Chloroethylnitrosourea-derived Ethano Cytosine and Adenine Adducts are Substrates for *Escherichia coli* Glycosylases Excising Analogous Etheno Adducts

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Running title:

Excision of Ethano dC and dA Adducts by *E. coli* Glycosylases

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Abbreviations: EC, 3,N^4-ethanocytosine: EA, 1,N^6-ethanoadenine; εC, 3,N^4-ethenocytosine; εA, 1,N^6-ethenoadenine; AP, apurinic/apyrimidinic; CNU, chloroethylnitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU: 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; BER, base excision repair; Mug, mismatch-specific uracil-DNA glycosylase; AlkA, *E. coli* 3-methyladenine DNA glycosylase II; APNG, alkylpurine-DNA-*N*-glycosylase; TDG, thymine-DNA glycosylase; Endo IV, endonuclease IV; Exo III, exonuclease III; APE1, the major human AP endonuclease; AGT, O^6-alkylguanine-DNA alkyltransferase; pol, polymerase; MD, molecular dynamics; RMSD, root mean square deviation.

Key words: Chloroethylnitrosoureas; ethano adducts; etheno adducts; DNA repair; glycosylases

Abstract

Exocyclic ethano DNA adducts are saturated etheno ring derivatives formed mainly by therapeutic chloroethylnitrosoureas (CNUs), which are also mutagenic and carcinogenic. In this work, we report that two of the ethano adducts, 3,N^4-ethanocytosine (EC) and 1,N^6-ethanoadenine (EA), are novel substrates for the *Escherichia coli* mismatch-specific uracil-DNA glycosylase (Mug) and 3–methyladenine DNA glycosylase II (AlkA), respectively. It has been shown previously that Mug excises 3,N^4-ethenocytosine (εC) and AlkA releases 1,N^6-ethenoadenine (εA). Using synthetic oligonucleotides containing a single ethano or etheno adduct, we found that both glycosylases had a ~20-fold lower excision activity toward EC or EA than that toward their structurally analogous εC or εA adduct. Both enzymes were capable of excising the ethano base paired with any of the four natural bases, but with varying efficiencies. The Mug activity toward EC could be stimulated by *E. coli* endonuclease IV and, more efficiently, by exonuclease III. Molecular dynamics (MD) simulations showed similar structural features of the etheno and ethano derivatives when present in DNA duplexes. However, also as shown by MD, the stacking interaction between the EC base and Phe 30 in the Mug active site is reduced as compared to the εC base, which could account for the lower EC activity observed in this study.

3. . **Indroduction**

Chloroethylnitrosoureas (CNUs), such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), have been used in the treatment of certain types of cancers [1,2]. However, these agents are also mutagenic and carcinogenic by virtue of their ability to modify DNA [3-5]. Three types of base modifications from the reaction of these bifunctional alkylating agents with DNA have been described: monosubstituted purine bases (either a hydroxyethyl or a haloethyl group), exocyclic ethano bases and cross-links [1,2]. The exact role of these lesions in the cellular response to these antitumor agents is still poorly understood. However, the therapeutic effect of CNUs appears to correlate with the cytotoxicity which is mainly attributed to the dC-dG cross-link 1-(3-cytosinyl)-2-(1-guanyl)ethane [6], whereas the other modifications such as ethano bases could lead to mutagenic and carcinogenic effects. The studies on the dC-dG cross-link have shown that its cellular formation and repair by O^6-alkylguanine-DNA alkyltransferase (AGT) is related to tumor cell resistance to the nitrosoureas used [7].

Several stable ethano adducts have been identified upon reaction with DNA by CNUs, which include 3,N^4-ethanocytosine (EC), 1,N^6-ethanoadenine (EA), and N^2,3-ethanoguanine (EG) [1,2]. Chemically, these adducts closely resemble their corresponding etheno adducts which are formed by unrelated environmental compounds such as vinyl chloride and ethyl

carbamate, or through the process of lipid peroxidation [8]. The difference between an ethano and etheno adduct is in the saturation of the C7=C8 double bond in the 5-membered exocyclic ring (see Figure 1).

Thus far, very few studies have been undertaken to determine the mutagenic potential of these ethano adducts *in vitro* and *in vivo*, although the immediate consequences of these adducts on replication, if unrepaired, are anticipated to be polymerase blockage, base substituions or frameshift deletions since the exocyclic ring disrupts Watson-Crick hydrogen bonding. EC, when synthesized and site-specifically incorporated into an oligonucleotide [9], was found to be primarily a blocking lesion with miscoding potentials when replicated by *E. coli* DNA polymerase I [10]. Recently, an oligonucleotide with a site-specific EA was also synthesized using phosphoramidite chemistry [11]. Subsequent studies by Hang *et al.* using several mammalian DNA polymerases such as pol α, β, η, and ι, showed that although there is a measurable extent of error-free nucleotide incorporation, all these polymerases primarily misinsert opposite EA, suggesting that the adduct is mutagenic [12].

The first repair study on an ethano adduct was performed by Habraken *et al.* who reported the release of N^2,3-EG by the purified *E. coli* AlkA protein (3-methyladenine DNA glycosylase II) [13]. The same enzyme was also found to act on its structural analogue, N^2,3-

ethenoguanine (N^2,3-εG) [14]. Recently, we found that the human alkylpurine-DNA-*N*-glycosylase (APNG), a functional homologue of AlkA, excises the EA adduct at a rate that was considerably lower than that for εA excision [15]. Over the years, in addition to AGT, the potential role of DNA glycosylases in protecting cells from CNUs has been studied [16-20]. It seems that, at least in some of these studies, DNA glycosylases such as APNGs could contribute to such protection, but the molecular mechanisms have not yet been clear.

In contrast to the ethano adducts, there is considerably more information on the mutagenicity and repair of their etheno counterparts. The activity excising 3,N^4-ethenocytosine (εC), a highly mutagenic lesion [21], was first identified in HeLa cells [22]. This activity has now been identified as the thymine-DNA glycosylase (TDG) in humans [23-25] and its homologue in *E. coli*, mismatch-specific uracil-DNA glycosylase (Mug) [23-25]. Both enzymes were so named since they were originally found to remove uracil and/or thymine from DNA duplexes when paired with guanine [26-29]. The recombinant human TDG also excises T from O^6-methyl G•T and 2-amino-6-(methylamino)purine•T mispairs [30]. Recently, Mug protein was also found to efficiently remove 8-(hydroxymethyl)-3,N^4-ethenocytosine (8-HM-εC) [31], a potential product resulting from glycidaldehyde reaction. Most recently, human TDG was shown to excise an oxidized base, thymine glycol (Tg),

from a Tg•G mispair [32]. These data indicate that the Mug/TDG proteins have a broad substrate specificity.

1,N^6-ethenoadenine (εA), also a highly mutagenic lesion [21], is a substrate for *E. coli* AlkA protein as well as its homologues in yeast and mammalian cells [24,33]. These proteins represent a family of enzymes with probably the broadest substrate range among DNA glycosylases covering a structurally diverse group of DNA lesions [34,35]. Recently, 1,N^2-ethenoguanine (1,N^2-εG) was found to be efficiently excised by *E. coli* Mug and also a substrate for human APNG, which is structurally unrelated to Mug [36]. In another study, Mug could also remove εA but with extremely low efficiency [37]. These results demonstrated that there is certain degree of overlap in specificities of the AlkA/APNG and Mug/TDG families, but in general they recognize purine and pyrimidine lesions, respectively.

Recent rapid progress in crystallizing DNA glycosylases has greatly enhanced our knowledge of how these enzymes selectively recognize chemically modified bases. Both Mug [38] and AlkA [39,40] have been crystallized and several co-crystal structures also solved [41,42]. These studies showed that both enzymes bend DNA and flip out the damaged base into the active site where the glycosylic bond cleavage takes place. From the detailed

analysis of these protein and protein-DNA structures, attempts have been made to find general structural requirements for recognition of all the known substrates as well as specific interactions for an individual substrate.

In monofunctional glycosylase (*e.g*. Mug or AlkA)-initiated base excision repair (BER) in *E. coli*, the apurinic/apyrimidinic (AP) site produced by a glycosylase is further processed by either exonuclease III (Exo III) or endonuclease IV (Endo IV) [35,43]. The latter two enzymes have similar substrate specificities, but do not share sequence homology [35]. Exo III is the major AP endonuclease of *E. coli* and is responsible for about 90% of the AP activity in *E. coli* crude extracts [44-46]. The interaction between DNA glycosylases and AP endonucleases has been of considerable interest in recently years [43]. It has been found that, similar to several other glycosylases, the Mug protein acts toward its substrates in a single turnover mode as a result of its strong affinity for the product AP site [47]. Addition of Endo IV can enhance the Mug activity by promoting dissociation of the tightly bound Mug protein from the AP-containing DNA, leading to a significantly increased turnover of the glycosylase [31,47].

In this work, we investigated the recognition and excision of EC and EA in defined oligonucleotides using purified *E. coli* DNA glycosylases. The focus was on whether the two glycosylases that are known to recognize the etheno adducts, Mug and AlkA protein,

also act on the closely related ethano adducts. If so, what are their relative excision efficiencies and can these activities be stimulated by a 5' AP endonuclease. In order to gain structural insight into the observed enzymatic recognition and differences in repair efficiency, molecular dynamics (MD) simulations were performed of the oligonucleotides containing a modified base as well as Mug protein complexed to an EC- or εC-containing duplex.

2. Materials and Methods

2.1. Oligodeoxynucleotide substrates

The 3,N^4-ethano-dC and 3,N^4-etheno-dC phosphoramidites were both purchased from Chem-Master International Inc (East Setauket, NY) and the 15-mer oligonucleotides containing a site-directed EC or εC adduct (at the 8th position, see Figure 2A for sequence information) were synthesized and HPLC-purified by Operon, Inc. (Alameda, CA). Synthesis of the 1,N^6-ethano-dA phosphoramidite and its site-specific incorporation into defined oligonucleotides was previously described by Maruenda *et al.* [11]. 1,N^6-Etheno-dA phosphoramidite was purchased from Glen Research (Sterling, VA). Both derivatives were placed at the 6th position from the 5'-end of a 25-mer sequence using an Applied Biosystems Model 394 automated DNA synthesizer (see Figure 3A sequence). The unmodified 25-mer and 15-mer controls and the four complementary strands with each of the four normal bases

opposite the modified base were also synthesized and HPLC-purified by Operon Inc. All the oligomers, modified and unmodified, were also subjected to 20% denaturing PAGE purification.

2.2. Repair enzymes

E. coli Mug protein, MutY protein, formamidopyrimidine-DNA glycosylase (Fpg protein), Endo III, Endo VIII and Endo IV were from Trevigen (Gaithersburg, MD). *E. coli* AlkA protein was a gift from Dr. Tim O'Connor (Beckman Research Institute, Duarte, CA). *E. coli* uracil-DNA glycosylase (Ung) was from Amersham Pharmacia Biotech (Piscataway, NJ). Exo III was from New England Biolabs (Beverly, MA).

2.3. Enzymatic assays

An oligomer cleavage assay [31] was used to test glycosylase-mediated cleavage of radiolabeled oligomers containing a site-directed exocyclic base. Briefly, oligonucleotides, modified and control, were 5'-end labeled with [γ-^{32}P] ATP (specific activity 6,000 Ci/mmol; 1 Ci = 37 GBq, Amersham Pharmacia Biotech, Piscataway, NJ) and annealed to a complementary strand in a molar ratio of 1 to 1.5. For detection of Mug activity, the reaction mixtures contained ^{32}P-end labeled 15-mer duplex in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin and varying

amounts of Mug protein in a total volume of 10 μl. Incubations were carried out for various lengths of time at 37 °C and stopped by heating the samples at 95-100 °C for 3 min and then placing on ice. For AlkA reactions, the standard mixtures (10 μl) contained ^{32}P-labeled 25-mer oligomer duplex in 10 mM HEPES-KOH (pH 7.4), 100 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mg/ml acetylated bovine serum albumin and varying amounts of AlkA protein. For both Mug and AlkA reactions, a 5' AP endonuclease was then added to the reactions, which were incubated at 37 °C for a further 20 min. In this step, the AP site, which was formed as a result of DNA glycosylase action, was hydrolyzed on the 5' side by the added AP endonuclease. Note that for AP endonuclease enhancement assay, either Exo III or Endo IV was initially added to the reaction mixture, prior to the addition of a glycosylase. The reactions were finally stopped by adding an equal amount of a F/E solution (90% formamide plus 50 mM EDTA) to the reaction mixture which was heated at 95-100 °C for 3 min. Reaction products were resolved on a 12% denaturing PAGE with a 5'-end labeled appropriate oligomer marker(s). For band quantitation, the Bio-Rad FX Molecular PhosphorImager and Quantity One software (version 4.0.1) were used.

2.4. Molecular modeling

A set of force-field parameters for the etheno adducts of dC and dA and ethano adduct of dA were previously developed using *ab initio* quantum mechanical calculations which

was reported in our earlier publications [15,31,48]. In this work, the EC adduct was built by modifying the C7=C8 double bond at the exocyclic ring into a single bond. The coordinates for EC were geometry optimized and atom-centered charges were obtained from the *ab initio* Hartree-Fock calculations using 6-311G* polarization basis set in Spartan 5.0 suite (Wavefunction, Inc., Irvine, CA). The dC and dA adducts were incorporated into 15-mer DNA duplexes opposite G and T, respectively. The DNA sequence used was a truncated form of the same 25-mer duplex for the biochemical studies in this paper. A 2 ns molecular dynamics (MD) simulation with the explicit solvent and counterions was performed using the Amber 7.0 force field [49] (for details on equilibration and production procedures see Guliaev et al. [48]). The stability of the simulations was evaluated by calculating the root mean square deviation (RMSD) values, which showed a plateau for all structures after first 500 ps, relative to the coordinates of the initial (energy minimized) structures. The representative structures of the adduct-containing base pairs (EC•G, εC•G, EA•T and εA•T) were generated by averaging the last 1.5 ns of MD.

The εC-DNA/Mug and EC-DNA/Mug complexes were generated using the crystallographic coordinates for *E. coli* Mug co-crystallized with the oligonucleotide containing 1-(2'-deoxy-2'-fluoro-B-β-arbinofuranosyl)-uracil (βFU) (5'-CGCGAG-βFU-

TCGCG-3') [42]. The βFU moiety was replaced with the geometry optimized cytosine adducts (εC or EC). The resulting complexes were solvated and subjected to a series of the MD runs, which include several steps of equilibration holding solute fixed and a 2 ns of unrestrained production runs (for procedure details see Guliaev et al. [15]). For all MD simulations, the electrostatic interactions were calculated with the particle-mesh-Ewald method [50,51] using 1 Å charge grid spacing with B-spline interpolation and sum tolerance of 10^{-6} Å. A 12 Å cutoff was applied to the Lennard-Jones interactions and SHAKE algorithm was used to all X-H bonds [52] with the 2 fs time step. The final structures representing the conformational family of the DNA/Mug complexes were generated by averaging the MD trajectories based on RMSD profiles (from 0.6 ns to 2 ns). The atom coordinates were stored every 1ps.

The DNA structures were analyzed using CURVES 5.1 software [53] and visualized with INSIGHT II (Biosym/MSI, San Diego, CA). The MD trajectories for the DNA/enzyme complexes were processed using the analytical modules of AMBER 7.0 and Visual Molecular Dynamics (VMD) program [54]. All calculations were performed on a Silicon Graphics Origin 2000 server interfaced with a dual processor Octane workstation (Silicon Graphics Inc., Mountain View, CA). The 3D figures were generated using the VMD and Raster3D [55] software.

3. Results

3.1. Recognition and excision of EC by the E. coli Mug protein

We first tested the excision of EC by the Mug protein which was reported to recognize the analogous εC adduct. As shown in Figure 2A, Mug catalyzed a protein-dependent cleavage of the 15-mer containing an EC (lanes 4 to 9, left). However, the extent of excision of EC was much less than that of the corresponding εC adduct (lanes 11 to 16, right). Figure 2A also demonstrated that the cleavage product from the EC-containing 15-mer oligonucleotide after a 5' AP endonuclease (HAP1) treatment was a ^{32}P-labeled 7-mer, which is the expected product resulting from the 5' hydrolysis of an AP site at the 8th position by a 5' AP endonuclease. The time course of excision of EC from the duplex by Mug is shown in Figure 2B, in which the comparison was also made between the rates of EC and εC excision under the same assay conditions. Excision of EC was approximately 20 times slower than that of εC.

We further tested the effect of the opposite base on the specific glycosylase activity toward EC, as it has previously been shown that a glycosylase activity can be significantly affected by the nature of the base opposite a damaged base (*e.g.* [25,31,56]). As shown in Figure 2C, Mug was capable of cleaving EC when the adduct being paired with A, G, C, or T, but differed in its specificity toward these modified base pairs. The Mug protein

preferentially excised EC from EC•G and had lower activity toward EC when the opposite base was a pyrimidine base, T or C.

In order to examine whether there is any other known glycosylase(s) which may also possess activity toward the EC adduct, we tested various available *E. coli* DNA glycosylases. This includes both monofunctional glycosylases (AlkA, Ung, MutY) and glycosylases/AP lyases (Fpg, EndoIII and EndoVIII). Under the experimental conditions used, Mug appears to be the only glycosylase to specifically excise EC among the enzymes tested (data not shown).

3.2. Recognition and excision of EA by E. coli AlkA protein

The AlkA protein, which has been known to excise εA, was also shown to excise EA. Figure 3A shows the cleavage of the 25-mer EA-oligomer as a function of protein concentrations (lanes 3 to 6), which leads to a 5-mer product, the same size as that from the εA cleavage. Similar to EC vs. εC activity of Mug, the extent of EA excision by AlkA was much less than that of εA (lane 4 and lane 8 contained same amount of AlkA, 15 ng). Kinetics of the AlkA activity (Figure 3B) shows that excision of EA was about 20 times slower than that of εA. In separate experiments, addition of a second aliquot of fresh AlkA protein during the course of reaction could increase the rate and extent of excision of EA

(data not shown). Similar to Mug, the activity of AlkA was also influenced by the nature of base pairing at the adduct site (Figure 3C). When other available DNA glycosylases (see 3.1.) were examined for an ethano activity, the AlkA protein was the only glycosylase in *E. coli* that acted on EA under the assay conditions used (data not shown). There was no detectable overlapping of activities toward EC or EA between the Mug and AlkA protein.

3.3. Stimulation of Mug activity by 5' AP endonucleases

E. coli Endo IV has been shown to be able to stimulate Mug activity toward such substrates as U•G, εC•G and 8-HM-εC•G [47,31]. In this work, Endo IV showed a similar effect on the excision of EC by Mug. As shown in Figure 4A, the Mug activity toward EC was increased by ~2.3-fold after co-incubation with Endo IV for 30 min. In addition, we demonstrated that Exo III could also stimulate the Mug activity against EC (Figure 4A). Moreover, the stimulation of the Mug activity by Exo III was much higher (~6.2-fold after 30 min) than that by Endo IV when equimolar amount of proteins were used (6.4 nM for both Endo IV and Exo III). In these experiments, the molar ratio of Mug to oligomer substrate was chosen to be under protein-limiting condition (1.2 nM Mug and 3.2 nM oligomer duplex) in order to observe enzyme turnover. Figure 4B showed that the stimulation of Mug EC activity by Exo III was strongly dependent on the Exo III concentrations used. When 1-, 4- or 16-fold Exo III was added with a constant Mug concentration (1.2 nM), the rate of EC excision by Mug increased accordingly. Note that

neither Endo IV nor Exo III had any detectable effect on the EC adduct (data not shown). In the case of EA, similar experiments are not included since a much higher concentration of the AlkA protein had to be used in order to obtain measurable excision activity through denaturing PAGE and subsequent phosphorImaging, which would lead to single turnover reactions.

3.4. Structural studies using molecular dynamics simulations

3.4.1.Conformation of the DNA duplexes at the lesion site

Figure 5 shows conformations of the geometry optimized structures of εC and EC (Figure 5A) and the structures of the adduct-containing base pairs (Figure 5B). For both ethano dC and dA adducts, the change from the double bond at the C7=C8 position to a single bond changed the conformation of the extra exocyclic ring. Saturation of the extra ring resulted in displacement of one of the ring carbons outside the plane (C7 for both EC and EA), which produces the envelop form of the 7,8-dihydro imidazole ring (Figure 5A). The displacement of the C7 carbon in EC was slightly larger than in EA [15] and can be explained by the lower degree of conjugation in the EC adduct.

The structural differences between the etheno and ethano adducts did not change conformation of the lesion in our modeling study (Figure 5B) and were previously reported in our molecular modeling work for the εA and EA adducts [15]. No hydrogen bonds were

observed between bases in the εA•T and EA•T pairs (Figure 5B). Both adducts adopted an *anti* orientation and were displaced toward the major groove, forming a non-planar base pair with the opposite T. The sugar pucker of the EA and εA adducts falls into the C2'endo range. The etheno and ethano derivatives of dC were also paired in a similar way with the opposite dG. However, a single bifurcated hydrogen bond was formed between the adducted base-pairs (Figure 5B, yellow dotted lines). The distinct characteristic of the εC•G pair was the high shear value, which was previously reported by NMR, molecular modeling and recently by crystallographic studies [15,31,57-59]. Such a sheared base-pair alignment was also observed for the EC•G pair in our calculations. The sugar conformation of the ethano and etheno adducts of dC falls into a C3'-endo range. The overall DNA conformation for the etheno and ethano containing duplexes remained in the B-DNA family during the entire course of simulation.

3.4.2. DNA/Mug complexes

The availability of the crystal structure of Mug complexed to DNA-containing a non-hydrolysable substrate analog βFU [42], enabled us to use it in our simulations. It has already been proposed that εC can be easily accommodated in the Mug active site [42]. In this work, the stability of εC at the active site was confirmed by performing the MD simulation of the Mug-εC substrate complex. The structure generated by the replacement

of the βFU moiety with εC was stable during the entire course of simulation. The εC base remained stacked against the side chain of the phenylalanine residue (Phe 30), thus occupying hyrdophobic space at the bottom of the binding pocket (Figure 6A). The position of εC at the enzyme active site was stabilized by 2 hydrogen bonds. The peptide NH of Ile 17 forms a hydrogen bond to the O2 carbonyl of εC and NH of Phe 30 forms a hydrogen bond with the N4 nitrogen of the εC exocyclic ring. As proposed earlier, the etheno moiety of εC makes a favorable van der Waals interaction with Cα of Gly 16 [42]. The replacement of εC with EC in the Mug active site did not affect the position of the adduct relative to the amino acids in the binding pocket. The averaged minimized structure produced by MD simulation showed that the EC moiety can also be favorably accommodated at the enzyme active site. The position of this adduct was stabilized by the same hydrogen bonds as reported for εC [42]. However, the presence of the two extra hydrogens and non-planar conformation of the exocyclic ring influenced the conformation of Phe 30. The side chain of Phe 30 in the EC-DNA/Mug complex rotated by 10º along the Cα-Cβ-C1-C2 torsion angle from its position in the εC-DNA/Mug, thus reducing the stacking interaction between Phe 30 and the EC moiety (shown by yellow arrow in Figure 6B).

4. Discussion

The data shown in this work is, to our knowledge, the first demonstration that the two ethano adducts, EC and EA, are substrates for *E. coli* Mug protein and AlkA protein, respectively. These exocyclic adducts are produced by the reaction of DNA bases with CNUs such as BCNU and CCNU [1,2]. Although their biological role in causing cytotoxicity and/or carcinogenesis is still not clear, there is some *in vitro* biochemical evidence [10,12] suggesting that these lesions may block DNA synthesis and/or miscode in DNA replication.

The Mug/TDG proteins have relatively broad substrate specificities, mainly in repair of modified/altered pyrimidine bases. The current finding that CNU-derived EC adduct is also a substrate for the Mug protein extends its substrate range. To date there are several exocyclic adducts that have been found to be excised by the Mug protein but with markedly varied efficiencies. These include εC [23,25], 8-HM-εC [31], EC [this work], 1,N^2-εG [36] and εA [37], with εC being the best substrate. This suggests that Mug may recognize certain common feature(s) of these structurally related exocyclic adducts and could be one of the primary glycosylases responsible for repair of this important group of DNA lesions.

The AlkA/APNG proteins also excise a large structurally diverse group of substrates and their role in repair of etheno adducts drew much attention in 1990s since these lesions are so different from their originally identified substrates such as N-3- and N-7-alkylated bases [60]. The AlkA protein is known to recognize a number of base modifications produced by CNUs thus far, including several 7-alkylguanines, N^2,3-EG and the cross-link, 1,2-bis(7-guanyl)ethane [13,61,62], and now EA from this work. However, whether any of these activities has physiological importance is not yet known.

It is also noticed that the Mug protein usually has higher activities towards its exocyclic base substrates that does the AlkA protein. Comparison of the kinetic constants published previously shows that Mug removes εC from εC•G pair in DNA much more efficiently (k_{cat}/K_m=380 min^{-1} μM^{-1}) [24] than the excision of εA from εA•T by AlkA (k_{cat}/K_m=0.001 min^{-1} μM^{-1}) [25]. In this study, the data in Figures 2 and 3 also show clearly that the initial rates of excision of EC/εC by Mug are much faster than excision of EA/εA by AlkA, as judged by nM of modified bases cleaved per nM protein/min.

To date, there is no clear evidence for the *in vivo* repair of CNU-induced modifications by a specific glycosylase that could increase cellular resistance to these agents. It is now well established that AGT can prevent formation of the dC-dG cross-link in DNA, thus reducing

the toxicity of CNUs [63]. However, data from various studies on the role of APNGs in such cellular protection are controversial (e.g. [16-20]). Mug/TDG, which removes or is expected to remove EC, respectively, might also be involved in such a function. Moreover, two other glycosylases, Fpg and α-hOgg1, have been shown to substantially protect mammalian cells against BCNU damage [64], possibly due to their repair of ring-opened secondary lesions derived from BCNU damage.

It has been known that for a number of glycosylases, the presence of a 5' AP endonuclease can stimulate their activity [43], although such interaction and its mechanism is still not fully understood. Among these enzymes is the Mug protein, which is basically a single turnover enzyme [47]. In this study, we found that *E. coli* Endo IV could stimulate the EC activity of Mug by more than two-fold. We have also shown that the stimulation of Mug activity occurred with another 5' AP endonuclease in *E. coli*, Exo III. Moreover, Exo III had a 2.7-fold higher stimulatory effect on the EC activity than did Endo IV (Figure 4A). Considering the fact that Exo III is the major 5' AP endonuclease *in E. coli*, the overall stimulatory effect *in vivo* by Exo III would be expected to be greater than the Endo IV-mediated turnover of Mug protein. If such an effect does occur *in vivo*, the lower EC activity of Mug as compared to the εC activity, would be significantly enhanced by these AP endonucleases. Verification of these biochemical results must await for further studies using mutants lacking Exo III or Endo IV.

A long-standing question regarding these glycosylases is what determines their substrate specificity as well as excision efficiency [34]. The substantial differences in rate of excision of ethano vs. etheno dC and dA adducts prompted us to look further into the structural basis for such biochemical findings using molecular dynamics (MD) simulations of a Mug/EC-DNA complex, based on the crystallographic coordinates for Mug co-crystallized with βFU-DNA [42].

First, MD simulations did not reveal any specific conformational features of the oligonucleotides containing a single adduct which could indicate the effect of the ethano vs. etheno adducts on the lesion conformation in the DNA duplex. Both EC and εC showed similar structural motifs, when opposite G (Figure 5B). Similar to the dC derivatives, molecular modeling did not reveal any distinct base-pairing features between EA•T and εA•T base pairs. It appears that the initial enzyme recognition could be similar for the etheno and ethano adducts, assuming that the enzyme can detect a specific structural motif in the DNA duplex. For example, it was proposed by Cullinan *et al.* that the sheared base-pairs could be a structural feature for recognition by Mug/TDG [58,65].

MD simulation of the EC-DNA/Mug complex showed that EC can be accommodated at the enzyme active site, but with a minor conformational change of Phe 30, as compared to the εC-DNA/Mug complex (Figure 6B). The observation of rotation of the side chain of Phe 30 indicates that the EC moiety imposes some steric constraint on the Mug active site. The energy penalty produced by this less favorable interaction could reduce the ability of EC to be accommodated at the Mug binding site, as compared with εC, and could contribute to the observed difference in excision efficiency between EC and εC. The saturation of the imidazole ring in the EA and EC derivatives also partially reduced the stacking ability of these adducts, as compared to the etheno adducts which favor the π–π interactions with the neighboring bases or residues at the enzyme active site. Moreover, the extra hydrogens at the C7 and C8 position of the ethano ring, as compared to the etheno ring, contribute additional van der Waals surface area. This could cause a greater difficulty in initiating induced fit binding of the EC residue at the enzyme active site. Similarly, the change in conformation of the EA extra ring, as compared to that of εA, may also disfavor the binding of EA to the AlkA active site pocket, particularly its stacking with Trp 272, which should stabilize the extra-helical position of the adduct [39-41,66]. A weaker stacking ability of EA in the active site could contribute to the lower efficiency of the EA activity. However, the open structure of the AlkA binding pocket makes it more difficult to speculate regarding its specificity, which might result from factors other than binding

selectivity. More detailed conformational studies of the AlkA/substrate complexes are needed for a better understanding of such mechanisms.

Acknowledgements

We would like to thank Dr. Tim O'Connor for the AlkA protein and Dr. Tracey A. Barrett for providing crystallographic coordinates for the Mug complexed to βFU-containing DNA. This work was supported by NIH grants CA72079 (to B.H.) and CA47723 (to B.S) and was administrated by the Lawrence Berkeley National Laboratory under Department of Energy contract DE-AC03-76SF00098.

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Figure Legends

Figure 1. Chemical structures of ethano and etheno dC and dA adducts.

Figure 2. A. Protein-dependent cleavage by *E. coli* Mug of oligonucleotide containing an ethano dC adduct. Various amounts of Mug protein (0-2.4 ng) (lanes 3-9) were reacted with ^{32}P-end labeled 15-mer oligomer containing a single EC (3.2 nM) for 30 min at 37^{o}C. The sequence of the 15-mer is shown on the left side of the autoradiogram. X represents the location of the adduct. The reaction products (7-mer) were separated from the uncleaved 15-mer using 12% denaturing PAGE and visualized using a phosphorimager (see Materials and Methods). N: control oligomer duplex; B. Time course of removal of EC/εC from the 15-mer duplex by Mug. The base opposite the adduct is G. The ^{32}P-end labeled oligomer duplexes (3.2 nM) were reacted with 1.2 nM Mug (for EC excision) or 0.04 nM (for εC excision) for varying times at 37^{o}C. The rates of excision were calculated as nM oligomer substrate cleaved per ng protein. Note that in the plot the rates are presented using broken scales. C. Effect of opposite base on the excision of EC by Mug protein. 5' ^{32}P-end labeled oligonucleotide containing an EC was annealed to complementary strands with each of the four bases opposite the adduct. The standard reaction conditions were used for this experiment. The incubation time was 15 min at 37^{o}C. The excision rate of the oligomer

duplex containing EC•G pair was arbitrarily assigned as 100% and the activities of the other three mispairs were then plotted as percent of its activity. Each bar represents three or four sets of data.

Figure 3. A. Protein-dependent cleavage by *E. coli* AlkA of an EA-containing oligonucleotide. Increasing concentrations of AlkA (0-120 ng) (lanes 3-6) were incubated with ^{32}P-end labeled 25-mer oligomer containing EA annealed to a complementary strand (2 nM) for 30 min at 37^{o}C. Note that for εA•T-containing 25-mer duplex only 15 ng AlkA protein was used in lane 8. Lanes 1, 3 and 7 contained buffer only. B. Time course of excision of EA by AlkA. The ^{32}P-end labeled oligomer duplexes (2 nM) were reacted with 100 nM AlkA (for EA excision) or 12.5 nM (for εA excision) for varying times at 37^{o}C. C. Effect of opposite base on the excision of EA by AlkA. Details see Figure 2.

Figure 4. Stimulation of Mug activity by *E. coli* Endo IV or Exo III. A: 3.2 nM ^{32}P-end labeled 15-mer duplex was incubated with 1.2 nM Mug protein for varying time 37^{o}C (bottom curve). In separate reactions, 6.4 nM Endo IV or Exo III was co-incubated with 1.2 nM Mug protein (middle and top lines). B: Concentration-dependent stimulation of Mug activity toward EC by Exo III. 3.2 nM of ^{32}P-end labeled 15-mer duplex was incubated with 1.2 nM Mug protein with or without Exo III (bottom line) at 37^{o}C. The concentrations of

Exo III used are shown above each plot. Exo III alone did not show any detectable effect on the oligomer containing an EC (data not shown). All the reactions were stopped at various time points by heating at 95-100 ^{o}C for 3 min. 0.1 U Endo IV was then added to all samples for 20 min at 37^{o}C, and reactions were terminated by adding a F/E solution.

Figure 5. A) Geometry optimized structure of εC and EC. The saturation results in the envelope form and an additional van der Waals surface (shown in light blue) of the EC exocyclic ring, as compared to εC. B) Structures for the εC•G, EC•G, εA•T and EA•T lesions produced by 2 ns MD simulation. The yellow dotted line indicates the hydrogen bond pattern for the EC•G and εC•G base pairs. The etheno and ethano derivatives of the dA (EA and εA) do not form hydrogen bonds with the opposite T.

Figure 6. A) Plane to plane stacking between Phe 30 (in magenta) and εC (colored by atoms) in the active site of the Mug complexed with εC-DNA produced by 2ns MD simulation. Surface representation shows selective amino acid residues from the enzyme active site. B) Stacking interaction between Phe 30 (in magenta) and EC (colored by atom) in the active site of the Mug complexed with EC-DNA produced by 2ns MD simulation. Note the rotation of the Phe 30 side chain (indicated by yellow arrow) induced by the non-planar conformation of the EC base as compared to the εC base.