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An HPLC-tandem mass spectrometry method for simultaneous detection of alkylated base excision repair products

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Abstract

DNA glycosylases excise a broad spectrum of alkylated, oxidized, and deaminated nucleobases from DNA as the initial step in base excision repair. Substrate specificity and base excision activity are typically characterized by monitoring the release of modified nucleobases either from a genomic DNA substrate that has been treated with a modifying agent or from a synthetic oligonucleotide containing a defined lesion of interest. Detection of nucleobases from genomic DNA has traditionally involved HPLC separation and scintillation detection of radiolabeled nucleobases, which in the case of alkylation adducts can be laborious and costly. Here, we describe a mass spectrometry method to simultaneously detect and quantify multiple alkylpurine adducts released from genomic DNA that has been treated with *N*-methyl-*N*-nitrosourea (MNU). We illustrate the utility of this method by monitoring the excision of *N*3-methyladenine (3mA) and *N*7-methylguanine (7mG) by a panel of previously characterized prokaryotic and eukaryotic alkylpurine DNA glycosylases, enabling a comparison of substrate specificity and enzyme activity by various methods. Detailed protocols for these methods, along with preparation of genomic and oligonucleotide alkyl-DNA substrates, are also described.

Keywords

base excision repair; DNA glycosylase; alkylation; methylpurine; mass spectrometry

1. Introduction

Alkylation DNA damage is produced by a large number of environmental toxins, endogenous methyl donors, and chemotherapeutic agents [1], and thus the mechanisms of alkyl-DNA toxicity and mutagenicity are of great interest. Much of our understanding of the generation and enzymatic repair of DNA alkylation comes from the use of laboratory alkylating agents, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), *N*-methylnitrosourea (MNU), methylmethanesulfonate (MMS), and dimethylsulfate (DMS), which methylate

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DNA to produce *N*3- and *N*7-methylpurines (3mA, 3mG, 7mA, 7mG), *O*⁶-methylguanine (*O*⁶mG), *M*1-methyladenine (1mA) and to a lesser extent *N*3-methylcytosine (3mC), *O*²-methylcytosine, and *O*⁴-methylthymine (Figure 1B) [2]. *O*⁶mG is demethylated by alkyltransferases Ada/MGMT, 1mA and 3mC are repaired by oxidative demethylases AlkB/ALKBH, and the remaining *N*3- and *N*7-methylpurines and *O*²- and *O*⁴-methylpyrimidines are repaired by various DNA glycosylases [3].

DNA glycosylases maintain genome integrity by initiating base excision repair (BER) of the large number of aberrant nucleobases that arise from deamination, oxidation, and alkylation [4]. These enzymes are specialized for particular types of damage, and all catalyze the hydrolysis of the *N*-glycosidic bond to generate a free nucleobase and an apurinic/aprimidinic (AP) site (Figure 1A). Some (bifunctional) DNA glycosylases contain a nicking (AP lyase) activity in addition to the base excision activity [5]. The resulting AP site is processed by AP endonuclease, DNA polymerase, and DNA ligase activities to restore undamaged DNA as part of the BER pathway [6]. DNA glycosylase activity was first demonstrated by Tomas Lindahl with the enzymatic release of free uracil from single- and double-stranded DNA [7]. Shortly thereafter, other bacterial and human DNA glycosylases were discovered that excise 3mA [8-14], as well as hypoxanthine [15, 16] and a variety of oxidized bases (e.g., thymine glycol, 8-oxoguanine) [17, 18], from DNA.

There are two predominant assays to monitor base excision activity of DNA glycosylases (Figure 2). The more common method involves liberation of a single, defined lesion that has been chemically or enzymatically incorporated into an oligonucleotide [19-28] (Figure 2A). AP sites generated by the glycosylase are nicked by alkali treatment or, in the case of the bifunctional enzymes, by intrinsic lyase activity, and the substrate and product fragments separated electrophoretically to quantify the ratio of substrate/product. Fragments have also been analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [29]. The oligonucleotide-based glycosylase assay enables precise characterization of binding and catalysis of a specific lesion under well-defined conditions and nucleotide sequence contexts, and takes advantage of automated chemical DNA synthesis technology and availability of a large number of commercially available phosphoramidite precursors. This assay has therefore become a powerful method to characterize stable lesions, such as 8-oxoguanine, uracil, 5-methylcytosine, and 1,*N*⁶-ethenoadenine [19, 21, 26, 30, 31]. However, some modifications, most notably *N*3- and *N*7-substituted alkylpurines, are prone to spontaneous depurination or ring opening under the conditions required for oligonucleotide synthesis and purification [32, 33]. In addition, the oligonucleotide assay is not amenable to high-throughput analysis of multiple lesions.

Another common method involves excision of nucleobases from genomic DNA that has been pre-treated with an oxidizing or alkylating agent, followed by ethanol precipitation of the DNA and high performance liquid chromatography (HPLC) separation of the soluble (nucleobase) fraction [13, 34-39] (Figure 2B). This substrate has the advantage of allowing multiple types of alkylated bases to be evaluated and has been useful for characterizing lesions not amenable to chemical synthesis, such as the relatively labile 3mA [38, 40]. The glycosylase activities and substrate preferences of the alkylpurine DNA glycosylases were initially characterized by scintillation detection of [³H]-methylbases liberated from genomic DNA that had been pre-treated with a radiolabeled laboratory methylating agent such as *N*-[³H]-methyl-*N*-nitrosourea (MNU) [13, 34-37]. However, these reagents have become costly and pose an environmental hazard.

Tandem mass spectrometry (MS/MS) circumvents the need for radioactive reagents while enabling highly selective and sensitive quantitation of nucleobases, nucleosides, and nucleotides [41-44]. Mass spectrometry approaches have been used to identify modified

nucleoside metabolites from rat liver [45], establish the spectrum of lesions produced in genomic DNA by oxidizing [46, 47] and alkylating agents [48-51], and study oxidative damage repair [29, 47, 52-54]. To our knowledge, HPLC-MS/MS techniques capable of simultaneously detecting alkylated DNA adducts present in DNA [44, 48, 50, 51] have not been utilized to assay the excision activity of DNA glycosylases.

The method described here involves reverse-phase HPLC separation of nucleobase products, followed by positive ion electrospray ionization [ESI(+)] mass spectrometric detection in multiple reaction monitoring (MRM) mode (Figure 2B). We show that the four common methylpurines formed by reaction of DNA with MNU (7mG, 3mA, O⁶mG, and 1mA) can be fully resolved based on their chromatographic retention times and MRM transitions. We illustrate the utility of the method by comparing the substrate specificities of five alkylpurine DNA glycosylases for 3mA and 7mG and characterizing the kinetics of base excision for the DNA glycosylase AlkD. We also present representative data for the commonly used oligonucleotide base excision assay, along with detailed protocols for preparation and use of the different methylated DNA substrates used in the various glycosylase assays.

2. Materials and Methods

Methods for chemical synthesis of deuterated nucleobase standards *d*₃-1mA, *d*₃-7mG, and *d*₃-O⁶mG (Figure S1) and detailed, step-by-step protocols for glycosylase substrate preparation and base excision assays are provided in the Supplemental Information.

2.1. Base excision assay using methylated genomic DNA and HPLC-MS/MS detection

2.1.1. Preparation of a methylated genomic DNA substrate—Methylated genomic DNA substrate was prepared by incubating 1 mg phenol:chloroform-purified calf thymus DNA and 1 μmol MNU in 0.3 M sodium cacodylate/0.1 M sodium perchlorate (pH 8.3) in the dark for 8 h at room temperature. The DNA was ethanol precipitated and the fibers washed with cold ethanol, resuspended in 1 ml Tris-EDTA pH 8.0 (TE) buffer, and dialyzed against additional TE buffer to remove any spontaneously hydrolyzed methyladducts. Final yield was 0.8 mg DNA as determined by UV absorbance.

2.1.2. Excision of methylated bases from genomic DNA—In a 50 μl reaction, 10 μg of the methylated genomic DNA substrate (step 2.1.1) was incubated with either 5 N HCl for 1 h at 37°C (to determine the upper limit of methylated bases present) or with 0-20 μM enzyme at 37°C for varying amounts of time. The time course of HCl depurination shown in Table S1 was performed using 0.5 N HCl at 70°C for 0.5-6 h. Enzymatic reactions contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, 100 mM KCl, 10 mM dithiothreitol (DTT), 2 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mg/ml bovine serum albumin (BSA). Reactions were terminated by addition of 50 μl stop buffer (0.5 mg/ml salmon DNA, 1 mg/ml BSA, 1 M NaCl) containing 10 μM of each deuterated nucleobase standard (*d*₃-1mA, *d*₃-3mA, *d*₃-7mG, *d*₃-O⁶mG), followed by ethanol precipitation of the DNA. The supernatant was evaporated to dryness and the residue reconstituted in 50 μl ultrapure water and transferred to a 200-μl silanized autosampler vial equipped with a Teflon-lined bonded rubber septum in preparation for HPLC-MS/MS analysis.

2.1.3. Detection of excised bases by HPLC-MS/MS

2.1.3.1. Instrumentation: Sample analyses were carried out using a Waters Acquity UPLC system (Milford, MA), made up of a binary solvent manager, a refrigerated sample manager, and a heated column manager. MS/MS detection was performed using a ThermoElectron TSQ Quantum Ultra triple-stage quadrupole mass spectrometer (San Jose, CA) equipped

with an *Ion Max* source housing, an ESI probe, and a 50 μ m interior diameter stainless steel capillary.

2.1.3.2. Preparation of calibration standards: Calibration of the HPLC-MS/MS response was performed with standard solutions of 1mA, 3mA, 7mG, and O⁶mG over a range of 50-4,000 nM for each compound. A stock solution of 1mA was prepared at 1 mM in 10 mM ammonium acetate. A stock solution of 3mA was prepared at 1 mM in water. Stock solutions of O⁶mG and 7mG were prepared at 800 μ M and 39 μ M, respectively, in methanol. In order to account for matrix effects, stock analyte solutions were diluted in 50 μ l mock glycosylase reactions containing 5 μ M AlkD D113N, 50 mM HEPES pH 7.5, 100 mM KCl, 10 mM DTT, 2 mM EDTA, and 0.1 mg/ml BSA. After incubating at 37°C for 1 h, 50 μ l stop buffer containing 10 μ M of each deuterated standard was added and samples were processed as described above (step 2.1.2) in preparation for HPLC-MS/MS analysis. Stock solutions of *d*₃-1mA, *d*₃-3mA, *d*₃-O⁶mG were prepared at 100 μ M in water. A stock solution of *d*₃-7mG was prepared at 100 μ M in methanol.

Extraction recovery was determined by preparing two sets of 50 μ l mock glycosylase reactions: the first containing 1 μ M of each methylpurine and the second lacking analyte. After incubating at 37°C for 1 h, 50 μ l stop buffer containing 10 μ M of each deuterated standard was added and samples were processed as described above (step 2.1.2) in preparation for HPLC-MS/MS analysis. Mock glycosylase samples previously lacking analyte were spiked with 1 μ M of each methylpurine after resuspension in water.

2.1.3.3. HPLC: A SymmetryShield RP₁₈ column [2.1 $\times$ 150 mm, 3.5 μ m particle size, Waters] equipped with an Acquity UPLC in-line stainless steel filter unit (0.2 μ m, Waters) was used for all chromatographic separations. The column and autosampler tray temperatures were 25°C and 10°C, respectively. Mobile phases were made up of 0.2% perfluoropentanoic acid in (A) H₂O and in (B) MeOH:H₂O (95:5). Gradient conditions were as follows: 0-1 min, B = 0%; 1-8 min, B = 0-25% (curve 8); 8-9 min, B = 25-100% (curve 8); 9-10 min, B = 100-0% (curve 8); and 10-16 min, B = 0%. The flow rate was maintained at 0.4 ml/min. A software-controlled divert valve was used to transfer eluent to waste from 0-3 min and from 11-16 min. The sample injection volume was 10 μ l. The autosampler injection valve and the sample injection needle were flushed and washed sequentially with 1 ml mobile phase B and 1 ml mobile phase A before each injection.

2.1.3.4. MS/MS, data acquisition, and processing: The mass spectrometer was operated in positive ion mode and quantitation was based on multiple reaction monitoring (MRM) of methylated adenines and guanines. Product ion mass spectra were acquired in the 30-200 *m/z* range, and each methylated base was readily identified by its characteristic precursor ion and major product ion. Full scan MS in positive ion electrospray mode (Supplemental Figures S2-S5) gave a major protonated molecular ion with *m/z* 150 [M + H]⁺ for each adenine analogue, *m/z* 166 [M + H]⁺ for each guanine analogue, *m/z* 153 [M + H]⁺ for *d*₃-1mA and *d*₃-3mA, and *m/z* 169 [M + H]⁺ for *d*₃-7mG and *d*₃-O⁶mG. Collision induced dissociation (CID) of *m/z* 150 of protonated 1mA and 3mA gave product ions with *m/z* 109 [M + H - 41]⁺ and 123 [M + H - 27]⁺, respectively. CID of *m/z* 166 of protonated 7mG and O⁶mG gave product ions with *m/z* 124 [M + H - 42]⁺ and 134 [M + H - 32]⁺, respectively. CID of *m/z* 153 of protonated *d*₃-1mA and *d*₃-3mA gave a product ion with *m/z* 109 [M + H - 44]⁺ and 82 [M + H - 27]⁺, respectively. CID of *m/z* 169 of protonated *d*₃-7mG and *d*₃-O⁶mG gave product ions with *m/z* 127 [M + H - 42]⁺ and 70 [M + H - 32]⁺, respectively. The mass transitions (precursor to product) monitored were 150 → 109 for 1mA, 150 → 123 for 3mA, 166 → 124 for 7mG, 166 → 134 for O⁶mG, 153 → 109 for *d*₃-1mA, 153 → 82 for *d*₃-3mA, 169 → 127 for *d*₃-7mG, and 169 → 70 for *d*₃-O⁶mG. Although 1mA, 3mA, 7mG, and O⁶mG do not have unique *m/z* transitions, chromatographic separation (Figure

S6) allowed for accurate quantitation. Data collection parameters were as follows: spray voltage, 4500 eV; capillary temperature, 300°C; sheath gas, 30 psi; and collision energies, varied. Data acquisition and quantitative spectral analysis were performed using Thermo-Finnigan Xcalibur version 1.3 and Thermo-Finnigan LCQuan version 2.5.5, respectively. Standard curves were prepared by plotting observed peak area ratios (analyte peak area / internal standard peak area) against corresponding known quantities of 7mG, 3mA, O⁶mG, and 1mA (Figure S7). Analyte amounts in experimental samples were determined by comparing observed peak area ratios to the standard curves.

2.2. Base excision assay using a defined oligonucleotide substrate

2.2.1. Preparation of oligonucleotide substrates

2.2.1.1. Chemical synthesis of stable lesion-containing oligonucleotides:

Oligonucleotides containing stable lesions [1, N⁶-ethenoadenine (A), 1mA] were chemically synthesized by Integrated DNA Technologies or Midland Certified using phosphoramidite precursors (Glen Research). 25mer oligonucleotide [d(GACCACTACACCXATTTCCTTACAAC)] containing a centrally located lesion (X) was either [³²P]-labeled or synthesized with 6-carboxyfluorescein (FAM) at the 5' end and annealed to 2-3-fold molar excess of complementary oligonucleotide [d(GTTGTAAGGAATTGGTGTAGTGGTC)] to produce a 5 μM ³²P-DNA or a 50 μM FAM-DNA stock. [³²P]-oligos were subsequently purified by phenol:chloroform extraction and a G-25 spin column.

2.2.1.2. Enzymatic synthesis of 7mG-containing oligonucleotides: 7mG was

enzymatically incorporated into DNA duplexes based on a method previously described by Asaeda *et al* [30]. A 5'-FAM labeled oligonucleotide primer [d(GACCACTACACC)] was annealed to 3-fold excess of complementary oligonucleotide [d(GTTGTAAGGAATCGGTGTAGTGGTC)], which contained only one cytosine (underlined) in the single-stranded region of the template. Primer-template was extended using DNA polymerase I Klenow fragment (New England Biolabs) and a 4-fold excess of 2'-deoxy-7-methylguanosine 5'-triphosphate (d7mGTP, Sigma-Aldrich) over dCTP, dTTP, and dATP. Extension reactions (20 μl) were carried out at room temperature for 5 min and contained 10 μM primer-template, 400 μM d7mGTP, 100 μM d(C/T/A)TP, 1 U Klenow fragment, and polymerase buffer [66 mM Tris-HCl (pH 7.6), 6.6 mM NaCl, and 1.5 mM β-mercaptoethanol]. Reactions were quenched with 5 mM EDTA and the DNA was purified by phenol:chloroform extraction and exchanged into TE buffer using a G-25 spin column.

2.2.2. Excision of 7mG from an oligonucleotide substrate—Working stocks of FAM-labeled 7mG-oligonucleotides (step 2.2.1.2) were diluted to 500 nM in AAG activity buffer [50 mM sodium acetate (pH 6.0), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.1 mg/ml BSA]. Glycosylase reactions (80 μl) contained 5 μM 79AAG, 100 nM FAM-DNA, and AAG activity buffer, and were incubated at 37°C. At various times, 8 μl aliquots were added to 2 μl 1 M sodium hydroxide and heated at 70°C for 2 min. Samples were denatured by addition of 10 μl formamide/dye loading buffer [80% (w/v) formamide, 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol, and 10 mM EDTA pH 8.0] and heating at 70°C for 5 min prior to running on a 20% acrylamide/8M urea sequencing gel. Gels were run at 40 W for 45 min in 0.5× TBE buffer (45 mM Tris, 45 mM borate, and 1 mM EDTA pH 8.0) and imaged on a Typhoon Trio Variable Mode Imager (GE Healthcare) in fluorescence mode using a 532-nm green laser and 526-nm emission filter.

2.3. Protein purification

Human AAG catalytic domain, which lacks 79 residues from the amino terminus (79AAG) [55], *Saccharomyces cerevisiae* MAG [26], *Salmonella typhi* TAG wild-type and E38A mutant [40], and *Bacillus cereus* AlkD wild-type and D113N mutant [27] were purified as described previously.

The AlkC gene was cloned from *B. cereus* genomic DNA (ATCC 14579) into a modified pET-19b expression vector (Novagen) that contains a Rhinovirus 3C (PreScission protease) cleavable N-terminal His₁₀-Tag. The vector was transformed into *E. coli* Rosetta cells and was overexpressed overnight at 16°C upon addition of 0.1 mM IPTG. Cells were harvested in 50 mM Tris-HCl (pH 8.5), 500 mM NaCl, and 10% glycerol and lysed with an Avestin Emulsifer C3 homogenizer operating at ~20,000 psi. His₁₀-AlkC protein was purified using Ni-NTA (Qiagen) affinity chromatography. Following cleavage of the His₁₀ tag, AlkC was further purified by heparin affinity and gel filtration chromatography to >99% homogeneity. Protein was concentrated to 2.4 mg/ml and stored in 20 mM Tris-HCl (pH 8.5), 200 mM NaCl, 10% glycerol, 2 mM DTT, and 0.1 mM EDTA.

3. Results

We set out to design a mass spectrometry method to simultaneously detect the various methylated nucleobases liberated from genomic DNA as a result of DNA glycosylase activity. Treatment of double-stranded DNA with MNU produces several methylated nucleobases (Figure 1), with 7mG comprising 66% of the total methylated DNA, 3mA 8%, O⁶mG 6%, 7mA 2%, 1mA 1%, and the other possible methylbases each comprising <1% (totaling ~4%). The remaining 12% methylated DNA products are phosphodiester backbone modifications [2]. We incubated MNU-treated calf thymus DNA with either mild acid for complete depurination or alkylpurine DNA glycosylases for more selective methylbase excision. The released methylpurines were separated and quantified by reversed-phase HPLC and ESI(+) MS/MS in MRM mode to obtain the highest possible selectivity (Figure 2B). Nucleobases were quantified relative to internal deuterated standards that were added prior to chromatographic separation. We found that addition of an ion-pairing agent, perfluoropentanoic acid, to the mobile phase increased the retention times of the methylated bases and improved the HPLC separation. Direct infusion of methylated nucleobase standards allowed for tuning of the ESI source and optimization of MRM parameters.

3.1. Analytical figures of merit

We first determined response calibration parameters, analyte percent recovery, and lower limits of quantitation using mock glycosylase reactions containing an inactive AlkD D113N mutant [27, 56] and known amounts of analytes, but lacking calf thymus DNA. The limits of detection and lower limits of quantitation were 500 fmol for all four bases measured. Signal responses for all methylpurines were linear over 0.5-40 pmol (injected amounts, 50-4,000 nM) with all correlation coefficients (R^2) over 0.99 (Figure S7). Loss of analytes during sample processing was minimal as extraction recovery values were $100 \pm 7\%$ for 1mA, $95 \pm 6\%$ for 3mA, $96 \pm 6\%$ for 7mG, and $101 \pm 10\%$ for O⁶mG.

We next quantitated the total amount of each methylbase present in the MNU-treated DNA by acid depurination. Two concentrations of substrate were tested, and 1mA, 3mA, 7mG, and O⁶mG products were measured over four different times from 0.5-6 h (Table S1). Depurination of all four bases from 3 μ g DNA was complete after 30 min. However, using 10 μ g DNA, 3mA depurination was complete after 30 min, but 3 h was needed for maximum 1mA and 7mG hydrolysis. Interestingly, the amounts of O⁶mG decreased over time, suggesting that the O⁶mG nucleobase was being degraded from acid treatment, either before or after excision from DNA. In support of this, O⁶mG could not be detected from

DNA treated with 5 N HCl. The relative amounts of total nucleobases detected from 3 μ g and 10 μ g DNA were 23:5:3:1 and 47:12:4:1 (7mG:3mA:O⁶mG:1mA), respectively. Thus, on average the adenine adducts were consistent with previously reported values of MNU-treated DNA (66:8:6:1) while the guanine adducts were slightly underestimated [2]. When acid depurination of 10 μ g DNA was performed with 5 N HCl at 37°C for 1 h, the ratio of 7mG:3mA was 7.5:1 (Table S2), nearly identical to that previously reported, whereas 1mA levels were slightly higher than expected (3.3:1 3mA: 1mA)

3.2. Substrate selection by alkylpurine DNA glycosylases

We tested the utility of the HPLC-MS/MS method by measuring 3mA and 7mG excised from MNU-treated genomic DNA by several well-characterized DNA glycosylases from various organisms (Figure 3A, Table S2). Eukaryotic and prokaryotic alkyl-DNA glycosylases are known to span a wide range of substrate specificities [4, 57]. Human AAG/MPG [16, 58], *S. cerevisiae* MAG [59, 60], and *E. coli* AlkA [14, 32] excise a broad range of alkylated and deaminated bases [26, 37, 61-63], while *B. cereus* AlkC and AlkD have intermediate specificities for cationic lesions, including 3mA and 7mG [27, 38, 56, 64], and *E. coli* TAG [12] is highly specific for 3mA [39, 40]. As negative controls, we tested an AlkD D113N mutant, which reduces 7mG activity 100-fold relative to wild-type AlkD, to levels indistinguishable from spontaneous 7mG depurination, as well as TAG E38A, which reduces 3mA activity 300-fold with respect to wild-type TAG [27, 40].

As expected, after 1 h at 37°C, all wild-type enzymes released the maximal or near maximal amount of 3mA (Figure 3A, Table S2). The TAG E38A and AlkD D113N mutants showed significantly reduced 3mA activity, similar to that previously reported for TAG E38A [40] and consistent with the observed catalytic impairment of AlkD D113N [27, 56, 64]. AAG, MAG, and AlkD showed robust 7mG activity, while TAG and AlkC displayed relatively weak removal of 7mG, again as expected. Consistent with previous results from oligonucleotide substrates [27, 64], the AlkD D113N mutant exhibited greatly decreased 7mG activity. Minimal amounts of 3mA and 7mG were detected in a no-enzyme control, with ~6% nucleobases released compared to acid depurination.

3.3. Characterization of enzymatic activity by HPLC-MS/MS

We further investigated the utility of the mass spectrometry method by monitoring enzyme concentration dependence and kinetics of base excision from the genomic substrate. Using AlkD, we measured the release of 3mA and 7mG as a function of enzyme concentration (Figure 3B). Each methylpurine showed a different concentration dependency. 3mA was completely excised with 0.5 μ M AlkD, with an apparent dissociation constant ($K_{1/2}$) of 2.7×10^{-8} M, whereas at least 10-fold more enzyme ($>5 \mu$ M) was required to completely remove 7mG ($K_{1/2} = 1.5 \times 10^{-6}$ M). These results are consistent with previously reported values using [³H]-MNU-treated calf thymus DNA [38]. Additionally, the 7mG data is in good agreement with the $K_{1/2}$ determined using an oligonucleotide-based assay [64]. We next followed AlkD-catalyzed release of 3mA and 7mG over time (Figure 3C). Under the conditions tested, AlkD completely excised 3mA within 24 min, with an observed second-order rate constant (k_{obs}) of $4.0 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. AlkD-catalyzed excision of 7mG from genomic DNA was 150-fold slower ($k_{\text{obs}} = 2.7 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$) than that of 3mA, and about 6-fold slower than excision of 7mG from an oligonucleotide substrate under comparable conditions [64].

3.4. Excision of 7mG from an oligonucleotide substrate

To illustrate the oligonucleotide based base excision assay, we monitored base excision from a defined oligonucleotide substrate using denaturing polyacrylamide electrophoresis. As previously reported, it is possible to prepare a defined 7mG-containing oligonucleotide substrate that can be used for DNA glycosylase assays [26-28, 30, 55]. We enzymatically

incorporated 7mG into the central position of a 25mer duplex and monitored release of the 7mG under single turnover conditions by the catalytic domain of human AAG (79AAG) (Figure 4). At time zero, about 28% of the total DNA was in the product form, consistent with the propensity of spontaneous depurination of 7mG (Figure 4A) [64]. The k_{cat} for removal of 7mG by 79AAG was $(3.6 \pm 0.1) \times 10^{-4} \text{ s}^{-1}$, about 5-fold slower than a previously reported value under similar conditions [28].

4. Discussion

Here, we describe biochemical methods to assay *in vitro* DNA glycosylase activities, focusing on the preparation of alkylpurine DNA substrates and a new mass spectrometry method we have developed to quantify multiple alkylpurine bases liberated from a genomic DNA substrate. Other HPLC-MS/MS methods have been developed to simultaneously detect alkylated DNA adducts present in DNA [44, 48, 50, 51]. The method developed here expands on previous reports by examining the enzymatic excision of methylated adducts from DNA by alkylpurine DNA glycosylases. We show that this method is consistent with results from other DNA glycosylase assays, can be used to perform simultaneous kinetic characterization of base excision activity toward multiple nucleobase substrates, and has the potential to uncover new activities.

We measured 3mA and 7mG excision activities for several well-characterized DNA glycosylases and compared the results from previous work. This allowed us to compare MS/MS detection versus [^3H]-scintillation detection and kinetic parameters from genomic versus 7mG-containing oligonucleotide substrates. Firstly, the relative activities of AAG, MAG, TAG, AlkC, and AlkD for 3mA and 7mG are consistent with the literature [28, 38, 40, 55, 63-65]. Secondly, there is no significant difference between MS/MS and [^3H]-scintillation detection of 3mA and 7mG (as judged by TAG and AlkD activities, respectively) from genomic DNA [38, 40]. Thirdly, the AlkD concentration-dependent release of both 3mA and 7mG from genomic DNA is comparable between [^3H]-scintillation and MS/MS modes of detection [38]. There was a modest (6-fold) difference in the kinetics of 7mG excised from genomic versus oligonucleotide DNA substrates [27, 64], a difference most likely a result of the excess non-specific binding sites present in the genomic DNA substrate. Nonetheless, as previously shown for [^3H] detection [40, 66], our results confirm that MS/MS detection is amenable to characterization of enzyme kinetics.

In addition to the ability to detect multiple nucleobase products, the use of genomic DNA allows the ability to probe less stable lesions that cannot be incorporated into oligonucleotides. Of interest is 3mA, a product of endogenous methyl donors (e.g., S-adenosylmethionine) [67, 68] and alkylating agents used in chemotherapy [69, 70]. Due to the short half-life of 3mA in DNA [71], it has not been possible to prepare purified oligonucleotides containing a single 3mA modification suitable for use in biochemical assays, although methylsulfonate derivatized lexitropsin (Me-Lex) peptides have been useful to study the biological effects of 3mA [72, 73]. The data presented here represents the first kinetic characterization of 3mA release by AlkD, and reveals that AlkD excises 3mA at a rate two orders of magnitude greater than 7mG, suggesting that 3mA may be the preferred substrate of AlkD. In addition, 3mA excision by AlkD is 6-10-fold faster ($k_{obs} = 4.0 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) than by 3mA glycosylases TAG ($k_{obs} = 3.9 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) and MagIII ($k_{obs} = 6.4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) [40, 66].

We believe our method will be invaluable in providing detailed information about substrate specificities among closely related glycosylases. Simultaneous detection of nucleobase products will enable high-throughput structure-function analysis of substrate specificity, including evaluation of mutants that are predicted to alter substrate specificity, since amino

acid substitutions may differentially affect activity toward each lesion. Although neither 1mA nor O⁶mG are typical glycosylase substrates, we included them in this analysis because they are produced at significant levels by various laboratory methylating agents. It is conceivable that newly discovered or mutant glycosylases could have activity toward these nucleobases, as previously observed for 1mA excision by *Archaeoglobus fulgidus* AlkA [25]. Finally, we note that detection of methylated bases by HPLC/MS-MS is not confined to genomic DNA substrates. We are able to detect 1mA excised from a 25mer oligonucleotide by acid depurination (data not shown). Thus, this MS/MS method may be more generally applied to other DNA repair activities, such as 1mA conversion to adenine by ALKB orthologs [74].

5. Conclusions

DNA glycosylase (base excision) activity can be monitored from oligonucleotides containing a single lesion or from genomic DNA containing a large number of different lesions. The oligonucleotide assay is more amenable to precise control of experimental parameters and single-turnover kinetics, while also being relatively inexpensive and easy to implement, but is limited to more stable lesions. Conversely, the genomic DNA assay allows simultaneous evaluation of enzymatic activity from DNA containing multiple types of potential substrates, including less stable lesions, but suffers from increased cost and difficulty of implementation. Use of radiolabeled methylating agents like [³H]-MNU have become prohibitive, necessitating alternative detection methods of non-radiolabeled adducts. We demonstrate the use of a robust HPLC-MS/MS method for detecting DNA glycosylase nucleobase products, which will enable high-throughput analysis of substrate specificities and activities of alkylation specific DNA repair proteins. The required nucleobase standards are either commercially available (1mA, 3mA, 7mG, O⁶mG, *d*₃-3mA, *d*₃-O⁶mG) or straightforward to synthesize (*d*₃-1mA, *d*₃-7mG, *d*₃-O⁶mG), and we believe our method should be accessible to most laboratories that have HPLC-MS/MS capabilities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

3mA	<i>N</i> 3-methyladenine
7mG	<i>N</i> 7-methylguanine
1mA	<i>N</i> 1-methyladenine
A	1, <i>N</i> ⁶ -ethenoadenine
MAG	methyladenine DNA glycosylase
TAG	<i>E. coli</i> 3-methyladenine DNA glycosylase I
AlkA	<i>E. coli</i> 3-methyladenine DNA glycosylase II
AAG	human alkyladenine DNA glycosylase
FAM	6-carboxyfluorescein
MNU	<i>N</i> -methyl- <i>N</i> -nitrosourea
MNNG	<i>N</i> -methyl- <i>N</i> -nitro- <i>N</i> -nitrosoguanidine
MMS	methylmethanesulfonate
DMS	dimethylsulfate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Tris	tris(hydroxymethyl)aminomethane
DTT	dithiothreitol
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetic acid
HPLC	high performance liquid chromatography
MS/MS	tandem mass spectrometry
MRM	multiple reaction monitoring
CID	collision induced dissociation
ESI	electrospray ionization
ESI(+)	positive ion mode electrospray ionization

Highlights

- Mass spectrometric detection of multiple alkyl-DNA adducts excised by glycosylases
- Use of HPLC-MS/MS to characterize glycosylase activities
- Methods for preparation of alkylated genomic DNA and oligonucleotides
- Comparison of methods to characterize alkylpurine DNA glycosylase activities

Figure 1. Alkylpurine DNA glycosylase reaction and substrates. (A) Schematic of the DNA glycosylase reaction. (B) Structures of the major methylated purines produced by laboratory alkylating agents. Percentages refer to the relative amounts of methylpurines produced by MNU treatment [2].

Figure 2.

Two methods for monitoring DNA glycosylase base excision activity. (A) Oligonucleotide assay. A ^{32}P - or fluorescently labeled (star) oligonucleotide containing a modified nucleobase (asterisk) is incubated with a DNA glycosylase to generate an AP site. At various time points, aliquots of the reaction are mixed with NaOH and heated to nick any AP sites. The substrate (S) and product (P) bands are separated by denaturing polyacrylamide gel electrophoresis, shown schematically to the right. (B) Genomic DNA assay. Genomic DNA is treated with a methylating agent (e.g., *N*-methyl-*N*-nitrosourea, MNU) to produce a spectrum of methylated nucleobases (gray ovals), some of which are excised from the DNA backbone by DNA glycosylases. The glycosylase reaction is quenched and spiked with deuterated internal standards (dark gray ovals) before the genomic DNA is ethanol precipitated and the soluble fraction containing free methylbases is subjected to HPLC/MS-MS. Ions were generated in positive ion electrospray ionization [ESI(+)] mode. MS/MS detection was based on multiple reaction monitoring of highly selective collision induced dissociation (CID) transitions with a triple quadrupole mass spectrometer.

Figure 3.

Characterization of base excision from genomic DNA using HPLC-MS/MS. (A) Comparison of 3mA (black bars) and 7mG (gray bars) excision activities of several alkylpurine DNA glycosylases. Methylated genomic DNA was incubated with either 5 N HCl or 5 μ M glycosylase for 1 h at 37°C. Enzymes used: human 79AAG, *S. cerevisiae* MAG, *S. typhi* TAG wild-type and E38A mutant, *B. cereus* AlkC, and *B. cereus* AlkD wild-type and D113N mutant. The numbers used to generate this graph are provided in Supplemental Table S2. (B) Enzyme concentration dependence of 3mA (black circles) and 7mG (gray squares) excision by AlkD. Reactions containing 0.01–5 μ M AlkD were incubated at 37°C for 1 h. (C) Time dependence of 3mA (black circles) and 7mG (gray squares) excision by AlkD. Reactions containing 0.5 μ M AlkD were incubated at 37°C for 1 min to 10 h. Reaction mixtures were equilibrated at 37°C for 3 min prior to the addition of enzyme.

Figure 4.

Excision of 7mG from an oligonucleotide substrate by human 79AAG. Reactions contained 5 μ M 79AAG, 100 nM FAM-DNA, 50 mM sodium acetate (pH 6.0), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.1 mg/ml BSA. (A) Denaturing polyacrylamide gel showing 25mer 7mG-DNA (S) and AP-nicked 13mer product (P) at various reaction times. (B) Quantitation of the data in panel A.