Alteration of Arginine-128 to Alanine Abolishes the Ability of Human O^6-Alkylguanine-DNA Alkyltransferase To Repair Methylated DNA but Has No Effect on Its Reaction with O^6-Benzylguanine†

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ABSTRACT: O^6-Alkylguanine-DNA alkyltransferase (AGT) is a DNA repair protein that removes the promutagenic O^6-methylguanine lesion from DNA. In order to obtain more information about the mechanism of action of AGT, two conserved residues in a putative DNA binding domain were changed by site-directed mutagenesis, and the abilities of the mutant proteins to bind to DNA, to repair methylated DNA, and to convert O^6-benzylguanine to guanine were examined. The alteration of arginine-128 to alanine (R128A) reduced the AGT activity toward methylated DNA substrates by a factor of more than 1000 but did not decrease the rate of reaction with O^6-benzylguanine. The change of residue tyrosine-114 to glutamic acid (Y114E) completely abolished the ability to repair O^6-methylguanine in DNA in the assays used showing that this was reduced by >15 000-fold, but the ability to convert O^6-benzylguanine to guanine was reduced by only 60-fold. Alteration of this residue to alanine (Y114A) reduced activity toward methylated DNA by >1000-fold and toward O^6-benzylguanine by about 80-fold. Neither the R128A nor the Y114E mutant AGT were able to compete with the control AGT for the repair of methylated DNA whereas the inactive mutant, C145A, in which the cysteine acceptor site is changed to alanine, competed effectively in this assay. These results suggest that the residues arginine-128 and tyrosine-114 are involved in the DNA binding properties of the AGT. The ability of the AGT proteins to form stable complexes with DNA was therefore examined by measuring the retardation of DNA during electrophoresis. The mutant Y114E did not form complexes with either single-stranded or double-stranded M13 DNA or with an oligodeoxynucleotide 16-mer in a single-stranded or duplex form. Mutant R128A did form a well retarded complex with double-stranded M13 DNA but did not form such a complex with single-stranded M13 DNA or with the single-stranded 16-mer. Some complex formation occurred with the double-stranded 16-mer, but this was less stable than the complex formed by control AGT. These results provide direct evidence that the domain of the AGT containing residues 114 and 128 is involved in DNA binding. The results with the mutant R128 further suggest that a single-stranded region is generated during the AGT reaction and that arginine-128 is involved in binding this single-stranded region in a conformation that allows alkyl transfer to occur.

Adducts at the O^6-position of guanine in DNA have been implicated in both the promutagenic and the toxic effects of alkylating agents (Saffhill et al., 1985; Yarosh, 1985; Pegg, 1990; Singer & Essigmann, 1991). These adducts are repaired and the DNA restored to its original state by the action of a single protein, O^6-alkylguanine-DNA alkyltransferase (AGT)[1] (Lindahl et al., 1988; Demple, 1990; Mitra & Kaina, 1993; Pegg et al., 1995). The AGT protein binds to DNA and brings about the transfer of the alkyl group from the DNA to a cysteine residue within the protein sequence. The same protein acts as both a transferase and an alkyl acceptor. This rupture of an ether bond and the formation of a thioether in the protein overcomes a significant energy barrier, but the AGT is not a classical enzyme since it acts only once. The alkylcysteine formed in the reaction is not converted back to cysteine. AGT is therefore important both as a means of protection against alkylation damage and as a model protein for studies of interaction with and modification of DNA.

The mechanism by which AGT repairs DNA is not well understood at present but is thought to involve an S_N2 reaction in which the cysteine acceptor displaces the alkyl group from the guanine (Spratt & de los Santos, 1992; Spratt & Campbell, 1994; Pegg et al., 1995). The formation of a thiolate anion, which acts as the nucleophile, has been proposed, and the generation of this thiolate may be facilitated by the action of a neighboring histidine group (Demple, 1990). The structure of an AGT from *Escherichia coli* called Ada-C has been determined by X-ray crystallography (Moore et al., 1994; Moody & Moore, 1995). In this structure, the cysteine acceptor site is buried, and the protein must undergo a conformational change on binding DNA in order to provide access of the O^6-alkylguanine to

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[1] Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase (EC 2.1.1.63); BG, O^6-benzylguanine; RP-HPLC, reversed-phase high-pressure liquid chromatography; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

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the active site. It has been proposed that this conformational change leads to rupture of a bond between a glutamic acid residue and the histidine adjacent to the cysteine acceptor site that allows this histidine to participate in the formation of the thiolate anion (Moore et al., 1994). There is no direct experimental evidence in favor of this mechanism, but the histidine and glutamic residues are conserved in all known AGT sequences and mutation of these residues leads to a loss of AGT activity and extreme instability of the AGT protein (Ling-Ling et al., 1992; Ihara et al., 1994; Crone et al., 1994; Rafferty et al., 1994). Evidence that the AGT does undergo a conformational change on binding DNA has been provided by studies using circular dichroism and fluorescence anisotropy (Takahashi et al., 1990; Chan et al., 1993).

DNA binding is not absolutely essential for AGT activity since it is known that O^6-methylguanine free base is demethylated (Karran, 1985; Dolan et al., 1985; Yarosh et al., 1986; Spratt & de los Santos, 1982). However, the rate of reaction with this substrate is extremely slow. More complete investigations with a low molecular weight substrate for the AGT have been carried out with O^6-benzylguanine (BG). Detailed studies with the human AGT have shown that BG is converted to guanine with a stoichiometric formation of *S*-benzylcysteine at the cysteine acceptor site and concurrent inactivation of the AGT (Dolan et al., 1990; Pegg et al., 1993). BG therefore provides a useful substrate to investigate the mechanism of the AGT reaction in the absence of DNA and to examine separately the effects of DNA and substrate binding. The rate of reaction with BG is increased about 6-fold in the presence of DNA or of oligodeoxynucleotides containing more than eight nucleotides (Goodtzova et al., 1994).

The structure of the Ada-C AGT determined by X-ray crystallography contains a helix−turn−helix−helix domain that resembles some DNA binding motifs (Moore et al., 1994). This region is quite far from the active site cysteine but does contain a considerable number (12) of the 22 amino acid residues that are conserved in all of the 11 known AGT amino acid sequences (Pegg et al., 1995). The only previously reported AGT mutant protein with a mutation located in this region is the conversion of trytophan-100 to alanine (W100A) or glutamic acid (W100E). These changes had no effect on any of the properties of the AGT studied (Crone & Pegg, 1993; Crone et al., 1994).

In order to investigate in more detail if this domain is involved in DNA binding and AGT activity, we have now produced mutants in this region by site-specific mutagenesis, expressed the mutated proteins in *E. coli*, and studied the properties of these proteins with regard to the repair of methylated DNA, the reaction with BG, and the binding to single- and double-stranded DNA. The positions altered were arginine-128 and tyrosine-114. These residues were selected for mutation because they are conserved in all known AGT sequences and are located on the surface of the protein (Moore et al., 1994; Moody & Moore, 1995). They were changed to alanine residues to examine the requirement for the amino acid side chains, and, in addition, tyrosine-114 was changed to an acidic residue since this change might prevent DNA binding. It was found that the conversion of arginine-128 to alanine led to an alkyltransferase protein (R128A) which had lost activity toward O^6-methylguanine in DNA but was still able to react with BG at a normal (actually slightly greater) rate. This protein had a greatly reduced binding to single-stranded DNA. The implications of these results with regard to the mechanism of action of the AGT are discussed.

MATERIALS AND METHODS

Materials. All oligodeoxynucleotides were made in the Macromolecular Core Facility, Hershey Medical Center, by using a Milligen 7500 DNA synthesizer. *E. coli* strain GWR109 (Rebeck & Samson, 1991) was generously provided by Dr. L. Samson, Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, MA 02115. Restriction enzymes were purchased from GIBCO BRL (Gaithersburg, MD) and New England Biolabs (Beverly, MA). Most of the biochemical reagents were purchased from Sigma (St. Louis, MO). Plasmid pGem-3Zf(+) and T4 polynucleotide kinase were purchased from Promega Corp. (Madison, WI). [8-^{3}H]BG (0.34 Ci/mmol) was produced by catalytic tritium exchange and purified as previously described (Pegg et al., 1993). *N*-[^{3}H]-Methyl-*N*-nitrosourea and [α-^{32}P]ATP (3000 Ci/mmol, 10 mCi/mL) was obtained from Amersham Corp. (Arlington Heights, IL). The listed specific activity of this material was 18.8 mCi/mmol, but determinations of the specific activity of the O^6-methylguanine present in a hydrolyzed sample of the DNA and comparison with other samples of labeled methylated DNA indicated that the specific activity of the was actually 5.9 mCi/mmol. O^6-Benzyl[8-^{3}H]guanine (0.34 mCi/mmol) ([8-^{3}H]BG) was prepared by catalytic tritium exchange of O^6-benzylguanine with tritiated water by Amersham Corp. and was purified by RP-HPLC as previously described (Pegg et al., 1993).

Generation of Mutant AGTs. The construction of mutants C145A and W100A has been described previously (Crone & Pegg, 1993). The construction of mutants Y114E, Y114A, and R128A was carried out by the polymerase chain reaction (Landt et al., 1990) as described previously (Crone et al., 1994) using as mutagenic primers 5′-CGGAGAAGT-GATTTCTGAACAGCAATTAGCAGCC-3′ for Y114E, 5′-CGGAGAAGTGATTTCTGCCCAGCAATTAG-CAGCC-3′ for Y114A, and 5′-CCCAAAGCCGCGGCAG-CAGTGGGAGGAGC-3′ for R128A. The entire coding sequence of the AGT was checked to ensure that only the desired mutations were present.

Purification and Assay of Alkyltransferase. The recombinant human alkyltransferase and mutants W100A, Y114E, Y114A, R128A, and C145A were expressed in *E. coli* and purified as previously described using Polymin P precipitation, ammonium sulfate fractionation, and chromatography on Mono-S (Pegg et al., 1993) 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 about 22 000 when analyzed by SDS−PAGE.

Enzyme 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 (Dolan et al., 1990). The substrate contained 6700 cpm of ^{3}H/μg of DNA of which about 13% was O^6-[^{3}H]methylguanine. Various amounts of

purified AGT proteins were incubated with 7 μg of [^{3}H]-methylated DNA substrate containing 7000 cpm as O^6-[^{3}H]-methylguanine and 0.36 mg of carrier calf thymus DNA in 50 mM Tris, pH 7.5, 5 mM dithiothreitol, and 0.1 mM EDTA in a total volume of 1 mL, and the mixture was incubated in a 37 °C water bath for 30 min. The DNA was then precipitated with 0.25 N perchloric acid at 4 °C and hydrolyzed by heating in 0.5 mL of 0.1 N HCl at 70 °C for 30 min. This procedure was repeated, and the combined hydrolysates were taken, neutralized by the addition of 0.15 mL of 1 M Tris, and filtered through a circular 0.45 μm pore microfilter. The labeled 7-methylguanine and O^6-methylguanine present were then separated by HPLC on a Beckman Ultrasphere ODS column (25 cm × 0.46 cm) using isocratic elution at 37 °C with 0.05 M ammonium formate, pH 4.5, containing 12% methanol. The eluate was monitored for radioactivity by mixing with 3.5 parts of Flow-scint III, Packard (Meriden, CT) and passing through a Radiomatic Flo-One/βeta A-140A radioactivity monitor (Packard Instruments, Downers Grove, IL). The counting efficiency was 35%.

Reaction of AGT with BG. Measurements of [8-^{3}H]-guanine formation from [8-^{3}H]BG were carried out using various amounts of the AGT or mutant proteins in an assay mixture consisting of 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA and 5 mM dithiothreitol in a volume of 0.25 mL in the presence or absence of DNA as indicated. The formation of labeled product was stopped by the addition of 0.6−0.8 mL of the same buffer containing 0.2 mM guanine and 0.2 mM O^6-benzylguanine. Aliquots were then separated by RP-HPLC on a Beckman Ultrasphere ODS column (25 cm × 4.6 mm) using isocratic elution at a temperature of 36 °C with a buffer of equal parts methanol and 0.05 M ammonium formate, pH 4.5. The eluate from the HPLC was monitored for radioactivity by mixing with 3.5 volumes of Flow Scint III and passing through a Radiomatic Flo-One,Beta A-140A radioactivity monitor (Packard Instruments).

Gel Mobility Shift Assays. Gel mobility shift assays with M13 mp18 ssDNA and dsDNA (M13 mp18 RF, linearized with *Eco*RI) were carried out following the procedure of Lohman et al. (1986) with slight modifications. For assay, 200 ng of DNA was incubated with different amounts of control or mutant AGT proteins in buffer A (50 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mg/mL bovine serum albumin) containing 100 mM NaCl in a 10 μL reaction volume at 37 °C for 10 min. The samples were then mixed with 1 μL of 50% glycerol containing 0.04% bromophenol blue and loaded onto a 0.5% agarose gel in 40 mM Tris-acetate, pH 8.0, and 1 mM EDTA and subjected to electrophoreses at 6 V/cm constant voltage for 3 h at room temperature. The gel was then stained with 0.5 μg/mL ethidium bromide in water and photographed under ultraviolet illumination.

For assays using an oligodeoxynucleotide, a 16-mer, 5′-GACTGACTGACTGACT-3′, and its complementary reverse strand, 5′-AGTCAGTCAGTCAGTC-3′, were end-labeled with [^{32}P]ATP using T4 polynucleotide kinase. The oligonucleotide duplex DNA was formed by mixing equal concentrations of both the forward and reverse strands and boiling for 3 min followed by gradual cooling to room temperature. The DNA−protein complex formation between the oligonucleotide DNA and the AGT protein was analyzed by electrophoretic mobility shift of the oligonucleotide DNA as a result of its binding to the protein. The binding reactions were carried out in 10 μL reaction volumes in buffer A containing 100 fmol of the single-stranded or double-stranded [^{32}P]labeled-oligonucleotide DNA, a 150 molar excess of unlabeled oligonucleotide DNA, and 4.8 μg of wild-type or mutant AGT protein in the absence and presence of 100 mM NaCl. After incubation at 37 °C for 10 min, the samples were mixed with 1 μL of 50% glycerol containing 0.04% bromophenol blue. The DNA−protein complexes were then resolved on pre-electrophoresed 8.0% polyacrylamide gels (7.6% acrylamide and 0.4% bisacrylamide) for 2 h at 140 V in 90 mM Tris-borate, pH 8.0, and 1 mM EDTA. After electrophoresis the gels were exposed on Kodak X-OMAT film with intensifier screens. The complex formation between the oligonucleotide DNA and the protein was quantified by scanning the gels for radioactivity on Betascope 603 blot analyzer (Betagen, Waltham, MA). The amount of radioactivity in the unbound oligonucleotide incubated with wild-type or different mutant AGT proteins was calculated and compared with the radioactivity in the free oligonucleotide incubated in the absence of any protein.

RESULTS

Control AGT and mutant AGTs containing the R128A, Y114A, and Y114E alterations were produced by site-directed mutagenesis and expressed in *E. coli* strain GWR109. These mutant AGT proteins were expressed as well as the control human AGT as determined by Western blots of crude cells extracts developed with antibodies to AGT. All three proteins were purified to homogeneity from large scale cultures. The purified proteins were then tested for the ability to repair methylated DNA by incubation with a substrate of calf thymus DNA that had been methylated by reaction with *N*-[^{3}H]methyl-*N*-nitrosourea. The amount of O^6-[^{3}H]methylguanine that was removed from the substrate in a 30 min incubation with various amounts of these proteins is shown in Figure 1A. The R128A and Y114A mutants were much less active than the control AGT. About 0.02 μg of control protein was needed to remove 50% of O^6-[^{3}H]methylguanine whereas about 40 μg of the Y114A mutant and 50 μg of the R128A mutant AGT was needed for this level of repair. The Y114E mutant protein was completely inactive with no detectable loss of O^6-[^{3}H]methylguanine when 300 μg of this mutant AGT protein was added.

A time course of the repair of methylated DNA by the control, Y114A, and R128A mutant AGTs is shown in Figure 1B. Although the substrate was almost completely repaired by 0.1 μg of the control AGT in 5 min, there was no detectable repair in 1 h with 0.25 μg of the R128A protein, and even when 70 μg of the mutant AGT was added, the reaction had gone to only 50% of completion within 30 min. Mutant Y114A also repaired methylated DNA much more slowly than the control (Figure 1B).

The ability of the R128A and Y114E mutant AGTs to act on BG was examined by incubation with 8-[^{3}H]BG and measurement of the production of 8-[^{3}H]guanine (Figure 2). Comparison of the effects of various amounts of the proteins to bring about this reaction showed that there was little difference between the R128A mutant and the control AGT in this reaction with the R128A mutant being slightly more active (Figure 1B). This was confirmed by an experiment measuring the time course of the reaction with 5 μg of the

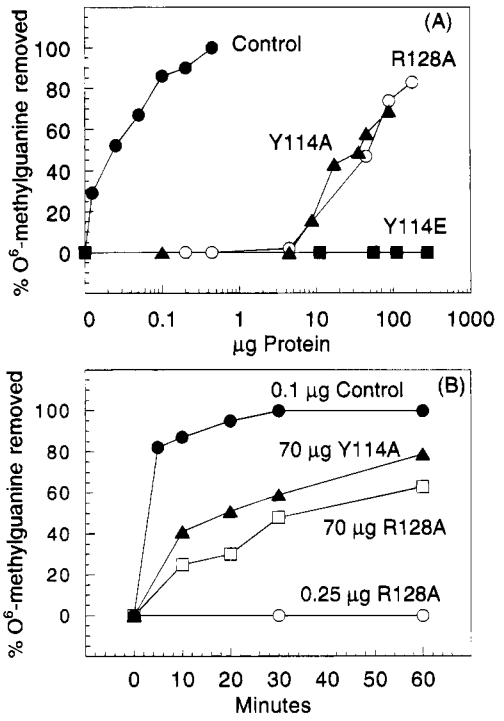

FIGURE 1: Activity of control, R128A, and Y114E mutant AGTs on methylated DNA. The activity was measured by assaying the removal of O^6-[^{3}H]methylguanine from [^{3}H]methylated DNA substrate and expressing the results as the percentage of O^6-methylguanine removed. Panel A shows the results with varying amounts of the control (●), R128A (○), Y114A (▲), and Y114E AGT (■) proteins after a 30 min incubation. Panel B shows the results for incubation of 0.1 µg of control (●), 0.25 µg of the R128A mutant (○), and 70 µg of the R128A mutant (□) or Y114A (▲) mutant AGTs for various times up to 60 min.

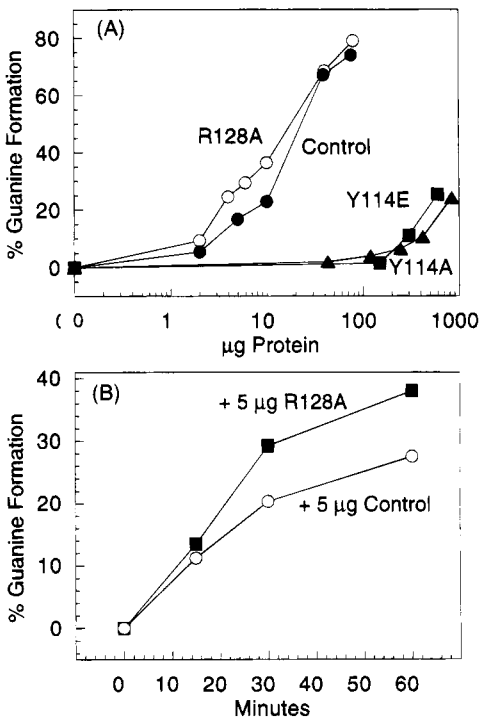

FIGURE 2: Activity of control, R128A, and Y114E mutant AGTs on BG. The activity was measured by assaying the conversion of [8-^{3}H]BG into [8-^{3}H]guanine and expressing the results as the percentage of BG converted. Panel A shows the results with varying amounts of the control (●), R128A (○), Y114A (▲), and Y114E AGT (■) proteins after a 20 min incubation. Panel B shows the results for incubation of 5 µg of control (○) or R128A mutant AGT (■) for various times up to 60 min.

control and R128A mutant AGT (Figure 1B) which also showed that the R128A mutant was slightly more effective than the control. The Y114E and Y114A mutant AGTs were a great deal less active than the control in producing 8-[^{3}H]-guanine, and about 60 and 80 times more protein, respectively, was needed to get an equal amount of guanine production (Figure 2A). However, the Y114E mutant AGT protein clearly did react with BG in contrast to its inability to repair methylated DNA.

These results indicate that the mutation R128A alters the ability of the AGT to react with a DNA substrate without affecting the ability to bind and react with a low molecular weight substrate such as BG. One explanation for this would be that the DNA binding domain of the protein requires arginine-128 in order to interact with the DNA substrate. This hypothesis was tested by studying the ability of these mutant alkyltransferases to compete with the wild-type AGT for repair of methylated DNA. A mutant AGT where the cysteine-145 acceptor site was changed to an alanine (C145A) (Crone & Pegg, 1993) was used as an example of an inactive AGT protein in which the DNA binding domain is not affected. The active control AGT protein was incubated with [^{3}H]methylated DNA for 30 min alone or in the presence of each of the mutant proteins C145A, R128A, and Y114E, and the extent of repair of O^6-[^{3}H]methylguanine was determined (Figure 3). A reduction of the repair by the control AGT when measured in presence of mutant should reflect the ability of mutant protein to compete with control for the DNA substrate. The presence of the C145A mutant protein did produce the expected decrease, but there

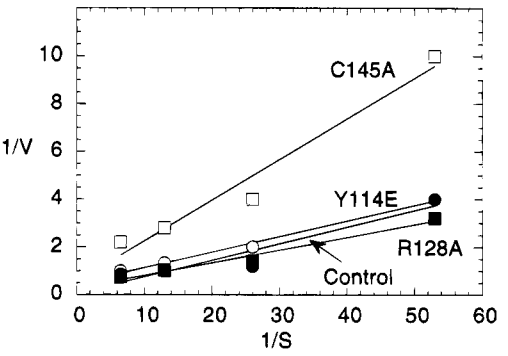

FIGURE 3: Competition of control and C145A, R128A, and Y114E for binding to the methylated [^{3}H]DNA substrate. Reactions were carried out to measure the repair of [^{3}H]methylated DNA with the control AGT alone (●), and the control AGT plus 5 µg of C145A (□), plus 5 µg of Y114E (○), or plus 5 µg of R128A (■) proteins for 30 min. The reciprocal of the amount of repair (V) was then plotted against the reciprocal of the amount of AGT protein added (S).

was no effect of the R128A and Y114E mutant AGTs. This indicates that these mutant AGT proteins are unable to prevent the binding of control AGT to DNA. The most probable reason for this failure is that these mutant AGT proteins have a much lower affinity for DNA binding than the control AGT protein.

The DNA binding properties of the AGT proteins (C145A, Y114E, R128A, and W100A) were then tested more directly using a gel shift assay (Figures 4 and 5). Experiments carried out with dsDNA and ssDNA from M13 are shown in Figure 4. When incubated with M13 ssDNA, there was formation of stable and well retarded M13 ssDNA−protein complexes

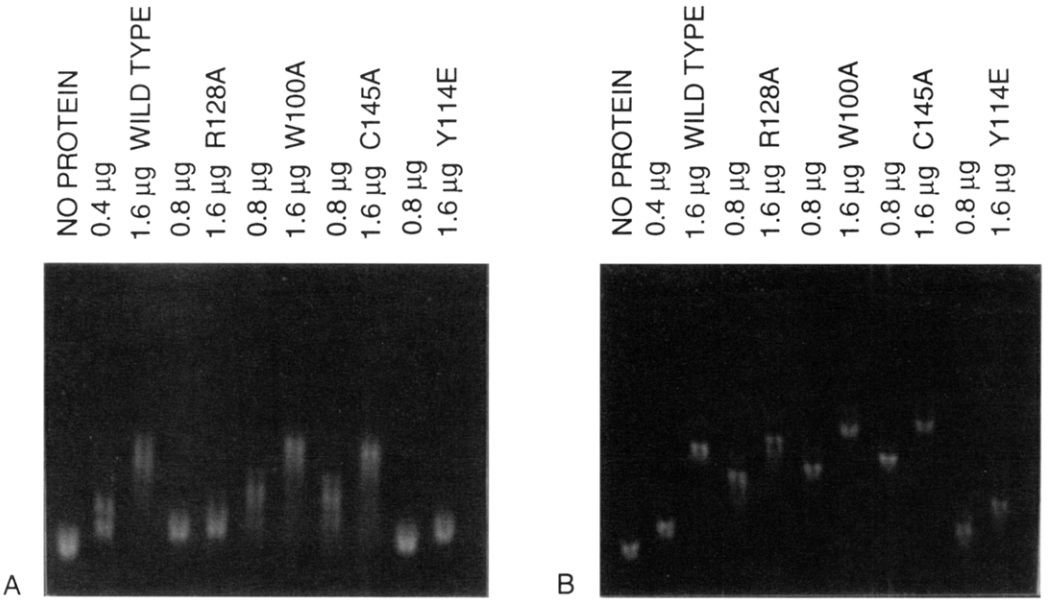

FIGURE 4: Binding of control and mutant AGTs to M13 DNA. Gel mobility shift assays were carried out with M13 ssDNA (panel A) and M13 dsDNA (panel B). Each reaction mixture contained 200 ng of DNA and the indicated amount of either wild-type or mutant AGT protein in a 10 μL volume. After incubation at 37 °C for 10 min, the nucleoprotein complexes were resolved on a 0.5% agarose gel and stained with ethidium bromide.

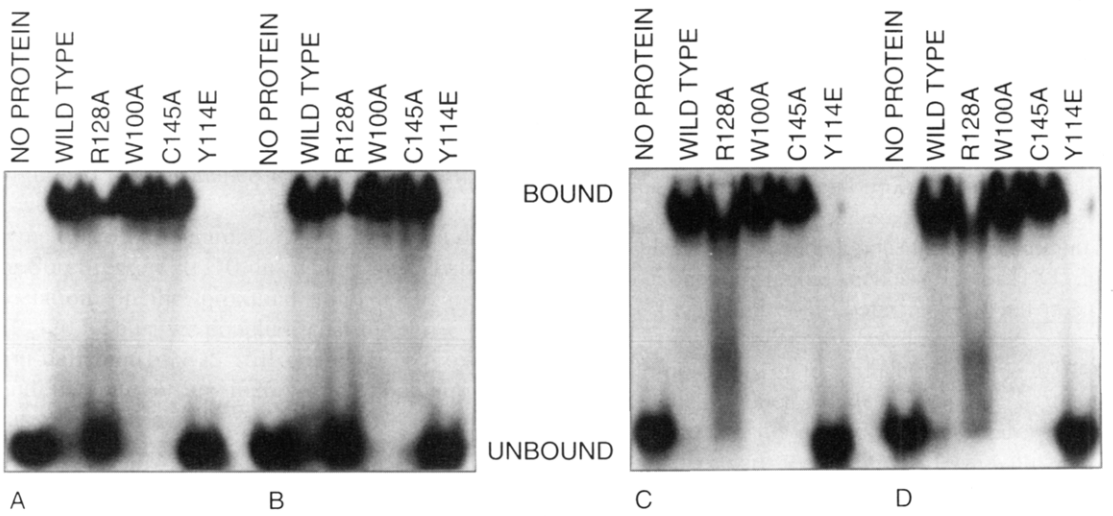

FIGURE 5: Binding of control and mutant AGTs to oligodeoxynucleotides. Gel mobility shift analysis of AGT binding to a single-stranded 16-mer (panels A and B) or a 16-mer duplex (panels C and D) were carried out. Each reaction contained 100 fmol of ^{32}P-labeled oligodeoxynucleotide, 15 pmol of unlabeled oligonucleotide, and 4.8 μg of wild-type or mutant AGT protein in a 10 μL volume containing contained either no additional salt (panels A and C) or 100 mM NaCl (panels B and D). After incubation at 37 °C for 10 min, the nucleoprotein complexes were resolved on 8% nondenaturing polyacrylamide gels and autoradiographs developed.

with wild-type, W100A, and C145A mutant AGT proteins, and the complexes showed an increase in retardation with increase in the amount of protein (Figure 4A). However, the ssDNA incubated with mutant proteins R128A and Y114E was not retarded as effectively as the ssDNA incubated with wild-type, W100A, or C145A proteins and migrated close to the DNA incubated without any protein. When the same experiment was carried out with M13 dsDNA, protein complexes were observed for all the proteins except for the mutant Y114E (Figure 4B). The mutant AGT protein R128A did form a DNA−protein complex with dsDNA despite the failure to form such a complex with ssDNA. The mutant AGT Y114A showed only a slight reduction in the binding to ssDNA and dsDNA (results not shown).

Gel retardation assays with these proteins were also carried out with single-stranded and duplex 16-mer oligodeoxynucleotides that were end-labeled with ^{32}P (Figure 5). The binding reactions were done in the absence (Figure 5, panels A and C) and presence of 100 mM NaCl (Figure 5, panels B and D). There was no effect of salt on the complex formation by the AGT preparations (Figure 5 and Table 1).

Wild-type, W100A, and C145A mutant proteins formed stable complexes with both single-stranded and double-stranded 16-mer (Figure 5), and virtually all of the input DNA was in the complex as less than 10% of the input radioactivity was observed in the unbound region (Table 1). The Y114E mutant protein failed to form any discrete complex with either single- or double-stranded 16-mer, and >90% of the input radioactivity was recovered in the unbound region. The mutant R128A showed different binding behavior with the single- and double-stranded oligodeoxynucleotide. Although this mutant did not form a clear band of a discrete complex with double-stranded 16-

Table 1: Binding of Control and Mutant AGTs to 16-mer Oligodeoxynucleotides

	percent of oligodeoxynucleotide unbound[a]			
	single-stranded 16-mer		double-stranded 16-mer	
protein	+100 mM NaCl	no addition	+100 mM NaCl	no addition
none	100	100	100	100
wild type	12	8	10	12
R128A	78	76	12	16
W100A	10	10	5	4
C145A	10	9	8	8
Y114E	92	96	98	94

[a] The amount of radioactivity present in the position of the unbound oligodeoxynucleotide in the presence of the proteins shown was compared to that found in this position when no AGT protein was added.

Table 2: Effect of DNA on Production of Guanine from BG by Control and R128A AGTs

	guanine formation[a]			
	by R128A AGT		by control AGT	
DNA addition	(cpm/ μg)	(fold increase)	(cpm/ μg)	(fold increase)
none	1057		750	
20 μg of calf thymus DNA	4990	(4.7)	4382	(5.8)
512 pmol of 16-mer duplex	4077	(3.9)	3659	(4.9)
51 pmol of 16-mer	1499	(1.4)	1953	(2.6)
205 pmol of 16-mer	1889	(1.8)	2206	(2.9)
1025 pmol of 16-mer	3321	(3.2)	4380	(5.8)

[a] Guanine formation was measured as described in the legend to Figure 2 in 0.25 mL assays containing the DNA shown and 2−4 μg of AGT protein which were incubated for 20 min at 37 °C. Results are shown from a representative experiment. Similar results were obtained in three different assays.

mer (Figure 5C,D), little of the labeled 16-mer was observed in the unbound form (Table 1) suggesting that the complex formed was less stable than the complex formed by the wild-type AGT. However, when this R128A protein was incubated with the single-stranded 16-mer, most (78%) of the input radioactivity was observed in the unbound area (Figure 5A,B and Table 1), clearly indicating the failure of this protein to form stable complexes with single-stranded nucleic acid.

Previous studies have indicated that the formation of guanine from BG by human AGT is stimulated by the addition of DNA and that either dsDNA or ssDNA is effective in bringing about this stimulation (Goodtzova et al., 1994). The rate of conversion of [^{3}H]BG to [^{3}H]guanine by the R128A mutant was increased by the addition of a large amount (20 μg) of calf thymus DNA by a factor of about 5, which is only slightly less than the 6-fold stimulation of the control AGT (Table 2). Similar results were obtained when the 16-mer duplex DNA was added with a 4-fold stimulation of the R128A and 5-fold stimulation of the control. However, when the single-stranded 16-mer was added, the activity of R128A was increased considerably less than that of the control AGT particularly at low concentrations of the 16-mer (Table 2).

DISCUSSION

Our results support the suggestion by Moore et al. (1994) that the AGT region containing amino acids tyrosine-114 and arginine-128 is involved in the DNA binding properties of the AGT. In the structure of the Ada-C AGT derived by X-ray crystallography, the sequence equivalent to that from amino acids phenylalanine-94 to asparagine-137 in the human sequence forms a helix−turn−helix−helix domain that resembles some DNA binding motifs (Moore et al., 1994). Our observations that the mutation R128A greatly reduces the ability to repair methylated DNA without diminishing the ability to react with BG are consistent with the hypothesis that this arginine residue plays a role in the binding of the DNA substrate.

The results with the Y114E and Y114A mutants suggest that this residue is also located in a region critical for DNA binding but may not itself play a critical role in this binding. Tyrosine-114 is clearly important for the activity of the AGT protein since these mutants were completely inactive in repairing methylated DNA (Y114E) or had activity reduced by >1000-fold (Y114A) and had greatly reduced activity with BG (by 60- and 80-fold, respectively). However, only the change of Y114E drastically affected the binding to DNA. It is possible that the presence of an acidic residue at position 114 interferes with DNA binding directly or by interacting with arginine-128.

Experiments with proteins produced by site-directed mutagenesis can always be criticized on the grounds that the changes made may lead to a general change in the protein structure which produces an observed change rather than to a specific requirement for the altered amino acid. In the absence of a crystal structure for our mutants, this possibility cannot be absolutely ruled out, but it is very unlikely for several reasons. First-, the R128A, Y114A, and Y114E mutant AGT proteins could be purified using exactly the same procedure as that for the wild-type AGT, and the purified proteins were eluted in the identical fractions from the Mono-S column and once purified these mutant proteins were as stable as the wild-type AGT. Secondly, as described above, the mutant proteins were able to react with BG. This argument is particularly compelling for the R128A mutant which was fully active with BG, but even the Y114E mutant has significant activity with BG and this protein was totally inactive in repairing methylated DNA, both in the *in vitro* experiments reported here and in studies in which its ability to protect an ada$^-$ogt$^-$ strain from the toxic effects of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine was measured (T. M. Crone and A. E. Pegg, unpublished observations). The latter is a very sensitive test which has indicated activity in a number of AGT mutants including mutations at asparagine-137, histidine-146, arginine-147, and glutamic acid-172 which are much too unstable for purification or even to give activity in broken cell preparations (Rafferty et al., 1994; Pieper et al., 1994; Crone et al., 1994). Thirdly, the R128A and Y114E mutant AGT proteins were stable in the *E. coli* cells expressing them. This contrasts sharply with the effects of mutations at some other conserved residues in the AGT including asparagine-137, histidine-146, arginine-147, and glutamic acid-172. These mutant AGT proteins have very short half-lives when expressed in *E. coli* (T. M. Crone and A. E. Pegg, unpublished observations), suggesting that these mutations bring about a destabilization of the protein structure.

Although the binding of ssDNA by the R128A mutant was clearly reduced in the gel shift assays shown in Figures 4 and 5, there must be some interaction of this protein with

single-stranded DNA since BG conversion to guanine was enhanced by the presence of a 16-mer (Table 2). This discrepancy may be accounted for if the binding to the 16-mer is insufficient to allow the complex to survive the conditions used in the gel shift experiments. A detailed study of the affinity of the control and R128A AGT mutants to bind to DNA using fluorescence anisotropy and other biophysical techniques is needed to fully evaluate this binding.

The inability of the mutant Y114E to bind to either dsDNA or ssDNA suggest that this change completely disrupts the DNA binding capacity of the AGT protein. The behavior of the R128A mutant is more interesting since this protein was able to bind to M13 dsDNA under the conditions of our gel shift experiments and showed some ability to bind to the double stranded 16-mer although this binding was less stable than that of the control AGT. However, the R128A mutant AGT failed to bind to the ssDNA from M13 or to the single-stranded 16-mer. Although it is well established that the most favored substrate for the AGT is a double-stranded DNA molecule (Demple, 1990; Mitra & Kaina, 1993; Pegg et al., 1995), it is likely that the protein must lead to a local melting or other distortion of the DNA structure in order to bring about the alkyl transfer. Recent studies with purified human and *E. coli* AGTs have demonstrated the rapid repair of O^6-methylguanine in single-stranded oligodeoxynucleotides containing more than eight residues (Liem et al., 1993). Studies of the repair of oligodeoxynucleotide duplexes containing O^6-methylguanine analogs also suggested that the duplex opens up in order for the reaction to occur (Spratt & Campbell, 1994). At this stage in the AGT reaction, the bound portion of the DNA substrate may be essentially single-stranded, and it appears that it is in the retention of the substrate at this stage that the R128A mutant is defective. These results suggest that binding of the AGT to DNA does induce a change in the structure of the DNA as well as of the AGT protein. The possibility that this distorts the structure of the DNA in a manner that flips out the methylated base placing it in a correct orientation for repair in an analogous manner to the action of the M. *Hha*I DNA-methyltransferase at its target cytosine (Klimasauskas et al., 1994; Roberts, 1994) deserves serious consideration.

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