**Differential repair of etheno-DNA adducts by bacterial and human AlkB proteins**

Daria Zdżalik1,¶, Anna Domańska1,2,¶, Paulina Prorok2,¶, Konrad Kosicki1, Erwin van den Born3, Päl Ø. Falnes3, Carmelo J. Rizzo4, F. Peter Guengerich5, Barbara Tudek1,2 \*

1Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, Warsaw, Poland

2Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland,

3Department of Biosciences, University of Oslo, Oslo, Norway

4Department of Chemistry, Center in Molecular Toxicology, and Vanderbilt Ingram Cancer Center, Vanderbilt University, Nashville, Tennessee, USA

5Department of Biochemistry, Center in Molecular Toxicology, and Vanderbilt Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

**DNAR 14-00175**

\*Corresponding author:

Barbara Tudek,

Institute of Biochemistry and Biophysics,

Polish Academy of Sciences,

Pawińskiego 5a,

02-106 Warsaw, Poland

tel: (+4822) 592-3334; fax: (+4822) 592-2190

e-mail: tudek@ibb.waw.pl

¶These authors contributed equally to this work

**Key words:** AlkB, etheno adducts, 1,*N*6-ethenoadenine, 3,*N*4-ethenocytosine, 1,*N*2-ethenoguanine, DNA repair

**Abbreviations:** ε-etheno; εA - 1,*N*6-ethenoadenine; εC - 3,*N*4-ethenocytosine; 1,*N*2-εG - 1,*N*2-ethenoguanine; 1-meA - 1-methyladenine; 3-meC, 3-methylcytosine

**Abstract**

AlkB proteins are evolutionary conserved Fe(II)/2-oxoglutarate-dependent dioxygenases, which remove alkyl and highly promutagenic etheno(ε)-DNA adducts, but their substrate specificity has not been fully determined. We developed a novel assay for the repair of ε-adducts by AlkB enzymes using oligodeoxynucleotides with a single lesion and specific DNA glycosylases and AP-endonuclease for identification of the repair products. We compared the repair of three ε-adducts, 1,*N*6-ethenoadenine (εA), 3,*N*4-ethenocytosine (εC) and 1,*N*2-ethenoguanine (1,*N*2-εG) by nine bacterial and two human AlkBs, representing four different structural groups defined on the basis of conserved amino acids in the nucleotide recognition lid, responsible for the enzyme binding to the substrate.

Two bacterial AlkB proteins, MT-2B (from *Mycobacterium tuberculosis*) and SC-2B (*Streptomyces coelicolor*) did not repair these lesions in either double-stranded (ds) or single-stranded (ss) DNA. Three proteins, RE-2A (*Rhizobium etli*), SA-2B (*Streptomyces avermitilis*), and XC-2B (*Xanthomonas campestris*) efficiently removed all three lesions from the DNA substrates. Interestingly, XC-2B and RE-2A are the first AlkB proteins shown to be specialized for ε-adducts, since they do not repair methylated bases. Three other proteins, EcAlkB (*Escherichia coli*), SA-1A, and XC-1B removed εA and εC from ds and ssDNA but were inactive towards 1,*N*2-εG. SC-1A repaired only εA with the preference for dsDNA. The human enzyme ALKBH2 repaired all three ε-adducts in dsDNA, while only εA and εC in ssDNA and repair was less efficient in ssDNA. ALKBH3 repaired only εC in ssDNA. Altogether, we have shown for the first time that some AlkB proteins, namely ALKBH2, RE-2A, SA-2B and XC-2B can repair 1,*N*2-εG and that ALKBH3 removes only εC from ssDNA. Our results also suggest that the nucleotide recognition lid is not the sole determinant of the substrate specificity of AlkB proteins.

**Introduction**

AlkB proteins belong to the superfamily of Fe(II)/2-oxoglutarate-dependent dioxygenases, and the first AlkB member was discovered in *Escherichia coli* (EcAlkB) [[1-5](#_ENREF_1)]. EcAlkB is a nucleic acid repair enzyme that hydroxylates alkyl groups bound to the nitrogen atoms at position 1 of purines and 3 of pyrimidines. The resulting unstable hydroxylalkyl group is then spontaneously released as an aldehyde, which leads to the regeneration of the unmodified base (Fig. 1) [[2](#_ENREF_2),[3](#_ENREF_3)]. The main substrates repaired by EcAlkB are 1-methyladenine (m1A) and 3-methylcytosine (m3C) in single-stranded (ssDNA) and double-stranded DNA (dsDNA) [[2](#_ENREF_2),[3](#_ENREF_3),[6-8](#_ENREF_6)]. Intriguingly, EcAlkB is also able to remove methyl lesions from RNA, suggesting a role for AlkB proteins in RNA repair [[9-12](#_ENREF_9)]. In addition to the methyl lesions, EcAlkB repairs larger groups, e.g. ethyl, hydroxyethyl, propyl, hydroxypropyl, and some exocyclic DNA adducts (ε-adducts) [[13-15](#_ENREF_13)].

Homologues of EcAlkB are present in numerous bacteria, eukaryotes, and certain plant RNA viruses. Nine AlkB homologues have been identified in humans: ALKBH1-ALKBH8 (AlkB homologue) and fat mass and obesity-associated protein FTO [[1](#_ENREF_1),[16-18](#_ENREF_16)]. ALKBH2 and ALKBH3 are the sole human homologues that complement AlkB deficiency in *E. coli* regarding reactivation of ssDNA phages in response to methylating agents (e.g., methyl methane sulfonate (MMS) [[9](#_ENREF_9),[13](#_ENREF_13)]. ALKBH3 demethylates m1A and m3C in ssDNA and RNA but is inactive towards these lesions in dsDNA [[8-10](#_ENREF_8),[19-21](#_ENREF_19)]. ALKBH2 repairs alkylated bases only in DNA, preferentially in dsDNA [[9](#_ENREF_9),[19](#_ENREF_19),[20](#_ENREF_20)]. Among vertebrate AlkB proteins, only ALKBH2 is considered a true repair enzyme, since ALKBH2-null mice accumulate m1A in their genomes, but ALKBH3-null mice do not [[22](#_ENREF_22)]. Recently, the function of ALKBH proteins has been expanded beyond nucleic acid repair. FTO and ALKBH5 were found to demethylate *N*6-methyladenine in mRNA [[23-25](#_ENREF_23)], whereas ALKBH8 catalyzes the final two step modifications of wobble uridine in tRNA anticodons [[26-29](#_ENREF_26)]. Finally, ALKBH1 and ALKBH4 have been proposed to demethylate lysine in histone H2A and actin, respectively [[30](#_ENREF_30),[31](#_ENREF_31)].

AlkB proteins also repair the highly mutagenic exocyclic ε-adducts [[15](#_ENREF_15),[32-36](#_ENREF_32)], e.g. 1,*N*6-ethenoadenine (εA) and 3,*N*4-ethenocytosine (εC). During the AlkB-mediated repair by oxidative dealkylation**,** the etheno bridge is converted to an epoxide and the product undergoes non-enzymatic hydration to a glycol which is then released as glyoxal (Fig. 1B) [[32](#_ENREF_32),[34](#_ENREF_34)]. These lesions, as well as *N*2,3-ethenoguanine (*N*2,3-εG), and 1,*N*2-ethenoguanine (1,*N*2-εG) are formed in the reaction of a variety of reactive bifunctional electrophilic agents with a nitrogen atom of the DNA base, followed by dehydration and ring closure [[37](#_ENREF_37),[38](#_ENREF_38)]. These ubiquitous lesions are generated in cellular DNA either by endogenous metabolic processes, e.g., oxidative stress-induced lipid peroxidation, or by the reaction of the DNA with diverse chemical carcinogens, e.g., bioactivated derivatives of vinyl chloride or urethane [[39-42](#_ENREF_39)]. Since the additional exocyclic ring disrupts the Watson-Crick hydrogen bonding, the ε-lesions generate a broad spectrum of base substitutions (transitions and transversions) or frameshift mutations [[41](#_ENREF_41),[43](#_ENREF_43)]. It is estimated that in mammalian DNA from 14% to 60% of ε-adducts give rise to mutations, while only 3% of 8-oxoguanine residues are pro-mutagenic in cells with functional DNA repair [[44-46](#_ENREF_44)]. These promutagenic properties of ε-lesions strongly underlies their contribution to carcinogenesis in mammals [[47-49](#_ENREF_47)].

 It is commonly believed that the main mechanism for removal of the ε-adducts from DNA is base excision repair (BER) initiated by specific DNA glycosylases. In mammaliancells εA and 1,*N*2-εG are removed by *N*-methylpurine-DNA glycosylase (ANPG) [[50](#_ENREF_50),[51](#_ENREF_51)], and εC is repaired by mismatch-specific thymine-DNA glycosylase (TDG) [[52](#_ENREF_52),[53](#_ENREF_53)]. In addition to TDG, a protein that binds methylated CpG islands (MBD4/MED1) and uracil-DNA glycosylase specific for ssDNA (SMUG1) are also capable of excising εC from DNA [[54](#_ENREF_54),[55](#_ENREF_55)]. In *E. coli*, *N*2,3-εG is excised by alkyl-*N*-purine-DNA glycosylase AlkA, which also excises εA, but with a very poor efficiency [[50](#_ENREF_50),[56](#_ENREF_56)]. Mismatch-specific uracil-DNA glycosylase, Mug excises from the DNA εC and 1,*N*2-εG [[51](#_ENREF_51),[53](#_ENREF_53),[57](#_ENREF_57)]. Recently it has been shown that εA and εC can also be repaired via an alternative nucleotide incision repair (NIR) pathway, in which human AP endonuclease APE1 makes an incision 5´ to the ε-base in a DNA glycosylase-independent manner [[58](#_ENREF_58)]. Additionally, εA and εC are also removed from the DNA *in vitro* by EcAlkB and ALKBH2 [[15](#_ENREF_15),[32-36](#_ENREF_32)].

AlkB proteins are widespreadamongbacteria, and some bacterial species possess two or three AlkBs, but their substrate specificities are poorly characterized. Based on sequence alignment and phylogeny analysis bacterial AlkB proteins have been divided into four groups 1A, 1B, 2A, and 2B each characterized by the presence of specific conserved amino acid residues in the nucleotide recognition lid, which has been postulated to specify substrate recognition, at least partially [[59](#_ENREF_59)].

The main goal of this work was to gain insight into repair of ε-adducts by bacterial and human AlkB proteins. To reach this goal we developed a new, sensitive, and time and cost effective BER-mediated assay, and analyzed the ability of nine bacterial and two human AlkB proteins representing all four groups mentioned above (1A, 1B, 2A, and 2B) to remove from the DNA εA, εC and 1,*N2*-εG *in vitro* in ss and dsDNA. To the best of our knowledge repair of 1,*N2*-εG has not been presented before, and for several of the studied enzymes repair of εC was not studied as well. In this newly developed assay the DNA with a single lesion is subjected to AlkB protein, and subsequently digested with an appropriate DNA glycosylase excising the damaged base and AP-endonuclease, which cleaves the DNA at the site of the lesion if the damage was not repaired by AlkB protein.

The majority of the bacterial AlkBs tested were able to repair ε-lesions. Three of the proteins analyzed, SA-2B (*Streptomyces avermitilis*), XC-2B (*Xanthomonas campestris*), and RE-2A(*Rhizobium etli*) appear to be specialized towards ε-adducts, since exhibited significant activity against all the ε-adducts tested without being active towards methylated bases. Altogether, our data suggest that AlkB-mediated removal of ε-lesions could be an important mechanism responsible for the repair of these lesions in some bacteria. Additionally, we analyzed the *in vitro* repair of εA, εC, and 1,*N*2-εG in ss and dsDNA by human ALKBH2 and ALKBH3. We confirmed that ALKBH2 is active towards εA and εC in dsDNA and showed a lower activity against these lesions in ss DNA. Finally, we have shown for the first time that ALKBH2 repairs 1,*N*2-εG in dsDNA and ALKBH3 εC in ssDNA.

**Materials and Methods**

**Plasmids and protein expression**

pET-28a(+)-derived plasmids encoding N-terminally 6×His-tagged bacterial and human AlkB proteins were obtained as described previously [[9](#_ENREF_9),[59](#_ENREF_59)], and were used to transform *E. coli* strain BL21-CodonPlus(DE3)-RIPL (Stratagene, La Jolla, CA, USA). Bacteria were cultured and when the optical density reached 1 measured at 600 nm (OD600), expression of recombinant proteins was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and incubation was continued at 16 °C for 16 h. Then, the bacteria were harvested by centrifugation at 5000 × g for 10 min at 4 °C and stored at -80 ºC until use.

**Purification of AlkB proteins**

Bacterial pellets were resuspended in a buffer containing 50 mM sodium phosphate (pH 7.0), 150 mM NaCl, 5 mM β-mercaptoethanol, 0.5% Nonidet NP-40 detergent(v/v), 10% glycerol (v/v), 5 mM imidazole, and EDTA-free Complete-Protease Inhibitor (Roche). The cells were lysed by the addition of lysozyme to a final concentration of 1 mg/mL, incubation on ice for 30 min, and subsequent sonication with three 11 W pulses of 30 s(with 30 s intervals). Cell debris was removed by centrifugation at 12,000 × g for 10 min at 4 °C. The resulting supernatant was directly mixed with HisPur™ Cobalt Resin (Thermo Scientific) and recombinant proteins were obtained by a single affinity purification step according to the manufacturer’s instructions. Protein purity and yield were assessed by 15% (w/v) SDS–PAGE followed by Coomassie Brilliant Blue (R-250) staining of the gel. Fractions containing AlkB proteins were pooled and dialysed in a buffer containing 50 mM Tris-HCl pH 7.0, 100 mM NaCl. Then 1 volume of storage buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50% glycerol (v/v) was added, and the concentration of purified proteins was estimated using a Bradford reagent (Sigma) assay.

**Preparation of 32P-labeled oligodeoxynucleotide substrates**

The oligodeoxynucleotide substrates containing a single εA (40-mer, 5'-d(GCTACCTACCTAGCGACCTεACGACTGTCCCACTGCTCGAA)-3', **Eurogentec S.A.,** Belgium), εC (25-mer, 5'-d(ATTCTCGTTAGGATεCGCGTCAAGCC)-3', Chemgenes Corporation, USA), or 1,*N*2-εG (18-mer, 5´-(TCACεGAATCCTTACGAGCCCCC)-3´, [[60](#_ENREF_60),[61](#_ENREF_61)] were 32P-labeled at the 5´ end by T4 polynucleotide kinase (Takara) with an excess of [γ-32P]ATP (3000Ci/mmol, Hartmann Analytic). Radiolabeled oligodeoxynucleotides were purified from unincorporated radioactivity using Micro Bio-Spin P-30 columns (Bio-Rad) according to the manufacturer’s instructions. The 32P-labeled oligodeoxynucleotides, when necessary, were annealed to their complementary oligonucleotides (present in a two-fold molar excess) by incubation for 2 min at 90 ºC and subsequent cooling to room temperature. Formation of duplexes was verified by nondenaturing PAGE.

***In vitro* repair assay of DNA etheno adducts**

Repair reactions of the ε-adducts in DNA with bacterial AlkB proteins were performed by incubating 100 pmol of the appropriate bacterial AlkB protein with 1 pmol of ss or ds 32P-labeled DNA oligodeoxynucleotide at 37 °C for 30 min in a 50 μL reaction mixture containing 50 mM Tris-HCl (pH 7.0), 2 mM ascorbic acid, 1 mM 2-oxoglutarate, and 80 μM (NH4)2Fe(SO4)2. The repair activity of the ε-adducts by ALKBH2 and ALKBH3 was estimated by incubating 1 pmol of the appropriate 32P-labeled oligodeoxynucleotide substrate with different amounts of the tested protein (10, 25 and 50 pmol) using the reaction conditions as above. Additionally, to determine specific repair activity of ALKBH2 and ALKBH3 towards etheno lesions, reactions with increasing amount of recombinant proteins and various timesofincubation were performed as indicated in the Results section. All AlkB-mediated reactions were stopped by incubation at 65°C for 20 min. The AlkB protein was removed by addition of 1 μL of proteinase K (20 mg/mL, Sigma-Aldrich) to the reaction and further incubation for 15 min at 42 °C followed by heat-inactivation of proteinase K at 90 °C for 10 min. When ssDNA substrates were used in the reaction, prior to the BER enzyme digestion the complementary DNA oligodeoxynucleotide was added and annealed as described.

In order to distinguish the repaired oligodeoxynucleotide from the unrepaired one, the 32P-labeled DNA was incubated with the excess of the appropriate DNA glycosylase and the resulting abasic site was cleaved with the excess of human AP-endonuclease1(APE1). The amounts of DNA glycosylases and AP-endonuclease necessary to completely cleave oligodeoxynucleotide at the site of the lesion were verified by applying a series of enzymes dilutions in reaction buffers supplemented with BSA (0.1 mg/mL). Briefly, 5 μL of AlkB reaction mixture was incubated for 30 min at 37 °C with human alkyl*-*N-purine-DNA glycosylase (ANPG) for εA or with *E. coli* uracil-DNA glycosylase Mug for εC and 1,*N*2-εG, followed by cleavage with the excess of APE1 for 30 min at 37 °C. Buffers for DNA glycosylases contained 70 mM Tris-HCl pH 7.8, 1 mM EDTA pH 8, 100 mM KCl, 5 mM β-mercaptoethanol, 100 μg/ml BSA, while for HAP-1 endonuclease, 20 mM Tris-HCl pH 7.5, 0.1 mM EDTA pH 8.0, 50 mM KCl, 5 mM MgCl2, 1 mM β-mercaptoethanol, 100 μg/ml BSA. Two controls were performed for each reaction: a negative control containing untreated oligodeoxynucleotide, and a positive control of the activity of the BER enzymes with the oligodeoxynucleotide subjected to the action of ANPG/Mug and APE-1 without previous AlkB treatment. Finally, the reaction products were separated by denaturing 20% (w/v) PAGE containing 7 M urea. The scheme of the method is shown in Fig. 2. A digital image of the separated radioactive oligodeoxynucleotides was captured by phosphorimaging using FLA-7000 (Fujifilm), and the radioactivity of the bands was quantified using Multi Gauge Analysis software (Fujifilm).

Since we and others (Speina E, Ciesla JM, Wojcik J, Bajek M, Kusmierek JT, Tudek B., The pyrimidine ring-opened derivative of 1,N6-ethenoadenine is excised from DNA by the Escherichia coli Fpg and Nth proteins. J Biol Chem. 2001 Jun 15;276(24):21821-7.; Basu AK, McNulty JM, McGregor WG., Solution conformation and mutagenic specificity of 1,N6-ethenoadenine. IARC Sci Publ. 1999;(150):325-33. ) have previously shown that ethenoadenine decomposes slowly into derivatives, which are not recognized by ANPG glycosylase, excising ethenoA, we have taken this into consideration during calculation of the results. In each experiment the negative control reaction without AlkB protein was performed, and per cent of “repair” was calculated also for this negative control reaction. Per cent of oligodeoxynucleotide in apparently “repaired band” was then subtracted from the per cent of repair by each AlkB protein. Such procedure enabled to avoid false results as this background was included during calculation of the per cent of *in vitro* AlkB-mediated repair.

***In vivo* repair of DNA etheno adducts by the studied enzymes**

pJB658-derived plasmids bearing the studied AlkB enzymes were prepared as described before [29]. Cultures of *E. coli* strain HK 82/F’ which were transformed with pJB658-derived plasmids bearing the studied AlkB enzymes were induced with 2 mM toluic acid (firma??) at OD600=0.1, and further grown until they reached A600=0.5. Bacteria in 500 μl of the culture were pelleted, resuspended in 250 μl of M9 minimum salt medium, and then mixed with an equal volume of minimum salt medium containing an appropriate concentration of chloroacetaldehyde (CAA). Bacteria were incubated with CAA for 30 min in 37ºC, subsequently diluted 10 000-fold and plated at LB plates. Colonies were counted after 24 h incubation at 37ºC.

**Results**

***In vitro* repair of etheno lesions in ss and dsDNA by bacterial AlkB proteins**

We studied the repair activity of nine bacterial AlkB proteins (Table 1) towards εA, εC, and 1,*N*2-εG using a newly developed DNA glycosylase/AP-endonuclease digestion assay. The N-terminally 6×His-tagged bacterial and human AlkB proteins cloned into pET-28a(+)-derived plasmids were overexpressed in *E. coli* strain BL21-CodonPlus(DE3)-RIPL strains and purified by affinity chromatography on HisPur™ Cobalt Resin. The purity of received enzymes was verified by PAGE, and is shown in Supplementary data, Fig. S1. Most of the studied AlkBs were homogenous, only three of them, MT-2B, RE-2A and SC-2B contained one or few additional faint protein bands. The proteins chosen represented all four structural AlkB groups (1A, 1B, 2A, and 2B), distinguished on the basis of the amino acid sequence of their nucleotide-recognizing domains. Previously, [van den Born et al. [59](#_ENREF_59)] showed that the majority of bacterial AlkB proteins studied in the present work remove methyl groups from m1A and m3C. They also found that AlkBs from *Xanthomonas campestris* (XC-1B and XC-2B) and one from *Rhizobium etli* (RE-2A) displayed a high repair activity towards εA in both ss and dsDNA. Interestingly, XC-2B and RE-2A showed negligible repair activity towards the methyl lesions, suggesting that those enzymes might have evolved to repair bulky alkylated DNA adduct such as ε-lesions [[59](#_ENREF_59)]. In this study we further characterized the repair potential of these AlkBs towards two additional DNA ε-adducts, εC and 1,*N*2-εG, using the novel assay with BER enzymes described in Methods (Fig. 2). Since [van den Born et al. [59](#_ENREF_59)] characterized the εA repair activity utilizing an established digestion assay based on the methylation*-*sensitive restrictase DpnII [[22](#_ENREF_22)], we additionally reexamined the repair of this lesion but using our BER-based assay. The recombinant proteins were incubated with 5´-[32P]-labeled oligodeoxynucleotides containing a single εA, εC, or 1,*N*2-εG. The AlkB repair of the lesions was visualized by DNA glycosylase treatment followed by the cleavage of the resulting abasic sites with AP-endonuclease (Fig. 2), and the obtained results are shown in Fig. 3.

We found that AlkB from *E. coli* (EcAlkB), which belongs to group 1A, exhibits a substantial repair activity towards εA and εC in both ss and dsDNA, but negligible activity towards 1,*N*2-εG and only in ssDNA. The second AlkB protein tested belonging to group 1A, SA-1A from *S. avermitilis*, showed similar activity, albeit it was slightly lower towards εC in comparison with EcAlkB. Unexpectedly, the third protein from that group, SC-1A from *S. coelicolor*, removed only εA and was more active on the dsDNA substrate. XC-1B from *X. campestris*, the only AlkB protein from group 1B studied in this work, was active towards εA and εC and clearly preferred dsDNA. The AlkB proteins from group 2B showed a strongly divergent ε-base repair capacity. Those from *M. tuberculosis* (MT-2B) and *S. coelicolor* (SC-2B) were inactive towards all three ε-adducts examined, whereas SA-2B from *S. avermitilis* and XC-2B *X. campestris* were very active towards all the ε-bases tested in both ss and dsDNA. Finally, the *R. etli* (RE-2A) AlkB protein from group 2A exhibited substantial repair activity towards εA and εC in both ss and dsDNA, and towards 1,*N*2-εG in dsDNA.

***In vitro* repair of etheno lesions in ss and dsDNA by human ALKBH2 and ALKBH3 proteins**

Repair of ε-adducts by human AlkB proteins has been studied by several laboratories; however, conflicting results have been obtained. Repair of εA and εC by ALKBH2 was demonstrated by [Ringvoll et al. [35](#_ENREF_35)] and [Fu and Samson [33](#_ENREF_33)], respectively. The repair specificity for ALKBH3 is more controversial. While [Mishina et al. [34](#_ENREF_34)] reported that human ALKBH3 repaired εA *in vitro* in the specific trinucleotide substrate T(εA)T, albeit with a much lower activity than *E. coli* AlkB, [Ringvoll et al. [35](#_ENREF_35)] did not confirm this finding in mammalian cells. Because of these discrepancies and the general paucity of data on those enzymes, we decided to study the repair activity of ALKBH2 and ALKBH3 towards εA, εC and 1,*N*2-εG in ss and dsDNA substrates in more detail using a BER enzyme-based assay. First, in order to check if ALKBH2 and ALKBH3 are able to remove ε-adducts *in vitro,* we incubated 5´-[32P]-labeled ss or dsDNA oligodeoxynucleotides containing εA, εC, or 1,N2-εG with 10, 25, or 50 pmol of each recombinant protein. As shown in Fig. 4, ALKBH2 repaired εA and εC in both ss and dsDNA and 1,*N*2-εG in dsDNA. Similar to [Ringvoll et al. [35](#_ENREF_35)], we did not observe any repair activity of ALKBH3 with εA; 1,*N*2-εG was not removed by this protein either. However, we observed that ALKBH3 repaired εC in ssDNA (Fig, 4).

To characterize the repair efficiency of ALKBH2 and ALKBH3 towards ε-lesions in more detail, we performed kinetic analysis of the repair reactions. The reaction duration and the amount of the recombinant protein were chosen to give a linear reaction rate. ALKBH2 exhibited a considerably higher repair activity against εA and εC in ds DNA than in ss DNA (Fig. 5). Thus, likewise to the methyl lesions, the ε-adducts are preferentially repaired by ALKBH2 in dsDNA. The ALKBH2 activity against these two ε-lesions in ds DNA was very similar, in both cases reaching a plateau of ~70% of the total repaired substrate after 5 min of incubation with 1 pmol of the enzyme in the reaction (Fig. 5). The measured specific activities towards εA and εC of ALKBH2 were almost identical, 69 and 66 fmol•min-1•pmol enzyme-1, respectively (Table 2). In ssDNA, ALKBH2 repaired εA about 2-fold more efficiently than εC, with specific activities of 21 and 11 fmol•min-1•pmol protein-1, respectively (Table 2). The repair of εA in ssDNA reached a plateau of 70% of the repaired substrate after 10 min of incubation with 6 pmol of the enzyme, and the repair of εC in ssDNA reached only a plateau of ~50% of the repaired substrate after 20 min with 6 pmol of the enzyme (Fig. 5). The ALKBH2-mediated repair of 1,*N*2-εG in dsDNA was an order of magnitude less efficient than the repair of εA and εC with the specific activity being 5 fmol•min-1•pmol ALKBH2-1 (Table 2). A plateau of ~60% of the repaired substrate was reached after 30 min of incubation at ~6 pmoles of the enzyme (Fig. 5). For ALKBH3, we studied only the repair of εC in ssDNA, because the earlier qualitative analysis showed that other ε-lesions were not repaired by that enzyme. The rate of εC repair was rather low, with a specific activity of 6 fmol•min-1•pmol ALKBH3-1 (Table 2), and a plateau was reached at only ~35% of the repaired substrate after 20 min of incubation with 8 pmol of the enzyme (Fig. 5).

**Bacterial survival assays**

The *in vivo* importance of reported *in vitro* repair activities of nine bacterial AlkBs was partially addressed by van den Born and coworkers [29] who tested *in vivo* repair of etheno lesions in ssDNA by these AlkBs, and showed that expression of SA-1A, SC-1A, RE-2A, SA-2B and XC-2B proteins in EcAlkB-deficient *E. coli* complemented its repair deficiency with activity comparable to EcAlkB. We have extended these studies on the effect of repair in dsDNA. A set of *alkB* deficient *E. coli* strains, bearing plasmids coding for the studied AlkB proteins was constructed, and sensitivity of these strains to chloroacetaldehyde was verified (Fig. 6). AlkB deficient strain (HK 82/F’) was more sensitive to CAA than the strain bearing AlkB from *E. coli* (Fig. 6). Also all other studied enzymes increased viability of AlkB-deficient *E. coli* strain upon treatment with CAA. This was surprising, since two AlkBs, MT-2B and SC-2B did not repair any ε-adduct *in vitro* or repair was very inefficient. Similar complementation was observed by van den Born and coworkers [29] when ssDNA of M13 phage damaged with CAA was transformed into *E. coli* expressing AlkBs studied here. This might suggest that even a remnant activity of AlkB is sufficient *in vivo* to protect bacteria from deleterious effects of ε-adducts.

**Discussion**

The contribution of different repair systems to the removal of highly mutagenic ε-adducts from DNA has not been fully characterized. The mutation spectra in repair mutants of *E. coli* exposed to chloroacetaldehyde (CAA), which efficiently creates ε-adducts of A, C, and G, suggest that their repair is executed by both base excision repair and AlkB-mediated oxidative dealkylation [[36](#_ENREF_36)]. However, the rates of repair of particular lesions induced by CAA have not been established, and the removal of 1,*N*2-εG has not been investigated thus far. The latter, although a minor lesion in dsDNA, has strong mutagenic potential, and is introduced by chemical carcinogens to ssDNA with a similar frequency as *N*2,2-εG. The difficulties in establishing the repair rate of ε-adducts by AlkBs derive from the fact that not all substrates are available commercially, the existing methods are labor and time consuming, and assays require large amounts of substrates and enzymes. We have established a new BER enzyme-based assay for quantifying the repair of ε-lesions in DNA by AlkB proteins. In this assay, following incubation with an AlkB protein the DNA substrates are treated with an appropriate DNA glycosylase and the resulting abasic sites are cleaved with AP-endonuclease*.* The DNA glycosylase/AP endonucleasecleavage occurs only if the ε-lesion is not repaired by oxidative dealkylation mediated by the AlkB protein. The method elaborated by us for measuring AlkB activity against ethenoadducts is an alternative to HPLC and ion-exchange chromatography or C-18 columns, as well as to restriction nuclease methods. Its simplicity and speed enables to test many AlkB enzymes at once. In comparison to HPLC method it needs much less expensive substrate (about 100 – 500 times less), and either less or the same amount of AlkB enzyme [15]. In comparison to restriction nucleases method, BER-mediated assay needs less enzyme (1 pmol *vs* 100 pmol in restriction nucleases analysis [22, 29]). In addition, restriction nuclease method requires that modified base is incorporated in a specific sequence recognized by this nuclease, while in BER-mediated assay any sequence can be modified, and sequence-specificity of AlkB enzymes can be investigated. Limitation of the method is availability of BER enzymes recognizing the studied lesions. In practice, the method is currently limited to etheno-DNA adducts.

We established the *in vitro* repair activity towards εA, εC, and 1,*N*2-εG in ss and dsDNA of nine bacterial AlkBs representing each of the four structural groups defined earlier. The majority of the bacterial AlkB proteins tested, similar to EcAlkB, remove εA and εC from DNA. This *in vitro* repair activity towards ε-adducts suggests that AlkB proteins constitute an alternative repair mechanism to the BER pathway for removing ε-lesions from genomic DNA *in vivo*. The conclusion that EcAlkB plays a major role in the DNA repair in *E. coli* is further supported by the fact that it decreases the AT→TA mutations generated by εA [[62](#_ENREF_62)]. Furthermore, because the bacterial AlkBs tested were active towards ε-adducts in ssDNA, they could also play an essential role in repairing ε-lesions in the replication forks and transcription bubbles during replication and transcription, respectively. It is worth mentioning that an earlier study showed that although SA-1A and SC-1A proteins efficiently reactivated CAA-treated M13 ssDNA *in vivo*, they displayed negligible levels of εA repair *in vitro* in ss and dsDNA substrates using a DpnII-based method [[59](#_ENREF_59)]. In contrast, using our BER digestion assay we found that these proteins in fact are able to remove εA from the DNA with similar efficiency to that of EcAlkB. The *in vitro* repair activity of SA-1A and SC-1A towards εA (and for SA-1A also εC), demonstrated here, explains their ability to reactivate CAA-treated M13 *in vivo*.

The present study showed for the first time that AlkB proteins can also repair the minor, but highly mutagenic 1,*N*2-εG adduct. The three bacterial AlkBs, i.e SA-2B, XC-2B and RE-2A, displayed a high repair activity against all the ε-adducts tested. They are the first AlkBs for which such a substantial activity against εA, εC, and 1,*N*2-εG has been shown. In addition, XC-2B and RE-2A are the first AlkBs that show repair activity toward εA, εC, and 1,*N*2-εG without displaying any activity towards 1-meA and 3-meC. *Xanthomonas campestris*, *Streptomyces avermitilis,* and *Rhizobium etli* are soil bacteria, and therefore they can be exposed to industrial pollutants present in the soil. It has been shown that some common industrial solvents, e.g. tetrachloroethylene, which are among the most frequent contaminants found in groundwater, can be biotransformed to vinyl chloride [[63](#_ENREF_63)]. Given this, it seems plausible that XC-2B, SA-2B, and RE-2A AlkB proteins might have evolved to protect these soil bacteriaagainst DNA ε-adducts introduced by vinyl chloride generated in the soil.

It has been suggested that the amino acids composition of nucleotide recognition lid determines the substrate specificity of AlkB proteins. Accordingly, AlkB proteins belonging to the same group are expected to have similar repair profiles against diverse DNA lesions. However, we observed that proteins from group 2B displayed substantial differences in their repair activities towards the ε-adducts; SA-2B and XC-2B efficiently removed all the ε-adducts tested, while MT-2B andSC-2B were not active against these lesions, and [van den Born et al. [59](#_ENREF_59)] observed divergent repair activities of methyl lesions among this group of proteins. Also the AlkBs from group 1A displayed contrasting activities against εC, because SA-1A and EcAlkB removed εC whereas SC-1A did not. Altogether, the results obtained here (as well as the data presented by [van den Born et al. [59](#_ENREF_59)]) indicate that AlkB proteins belonging to a given group can in fact show diverse repair activities against given DNA lesions. Thus, it is not possible to predict the substrate specificity of AlkB proteins based solely on their classification according to the amino acid composition of their nucleotide recognition lid. Indeed, recent studies have shown that substrate recognition by EcAlkB is also determined by aminoacids in the active site, which is located in enzyme flexible loop between aminoacids K134 to L139 [Zhu C and Yi C, Angew Chem Int Ed Engl. 2014 Apr 1;53(14):3659-62]. The mechanism of repair of propano-type adducts by AlkB from *E. coli* involved several stages, during which transient complexes are formed with ring-opened aldehydic form and ring-closed form. AlkB repairs ring-opened form [Singh et al., Chem Res Toxicol. 2014 Sep 15;27(9):1619-31]. Finally the efficiency of catalysis is determined by the dynamics of a specific conformational transition, which facilitates the proper sequencial order of substrate binding to AlkB, that is oxidation of 2-oxoglutarate, formation of enzyme-bound oxyferryl intermediate, and hydroxylation of an alkylated nucleobase [Ergel et al., J Biol Chem. 2014 Oct 24;289(43):29584-601]. Thus, our studies are consistent with the notion that substrate binding and efficiency of catalysis depend on the structure of nucleotide recognition lid, active site and the dynamics of enzyme conformational change.

It has been demonstrated that human ALKBH2, similar to the *E. coli* AlkB protein, is able to repair εA and εC in DNA [[33](#_ENREF_33),[35](#_ENREF_35)]. Using our BER-based digestion assay we confirmed here that ALKBH2 exhibits an *in vitro* activity towards εA and εC in ss and dsDNA. Similar to methylated bases, ALKBH2 repaired εA and εC more efficiently in dsDNA than in ssDNA. Based on the ability of recombinant ALKBH2 to remove εA and εC observed by us and others [[33](#_ENREF_33),[35](#_ENREF_35)], it seems reasonable that this enzyme could be engaged in the repair of these lesions *in vivo*. However, it has been demonstrated that *Alkbh2-/-*mice, in contrast to *Anpg-/-* mice, do not accumulate εA in genomic DNA [[35](#_ENREF_35)]. Given this, it seems that the direct reversal of εA by ALKBH2 is not sufficient to remove endogenously generated εA, and the main enzyme responsible for removing this lesion from DNA *in vivo* is the glycosylase ANPG [[35](#_ENREF_35)]. However, further studies in *Alkbh2-/-/Anpg-/-* double mutant mice are needed to elucidate fully the importance of ALKBH2-mediated repair of εA *in vivo*. Likewise, the *in vivo* significance of εC repair by recombinant ALKBH2 observed here, as well as by [Fu and Samson [33](#_ENREF_33)] needs further experimental verification. Some insight into this problem was recently provided by the study of [Calvo et al. [64](#_ENREF_64)], in which a knockout of three enzymes ANPG, ALKBH2, and ALKBH3 made mice particularly susceptible to inducers of colon inflammation, and all mice died following the first instillation of a pro-inflammatory compound. It has been established that εC can be excised from DNA *in vitro* by three different DNA glycosylases: TDG [[52](#_ENREF_52),[53](#_ENREF_53)], MBD4/MED1 [[55](#_ENREF_55)], and SMUG1[[54](#_ENREF_54)]. However, the primary substrates of these glycosylases are oxidation and deamination products of 5-methylcytosine in a CpG context [[65](#_ENREF_65),[66](#_ENREF_66)]. Given this, it seems likely that another repair enzyme responsible for εC repair *in vivo* exists, and ALKBH2 could be this one. We further found that recombinant ALKBH2 can remove 1,*N*2-εG from dsDNA. However, an ALKBH2-mediated repair of 1,*N*2-εG does not seem to play a major physiological role, because the *in vitro* activity of ALKBH2 against this lesion was considerably lower than the corresponding activity towards εA or εC. Finally, in agreement with the results of [Ringvoll et al. [35](#_ENREF_35)], we showed that recombinant ALKBH3 does not remove εA from DNA. These data are in contrast with the results reported by [Mishina et al. [34](#_ENREF_34)] who reported that ALKBH3 was able to repair εA *in vitro*. However, it should be mentioned that those authors incubated ALKBH3 with the DNA substrates for 16 h under non-physiological conditions (pH 6.0) [[34](#_ENREF_34)]. Notably, we observed a weak repair activity of ALKBH3 against εC in ss DNA but doubt that it has a biological significance.

In conclusion, we have shown that nine of eleven AlkB proteins studied (7 bacterial and 2 human) are able to repair ε-lesions *in vitro*. Additionally, we posit that DNA ε-adducts could be the primary substrates for some bacterial AlkBs *in vivo* since they exhibit a marked *in vitro* repair activity towards ε-lesions and lacking such activity towards DNA methyl lesions.

**Acknowledgments**

We would like to thank Prof. Jan Fronk for critical reading of the manuscript. This work was financed by grant from Ministry of Science and Higher Education N303 819540 (BT) and National Institutes of Health grants R01 ES010546 (FPG), P01 ES05355 (CJR), P01 CA160032 (CJR), P30 ES00267 (CJR. and FPG) and P30 CA068485 (C.J.R. and FPG).

**References**

1. Aravind L, Koonin EV (2001) The DNA-repair protein AlkB, EGL-9, and leprecan define new families of 2-oxoglutarate- and iron-dependent dioxygenases. Genome Biol 2: RESEARCH0007.

2. Falnes PØ, Johansen RF, Seeberg E (2002) AlkB-mediated oxidative demethylation reverses DNA damage in *Escherichia coli.* Nature 419: 178-182.

3. Trewick SC, Henshaw TF, Hausinger RP, Lindahl T, Sedgwick B (2002) Oxidative demethylation by *Escherichia coli* AlkB directly reverts DNA base damage. Nature 419: 174-178.

4. Kataoka H, Sekiguchi M (1985) Molecular cloning and characterization of the *alkB* gene of *Escherichia coli*. Mol Gen Genet 198: 263-269.

5. Kataoka H, Yamamoto Y, Sekiguchi M (1983) A new gene (*alkB*) of *Escherichia coli* that controls sensitivity to methyl methane sulfonate. J Bacteriol 153: 1301-1307.

6. Delaney JC, Essigmann JM (2004) Mutagenesis, genotoxicity, and repair of 1-methyladenine, 3-alkylcytosines, 1-methylguanine, and 3-methylthymine in *alkB Escherichia coli*. Proc Natl Acad Sci U S A 101: 14051-14056.

7. Falnes PØ (2004) Repair of 3-methylthymine and 1-methylguanine lesions by bacterial and human AlkB proteins. Nucleic Acids Res 32: 6260-6267.

8. Koivisto P, Robins P, Lindahl T, Sedgwick B (2004) Demethylation of 3-methylthymine in DNA by bacterial and human DNA dioxygenases. J Biol Chem 279: 40470-40474.

9. Aas PA, Otterlei M, Falnes PØ, Vagbo CB, Skorpen F, et al. (2003) Human and bacterial oxidative demethylases repair alkylation damage in both RNA and DNA. Nature 421: 859-863.

10. Ougland R, Zhang CM, Liiv A, Johansen RF, Seeberg E, et al. (2004) AlkB restores the biological function of mRNA and tRNA inactivated by chemical methylation. Mol Cell 16: 107-116.

11. Vagbo CB, Svaasand EK, Aas PA, Krokan HE (2013) Methylation damage to RNA induced in vivo in *Escherichia coli* is repaired by endogenous AlkB as part of the adaptive response. DNA Repair (Amst) 12: 188-195.

12. van den Born E, Omelchenko MV, Bekkelund A, Leihne V, Koonin EV, et al. (2008) Viral AlkB proteins repair RNA damage by oxidative demethylation. Nucleic Acids Res 36: 5451-5461.

13. Duncan T, Trewick SC, Koivisto P, Bates PA, Lindahl T, et al. (2002) Reversal of DNA alkylation damage by two human dioxygenases. Proc Natl Acad Sci U S A 99: 16660-16665.

14. Koivisto P, Duncan T, Lindahl T, Sedgwick B (2003) Minimal methylated substrate and extended substrate range of *Escherichia coli* AlkB protein, a 1-methyladenine-DNA dioxygenase. J Biol Chem 278: 44348-44354.

15. Maciejewska AM, Poznanski J, Kaczmarska Z, Krowisz B, Nieminuszczy J, et al. (2013) AlkB dioxygenase preferentially repairs protonated substrates: specificity against exocyclic adducts and molecular mechanism of action. J Biol Chem 288: 432-441.

16. Gerken T, Girard CA, Tung YC, Webby CJ, Saudek V, et al. (2007) The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. Science 318: 1469-1472.

17. Kurowski MA, Bhagwat AS, Papaj G, Bujnicki JM (2003) Phylogenomic identification of five new human homologs of the DNA repair enzyme AlkB. BMC Genomics 4: 48.

18. Sanchez-Pulido L, Andrade-Navarro MA (2007) The FTO (fat mass and obesity associated) gene codes for a novel member of the non-heme dioxygenase superfamily. BMC Biochem 8: 23.

19. Falnes PØ, Bjoras M, Aas PA, Sundheim O, Seeberg E (2004) Substrate specificities of bacterial and human AlkB proteins. Nucleic Acids Res 32: 3456-3461.

20. Lee DH, Jin SG, Cai S, Chen Y, Pfeifer GP, et al. (2005) Repair of methylation damage in DNA and RNA by mammalian AlkB homologues. J Biol Chem 280: 39448-39459.

21. Mishina Y, Lee CH, He C (2004) Interaction of human and bacterial AlkB proteins with DNA as probed through chemical cross-linking studies. Nucleic Acids Res 32: 1548-1554.

22. Ringvoll J, Nordstrand LM, Vagbo CB, Talstad V, Reite K, et al. (2006) Repair deficient mice reveal mABH2 as the primary oxidative demethylase for repairing 1meA and 3meC lesions in DNA. EMBO J 25: 2189-2198.

23. Fu Y, Jia G, Pang X, Wang RN, Wang X, et al. (2013) FTO-mediated formation of *N*6-hydroxymethyladenosine and *N*6-formyladenosine in mammalian RNA. Nat Commun 4: 1798.

24. Jia G, Fu Y, Zhao X, Dai Q, Zheng G, et al. (2011) *N*6-methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. Nat Chem Biol 7: 885-887.

25. Zheng G, Dahl JA, Niu Y, Fedorcsak P, Huang CM, et al. (2013) ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. Mol Cell 49: 18-29.

26. Fu D, Brophy JA, Chan CT, Atmore KA, Begley U, et al. (2010) Human AlkB homolog ABH8 Is a tRNA methyltransferase required for wobble uridine modification and DNA damage survival. Mol Cell Biol 30: 2449-2459.

27. Fu Y, Dai Q, Zhang W, Ren J, Pan T, et al. (2010) The AlkB domain of mammalian ABH8 catalyzes hydroxylation of 5-methoxycarbonylmethyluridine at the wobble position of tRNA. Angew Chem Int Ed Engl 49: 8885-8888.

28. Songe-Moller L, van den Born E, Leihne V, Vagbo CB, Kristoffersen T, et al. (2010) Mammalian ALKBH8 possesses tRNA methyltransferase activity required for the biogenesis of multiple wobble uridine modifications implicated in translational decoding. Mol Cell Biol 30: 1814-1827.

29. van den Born E, Vagbo CB, Songe-Moller L, Leihne V, Lien GF, et al. (2011) ALKBH8-mediated formation of a novel diastereomeric pair of wobble nucleosides in mammalian tRNA. Nat Commun 2: 172.

30. Li MM, Nilsen A, Shi Y, Fusser M, Ding YH, et al. (2013) ALKBH4-dependent demethylation of actin regulates actomyosin dynamics. Nat Commun 4: 1832.

31. Ougland R, Lando D, Jonson I, Dahl JA, Moen MN, et al. (2012) ALKBH1 is a histone H2A dioxygenase involved in neural differentiation. Stem Cells 30: 2672-2682.

32. Delaney JC, Smeester L, Wong C, Frick LE, Taghizadeh K, et al. (2005) AlkB reverses etheno DNA lesions caused by lipid oxidation in vitro and in vivo. Nat Struct Mol Biol 12: 855-860.

33. Fu D, Samson LD (2012) Direct repair of 3,*N*4-ethenocytosine by the human ALKBH2 dioxygenase is blocked by the AAG/MPG glycosylase. DNA Repair (Amst) 11: 46-52.

34. Mishina Y, Yang CG, He C (2005) Direct repair of the exocyclic DNA adduct 1,*N*6-

ethenoadenine by the DNA repair AlkB proteins. J Am Chem Soc 127: 14594-14595.

35. Ringvoll J, Moen MN, Nordstrand LM, Meira LB, Pang B, et al. (2008) AlkB homologue 2-mediated repair of ethenoadenine lesions in mammalian DNA. Cancer Res 68: 4142-4149.

36. Maciejewska AM, Ruszel KP, Nieminuszczy J, Lewicka J, Sokolowska B, et al. (2010) Chloroacetaldehyde-induced mutagenesis in *Escherichia coli*: the role of AlkB protein in repair of 3,*N*4-ethenocytosine and 3,*N*4-α-hydroxyethanocytosine. Mutat Res 684: 24-34.

37. Bolt HM (1988) Roles of etheno-DNA adducts in tumorigenicity of olefins. Crit Rev Toxicol 18: 299-309.

38. Guengerich FP (2003) Cytochrome P450 oxidations in the generation of reactive electrophiles: epoxidation and related reactions. Arch Biochem Biophys 409: 59-71.

39. Chung FL, Pan J, Choudhury S, Roy R, Hu W, et al. (2003) Formation of trans-4-hydroxy-2-nonenal- and other enal-derived cyclic DNA adducts from omega-3 and omega-6 polyunsaturated fatty acids and their roles in DNA repair and human p53 gene mutation. Mutat Res 531: 25-36.

40. Marnett LJ (2000) Oxyradicals and DNA damage. Carcinogenesis 21: 361-370.

41. Singer B., Bartsch H. (1999) Exocyclic DNA adducts in mutagenesis and carcinogenesis. Lyon: IARC Press, IARC Scientific Publication No 150.

42. Winczura A, Zdzalik D, Tudek B (2012) Damage of DNA and proteins by major lipid peroxidation products in genome stability. Free Radic Res 46: 442-459.

43. Barbin A (2000) Etheno-adduct-forming chemicals: from mutagenicity testing to tumor mutation spectra. Mutat Res 462: 55-69.

44. Basu AK, Wood ML, Niedernhofer LJ, Ramos LA, Essigmann JM (1993) Mutagenic and genotoxic effects of three vinyl chloride-induced DNA lesions: 1,*N*6-ethenoadenine, 3,*N*4-ethenocytosine, and 4-amino-5-(imidazol-2-yl)imidazole. Biochemistry 32: 12793-12801.

45. Pandya GA, Moriya M (1996) 1,*N*6-Ethenodeoxyadenosine, a DNA adduct highly mutagenic in mammalian cells. Biochemistry 35: 11487-11492.

46. Moriya M, Zhang W, Johnson F, Grollman AP (1994) Mutagenic potency of exocyclic DNA adducts: marked differences between *Escherichia coli* and simian kidney cells. Proc Natl Acad Sci U S A 91: 11899-11903.

47. Barbin A, Bartsch H, Leconte P, Radman M (1981) Studies on the miscoding properties of 1,*N*6-ethenoadenine and 3,*N*4-ethenocytosine, DNA reaction products of vinyl chloride metabolites, during in vitro DNA synthesis. Nucleic Acids Res 9: 375-387.

48. Miller JA, Miller EC (1983) The metabolic activation and nucleic acid adducts of naturally-occurring carcinogens: recent results with ethyl carbamate and the spice flavors safrole and estragole. Br J Cancer 48: 1-15.

49. Nair J, Sone H, Nagao M, Barbin A, Bartsch H (1996) Copper-dependent formation of miscoding etheno-DNA adducts in the liver of Long Evans cinnamon (LEC) rats developing hereditary hepatitis and hepatocellular carcinoma. Cancer Res 56: 1267-1271.

50. Saparbaev M, Kleibl K, Laval J (1995) *Escherichia coli*, *Saccharomyces cerevisiae*, rat and human 3-methyladenine DNA glycosylases repair 1,*N*6-ethenoadenine when present in DNA. Nucleic Acids Res 23: 3750-3755.

51. Saparbaev M, Langouet S, Privezentzev CV, Guengerich FP, Cai H, et al. (2002) 1,*N*2-ethenoguanine, a mutagenic DNA adduct, is a primary substrate of *Escherichia coli* mismatch-specific uracil-DNA glycosylase and human alkylpurine-DNA-N-glycosylase. J Biol Chem 277: 26987-26993.

52. Hang B, Medina M, Fraenkel-Conrat H, Singer B (1998) A 55-kDa protein isolated from human cells shows DNA glycosylase activity toward 3,*N*4-ethenocytosine and the G/T mismatch. Proc Natl Acad Sci U S A 95: 13561-13566.

53. Saparbaev M, Laval J (1998) 3,*N*4-ethenocytosine, a highly mutagenic adduct, is a primary substrate for *Escherichia coli* double-stranded uracil-DNA glycosylase and human mismatch-specific thymine-DNA glycosylase. Proc Natl Acad Sci U S A 95: 8508-8513.

54. Kavli B, Sundheim O, Akbari M, Otterlei M, Nilsen H, et al. (2002) hUNG2 is the major repair enzyme for removal of uracil from U:A matches, U:G mismatches, and U in single-stranded DNA, with hSMUG1 as a broad specificity backup. J Biol Chem 277: 39926-39936.

55. Petronzelli F, Riccio A, Markham GD, Seeholzer SH, Genuardi M, et al. (2000) Investigation of the substrate spectrum of the human mismatch-specific DNA N-glycosylase MED1 (MBD4): fundamental role of the catalytic domain. J Cell Physiol 185: 473-480.

56. Matijasevic Z, Sekiguchi M, Ludlum DB (1992) Release of *N*2,3-ethenoguanine from chloroacetaldehyde-treated DNA by *Escherichia coli* 3-methyladenine DNA glycosylase II. Proc Natl Acad Sci U S A 89: 9331-9334.

57. Gallinari P, Jiricny J (1996) A new class of uracil-DNA glycosylases related to human thymine-DNA glycosylase. Nature 383: 735-738.

58. Prorok P, Saint-Pierre C, Gasparutto D, Fedorova OS, Ishchenko AA, et al. (2012) Highly mutagenic exocyclic DNA adducts are substrates for the human nucleotide incision repair pathway. PLoS One 7: e51776.

59. van den Born E, Bekkelund A, Moen MN, Omelchenko MV, Klungland A, et al. (2009) Bioinformatics and functional analysis define four distinct groups of AlkB DNA-dioxygenases in bacteria. Nucleic Acids Res 37: 7124-7136.

60. Goodenough AK, Kozekov ID, Zang H, Choi JY, Guengerich FP, et al. (2005) Site specific synthesis and polymerase bypass of oligonucleotides containing a 6-hydroxy-3,5,6,7-tetrahydro-9H-imidazo[1,2-a]purin-9-one base, an intermediate in the formation of 1,*N*2-etheno-2'-deoxyguanosine. Chem Res Toxicol 18: 1701-1714.

61. Langouet S, Muller M, Guengerich FP (1997) Misincorporation of dNTPs opposite 1,*N*2-ethenoguanine and 5,6,7,9-tetrahydro-7-hydroxy-9-oxoimidazo[1,2-α]purine in oligonucleotides by *Escherichia coli* polymerases I exo- and II exo-, T7 polymerase exo-, human immunodeficiency virus-1 reverse transcriptase, and rat polymerase β. Biochemistry 36: 6069-6079.

62. Maciejewska AM, Sokolowska B, Nowicki A, Kusmierek JT (2011) The role of AlkB protein in repair of 1,*N*6-ethenoadenine in *Escherichia coli* cells. Mutagenesis 26: 401-406.

63. Vogel TM, McCarty PL (1985) Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl Environ Microbiol 49: 1080-1083.

64. Calvo JA, Meira LB, Lee CY, Moroski-Erkul CA, Abolhassani N, et al. (2012) DNA repair is indispensable for survival after acute inflammation. J Clin Invest 122: 2680-2689.

65. Morera S, Grin I, Vigouroux A, Couve S, Henriot V, et al. (2012) Biochemical and structural characterization of the glycosylase domain of MBD4 bound to thymine and 5-hydroxymethyuracil-containing DNA. Nucleic Acids Res 40: 9917-9926.

66. Sjolund AB, Senejani AG, Sweasy JB (2013) MBD4 and TDG: multifaceted DNA glycosylases with ever expanding biological roles. Mutat Res 743-744: 12-25.

**Figure Legends**

**Figure 1. The mechanism of AlkB-mediated DNA repair through oxidative dealkylation of 1-methyladenine (m1A) (A) and 1,*N*6-ethenoadenine (εA) (B).**

**Figure 2. Schematic representation of the BER enzymes digestion assay for visualization of AlkB-mediated repair of ε-lesions.** 32P-labeled ssDNA or dsDNA oligodeoxynucleotides containing single ε-lesion are incubated with AlkB protein. Subsequently, to visualize the AlkB-mediated repair, oligodeoxynucleotides are treated with the appropriate DNA glycosylase (G) followed by AP endonuclease1 (AP*)*. Reaction products are separated by denaturing PAGE and visualized by phosporimaging. The dashed line indicates the complementary, lesion-free unlabeled oligodeoxynucleotide, which is either present during the repair reaction (dsDNA repair) or is added post-repair (ssDNA repair). G/AP cleavage of the DNA substrate occurs only if the ε-lesion is not removed. A repaired substrate will appear as a slower-migrating band on PAGE.

Figure 3. *In vitro* repair activity of bacterial AlkB proteins towards DNA ε-adducts. (A) 1 pmol of 32P-labeled oligodeoxynucleotide was incubated with 25 or 50 pmol of tested AlkB protein for 30 min at 37 °C, digested with the excess of an appropriate DNA glycosylase (G) and AP endonuclease (AP), and separated by 20% (w/v) denaturing PAGE. (B) Quantification of results from experiments exemplified in A. Values represent the means ± SD of three independent experiments.

**Figure 4. *In vitro* repair activity of human ALKBH2 and ALKBH3 towards DNA ε-adducts.** ss or ds 32P-5’-labeled oligodeoxynucleotides (1 pmol), containing εA, εC or 1,*N*2-εG, were incubated with 10, 25, or 50 pmol of recombinant ALKBH2 or ALKBH3 for 30 min at 37 °C, digested with the appropriate DNA glycosylase (G) and AP endonuclease (AP), separated by 20% (w/v) denaturing PAGE and labeled DNA was visualized by phosphorimaging.

Figure 5. Comparison of *in vitro* repair efficiency of DNA ε-adducts by recombinant human ALKBH2 and ALKBH3. (A) ss or ds 32P-end-labeled oligonucleotidescontaining εA, εC, or 1,*N*2-εG (1 pmol) were incubated with increasing amounts of recombinant ALKBH2 or ALKBH3 for the indicated time at 37 °C, then digested with the appropriate DNA glycosylase (G) and AP endonuclease (AP), separated by 20% (w/v) denaturing PAGE. Radioactive DNA was visualized by phosphorimaging. (B) Quantification of results from experiments shown in A. Each data point shows the mean of three independent measurements, error bars indicate SD. Calculated specific repair activities are given in Table 2.

**Figure 6. Effect of complementation of alkB mutation in *E. coli* by nine bacterial AlkB homologs, upon treatment with chloroacetaldehyde.**

**Tables**

**Table 1. AlkB proteins tested in the study**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Origin** | **AlkB** | **Accession No.** | **Gi number** | **Size (aa)** |
| *Escherichia coli* | EcAlkB | NP\_754639 | 26248599 | 216 |
| *Mycobacterium tuberculosis* | MT-2B | NP\_335465 | 15840428 | 205 |
| *Rhizobium etli* | RE-2A | YP\_472140 | 86360251 | 189 |
| *Streptomyces avermitilis* | SA-1A | NP\_822618 | 29827984 | 222 |
| *Streptomyces avermitilis* | SA-2B | NP\_822306 | 29827672 | 208 |
| *Streptomyces coelicolor* | SC-1A | NP\_625335 | 21219556 | 216 |
| *Streptomyces coelicolor* | SC-2B | NP\_631357 | 21225578 | 210 |
| *Xanthomonas campestris* | XC-1B | NP\_636023 | 21230106 | 201 |
| *Xanthomonas campestris* | XC-2B | NP\_636458 | 21230541 | 211 |
| *Homo sapiens* | ALKBH2 | NP\_001001655 | 48717226 | 261 |
| *Homo sapiens* | ALKBH3 | NP\_631917 | 21040275 | 286 |

**Table 2. Specific repair activity of human recombinant ALKBH2 and ALKBH3 proteins against etheno lesions**

|  |  |  |  |
| --- | --- | --- | --- |
| **Protein** | **ε-lesion** | **ss/ds****DNA** | **Specific repair activity****[fmol of product•min**-1**•pmol protein**-1**]** |
| ALKBH2 | εA | ss | 21.4 ± 3.3 |
| ds | 69.4 ± 3.6 |
| εC | ss | 11.1 ± 2.7 |
| ds | 65.8 ± 1.2 |
| εG | ds | 4.6 ± 1.3 |
| ALKBH3 | εC | ss | 5.8 ± 1.1 |