Investigation of the Role of Tyrosine-114 in the Activity of Human O^6-Alkylguanine-DNA Alkyltranferase†

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ABSTRACT: Tyrosine-114 is one of 13 totally conserved amino acids in all known sequences of O^6-alkylguanine-DNA alkyltransferase (AGT). The importance of this amino acid in repair of alkylated DNA by AGT was studied by changing it to phenylalanine (F), alanine (A), threonine (T), or glutamic acid (E) in human AGT. The activities of the mutant proteins were then compared to those of the wild type with regard to abilities to do the following: (a) protect *Escherichia coli* from the methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG); (b) repair methylated DNA in vitro; (c) bind to oligodeoxynucleotides containing O^6-methylguanine; and (d) react with the low molecular weight pseudosubstrate, O^6-benzylguanine. When expressed at high levels in *E. coli* strain GWR109, lacking endogenous AGT, the wild type and the Y114F mutant were highly effective in reducing mutations and cell killing by MNNG. The Y114A mutant had a much smaller protective effect, and mutants Y114T and Y114E were inactive. Purified preparations of all four AGT mutants showed an approximately similar degree (74−120-fold) of reduction in the rate of reaction with O^6-benzylguanine. In contrast, the degree of reduction in activity toward methylated DNA substrates in vitro varied according to the mutation with the more conservative Y114F producing only a 30-fold reduction and the most drastic change of Y114E abolishing activity completely. Alteration Y114A produced a 1000-fold reduction whereas Y114T reduced activity by 10000-fold. All of the mutations affected the binding of AGT to single- or double-stranded oligodeoxynucleotides containing O^6-methylguanine. The extent of increase in the K_d varied according to the amino acid with 2−5-fold (F), 7−11-fold (A), 167−200-fold (T), and 600−1000-fold (E) increases. These results are consistent with tyrosine-114 playing a role both in the binding of AGT to its DNA substrate and in facilitating the transfer of the alkyl group. It is probable that AGT resembles other DNA repair proteins in bringing about a "flipping out" of the target base from the DNA helix. Tyrosine-114 is therefore an excellent candidate for a key role in the interaction with the flipped O^6-methylguanine. The results also show that when large amounts of AGT are produced in the cell, substantial decreases in the efficiency with which AGT can repair methylated DNA do not prevent the ability to protect *E. coli* from toxic alkylating agents. Mutant Y114F, whose activity was reduced by 30-fold, was equal to wild-type AGT in bringing about this protection.

O^6-Alkylguanine DNA alkyltransferase (AGT)[1] participates in the repair of DNA lesions formed by monofunctional alkylating agents (*1*−*4*). Among these lesions, the minor alkylation products O^6-alkylguanine and O^4-alkylthymine are most likely to be responsible for the induction of the mutations and the initiation of the tumors due to their mispairing properties (*5*). AGT repairs these lesions by transferring the alkyl group to an active site cysteine residue of the protein itself, resulting in its inactivation and restoration of the DNA.

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1   MDKDCEMKRT TLDSPLGKLE LSGCEQGLHE IKLLGKGTSA
41  ADAVEVPAPA AVLGGPEPLM QCTAWLNAYF HQPEAIEEFP
81  VPALHHPVFQ QESFTRQVLW KLLKVVKFGE VISYQQLAAL
121 AGNPKAARAV GGAMRGNPVP ILIPCHRVVC SSGAVGNYSG
161 GLAVKEWLLA HEGHRLGKPG LGGSSGLAGA WLKGAGATSG
201 SPPAGRN
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FIGURE 1: Sequence of human AGT. The residues that are totally conserved in all known AGT sequences are shown in bold.

AGTs have been found in many living systems, including prokaryotes and eukaryotes. At least 21 DNA sequences coding for AGTs have now been reported. There are 13 absolutely invariant residues in these sequences (Figure 1). As expected, the cysteine acceptor residue is one of these along with the surrounding 3 amino acids in the sequence PCHR. Only one crystal structure for AGTs is available: that of the *Escherichia coli* Ada-C protein (*6*, *7*). This structure shows that the cysteine acceptor is buried in the protein and suggests that a change in protein conformation

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[1] Abbreviations: AGT, human O^6-alkylguanine-DNA alkyltransferse (EC 2.1.1.63); MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; ss, single-stranded; ds, double stranded.

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is necessary in order to permit access of the target O^6-alkylguanine. Conformational changes in the protein as a result of binding to DNA have been detected by using CD and fluorescent anisotropy (*8*−*11*), but the extent of these changes is not yet known.

Binding to DNA is not absolutely necessary for the activity. AGT is able to act on low molecular weight substrates such as O^6-methylguanine and O^6-benzylguanine in addition to DNA alkylated at the O^6 position of guanine (*4*). However, the rate of repair is much slower with the free bases. Additional evidence of conformational changes in AGT upon binding to DNA was provided by studies showing that DNA stimulates the ability of the human AGT to react with O^6-benzylguanine (*12*).

Two models for the binding of AGT to a DNA substrate have been proposed. The Ada-C protein structure contains a helix-turn-helix domain that resembles known DNA binding motifs, and it was suggested that binding to this region results in a swiveling of the C-terminal helix to expose the DNA lesion to the active site (*6*, *7*). This mechanism is plausible but requires a substantial change in the protein conformation. An alternative model has been put forward on the basis of the similarity between AGT sequences and that of the DNA binding domain of mu transposase (*13*). Studies using NMR have shown that mu transposase binds to DNA using a novel winged helix-turn-helix structure (*14*). This model requires only a small conformational change in the AGT protein but a larger change in the DNA and is consistent with a base flipping mechanism in which the O^6-alkylguanine is flipped out of the DNA helix to permit the reaction to occur (*13*). Such base flipping is now well-established for DNA repair enzymes (*15*−*19*) and has been suggested for AGT (*20*−*23*). Although there is currently no direct proof for this hypothesis, results from experiments of the binding of AGT to DNA are consistent with such a mechanism. Alterations in the DNA conformation after AGT binding consistent with local melting have been detected by near-UV CD spectral changes (*10*). Studies of the repair of double-stranded (ds) oligodeoxynucleotides containing O^6-methylguanine analogs suggested that the duplex has to open up in order for the reaction to take place (*24*). Footprinting experiments also suggest that protein binding causes a twist in the DNA strand containing the O^6-methylguanine (*25*). Another study has demonstrated a different ability of human AGT to bind to single-stranded (ss) and ds DNA (*26*) and that AGT is likely to function as a DNA melting protein (*26*).

At present, no definitive studies in which the structure of AGT bound to a DNA substrate is determined by X-ray crystallography or NMR are available, and it is not possible to choose definitively between the two models described above. Both models suggest that the region including residues 94−137 is involved in DNA binding. This region contains 6 of the 13 totally conserved residues described above (Figure 1). These include tyrosine-114. A very limited previous study has suggested that this residue is of importance in the action of AGT since its mutation to glutamic acid abolished activity toward methylated DNA and significantly reduced the ability to react with O^6-benzylguanine (*27*).

In the present work, a series of mutations have been made at position 114 in human AGT and the properties of the mutant AGT protein compared to wild type with respect to the ability to protect cells from methylating agents, to bind to and repair methylated DNA, and to react with O^6-benzylguanine. The results are consistent with tyrosine-114 playing a major role in both the DNA binding and alkyl transfer reactions of AGT and are discussed in terms of the models of AGT described above and the mechanism of DNA repair.

MATERIALS AND METHODS

Materials. All restriction enzymes and oligodeoxynucleotides were purchased from Gibco BRL (Gaithersburg, MD) and New England Biolabs (Beverly, MA). *E. coli* GWR109 strain (B/r *his thy* ogt-1::Kan^r Δ*ada-alkB*) was a generous gift from Dr. L. Samson (*28*). Most of the biochemical reagents were purchased from Sigma (St. Louis, MO). (8-^{3}H)-(Benzylguanine (0.34 Ci/mmol) was made by catalytic tritium exchange of O^6-benzylguanine with tritiated water and purified by RP-HPLC as previously described (*29*). *N*-(^{3}H)-Methyl-*N*-nitrosourea (18.8 mCi/mmol) and [γ-^{32}P]-ATP (3000 Ci/mmol, 10 mCi/mL) were obtained from Amersham (Arlington Heights, IL).

Generation of Mutant AGTs. Plasmid pINAGT, which expresses the human AGT in *E. coli*, was produced by inserting the human cDNA coding sequence into the *E. coli* expression vector pINIII-A3(*lpp*$^{P-5}$) using the *Eco*RI and *Bam*HI sites in the vector and PCR to generate the appropriate sites in the cDNA as previously described (*30*). The pINAGT was digested with *Eco*RI and *Bam*HI, and the resulting fragment containing the human AGT amino acid coding squence was inserted into pGEM-3Zf(+). This plasmid, termed pGEMAGT, was used to generate the mutations. The human AGT mutants Y114F, Y114A, Y114T, and Y114E were made by PCR as previously described (*30*). The primers used to generate the mutations were the following: 5′-CGGAGAAGTGATTTCT**NNN**CAG-CAATTAGCAGCC-3′, where **NNN** were **GCC** for Y114A, **GAA** for Y114E, **ACC** for Y114T, and **TTC** for Y114F. The entire coding sequence for all of the mutated AGTs was verified through DNA sequencing.

Protein Expression and Purification. The recombinant human alkyltransferase and mutants Y114F, Y114A, Y114T, and Y114E were expressed in *E. coli* GWR109 and purified as previously described using Polymin P precipitation, ammonium sulfate fractionation, and chromatography on Mono-S (*29*) with the addition of a gel-filtration step using a Superose 12HR 10/30 column (Pharmacia/LKB Biotechnology) equilibrated with 50 mM Hepes, pH 8.0, containing 1 mM EDTA and 3 mM dithiothreitol and eluted with the same buffer at a flow rate of 0.5 mL/min. The AGT protein eluted at 28.4 min. The final AGT proteins obtained by this procedure gave a single band of M_r at about 22 000 when analyzed by SDS−PAGE. AGT activity against a DNA substrate containing O^6-methylguanine was assayed using calf thymus DNA, which had been methylated by reaction with *N*-[^{3}H]methyl-*N*-nitrosourea essentially as described (*31*).

Reaction of AGT with O^6-Benzylguanine. Measurements of (8-^{3}H)-guanine formation from (8-^{3}H)-benzylguanine were carried out using various amounts of the AGT or mutant proteins in the presence or absence of 16-mer oligodeoxy-

nucleotide (5′-GACTGACTGACTGACT-3′) as previously described (27).

Alkylation-Induced Mutation and Cell Killing. To measure cell killing, GWR109 cells containing the pINAGT plasmid or mutants were grown overnight in 5 mL of LB broth containing 50 μg/mL ampicillin, 50 μg/mL kanamycin, and 0.3 mM IPTG. Cultures of 5 mL of the same medium were inoculated with 50 μL from overnight cultures and grown in 50-mL conical tubes agitated at 150 rpm in a 37 °C water bath. MNNG at concentrations ranging from 0 to 20 μg/mL was added when the OD_{600} reached 0.7. Cultures were agitated at 250 rpm for 30 min at 25 °C as previously described (32). Dilutions ranging from 1:100 to 1:10000 were plated on LB plates supplemented with 50 μg/mL ampicillin and 50 μg/mL kanamycin. After incubation for 16−20 h at 37 °C, surviving colonies were counted.

To measure mutations, GWR109 cells harboring pINAGT or pINY114 mutants were grown in 10 mL of M9 media containing 50 μg/mL ampicillin and 50 μg/mL kanamycin with agitation at 220 rpm at 37 °C until OD_{600} was 0.7. The cultures were then pelleted and suspended in M9 media and treated with various concentrations of MNNG at 25 °C for 1 h. The cells were concentrated in M9 salts, diluted, and plated either on minimal plates containing histidine (40 μg/mL), ampicillin (50 μg/mL), and kanamycin (50 μg/mL) to estimate the number of surviving cells or on minimal plates lacking histidine to estimate the number of his^+ revertants. The mutation frequencies are expressed as the number of induced his^+ revertants per 10^8 surviving cells.

Kinetics of Alkyltransferase Reaction. The kinetics of methyl transfer were followed by quantitating the (^{3}H)-methylated AGT produced using a (^{3}H)-methylated DNA substrate (*33*). The concentrations of AGT protein used were determined in preliminary experiments as those giving readily measurable rates under the assay conditions. The reaction mixture (1 mL) for each time point contained AGT [0.01 μg (4.5×10^{-10} M) of wild type or 0.16 μg (7.3×10^{-9} M) of Y114F or 3.0 μg (1.4×10^{-7} M) of Y114A], 3.6×10^{-10} M O^6-(^{3}H)-methyl G in (^{3}H)-methylated DNA substrate, and 50 μg of cold calf thymus DNA in a buffer containing 50 mM Tris-HCl (pH 7.6), 5 mM dithiothreitol, and 0.5 mM EDTA. Since the reaction was found to be second-order, the rate constant was determined by the following equation:

$$kt = 1/C^0_a - C^0_b \ln(C^0_b(C^0_a - C^0_c)/C^0_a(C^0_b - C_c))$$

where C^0_a is the initial concentration of the AGT protein, C^0_b is the initial concentration of the methylated DNA substrate, and C_c is the concentration of methylated AGT formed at a given time t (*34*, *35*).

Electrophoretic Mobility Shift Assay. Oligodeoxynucleotides are (16-mers) of sequence 5′-AACAGCCATATXGCCC-3′ where X represents O^6-methylguanine or G was 5′ end labeled using γ-^{32}P (ATP) and T4 polynucleotide kinase and then annealed to the complementary oligodeoxynucleotide of 5′-GGGCCATATGGCTGTT-3′ sequence. Increasing concentrations of AGT protein were mixed with 10 nM (^{32}P)-labeled DNA probe in a 20-μL reaction mixture containing 20 mM Tris-HCl (pH 7.6), 5 mM dithiothreitol, 0.1 mM EDTA, and 3% glycerol. The reaction mixtures were incubated for 10 min on ice and loaded on to pre-run, pre-equilibrated (4 °C) 6% (75:1 acrylamide−bis) polyacrylamide gels and electrophoresed in 10 mM Tris-acetate, 0.25 mM EDTA (pH 7.6) buffer for 100 min at 125 V. The gels were vacuum dried, and the amount of radioactivity in the unbound and bound DNA probe was quantified by exposing the dried gel to PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Under conditions of excess protein, the apparent dissociation constant (K_d) was defined as the protein concentration required for half-maximal binding (*36*).

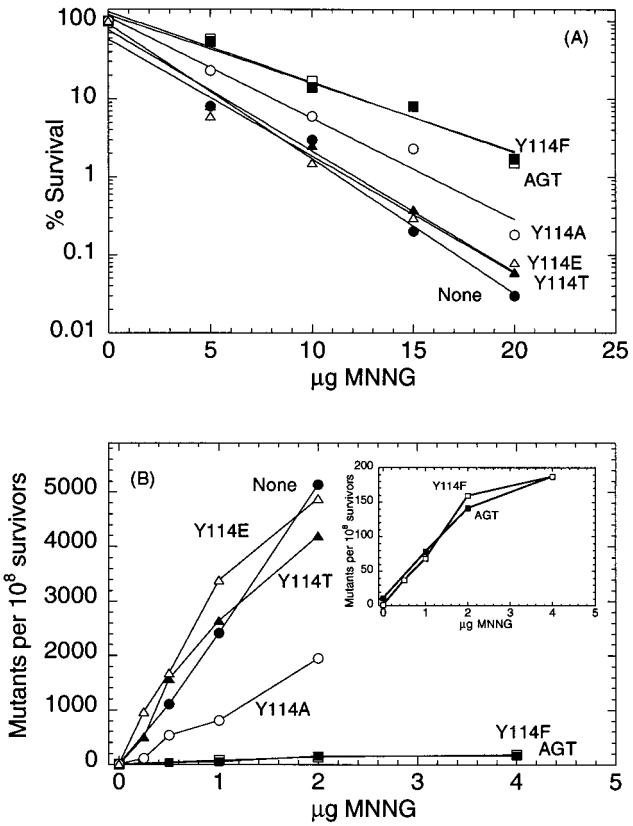

FIGURE 2: Effect of expression of AGT mutants on sensitivity of *E. coli* to MNNG. Panel A shows the results for the survival of GWR109 cells expressing no AGT (solid circles), wild-type AGT (open squares), Y114E (open triangles), Y114T (solid triangles), Y114A (open circles), and Y114F (filled squares). Panel B shows the production of mutants in the same cells. The insert in panel B shows the results for wild type and Y114F with a reduced scale.

RESULTS

All four (Y114F, Y114A, Y114T, and Y114E) AGT mutants were made by site-directed mutagenesis and expressed in GWR109 cells that lack endogenous alkyltransferase activity (*28*). The sensitivity of these cells to killing and to mutagenesis by the methylating agent MNNG was then examined and compared to that of GWR109 cells without AGT expressed and of GWR109 cells expressing wild-type AGT (Figure 2). As expected, the control GWR109 cells were very sensitive to MNNG, and expression of the wild-type AGT provided substantial protection from both its killing (Figure 2a) and its mutagenic (Figure 2b) effects. Results with the mutant Y114F were identical to those with wild-type AGT (Figure 2). Mutant Y114A was less effective than mutant Y114F but gave a clear protection. However, mutants Y114E and Y114T produced little if any protection (Figure 2).

In order to investigate the effect of these mutations on the activities of the respective AGT mutant proteins toward

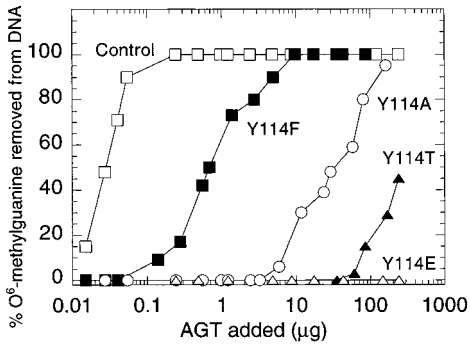

FIGURE 3: Removal of O^6-methylguanine from methylated DNA by purified AGT and mutants. The amounts of protein shown were incubated with (^{3}H)-methylated calf thymus DNA for 30 min, and the extent of removal of O^6-methylguanine was determined. Results are shown for wild-type AGT (open squares), Y114F (filled squares), Y114A (open circles), Y114T (solid triangles), and Y114E (open triangles) using protein amounts from 1.5 ng to 240 μg.

Table 1: Effect of Mutations on the Ability of AGT To Repair Methylated DNA in Vitro

protein added	amount of protein needed for 50% removal of O^6-methylguanine in 30 min (μg)	rate constant ($\times 10^{-6}$ M^{-1} min^{-1})
wild type	0.03	74
Y114F	0.7	2.6
Y114A	20	0.12
Y114T	300	nd[b]
Y114E	no removal[a]	nd[b]

[a] Tested with 2 mg of AGT protein. [b] nd, not done.

methylated DNA in vitro, the proteins were purified to homogeneity and tested for their ability to remove O^6-(^{3}H)-methylguanine from (^{3}H)-methylated calf thymus DNA substrate. Assays were carried out using various dilutions of the proteins, and the extent of repair of the substrate in 30 min at 37 °C was plotted against the amount of protein added (Figure 3). These results were used to obtain the amount of protein needed to repair 50% of the substrate in the 30-min incubation period (Table 1). All of the mutant AGT proteins were less active than the wild type, but their activities differed greatly. About 30 ng of wild-type AGT was required for 50% removal of O^6-(^{3}H)-methylguanine, and this value was increased about 25-fold in mutant Y114F, about 700-fold in mutant Y114A, and about 10000-fold in mutant Y114T. Y114E had no detectable activity when assayed even when 2 mg of protein was used. More detailed studies measuring the rate constants for the repair by wild type and mutants Y114F and Y114A were carried out essentially as previously described by measuring the amount of reaction as a function of time (Figure 4). The Y114F mutation reduced the rate constant by about 28-fold, and the Y114A mutation produced about a 630-fold reduction (Table 1). The Y114T mutant was not sufficiently active for the rate constant to be measured accurately.

The DNA binding properties of the mutant proteins were tested using a gel shift assay with a 16-mer oligodeoxynucleotide containing or lacking an O^6-methylguanine residue. Binding was measured with both a ss oligodeoxynucleotide and a ds oligodeoxynucleotide using polyacrylamide gel electrophoresis to separate the bound and unbound oligodeoxynucleotides. Representative results are shown for Y114A and for C145A mutant AGTs in Figure 5a. The

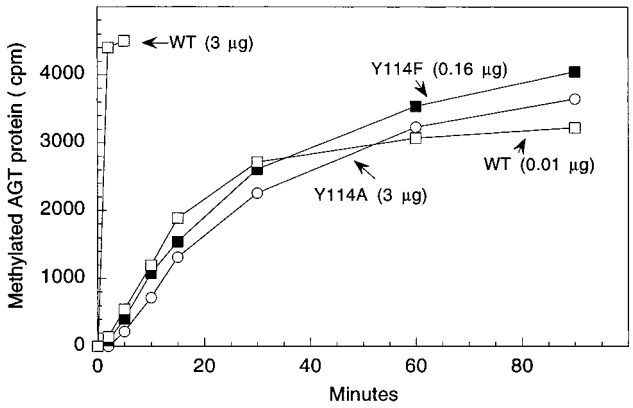

FIGURE 4: Rate of repair of O^6-methylguanine in methylated DNA by wild-type, Y114F, and Y114A AGT. Time courses of the transfer of (^{3}H)-methyl groups for DNA to AGT were carried out as described under Materials and Methods. Representative results are shown for wild-type (WT) AGT (0.01 μg of protein), Y114F (0.16 μg), and Y114A (3 μg) as indicated. The second-order rate constant was then calculated from this data as described under Materials and Methods. Results for 3 μg of wild-type AGT are also shown for comparison.

Table 2: Effect of Mutations on the Ability of AGT To Bind to Oligodeoxynucleotides Containing O^6-Methylguanine

protein used	apparent K_d (μM) for 16-mer oligodeoxynucleotides[a]			
	ds m^{6}G-16mer	ss m^{6}G-16mer	ds 16-mer	ss 16-mer
wild type	*b*	*b*	0.61	1.1
C145A	0.13	0.70	0.69	0.90
Y114F	0.63[c]	1.3	2.3	1.4
Y114A	0.95	8.0	2.7	3.4
Y114T	26	117	39	158
Y114E	138	428	nd[d]	n[d]

[a] The 16-mer oligodeoxynucleotides used were the following: ds m^{6}G-16mer, 5′-AACAGCCATATm^{6}GGCCC-3′ annealed to 5′-GGGCCATATGGCTGTT-3′; ss m^{6}G-16mer, 5′-AACAGCCATATm^{6}GGCCC-3; ds 16-mer, 5′-AACAGCCATATGGCCC-3′ annealed to 5′-GGGCCATATGGCTGTT-3′; ss 16-mer, 5′-AACAGCCATATGGCCC-3′. [b] Cannot be measured due to reaction with substrate. [c] It is possible that this value is overestimated due to the reaction of the Y114F AGT with the ds m^{6}G-16mer during the binding analysis. [d] nd, not done.

C145A mutant AGT was used because it was not possible to prevent the wild-type AGT from reacting with the substrate during the assay conditions even though the time of exposure was kept short and the binding was carried out at reduced temperature. Binding assays with wild-type protein and the 16-mer containing O^6-methylguanine therefore gave a smear when analyzed by polyacrylamide gel electrophoresis (results not shown). The C145A mutant AGT is unable to accept methyl groups but is similar to wild type in all other properties that have been examined including binding to DNA (*25*, *32*). Apparent dissociation constants were calculated from plots of the fraction of the oligodeoxynucleotide bound against the AGT concentration (Figure 5b) and are shown in Table 2.

The apparent K_d for binding of AGT to a ds oligodeoxynucleotide was about 5 times less when it contained an O^6-methylguanine residue (Table 2) confirming that the AGT has a higher affinity for DNA when a substrate lesion is present (*25*). It is also of interest that the K_d values for binding to a ss oligodeoxynucleotide containing O^6-methylguanine were about 5 time higher than for a ds substrate. This is in agreement with previous studies showing that ds

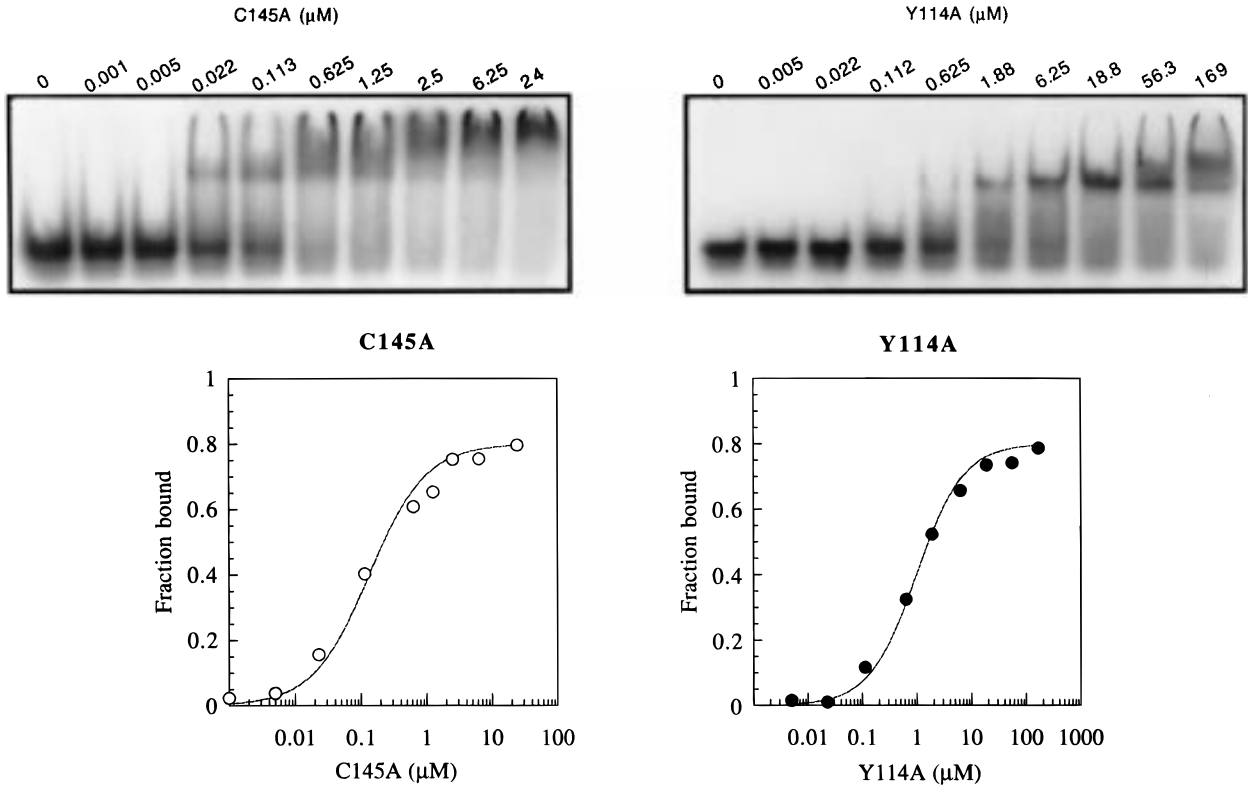

FIGURE 5: Binding of AGT to a 16-mer oligodeoxynucleotide containing O^6-methylguanine. Binding of C145A and Y114A mutant AGT proteins to a ds oligodeoxynucleotide containing O^6-methylguanine was analyzed by an electrophoretic mobility shift assay. Increasing concentrations of AGT mutant proteins C145A (0.001−24 μM) and Y114A (0.005−169 μM) were titrated into binding reaction mixtures containing 10 nM (^{32}P)-labeled oligodeoxynucleotide and the products separated as described under Materials and Methods. Panel A shows the gel analysis. The lane marked 0 contained the oligodeoxynucleotide in the absence of protein, and the amount of mutant AGT protein added is shown above each of the other lanes. Panel B shows graphs of the fraction of bound oligodeoxynucleotide in each lane plotted as a function of the log of the AGT concentration. The apparent K_d values shown in Table 2 were obtained from the protein concentration giving half-maximal binding in these plots for C145A and Y114A. Similar experiments were used to obtain K_d values for Y114F, Y114T, and Y114E and similar experiments using a ss oligodeoxynucleotide containing O^6-methylguanine or ds or ss oligodeoxynucleotide containing guanine to obtain the other K_d values shown in Table 2.

methylated DNA is the preferred substrate for the AGT protein (*1*, *4*, *37*,*38*). In contrast to the effect with ds 16-mers, the presence of an O^6-methylguanine made little difference to the binding of AGT to ss 16-mer oligodeoxynucleotides (Table 2).

All of the mutations at Y114 affected the binding of AGT to DNA, but the extent depended on the nature of the substitution. When the ds oligodeoxynucleotide containing O^6-methylguanine was used, the alteration to Y114F produced a 5-fold increase in the apparent K_d and Y114A produced a 7-fold increase whereas Y114T and Y114E had a much greater effect giving a 200-fold and a 1000-fold increase, respectively. Approximately similar increases were also observed when the ss oligodeoxynucleotide containing O^6-methylguanine was used, although in this case the apparent K_d for Y114F was only increased by 2-fold. (It is possible that the value for the K_d for this mutant with the ds substrate is somewhat overestimated due to some degree of reaction under the binding conditions since the rate of repair by this mutant is only 25-fold less than with the wild type.) However, it is clear that the decreased affinity for DNA cannot account for all of the reduction in the activity of these mutants.

O^6-Benzylguanine, which was first described as a potent inhibitor of mammalian AGTs (*31*), has been shown to act by serving as a substrate for the protein forming *S*-benzylcysteine at the active site with the release of stoichiometric amounts of guanine (*29*). Since O^6-benzylguanine is a free base, its reaction with the AGT does not involve the DNA binding properties of AGT.

Table 3: Effect of Mutations on the Ability of AGT To React with O^6-Benzylguanine

protein added	guanine formation[a] (cpm/μg of protein)	fold stimulation by DNA[b]	
		20 μg	80 μg
wild type	2137 ± 330 (7[c])	5.4	5.2
Y114F	18 ± 2 (6)	2.5	2.4
Y114A	25 ± 7 (6)	2.0	1.8
Y114T	19 ± 4 (6)	1.5	1.5
Y114E	29 ± 6 (4)	1.0	1.0

[a] Measured in the absence of DNA in a 20-min incubation. [b] The oligodeoxynucleotide used was 5′-GACTGACTGACTGACT-3′. [c] Number of observations.

The ability of WT AGT and Y114 mutant proteins to convert O^6-benzylguanine into guanine was therefore examined by measuring the rate of formation of (^{3}H)-guanine from O^6-(^{3}H)-benzylguanine (Table 3). All of the mutants showed a greatly reduced ability to react with O^6-benzylguanine. The degree of reduction was approximately the same in all mutants ranging from a 120-fold reduction in Y114F to a 75-fold decrease in Y114E. These results indicate that the tyrosine-114 is likely to play a role in the alkyl group transfer as well as in DNA binding.

Previous studies have shown that binding to DNA causes a conformational change in AGT protein structure that increases its rate of reaction with O^6-benzylguanine (*12*). Such stimulation can be brought about by the addition of either ds calf thymus DNA or oligodeoxynucleotides containing 8 or more nucleotides (*12*). As shown in Table 3, the amount of guanine formation from O^6-benzylguanine was increased by 5.4-fold when wild-type AGT was, used whereas mutants Y114F, Y114A, and Y114T showed only 2.5-, 1.9-, and 1.5-fold increases, respectively. No activation was observed for Y114E mutant.

DISCUSSION

The limited number of highly conserved residues in AGT squences focuses attention on these sites as critical for the stability or reaction of the protein. Of these residues, studies by site-directed mutagenesis have indicated that the residues asparagine-137 (*39*, *40*), histidine-146, arginine-147 (*40*−*42*), and glutamic acid-172 (*40*, *43*) play important structural roles in the protein, which has very limited activity and stability in their absence. The AGT protein with mutations at these sites, therefore, could not be purified (*40*). In contrast, AGT mutants in which positions cysteine-145 (*25*, *32*, *44*), arginine-128 (*27*), lysine-165 (unpublished observations), or tyrosine-114 (this paper) are converted to alanine are stable and their properties can be studied using homogeneous preparations. Cysteine-145 functions as the acceptor site, and both arginine-128 and lysine-165 appear to be involved in DNA binding. However, it is shown in the present paper that tyrosine-114 appears to play a role in both alkyl transfer and substrate binding. Although changing an amino acid in a protein may generate unpredictable alterations in the protein structure, there was no indication that this was the case with any of the mutants studied here in which tyrosine-114 was converted to phenylalanine, alanine, threonine, or glutamic acid. All of these proteins were stable when expressed in *E. coli* and on storage in vitro and were readily purified from the supernatant fraction of the bacterial extracts.

Our results confirm preliminary findings with the inactive Y114E mutant (*27*), suggesting that tyrosine-114 plays a critical role in the DNA repair alkyl transfer reaction brought about by AGTs. The values in Table 1 show that there is a good correlation between the second-order rate constant for the repair of methylated DNA and the amount of protein needed for 50% repair of the DNA substrate. Both estimates of the efficiency of the AGT reaction indicate that the alteration of tyrosine-114 greatly reduces the effectiveness of the protein. The extent of reduction depended on the substitution with the conservative alternation to phenylalanine giving a 25-fold reduction, whereas alteration to alanine produced about a 600-fold reduction. The more drastic alterations to threonine and to glutamic acid reduced activity by 10000-fold and to beyond the limit of detection (about 30000-fold), respectively.

Furthermore, although this residue is localized in the region envisaged as a DNA binding domain in both models for the AGT−substrate binding (*6*, *13*), its role is not limited to this. All of the mutations studied reduced the rate of the alkyl transfer reaction with O^6-benzylguanine by about two orders of magnitude (Table 3). This included the conservative change to phenylalanine. The DNA binding properties of this mutant were, in fact, only slightly reduced from that of wild-type AGT (or its C145A surrogate) (Table 2), and the decrease in the rate of alkyl transfer seen with O^6-benzylguanine is more than sufficient to account for all of the reduction in the rate constant for the repair of methylated DNA (Table 1). In contrast, the more drastic change to alanine produced a greater reduction in the ability to bind to DNA containing O^6-methylguanine (particularly with the ss substrates), and it appears that both the decrease in binding to DNA and the decrease in alkyl transfer rates contribute to the 600-fold reduction in ability of the protein to repair methylated DNA.

Similarly, the Y114T mutant shows about a two order of magnitude decline in ability to bind DNA as well as a similar decrease in its ability to transfer benzyl groups from O^6-benzylguanine. The combined effects of the alterations correlate well with a decline in the repair of methylated DNA by a factor of about 10 000. The Y114E mutation reduces DNA binding by at least three orders of magnitude, and this combined with the large reduction in alkyl transfer rate renders the reaction with methylated DNA undetectable in our assay system.

Tyrosine-114 is one of two exposed residues (the other is arginine-128) identified in the putative DNA binding domain from the crystal structure of the Ada-C protein (*6*). Our results suggest that the aromatic ring portion of the tyrosine side chain is involved in DNA binding but that the entire side chain including the hydroxyl group is required for efficient alkyl transfer. In the winged helix-turn-helix model for AGT binding to DNA based on the mu transposase structure, tyrosine-114 is placed close to the flipped out O^6-methylguanine base (*13*). This could enable the tyrosine residue to facilitate the alkyl transfer reaction.

AGT plays a crucial role in the resistance to chemotherapeutic alkylating agents that form a toxic lesion at the O^6 position of guanine in DNA. *E. coli* strain GWR109, which we used in our experiments, lacks endogenous alkyltransferase activity and is, therefore, very sensitive to MNNG. This provides a very sensitive assay for AGT activity in vivo and was used to test the intracellular activity of all the mutant AGTs. The results are in general agreement with those obtained by assay of the purified mutant AGT proteins in vitro. However, it is noteworthy that even though the Y114F mutant AGT clearly has an impaired ability to repair methylated DNA in vitro, the expression of this mutant in *E. coli* was able to protect the cells from the toxic effects of MNNG as effectively as wild-type AGT. A plausible explanation for this finding is that the rate of repair of methylated DNA by AGT is sufficiently fast compared to the cell division rate to provide protection even when the rate is reduced by the Y114F mutation. Even the 600-fold reduction in the in vitro activity seen in the Y114A mutant does not abolish protection of *E. coli* from MNNG entirely, although this mutant was much less effective than wild-type AGT (Figure 2). These results are consistent with the previous report that some poorly active or unstable AGT mutants were able to provide some protection in vivo from killing by MNNG (*40*). These observations provide an explanation for the recovery of AGT mutants which are clearly not as active as wild-type AGT in screening protocols designed to isolate O^6-benzylguanine-resistant mutants by

expression of a library of mutant sequences in *E. coli* (*33*, *45*, *46*).

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