

Research Article

Repair of DNA Alkylation Damage by the *Escherichia coli* Adaptive Response Protein AlkB as Studied by ESI-TOF Mass Spectrometry

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DNA alkylation can cause mutations, epigenetic changes, and even cell death. All living organisms have evolved enzymatic and non-enzymatic strategies for repairing such alkylation damage. AlkB, one of the *Escherichia coli* adaptive response proteins, uses an α -ketoglutarate/Fe(II)-dependent mechanism that, by chemical oxidation, removes a variety of alkyl lesions from DNA, thus affording protection of the genome against alkylation. In an effort to understand the range of acceptable substrates for AlkB, the enzyme was incubated with chemically synthesized oligonucleotides containing alkyl lesions, and the reaction products were analyzed by electrospray ionization time-of-flight (ESI-TOF) mass spectrometry. Consistent with the literature, but studied comparatively here for the first time, it was found that 1-methyladenine, 1,*N*⁶-ethenoadenine, 3-methylcytosine, and 3-ethylcytosine were completely transformed by AlkB, while 1-methylguanine and 3-methylthymine were partially repaired. The repair intermediates (epoxide and possibly glycol) of 3,*N*⁴-ethenocytosine are reported for the first time. It is also demonstrated that *O*⁶-methylguanine and 5-methylcytosine are refractory to AlkB, lending support to the hypothesis that AlkB repairs only alkyl lesions attached to the nitrogen atoms of the nucleobase. ESI-TOF mass spectrometry is shown to be a sensitive and efficient tool for probing the comparative substrate specificities of DNA repair proteins *in vitro*.

1. Introduction

Nuclear and mitochondrial DNA is vulnerable to chemical modification from exogenous and endogenous sources. Exogenous sources include radiation, oxidation and alkylation by organic and inorganic chemical agents; endogenous cellular processes that contribute to the burden of genomic damage include enzyme-promoted oxidation, alkylation, and deamination of DNA [1, 2]. Because DNA has many potential nucleophilic reaction sites, such as the nitrogen and oxygen atoms on the bases and the oxygen atoms on

the sugar and phosphodiester backbone, small alkylating agents from the environment or from internally generated sources form a remarkably vast array of covalent alkyl-DNA adducts [3]. These adducts challenge the cell in several ways. They can cause mutations and hence irreversibly reprogram the destiny of a somatic or germ cell. They jeopardize the epigenetic pattern that imprints long term gene regulation. Moreover, adducts can block DNA and RNA synthesis, inhibit mRNA transcription and translation, and lead to strand breaks. To avoid the undesired effects of alkyl-DNA lesions, living systems have developed a host of DNA repair

systems that act as front line defenses against the threats that these adducts pose to cellular welfare [1, 2].

The cellular response of *E. coli* to alkylation is an intricate and fascinating system that includes a specific sensor of DNA alkylation burden (monitored as the alkyl phosphotriester concentration) that, when triggered, results in the expression of four proteins that afford a robust global defense against a broad array of alkyl DNA adducts [4–6]. When *E. coli* experiences a low dose of a methylating agent, the alkylation damage to DNA creates a signal for transcriptional activation of the genes encoding the Ada, AlkA, AlkB, and AidB proteins, each of which combats selective features of the alkylation threat. The accumulation of those four proteins affords *E. coli* resistance to the mutagenicity and toxicity of subsequent higher doses of the alkylating agent [7]. This process in *E. coli* is called the adaptive response, which is activated by the Ada protein following its alkylation by the aforementioned DNA backbone alkyl phosphotriester that serves as the cellular barometer for alkylation stress [4, 5]. The AlkA enzyme is a DNA glycosylase and works against selected alkylated bases, such as 3-methyladenine and 7-methylguanine, among others [2]. The AidB protein is proposed to be a defensive operative that sequesters or chemically inactivates alkylating agents before they can react with DNA; its exact enzymatic activity and mechanism, however, are still unknown [8]. The AlkB protein was discovered to be an α -ketoglutarate- and Fe(II)-dependent oxidizing enzyme that can efficiently repair various DNA and RNA alkyl lesions [9, 10] (Figure 1). The reported substrate scope for the AlkB enzyme encompasses either simple methylated or ethylated DNA lesions, such as 1MeA, 3MeC, 3EtC, 1MeG, and 3MeT, or even lesions with bridged two-carbon units, such as eA, eC, and ethanoA [2, 4, 11, 12] (Figure 2). AlkB has many mammalian homologs, including ABH1 through ABH8 and FTO, some of which have also been proposed or proved to act upon alkylated DNA and RNA [2, 4, 11, 12]. Since the discovery of the enzymatic properties of AlkB, research on this protein and its homologs has become a fertile research area.

In this work we utilized chemical synthesis to prepare structurally defined lesions at specific sites within oligonucleotides. The site-specifically modified oligonucleotides were incubated with purified AlkB protein to allow a head-to-head comparison of the substrate properties of various alkyl lesions in the same DNA sequence context. This method enabled us to monitor efficiently the ability of AlkB to repair alkylation damage by a mass spectrometry tool employed by us earlier in a limited manner with three DNA lesions [13, 14]. This tool is demonstrated to have promise for the high throughput comparative analysis of multiple and diverse DNA repair substrates.

2. Materials and Methods

2.1. Oligonucleotide Synthesis. Oligonucleotides containing the lesions in Figure 2 were made using solid-phase phosphoramidite chemistry, and were deprotected, purified and characterized as described previously [13, 15–17]. For all nine lesions, the 16 mer oligonucleotide

sequence was 5'-GAAGACCTXGGCGTCC-3', ($X = \text{lesion}$); for $O^6\text{MeG}$, an additional sequence was made (5'-GAAGACCGXTGCGTCC-3', $X = O^6\text{MeG}$). The calculated MWs of the oligonucleotides are shown in Table 1. DNA concentration was determined by UV absorbance using the extinction coefficients (ϵ) at 260 nm (For any alkylated base, we substituted its extinction coefficient with the extinction coefficient of its unmodified counterpart due to the small difference between the values in the context of 16 mer DNA.).

2.2. In Vitro Reactions of Lesions with AlkB. All assays were carried out with AlkB Δ N11, a truncated form of AlkB in which the first eleven residues are deleted. AlkB Δ N11 was purified as described [14] and shown previously to have similar activity to wild-type protein [14, 18]. All AlkB reactions used similar conditions as previously described [13], the major change being that all the reactions were performed in HEPES buffer. Reactions were performed at 37°C in 45 mM HEPES (pH 8.0), 67 μM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 0.9 mM α -ketoglutarate and 1.8 mM ascorbate. DNA 16 mer substrates were as described in the previous section. Reactions were conducted with 5 μM DNA (see Figures 3-5 for detailed information) and with or without 2.5 μM AlkB in a 10 μL volume. Reaction mixtures were kept on dry ice until ESI-TOF MS analysis.

2.3. LC-ESI-TOF/MS Analysis. Oligonucleotide analyses were performed on an Agilent ESI-TOF mass spectrometer (Palo Alto, CA). ESI was conducted using a needle voltage of 3.5 kV. Nitrogen gas was used with a setting of drying 10 L/min and setting of nebulizer 15 psig and a heated capillary at 325°C. Liquid chromatographic separations were performed using a Zorbax SB-Aq column (2.1 \times 150 mm; 3.5 μm ; Agilent Technologies, Palo Alto, CA) with a flow rate of 0.2 mL/min. Solvent A was 10 mM ammonium acetate in water and solvent B was 100% acetonitrile. A linear gradient was performed in the following steps: 2% to 30% B over 30 minutes, 30% to 2% B over 5 minutes, and 2% to 2% B over 10 minutes. Data analyses were performed using Agilent MassHunter Workstation software. For each individual lesion, we assumed the 16 mer DNA oligo with the lesion and the oligo with the undamaged counterpart have identical extinction coefficients. In an effort to achieve unbiased quantification of the repair reaction, we first extracted the UV chromatogram by using two m/z values that bracket all expected oligonucleotide species. The time interval encompassing all extracted UV peaks was used to generate the ion chromatogram containing all expected oligonucleotide species. We found the -4 charge envelope of each target oligonucleotide. Then we chose the highest peak in each envelope to generate oligonucleotide-specific UV profiles, from which we selected the maximum UV absorbance at 260 nm to extract the ion abundance, whose maximum peak in the envelope was used to calculate the relative ratio between the oligonucleotides with the lesion or the undamaged/transformed counterpart.

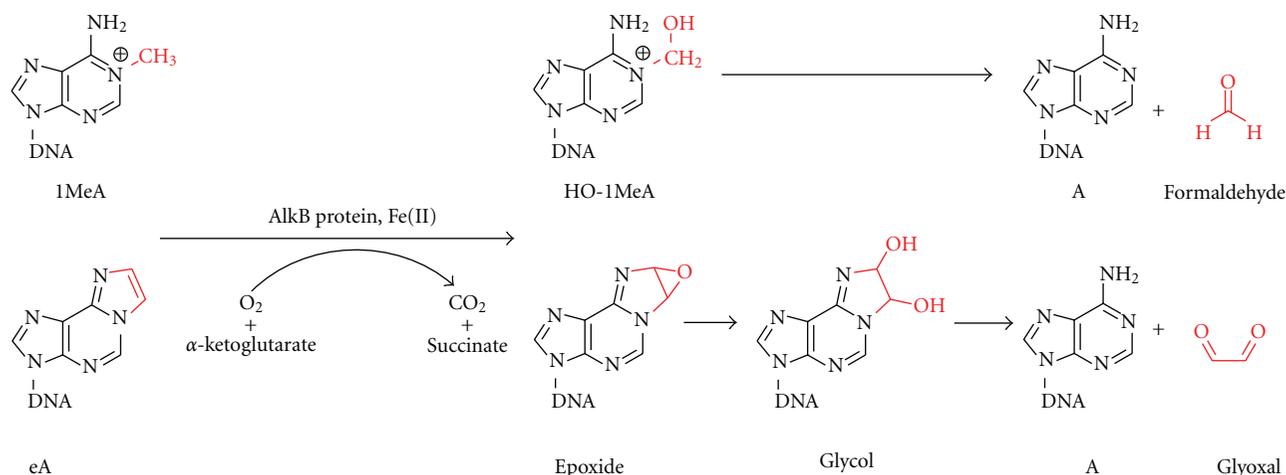


FIGURE 1: Proposed mechanisms and examples (1MeA and eA) of AlkB-mediated enzymatic repair of alkyl DNA lesions.

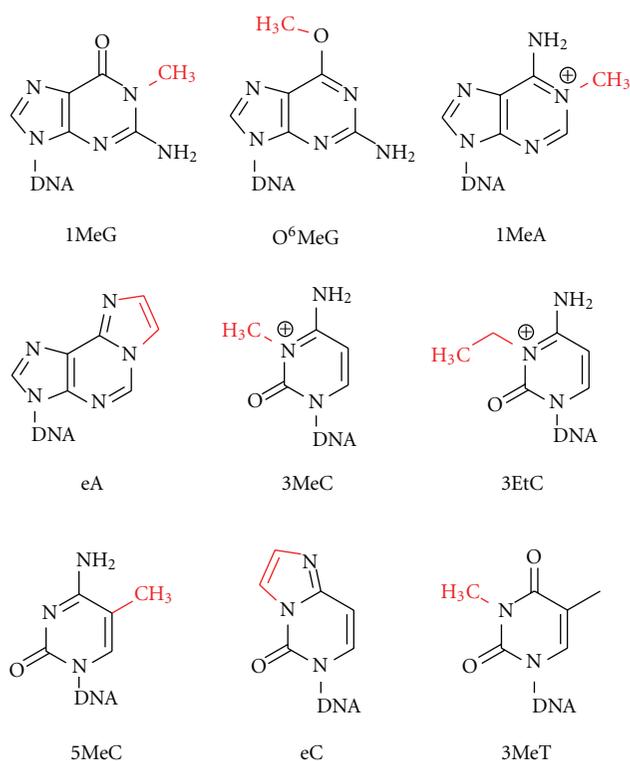


FIGURE 2: Chemical structures and abbreviations of DNA lesions screened as possible repair substrates for the AlkB protein using ESI-TOF mass spectrometry. The repair target within each base is highlighted in red.

3. Results

Nine 16 mer oligonucleotides were chemically synthesized containing the alkyl-DNA adducts shown in Figure 2. The sequence used was 5'-GAAGACCTXGGCGTCC-3'; the flanking sequence of each lesion (X) was identical, fixing the sequence context for the repair reaction. The 16 mers with each lesion were incubated in HEPES buffer with the known

cofactors for the AlkB reaction: α -ketoglutarate, iron in the +2 oxidation state, and ascorbate (see Section 2 for details). For each lesion, two sets of experiments were conducted, one with the AlkB protein and one in the reaction buffer without the AlkB protein. Following the repair reaction, ESI-TOF mass spectrometry was used to detect the reaction products. The 16 mer oligonucleotides demonstrated a good signal in the -4 charge envelope of the ESI-TOF spectra. To give one example to illustrate the method of analysis, the molecular weight (MW) of the 1MeG lesion in the 16 mer is calculated as 4918.87 Daltons for the neutral species, and the MW of its monoisotopic peak (all ¹²C, ¹⁴N, etc) when migrating with 4 negative charges (the -4 charge envelope) in the electric field of the spectrometer is *calculated* as having an *m/z* of 1228.71 (all MW information is shown in Table 1). The multiple peaks in each -4 charge envelope reflect the number of ¹³C or other heavier isotopes (Figure 3). Again using 1MeG as the example, its monoisotopic peak is *observed* as 1228.62. This experimental result is considered consistent with the theoretical calculation. The next peak in that envelope has an *m/z* of 1228.87, 0.25 amu larger than the 1228.62 peak, which indicates a species containing ¹³C, ¹⁵N or another isotope that adds a nominal mass of 1.0 to the total weight of the 16 mer (e.g., 0.25 = *m/z* = one ¹³C/a charge state of 4). Additional peaks in the spectrum represent additional heavy isotopes within the parental 16 mer.

3.1. N1-Methylguanine. N1-Methylguanine (1MeG) (Figure 2) has been found both *in vitro* [19] and *in vivo* [20]. The glycosylase AAG can repair the 1MeG lesion *in vitro* [21]. Our group and others have observed that the AlkB protein can repair 1MeG both *in vivo* and *in vitro* [15, 22]. In the *in vivo* assay, AlkB can partially overcome the strong block of 1MeG to replication (lesion bypass increases from 2% in AlkB- cells to 16% in AlkB+ cells). Similarly, AlkB causes a reduction in the mutagenicity of 1MeG from a high frequency of 80% in AlkB- cells to 4% in AlkB+ cells [15].

In the *in vitro* incubation reaction without AlkB protein, we observed the monoisotopic peak of the 1MeG 16mer

TABLE 1: Calculated and observed monoisotopic molecular weight of oligonucleotides and intermediates present in the AlkB repair reactions.

Lesion or base	MW (calculated)	m/z (calculated)		m/z (observed)
	of neutral species	−4 charge monoisotopic peak		−4 charge monoisotopic peak
G	4904.86	1225.21		1225.11
1MeG	4918.87	1228.71		1228.61/2
O ⁶ MeG	4918.87	1228.71		1228.62/3
A	4888.86	1221.21		1221.12/33
1MeA	4902.88	1224.71		1224.67
eA	4912.86	1227.21		1227.22/4
eA epoxide	4928.86	1231.21		1231.23
eA glycol	4946.87	1235.71		1235.72
C	4864.85	1215.20		1215.20/1
3MeC	4878.87	1218.71		1218.72
3EtC	4892.88	1222.21		1222.23
5MeC	4878.87	1218.71		1218.73
eC	4888.85	1221.20		1221.21/2
eC epoxide	4904.84	1225.20		1225.19
eC glycol	4922.86	1229.71		1229.69
T	4879.85	1218.95		1218.97
3MeT	4893.87	1222.46		1222.49

oligonucleotide for the −4 charge state at m/z of 1228.62 (Figure 3(a)), which correlates well with the calculated value for the −4 charge monoisotopic peak (m/z = 1228.71). In the presence of the AlkB protein (Figure 3(b)), we found 47% of the 1MeG was repaired to the undamaged guanine base (−4 charge m/z = 1225.11) and 53% of the 1MeG (−4 charge m/z = 1228.61) was unchanged. Whereas many other lesions, such as 1MeA, 3MeC, 3EtC, or eA [13, 15], are fully transformed, 1MeG is not. It is noteworthy that its incomplete repair observed here is also in line with studies done on the same lesion *in vivo*, which showed that 1MeG has a relatively low efficiency of lesion bypass (16% for AlkB+ *E. coli*) [15].

3.2. O⁶-Methylguanine. O⁶-Methylguanine (O⁶MeG) (Figure 2) is formed when the genome is under alkylation attack [23] and is an especially potent driver of G → A transition mutations [24]. O⁶MeG is formed both endogenously [25, 26] and exogenously [27]. *E. coli* has two repair proteins for such damage. The constitutive Ogt and the inducible Ada proteins reverse methylation damage by transferring the methyl group to one of the internal cysteine residues on each protein. This transfer is a stoichiometric (“suicidal”) reaction that irreversibly inactivates the repair proteins [5]; thus, Ogt and Ada are not true enzymes since their active sites cannot be regenerated. The mismatch repair (MMR) pathway has also been reported to play an important role in the response to O⁶MeG [28]. In the present work, it was our goal to test whether AlkB can also act upon and possibly repair O⁶MeG.

For the O⁶MeG lesion, we synthesized two sets of 16mers and named them as TXG and GXT according to the neighboring nucleosides (see Section 2). In the mass spectra of both TXG (Figure 3(c)) and GXT (Figure 3(e)) in the absence of the AlkB enzyme, we observed m/z values of 1228.62 (TXG) and 1228.63 (GXT) for the −4 charge

monoisotopic peak, which are in good agreement with the calculated m/z of 1228.71. For the reactions with AlkB protein, we did not observe any repaired product nor any structural modifications (as shown below, AlkB sometimes fully removes the alkyl group but it sometimes can create stable oxidized products that are distinct from both the starting material and an unmodified base product). The observed products of the reaction were identical to the starting material: m/z of 1228.62 for TXG (Figure 3(d)) and of 1228.62 for GXT (Figure 3(f)). These results indicate O⁶MeG is not a substrate for AlkB in the *in vitro* experiments under the reaction conditions tested, which provide for full transformation of other lesions, such as 1MeA and eA.

3.3. N1-Methyladenine. N1-Methyladenine (1MeA) (Figure 2) is formed mainly in single-stranded DNA by alkylating agents and has been detected *in vitro* [3, 9, 10, 19, 29–31] and *in vivo* [3, 32–35]. These alkylating agents include S_N2 agents, such as methylmethanesulfonate (MMS) and the naturally occurring methyl halides [5]. The AlkB enzyme repairs 1MeA both *in vitro* and *in vivo* [15]. Studies of 1MeA *in vivo* reveal that the lesion imposes a severe block to DNA replication in AlkB− cells, which is completely removed in AlkB+ cells. These results underscore the physiological relevance of the AlkB system for countering the toxicity of this base. While very toxic, 1MeA is only weakly mutagenic in both AlkB− and AlkB+ cells [15].

In our *in vitro* study of 1MeA repair, the lesion was completely repaired to the undamaged base adenine (Figure 3(h), m/z = 1221.12), while no change occurred in the absence of the AlkB protein (Figure 3(g), m/z 1224.67). The *in vitro* results confirm that 1MeA is a good substrate for AlkB. Moreover, the current observations correlate very well with the strong reparability of 1MeA suggested by earlier *in vivo* lesion bypass studies [15].

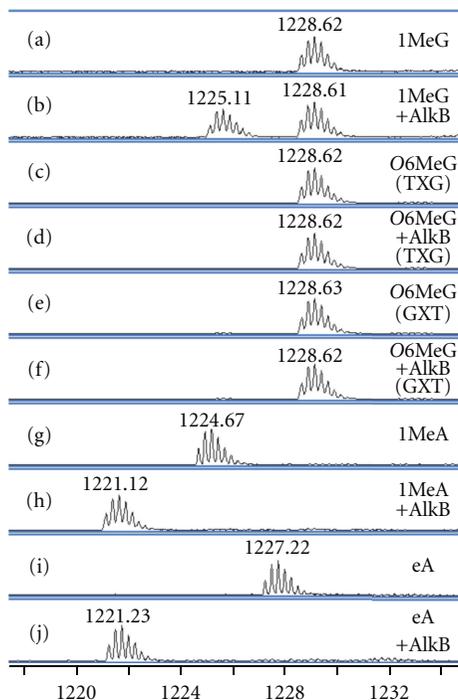


FIGURE 3: ESI-TOF analysis of 16mer reactants and products from the *in vitro* incubation reactions of alkylated lesions and AlkB protein. Data represent the -4 charge envelopes; multiple ion mass peaks associated with each envelope reflect mostly the number of ^{13}C atoms in each -4 charged packet. The monoisotopic peak (all ^{12}C , ^{14}N , etc.) value is labeled above each peak envelope. (a) 1MeG; (b) 1MeG + AlkB; (c) O^6MeG (TXG); (d) O^6MeG (TXG) + AlkB; (e) O^6MeG (GXT); (f) O^6MeG (GXT) + AlkB; (g) 1MeA; (h) 1MeA + AlkB; (i) eA; (j) eA + AlkB.

3.4. $1, N^6$ -Ethenoadenine. The formation of $1, N^6$ -ethenoadenine (eA) (Figure 2) may result from the reaction of adenine with products of unsaturated lipid peroxidation [36–39] or arise from normal physiological conditions in both rodents and humans [40, 41]. The eA lesion is believed to be a good biomarker of inflammation and oxidative stress. This lesion may also be formed by exposure to the common industrial agent vinyl chloride and its metabolites, such as chloroacetaldehyde [42]. In duplex DNA, eA can be repaired by the base excision repair (BER) pathway *in vitro* [21, 43] and *in vivo* [44, 45]. Recently, it was shown that AlkB and its human homolog ABH2 and ABH3 can repair eA *in vitro* [13, 46, 47]. The direct reversal mechanism is also likely to be operative *in vivo*. In AlkB $-$ cells, eA is 35% mutagenic, but less than 0.3% mutagenic in AlkB $+$ cells [13].

In our present *in vitro* study using mass spectrometry to monitor the repair reaction, the oligonucleotide with eA was observed at m/z of 1227.22 for its -4 charge monoisotopic peak in the absence of AlkB (Figure 3(i)). In the presence of AlkB, the eA lesion was mostly converted to the undamaged product, adenine ($m/z = 1221.23$, Figure 3(j)). The m/z difference at the -4 charge state between the eA and A is 5.99 Daltons, which corresponds to the ~ 24 Dalton MW difference (two carbon atoms) of

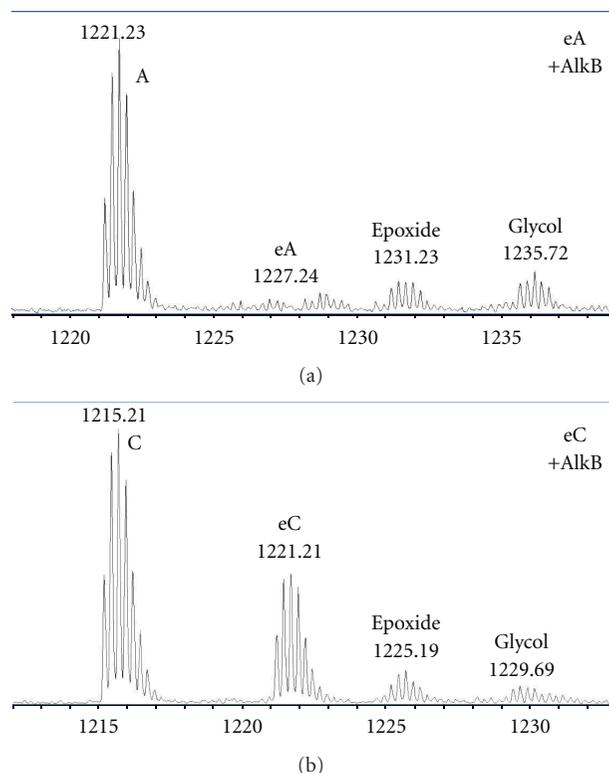


FIGURE 4: ESI-TOF analysis of 16mer reactants and products from the *in vitro* incubation reactions of eA and eC lesions with the AlkB protein. Data represent the -4 charge envelopes; multiple ion mass peaks associated with each envelope reflect mostly the number of ^{13}C atoms in each -4 charged packet. The monoisotopic peak (all ^{12}C , ^{14}N , etc.) value is labeled above each peak envelope. (a) eA + AlkB; (b) eC + AlkB.

the two products. We also observed peak clusters consistent in MW with epoxide and glycol intermediates (Figures 1 and 4(a)), which are consistent with previous observations [13].

3.5. N^3 -Methylcytosine and N^3 -Ethylcytosine. N^3 -Methylcytosine (3MeC) (Figure 2) has been detected both *in vitro* [3, 9, 19, 29–31, 48, 49] and *in vivo* [3, 20, 34, 49]. The corresponding ethyl homolog, N^3 -ethylcytosine (3EtC) (Figure 2), also has been detected *in vitro* [3, 29] and *in vivo* [3, 50]. In *E. coli*, the AlkB protein has good activity against 3MeC and 3EtC both *in vitro* and *in vivo* [9, 10, 15]. The mutation frequency of 30% for both 3MeC and 3EtC in AlkB $-$ cells dropped to $<2\%$ in AlkB $+$ cells [15].

The m/z values of the starting material for 3MeC and 3EtC in the ESI-TOF mass spectra were 1218.72 (Figure 5(a)) and 1222.23 (Figure 5(c)), respectively. After incubation with AlkB protein, the 3MeC and 3EtC residues were completely repaired to the undamaged cytosine (m/z of 1215.21 for 3MeC, Figure 5(b); and of 1215.20 for 3EtC, Figure 5(d)). The complete *in vitro* repair of these lesions establishes both as good substrates for AlkB, consistent with findings from previous *in vivo* bypass and mutagenesis assays [15].

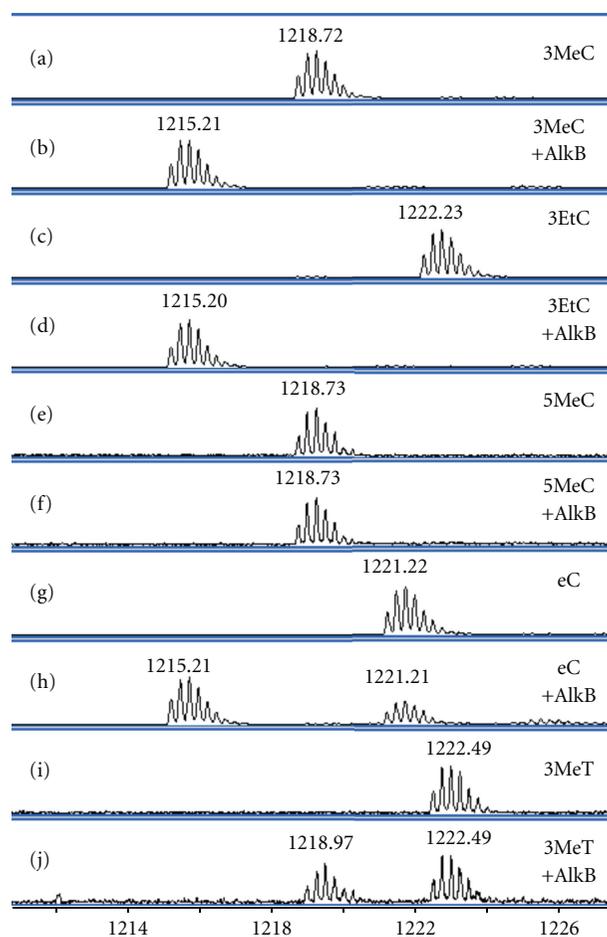


FIGURE 5: ESI-TOF analysis of 16 mer reactants and products from the *in vitro* incubation reactions of alkylated lesions and AlkB protein. Data represent the -4 charge envelopes; multiple ion mass peaks associated with each envelope reflect mostly the number of ^{13}C atoms in each -4 charged packet. The monoisotopic peak (all ^{12}C , ^{14}N , etc.) value is labeled above each peak envelope. (a) 3MeC; (b) 3MeC + AlkB; (c) 3EtC; (d) 3EtC + AlkB; (e) 5MeC; (f) 5MeC + AlkB; (g) eC; (h) eC + AlkB; (i) 3MeT; (j) 3MeT + AlkB.

3.6. 5-Methylcytosine. 5-Methylcytosine (5MeC) (Figure 2) is the naturally methylated form of cytosine seen in prokaryotes and eukaryotes. This methylation does not disturb the Watson-Crick base pairing ability of cytosine, and it is not recognized as DNA damage. 5MeC is an important epigenetic biomarker for mammalian development. It also occurs in bacterial systems as a marker for restriction endonucleases and as a marker used in a specialized type of mismatch repair [51]. Recently, researchers found TET1, an α -ketoglutarate- and Fe(II)-dependent enzyme, that can oxidize 5MeC to 5-hydroxymethylcytosine [52]. We assessed the ability of AlkB to act similarly on 5MeC.

In the reaction without the AlkB protein, the monoisotopic -4 charge peak of 5MeC appeared at $m/z = 1218.73$ (Figure 5(e)). In the incubation reaction with 5MeC plus AlkB, the only peak in the spectrum corresponded to 5MeC ($m/z = 1218.73$, Figure 5(f)). No peak corresponding to

5-hydroxymethylcytosine or the fully demethylated product, cytosine, was observed, indicating that 5MeC is not an AlkB substrate under the conditions of analysis.

3.7. 3, N^4 -Ethenocytosine. 3, N^4 -Ethenocytosine (eC) (Figure 2) is produced from the same precursors and by the same pathways that generate eA in DNA [36–38, 53]. The BER pathway (human thymine-DNA-glycosylase (hTDG) in human and double-stranded uracil-DNA-glycosylase (dsUDG) in *E. coli*) has been shown to suppress the occurrence of this adduct [44, 53]. In *E. coli*, AlkB has a modest effect on eC toxicity, but reduces the mutation of eC from 82% in AlkB $-$ cells to 37% in AlkB $+$ cells [13].

In our *in vitro* studies, the reaction of eC oligonucleotide without the AlkB protein gave a -4 envelope signal at $m/z = 1221.22$ (Figure 5(g)). The reaction with AlkB showed eC to be a significant substrate for AlkB protein, although the lesion was not fully repaired under conditions that fully repaired other adducts, such as 1MeA and 3MeC. Only 67% of the eC lesion was consumed (to cytosine, epoxide, and glycol) and 33% of the eC remained intact (signal at $m/z = 1221.21$, Figure 5(h)). We also observed a small amount of the epoxide and possibly glycol products (Figure 4(b)), comparable to the products found in the eA repair reaction by AlkB [13]. These results strongly support the conclusions from earlier *in vivo* experiments, and further show that eC may be repaired by AlkB via an epoxide intermediate, which is hydrolyzed to a glycol and then may be released as glyoxal (Figure 1). This ESI-TOF analysis of eC with AlkB is the first demonstration of the detailed mechanism and intermediates of this lesion repaired by the AlkB protein *in vitro*.

3.8. N3-Methylthymine. N3-Methylthymine (3MeT) (Figure 2) has been found both *in vitro* [3, 19, 29, 31, 54] and *in vivo* [54, 55] and is formed through the reaction of thymine with S_N2 alkylating agents such as MMS. This adduct has been reported as a very weak substrate for AlkB *in vivo* [15]. Recently, another α -ketoglutarate- and Fe(II)-dependent demethylase, FTO (fat mass and obesity associated) protein has been reported as an enzyme that can efficiently repair 3MeT in ssDNA [12, 56, 57].

Our *in vitro* experiments confirm the ability of AlkB to effectuate the partial repair of 3MeT as shown in the *in vivo* assays. In the absence of the AlkB protein, the -4 envelope monoisotopic peak of 3MeT appeared at m/z of 1222.49 (Figure 5(i)). In the reaction with AlkB protein, 47% of 3MeT was converted to thymine ($m/z = 1218.97$, Figure 5(j)), leaving 53% unrepaired ($m/z = 1222.49$, Figure 5(j)).

4. Discussion

4.1. Advantages of Chemical Synthesis and ESI-TOF Mass Spectrometry. In the *in vitro* experiments described above, purified AlkB protein was incubated with oligonucleotides containing individual DNA lesions within the same sequence context. There are two ways commonly used to generate alkyl lesions in DNA. The first is by treatment of an unmodified oligonucleotide of known sequence with alkylating agents,

such as MMS and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), to generate a spectrum of alkyl lesions within the oligomer. The second approach, which was used in this work, utilizes chemical synthesis to incorporate a modified DNA base into a defined sequence, thus providing homogeneous targeted damage to be used as a substrate for repair, replication, or other biochemical assays. All nine lesions investigated in this paper were synthesized in the same 16 mer background, thus eliminating sequence context as a variable for rank-ordering the extent to which each lesion may be repaired by the AlkB protein. We then utilized high-resolution liquid chromatography-mass spectrometry (LC-MS) to detect the reaction products. The ESI-TOF mass spectrometry method utilized here allowed us to achieve good signal with only 20 pmol of oligonucleotide, establishing it as a viable tool for DNA repair studies.

4.2. Substrate Scope for AlkB and Correlation of In Vitro Data with In Vivo Results. As reported in the above sections, we observed that the AlkB protein can repair different alkyl lesions to varying extents. Under the current experimental condition, good substrates for AlkB were 1MeA, eA, 3MeC, and 3EtC, which were completely repaired or transformed after a one-hour incubation at 37°C, which mimics human physiological temperature. The 1MeG, eC, and 3MeT lesions are less efficient substrates for AlkB under the conditions tested, and the extent of their transformation varied from 47% to 67%. The repair efficiency trends from the *in vitro* experiments performed here are in line with previously published *in vivo* results from lesion bypass and mutagenesis assays performed in our laboratory using single-stranded viral genomes site-specifically modified with each lesion, which were passaged through AlkB⁻ and AlkB⁺ *E. coli* [13, 15]. The oxygen-attached lesion O⁶MeG in two sequence contexts and the carbon-attached lesion 5MeC were not repaired or transformed at all in the AlkB reactions under the conditions tested. These results, combined with others in the literature, suggest that AlkB repairs only those alkyl-DNA lesions that are attached via the nitrogen atoms of the bases and not lesions bound to carbon or oxygen atoms.

5. Conclusions

There were two goals to the present study. The first was to expand our earlier study of DNA repair in which we used ESI-TOF to study repair of eA [13]. That work was a systematic enzymatic analysis including demonstration that this tool can capture real-time snapshots of the repair reaction as it progresses from starting material, through intermediates, and to products. In the present work, for the sake of simplicity, one time point was chosen (that at which eA repair was complete in the previous kinetic analysis). At that time point (one hour), the ESI-TOF method is shown to be of great value as a tool for rapid comparison of many structurally different substrates. Having shown the potential of this technology for real-time monitoring of DNA

repair reactions, future studies can use ESI-TOF and isotope-labeling with multiple time points for each lesion to define more accurately the kinetic parameters of their individual repair reactions.

The second goal of this work was to probe, more completely and systematically than in the past, the structural requirements of the AlkB enzyme. Among the nine lesions studied, we present direct new evidence for AlkB repair of eC by a direct reversal mechanism, and buttress the growing body of evidence that lesions connected to bases at nitrogens are the major targets of this enzyme. Similar analyses using the same substrate library as in the present study could be performed using the eight known human AlkB homologs as well as the FTO protein, TET1, and other α -ketoglutarate- and Fe(II)-dependent enzymes. Such studies will help further to define how this class of proteins helps to protect the genome from DNA damage or helps to modify gene expression programs.

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