A comparison of two DNA base excision repair glycosylases from *Arabidopsis thaliana*[#]

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Abstract

Plants contain the genes for both formamidopyrimidine-DNA glycosylase (FPG) and oxoguanine glycosylase (OGG). These enzymes play analogous roles in the base excision repair pathways of bacteria (FPG) and archaea, yeast, and mammals (OGG). Why have plants retained both genes? We tested one hypothesis by comparing the specificities of *Arabidopsis* FPG and OGG purified from *Escherichia coli* expression clones. Using depurinated DNA as substrate, the specific activity of *Arabidopsis* FPG was higher than that of *Arabidopsis* OGG. Using DNA oxidized by treatment with light in the presence of methylene blue, the specific activities of *Arabidopsis* FPG and OGG were equal. Using an oligonucleotide containing one oxoguanine (paired with C) and labeled with fluorescein, the specific activity of *Arabidopsis* OGG was greater than that of either FPG. The results support the hypothesis that genes for the two enzymes have been retained during evolution of plants for their specialized enzyme activities.

Keywords: Base excision repair; 7,8-dihydro-8-oxoguanine; Formamidopyrimine-DNA glycosylase; FPG; Oxoguanine glycosylase; OGG

Introduction

DNA oxidation may occur when the DNA reacts with reactive oxygen species, including singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radical [1]. It may occur, as with gamma irradiation, through electron loss to form DNA free radicals, which react with oxygen or other molecules. The oxidation of bases is particularly significant from a genetic standpoint, since modification can alter base-pairing. A major DNA oxidation product is 7,8-dihydro-8-oxoguanine (Go), which pairs equally well with C and A, leading to C → A and G → T transversions.

Go, like many other modified bases, is removed and replaced through the base excision repair pathway. The process is initiated by the removal of the base from deoxyribose by a DNA glycosylase. In bacteria, this enzyme is formamidopyrimidine-DNA glycosylase (FPG), named for its alternative substrate, a ring-opened purine [2]. In yeast and mammals, an analogous (but not homologous) enzyme, oxoguanine glycosylase (OGG) performs the same function [3]. So far, only plants are known to have both

enzymes [4-6]. It is likely that during evolution plants, like other eukaryotes, received the gene for OGG from archaeal ancestors; and they obtained the gene for FPG from bacteria during the endosymbiotic conversion of the bacteria into plastids.

It is not known why plants have retained the genes for both OGG and FPG, two enzymes with apparently redundant functions. It is possible that the two enzymes have evolved different specificities. Alternatively, they may be localized in different organelles. These are not mutually exclusive hypotheses. They might, because of different localization, be specialized for maximum efficiency on different DNA structures or most prevalent types of damage. Here we compare the activities of FPG-1, the most active form of FPG, and OGG from *Arabidopsis thaliana* and demonstrate significant differences in their specificities toward various DNA substrates.

Materials and Methods

Clones. Gao and Murphy [7] described the isolation of FPG cDNAs from an *Arabidopsis thaliana* flower cDNA library [8]. The same library and techniques were used to identify an OGG cDNA clone. The base sequence of the OGG cDNA was identical to Genbank accession no. **AJ277400** [4].

Expression and storage of enzymes. The cDNA for *A. thaliana* FPG-1 was modified for optimum expression in *E. coli* by adjusting the codons for the N-terminal six amino acid to match *E. coli* preferences. The cDNA was inserted into pET28b to provide a his_6 amino acid sequence at the C-terminal end, as described by Gao and Murphy [7]. The cDNA for OGG was inserted into pET28b in the same fashion. *E. coli* BL21(DE3) was used as the expression host for both pET vectors. The FPG-1-containing strain was grown at 37ºC to an A_{600} of 0.6, induced with 0.4 mM IPTG, and incubated at the same temperature for 4 h. The OGG-containing strain was grown at 37ºC, induced, and incubated at 14ºC for 24 h. Cells collected by centrifugation were lysed by sonication in 50 mM Na phosphate, pH 8.0 containing 300 mM NaCl and 10 mM imidazole. The extracts were clarified by centrifugation, and the his_6-tagged protein was purified with NiNTA-gel (Qiagen, Inc., Santa Clarita, CA) using the binding and elution protocols provided by the manufacturer. The eluate of FPG-1 was stored in the elution buffer (50 mM Na phosphate, pH 8.0 containing 300 mM NaCl and 250 mM imidazole) at 4ºC or in the same buffer diluted with 50% glycerol at -20ºC. The eluate of OGG precipitated at 4ºC but was stable in 50% glycerol at -20ºC.

E. coli FPG was obtained from PharMingen, Inc. (San Diego, CA) and stored in 25 mM Tris HCl, pH 7.6, 50 mM NaCl, 0.5 mM DTT, and 50% glycerol at -80ºC.

Substrates and assays. Plasmid pUC19 was depurinated by heating it at 70ºC for 1 h in 10 mM Na citrate buffer, pH 5.0, containing 0.1 M NaCl. For photosensitization, pUC19 was diluted to a volume of 400 μl in 25 mM N-2-hydroxythylpiperazine-N'-2-ethane-sulfonic acid (HEPES)-NaOH buffer, pH 7.1 containing 0.5 mM EDTA, 0.5 mM dithiothreitol (HE buffer). Methylene blue, 1 μM, was added and the solution was illuminated for 5 min with white light at approximately 1050 μmol m^{-2} s^{-1}. After either treatment, the DNA was precipitated with ethanol, dissolved in water, reprecipitated, dried, and redissolved in water.

For a typical assay of cleavage of supercoiled plasmid, approximately 40 fmol of depurinated or methylene-blue/light-treated plasmid was combined with enzyme. For titrations, samples of enzyme were serially diluted in NaCl (calculated to provide 150 mM final concentration) before HE buffer and substrates were added and the total volume made to 10 μl. The reaction mixtures were incubated at 37ºC for 30 min. OGG-containing samples were heated for 2 min at 65ºC to inactivate the enzyme. (The heat step was found to be optional for assays of FPG.) Samples were subjected to electrophoresis through 1% agarose in 0.04M Tris-acetate, 1 mM EDTA (TAE). The gels were incubated in ethidium bromide, washed with water, and visualized under UV light with an Alpha Innototech ChemiImager 5500 system. Quantification of supercoiled and open-circle plasmid bands was accomplished using the 1-D

line analysis of AlphaEaseFC™ software supplied with the ChemiImager system. To visualize the action of the enzymes on plasmids with different degrees of supercoiling, the reaction mixtures were subjected to electrophoresis through 1.2% agarose in 50 mM Tris, 160 mM glycine containing 1.5 mg l^{-1} chloroquin.

Gao and Murphy [7] described the oligonucleotide containing a single 8-oxo-G base and a 3'-terminal fluorescein (5'AGCGGCCATCGGATACCGTCGoACCTCGAGGAA TTCCF3', Go = 8-oxo-G, F = fluorescein) and its complement (C opposite Go). For assay of cleavage, 2.5 pmol of oligonucleotide was combined with an equal amount of its complement in HE buffer and mixed with 150 mM NaCl and enzyme in a total volume of 7 μl. The reaction was incubated at 37ºC and stopped by heating as described above. Two μl of formamide were added, and the mixture was subjected to electrophoresis in 15% acrylamide gel containing 7 M urea. The degree of cleavage was measured by visualization and quantitation of the fluorescein label.

Results

Cleavage of depurinated DNA.

The cleavage of depurinated DNA by FPG was strongly dependent on salt concentration (Fig. 1). With a minimum amount of NaCl, 20 mM, there was no significant amount of cleavage; the activity was greatly increased with 50 mM NaCl and peaked at 100-150 mM NaCl. An increase to 300 or 500 mM NaCl inhibited activity. In contrast OGG showed little stimulation of activity with added NaCl and was inhibited only above 300 mM NaCl.

Although both FPG and OGG cleaved depurinated pUC19 DNA, there was a striking difference in the specific activities shown by these two enzymes (Fig 2). *Arabidopsis* FPG gave detectable cleavage with as little as 0.01 ng (0.2 fmol) of enzyme and produced maximal cleavage with 1 ng (20 fmol). *E. coli* FPG was slightly less active, requiring 3 to 10 times more enzyme for the same degree of cleavage. In contrast, *Arabidopsis* OGG was substantially less active, requiring 10 ng (250 fmol) of enzyme for detectable cleavage and 100-300 ng for maximal cleavage. These observations held true for both depurinated pUC19 monomer and dimer. However, FPG was able to cleave only about 60-70% of pUC19 monomer. In two out of three experiments, OGG cleaved a larger fraction of the monomer, suggesting that OGG recognized more damaged sites than FPG.

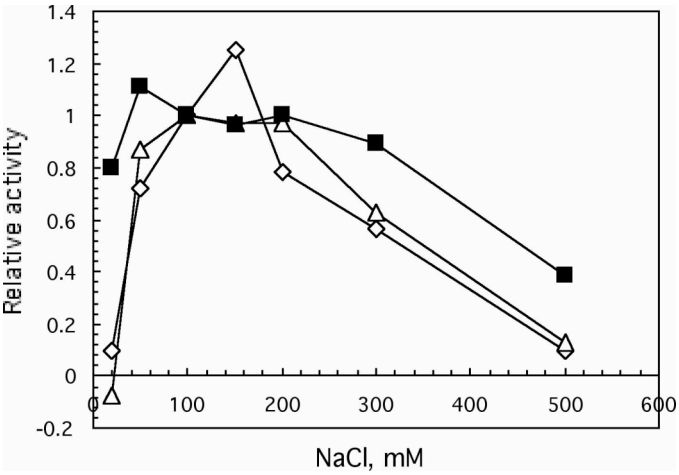

Fig. 1. Activity of *Arabidopsis* FPG and OGG in cleaving depurinated supercoiled plasmid DNA as a function of salt concentration. Data represent means for two independent preparations of FPG (triangle, n=2; diamond, n=3) and one preparation of OGG (filled circle, n=3). The values in each experiment were normalized to a value of 1.0 at 100 mM NaCl. The average SE was 0.11; error bars show representative values of SE.

The difference in the activity of FPG and OGG did not relate to the supercoiling of the substrate. An analysis of the depurinated pUC19 that remained after treatment with FPG and OGG, using chloroquin to separate different degrees of supercoiling, showed no preferential cleavage of any isomer of plasmid with either enzyme (data not shown).

Cleavage of DNA treated with methylene blue and light.

Irradiation of DNA with white light in the presence of methylene blue produces a mixture of altered bases, including 8-oxoguanine and FapyG (2,6-diamido-4-hydroxy-5-

formamidopyrimidine), both of which are removed by *E. coli* FPG [2], which presumably cleaves the DNA at those sites. Although our pUC19 substrate was treated with less methylene blue (1 μM vs 10 μM) and light (5 min vs 60 min) than that of Boiteux et al. [2], it was efficiently cleaved by both FPG and OGG (Fig .3). With this substrate, *E. coli* FPG was the most active, producing detectable cleavage with just over 0.1 ng of enzyme. *Arabidopsis* FPG and OGG had similar activities, both less than that of the *E. coli* enzyme, requiring from 1 to 10 ng of enzyme for detectable cleavage.

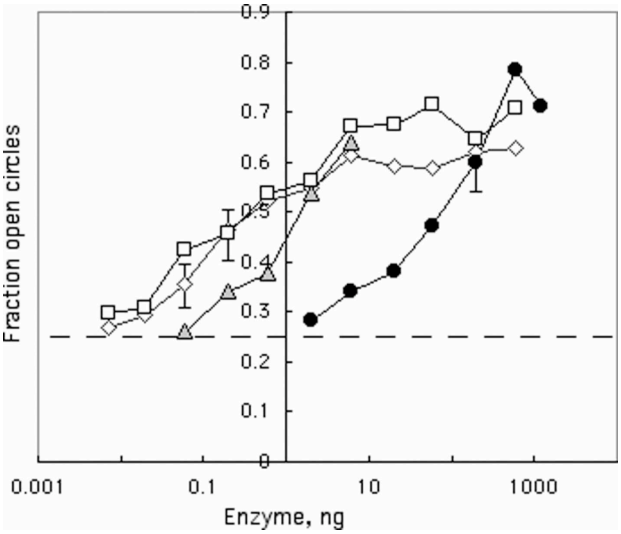

Fig. 2. Activity of *Arabidopsis* FPG and OGG in cleaving depurinated supercoiled plasmid DNA. Data represent means for two independent preparations of *Arabidopsis* FPG (open square, n=3; diamond, n=4), one preparation of *Arabidopsis* OGG (filled circle, n=3), and *E. coli* FPG (gray triangle, n=1). The amount of substrate (pUC19) was 40 fmol; the reaction proceeded for 30 min at 37ºC. The dashed line shows the fraction of open circles in the absence of enzyme. The SE for the *Arabidopsis* enzymes averaged 9% of the mean; error bars show representative values of SE.

Cleavage of the 8-oxo-G-containing oligonucleotide.

Ohtsubo et al. [6] reported the ability of *E. coli* and *Arabidopsis* FPG to cleave an oligonucleotide containing 8-oxo-G (and paired with a complementary strand containing C opposite the 8-oxo-G), and this observation with *Arabidopsis* FPG has been confirmed [7, 9]. The earlier experiments by all three groups used ^{32}P-labeled DNA to measure activities at the femtomole level. In the present experiments, we made use of a fluorescein label on the Go-containing strand, which decreased the

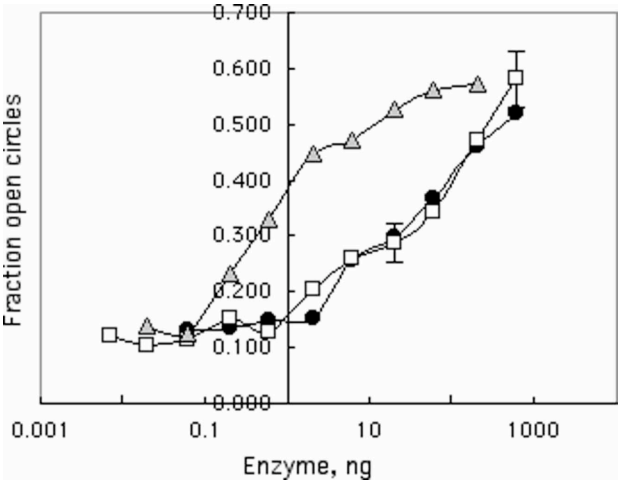

Fig. 3. Activity of *Arabidopsis* FPG and OGG in cleaving supercoiled plasmid DNA treated with light in the presence of methylene blue. Data represent means for *Arabidopsis* FPG (open square, n=3), *Arabidopsis* OGG (filled circle, n=1), and *E. coli* FPG (gray triangle, n=1). The amount of substrate (pUC19) was 40 fmol; the reaction proceeded for 30 min at 37ºC. The error bar shows representative values of SE for FPG.

sensitivity of the assay and allowed a clear distinction between FPG and OGG (Fig. 4). In this system, we did not detect cleavage activity by either *E. coli* FPG (5 or 50 ng) or *Arabidopsis* FPG (360 or 3600 ng). However, OGG cleaved the oligonucleotide, 100 ng (2.5 pmol) of enzyme giving approximately 90% cleavage (2 pmol) in 2 h at 37ºC.

Discussion

There are differences between our in vitro systems for detecting glycosylase activity and the environment *in vivo* that we must acknowledge. First is the possibility that the physical size or structure of the assay DNA might differentiate between the enzymes in a way that DNA *in vivo* does not. A direct test indicated that the degree of supercoiling of assay plasmid DNA did not affect either FPG or OGG activity on depurinated DNA. It is still possible that the small size of the Go-containing oligonucleotide reduced its recognition as a substrate by FPG, but not by OGG. It has been

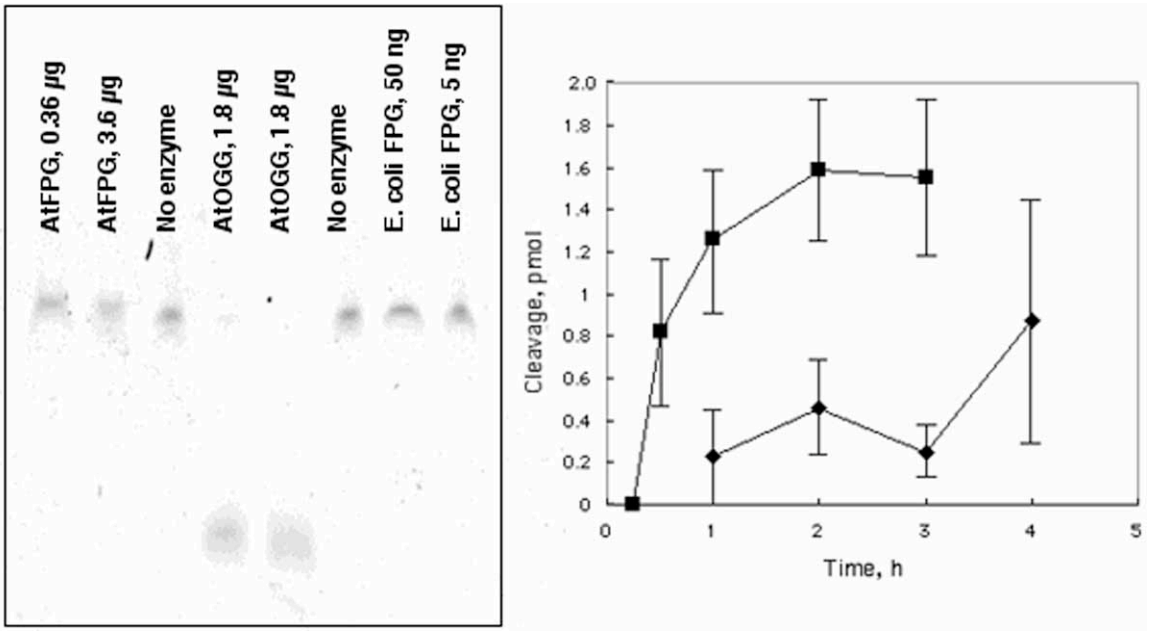

Fig. 4. Cleavage of a Go-containing oligonucleotide by OGG. Left: Representative gel. Cleaved and uncleaved chains were separated by acrylamide electrophoresis and detected by their fluorescein tags. Right: 2.5 pmol of substrate were incubated with 1.25 pmol (diamonds, n=3) or 2.5 pmol (squares, n=4) of enzyme at 37ºC for the indicated time. Data points show means±SE.

suggested that the dissociation rate of OGG from DNA is very slow, much slower than that of FPG; it is possible that this difference could be exaggerated with low-molecular size DNA.

Another difference might involve protein cofactors that associate with glycosylase in vivo but are lacking in our in vitro systems.

A third difference arises because the enzymes we tested were modified with C-terminal his_6 "tags" to facilitate their purification from *E. coli*. Although the C-terminal ends were clearly not critical for activity, it is possible that they modified the specificity of the enzymes. However, Rabow and Kow [10], working with *E. coli* FPG, found that enzyme with C-terminal histidines was similar in specificity and specific activity to native enzyme.

With the preceding provisos, the results of our experiments show that in our experimental systems *Arabidopsis* OGG is as active as FPG (methylene blue-light treated plasmid DNA, Fig. 3) or more active than FPG (oligonucleotide, Fig. 4) in recognizing Go. In contrast, FPG is more active in recognizing depurinated sites in DNA (Fig. 2). This supports the hypothesis that genes for the two enzymes have been retained in plants during evolution for their specialized enzyme activities. Morales-Ruiz et al. [9] found that the excision kinetics of *Arabidopsis* OGG were different from those of other species, supporting the idea that the activity of the enzyme has evolved in the plant cell environment.

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