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Characterization of DNA glycosylase activity by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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ABSTRACT

The DNA of all organisms is persistently damaged by endogenous reactive molecules. Most of the single-base endogenous damage is repaired through the base excision repair (BER) pathway that is initiated by members of the DNA glycosylase family. Although the BER pathway is often considered to proceed through a common abasic site intermediate, emerging evidence indicates that there are likely distinct branches reflected by the multitude of chemically different 3′ and 5′ ends generated at the repair site. In this study, we have applied matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS) to the analysis of model DNA substrates acted on by recombinant glycosylases. We examine the chemical identity of several possible abasic site and nicked intermediates generated by monofunctional and bifunctional glycosylases. Our results suggest that the intermediate from endoIII/Nth might not be a simple β-elimination product as described previously. On the basis of ^{18}O incorporation experiments, we propose a new mechanism for the endoIII/Nth family of glycosylases that may resolve several of the previous controversies. We further demonstrate that the use of an array of lesion-containing oligonucleotides can be used to rapidly examine the substrate preferences of a given glycosylase. Some of the lesions examined here can be acted on by more than one glycosylase, resulting in a spectrum of damaged intermediates for each lesion, suggesting that the sequence and coordination of repair activities that act on these lesions may influence the biological outcome of damage repair.

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The DNA of all organisms is persistently damaged and must be repaired continuously. It is estimated that the DNA of higher organisms is damaged between 10^4 and 10^5 times per cell per day under normal physiological conditions, and this number can be increased substantially by physiological and chemical stress [1,2]. Most endogenous damage involves single-base adducts that are repaired by the base excision repair (BER)[1] pathway. The BER pathway is initiated by one of a series of lesion-selective glycosylases that remove the damaged base [3–6]. The monofunctional glycosylases remove the damaged base, generating an abasic site, whereas the bifunctional glycosylases generate an abasic site that is then converted directly to a single-strand break [7]. The abasic site generated by the monofunctional glycosylases can be converted to a single-strand break by apurinic/apyrimidinic (AP)–endonuclease or by the AP lyase activity of one of the bifunctional glycosylases. Sugar fragments at the repair site are then removed by one of several possible activities, generating a 3′ hydroxyl and a 5′ phosphate that serve as the substrate for repair synthesis ending with ligation of the repair gap [8–13].

The endogenous single-base lesions include more than 30 oxidized, hydrolyzed, and methylated bases. The glycosylases of the BER pathway generally recognize groups of lesions, exploiting properties such as reduced thermodynamic stability and altered substituent inductive properties, shapes, sizes, and hydrogen-bonding properties of functional groups to locate and distinguish damaged bases from normal bases [14–19]. Although the types of endogenous damage are complex, repair via the BER pathway proceeds through a common abasic site intermediate. Emerging data suggest, however, that there may be subpathways through which lesions may be channeled, potentially resulting in distinct biological outcomes [20–25].

Experimental studies aimed at examining various steps of BER generally rely on incubation of oligonucleotides with repair

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[1] *Abbreviations used:* BER, base excision repair; AP, apurinic/apyrimidinic; MALDI–TOF–MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; U, uracil; FU, 5-fluorouracil; T, thymine; ClU, 5-chlorouracil; HmU, 5-hydroxymethyluracil; FoU, 5-formyluracil; HPLC, high-performance liquid chromatography; GC, gas chromatography; [γ-^{32}P]ATP, adenosine-5′-[γ-^{32}P]triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; UNG, uracil–DNA glycosylase; Fpg, formamidopyrimidine DNA glycosylase; Nth, *E. coli* endonuclease III; endoIII, *E. coli* endonuclease III; hOGG1, human oxoguanine glycosylase 1; MUG, mispaired uracil–DNA glycosylase; TDG, thymine DNA glycosylase; hSMUG1, single-stranded selective monofunctional uracil–DNA glycosylase; APE1, AP endonuclease 1; 3-HPA, 3-hydroxypicolinic acid; 3′-dRP, 3′-deoxyribose phosphate; oxoG, 8-oxoguanine; 5′-dRP, 5′-deoxyribose phosphate; C, cytosine; PNK, polynucleotide kinase.

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proteins followed by the resolution of ^{32}P-labeled fragments by gel electrophoresis [11,12,15–17]. Although gel methods are well established, such methods alone cannot reveal the chemical identities of the DNA fragments generated by the BER proteins. Alternatively, analysis by mass spectrometry can provide chemical identification of the repair intermediates. Although matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS) has been widely applied to the examination of proteins and peptide fragments, substantially less work has been done with oligonucleotides.

In this study, we designed and synthesized 18-mer oligonucleotides, each containing one of a series of eight known endogenous damage products. Each lesion was placed at a defined site so that the masses of the resulting fragments generated by repair proteins would be distinct and resolvable. Oligonucleotides were incubated with BER proteins and examined in parallel by traditional gel methods and by MALDI–TOF–MS. Our results confirm the proposed mechanism of action of most BER proteins examined. However, our results indicate that endoIII/Nth does not generate the expected product of β-elimination. Alternatively, we propose a new mechanism that is supported by the results of enzymatic cleavage of oligonucleotides in the presence of ^{18}O-enriched water. Our results demonstrate that the intermediates of BER are indeed chemically complex and support suggestions that BER may have multiple subpathways.

Materials and methods

Oligonucleotide synthesis and characterization

Oligonucleotides were prepared by solid-phase synthesis methods as described previously [26]. The sequences of the oligonucleotides used in this study, as well as the modified base of each 18-mer oligonucleotide, are shown in Table 1. The complementary strand for most of the lesions examined here was 5′-TTTCGTGGCC**G**GCCTCGATT-3′ (protonated monoisotopic mass 6089 amu), where the damaged or modified pyrimidine was paired opposite **G**. The complementary strand for the oligonucleotide oxoG was 5′-TTTCGTGGCC**T**GCCTCGATT-3′ (protonated monoisotopic mass 6064 amu), where the oxoG lesion was paired opposite **T**. In all cases, oligonucleotides formed duplexes containing approximately 14 paired bases with potentially unpaired bases at the 3′ and 5′ duplex ends. The mass of the complementary strand was increased by the addition of two terminal bases so that it did not interfere with mass spectral observation of the lesion-containing oligonucleotides or their fragments.

The phosphoramidites for uracil (U), 5-fluorouracil (FU), thymine (T), adenine, and guanine were obtained from Glen Research (Sterling, VA, USA). The phosphoramidites for 5-chlorouracil (ClU) [27], 5-hydroxymethyluracil (HmU) [28], and 5-formyluracil (FoU) [29] were prepared as described previously. Following synthesis, oligonucleotides were deprotected with concentrated aqueous ammonia (28–30% as NH_3) at 60 °C for 12 h. Oligonucleotides containing potentially labile bases were synthesized using labile protecting groups and deprotected with potassium carbonate in methanol [30]. Oligonucleotides were purified with Poly-Pak II cartridges or by high-performance liquid chromatography (HPLC) and further characterized by gas chromatography (GC)–MS following acid hydrolysis and conversion to the trimethylsilyl ethers [31,32] and also by MALDI–TOF–MS [33,34].

Oligonucleotide labeling and annealing

5′-End radiolabeling was performed using adenosine 5′-[γ-^{32}P]triphosphate ([γ-^{32}P]ATP, MP Biomedical, Costa Mesa, CA, USA) and T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA) under conditions recommended by the enzyme supplier. Labeled mixtures were subsequently centrifuged through G-50 Sephadex columns (Boehringer Mannheim, Indianapolis, IN, USA) to remove excess unincorporated nucleotide. Labeled single-stranded oligonucleotides were annealed to a twofold molar excess of unlabeled complementary strand opposite G except that oxoG was paired with T in buffer depending on the glycosylase enzyme that was used for the reaction. UNG and endoIII buffer contain 20 mM Tris–HCl, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM $MgCl_2$ at pH 8.0; Fpg buffer (10 mM Bis–Tris–propane–HCl, 10 mM $MgCl_2$, and 1 mM DTT, pH 7.0); hOGG1 buffer (10 mM Tris–HCl, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT, pH 7.9); TDG buffer (10 mM Hepes–KOH [pH 7.4], 100 mM KCl, and 10 mM EDTA); MUG buffer (20 mM Tris–HCl [pH 8.0], 0.1 mg/ml bovine serum albumin [BSA], 1 mM EDTA, and 1 mM DTT); hSMUG1 buffer (20 mM Tris–HCl [pH 8.0], 1 mM EDTA, 1 mM DTT, 50 mM NaCl, and 0.1 mg/ml BSA). The mixture was heated to 95 °C, except FoU oligo (75 °C), for 1 min and cooled slowly to room temperature.

Enzymatic reactions analyzed by gel electrophoresis

UNG (uracil–DNA glycosylase, *Escherichia coli*), Fpg (formamidopyrimidine DNA glycosylase, *E. coli*), Nth (endonuclease III, *E. coli*), and hOGG1 (human oxoguanine glycosylase 1) were purchased from New England Biolabs (Beverly, MA, USA). MUG (mispaired uracil–DNA glycosylase, *E. coli*) and TDG (thymine DNA glycosylase, *Methanobacterium thermoautotropicum*) were obtained from Trevigen (Gaithersburg, MD, USA). hSMUG1 (single-stranded selective monofunctional uracil–DNA glycosylase, human) was cloned and purified by our lab as described previously [35]. DNA substrates (500 fmol/reaction) were incubated with recombinant proteins (5 pmol/reaction except for endoIII, which was at 500 fmol/reaction) for 2 h at 37 °C in the reaction buffer depending on the enzyme buffer recommended by the manufacturer in a total volume of 10 μl. Reactions were stopped by adding 5 μl of 0.1 M NaOH and an equal volume of Maxam–Gilbert loading buffer (98% formamide, 0.01 M EDTA, 1 mg/ml xylene cyanole, and 1 mg/ml bromophenol blue), and 50 pmol of modified base-containing oligonucleotide was added as a competitor. The backbone

Table 1
Sequences of oligonucleotides, theoretical monoisotopic masses of 18-mer oligonucleotides, and masses after base removal, β-elimination, and β-δ-elimination.

	Oligonucleotide sequence	Monoisotopic mass $(M+H)^+$	Base removal $(M+H)^+$	β-Elimination $(M+H)^+$	Hydrolysis $(M+H)^+$	β-δ-Elimination $(M+H)^+$	3′-OH $(M+H)^+$
1	5′-TTTTCGAGGCCGGCCACG-3′	5489.94	5396.94	3211.54	3229.54	3113.54	3033.54
2	5′-TTGTCGAGGCTGGCCACG-3′	5529.95	5421.95	3236.55	3254.55	3138.55	3058.55
3	5′-CTGTCGAGGCUGGCCACG-3′	5500.94	5406.94	3221.55	3239.55	3123.55	3043.55
4	5′-CGGTCGAGGCFUGGCCACG-3′	5543.93	5431.93	3246.56	3264.56	3148.56	3068.55
5	5′-CCCTCGAGGCClUGGCCACG-3′	5479.89	5351.89	3166.55	3184.55	3068.55	2988.54
6	5′-TGGTCGAGGCFoUGGCCACG-3′	5568.94	5446.94	3261.56	3279.56	3163.56	3083.55
7	5′-AGGTCGAGGCHmUGGCCACG-3′	5579.96	5455.96	3270.57	3288.57	3172.57	3092.57
8	5′-CCTTCGAGGCoxoGGGCCACG-3′	5515.95	5366.95	3181.54	3199.54	3083.54	3003.54