et al.*, 2000a), or BRCA1 and BRCA2 (Le Page *et al.*, 2000b) were reported to be required or necessary in transcription-coupled repair of 8-oxo-G. In contrast, Poly (ADP-ribose) polymerase-1 was required for completing repair synthesis of non-transcribed strand (Le Page *et al.*, 2003). So, machinery for repair synthesis of 8-oxo-G-containing DNA in mammalian cells may be much more complicated and differently act in a transcription- and/or replication-dependent manner. Therefore, contribution of several repair pathways under different situations should be understood in the context of physiologic and pathologic significance.

Acknowledgement

The authors thank Dr. Kasai at the University of Occupational and Environmental Health, Japan for the gift of oligonucleotides containing 8-oxo-G.

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