DNA-binding Mechanism of $O^6$-Alkylguanine-DNA Alkyltransferase

EFFECTS OF PROTEIN AND DNA ALKYLATION ON COMPLEX STABILITY*

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The mutagenic and cytotoxic effects of many endogenous and exogenous alkylating agents are mitigated by the actions of $O^6$-alkylguanine-DNA alkyltransferase (AGT). In humans this protein protects the integrity of the genome, but it also contributes to the resistance of tumors to DNA-alkylating chemotherapeutic agents. Here we report properties of the interaction between AGT and short DNA oligonucleotides. We show that although AGT sediments as a monomer in the absence of DNA, it binds cooperatively to both single-stranded and double-stranded deoxyribonucleotides. This strong cooperative interaction is only slightly perturbed by active site mutation of AGT or by alkylation of either AGT or DNA. The stoichiometry of complex formation with 16-mer oligonucleotides, assessed by analytical ultracentrifugation and electrophoretic mobility shift assays, is 4:1 on single-stranded and duplex DNA and is unchanged by several active site mutations or by protein or DNA alkylation. These results have significant implications for the mechanisms by which AGT localizes and interacts with repairable alkyl lesions to effect DNA repair.

$O^6$-Alkylguanine-DNA alkyltransferase is a ubiquitous repair protein that plays a vital role in minimizing the mutagenic effects of alkylating agents (1–4). It catalyzes the stoichiometric transfer of a variety of alkyl substituents from the $O^6$-position of guanine to an active site cysteine, preventing incorrect base pairing caused by these adducts. More than 100 alkyltransferases are now known, and the crystal structures are available for three family members: the Ada-C protein from *Escherichia coli* (5), the human alkyltransferase (hAGT)$^1$ (6), and the protein from the thermophilic archaeon, *Pyrococcus kodakaraensis* (7). All of the known alkyltransferases lack the ability to dealkylate themselves, and no dealkylation activity has been found in cell extracts to date. On this basis, it is widely thought that alkyltransferase participates in a single reaction and is then irreversibly inactivated. Given the apparently nonenzymatic nature of the protein, the protection afforded by alkyltransferase is likely to depend on the regulation of its synthesis and degradation and on its ability to efficiently locate repairable lesions throughout the genome.

The mechanisms by which AGT interacts with adduct-containing and adduct-free DNAs are poorly understood. Two contrasting mechanisms have been proposed to date. In the first, single AGT proteins bind normal and lesion-containing DNA, and the distribution of AGT between normal and lesion sites depends on a difference in binding affinity. This model is consistent with the observation that a single AGT monomer is necessary and sufficient to dealkylate a single $O^6$-alkyl guanine adduct within a DNA duplex (3). It is supported by the observation that single AGT-DNA complexes are detected by gel shift assay when AGT binds short DNA molecules. These complexes have been interpreted as having a 1:1 AGT-DNA stoichiometry, and the binding affinities have been calculated based on that assumption (8). The second mechanism was proposed when it was found that some AGT-DNA complexes have stoichiometries greater than 1:1 and form without the accumulation of detectable binding intermediates (9). This pattern strongly suggests a cooperative binding mechanism for AGT.

Here we more thoroughly characterize the cooperative binding mechanism. We show that it functions on both single-stranded and duplex 16-mer DNAs and with unmodified and alkylated hAGTs. In all cases the stoichiometries of hAGT:16-mer complexes were 4:1. In this binding mode, hAGT discriminates poorly between lesion-containing and lesion-free DNA. Together these results support a novel model of binding site search and recognition that involves the cooperative formation and productive movement of multi-protein complexes.

**EXPERIMENTAL PROCEDURES**

*Reagents—* $T_4$ polynucleotide kinase was purchased from New England Biolabs, and $[\gamma^{25}P]ATP$ was purchased from PerkinElmer Life Sciences. Acrylamide and $N,N'$-methylene bisacrylamide were purchased from Aldrich. $O^6$-Methylguanine and $O^6$-benzylguanine were generously provided by Dr. R. C. Moschel (ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD).

AGT Protein—Recombinant human AGT (wild type and C145A mutant proteins) were prepared as previously described (10). Both of the proteins were homogeneous as judged by electrophoresis (not shown). The wild type protein was 100% active in debenzoylating and adduct-free DNAs are poorly understood. Two contrasting mechanisms have been proposed to date. In the first, single AGT proteins bind normal and lesion-containing DNA, and the distribution of AGT between normal and lesion sites depends on a difference in binding affinity. This model is consistent with the observation that a single AGT monomer is necessary and sufficient to dealkylate a single $O^6$-alkyl guanine adduct within a DNA duplex (3). It is supported by the observation that single AGT-DNA complexes are detected by gel shift assay when AGT binds short DNA molecules. These complexes have been interpreted as having a 1:1 AGT-DNA stoichiometry, and the binding affinities have been calculated based on that assumption (8). The second mechanism was proposed when it was found that some AGT-DNA complexes have stoichiometries greater than 1:1 and form without the accumulation of detectable binding intermediates (9). This pattern strongly suggests a cooperative binding mechanism for AGT.

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gested protein, in which the conversion of the fragment containing the active site cysteine (Gly$^{39}$-Arg$^{147}$, $M_{predicted} = 1314.72$, $m/z_{observed} = 1314.72$).

This paper is available on line at http://www.jbc.org

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1315.82 to its methylated derivative \( M_{\text{predicted}} = 1328.73 \) and desalted derivative \( M_{\text{predicted}} = 1391.80 \) (1393.66) was observed. Complete conversion of hAGT to the alkyl form (as monitored by mass spectrometry) eliminated detectable alkyl transferase activity assayed with \(^3\text{H}\)methyl calf thymus DNA as substrate (results not shown) (14). Human AGT undergoes a conformational change upon alkylation that reduces its \textit{in vivo} and \textit{in vitro} half-life (14). Accordingly, the samples were used immediately following alkylation to avoid problems of instability.

Human AGT concentrations were measured both with the BCA dye binding assay (15) and spectrophotometrically, using a molar absorption coefficient, \( \varepsilon_{280} = 1.04 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \) (per base pair) for duplex samples and \( \varepsilon_{280} = 3.93 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \) for single-stranded samples.

Electrophoretic Mobility Shift Assays—The binding reactions were carried out at 20 ± 1°C in 10 mM Tris (pH 7.6), 1 mM dithiothreitol, and 10 μg/ml bovine serum albumin, supplemented with NaCl as indicated. Protein-DNA complexes were formed by adding appropriate amounts of hAGT to solutions containing \(^3\text{P}\)-labeled oligodeoxyribonucleotides. The mixtures were equilibrated at 20 ± 1°C for 30 min. Duplicate samples incubated for longer periods gave identical results, indicating that equilibrium had been attained. Electrophoresis was performed using 10% polyacrylamide gels (acrylamide: bisacrylamide = 29:1) cast, and run at 8 W/cm in buffer consisting of 10 mM Tris acetate (pH 7.6) supplemented with NaCl to match the conductivity of the protein-DNA samples. Autoradiograms were obtained with Kodak X-Omat Blue XB-1 film exposed at 4°C.

Analytical Ultracentrifugation—hAGT protein and oligodeoxyribonucleotides were dialyzed against 10 mM Tris (pH 7.6), 1 mM DTT, 1 mM EDTA, 100 mM NaCl. Analytical ultracentrifugation was performed at 20 ± 0.1°C in a Beckman XL-A centrifuge using an AN 60 Ti rotor.

In many cases the association constant was also evaluated by direct titration. hAGT protein was directly added to \(^3\text{P}\)-DNA solutions (typical concentration \( 10^{-7} \) M), and the samples were analyzed by native gel electrophoresis. The free protein concentration \( [P] \) was estimated using the approximation \( A(P) = [P] - n[P,D] \) in which \( [P] \) is the total hAGT concentration in the reaction mixture, and an initial value of \( n = 4 \) was assumed on the basis of our sedimentation equilibrium experiments (see Fig. 1). For the highly cooperative formation of a 4:1 complex under conditions of large protein excess, the fractional saturation \( Y \) is given by

\[
Y = \frac{[P,D]}{[P] + [P,D]} = \frac{[P]^+}{K_c + [P]^+} \quad \text{(Eq. 2)}
\]

Estimates of \( K_c \) were obtained by fitting Equation 2 to the experimentally determined dependence of \( Y \) on \([P]\).

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The binding reactions were measured spectrophotometrically, using a molar extinction coefficient, \( \varepsilon_{280} = 1.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \) (per base pair) for duplex samples and \( \varepsilon_{280} = 1.04 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \) (per base) for single-stranded samples.

\[
\ln \left[ \frac{[P,D]}{[D]} \right] = n \ln [P] + \ln K_c \quad \text{(Eq. 1)}
\]

\( \ln \left[ \frac{[P,D]}{[D]} \right] \) is the binding ratio, \( n \) is the stoichiometric ratio, \( K_c \) is the association constant.

\[
Y = 1 - \exp \left( \sigma_r (r^2 - r_0^2) \right) + Y_0 \quad \text{(Eq. 3)}
\]

Here \( r \) is the radial distance, \( \sigma_r \) is the absorbance at radial position \( r \), and \( Y_0 \) is the absorbance of the nth species at the reference radius \( r_0 \). The parameter \( \sigma_r \) is the reduced molecular weight \( [P] = M_0 (1 - e^{2p})/2p\). \( M_0 \) is the molecular weight of the nth species, \( p \) its partial specific volume, \( \rho \) is the solvent density, \( \omega \) is the rotor angular velocity, \( R \) is the gas constant, \( T \) is the absolute temperature, and \( \epsilon \) is the base-line offset. Solvent density \( (1.004 \text{ g/ml}) \) was measured using a Mettler density meter. The partial specific volume of hAGT \( (0.744 \text{ ml/g}) \) was calculated by the method of Cohn and Edsall (21), using partial specific volumes of amino acids tabulated by Laue et al. (22).

For a system in which hAGT protein is in binding equilibrium with DNA according to the mechanism \( nP + D = P_n D \), Equation 3 becomes...
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### RESULTS

**Wild Type and Representative Mutant hAGT Proteins Are Monomeric**—Solutions containing hAGT protein at two nominal concentrations (3.6 and 13.7 μM) were brought to sedimentation equilibrium at three different centrifuge speeds (22,000, 31,000, and 43,000 rpm). A representative data set for a 13.7 μM sample of His6-tagged C145S hAGT taken at 43,000 rpm and 20 °C is shown in Fig. 1 (curve A). The *solid line* is the result of fitting the single-species version of Equation 3 (n = 1) to the data. This fit returned a value of $M_p = 21,800 ± 400$, which is in excellent agreement with the monomer molecular weight derived from the protein sequence ($M_p = 21,860$). The small, uniformly distributed residuals indicate that the monomer model is consistent with the data over the entire concentration range present in the centrifuge cell. Extension of the model to include oligomers of hAGT (Equation 5 with $n > 1$ and $M_n = nM_p$) did not improve the quality of the fit as judged by the correlation coefficient or by the magnitude of the residuals (result not shown). Similar results were obtained for wild type hAGT ($M_p = 21,500 ± 200$), C145A hAGT ($M_p = 21,400 ± 200$), His6-tagged wild type hAGT ($M_p = 22,000 ± 500$), and His6-tagged C145A hAGT ($M_p = 21,800 ± 200$). These molecular weights agree well with values predicted from protein se-

### Table I

<table>
<thead>
<tr>
<th>Protein</th>
<th>Single-stranded 16-mer DNA</th>
<th>Double-stranded 16-mer DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stoichiometry</td>
<td>$K_r/10^{25}$ M$^{-4}$</td>
</tr>
<tr>
<td>野型hAGT</td>
<td>$3.89 ± 0.2^a$</td>
<td>0.145 ± 0.024</td>
</tr>
<tr>
<td></td>
<td>$3.90 ± 0.4^r$</td>
<td>0.154 ± 0.019</td>
</tr>
<tr>
<td></td>
<td>$4.02 ± 0.10^r$</td>
<td>$0.125 ± 0.012$</td>
</tr>
<tr>
<td></td>
<td>$3.94 ± 0.3^r$</td>
<td>0.121 ± 0.014</td>
</tr>
<tr>
<td>C145A hAGT</td>
<td>$3.99 ± 0.06^r$</td>
<td>$0.300 ± 0.079$</td>
</tr>
<tr>
<td></td>
<td>$3.82 ± 0.2^r$</td>
<td>0.305 ± 0.045</td>
</tr>
<tr>
<td></td>
<td>$3.99 ± 0.10^r$</td>
<td>$0.269 ± 0.059$</td>
</tr>
<tr>
<td>His$_6$-Wild type hAGT</td>
<td>$3.99 ± 0.10^r$</td>
<td>$0.274 ± 0.076$</td>
</tr>
<tr>
<td></td>
<td>$3.82 ± 0.10^r$</td>
<td>0.688 ± 0.098</td>
</tr>
<tr>
<td></td>
<td>$3.84 ± 0.10^r$</td>
<td>0.697 ± 0.013</td>
</tr>
<tr>
<td></td>
<td>$3.98 ± 0.07^r$</td>
<td>$3.94 ± 0.08^r$</td>
</tr>
</tbody>
</table>

* Formation constants for the 4:1 AGT-DNA complex are in units of $10^{-25}$ M$^{-4}$. The values given in parentheses are monomer-equivalent association constants (units of $10^{-4}$ M$^{-1}$).
* Stoichiometry determined by sediment dilution method.
* Stoichiometry determined by continuous variation analysis.
* Stoichiometry determined by sedimentation equilibrium.
* Association constant determined by serial dilution method.
* Association constant determined by direct titration method, assuming a binding stoichiometry.

$$A(r) = \alpha_0 \exp(\alpha_r(r - r_0)^2) + \alpha_0 \exp(\alpha_{r,D}(r - r_0)^2) + \alpha \exp(\alpha_{c,D}(r - r_0)^2) + \alpha (\text{Eq. 4})$$

Here, most terms are defined as for Equation 3; $\alpha_0$ and $\alpha_{r,D}$ are absorbances of DNA and protein-DNA complex at $r_0$, the reduced molecular weights of DNA and protein-DNA complex are given by $\alpha_0 = \alpha_0 + \alpha_{r,D}(1 - \alpha_0)\phi_2(2\phi_2RNT)$ and $\alpha_{r,D} = (nM_p + M_p)1 - \alpha_0\phi_2(2\phi_2RNT)$, and $n$ is the protein-DNA ratio of the complex. In this analysis, the known molecular weights of recombiant hAGT proteins (21,614 ± $M_p$ ≤ 21,876) and DNA ($M_D = 4,881$ for single-stranded DNA and $M_D = 9,762$ for double-stranded DNA) were used as constants. The partial specific volume of NaDNA at 0.1 m NaCl (0.520 ml/g) was estimated by interpolation of the data of Cohen and Eisenberg (23). Partial specific volumes of each of protein-DNA complexes were estimated using Equation 5.

$$\phi_{r,D} = (nM_p + M_p)$$

$\phi_{r,D}$ is the stoichiometric ratio of protein to DNA in the complex. Equation 5 is based on the assumption that there is no significant change in partial specific volumes of the components upon association. Although we do not know whether such a volume change occurs, it seems reasonable that values of $\phi_{r,D}$ for complexes containing a large mass proportion of protein (like those analyzed here) should reflect that proportion. Equation 4 was used in a global analysis of multiple data sets obtained at different macromolecular concentrations and/or rotor speeds (24). In this method, the values of $\alpha_0$, $\alpha_r$, $\alpha_{c,D}$, and $\alpha$ are unique to each sample, but the value of $n$ must be common to all of the data sets. Nonideality was not considered, because there was no evidence of nonideal effects.

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**Fig. 2.** Serial dilution analysis of the interaction between hAGT and 16-mer oligonucleotides. A, electrophoretic mobility shift assay. The initial mixture contained His$_6$-tagged C145S hAGT (3.5 μM) and double-stranded 16-mer (0.8 μM duplex) in 10 mM Tris (pH 7.6), 1 mM DTT, and 10 μg/ml bovine serum albumin (lane a). Serial dilutions were performed using the same buffer (dilution factor 0.854:step; lanes b–m). The solid lines represent linear least squares fits of Equation 1 to data obtained for wild type hAGT with single-stranded DNA (●) and double-stranded DNA (▲). For single-stranded 16-mer, this analysis returned $n = 3.84 ± 0.2$ and $K_{PD} = 6.9 ± 1.0 × 10^2$ M$^{-1}$. For double-stranded 16-mer, $n = 3.96 ± 0.1$ and $K_{PD} = 4.3 ± 0.9 × 10^3$ M$^{-1}$.
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Continuous variation analysis of hAGT-DNA complexes. A, electrophoretic mobility shift assay of the binding of wild type hAGT to single-stranded 16-mer DNA. The total macromolecular concentration was fixed ([hAGT] + [DNA] = 6 × 10⁻⁶ m) with the mole fraction varied regularly in increasing increments of protein across the gel lanes a–n. Binding was carried out at 20 ± 1 °C in 10 mM Tris (pH 7.6), 1 mM DTT, and 10 μg/ml bovine serum albumin. Electrophoresis was performed as described under “Experimental Procedures.” Band B, bound DNA; band F, free DNA. B, job plot of wild type hAGT binding single-stranded 16-mer DNA. The data were from A and replicate experiments. The error bars (most obscured by data symbols) indicate the range of values obtained in three parallel experiments. The solid lines are least squares fits to rising and falling subsets of the data. Their intersection yields a binding stoichiometry of 3.9.

AGT Forms 4:1 Complexes with a Single-stranded Oligodeoxyribonucleotide 16-mer and Its Cognate Duplex—Mixtures containing hAGT and single-stranded DNA were brought to sedimentation equilibrium at four different centrifuge speeds (11,000, 15,000, 20,500, and 27,000 rpm). Representative data are shown in Fig. 1 (curve B); the solid curve represents the global fit of Equation 4 to the data ensemble. The small, uniformly distributed residuals indicate that the simple mechanism nP + D ⇌ P₄D, with n = 3.98 ± 0.07 is consistent with the data. Sedimentation models with additional species did not fit the data significantly better than Equation 4 (results not shown). This outcome is intriguing because it suggests that the n = 4 complex forms without significant accumulation of intermediates of lower stoichiometry. This interpretation is supported by the gel mobility shift experiments described below. Parallel experiments carried out with wild type and C145A, His₉ wild type, His₉-C145A, and His₈-C145S hAGTs returned closely similar protein-DNA stoichiometries (Table 1), indicating that neither modification of the active site cysteine nor presence of a C-terminal His₉ tag alters the stoichiometry of association.

Measurement of binding affinities by direct titration. Interaction of single-stranded 16-mer DNA with hAGT. A, binding carried out at 20 ± 1 °C in 10 mM Tris (pH 7.6), 1 mM DTT, 10 μg/ml bovine serum albumin. B, binding carried out at 20 ± 1 °C in the same buffer as that shown in A, adjusted to contain 70 mM NaCl. All of the samples contained 8.75 × 10⁻⁷ m DNA; the samples in lanes b–l of each gel contained increasing concentrations of His₉-tagged C145A hAGT. Electrophoresis was performed as described under “Experimental Procedures.” Band B, bound DNA; band F, free DNA. C, binding isotherms for hAGT. The measurements were performed in triplicate. The error bars indicating the ranges of values are obscured by the data symbols. The smooth curves are fits of Equation 2 to the data, returning association constants (Kₐₚₖ) of 2.7 ± 0.6 × 10⁵ M⁻¹ in 10 mM Tris buffer without added KCl and 1.7 ± 0.5 × 10² M⁻¹ in 10 mM Tris, 70 mM KCl buffer, respectively.

Similar experiments were carried out with hAGT and a 16-bp duplex DNA. Samples were brought to sedimentation equilibrium at 11,000, 15,000, 20,500, and 27,000 rpm. Representative data are shown in Fig. 1 (curve C); the solid curve represents the global fit of Equation 4 to the data ensemble. As before, the high quality of the fit indicates that the simple mechanism nP + D ⇌ P₄D, with n = 3.94 ± 0.08 is consistent with the data. Inclusion of additional species in the sedimentation model did not improve the quality of the fit (results not shown), suggesting that, as with single-stranded DNA, stoichiometric intermediates are not present in significant concentrations. This suggestion is supported by gel shift results (dis-
The mobility shift assay method for the active site mutant C145A stoichiometry values were obtained by the electrophoretic well with ones obtained by analytical ultracentrifugation, stranded DNA and 3.99

The association stoichiometry. As shown in Fig. 3 and summarized below). The fact that the hAGT stoichiometry is the same, within error, for both single-stranded and duplex 16mers is intriguing, because it suggests that factors determining stoichiometry may not be sensitive to the association state of the DNA.

Electrophoretic mobility shift assays (25) were performed to explore a range of hAGT and DNA concentrations below those accessible in the analytical ultracentrifuge. The binding of hAGT to DNA produced a single mobility-shifted complex at all protein and DNA concentrations that gave detectable binding (Fig. 2A). This binding pattern is consistent with a mechanism of the type nP + D ⇌ PnD in which the maximum stoichiometry (n) is reached without accumulation of significant concentrations of intermediates (19). The dependence of ln([P]/[nD]/[D]) on ln(P) is shown in Fig. 2B. The values of stoichiometry and Ks were calculated from these data as described under “Experimental Procedures.” The stoichiometry values most consistent with the data for His6-tagged C145S hAGT binding to 16-mer are 3.84 ± 0.2 for single-stranded DNA and 3.99 ± 0.1 for double-stranded DNA. These stoichiometry values (summarized in Table I) agree well with ones obtained by analytical ultracentrifugation, despite a difference in the salt concentration of the buffers used in the two techniques (serial dilution assays, −10 mM; sedimentation equilibrium assays, −110 mM). Closely similar stoichiometry values were obtained by the electrophoretic mobility shift assay method for the active site mutant C145A hAGT as well as His6-wild type hAGT and His6-C145A hAGT (Table I), supporting the conclusion that neither the C145A modification of the active site nor the presence of a C-terminal His6 tag has a significant effect on the stoichiometry of these interactions.

Binding stoichiometries were also measured by the continuous variation (Job plot) method (26). With the input concentrations of protein and DNA ([P]0 + [D]0) held constant, the ratio of [P]0/[D]0 that yields the greatest concentration of complex (the optimal combining ratio) is a measure of the association stoichiometry. As shown in Fig. 3 and summarized in Table I, this method returns hAGT-DNA stoichiometries close to 4:1, in good agreement with values obtained by the serial dilution and analytical ultracentrifuge methods. Taken with the fact that higher stoichiometry complexes are readily observed with larger DNAs (9), the absence of detectable complexes with stoichiometries greater than 4:1, even at high [hAGT], suggests that this stoichiometry represents protein saturation for both single-stranded and duplex 16-mer DNAs. Together, the presence of free DNA in equilibrium with the 4:1 complex and the absence of complexes with protein:DNA ratios <4:1, suggest that hAGT binds cooperatively to both single-stranded and double-stranded DNAs. Because free hAGT is monomeric (Fig. 1), this pattern suggests that the protein assembly forms on DNA and not in free solution prior to DNA binding.

**Equilibrium Constants Depend Only Weakly on DNA Secondary Structure, Sequence Changes at the Active Site, or the Presence of a C-terminal His6 Tag**—Association constants for the interaction of methylated 16-mer oligodeoxyribonucleotides with hAGT proteins (best seen by comparison of the effective monomer association constants given in Table II) is an especially interesting result. It indicates that neither the C145A mutation of the active site nor the presence of a C-terminal His6 tag has a significant effect on DNA binding affinity under our assay conditions. A similar result with active site cysteine mutants was previously obtained for untagged C145A and C145S proteins under different binding conditions, by Hazra et al. (28). Under our experimental conditions, hAGT appears to bind double-stranded DNA with slightly higher affinity than single-stranded DNA (best seen by comparison of the effective monomer association constants given in Table II). In addition, the presence of a methylated guanine nucleotide also enhances hAGT binding, and that enhancement is slightly more pronounced in single-stranded DNA than in double-stranded DNA (Table II). However, the small differences in affinity and the closely similar stoichiometries suggest that the overall mechanism of hAGT interaction is altered little by changes in DNA secondary structure or alkylation, hAGT active site mutation, or the presence of a C-terminal His6 affinity tag.

**DNA Interactions of Alkylated hAGT**—Proteolysis is the ultimate fate of alkyl-AGT (29), but it is not known whether the alkyl-protein plays a role in DNA repair prior to degradation. Studies were undertaken to examine the DNA binding proper-
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Fig. 5. Analysis of the interaction of alkylated hAGT with and double-stranded DNA. A, titration of the 16-mer duplex with Cys\(^{154}\) methyl hAGT (Me AGT). A 1 \(\mu\)g/ml stock of wild type hAGT was alkylated at the active site cysteine as described under “Experimental Procedures.” Binding was carried out at 20 ± 1 °C in 10 mM Tris (pH 7.6), 1 mM DTT, and 10 \(\mu\)g/ml bovine serum albumin. All of the samples contained 8.75 \(\times\) 10\(^{-7}\) M DNA; the samples in lanes b–l contained increasing concentrations of Me AGT. Electrophoresis was carried out as described under “Experimental Procedures.” Band B, bound DNA; band F, free DNA. B, binding isotherms; dependence of the fractional saturation of DNA (Y) on free [hAGT]; data from the experiment shown in A and from parallel titrations of double-stranded DNA with wild type hAGT and benzylated hAGT (Bz AGT; not shown). The solid lines are fits of Equation 2 to the data. Apparent monomer-equivalent dissociation constants \(K_{D}^{E}\) were 1.05 \(\pm\) 0.05 \(\times\) 10\(^{-6}\) M for wild type hAGT, 3.50 \(\pm\) 0.08 \(\times\) 10\(^{-6}\) M for Me AGT, and 4.40 \(\pm\) 0.05 \(\times\) 10\(^{-6}\) M for Bz AGT. C, job plots for Me AGT and Bz AGT binding to double-stranded 16-mer DNA. O, Me AGT; □, Bz AGT. Experiments like that shown in Fig. 5A were performed in triplicate using double-stranded 16-mer DNA as the substrate. The concentration ranges of AGT-DNA complexes are given by the error bars. The solid lines are least squares fits to rising and falling subsets of the data for Me AGT. The optimal combining ratio for Me AGT and Bz AGT with DNA are 4:1 and 4:2, respectively.

In band F, free DNA.

As described under “Experimental Procedures.” Electrophoresis was carried out as described under “Experimental Procedures.” Band B, bound DNA; band F, free DNA. B, binding isotherms; dependence of the fractional saturation of DNA (Y) on free [hAGT]; data from the experiment shown in A and from parallel titrations of double-stranded DNA with wild type hAGT and benzylated hAGT (Bz AGT; not shown). The solid lines are fits of Equation 2 to the data. Apparent monomer-equivalent dissociation constants \(K_{D}^{E}\) were 1.05 \(\pm\) 0.05 \(\times\) 10\(^{-6}\) M for wild type hAGT, 3.50 \(\pm\) 0.08 \(\times\) 10\(^{-6}\) M for Me AGT, and 4.40 \(\pm\) 0.05 \(\times\) 10\(^{-6}\) M for Bz AGT. C, job plots for Me AGT and Bz AGT binding to double-stranded 16-mer DNA. O, Me AGT; □, Bz AGT. Experiments like that shown in Fig. 5A were performed in triplicate using double-stranded 16-mer DNA as the substrate. The concentration ranges of AGT-DNA complexes are given by the error bars. The solid lines are least squares fits to rising and falling subsets of the data for Me AGT. The optimal combining ratio for Me AGT and Bz AGT with DNA are 4:1 and 4:2, respectively.

As summarized in Table III, alkylated hAGT proteins bind DNA, with somewhat lower affinity than nonalkylated proteins. The larger benzyl adduct causes a greater decrease in affinity than the methyl adduct, but neither modification has a profound effect on binding. Alkylated hAGT proteins bind \(O^6\)-alkylguanine-containing DNA; the affinities of these proteins for lesion-containing oligonucleotides are slightly but significantly elevated over that for lesion-free molecules (Table IV). Together with the observation that alkyl and native hAGT proteins form similar 4:1 complexes with 16-mer DNAs, the elevated affinity for \(O^6\)-alkylguanine-containing DNA suggests that the mechanism of binding is not greatly perturbed by protein alkylation.

DISCUSSION

\(O^6\)-Alkylguanine-DNA alkyltransferases reduce the mutagenicity of DNA-alkylating agents and enhance the resistance of tumor cells to chemotherapeutic agents (30). Despite these important functions, little is known of the mechanisms by which the human protein interacts with alkylated and nonalkylated DNAs. Most models of these interactions are based on results obtained with Ada, a two-domain bacterial protein that shares sequence similarity with human AGT (31). However, Ada has a simple, noncooperative DNA binding mechanism (32) quite distinct from the cooperative one that we have found for human AGT. This disparity may reflect a functional divergence of the eukaryotic and bacterial proteins (33). The differences in the binding mechanisms of hAGT and Ada suggest that caution should be used in modeling functions of the human protein on the basis of its well studied Ada homologue.

We have shown that hAGT is rigorously monomeric in free solution and that it forms multiprotein complexes with short DNA molecules (Ref. 9 and this work). Human AGT binds with a 4:1 stoichiometry to 16-nucleotide single strands and 16-bp duplexes, regardless of the DNA association state, the presence of repairable \(O^6\)-alkylguanine lesions, active site mutations (C154A and C154S), or the C-terminal His\(_6\) affinity tag. This binding mechanism is robust. Its qualitative features are not affected by changes in [NaCl] (Figs. 1, 2, and 4), temperatures between 4 and 30 °C, or the presence of divalent cations.5 The absence of detectable complexes with stoichiometries greater than 4 suggests that the 4:1 ratio represents saturation for 16-mer DNA. The presence of the 4:1 complex in equilibrium with free DNA and the absence of detectable complexes of lower stoichiometry indicate that the binding is highly cooperative. These results are particularly significant in view of previous studies in which association constants for AGT-DNA complexes were derived for assumed 1:1 binding models (8). For a cooperative binding mechanism, differences in the stability of the protein-DNA assembly may be due to differences in the intrinsic affinity of AGT for a given DNA or to differences in the stability of the AGT-AGT interaction. Our results are consistent with binding mechanisms in which the aggregate stability of the 4:1 complex is large enough to effectively mask any difference in affinity for single-stranded and duplex DNAs or any difference in affinity for alkylated and nonalkylated DNAs.

As shown in Tables III and IV, mutation of the active site cysteine to alanine or serine does not eliminate DNA binding cooperativity. This supports the conclusion (28) that conservative mutation of the active site cysteine has little effect on hAGT structure. Because such mutants lack alkyltransferase activity, they are useful for the study of interactions with \(O^6\)-alkylguanine-containing DNAs (see below). Analogous results were obtained with proteins in which residues 202–207 of hAGT were converted to histidines, forming a C-terminal His\(_6\),...
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### Table III
Association constants and binding stoichiometries for the interaction of 16-mer oligodeoxyribonucleotides with alkylated human AGT proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>O6-methylguanine</th>
<th>O6-methylguanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stoichiometry</td>
<td>$K_a/10^6\text{ M}^{-1}$</td>
<td>$K_{	ext{mon}}/10^5\text{ M}^{-1}$</td>
</tr>
<tr>
<td>Stoichiometry</td>
<td>$K_a/10^6\text{ M}^{-1}$</td>
<td>$K_{	ext{mon}}/10^5\text{ M}^{-1}$</td>
</tr>
<tr>
<td>Me AGT</td>
<td>3.88 ± 0.26</td>
<td>0.29 ± 0.059</td>
</tr>
<tr>
<td>Bz AGT</td>
<td>3.95 ± 0.24</td>
<td>0.174 ± 0.014</td>
</tr>
<tr>
<td>His$_2$AGT</td>
<td>3.97 ± 0.25</td>
<td>0.091 ± 0.008</td>
</tr>
<tr>
<td>His$_2$Bz AGT</td>
<td>3.89 ± 0.44</td>
<td>0.0859 ± 0.009</td>
</tr>
</tbody>
</table>

### Table IV
Association constants and binding stoichiometries for the interaction of oligodeoxyribonucleotides with alkylated human AGT proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Single-stranded 16-mer DNA containing an O6-methylguanine</th>
<th>Double-stranded 16-mer DNA containing an O6-methylguanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stoichiometry</td>
<td>$K_a/10^6\text{ M}^{-1}$</td>
<td>$K_{	ext{mon}}/10^5\text{ M}^{-1}$</td>
</tr>
<tr>
<td>Stoichiometry</td>
<td>$K_a/10^6\text{ M}^{-1}$</td>
<td>$K_{	ext{mon}}/10^5\text{ M}^{-1}$</td>
</tr>
<tr>
<td>Me AGT</td>
<td>3.90 ± 0.26</td>
<td>2.73 ± 1.6</td>
</tr>
<tr>
<td>Bz AGT</td>
<td>3.83 ± 0.3</td>
<td>2.61 ± 1.7</td>
</tr>
<tr>
<td>His$_2$Me AGT</td>
<td>3.93 ± 0.16</td>
<td>0.179 ± 0.059</td>
</tr>
<tr>
<td>His$_2$Bz AGT</td>
<td>4.03 ± 0.36</td>
<td>0.168 ± 0.016</td>
</tr>
<tr>
<td>His$_2$Bz AGT</td>
<td>3.89 ± 0.22</td>
<td>0.52 ± 0.021</td>
</tr>
<tr>
<td>His$_2$Bz AGT</td>
<td>3.92 ± 0.16</td>
<td>0.374 ± 0.021</td>
</tr>
<tr>
<td>4.00 ± 0.02</td>
<td>0.391 ± 0.008</td>
<td>2.50 ± 0.013</td>
</tr>
</tbody>
</table>

### Table V
Alterations in binding free energy as a consequence of DNA alkylation

<table>
<thead>
<tr>
<th>Protein preparation$^a$</th>
<th>Me AGT</th>
<th>Bz AGT</th>
<th>His$_2$AGT</th>
<th>His$_2$Bz AGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>C145A hAGT</td>
<td>His$_2$ C145A hAGT</td>
<td>His$_2$ C145S hAGT</td>
<td>Me AGT</td>
<td>Bz AGT</td>
</tr>
<tr>
<td>Δ$\Delta$G/kcal mol$^{-1}$</td>
<td>-10.68 ± 0.15</td>
<td>-10.89 ± 0.37</td>
<td>-11.02 ± 0.24</td>
<td>-8.55 ± 0.47</td>
</tr>
</tbody>
</table>

### Notes

$^a$ Formation constants for 4.1 AGT-DNA complex in units of 10$^{-6}$ M$^{-1}$, monomer association constants in units of 10$^{-6}$ M$^{-1}$ in parentheses.

$^b$ Stoichiometry determined by serial dilution method.

$^c$ Stoichiometry determined by continuous variation analysis.

$^d$ Association constant determined by serial dilution method.

$^e$ Association constant determined by direct titration method, assuming a binding stoichiometry of 4:1.

$^f$ Stoichiometry determined by serial dilution method.

$^g$ Stoichiometry determined by continuous variation analysis.

$^h$ Association constant determined by serial dilution method.

$^i$ Association constant determined by direct titration method, assuming a binding stoichiometry of 4:1.

$^j$ Stoichiometry determined by serial dilution method.

$^k$ Stoichiometry determined by continuous variation analysis.

$^l$ Association constant determined by serial dilution method.

$^m$ Association constant determined by direct titration method, assuming a binding stoichiometry of 4:1.

$^n$ Difference in association free energy for a single molecule of AGT protein within a 4:1 complex formed with methylated 16-bp DNA and that of a single protein within a 4:1 complex with nonalkylated 16-bp DNA. Individual free energies calculated from observed association calculated using $ΔG^0 = −RT\ln K$.

$^o$ Association constant determined by serial dilution method.

$^p$ Association constant determined by direct titration method, assuming a binding stoichiometry of 4:1.

$^q$ Stoichiometry determined by serial dilution method.

$^r$ Stoichiometry determined by continuous variation analysis.

$^s$ Association constant determined by serial dilution method.

$^t$ Association constant determined by direct titration method, assuming a binding stoichiometry of 4:1.

$^u$ Stoichiometry determined by serial dilution method.

$^v$ Stoichiometry determined by continuous variation analysis.

$^w$ Association constant determined by serial dilution method.

$^x$ Association constant determined by direct titration method, assuming a binding stoichiometry of 4:1.

$^y$ Difference in association free energy for a single molecule of AGT protein within a 4:1 complex formed with methylated 16-bp DNA and that of a single protein within a 4:1 complex with nonalkylated 16-bp DNA. Individual free energies calculated from observed association calculated using $ΔG^0 = −RT\ln K$.

$^z$ Association constant determined by serial dilution method.

$^{|}$ Association constant determined by direct titration method, assuming a binding stoichiometry of 4:1.

$^{\dagger}$ Stoichiometry determined by serial dilution method.

$^{\ddagger}$ Stoichiometry determined by continuous variation analysis.

$^*$ Association constant determined by serial dilution method.

$^†$ Association constant determined by direct titration method, assuming a binding stoichiometry of 4:1.

$\Delta$ Affinity tag. Although the DNA affinities of His$_2$-AGT proteins were slightly elevated when compared with the tag, the His$_2$ modification did not alter the binding stoichiometry or the cooperative nature of the interaction. This outcome suggests that the wild type sequence of the C-terminal residues (202-207) may not be a determinant of hAGT self-association or binding cooperativity. The elevated affinity observed with His$_2$-modified proteins is consistent with the notion that these residues might interact electrostatically with DNA.

Experiments designed to probe the effect of the DNA association state on binding affinity returned the striking result that hAGT displays little preference for duplex 16-mers over single-stranded 16-mers (summarized in Tables I and II).

This accords well with results obtained by competition assay using high molecular weight DNAs (9) but is a smaller difference than that found by Bender et al. (8) using 29-mer oligonucleotides. Because the apparent binding site size varies from a 4-nucleotide/hAGT monomer on a 16-nucleotide single-stranded DNA to an 8-nucleotide/hAGT monomer on an 80-nucleotide DNA (9), this contrast might reflect stochiometric or geometric differences between our complexes and those of Bender et al. (8). Alkylation of the active site cysteine has been shown to destabilize the native fold of hAGT (13). Recently, it has been proposed that an alkylation-mediated conformation change promotes DNA release from the active site (34). However,
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results shown above indicate that although alkylation is associated with a mild reduction in DNA affinity, methylated and benzyolated proteins retain significant binding activity. In addition, the cooperative binding mechanism appears to be unchanged by alkylation. Together, these observations raise the unexpected possibility that alkylated protein molecules participate in cooperative DNA binding in the DNA binding and repair activities of other hAGT molecules. It was not possible to measure the affinity of wild type hAGT for O6-methylguanine-containing DNAs, because of the rapid alkylation of the protein and dealkylation of the DNA, under native binding conditions. However, the modestly elevated affinities of C145A hAGT, His89-C145A hAGT, and His89C145S hAGT for DNAs containing O6-methyl guanine relative to nonalkylated DNAs (Tables I and II) are indications that hAGT possesses some specificity that may enhance its binding to lesion-containing sites. In view of the relatively small preference for O6-methylguanine-containing DNA (a factor of 3–4/monomer in our 4:1 complexes), it seems possible that cooperative interactions may provide an alternative mechanism for “scanning” segments of DNA that does not require much preferential binding to lesion sites. Processive binding of large cooperative units of hAGT molecules to long stretches of DNA may provide a mechanism by which alkyl adducts are located and repaired in an efficient manner, despite the low specificity of hAGT monomers for lesion sites. hAGT-containing repair complexes may potentially form wherever alkylation damage occurs, overcoming differences in intrinsic affinity based upon DNA sequences (9). Cooperativity may represent an essential feature of hAGT-DNA binding that enables efficient repair of a wide variety of lesions contained in any genomic sequence.

By the same token, the cooperative binding has the potential to mask differences in the intrinsic affinity of hAGT for competing binding sites on a DNA molecule. For example, in the binding of His89-C145A hAGT to O6-methylguanine-containing single stranded 16-mer, our data do not distinguish between a single specific high affinity interaction at the lesion site averaged with three nonspecific interactions and four identical interactions of modestly elevated affinity. In this example, binding to the lesion-containing oligomer compound is characterized by $n = 3.89 \pm 0.2, K_n = 37.3 \pm 22 \times 10^{24} M^{-4}$ (Table II), whereas the binding of the same protein to the homologous nonalkylated 16-mer is characterized by $n = 3.92 \pm 0.1, K_n = 0.269 \pm 0.059 \times 10^{24} M^{-4}$ (Table I). Assuming equipartition of free energy, the −138-fold difference in the average stabilities of these complexes corresponds to a difference of −3.4 in the average, monomer-scale association constants. However, if the 4:1 complex contains one protein bound with high affinity to the lesion site and three others bound nonspecifically (with $K_n$ equal to that of the protein for nonalkylated DNA), the entire 138-fold affinity difference between complexes on alkyl and nonalkyl DNAs might be a measure of the binding specificity of His89-C145A hAGT for an O6-methylguanine-containing site in a single-stranded DNA. Similarly large affinity differences are found for other mutant or alkylated hAGT proteins and double-stranded oligonucleotide (Table V).

Alone, the observed level of preferential binding cannot drive an efficient lesion search in a genome containing $10^{9}$ bp, like those present in many eukaryotes. Although it is possible that other (currently unknown) interactions provide the necessary specificity in vivo, we propose an alternative surveillance mechanism that requires additional components. High binding cooperativity may allow AGT to occupy any exposed DNA region, including those near replication forks. The movement of this available DNA with replication would produce a processive search for alkylated sites that does not require high lesion binding specificity. A greater understanding of the mechanism by which AGT interacts with target and nonspecific DNAs may ultimately lead to novel methods to control its activities for therapeutic purposes.

REFERENCES

DNA-binding Mechanism of O\textsuperscript{6}-Alkylguanine-DNA Alkyltransferase: EFFECTS OF PROTEIN AND DNA ALKYLATION ON COMPLEX STABILITY
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