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Reactions of Glyceraldehyde 3-Phosphate Dehydrogenase Sulfhydryl Groups with *bis*-Electrophiles Produce DNA-Protein Crosslinks but Not Mutations

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Abstract

The environmental contaminant 1,2-dibromoethane and diepoxybutane, an oxidation product of the important industrial chemical butadiene, are *bis*-functional electrophiles and known to be mutagenic and carcinogenic. One mechanism by which *bis*-electrophiles can exert their toxic effects is through the induction of genotoxic and mutagenic DNA–peptide crosslinks. This mechanism has been shown in systems overexpressing the DNA repair protein *O*⁶-alkylguanine DNA-alkyltransferase (AGT) or glutathione *S*-transferase and involves reactions with nucleophilic cysteine residues. The hypothesis that DNA-protein crosslink formation is a more general mechanism for genotoxicity by *bis*-electrophiles was investigated by screening nuclear proteins for reactivity with model monofunctional electrophiles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was identified as a candidate due to the nucleophilicity of two cysteine residues (Cys¹⁵² and Cys²⁴⁶) in reaction screens with model electrophiles (Dennehy, M. K. et al. (2006) *Chem. Res. Toxicol.* 19, 20–29). Incubation of GAPDH with *bis*-electrophiles resulted in inhibition of its catalytic activity, but only at high concentrations of diepoxybutane. *In vitro* assays indicated DNA-GAPDH crosslink formation in the presence of diepoxybutane, and *bis*-electrophile reactivity at Cys²⁴⁶ was confirmed using mass spectral analysis. In contrast to AGT, overexpression of human GAPDH in *Escherichia coli* did not enhance mutagenesis by diepoxybutane. We propose that the lack of mutational enhancement is in part due to the inherently lower reactivity of GAPDH toward *bis*-electrophiles as well as the reduced DNA binding ability relative to AGT, preventing the *in vivo* formation of DNA-protein crosslinks and enhanced mutagenesis.

Introduction

The mechanism of mutagenesis by *bis*-electrophiles such as 1,2-dibromoethane and diepoxybutane is of great interest because of the carcinogenic properties of these chemicals, as well as the potential for human exposure. 1,2-Dibromoethane was formerly used as a fuel additive and fumigant, and is an environmental contaminant, although industrial use of this chemical has been drastically reduced (1) (www.atsdr.cdc.gov/toxprofiles/tp37.html).

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Diepoxybutane is an oxidation product of the major industrial chemical butadiene, which is used in the synthesis of plastics and rubber and found in automobile exhaust and cigarette smoke (2) (www.atsdr.cdc.gov/toxprofiles/tp28.html). 1,2-Dibromoethane and diepoxybutane are both carcinogens in laboratory animals (3–7) and induce mutagenicity and toxicity in *in vitro* systems (8–10).

One mechanism by which these chemicals exert their mutagenic effects is through the crosslinking of peptides to DNA. This phenomenon was discovered in systems overexpressing *O*⁶-alkylguanine-DNA alkyltransferase (AGT)¹ (11–13) or GSH transferase (14), and the mechanism of crosslink formation between AGT and DNA has been extensively investigated for 1,2-dibromoethane (15–17). The nucleophilic cysteine residues of AGT (18) and GSH (activated by GSH transferase) activate *bis*-electrophiles by forming half-mustards, which can cyclize into unstable episulfonium ions (19). Reaction between highly electrophilic episulfonium ions and DNA results in the formation of DNA–peptide crosslinks. As a consequence of this reaction, *bis*-electrophiles also inhibit the activity of this DNA repair protein (15).

DNA-protein crosslinks are formed with many endogenous and exogenous chemicals, leading to highly persistent DNA lesions whose repair is poorly characterized (20–22). These large, bulky adducts are thought to disrupt normal DNA replication, producing genotoxic responses to the crosslinks (23, 24). Many chemotherapeutic agents induce genotoxicity by forming DNA-protein crosslinks. Identification of the specific proteins crosslinked to DNA by *bis*-electrophiles may lead to biomarker development for occupational and environmental exposure (24).

While mechanisms of crosslinking and misincorporation by *bis*-electrophiles have been extensively studied with GSH transferase/GSH and AGT (25), the formation of crosslinks involving other proteins has not been investigated. We sought to determine whether or not other peptides could enhance mutagenesis of *bis*-electrophiles through an AGT-like mechanism. In order to address this question, isolated human nuclear proteins reactive toward model electrophiles were identified in a screen, along with their nucleophilic sites (26). One protein found to be reactive with monofunctional electrophiles was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH is a glycolytic enzyme responsible for catalyzing the oxidation of glyceraldehyde 3-phosphate (G3P) to 1,3-bisphosphoglycerate. Recent studies provide evidence that GAPDH also functions within the nucleus during DNA replication and repair (27, 28). Cys¹⁵², located near the active site and a peripheral cysteine (Cys²⁴⁶) were identified as reactive nucleophiles with the monofunctional reagents (26, 29).

Using the AGT mechanism as a model, reactivity and crosslinking ability of GAPDH were investigated upon treatment with *bis*-electrophiles. Although GAPDH displayed several of the characteristics of AGT (15), enhancement of mutagenesis was not observed in *E. coli*

¹Abbreviations: AGT, *O*⁶-alkylguanine-DNA alkyltransferase; CID, collision-induced dissociation; G3P, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IOA, iodoacetamide; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; SRM, selective reaction monitoring.

cells. Our results show that reactivity toward *bis*-electrophiles and nuclear location does not necessarily predict the ability of a protein to enhance mutagenesis by these compounds.

Experimental Procedures

Materials

1,2-Dibromoethane, (1,2,3,4)-diepoxybutane (racemic), and CH_2Br_2 were purchased from Aldrich Chemical Co. (Milwaukee, WI). The oligonucleotide 5'-GGAGGAGGAGGAGGAG-3' was synthesized by Midland Certified (Midland, TX) and purified by denaturing gel electrophoresis. Purified human erythrocyte GAPDH was purchased from Sigma-Aldrich (St. Louis, MO) and *E. coli* recombinant human AGT was a gift of A. E. Pegg (Pennsylvania State Univ., Hershey, PA).

GAPDH Activity Assays

Activity assays were performed as previously described (30) using GAPDH. GAPDH was diluted to a concentration of 30 $\mu\text{g}/\text{mL}$ with the reaction buffer prior to incubation. Reactions (1.0 mL) containing 0.25 mM NAD^+ , 3.3 mM dithiothreitol, and 1 $\mu\text{g}/\text{mL}$ GAPDH in 15 mM sodium pyrophosphate buffer (pH 8.5) containing 30 mM sodium arsenate were treated with each *bis*-electrophile dissolved in DMSO for 30 min at 37 °C in a shaking water bath (the concentration of DMSO was <1%, v/v). Reactions were cooled to room temperature for 5 min before the addition of G3P to a final concentration of 0.5 mM. Activity was determined as the change in A_{340} observed for 30 s immediately following the addition of G3P, using a continuous assay in a Cary 14-OLIS spectrophotometer (On-Line Instrument Systems, Bogart, GA).

GAPDH activity of cell lysates was assessed by inducing 5 mL cultures in LB (starting at an OD_{600} of 0.5) for 3 h. Cultures were centrifuged and the pellets were resuspended in 1 mL of 15 mM sodium pyrophosphate buffer (pH 8.5) containing 30 mM sodium arsenate and sonicated for 15 s. Lysate (33 μL) was incubated with the same sodium pyrophosphate/arsenate buffer containing 0.25 mM NAD^+ and 3.3 mM dithiothreitol for 30 min at 37 °C while shaking. Samples were analyzed for activity as described above.

Mass Spectral Analysis

GAPDH (1 μg) was incubated with 10 mM 1,2-dibromoethane or diepoxybutane in 50 μL of 50 mM Tris-HCl (pH 7.6) buffer containing 0.1 mM EDTA for 1 h at 37 °C. Samples were reduced, alkylated, and digested with trypsin as previously described (26). Sample peptide solutions were desalted with P10 C_{18} ZipTips (Millipore, Billerica, MA) according to the manufacturer's instructions (www.millipore.com/userguides.nsf/docs/pr02358). Mass spectral analysis was performed in the positive ion mode by electrospray LC-MS/MS using a TSQ Quantum HPLC-ESI-MS instrument coupled to a Surveyor autosampler and Surveyor MS pump (ThermoElectron, San Jose, CA). Samples were separated as described elsewhere (31) using an ODS-AQ column (5 μm , 2.0 mm $\times$ 150 mm, YMC, Kyoto) with a 98:2:0.05:0.4 (v/v/v/v) mixture of H_2O , CH_3OH , $\text{CF}_3\text{CO}_2\text{H}$, and $\text{CH}_3\text{CO}_2\text{H}$. The Quantum mass spectrometer was equipped with a standard electrospray ionization source and fused silica capillary. The flow rate was maintained at 500 $\mu\text{L}/\text{min}$ and the source CID was used at

10 V. SRM mode analysis was performed for adducted peptides (m/z 759.9, 789.4 [$M+2H$] $^{2+}$) and the unmodified peptide (m/z 737.4 [$M+2H$] $^{2+}$). Mass spectral resolution was set to a peak width of 1.0 u for both precursor and product ions. Mass transitions (m/z 759.9 $\rightarrow$ 936.6 [$M+H$] $^+$, 737.4 $\rightarrow$ 991.1 [$M+H$] $^+$, and 789.4 $\rightarrow$ 996.0 [$M+H$] $^+$); at the specified collision energy (-26 , -24 , and -28 eV, respectively) were monitored for adducted peptides. Data were acquired in the profile mode. Xcalibur Software, version 1.3 (ThermoElectron), was used on a Dell Optiplex GX240 computer (Dell Computer, Round Rock, TX) running a Microsoft Windows 2000 operating system (Microsoft, Redmond, WA) to control all instruments and process data.

Gel Mobility Shift Assays

Gel mobility shift assays were performed as previously described using a 12% SDS (w/v) polyacrylamide gel and the 5'-end ^{32}P -labeled oligonucleotide 5'-GGAGGAGGAGGAGGAG-3' (15). Reactions (10 μ L) contained 2 μ g GAPDH and were incubated for 1 h at 37 °C with CH_2Br_2 , 1,2-dibromoethane, or diepoxybutane prior to analysis.

GAPDH cDNA Synthesis and Expression

The amino acid sequence and native cDNA sequence for human GAPDH was obtained via GenBank (NM_002046). Forty overlapping 40- to 50-mer oligonucleotides were synthesized (Operon, Huntsville, AL) based on DNAWorks results (<http://helixweb.nih.gov/dnaworks/>) (32). The codons were optimized automatically for *E. coli* codon preferences. Overlapping melting temperatures were designed to be 65 ± 3 °C. The 5'- and 3'-flanking sequences were 5'-CCGAATT-3' (sense) and 5'-GACCCCTGGATCCCGC-3' (sense) respectively. Flanking sequences were engineered to contain an *Eco*RI restriction site on the 5'-end and a *Bam*HI restriction site on the 3'-end of the gene. Polymerase cycling assembly and polymerase chain reaction (PCR) amplification of the gene was performed as described elsewhere (32). The PCR product was digested with *Bam*HI and *Eco*RI restriction enzymes (New England Biolabs, Ipswich, MA) and purified by agarose gel electrophoresis. The resulting fragment was ligated into a pINIII-A3(*lpp* $^{p-5}$) vector, which was also digested with *Eco*RI and *Bam*HI restriction enzymes. The ligation mixture was transformed into DH5 α *E. coli* cells and selected for ampicillin resistance on LB plates containing 50 μ g/mL ampicillin. Vectors were purified from surviving colonies with a QIAprep Miniprep kit (Qiagen, Valencia, CA), and restriction digestion with *Eco*RI and *Bam*HI was used to screen for ligated vectors. The GAPDH insert of a plasmid yielding approximately 7 and 1 kb DNA fragments upon restriction digestion was analyzed in the Vanderbilt DNA Sequencing facility using an Applied Biosystems Model 3700 fluorescence sequencing unit with a Taq dye terminator kit (PE Applied Biosystems, Foster City, CA). The sequenced plasmid was amplified and isolated from DH5 α cells and transformed into TRG8 *E. coli* cells, which were selected for with LB plates containing both ampicillin (50 μ g/mL) and kanamycin (50 μ g/mL). GAPDH expression was verified in cells induced with IPTG (0.2 mM) by immunoblot analysis using polyclonal human anti-GAPDH antibodies (Promega, Madison, WI) according to the manufacturer's instructions. Enhanced chemiluminescence (GE, Buckinghamshire, UK) was used to detect protein bands and expression was quantified with an Alpha Imager 3400 (Alpha Innotech, San Leandro, CA).

Cell Survival and Mutagenicity

TRG8 cells expressing AGT or containing the empty pINIII vector served as positive and negative controls, respectively. All strains were induced at an OD₆₀₀ of 0.6 and incubated at 37 °C while shaking for 3 h prior to *bis*-electrophile treatment. The toxic and mutagenic effects of treatment were assessed as described previously (13), but with culture dilutions of 1:10⁶ on *his*⁺ plates.

Electrophoretic Mobility Shift Assays

The DNA binding ability of GAPDH and purified human AGT was analyzed essentially as previously described (33) but using the ³²P-end labeled oligonucleotide 5'-GGAGGAGGAGGAGGAG-3' and 10% (w/v) native polyacrylamide gel. Running buffer containing 10 mM Tris acetate (pH 7.6) and 100 mM NaCl was circulated frequently to prevent buffer exhaustion while samples were run at 8 V/cm following a 30 min pre-run.

Results and Discussion

GAPDH Inhibition

Inhibition of GAPDH activity by sulfhydryl-reactive chemicals, e.g. iodoacetamide (IOA), has been recognized for some time and is known to be the result of alkylation at the active site cysteine (Cys¹⁴⁹) (30). *bis*-Electrophiles are also known to selectively alkylate sulfhydryls, making Cys¹⁵² (located near the active site) a potential target for *bis*-electrophile reactivity. The activity of GAPDH was assessed by monitoring changes in A₃₄₀ in the presence of G3P. Human erythrocyte GAPDH was incubated with various concentrations of CH₂Br₂, 1,2-dibromoethane, or diepoxybutane. A reduction in GAPDH activity was only observed for samples treated with high concentrations of diepoxybutane (Figure 1). The addition of 1,2-dibromoethane, CH₂Br₂, or only DMSO did not affect the ability of GAPDH to reduce NAD⁺. Inhibition was observed with diepoxybutane, but the concentration required for IC₅₀ was 10³-fold higher than the concentration of IOA needed for the same effect. AGT activity is also known to be more sensitive to treatment with diepoxybutane, with an IC₅₀ of 1.0 mM (17). The assays used to monitor GAPDH and AGT activity differ, but comparison of the diepoxybutane concentrations required at IC₅₀ for AGT (17) and GAPDH show a 10-fold difference.

MS Analysis of *bis*-Electrophile-Reactive Cysteine Residues

Crosslink formation between DNA and AGT was previously shown to be dependent on the reaction between the active site cysteine (Cys¹⁴⁵) and *bis*-electrophiles (15), and demonstrated using site-directed mutagenesis with C145A and C145S mutants. The active site cysteine (Cys¹⁴⁹) of GAPDH, also known to be nucleophilic and reactive toward monofunctional electrophiles, made this protein a potential candidate for reacting with *bis*-functional electrophiles (30). In order to identify *bis*-electrophile-reactive residues of GAPDH, MS analysis was used to probe treated protein for hydrolyzed 1,2-dibromoethane and diepoxybutane adducts. Analysis was focused on two tryptic peptides identified in the monofunctional electrophile screen (26), each containing a nucleophilic cysteine residue (Cys¹⁵² and Cys²⁴⁶). One peptide contained Cys¹⁵² as well as the active site cysteine Cys¹⁴⁹, while the second peptide contained the peripheral Cys²⁴⁶, which maps to the

interface of two monomers in the functional homotetramer (34). A 1,2-dibromoethane-induced hydroxyethyl adduct and diepoxybutane-induced trihydroxybutyl adduct (m/z 759.9 and 789.4, respectively) were observed only for the peptides containing Cys²⁴⁶ (Figures 2A, 2B). The ethylene elimination product from 1,2-dibromoethane was also only observed with the Cys²⁴⁶ peptide, while spectra corresponding to unmodified and carboxymethylated cysteines were observed for both peptides, thus confirming ionization of each peptide (data not shown). Selective reaction monitoring (SRM) was used to verify that 1,2-dibromoethane and diepoxybutane hydrolysis adducts are distinguishable due to the differential elution and reaction specificity for each *bis*-electrophile (see Supporting Information, Figure S-1).

GAPDH-DNA Crosslinking Assays

The mutagenic properties of *bis*-electrophiles were first shown to be enhanced in systems overexpressing GSH transferases in 1978 (14). Subsequently this phenomenon was also identified in systems overexpressing AGTs (11). *bis*-Electrophiles induced mutations in these systems through induction of DNA-GSH and DNA-AGT crosslinks by reaction between nucleophilic sulfhydryls, *bis*-electrophiles, and DNA (15, 19). In order to assess the ability of *bis*-electrophiles to form crosslinks between DNA and GAPDH, reactions containing ³²P-labeled 16mer oligonucleotides, GAPDH, and *bis*-electrophile were incubated at 37 °C for 1 h and analyzed for crosslinking using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The only significant crosslink formation between GAPDH and DNA was observed with diepoxybutane (Figure 3) and was dependent on incubation time and *bis*-electrophile concentration (data not shown). Treatment of crosslink reactions with hot piperidine resulted in the disappearance of the slower migrating band, consistent with the *N*⁷-guanine or *N*³-adenine crosslinks formed between AGT and DNA (16). As in the activity assays, very high concentrations (20 mM) of diepoxybutane were required to induce *in vitro* crosslink formation.

In contrast to the expected reactions at Cys¹⁵², *bis*-electrophile adducts were only observed with Cys²⁴⁶. Indeed, reactions between Cys²⁴⁶ and other cellular electrophiles have been previously reported (35). The lack of reactivity at Cys¹⁵² seemed inconsistent with the inhibition assay results. The possibility exists that reactions at the peripheral Cys²⁴⁶ result in an inhibitory structural change or protein-protein crosslink formation, which has been previously documented (36). Supporting this potential mechanism, silver staining of a polyacrylamide gel containing *bis*-electrophile-treated GAPDH indicated the presence of a high *M*_r band consistent with that of a GAPDH dimer (data not shown).

Overexpression of GAPDH in *E. coli* Cells and *bis*-Electrophile-Induced Mutagenesis

Based on the precedent of AGT work (15), human GAPDH was heterologously expressed in *E. coli* TRG8 cells engineered to lack endogenous *O*⁶-alkylguanine transferase background (*ogt*[−], *ada*[−]). Immunoblot analysis was used to verify overexpression of GAPDH in pINIII-hGAPDH-transformed cells through comparison with cells containing the empty pINIII vector (Figure 4A). Quantification of AGT expression by immunoblot analysis showed values (17 nM) similar to that of GAPDH expression (14 nM) 3 h after induction, showing that lack of mutagenesis was not due to lower protein levels. The lysate from cells overexpressing recombinant GAPDH also displayed 2-fold higher total GAPDH activity

(both *E. coli* and human enzymes) in comparison with control cells (1.00 ± 0.16 versus 0.52 ± 0.10 $A_{340} \text{ min}^{-1} \text{ mL}^{-1}$) providing evidence that an active form of this enzyme was being produced.² Toxicity and mutagenicity of diepoxybutane treatment utilized the pINIII-containing strain as controls due to some bleed-through expression of GAPDH in uninduced TRG8 cells (without IPTG). GAPDH over-expression did not significantly affect the growth rate of these cells (results not shown). Treatment of cells expressing AGT or GAPDH or containing empty pINIII vectors with diepoxybutane resulted in reduced survival for all strains, consistent with previous AGT studies (17) (Figure 4B).

In order to assess the effect of GAPDH overexpression on mutagenesis by diepoxybutane, treated cells were plated on restrictive plates lacking histidine and grown for several days, allowing selection of cells containing reversion mutations in the *hisG* gene. As previously observed, AGT expression in diepoxybutane-treated *E. coli* resulted in more *his*⁺ reversion mutants (17); however, GAPDH expression did not significantly affect the mutation rate (Figure 4C).

Reduced DNA-binding Ability of GAPDH in Comparison with AGT

Incubation of GAPDH with high concentrations of diepoxybutane was found to inhibit activity and induce *in vitro* DNA-protein crosslinks (Figures 1, 3) and MS analysis also confirmed the reactivity of nucleophilic Cys²⁴⁶ with *bis*-electrophiles (Figure 2). However, overexpression of GAPDH in cells treated with diepoxybutane did not enhance mutagenesis (Figure 4). GAPDH and AGT are both reactive with *bis*-electrophiles *in vitro*, but overexpression of these proteins in treated cells had very different consequences. In order to understand this apparent discrepancy, we considered differences between the inherent characteristics of each protein. One of the most obvious is that AGT is a nuclear protein known to bind and repair DNA (33, 39). Conversely, GAPDH is predominantly cytosolic although some nuclear functions have been proposed (27) and DNA-binding ability has been documented (40). One possibility is that the lack of mutation enhancement in treated cells overexpressing GAPDH is due to differing nuclear locations with respect to DNA during reactions with *bis*-electrophiles. Assessment of the differences in DNA binding ability between AGT and GAPDH indicated that AGT associates with DNA to a much greater extent than GAPDH (Figure 5), which may explain the lack of mutagenesis observed for GAPDH.

Conclusions

As in the case of AGT, treatment of GAPDH with diepoxybutane results in inhibition of activity and DNA-protein crosslink formation in *in vitro* assays in response to treatment with diepoxybutane. Although the reactivity of GAPDH with both diepoxybutane and 1,2-dibromoethane was validated with MS results, the overexpression of this protein in treated *E. coli* cells did not result in enhanced mutagenesis, in contrast with AGT (15). The lack of

²The fold increase in the total GAPDH activity is not as high as with AGT, because the endogenous activity is essential for the cells and cannot be deleted. The appropriate comparison is the total molar concentration, determined immunochemically (*vide supra*). However, the point is made that our data show that the heterologously expressed human GAPDH is catalytically active in *E. coli* cells, as expected from literature precedents (37, 38).

mutational enhancement may be due to the inherently lower DNA binding ability of GAPDH (Figure 5), which could reduce the efficiency of crosslink formation. In addition, the high concentration of diepoxybutane required to inhibit GAPDH activity (Figure 1) and induce crosslink formation (Figure 3) suggest that GAPDH is also inherently less reactive toward *bis*-electrophiles than AGT. These findings provide evidence that not all *bis*-electrophile-reactive proteins are capable of enhancing mutagenesis and suggest that *in vivo* crosslinking depend in part on the inherent characteristics of a protein. These studies also provide evidence that proteins display varying degrees of reactivity toward different *bis*-electrophiles, with AGT being more reactive toward 1,2-dibromoethane (17) and GAPDH selectively reacting with diepoxybutane. Other protein candidates are currently being investigated for their ability to react with and enhance mutagenicity of *bis*-electrophiles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Inhibition of GAPDH activity by diepoxybutane. Reactions (1.0 mL) containing 30 μ M GAPDH were incubated with various concentrations of the *bis*-electrophiles CH₂Br₂ (▲), 1,2-dibromoethane (▼), or diepoxybutane (■) in sodium pyrophosphate (15 mM) buffer containing 30 mM arsenic pyrophosphate, 250 μ M NAD⁺, and 3 mM dithiothreitol for 30 min at 37 °C in a shaking water bath. The change in A₃₄₀ was monitored for 30 s following the addition of 0.25 μ mol G3P and inhibition was compared with IOA (◆) treatment.

Figure 2.

MS analysis of Cys²⁴⁶ adducts formed from 1,2-dibromoethane or diepoxybutane-treated GAPDH. GAPDH (1 μ g) was incubated in AGT buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.1 mM dithiothreitol) with 10 mM 1,2-dibromoethane or diepoxybutane for 1 h at 37 °C and reduced, alkylated, and digested with trypsin. Tryptic peptides were separated and analyzed by MS. The hydrolyzed adducts resulting from incubation with (A) 1,2-dibromoethane or (B) diepoxybutane were identified as conjugated only to Cys²⁴⁶, resulting in peptides displaying m/z 759.4 and 789.9, respectively.

Figure 3.

In vitro formation of GAPDH-DNA crosslinks by diepoxybutane. Human GAPDH (2 μ g) was incubated with 32 P-labeled 16mer oligonucleotide in AGT buffer (50 mM Tris-HCl (pH 7.4), 0.1 mM dithiothreitol, and 1 mM EDTA) containing DMSO vehicle (<1%, v/v) (lane 1), 20 mM CH₂Br₂ (lane 2), 20 mM diepoxybutane (lane 3), or 20 mM 1,2-dibromoethane (lane 4) at 37 °C for 1 h. Samples were separated on a 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel, resulting in the formation of a distinct, slowly migrating band corresponding to a DNA-protein crosslink present in only diepoxybutane-treated sample.

Figure 4.

Lack of enhanced mutagenesis by diepoxybutane in cells overexpressing GAPDH. (A) Immunoblot analysis of GAPDH expression induction by TRG8 *E. coli* cells containing pINIII-hGAPDH vectors in comparison with those transformed with the empty pINIII vector showed significant production of hGAPDH. Expression of GAPDH (14 nM) was similar to that of AGT (17 nM) 3 h after induction. (B) TRG8 *E. coli* cells expressing GAPDH (▼) or AGT (▲) or containing the empty pINIII vectors (■) were treated with a range of diepoxybutane concentrations for 30 min at 37 °C prior to plating. The toxicity of diepoxybutane was shown to reduce the percent survivorship for all three strains. (C) Mutational frequency was scored as the number of *his*⁺ revertant colonies on histidine deficient plates per 10⁸ survivors.

Figure 5.

DNA-binding ability of GAPDH and AGT. Human GAPDH or human AGT (0 – 50 μ M) was incubated with 6 μ M 32 P-16mer in binding buffer (10 mM Tris (pH 7.6), 100 mM NaCl, 1 mM dithiothreitol, and 10 μ g/mL bovine serum albumin) for 30 min at 23 $^{\circ}$ C. Samples were separated on a native 10% (w/v) polyacrylamide gel at 8 V/cm and protein-DNA complexes were quantified using autoradiography.