Function of Domains of Human O^6-Alkylguanine-DNA Alkyltransferase†

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ABSTRACT: O^6-Alkylguanine-DNA alkyltransferase (AGT) is an important DNA repair protein that protects from alkylating agents by converting O^6-alkylguanine to guanine forming *S*-methylcysteine in the AGT protein. The crystal structure of human AGT shows clearly the presence of two domains. The N-terminal domain contains a bound zinc atom, and zinc binding confers a mechanistic enhancement to repair activity, but this domain has no known function. The C-terminal domain contains all residues so far implicated in alkyl transfer including the cysteine acceptor site (Cys145), the O^6-alkylguanine binding pocket, and a DNA binding domain. We have expressed and purified the two domains of human AGT separately. The C-terminal domain was totally inactive in vitro, but good activity forming *S*-alkylcysteine at Cys145 was obtained after recombination with the N-terminal domain via a freeze−thawing procedure. This suggests that the N-terminal domain plays a critical structural role in maintaining an active configuration of the C-terminal domain. However, this C-terminal domain alone had activity in protecting against the cytotoxic and mutagenic activity of the methylating agent, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) when expressed in *Escherichia coli* cells lacking endogenous AGT, suggesting that other proteins can fulfill this function. Remarkably, the free N-terminal domain of hAGT was able to repair O^6-alkylguanine in vitro via alkyl transfer provided that zinc ions were present. The N-terminal domain was also able to produce moderate protection from MNNG when expressed in *E. coli*. This cryptic Zn^{2+}-dependent DNA repair activity may be relevant to the evolution and function of AGTs.

O^6-Alkylguanine adducts are formed in DNA by many alkylating agents including important environmental carcinogens such as nitrosamines. These adducts are repaired by a unique single step mechansim mediated by O^6-alkylguanine-DNA alkyltransferases (AGTs[1]) (*1*−*5*). AGTs transfer the alkyl group from O^6-alkylguanine to a cysteine residue located within the protein, and each molecule can act only once since the *S*-alkylcysteine formed is not regenerated. This transfer protects against the mutagenic and cytotoxic effects of alkylating agents, and the content of AGT is a critical factor in determining cellular sensitivity to these agents (*6*, *7*). The AGT family members share a highly conserved −PCHRV− amino acid sequence surrounding the cysteine acceptor site and contain a number of other conserved amino acids in the active site and DNA binding domain of the protein. Using these motifs, the presence of an *AGT* gene has been identified in more than 100 different species from the three domains of life, Archaea, Bacteria, and Eukarya. However, it is not ubiquitous being absent from plants, *Schizosaccharomyces pombe* and *Deinococcus radiodurans*.

Escherichia coli contains two AGT genes *Ada* and *Ogt* (*8*, *9*). The *Ada* encoded protein is a bifunctional protein, which acts both as a transcriptional regulator activating genes dealing with alkylation damage and as an AGT that repairs O^6-methylguanine as described above. The two halves of this Ada protein can be expressed separately in functional forms (*10*). The Ada-C protein derived from residues 175−354 has been crystallized and its structure determined (*11*). The structure is generally similar to that of the two other AGTs for which structures are available, from human (*12*, *13*) and *Thermococcus kodakaraensis* KOD1 (*14*). The transcriptional regulator derived from residues 1−178 of Ada is activated via a methyl transfer from methylphosphotriesters in DNA to a cysteine residue, Cys69 (*15*, *16*). This cysteine is one of 4 cysteine residues that form a complex with a tightly bound zinc atom. This metal is needed for the activation of the cysteine to carry out methyl transfer (*16*−*19*). Methylation at Cys69 alters the protein structure in a way that greatly increases affinity to the regulatory DNA sequences in Ada responsive genes (*16*). *Salmonella typhimurium* contains a bifunctional protein similar to Ada, but *Bacillus subtilis* contains two separate proteins encoded by genes *adaA* and *adaB* corresponding to the transcriptional regulator and the AGT, respectively (*20*, *21*).

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[1] Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; hAGT, human AGT; IPTG, isopropyl β-D-thiogalactopyranoside; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; BG, O^6-benzylguanine; N-hAGT, the amino-terminal fragment composed of residues 1−91 of hAGT; C-hAGT, the carboxyl-terminal fragment of hAGT composed of residues 92−207; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; TAE−PAGE, Tris-acetate−polyacrylamide gel electrophoresis; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; TFA, trifluoroacetic acid; cAGT-2, AGT-2 from *C. elegans;* Fa-AGTendoV, AGT from *F. acidarmanus*.

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The three currently known AGT crystal structures all exhibit a similar two-domain structure with all the residues that form the helix−turn−helix DNA binding motif and the active site pocket being present in the C-terminal domain (*11*−*14*). This domain contains multiple highly conserved residues including those involved in nucleotide flipping and activation of the cysteine acceptor site [Cys145 in human AGT (hAGT)], which has a very low pK_a (*22*), to promote alkyl transfer. The N-terminal domains have virtually no similarity in amino acid sequence but are quite similar in three-dimensional structure. The N-terminal domain of the hAGT contains a bound zinc atom (*13*). This zinc is not present in the two known microbial AGT structures and is not essential for repair activity of hAGT, but its presence increases the rate of DNA repair (*23*). The mechanism of this effect is unclear since the Cys145 residue is some distance from the zinc atom and zinc binding may simply play a structural role in maintaining the protein structure in an optimal form for alkyl transfer.

To clarify the role of the N-terminal domain of hAGT we have expressed both the N-terminal and C-terminal domains separately. The C-terminal domain was totally inactive in vitro despite the presence of all residues so far implicated in AGT activity, but activity was restored when it was combined with the N-terminal domain in ways allowing the two fragments to renature together, suggesting that the N-terminal domain plays a critical structural role in maintaining the C-terminal domain in an active configuration. Remarkably, the N-terminal domain alone had a weak AGT activity in vitro that was totally zinc dependent. Both domains were able to provide protection from methylation damage when expressed in *E. coli*.

EXPERIMENTAL PROCEDURES

Materials. Oligodeoxyribonucleotides were purchased from Life Technologies Inc. (Gaithersburg, MD) or Qiagen Operon (Alameda, CA). *E. coli* XL1-blue bacterial strain and Pfu polymerase enzyme were purchased from Stratagene (La Jolla, CA). DNA isolation kits and the pQE-30 plasmid were obtained from Qiagen (Chatsworth, CA). Restriction enzymes *Eco*RI, *Nde*I, *Hin*dIII, and *Kpn*I were obtained from Invitrogen (Carlsbad, CA), and *Eco*47III and *Bam*HI were purchased from New England Biolabs (Beverly, MA). Ampicillin, isopropyl β-D-thiogalactopyranoside (IPTG), hemocyanin, calf thymus DNA, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), and most other biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Talon Metal Affinity IMAC Resin and T4-ligase with the Ligation Express kit were purchased from Clontech (Palo Alto, CA). Urea was purchased from Roche Diagnostics (Indianapolis, IN). Nitrocellulose filters (0.45 μm) were obtained from Millipore (Billerica, MA). O^6-Benzylguanine and oligodeoxyribonucleotides containing O^6-methyldeoxyguanosine or O^6-benzyldeoxyguanosine were generously provided by Dr. R. C. Moschel (ABL-Basic Research program, NCI-Frederick Cancer Research and Development Center, Frederick, MD) (*24*). [γ-^{35}S]ATPγS (10 mCi/mL) and Sephadex MicroSpin G-25 column were purchased from Amersham Biosciences.

Construction of pQE Plasmids for Expression of N-Terminal Domain and C-Terminal Domain of hAGT. Plasmids for the preparation of recombinant N-hAGT (residues 1−91) and C-hAGT (residues 92−207) were constructed from previously described plasmids pQE30-hAGT [which has an N-terminal $(His)_6$-tag in a sequence replacing the terminal M− with the sequence MRGS$(H)_6$GS− (*25*)] and pQE-hAGT (which has a C-terminal $(His)_6$-tag replacing residues 202−207 −PPAGRN with $(H)_6$ (*26*)]. PCR was used to isolate the relevant sequences. Primers 5′-d(TTGTGAGCGGATAACAAATTC)-3′ and 5′-d(ATACGTGGTACCTCATTGCTGGAAAAC)-3′ with a *Kpn*I site (underlined) following the stop codon were used as the sense and antisense primers for N-hAGT. Primers 5′-d(CACACAGAATTCATTAAAGAGAAATTAACTATGGAGTCGTTCACGCGT)-3′ with an *Eco*RI site (underlined) and 5′-d(GGATCTATCAACAGGAGTCC)-3′ were used as the sense and antisense primers for C-hAGT. The PCR was carried out using Pfu polymerase under the following conditions: initial denaturation for 2 min at 94 °C, 30 cycles of denaturation (30 s at 94 °C), annealing (30 s at 56 °C) and extension (1 min at 72 °C) followed by a final extension at 72 °C for 5 min. The PCR product and plasmid vector were digested with *Eco*RI and *Kpn*I enzymes, and the products were purified with QIAquick Gel Extraction kit (Qiagen). The fragments containing the truncated hAGT sequences were ligated into a pQE30 vector digested with the same enzymes. The resulting plasmids were used to transform XL1-Blue cells and termed pQE-N-hAGT and pQE-C-hAGT, respectively. The entire coding region of both plasmids was verified by DNA sequencing carried out by the Macromolecular Core Facility, Hershey Medical Center.

Plasmids for the production of the C5A and C24A mutants of N-hAGT were constructed from these mutants in pQE30-hAGT (*23*). The plasmids were cut with *Eco*RI and *Eco*47III, and the 311 bp fragment corresponding to the sequence encoding residues 1−84 of hAGT was inserted into pQE-N-hAGT digested with these enzymes.

Protein Purification. The XL1-Blue cells containing pQE-N-hAGT and pQE-C-hAGT plasmids were used for protein expression and purification with immobilized metal affinity chromatography essentially as previously described for pQE30-hAGT (*25*). Cells were grown in LB medium at 37 °C until an OD_{600} of 0.5−0.7 was reached. IPTG was added to 200 μM, and the cells were collected after 4 h. Cell pellets were lysed in 20 mM Tris-HCl pH 8.0, 250 mM NaCl, and 8 M urea buffer and centrifuged to remove cell debris. The supernatant fraction was collected and applied to a column containing Talon IMAC resin equilibrated with the same buffer. After the column was washed extensively with the buffer, the $(His)_6$-tagged protein was eluted with 200 mM imidazole in the same buffer. The fractions containing protein were pooled, and calf thymus DNA was added to a final concentration of 0.5−1 mg/mL. The protein was then dialyzed against decreasing concentrations of urea in 50 mM Tris-HCl pH 7.6, 100 mM NaCl, 5 mM dithiothreitol (DTT), 5% glycerol plus or minus 50 μM $ZnCl_2$ as indicated. The mixture was first dialyzed against the buffer containing 5 M urea for 12 h, followed by dialysis for 6−12 h against 2.5, 1.0, and 0 M urea in the above buffer. A final dialysis against 50 mM Tris-HCl pH 7.6, 100 mM NaCl, 5 mM DTT, and 5% glycerol was used to remove free zinc. The purified proteins were analyzed by SDS−PAGE on 12% gels.

Reconstitution of N-hAGT and C-hAGT. Recombination of the N-hAGT and C-hAGT was carried out by three methods: (a) direct mixing at 4 °C of equal amounts of the proteins purified separately as described above; (b) mixing of the purified denatured proteins in 20 mM Tris-HCl pH 8.0, 250 mM NaCl, and 8 M urea buffer followed by dialysis/renaturing against decreasing amounts of urea as described above; (c) direct mixing of equal amounts of the purified proteins at 4 °C followed by freezing at −20 °C for 1 day and thawing at 4 °C. This process was repeated up to 5 times.

AGT Activity Assays. Several methods were used. (a) AGT activity was measured by determining the transfer of [^{3}H]-methyl groups to AGT from [^{3}H]-methylated calf thymus DNA (*26*). The protein was incubated with 10 μg of [^{3}H]-methylated DNA substrate and 50 μg of hemocyanin in 1.0 mL of 50 mM Tris-HCl pH 7.6, 5 mM DTT, and 0.1 mM EDTA for 2 h at 37 °C. The [^{3}H]-methylated protein formed was collected on nitrocellulose filters and counted as described (*26*). At the counting efficiency used, 1 fmol of methyl groups transferred equaled 22 cpm.

(b) The AGT activity was measured by determining the loss of radioactivity from O^6-[^{3}H]methylguanine in the [^{3}H]-methylated DNA substrate (*24*). After incubation of the protein with 10 μg of [^{3}H]-methylated DNA substrate and 50 μg calf thymus DNA in 1.0 mL of 50 mM Tris-HCl pH 7.6, 5 mM DTT, and 0.1 mM EDTA for 2 h at 37 °C, 143 μL of 2 N perchloric acid was added to precipitate the DNA. The pellet was hydrolyzed in 0.5 mL of 0.1 N HCl at 70 °C for 30 min. After centrifugation, the supernatant from the hydrolysis was separated by isocratic high pressure liquid chromatography (HPLC) using 50 mM ammonium formate pH 4.5 in 20% methanol at a flow rate of 1 mL/min for 20 min at 21 °C on a Beckman reverse-phase C-18 column. The effluent was analyzed using a Packard Series A-100 radioactivity monitor.

(c) Assays were conducted using as substrates either double stranded 11-mer 5′-d(GCGCAm^{6}GAGTCG)-3′ annealed with its complement or the 16-mers 5′-d(AACAGGCCATATm^{6}GGCCC)-3′ and 5′-d(AACAGGCCATATb^{6}GGCCC)-3′ (where m^{6}G represents O^6-methyldeoxyguanosine and b^{6}G represents O^6-benzyldeoxyguanosine) (*27*). The protein was incubated with one of these substrates (100 pmol) in 0.1 mL of 50 mM Tris-HCl pH 7.6, 5 mM DTT, and 0.1 mM EDTA at 21 °C for 1 h. The reaction was stopped by addition of 11 μL of 10% SDS, and the samples were separated by HPLC on a Beckman reverse-phase C-18 column at 40 °C using a flow rate of 1 mL/min and a gradient of 10−40% methanol in 25 mM Na phosphate buffer pH 6.3 for 60 min.

(d) A 70-mer 5′-d(CACGGGACCGTACACGCAGTAGCCGATGACAGCTm^{6}G*CAGCTG*AGTAGCCGATGACGCACATGCCAGGGCAC)-3′ was used as a substrate for the assays. This substrate when converted to its double stranded form contains two adjacent sequences (underlined) that are potential sites for cleavage by *Pvu*II, one of which (italics) can be cleaved by this enzyme directly (*12*). The other site contains an O^6-methylguanine residue that prevents cleavage unless the substrate is repaired. The oligo and an unmethylated control were 5′-end labeled with [γ-^{35}S]ATPγS and T4 polynucleotide kinase at 37 °C for 1 h, heated at 65 °C for 20 min, and passed through a Sephadex MicroSpin G-25 column to remove unincorporated [γ-^{35}S]ATPγS. The [^{35}S]-labeled 70-mer was then mixed with 1.5× mol excess reverse complement at 100 °C and allowed to cool slowly to room temperature. The resulting double stranded substrate (6 pmol) was incubated with the protein in 30 μL of 10 mM Tris-HCl pH 7.6, 5 mM DTT, 0.1 mM EDTA for 1 h at 37 °C. After heating at 65 °C for 20 min, 3 μL of 10× *Pvu*II buffer (New England Biolabs) and 20 units of *Pvu*II were added followed by incubation for 1 h at 37 °C. The resulting 32-mer and 38-mer cleavage products were loaded onto 20% gels (37.5:1 acrylamide:bis-acrylamide) and separated by TAE−PAGE at 160 V for 3 h. The gel was fixed, vacuum-dried, and exposed to Kodak film for 20 h at −78 °C.

Inactivation of AGT Activity by O^6-Benzylguanine. The proteins tested were incubated with different concentrations of O^6-benzylguanine at 37 °C for 30 min in 0.5 mL of 50 mM Tris-HCl pH 7.6, 5 mM DTT, and 0.1 mM EDTA. The residual AGT activity was then determined by method (a) described above. The results were expressed as the percentage of the AGT activity remaining, and the graphs of remaining AGT activity against inhibitor concentration were used to calculate the ED_{50} value representing the amount of inhibitor needed to produce a 50% loss of activity.

MALDI-TOF MS Analysis. MS analysis was carried out by the MS/Proteomics Core Facility at the Penn State College of Medicine. The proteins were incubated with excess free O^6-benzylguanine in 50 mM Tris-HCl pH 7.6, 5 mM DTT, and 1 mg/mL calf thymus DNA for 1 h at 37 °C. Samples were dialyzed against 50 mM NH_4HCO_3 pH 8.0 for 18−24 h with the dialysis buffer changed every 6−8 h. The samples were then digested with modified trypsin in 50 mM NH_4HCO_3 pH 8.0 with 10% v/v acetonitrile for 3 h at 50 °C. The reaction was stopped by addition of 4 μL of glacial acetic acid, and the digested samples were evaporated and resuspended in 200 μL of deionized water 3 times to remove volatile digestion buffers. The final suspension was evaporated to 10 μL, and then 1.0% trifluoroacetic acid (TFA) was added to bring the final TFA concentration to 0.1%. Using a BioHit Multipipettor, ZipTip SCX tips were equilibrated three times with 10 μL of 0.1% TFA, and then the sample aliquots were pipetted up and down across the resin 15 times to bind and concentrate peptides. The bound peptides were washed five times with 10 μL of 0.1% TFA to remove salts, followed by elution of the bound peptides by carefully pipetting 2 μL of freshly prepared 5% NH_4OH:30% methanol up and down 4−5 times. The droplet containing the eluted peptides was deposited onto a polished stainless steel MALDI target plate (Applied Biosystems) and allowed to dry. After complete drying, each spot was overlaid with 0.6 μL of freshly prepared CHCA matrix (5 mg/mL recrystallized α-cyano-hydroxycinnamic acid:2 mg/mL $NH_4H_2PO_4$ in 50% acetonitrile:0.1% TFA). MALDI-TOF analyses were performed on a 4700 Proteomics Analyzer (Applied Biosystems), acquiring 1000 total shots/spectrum (25 shots each from 40 different regions of each spot) at a laser intensity of 1550. Spectra were collected for the *m*/*z* range between 400 and 4000, with a focus-mass setting of 1600 *m*/*z*. Spectra were initially calibrated using a plate-wide default calibration acquired immediately before the sample spectra were acquired, and then final calibration was done using hAGT peaks at 931.51, 1085.534, 1668.944, and 1906.007. The mass errors for these internal standard peaks after the calibration ranged from −1.76 ppm to 3.85 ppm.

Protection of E. coli from Damage Induced by MNNG. *E. coli* strain GWR109 cells that lack endogenous AGT activities (*28*) were transformed with plasmids pQE30, pQE30-hAGT, pQE-N-hAGT, or pQE-C-hAGT. The cells were grown overnight at 37 °C in 1 mL of LB medium containing 75 μg/mL ampicillin and 50 μg/mL kanamycin, and then 20 μL was inoculated into 0.98 mL of fresh LB medium. After growing for 2−2.5 h until OD_{600} reached 0.6−0.8, IPTG was added to a final concentration of 150 μM. After incubation for 30 min, the cultures were exposed to different amounts (0 to 15 μg/mL) of MNNG for 30 min. Appropriate dilutions of the culture were then spread on LB plates containing 75 μg/mL ampicillin and 50 μg/mL kanamycin. The plates were incubated for 16−20 h at 37 °C, and surviving colonies were scored. A survival curve was plotted against dose of MNNG. Mutation frequency was measured using a forward mutation assay to rifampicin resistance (*29*) essentially as previously described (*30*). To identify cells with a mutation in the *rpoB* gene, MNNG-treated cells were diluted and spread on LB plates containing 75 μg/mL ampicillin, 50 μg/mL kanamycin, and 100 μg/mL rifampicin. The number of viable cells was counted following incubation at 37 °C for 2 days. The mutation frequency was expressed as the number of *rpoB* mutants per 10^6 survivors, after adjusting to the level of surviving cells when grown on LB plates lacking rifampicin.

The expression level of the $(His)_6$-tagged proteins was determined by immunoblotting using a Penta His antibody (Qiagen, CA). Cell pellets were lysed in 50 mM Tris-HCl pH 7.6, 250 mM DTT, 1% SDS and heated at 100 °C for 7 min. The samples were resolved by 15% SDS−PAGE gel and electrotransferred to PVDF membranes (Pall life sciences, FL). Detection was carried out using the Penta His antibody as primary antibody with HRP linked anti mouse IgG at 1:2000 dilution as secondary antibody and the chemiluminescent reagent lumiGLO (Cell signal technology, MA).

RESULTS

Isolation of Domains of Human AGT. Plasmids were constructed for the production of the 91 amino acids forming the N-terminal domain of hAGT (N-hAGT) and the C-terminal domain formed by residues 92−207 (C-hAGT) in *E. coli*. Both proteins were predominantly insoluble but were readily solubilized in the presence of 8 M urea and purified in 8 M urea using $(His)_6$-tags. Full length hAGT protein was soluble when expressed at high levels in *E. coli* but was also purified using the denaturing conditions as a control. The proteins were then renatured by dialysis against decreasing concentrations of urea to refold the protein. To prevent the C-hAGT from aggregating and precipitating from solution, calf thymus DNA was added prior to refolding. Under these conditions, all three proteins were soluble and were readily obtained in a pure form (Figure 1).

Activity of Domains of hAGT. When tested for the ability to transfer radioactivity from a [^{3}H]-methylated DNA substrate to the protein, the C-hAGT was totally inactive. However, surprisingly the N-hAGT had a very weak but definite activity that was totally dependent on the refolding being carried out in the presence of zinc ions. Mutation of either Cys5 or Cys24, which are two of the residues that form a complex with zinc in the hAGT crystal structure, prevented the zinc-dependent alkyl transferase by N-hAGT (Table 1). Although the [^{3}H]-methylated-DNA substrate contains multiple methylated products including methylphosphotriesters, it is very likely that this alkyl transfer activity was due to the ability of the N-hAGT to act on O^6-methylguanine rather than other methylation products because the activity was totally abolished if the DNA substrate was pretreated with an excess of either hAGT or Ogt that removed virtually all of the O^6-methylguanine (data not shown).

Since the ability of N-hAGT to repair O^6-methylguanine was unexpected, several experiments were carried out to confirm this finding. First, the [^{3}H]-methylated-DNA substrate was incubated with an excess of N-hAGT, the DNA was subjected to weak acid hydrolysis to release purine bases, and the bases were then separated by HPLC. Prior to incubation or after incubation without N-hAGT there were 14 488 cpm in the peak corresponding to 7-methylguanine and 1573 cpm in the peak corresponding to O^6-methylguanine. After incubation with 83 pmol of N-hAGT, there was no change in the peak corresponding to 7-methylguanine (14 695 cpm) but the peak corresponding to O^6-methylguanine was reduced to 550 cpm. After incubation with 166

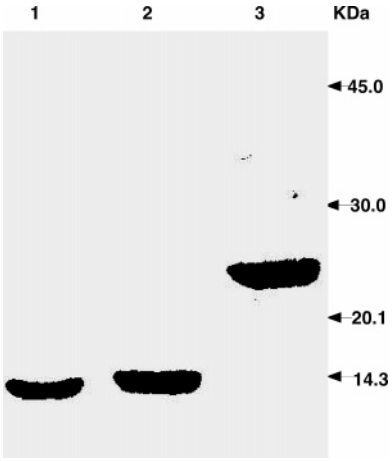

FIGURE 1: Purity of N-hAGT, C-hAGT, and hAGT. The proteins were isolated under denaturing conditions, renatured, and analyzed by SDS−PAGE as described under Experimental Procedures. The gels were stained with Coomassie brilliant blue. Lane 1: N-hAGT. Lane 2: C-hAGT. Lane 3: hAGT. Molecular markers shown on right side.

Table 1: Alkyl Transfer Activity of N-hAGT and C-hAGT

AGT added	addition during refolding	labeled protein formed (fmol/pmol AGT added)[a]
N-hAGT	none	0[b]
C-hAGT	none	0[b]
N-hAGT	Zn^{2+}	1.7
C-hAGT	Zn^{2+}	0[b]
N-hAGT (C5A)	Zn^{2+}	0[b]
N-hAGT (C24A)	Zn^{2+}	0[b]
N-hAGT + C-hAGT[c]	none	1.2
N-hAGT + C-hAGT[c]	Zn^{2+}	14.3
hAGT[c]	Zn^{2+}	68.2

[a] In every case where a value is given, sufficient protein was added to give at least 2000 cpm transferred, which is more than 10 times the background and was significantly different from background ($p < 0.001$). [b] No transfer was detected with 720 pmol of protein. [c] Refolding was carried out as described in the legend to Figure 4.

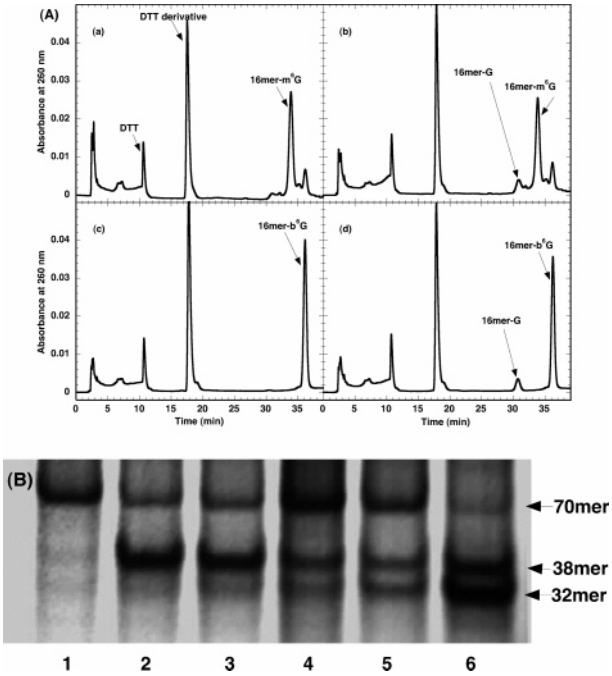

FIGURE 2: Repair of O^6-methylguanine by N-hAGT. Figure 2A shows the repair of 16-mers 5′-d(AACAGGCCATATm^{6}GGCCC)-3′ (panels a and b) or 5′-d(AACAGGCCATATb^{6}GGCCC)-3′ (panels c and d). After incubation of 250 pmol of these 16-mers with 3900 pmol of N-hAGT for 1 h at 21 °C (panels b and d) or incubation without N-hAGT (panels a and c), the products were separated by HPLC. Figure 2B shows the repair of 5′-[^{35}S]d-(CACGGGACCGTACACGCAGTAGCCGATGACAGCTm^{6}G-*CAGCTG*AGTAGCCGATGACGCACATGCCAGGGCAC)-3′. As described in Experimental Procedures, repair was demonstrated by the ability of *Pvu*II to cleave at the first site (underlined) to generate a 32-mer product. The 38-mer derived from *Pvu*II cleavage of the second site (underlined italics) acts as a control. Lane 1: reaction buffer containing no AGT and no *Pvu*II. Lane 2: reaction buffer containing *Pvu*II but no AGT. Lane 3: 50 pmol of N-hAGT plus *Pvu*II. Lane 4: 100 pmol of N-hAGT plus *Pvu*II. Lane 5: 200 pmol of N-hAGT plus *Pvu*II. Lane 6: 30 pmol of hAGT plus *Pvu*II. The ratio of the 38-mer to 32-mer was 4.45, 3.11, 1.00, 0.96, and 0.76 from lanes 2 to 6, respectively.

pmol of N-hAGT, there were 14 785 cpm in the peak corresponding to 7-methylguanine and no cpm above background in the peak corresponding to O^6-methylguanine. These results show clearly that the incubation with N-hAGT selectively removed O^6-methylguanine from the substrate DNA.

Second, the ability to convert either O^6-methyldeoxyguanosine or O^6-benzyldeoxyguanosine in DNA to deoxyguanosine by N-hAGT was confirmed by incubation of 16-mers [5′-d(AACAGGCCATATm^{6}GGCCC)-3′ or 5′-d(AACAGGCCATATb^{6}GGCCC)-3′] with the protein. As shown in Figure 2A, this led to the formation of 5′-d(AACAGGCCATATGGCCC)-3′.

Finally, the N-hAGT was incubated with a double stranded 70-mer containing two *Pvu*II sites, one of which contained an O^6-methylguanine preventing cleavage by this restriction enzyme. The strand containing O^6-methylguanine was labeled at the 5′ end. After incubation with *Pvu*II, this substrate generated only a labeled 38-mer product from the unblocked site. After incubation with either N-hAGT or intact hAGT, a labeled 32-mer product was also generated from cleavage at the second site which was revealed by conversion of the O^6-methylguanine to guanine (Figure 2B).

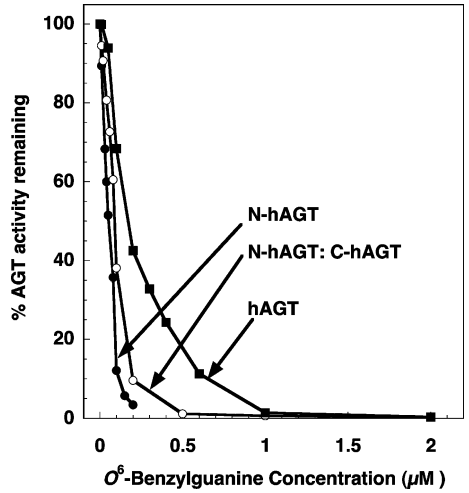

FIGURE 3: Inactivation of hAGT, N-hAGT:C-hAGT mixture, and N-hAGT by O^6-benzylguanine. Results are shown for hAGT (filled squares), N-hAGT:C-hAGT mixture (open circles), and N-hAGT (filled circles).

The possibility that the N-hAGT preparation was contaminated with either the Ada or the Ogt AGTs from *E. coli* is totally excluded by the finding shown in Figure 3 that the repair was blocked by preincubation with O^6-benzylguanine with an ED_{50} value of 50 nM. Neither of the *E. coli* AGTs is sensitive to inactivation by this inhibitor (*31*, *32*).

Reconstitution of Alkyl Transfer Activity by Combining Domains of Human AGTs. Mixing the isolated renatured N-hAGT and C-hAGT polypeptides together gave only a very weak alkyl transfer activity (Figure 4), but refolding the two denatured domains together after isolation in 8 M urea by combining them prior to dialysis against decreasing concentrations of urea did lead to some restoration of activity (Table 1 and Figure 4A). This restored activity was seen after refolding in the absence of zinc, a condition in which neither the N-hAGT nor the C-hAGT alone had any activity. A much greater activity was seen when refolding was carried out in the presence of zinc and the resulting activity of the combination of the N-hAGT and C-hAGT proteins was much greater than that from the N-hAGT alone (Table 1 and Figure 4B).

Although some alkyl transfer activity could be restored by renaturing the two domains together as described in the paragraph above, a much greater activity was obtained when the N-hAGT and C-hAGT proteins were isolated and renatured separately and then mixed by freeze−thawing twice as described under Experimental Procedures (Figure 4A). This procedure restored activity to a level about 50% of that seen with intact hAGT treated in the same way. These experiments were carried out without zinc addition. A much higher activity of both intact hAGT and the N-hAGT and C-hAGT reconstituted by freeze−thawing or renaturation from urea was obtained when zinc was included in the renaturation buffer (Figure 4B). However, the effect of zinc inclusion was greater on the intact N-hAGT (increased ca. 50-fold) rather than the combined N-hAGT and C-hAGT fragments (increased ca. 10-fold). Thus, the difference in activity between the intact protein and the recombined fragments was about 10-fold when zinc was present (Figure 4B).

The results described above were obtained by measuring the transfer of radioactivity from [^{3}H]-methylated-DNA

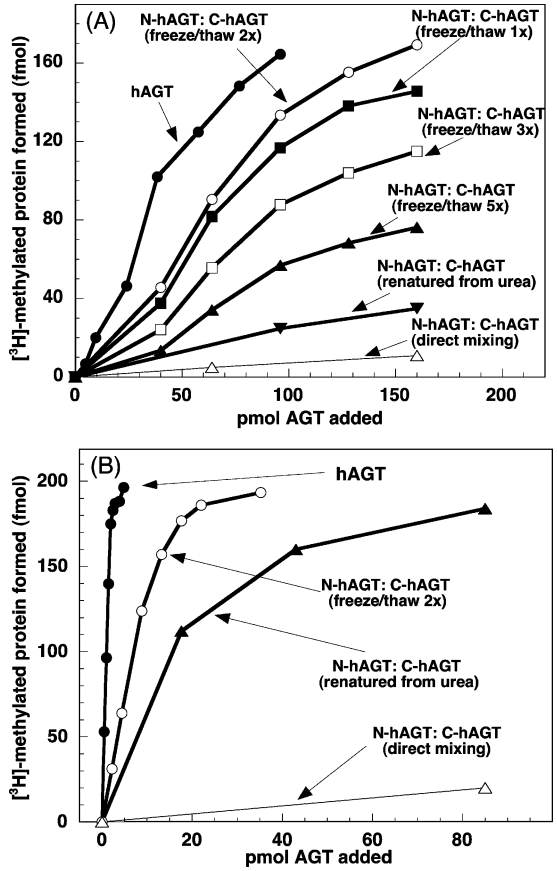

FIGURE 4: Reconstitution of alkyl transfer activity by combination of N-hAGT and C-hAGT. The proteins were combined as described in Experimental Procedures. Figure 4A shows the effects of intact hAGT and combinations of C-hAGT and N-hAGT prepared without zinc. Results are shown for intact hAGT (filled circles), and for N-hAGT:C-hAGT combined by direct mixing (open triangles); by renaturation from urea (inverted filled triangles); and by freeze−thawing using 1 cycle (filled squares), 2 cycles (open circles), 3 cycles (open squares), or 5 cycles (filled triangles). Figure 4B shows the effects of intact hAGT and combinations of C-hAGT and N-hAGT prepared with zinc. Results are shown for intact hAGT (filled circles) and for N-hAGT:C-hAGT combined by direct mixing (open triangles); by renaturation from urea (filled triangles) and by freeze−thawing using 2 cycles (open circles).

substrate to the protein using assay method (a) as described in Experimental Procedures. Specific activity toward O^6-methylguanine was confirmed using method (b) by showing the conversion of a double stranded substrate, 5′-d(GCGCAm^6-GAGTCG)-3′ annealed with its complement substrate, to the parent oligonucleotide containing guanine (Figure 5). These results confirm previous findings that the presence of a complexed zinc atom in the N-domain of hAGT is important for an optimal DNA repair activity (*23*).

As shown in Figure 3, the N-hAGT and C-hAGT complex reconstituted by freeze−thawing was also highly sensitive to inactivation by O^6-benzylguanine with an ED_{50} value of 90 nM. This is slightly lower than that found for the intact N-hAGT (220 nM). The latter value agrees with previous reports using protein that had not been isolated in a denatured form and then renatured (*24*, *31*).

MALDI-TOF analysis was used to show that reaction of O^6-benzylguanine with the reconstituted N-hAGT and C-hAGT fragments led to transfer of the benzyl group to Cys145. After reaction of either the intact hAGT or the reconstituted N-hAGT and C-hAGT fragments with O^6-benzylguanine followed by tryptic digestion, a peptide with *m*/*z* of 1406 appeared that corresponds to the peptide Gly136−Arg147 containing *S*-benzylcysteine, while the unmodified peptide with *m*/*z* of 1316 was reduced (Figure 6).

Effect of Domains of Human AGT in Protection of E. coli from Alkylation Damage. E. coli strain GWR-109, which lacks both *Ada* and *Ogt* AGT genes and is very sensitive to methylation damage, was used to assess the ability of the N-hAGT and C-hAGT domains to protect from MNNG. These domains and full length hAGT were expressed from the pQE-30 vector. Although both separate domains were less active than the hAGT [which as previously reported (*33*, *34*) gives almost complete protection over the dose range of MNNG used], they each provided statistically significant protection ($p < 0.01$) from the killing effect of MNNG (Figure 7A) and the induction of mutations by MNNG (Figure 7B). The presence of the N-hAGT resulted in more than a 10-fold increase in survival and reduction in mutants caused by MNNG. This protection was greatly reduced by the addition of 50 μM O^6-benzylguanine, which is consistent with the strong inactivation of the alkyl transfer activity of this N-hAGT as shown in Figure 3.

DISCUSSION

The three known AGT crystal structures (*11*, *13*, *14*) and the structure of the hAGT bound to a substrate (*12*) provide a highly plausible model for the mechanism of action of this protein. The DNA substrate is bound in a unique minor groove binding via a helix−turn−helix DNA binding motif. The substrate nucleotide is flipped into a binding pocket containing the cysteine acceptor site, which is located in a hydrophobic cleft defined by Met134 and the loop formed by Val155-Gly160. Within this pocket the *N*3 of O^6-methylguanine interacts with the hydroxyl of Tyr114 and the O^6 atom interacts with the hydroxyl of Ser159. The position of the O^6-methylguanine in the base stack is taken by Arg128 of the protein, which intercalates via the minor groove. Steric factors involving Tyr114 force rotation of the target nucleotide and thus play a major role in the required nucleotide flipping. The −SH group of the Cys145 acceptor site is very reactive and has a very low pK_a consistent with a thiolate anion (*22*). The activation of this residue is brought about by the interaction of Cys145 with a network of amino acids and a bound water linking residues Cys145, His146, Arg147, and Glu172 (*13*). Thus, all of the residues needed for the alkyl transfer reaction are present in the C-terminal domain of the protein. However, as shown in Table 1, this domain is totally inactive when expressed alone. Activity can be restored to the isolated C-hAGT fragment by providing the purified N-terminal domain but only if the two proteins are denatured and then allowed to renature together. Freeze−thawing was found to be an efficient way to accomplish this reconstruction and, under these conditions, the recombined fragments showed a good activity although it was still less than that produced by the intact hAGT protein treated in the same way. It seems probable that this difference is due to a reduced number of N-hAGT:C-hAGT molecules that are folded correctly.

The role of zinc in the activity of hAGT mediated through the Cys145 acceptor site is likely to be structural. All of the

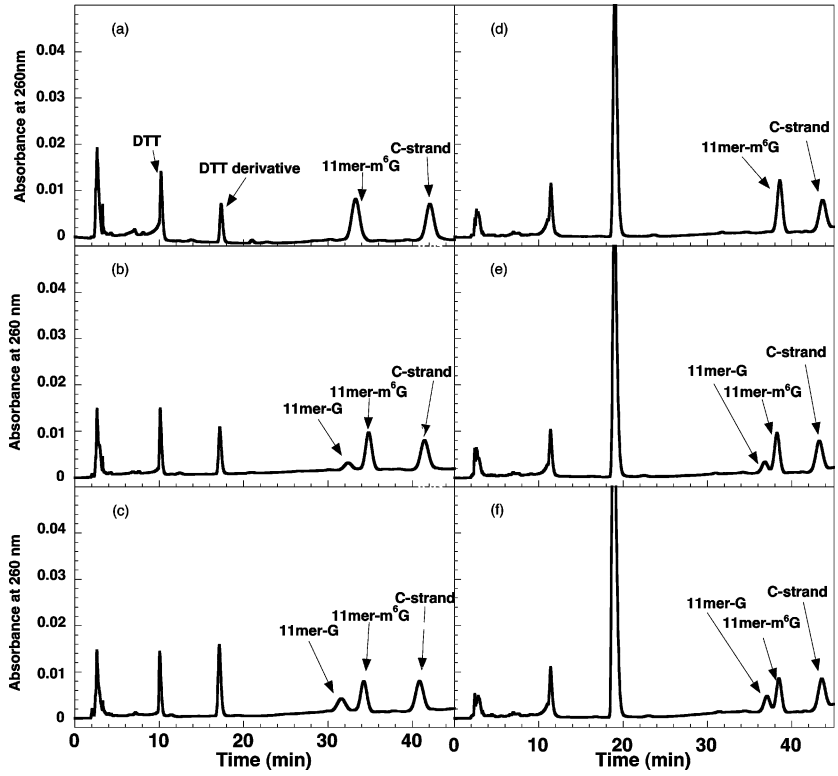

FIGURE 5: Repair of O^6-methylguanine by N-hAGT:C-hAGT mixture and hAGT. The hAGT and N-hAGT:C-hAGT mixture (combined by freeze−thawing) were prepared as in Figure 4B. A double stranded 11-mer 5′-d(GCGCAm^{6}GAGTCG)-3′ substrate (100 pmol) was incubated for 0 h (a and d) or 1 h (b, c, e, and f) with 52 pmol of h-AGT (a and b), 105 pmol of hAGT (c), 400 pmol of N-hAGT:C-hAGT mixture (d and e), or 600 pmol of N-hAGT:C-hAGT mixture (f), and the products were separated by HPLC. The slightly different retention times in samples d−f are due to the presence of DNA (60−90 μg) in the N-hAGT:C-hAGT mixture.

residues complexed with zinc (Cys5, Cys24, His29, and His85) are located in the N-terminal domain. The absence of zinc from the intact hAGT did not reduce the number of active AGT molecules but led to a 60-fold reduction in the rate constant for repair with no affect on DNA binding (*23*). Fluorescence studies indicated that zinc binding induced a small conformational change and enhanced hAGT stability as evidenced by resistance to the denaturing effects of urea (*23*). The present findings show that alkyl transfer activity can be restored from recombination of the N-hAGT and C-hAGT fragments even in the absence of zinc but that a much greater activity is achieved when the N-terminal fragment is prepared in the presence of zinc. This is consistent with the conclusion that the presence of zinc confers a mechanistic enhancement to repair activity via a structural effect on the N-terminal domain allowing it to interact with the C-terminal domain and maintain it in an optimal form for transfer activity. The fact that the presence of zinc during renaturation gave a greater enhancement with the intact hAGT protein that with the recombination of the two fragments may be due to a greater probability that the two portions of the protein come together in the preferred conformation when they are covalently joined.

It should be noted that there are two known naturally occurring active AGT variants that completely lack the N-terminal domain. These are cAGT-2 from *Caenorhabditis elegans* (*35*) and AGTendoV from *Ferroplasma acidarmanus* (*34*). Both of these proteins have a long extension at the carboxyl end of the AGT domain structure. In the case of cAGT-2, this sequence resembles histone 1C (*35*), and with AGTendoV, it is similar to endonuclease V (*34*). These sequences may provide the needed interaction to maintain the AGT domain in an active configuration. It is interesting that these two C-terminal extensions are capable of DNA binding. Although, as described above, the C-hAGT fragment contains all of the residues implicated in the helix−turn−helix DNA binding motif, the N-hAGT fragment does have the ability to bind to DNA as indicated both by direct observations using gel shift analysis (unpublished observations) and by the fact that it alone can repair DNA. Physiochemical studies of hAGT binding to DNA have shown that this binding is highly cooperative (*36*, *37*). The mechanism of this cooperative binding is not well understood, but the presence of two potential DNA binding regions located in two separate domains that have interactions with each other could be an essential component of the cooperativity.

The most unexpected finding arising from the experiments presented in this paper is that the N-terminal domain of hAGT alone has a weak DNA repair activity that is able to convert O^6-methylguanine into guanine. This conclusion is supported by multiple experiments shown in Figures 2−5, Figure 7, and Table 1. In in vitro experiments the protein led to (i) the transfer of radioactivity from methylated DNA to itself; (ii) a loss of O^6-methylguanine from DNA; and (iii) the production of guanine from sites occupied by O^6-methylguanine. The preparations of N-AGT used for these experiments were pure as shown by SDS−PAGE (Figure 1), but the remote possibility that they are contaminated with an *E. coli* AGT is ruled out by the potent inactivation of the N-hAGT activity by reaction with O^6-benzylguanine which does not inhibit the bacterial alkyltransferases (Figure 3). In in vivo experiments, the expression of N-hAGT led to a protection from killing by MNNG and to a reduction in the

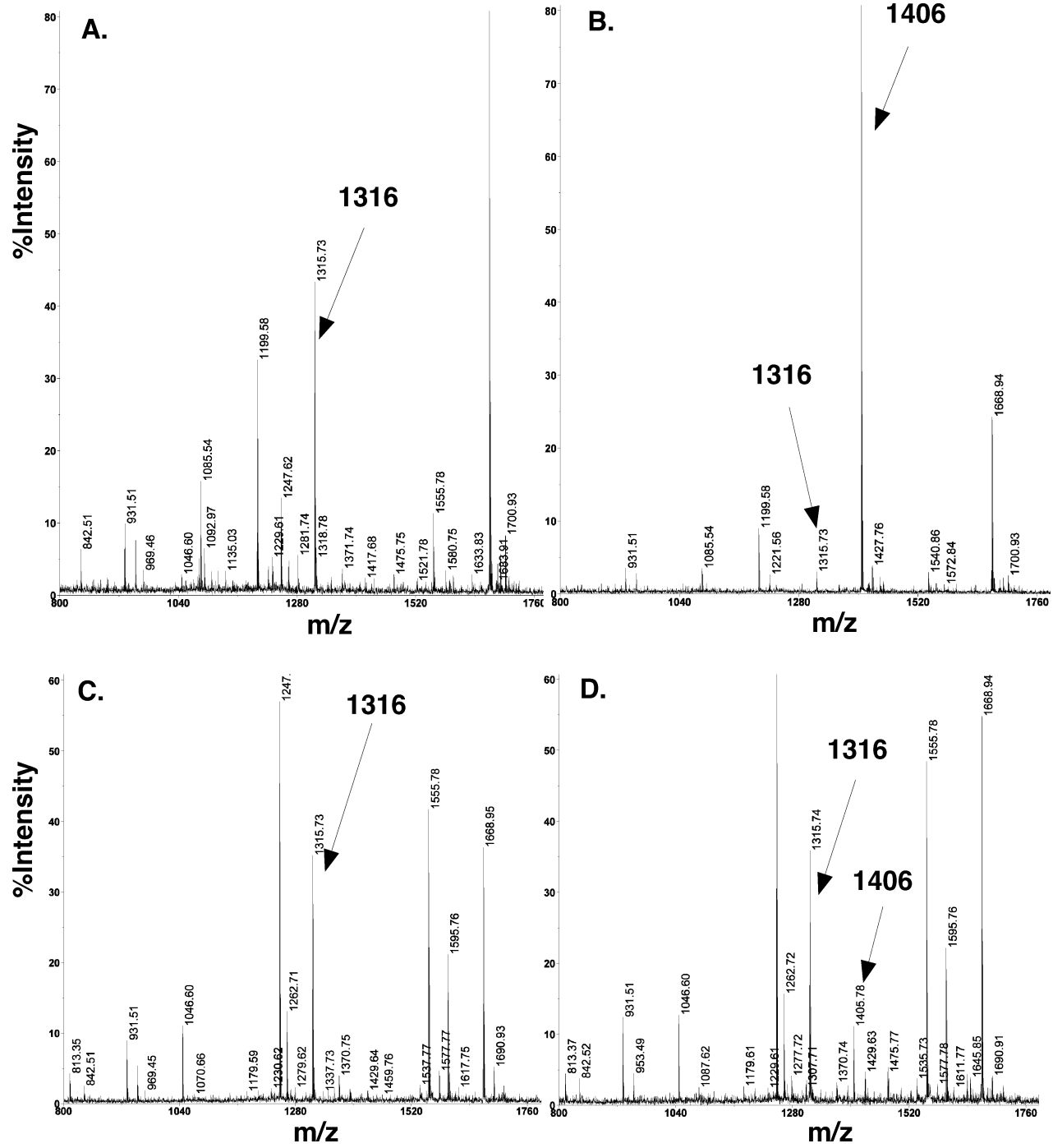

FIGURE 6: MALDI-TOF MS analysis of tryptic digests of hAGT and N-hAGT:C-hAGT mixture reacted with O^6-benzylguanine. hAGT (10 pmol) and N-hAGT:C-hAGT mixture (60 pmol) were reacted with 2 mM O^6-benzylguanine in 0.25 mL of 50 mM Tris HCl pH 7.6, 5 mM DTT, and 1 mg/mL calf thymus DNA for 1 h. The control and O^6-benzylguanine-treated samples were then trypsin-digested and prepared and analyzed by MALDI-TOF as described in Experimental Procedures. (A) Control (unmodified) hAGT. (B) O^6-Benzylguanine-modified hAGT. (C) Control (unmodified) N-hAGT:C-hAGT mixture. (D) O^6-Benzylguanine-modified N-hAGT:C-hAGT mixture. All the major peaks seen in the spectra correspond to expected tryptic fragments of hAGT and/or the N-hAGT:C-hAGT mixture, with peaks at ca. 932, 1086, 1248, 1316, 1556, 1668, and 1906 corresponding to the unmodified peptides of Thr10−Lys18, Gly194−Arg206, Glu166−Arg175, Gly136−Arg147, Leu19−Lys32, Val148−Lys165, and Phe108−Lys125, respectively. The larger peptides are not shown. The tryptic fragment containing the active (unmodified) Cys145 (Gly136−Arg147) produces an ion with *m*/*z* 1316 (see A, C). It produces an ion at *m*/*z* 1406 if Cys145 is modified by O^6-benzylguanine (see B, D).

number of mutants. Although O^6-methylguanine is not the only cytotoxic or mutagenic lesion produced by MNNG, it is a major factor in both the toxicity and the mutagenicity of this agent. The protection against MNNG provided by N-hAGT and the abrogation of this protection by the addition of O^6-benzylguanine to the cultures provide further support for the concept that N-hAGT does have a weak but real alkyl transfer activity.

The obligatory role for zinc in the activity of N-hAGT and the known ability of coordination with this metal to activate Cys69 for alkyl transfer from methylphosphotriesters in the *E. coli* N-Ada (*16*−*19*) suggests that the mechanism

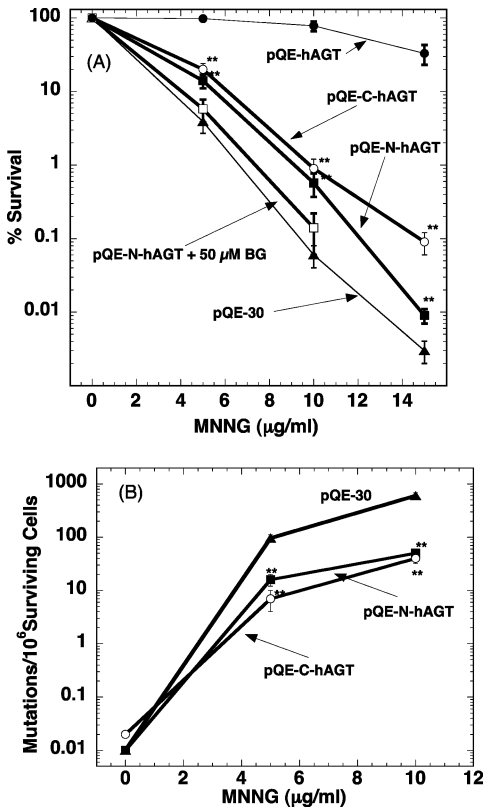

FIGURE 7: Protection of *E. coli* strain GWR109 from MNNG by hAGT, N-hAGT, and C-hAGT. *E. coli* strain GWR109 containing plasmids pQE-30 (filled triangles), pQE-N-hAGT (open squares and filled squares), pQE-C-hAGT (open circles), and pQE-hAGT (filled circles) were treated with MNNG as described in Experimental Procedures and the survival (A) and the incidence of rifampicin resistant mutants (B) determined. O^6-Benzylguanine (50 μM) was added to one of the cultures with plasmid pQE-N-hAGT (open squares). Results are shown ± SD for 5−8 separate experiments. Values that are statistically significant ($p < 0.01$) using Student's *t* test comparing responses at the same dose of MNNG between cells containing pQE30 with those containing pQE-N-hAGT or pQE-C-hAGT are marked with asterisks.

of DNA repair by N-hAGT may involve a cysteine activated in this way. The absence of activity in either the C5A or C24A mutants is consistent with this hypothesis. Both of these mutants in the intact full length hAGT protein also bind zinc less avidly than wild type (*23*), so the absence of activity in these mutants does not indicate which cysteine can be used as the acceptor. Unfortunately, we could not detect alkyl transfer to either Cys5 or Cys24 by MALDI-TOF analysis. This may be due either to the low activity of the N-hAGT fragment or to a problem detecting the expected tryptic peptide containing Cys5 (DCEMK) which is very small and contains two acidic residues and was not seen in the analysis of digests of unreacted N-hAGT or intact hAGT.

In vitro, the alkyl transfer activity of the N-hAGT may be cryptic and not contribute to the activity of hAGT. Perhaps the strongest evidence for this is the complete inactivity of the C145A and C145S mutants (*33*, *37*, *38*). Also, it has been shown that hAGT like other AGTs (*1*, *3*−*5*) acts with a stoichiometry close to 1 (*23*). After DNA repair leading to the formation of *S*-alkylcysteine at Cys145, the hAGT becomes unstable and is ubiquitinated and degraded (*38*, *39*). The trigger for this ubiquitination is unknown, but a significant change in conformation of hAGT occurs upon alkylation due to a steric clash between the *S*-alkylcysteine and Met134 (*13*). It is possible that alterations to the alkylated AGT either before or after ubiquitination or partial proteolysis would allow the second site to be used in vivo. This would be advantageous if it occurs under conditions where there is significant alkylation damage since more damage could be repaired prior to the synthesis of hAGT de novo.

The similarity of the cryptic zinc-activated repair activity of the N-hAGT to the repair activity of *E. coli* N-Ada also raises the possibility that this site in hAGT has evolved from a common zinc activated precursor, which was converted into the methylphosphotriester-activated transcriptional regulator in *E.* coli and related species and into a structural component of the AGTs in mammals that lack an adaptive response. Although this is rather speculative, it remains possible that alkylated hAGT does retain some gene-regulatory properties. Evidence that the methylated form of hAGT may influence estrogen-receptor regulated gene transcription has been presented (*40*). Also, despite the absence of a clear parallel with the adaptive response, which increases the gene products of the *Ada* regulon in *E. coli* many fold (*1*, *8*, *21*), there is some evidence that, in response to DNA damage, AGT may be moderately inducible in mammals. This induction varies with cell type and species (see refs *2*, *3*, *41*, and *42* and references therein). The AGT promoter contains 10 Sp1 transcription factor binding sites, two possible glucocorticoid response elements, and two AP-1 sites and may respond to glucocorticoids and p53 (*41*, *42*), but the extent to which AGT is specifically induced by alkylation damage remains unclear. The possibility that methylation of the amino terminal domain of hAGT may influence such transcription is therefore worthy of further investigation.

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