Reaction and Binding of Oligodeoxynucleotides Containing Analogues of O^6-Methylguanine with Wild-Type and Mutant Human O^6-Alkylguanine-DNA Alkyltransferase†

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ABSTRACT: O^6-Alkylguanine-DNA alkyltransferase (AGT) repairs DNA by transferring the methyl group from the 6-position of guanine to a cysteine residue on the protein. We previously found that the *Escherichia coli* Ada protein makes critical interactions with O^6-methylguanine (O^6mG) at the *N*1- and O^6-positions. Human AGT has a different specificity than the bacterial protein. We reacted hAGT with double-stranded pentadecadeoxynucleotides containing analogues of O^6mG. The second-order rate constants were in the following order ($\times 10^{-5}$ M^{-1} s^{-1}): O^6mG (1.4), O^6-methylhypoxanthine (1.6) > Se^6-methyl-6-selenoguanine (0.1) > S^6-methyl-6-thioguanine (S^6mG) (0.02) ≫ S^6-methyl-6-thiohypoxanthine (S^6mH), O^6-methyl-1-deazaguanine (O^6m1DG), O^6-methyl-3-deazaguanine (O^6m3DG), and O^6-methyl-7-deazaguanine (O^6-m7DG) (all <0.0001). Electrophoretic mobility shift assays were carried out to determine the binding affinity to hAGT. Oligodeoxynucleotides containing O^6mG, S^6mG and O^6m3DG bound to AGT in the presence of competitor DNA with K_d values from 5 to 20 μM, while those containing G, S^6mH, O^6-m1DG, and O^6m7DG did not (K_d > 200 μM). These results indicate that the 1-, N^2-, and 7- positions of O^6mG are critical in binding to hAGT, while the 3- and O^6-positions are involved in methyl transfer. These results suggest that the active site of *ada* AGT is more flexible than hAGT and may be the reason *ada* AGT reacts with O^4mT faster than hAGT.

O^6-Alkylguanine-DNA alkyltransferase (AGT)[1] repairs DNA by transferring a methyl group from the 6-position on guanine to a cysteine residue in the active site of the protein. The original guanine is regenerated, but the cysteine remains alkylated and, consequently, the protein is inactivated. This protein is found in many species. The amino acid sequences of the C-terminal domain of the *ada* protein, the *ogt* protein, and human AGT are similar, especially near the active site (*1*−*4*). The differences lead to different reactivities of the proteins. For example, the *ada* protein can repair O^4-methylthymine (O^4mT) more efficiently than mammalian proteins (*5*−*10*). The ability to repair different sizes of alkyl substituents is also altered. For example, human AGT can repair the bulky O^6-benzylguanine (*11*) and O^6-(4-(3-pyridyl)-4-oxobutyl)guanine (*12*) adducts while the *ada* protein cannot. Site-specific mutations of human and *ada* AGT suggest that steric constraints at the active site are responsible for the inability of the bacterial protein from reacting with O^6-benzylguanine (*13*−*15*). This factor, however, cannot account for the lesser reactivity of human AGT with O^4mT, as this base is smaller than O^6mG. An alternative hypothesis is that there are specific interactions between the human AGT and O^6mG that are not present between the ada AGT and O^6mG. For example, if human AGT makes a critical interaction with the 7-position of O^6mG, then reaction with O^4mT should be decreased. In O^4mT, there is a methyl group in the analogous position at the nitrogen at the 7-position of O^6mG. To test this hypothesis, oligodeoxynucleotides containing analogues of O^6mG (Scheme 1) were reacted with human AGT.

EXPERIMENTAL PROCEDURES

General. Scintillation spectroscopy was performed on a Beckman LS 9800 scintillation counter using Pico-Fluor 40 (Packard) as the cocktail. [^{3}H]Methylnitrosourea was synthesized from [^{3}H]acetic acid (*16*), which was purchased from Amersham. Wild-type and mutant AGT were purified as described (*17*). AGT was assayed by reaction with DNA which had been methylated with [^{3}H]MNU (*18*). Oligodeoxynucleotides of the sequence 5′-GGC GCT XGA GGC GTG-3′, in which X is an analogue of O^6mG, were synthesized and labeled with [^{32}P]phosphate as described (*19*). The oligodeoxynucleotides were annealed with a 10% excess of the complementary strand in which O^6mG was paired with cytosine.

Reaction between Oligodeoxynucleotides and AGT. The reactions between AGT and oligodeoxynucleotide substrates

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[1] Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; EMSA, electrophoretic mobility shift assay; O^6m1DG, O^6-methyl-1-deazaguanine; O^6m3DG, O^6-methyl-3-deazaguanine; O^6m7DG, O^6-methyl-7-deazaguanine; O^6mG, O^6-methylguanine; O^6mH, O^6-methylhypoxanthine; S^6mG, S^6-methyl-6-thioguanine; S^6mH, S^6-methyl-6-thiohypoxanthine; Se^6mG, Se^6-methyl-6-selenoguanine; Se^6mH, Se^6-methyl-6-selenohypoxanthine.

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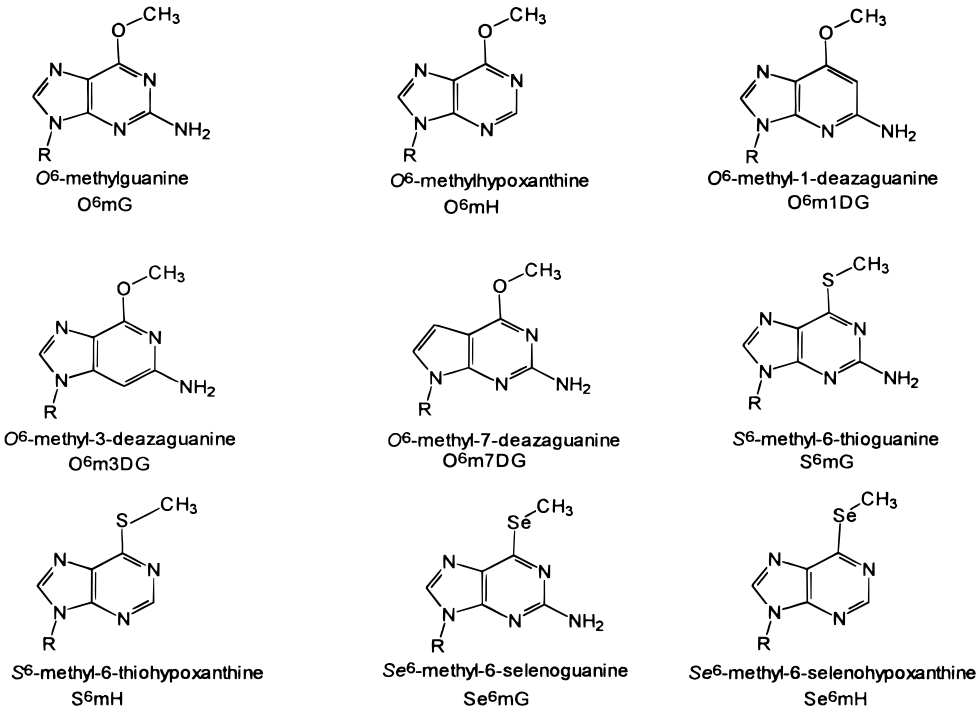
Scheme 1



were initiated by the addition of AGT (1−50 nM) to a 1 mL solution of ^{32}P-labeled oligodeoxynucleotide duplex (1 nM) in 20 mM Tris-HCl, 1, mM EDTA, 1 mM DTT, and 5% glycerol, pH 7.6 at 37 °C. After incubation at 37 °C, the reaction was quenched by the addition of 0.5 mL of 0.1 N NaOH to a 0.1 mL aliquot. The samples were kept frozen at −78 °C until analyzed by HPLC. In the inhibition studies, unlabeled DNA was incubated with AGT for 10 min prior to addition of the ^{32}P-labeled oligodeoxynucleotide. The progress of the reaction was analyzed by HPLC with a 4.6 × 250 mm Nucleopac Pa-100 (Dionex) strong anion-exchange column with a NaCl gradient in 10 mM NaOH (*20*). The reaction was analyzed by second-order kinetics as described (*19*).

Electrophoretic Mobility Shift Assay. Radiolabeled oligodeoxynucleotide (0.5 μL) was added to wtAGT or C145A (0−30 μM) in 25 mM Hepes, pH 7.8, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, and 10% glycerol containing 0 or 0.05 μg/μL salmon sperm DNA. The total volume was 10 μL. The oligodeoxynucleotide concentration was 0.025 μM for the modified strand and 2.5 μM for the complementary strand. When salmon sperm DNA was included, the reaction mixture was incubated for 3 h at 37 °C. When competitor DNA was not added, the incubation time was 10 min. The solution was then loaded on a 6% polyacrylamide gel (20 × 16 × 0.1 cm) in 0.5 × TBE and run at 200 V for 1 h. The gel was visualized with a Bio-Rad G250 Molecular Imager.

RESULTS

Repair of Modified O^6-Methylguanine. Previously, we concluded that AGT expressed from the *ada* gene of *Escherichia coli* interacts with the *N*^{2}-, *O*^{6}-, and 1-positions of *O*^{6}mG to effect methyl transfer (*18*, *19*). However, the human and bacterial proteins have slightly different substrate specificities (*5*−*12*). To determine whether the human protein interacts differently with *O*^{6}mG than *ada* AGT, we determined the second-order rate constants of the repair of oligodeoxynucleotides containing modified *O*^{6}mG. The results of these experiments are shown in Table 1. These rate constants were obtained by mixing an excess of AGT with an oligodeoxynucleotide duplex containing *O*^{6}mG or an analogue. The demethylation of the ^{32}P-labeled modified strand was monitored by anion-exchange chromatography (*19*, *20*). From the time course of the reaction, second-order reaction rates were determined.

Table 1: Reaction of AGT with Oligodeoxynucleotides Containing Analogs of O^{6}MG[a]

compd	*ada* AGT	human AGT
O^6-methylguanine	7.7 ± 0.9	1.4 ± 0.2
O^6-methylhypoxanthine	54 ± 7	1.6 ± 0.4
O^6-methyl-1-deazaguanine	0.022 ± 0.009	<0.001
O^6-methyl-3-deazaguanine	0.77 ± 0.25	<0.001
O^6-methyl-7-deazaguanine	0.31 ± 0.10	<0.001
S^6-methyl-6-thioguanine	0.017 ± 0.002	0.002 ± 0.001
S^6-methyl-6-thiohypoxanthine	<0.005	<0.001
Se^6-methyl-6-selenoguanine	0.044 ± 0.021	0.10 ± 0.04
Se^6-methyl-6-selenohypoxanthine	<0.005	0.0013 ± 0.0004

[a] k_2 is 10^5 M^{-1} s^{-1}. Reaction was run with AGT (1−50 nM), oligodeoxynucleotide duplex (1 nM) in 20 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 0.1 mg/mL BSA, 5% glycerol, pH 8.0 at 37 °C.

Human AGT reacted, with an oligodeoxynucleotide duplex containing *O*^{6}mG, at about 20% the rate of the *E. coli* protein. The relative reactivity with *S*^{6}mG was comparable with both proteins, the reaction of *S*^{6}mG with *ada* AGT was 450 times slower than with *O*^{6}mG, while with human AGT, *S*^{6}mG reacted 700-fold slower than *O*^{6}mG. *Se*^{6}mG reacted slightly more quickly with human protein than with the *ada* AGT. Thus, we concluded that substitution at the *O*^{6}-position did not result in significant differences between *ada* AGT and human AGT.

We previously found that *O*^{6}mH reacted 7-fold faster with *ada* AGT than with *O*^{6}mG prompting us to hypothesize that

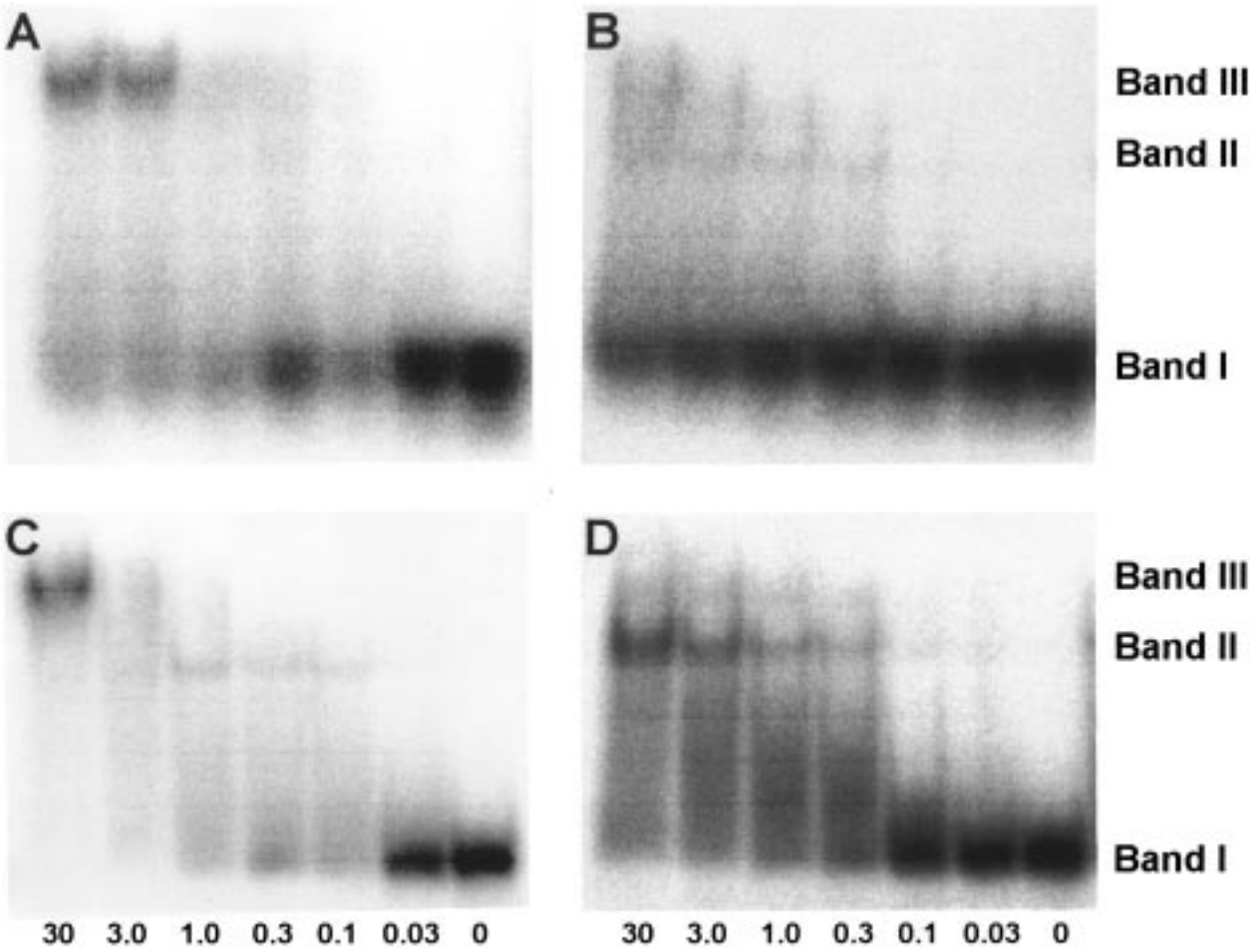


FIGURE 1: EMSA were carried out in in 25 mM Hepes, pH 7.8, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, and 10% glycerol at 37 °C. AGT (0−30 μM) was in excess over the oligodeoxynucleotide duplex (50 nM modified stand, 500 nM complementary strand). (A) The oligodeoxynucleotide duplex was that in which X = O^6m1DG. (B) AGT and oligodeoxynucleotide ($X = O^6$m1DG) duplex was also incubated with 50 μg/mL salmon sperm DNA. (C) The oligodeoxynucleotide duplex was that in which X = O^6m3DG. (D) AGT and oligodeoxynucleotide duplex ($X = O^6$m3DG) was also incubated with 50 μg/mL salmon sperm DNA. Band I is the free oligodeoxynucleotide duplex. Bands II and III are the oligodeoxynucleotide in a complex with AGT.

the helix may open up for methyl transfer to occur (*19*). This result, however, differs from that observed with human AGT, which reacted with O^6mG and O^6mH at approximately the same rate. This 7-fold rate difference is much less than that observed for substitutions on the 1-, 3-, and 7-positions.

Replacement of the ring nitrogens at the 1-, 3-, and 7-positions of O^6mG had a more drastic effect on the reaction with human AGT than *ada* AGT. Whereas, O^6m1DG, O^6-m3DG, or O^6m7DG reacted 10−100 times more slowly than with O^6mG with the *E. coli* protein, we did not detect any repair of these bases with the human AGT. The rate reduction was greater than 2000-fold. From these results, we concluded that the nitrogens at the 1-, 3-, and 7-positions are more essential for the repair of O^6mG by human AGT than by *ada* AGT.

Binding of AGT to O^6mG Analogues. Several of the O^6-mG analogues are not repaired by wild-type AGT. The lack of reactivity may be due to either (1) the inability of the O^6mG analogue to bind to the active site of AGT or (2) the analogue binds to the active site but the methyl group is not transferred. The binding of oligodeoxynucleotides containing O^6mG and analogues was evaluated with electrophoretic mobility shift assays (EMSA). Oligodeoxynucleotides were incubated with AGT and the oligodeoxynucleotide-protein binding was evaluated with PAGE. Typical gels are presented in Figure 1. Figure 1, panels A and B, show the binding of an oligodeoxynucleotide duplex containing O^6m1DG. As the AGT concentration is increased, the amount of free oligodeoxynucleotide (band I) is decreased and the amount of bound oligodeoxynucleotide (band III) is increased (Figure 1A). When competitor salmon-sperm DNA is added to the mixture, binding of the ^{32}P-labeled oligodeoxynucleotide to the protein is almost eliminated (Figure 1B). The same pattern arises whether wild-type or C145A AGT is used. Cysteine-145 is the active-site residue to which the methyl group on O^6mG is transferred. Thus, C145A cannot accept methyl groups from O^6mG, but it does bind to oligodeoxynucleotides containing O^6mG (*21*, *22*).

Figure 1, panels C and D, show the binding of an oligodeoxynucleotide duplex containing O^6m3DG. As the AGT concentration is increased, two retarded bands are observed (Figure 1C). A more mobile species (band II) is formed at lower AGT concentrations. As more AGT is added, this species is converted into a lower mobility species (band III). Salmon sperm DNA inhibits the formation of band III, more than band II (Figure 1D).

The guanine analogues can be divided into three groups based on the EMSA pattern. Oligodeoxynucleotides contain-

Table 2: Binding of AGT with Oligodeoxynucleotides Containing Analogs of O^6mG[a]

compd	wild-type		C145A	
	no DNA[b]	DNA[c]	no DNA[b]	DNA[c]
guanine	5.5 ± 1.3	>200	6.7 ± 0.8	>500
O^6-methylguanine	nd[d]	nd	2.6± 0.2	19 ± 3
O^6-methylhypoxanthine	nd	nd	3.7 ± 0.5	>500
O^6-methyl-1-deazaguanine	21 ± 1	>500	75 ± 14	>500
O^6-methyl-3-deazaguanine	5.3 ± 1.4	9.4 ± 1.9	7 ± 2	28 ± 5
O^6-methyl-7-deazaguanine	11 ± 2	43 ± 10	13 ± 2	>200
S^6-methyl-6-thioguanine	2.0 ± 0.3	5.3 ± 0.5	2.5± 0.5	24 ± 4
S^6-methyl-6-thiohypoxanthine	2.0 ± 0.2	18 ± 2	2.8 ± 0.4	>200

[a] K_d^{app} (μM). [b] AGT (0−30 μM), oligodeoxynucleotide duplex (50 nM modified stand, 500 nM complementary strand) in 25 mM Hepes, pH 7.8, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol. [c] Binding was measured in the presence of 0.05 μg/μL salmon sperm DNA. [d] Experiment was not done because AGT would react with the substrate.

ing G, O^6m1DG, and O^6mH form only band III when complexed with wild-type AGT or C145A. A very faint band II can be observed between AGT and duplexes containing O^6m7DG and S^6mH. Band II is most intense with oligodeoxynucleotides containing O^6mG, S^6mG, and O^6m3DG. Salmon sperm DNA inhibits formation of the slower moving protein−DNA complex (II) more than the faster complex (III).

The binding was analyzed by eqs 1 and 2, in which P represents AGT, D the oligodeoxynucleotide, and K_d the dissociation constant. Figure 1 shows that the AGT−oligodeoxynucleotide complex was not completely stable to the electrophoresis conditions. Therefore, the intensity of bands II and III do not reflect the total amount of ^{32}P-oligodeoxynucleotide bound to AGT just prior to electrophoresis. In our analysis of the binding, we have divided the intensity of the free nucleotide by the total radioactivity to obtain $[D]/[D]_T$, in which [D] is the free oligodeoxynucleotide concentration, and $[D]_T$ the total oligodeoxynucleotide duplex concentration. Equation 2 can be transformed to eq 3. We fitted the data, by nonlinear regression to obtain the K_d values. Since the AGT−DNA complex is somewhat unstable to the electrophoresis conditions the K_d values in Table 2 represent upper limits for the dissociation constant.

$$PD \rightleftarrows P + D \quad (1)$$

$$K_d = \frac{[P][D]}{[PD]} \quad (2)$$

$$\frac{[D]}{[D]_T} = \frac{K}{K_d + [P]_T} \quad (3)$$

Figure 2 shows typical binding curves of oligodeoxynucleotides with wtAGT. The K_ds for all the oligodeoxynucleotides fall within 2−20 μM for wtAGT and 2−75 μM for C145A. The modified guanine does not influence the binding very much. The DNA and not the O^6mG appears to govern the tightness of the binding. The binding curves are consistent with an AGT:DNA stoichiometry of 1:1. This result is different than that observed for single-stranded DNA, in which the stoichiometry between AGT and single-stranded DNA is 3.8:1 (*21*).

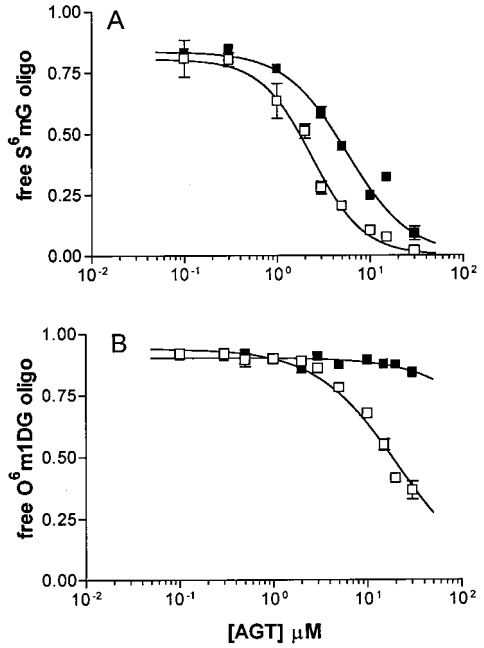


FIGURE 2: Plot of free oligodeoxynucleotide versus [AGT] in the presence (■) or absence (□) of salmon sperm DNA (50 μg/mL). The oligodeoxynucleotide contained either S^6mG (A) or O^6m1DG (B). Each point represents the mean of at least three determinations and the error bars are the standard deviations. The solid line is the best nonlinear least-squares fit to eq 3.

The presence of O^6mG or an analogue can influence the strength of the binding in the presence of competitor DNA. Figure 2A illustrates that the K_d for S^6mG is not greatly reduced in the presence of salmon sperm DNA; the K_d rises from 2.0 to 5.3 μM. In contrast, the binding of O^6m1DG with wtAGT is almost eliminated by salmon sperm DNA (Figure 2b).

Table 2 shows the K_d values for all the oligodeoxynucleotides. When salmon sperm DNA was added, oligonucleotides containing G showed an increase in K_d from 5.5 μM to 200 μM with wtAGT and 6.7 μM to >500 μM with C145A. This pattern was matched by O^6mH, O^6m1DG, O^6-m7DG, and S^6mH. The observation that these analogues mimic G suggests that AGT does not recognize these compounds as substrates for methyl transfer. The binding of AGT to oligodeoxynucleotides containing O^6mG, O^6-m3DG, and S^6mG are not affected as much by added salmon sperm DNA. The ability to form band II was proportional to the binding in the presence of salmon sperm DNA. The observation that O^6m3DG and S^6mG mimic the binding of O^6mG suggests that these compounds are recognized by AGT as substrates.

DISCUSSION

Altering the ring nitrogens of O^6mG affected the rate of reaction with human AGT much more than the *ada* protein. While *ada* AGT reacted 10−100-fold more slowly with oligodeoxynucleotides containing O^6m1DG, O^6m3DG, and O^6m7DG than with O^6mG (*19*), the rate of reaction of these O^6mG analogues with human AGT was undetectable. Thus, the rate of repair was decreased as least 2000-fold with human AGT. This observation suggests that these positions are more important for the repair of O^6mG by the human protein than by the bacterial protein.

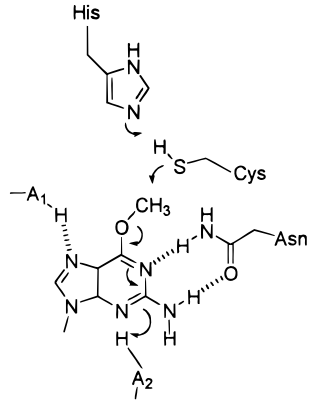
The lack of reactivity between AGT and O^6mG analogues may be due to (1) the inability of the substrate to bind to the active site or (2) the substrate binds but the methyl is not transferred. The binding of these oligodeoxynucleotides to wild-type and C145A human AGT was examined by EMSA. The binding affinity was only marginally affected by the analogue incorporated into the oligodeoxynucleotide, indicating that the DNA structure plays the major role in binding of oligodeoxynucleotides to AGT (Table 2). The poorest binding oligodeoxynucleotide contained O^6m1DG. This substrate has the Watson−Crick hydrogen-bonding face altered. Perhaps it caused the double-helix to be disordered such that it did not bind to AGT as well as double stranded DNA.

Major differences in binding of O^6mG analogues to AGT were observed when salmon sperm DNA was used to compete with the ^{32}P-labeled oligodeoxynucleotides for binding to AGT. The K_ds of oligodeoxynucleotides containing G, O^6mH, O^6m1DG, O^6m7DG, and S^6mH rose greater than 20-fold when an excess of salmon sperm DNA was added. Conversely, the binding of O^6mG, O^6m3DG, and S^6mG were increased less than 3-fold. O^6mG, O^6m3DG, and S^6mG also exhibited the most intense band IIs on the gel shift assays. We also found that salmon sperm DNA inhibited the formation of band III much more than band II in the EMSA. These observations suggest that O^6mG, O^6m3DG, and S^6mG bind to AGT in a different manner than the other substrates. Perhaps these substrates bind in an activated complex, in which O^6mG (or analog) is in position to react, transfer the methyl group to AGT. This hypothesis is similar to that proposed by Hazra et al. (*22*) in which O^6mG binds to AGT in an activated complex, while unmethylated DNA binds in a nonspecific manner. If our hypothesis is correct, O^6mG, S^6mG, and O^6m3DG have the correct features to be in this binding mode, while O^6mH, O^6m1DG, O^6m7DG, and S^6mH do not.

Although, S^6mG and O^6m3DG bind to AGT as well as O^6mG, their methyl groups are not transferred as fast. These results suggest that these positions are important for methyl transfer. Sulfur is larger than oxygen with covalent radii of 1.03 and 0.64 Å (*23*). Sulfur and oxygen also have different bond angles, 90° and 109°, respectively. These differences would alter the active-site geometry. The change in position can deleteriously affect the rate of methyl transfer. The importance of the 3-position of O^6mG is not as evident. The nitrogen can help neutralize the negative charge of the leaving group either by being protonated or by being an electron sink. Resonance structures of the leaving group guanine can be drawn in which the negative charge is localized on the O^6-, 1-, or 3-positions. All three positions are probably important in stabilizing the charge.

The ability of S^6mG and O^6m3DG to bind to AGT but not react implicates the 3- and O^6-positions as crucial for methyl transfer. The poor binding of O^6m1DG and O^6m7DG implicates the nitrogens at the 1- and 7-positions as being crucial in the binding of O^6mG to AGT. Although this analysis does not support a role of these positions in the methyl transfer step, we cannot rule out the possibility that these positions are also critical to the methyl transfer.

Scheme 2: Repair of O^{6}mG by human AGT



O^6mH does not bind to C145A in a complex stable enough to be observed by EMSA, but it does react. These results seem paradoxical. The amino group stabilizes the complex between AGT and O^6mG, but methyl transfer can occur without this tight binding mode. Perhaps there is an equilibrium between the O^6mG in the helix and in the binding pocket.

Scheme 2 illustrates our proposed mechanisms of reaction of human AGT with O^6mG. The N^2-, 1-, and 7-positions are involved in the binding of O^6mG to the active site of AGT. The interactions between these positions and AGT can stabilize the binding complex or they may be involved in flipping O^6mG out of the DNA helix and into the active site on AGT. Asn 138 is an essential active site residue at the bottom of the hydrophobic pocket of the C-terminal domain of *ada* AGT (*24*). This residue is totally conserved among species and mutation of this residue results in inactive AGT (*25*, *26*). We speculate that this residue in human AGT (Asn 137) may form a hydrogen bond to the 1- and N^2-positions of O^6mG.

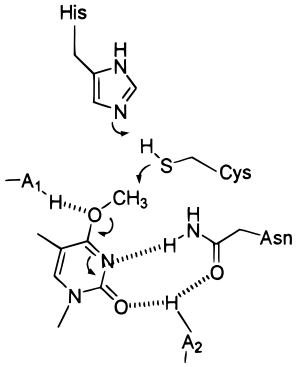
Methyl transfer can be accelerated by conversion of the active-site cysteine into a better nucleophile by deprotonation by the adjacent histidine (*27*). The negative charge on the leaving group guanine is stabilized by the electronegative nitrogens at the 1- and 3-positions, or by a proton transfer to the 3-position by an active-site residue (A_2).

Our conclusion that human AGT requires nitrogen atoms at the 3- and 7-positions of O^6mG is in contrast with that obtained with *ada* AGT (*18*, *19*). Since *ada* AGT reacted 100-fold more slowly with S^6mG and O^6m1DG and only 10-fold more slowly with O^6m3DG and O^6m7DG than with O^6mG, we previously concluded that the O^6- and 1-positions of O^6mG were essential for repair by *ada* AGT but that the 3- and 7-positions were not. These results, however, do not preclude any interactions between *ada* AGT and the 3- and 7-positions of O^6mG.

Differences in the reactivity of human and *ada* AGT with O^6m3DG and O^6m7DG may be due to differences in the flexibility of the active sites. In the repair of O^6mG, perhaps both human and *ada* AGT interact with O^6mG at the 1-, 3-, 7-, and O^6-positions, but *ada* AGT is flexible enough to accommodate perturbations at the 3- and 7-positions while human AGT is not.

The tighter interaction between human AGT and O^6mG, however, does not accelerate the rate of methyl transfer. In fact, the second-order rate constant for the repair of O^6mG in an oligodeoxynucleotide is 5-fold faster with *ada* AGT

Scheme 3: Repair of O^4mT by ada AGT



than human AGT (Table 1). These interactions may not increase the rate of reaction but may add specificity to the reaction, decreasing the rate of repair of O^4mT. Most reports have suggested that human AGT repairs O^6mG much faster than O^4mT (*5*−*10*), although a recent report has suggested that *ada* AGT repairs O^6mG at the same rate as O^4mT and human AGT repairs O^6mG only 37-fold faster than O^4mT (*28*).

If the active site were rigid, the 5-methyl group of O^4-mT would clash with the hydrogen bond donor A_1. Just as in the case for O^6m7DG and human AGT, the rate of reaction would be decreased. Scheme 3 illustrates how a flexible active site would be able to accommodate O^4mT as a substrate.

In conclusion, the mechanism of action of AGT is illustrated in Scheme 2. The 1-, 7-, and N^2-positions of O^6-mG are important for binding to human AGT, while the 3- and O^6-positions are involved in the methyl transfer. *ada* AGT has a similar mechanism, but the active site is looser and can accommodate perturbations in the substrate more readily than human AGT.

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