Interactions of Human O\(^6\)-Alkylguanine-DNA Alkyltransferase (AGT) with Short Single-stranded DNAs*

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The O\(^6\)-alkylguanine-DNA alkyltransferase (AGT) repairs O\(^6\)-alkylguanine and O\(^4\)-alkylthymine adducts in single-stranded and duplex DNAs. Here we characterize the binding of AGT to single-stranded DNAs ranging in length from 5 to 78 nucleotides (nt). Binding is moderately cooperative (37.9 ± 3.0 ≤ α ≤ 89.8 ± 8.9), resulting in an all-or-nothing association pattern on short templates. This cooperativity contrasts with the isolated binding seen in recent crystal structures of AGT-DNA complexes. The statistical binding site size \(S\) (mean = 5.2 ± 0.1) oscillates with increasing template length. The oscillation period (4.10 ± 0.02 nt/protein) is nearly identical to the binding site size obtained at the highest known binding density \((S = 4\) nt/protein) and is significantly smaller than the contour length \((8\) bp) occupied in crystalline complexes. A model in which AGT proteins overlap along the DNA contour is proposed to account for these features. Oscillations in intrinsic binding constant \(K_i\) and cooperativity factor \(α\) have the same frequency but are of opposite phase to \(S\) with the result that the most stable protein-protein and protein-DNA interactions occur at the highest packing densities. We hypothesize that modest binding cooperativity and high binding densities are adaptations that allow AGT to efficiently search for lesions in the context of chromatin remodeling and DNA replication.

DNA repair is crucial for the preservation of cell viability and genetic heredity in the presence of environmental, cellular, and chemical mutagens. The ubiquitous repair protein O\(^6\)-alkylguanine-DNA alkyltransferase* (AGT, also called O\(^6\)-methylguanine DNA methyltransferase) plays an essential role in maintaining genomic integrity by repairing O\(^6\)-alkylguanine and O\(^4\)-alkylthymine adducts that form in DNA exposed to alkylating agents (1–3). Both adducts are mutagenic and carcinogenic (1, 4, 5), whereas O\(^6\)-alkylguanine adducts are also strongly cytotoxic (6). Ironically AGT also protects cells against chemotherapeutic drugs that methylate or chloroethylate DNA (6, 7). Clinical trials of AGT inhibitors are underway in an attempt to increase the efficacy of DNA-alkylating drugs in cancer chemotherapy (8–10). Despite the interest focused on AGT as a result of its relevance to cancer, relatively little is known about its mechanisms of interaction with DNA and with proteins in its environment.

Human AGT is a monomeric protein (207 amino acids, \(M_r = 21,519\)) that is expressed constitutively in normal cells (3, 7, 11). It binds DNA with modest affinity, significant cooperativity, and little sequence specificity (12–14). In binding it discriminates only weakly between single-stranded and duplex structures (13, 14), and it repairs O\(^6\)-alkylguanine lesions in both single-stranded and duplex DNAs (15–17). In the repair reaction, each protein molecule catalyzes the transfer of a single alkyl group from the O\(^6\) position of guanine or O\(^4\) position of thymine to an active site cysteine (Cys\(^1\)-45) in the human protein. This reaction returns the DNA base to an unmodified state and permanently inhibits the alkyl acceptor activity of the enzyme, which is ultimately degraded (2, 18). As a consequence of this life cycle, the number of O\(^6\)-alkylguanine and O\(^4\)-alkylthymine adducts that can be repaired at one time depends on the steady-state cellular concentration of the unalkylated form of AGT (2, 3) and on its ability to partition between DNA lesion sites and the vast amount of genomic DNA in which they are embedded.

Three considerations motivate this characterization of AGT binding to single-stranded oligonucleotides. First, the enzyme binds single-stranded DNAs in vitro with affinities close to those observed with duplex DNAs (13, 14). Although repair rates have been found to be slower with single-stranded templates than with duplex (19), it remains possible that the repair of single-stranded templates might be a normal function of AGT within the cell. A better understanding of AGT interactions with single-stranded DNAs will contribute to our ability to test the novel possibility. Second, oligonucleotides containing O\(^6\)-alkylguanine offer advantages over the mononucleotide inhibitors currently under clinical trial. Important among these are improved water solubility, greater reactivity with AGT, and efficacy against O\(^6\)-benzylguanine-resistant AGT mutants (20, 21). A better understanding of the interactions of the enzyme

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* EC 2.1.1.63.

† The abbreviations used are: AGT, O\(^6\)-alkylguanine-DNA alkyltransferase; nt, nucleotide(s); EMSA, electrophoretic mobility shift assay.

6 Thus, every substrate is a suicide substrate.
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TABLE 1

Oligonucleotides

| Length | Sequence | $M_r$ |
|--------|----------|-------|
| nt     |          | Predicted$^a$ | Observed$^a$ |
| 5      | 5'-TGG TT-3' | 1,484 | 1,470 ± 285 |
| 7      | 5'-GGA GGG TGG G-3' | 2,218 | 2,170 ± 160 |
| 9      | 5'-AGC TC CTC ACT GCC G-3' | 2,701 | 2,650 ± 220 |
| 11     | 5'-GAC TGG TGT TTT TTT TTT TT T-3' | 3,310 | 3,430 ± 260 |
| 16     | 5'-GAC TGG TGT TTT TTT TTT TTT TT TTT TT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT T
as a guide and counted in a scintillation counter by the Cerenkov method. Similar results were obtained by densitometry of appropriately exposed autoradiographic films.

The serial dilution method (34, 35, 47) was used to obtain self-consistent estimates of binding stoichiometry (n) and the association constant (Ks). For the cooperative binding mechanism nP + D = PnD, the association constant is Ks = [PnD]/[D][P]n_free. Here P represents AGT protein, D represents DNA, and PnD represents the AGT-DNA complex of protein-DNA ratio n. Separating variables and taking logarithms gives Equation 1.

\[
\ln \left( \frac{[P_nD]}{[D]} \right) = n \ln[P]_{\text{free}} + \ln K_s
\] (Eq. 1)

Dilution of an AGT-DNA mixture changes [P_nD]/[D] by mass action while maintaining the ratio of [P]_{\text{total}} to [D]_{\text{total}}. The free protein concentration at each dilution step can be estimated using [P]_{\text{free}} = [P]_{\text{input}} - n[P_nD] starting with an initial value of n; Equation 1 is then used to calculate a new value of n from the DNA binding distribution. This value is then used to calculate a new estimate of [P]_{\text{free}}. These calculations are repeated recursively until values of n converge.

In many cases stoichiometries, association constants, and cooperativity parameters were evaluated by direct titration. Solutions of AGT protein (typically 5 × 10⁻⁷ M ≤ [AGT] ≤ 3 × 10⁻⁴ M) were added directly to [32P]DNA solutions (typically ~5 × 10⁻⁸ M), and samples were analyzed by native gel electrophoresis. Because the total concentration of protein binding sites on DNA was always significantly less than that of the protein, the approximation [P] = [P]_{\text{free}} was used. The dependence of binding density ν on the free protein concentration [P] was given by the McGhee-von Hippel isotherm (36) as modified by Record and co-workers (37) to account for finite lattice size (Equations 2 and 3).

\[
\frac{\nu}{[P]} = K(1 - s\nu) \left( \frac{(2\omega - 1)(1 - s\nu) + \nu - R}{2(\omega - 1)(1 - s\nu)} \right)^{n-1}
\] (Eq. 2)

\[
R = \left( \frac{(1 - (s + 1)\nu + \nu^2)(N - s + 1)}{2I - s\nu} \right) \frac{N - s + 1}{N}
\] (Eq. 3)

Here ν is the binding density (protein molecules/nucleotide), K is the equilibrium association constant for binding a single site, ω is the cooperativity parameter, N is the length of the DNA in nucleotides, and s is the size of the site (in nucleotides) that a protein molecule occupies to the exclusion of others. In this context, the cooperativity parameter ω is equal to the equilibrium constant for the process of moving a protein from an isolated site to a singly contiguous one or from a singly contiguous site to a doubly contiguous one. Thus, it is a measure of the population-averaged equilibrium constant for interaction between proteins occupying adjacent sites on the DNA.

**Analytical Ultracentrifugation**—Human AGT protein and oligodeoxoryribonucleotides were dialyzed against 10 mM Tris (pH 7.6), 1 mM dithiothreitol, 1 mM EDTA, 100 mM NaCl. Analytical ultracentrifugation was performed at 20 ± 0.1 °C in a Beckman XL-A centrifuge using an AN60 Ti rotor. Most scans were obtained at 252 nm to minimize protein contribution to the DNA signal (38), although some data sets were obtained at 260 nm. Equilibrium was held to be attained when scans taken 6 h apart were indistinguishable. Typically equilibrium times of 24 h met this criterion for AGT-DNA mixtures. Five scans were averaged for each sample at each wavelength and rotor speed.

For DNAs with small numbers of protein interaction sites, cooperative binding can be described by the simple mechanism nP + D = P_nD in which free DNA (D) is in equilibrium with saturated complex (P_nD), but intermediates with subsaturating protein stoichiometries are not present at significant concentrations. At sedimentation equilibrium, the radial distribution of absorbance for such a system is given by Equation 4.

\[
A(r) = a_P \exp[a_D(r^2 - r_0^2)] + a_D \exp[a_D(r^2 - r_0^2)]
\] + \alpha_{P,D} \exp[a_{P,D}(r^2 - r_0^2)] + e (Eq. 4)

Here A(r) is the absorbance at radial position r and a_P, a_D, and \alpha_{P,D} are absorbances of protein, DNA, and protein-DNA complex at the reference position r_0, and e is a base-line offset that accounts for radial position-independent differences in the absorbances of different cell assemblies. The reduced molecular weights of AGT protein, DNA, and protein-DNA complexes are given by a_P = M_p(1 - \tilde{v}_p)\omega^2/(2RT), a_D = M_D(1 - \tilde{v}_D)\omega^2/(2RT), and a_{P,D} = (nM_p + M_D)(1 - \tilde{v}_{P,D})\omega^2/(2RT). Here M_p and M_D are the molecular weights of protein and DNA, n is the protein:DNA ratio of the complex, ω is the solvent density, v is the rotor angular velocity, R is the gas constant, and T the temperature (kelvin). The partial specific volume of AGT (\tilde{v}_p = 0.744 ml/g) was calculated by the method of Cohn and Edsall (39) using partial specific volumes of amino acids tabulated by Laue et al. (40). The partial specific volume of single-stranded NaDNA at 0.1 M NaCl (0.502 ml/g) was estimated by interpolation of the data of Cohen and Eisenberg (41). Partial specific volumes of each protein-DNA complex were estimated using Equation 5.

\[
\tilde{v}_{P,D} = \frac{(nM_p\tilde{v}_p + M_D\tilde{v}_D)}{(nM_p + M_D)}
\] (Eq. 5)

This relationship is based on the assumption that there is no significant change in partial specific volumes of the components upon association. This approach has been used successfully for other protein-protein and protein-DNA interactions (42–44), although there are notable exceptions (45). Estimated with Equation 5, \tilde{v}_{P,D} is ~0.73 for the complexes described here. Accordingly a 1% error in our estimate of \tilde{v}_{P,D} results in an error in M_{P,D} of nearly 3%. However, a comparison of stoichiometries estimated by sedimentation equilibrium and EMSA methods (Table 2) suggests that errors in \tilde{v}_{P,D} are unlikely to be large. Equation 4 was used in global analysis of multiple data sets obtained at different macro-molecular concentrations and rotor speeds (46). In this method, the values of a_P, a_D, a_{P,D}, and e are unique to each sample, but the value of n must be common to all data sets. Terms accounting for non-ideal effects were not included because there was no evidence of non-ideality (see Table 1).
AGT Interactions with DNA

RESULTS

Determination of Stoichiometries—Human AGT binds single-stranded and duplex DNAs with significant cooperativity (13, 14). When short single-stranded DNAs are titrated with AGT, the major DNA species present at equilibrium are free DNA and the saturated protein-DNA complex (Fig. 1). The electrophoretic mobilities of these AGT complexes decrease with increasing DNA length (not shown), but protein-DNA stoichiometries cannot be reliably estimated from this effect (33, 35). Accordingly we used sedimentation equilibrium analyses to establish the stoichiometries of the protein-DNA complexes formed with single-stranded DNAs of 5, 7, 9, 11, 16, 30, and 78 nucleotides (representative data are shown in Fig. 2). In each case the data are fit by the sedimentation equation corresponding to the concerted binding model (nP + D ⇄ P,D; Equation 4). The small, symmetrical residuals (upper panels) indicate that this model is consistent with the mass distributions present in these samples. Molecular weights of protein-DNA complexes and free DNAs were obtained as parameters of these fits.9 The stoichiometry of each complex was inferred from the known molecular weights of the DNA and AGT protein, and the results are summarized in Table 2. The masses and stoichiometries of complexes formed with single-stranded 16-, 30-, and 78-mers were within error the same as values reported previously for analogous complexes formed with full-length human AGT protein prepared without the C-terminal His6 tag (13). These results confirm our previous conclusion that the presence of the C-terminal His6 tag has negligible effect on affinity and stoichiometry of AGT-DNA complexes (14). Under comparable solution conditions, increasing DNA length resulted in complexes of higher stoichiometry, indicating that for the DNA concentration range explored here and under conditions of AGT excess stoichiometry is limited by the number of binding sites available on each DNA.

Additional stoichiometry values were determined by native gel electrophoresis (EMSA) using the serial dilution procedure for the DNA concentration range explored here and under conditions of AGT excess stoichiometry is limited by the number of binding sites available on each DNA.

9 The excellent correspondence of the molecular weights observed for the free DNA species with those predicted from their sequences (summarized in Table 1) establishes that each DNA is single-stranded and demonstrates the absence of significant steric and electrostatic non-idealities under these solution conditions.
TABLE 2

| DNA length | M, (complex)a | Stoichiometry | S |
|------------|--------------|---------------|---|
| nt         |              |               |   |
| 5          | 23,746 ± 648 | 1.03 ± 0.03*  | 4.85 ± 0.15* |
| 7          | 24,697 ± 432 | 1.04 ± 0.02*  | 6.83 ± 0.15* |
| 9          | 44,632 ± 1,297 | 1.94 ± 0.06*  | 4.63 ± 0.13* |
| 11         | 43,080 ± 1,299 | 1.84 ± 0.06*  | 5.97 ± 0.19* |
| 16         | 91,770 ± 4,539 | 4.02 ± 0.21*  | 3.98 ± 0.15* |
| 22         | —a           | 3.89 ± 0.09b  | 4.11 ± 0.10* |
| 24         | —a           | 5.60 ± 0.30b  | 4.36 ± 0.44* |
| 30         | 123,632 ± 4,323 | 5.31 ± 0.15b  | 5.66 ± 0.15* |
| 41         | —a           | 5.32 ± 0.42b  | 5.63 ± 0.48* |
| 78         | 216,638 ± 6,268 | 8.90 ± 0.29b  | 8.76 ± 0.29* |

a Determined by sedimentation equilibrium analysis. The error ranges are 95% confidence limits.
b Determined by EMSA. The error ranges are 95% confidence limits.
c —, not determined.

FIGURE 3. Serial dilution analysis of the AGT complex with single-stranded 24-nt DNA. A, binding detected by EMSA. Sample a, 24-mer DNA (4.1 × 10−6 M) only. Sample b, 24-mer DNA (4.1 × 10−6 M) plus AGT (1.1 × 10−5 M). Samples c–l are sequential 1:1.33-fold dilutions of sample b. All samples were equilibrated in buffer consisting of 10 mM Tris (pH 7.6), 100 mM NaCl, 1 mM dithiothreitol, 0.05 mg/ml bovine serum albumin for 30 min at 20 °C prior to resolution on native gels as described under “Experimental Procedures.” B, graph of the dependence of ln[PnD]/[D] on ln[P] for three parallel experiments, starting with initial AGT:DNA ratios of 11.5 ( ), 15.3 ( ), and 24.1 ( ). The line represents a least squares fit to the data ensemble for the range about the midpoint of the reaction (−13.7 < ln([AGT]/M) < −12.0) with [AGT]mid calculated as described under “Experimental Procedures.” The slope equals 5.65 ± 0.21 for this subset of the data.

A representative experiment showing mass action dissociation of the AGT complex formed with a single-stranded 24-mer DNA is shown in Fig. 3A. As with forward titrations, no stoichiometric intermediates (expected to migrate between free DNA and saturated complex bands) were detectable. A graph of ln[PnD]/[D] as a function of ln[P] is shown in Fig. 3B. The slope of this graph around the midpoint of the binding reaction yields an apparent stoichiometry of 5.6 ± 0.5 and −n

\[
\ln[P_{\text{free, midpoint}}] = \ln K_n
\]

the natural logarithm of the association constant for the overall reaction. The stoichiometry values obtained for binding single-stranded 11-, 16-, 22-, 24-, 30-, and 41-mers are summarized in Table 2, and values of \( K_n \) are in Table 3. The agreement of stoichiometry values determined by EMSA with those obtained by sedimentation equilibrium indicates that the same complexes are detected by both methods and demonstrates that dissociation of complexes during electrophoresis is not a significant factor under the conditions that were used here.

**Binding Density Depends on DNA Length**—The number of nucleotide residues per protein molecule (S) is a measure of binding density that reflects the extent to which the DNA is occupied in a protein-DNA complex. When proteins are packed efficiently on templates of optimal length, S is minimized; when gaps occur in packing or partial length sites are present at DNA ends, \( S > S_{\text{min}} \) and the value of \( S \) overestimates the separation between the start points of successive binding sites. Such end effects are particularly severe when DNA templates are short (36, 37). We exploited this effect to estimate the size of the site occupied by AGT on single-stranded templates. As shown in Fig. 4A, S oscillates with increasing DNA length (L) for short templates (≤30 nt), whereas above this length, the separation between values of L that we tested is too great for oscillation to be evident. To determine the underlying period of the oscillation, the relation \( S = A \cos (BL + C) \) was used to model the dependence of S on L (Fig. 4B). In this equation, A is the amplitude of the oscillation, B is its angular frequency in degrees/nt, and C is an offset equal to the mean value of S. A fit of this relation to the data returned an angular frequency of \( B = 87.4 ± 0.4°/\text{nt} \), consistent with models in which successive binding sites are separated by 360/\( (87.4 ± 0.4°) = 4.1 ± 0.02 \) nt along the DNA contour. No other angles in the range 20° ≤ B ≤ 160° yield similarly small values of \( \chi^2 \) (Fig. 4C), indicating that there is only one plausible binding site frequency for this system.

The value of S is significantly greater for the 78-nt DNA than for any of the smaller single-stranded DNAs that we tested. Because incomplete occupancy of DNA ends should be equally possible for all DNAs, the less efficient packing of AGT on the 78-nt DNA may reflect the presence of gaps between groups of tightly packed protein molecules or a uniform but less tightly packed structure. Despite less efficient packing, the inclusion of complexes containing 41- and 78-nt DNAs in the frequency analysis returned an angle of 86.9 ± 0.5° in good agreement with the value obtained with shorter templates (result not shown). Thus, although the uniformity of packing may decay with increasing template length, the dominant picture remains one of a protein array in which the average separation between the start of two adjacent binding sites is ~4 nt. This spacing is significantly shorter than the ~8 bp/protein occupied in crystalline AGT complexes formed with duplex DNA (22, 48) and the opti-
TABLE 3
Association constants of AGT-DNA complexes

| DNA length nt | Serial dilution analysis | Scatchard analysis |
|---------------|--------------------------|-------------------|
|               | $K_i^b$/M$^{-1}$ (range)* | $K_{max}$/M$^{-1}$ | $K_i^b$ | $\omega$ | $K_i^b/\omega$ |
| 11            | $3.20 \times 10^{10}$ (9.79 $\times 10^{8}$–6.93 $\times 10^{10}$) | $3.33 \pm 1.79 \times 10^6$ | $3.04 \pm 0.29 \times 10^8$ | $37.9 \pm 3.0$ | $1.15 \pm 0.19 \times 10^2$ |
| 16            | $9.42 \times 10^{12}$ (3.11 $\times 10^{12}$–2.79 $\times 10^{12}$) | $8.05 \pm 0.26 \times 10^6$ | $1.59 \pm 0.21 \times 10^8$ | $89.8 \pm 8.9$ | $1.43 \pm 0.31 \times 10^2$ |
| 22            | $1.55 \times 10^{12}$ (7.34 $\times 10^{11}$–2.97 $\times 10^{12}$) | $4.42 \pm 0.88 \times 10^6$ | $8.93 \pm 1.31 \times 10^8$ | $43.0 \pm 6.6$ | $3.84 \pm 1.07 \times 10^2$ |
| 24            | $9.64 \times 10^{11}$ (1.61 $\times 10^{11}$–5.18 $\times 10^{11}$) | $5.35 \pm 1.98 \times 10^6$ | $1.38 \pm 0.10 \times 10^8$ | $61.7 \pm 3.7$ | $8.51 \pm 1.09 \times 10^2$ |
| 30            | $1.03 \times 10^{10}$ (2.17 $\times 10^{8}$–2.63 $\times 10^{10}$) | $4.53 \pm 1.55 \times 10^6$ | $1.02 \pm 0.15 \times 10^8$ | $49.3 \pm 5.3$ | $5.02 \pm 1.19 \times 10^2$ |
| 41            | $1.34 \times 10^{10}$ (1.67 $\times 10^{7}$–5.05 $\times 10^{8}$) | $4.14 \pm 0.69 \times 10^6$ | $1.25 \pm 0.05 \times 10^8$ | $54.5 \pm 2.8$ | $6.81 \pm 0.61 \times 10^2$ |

* Estimated using the relation $-n \log [P_{free}] = \log K_i$ obtained from Equation 1 when $\log [P_{D,D}]/[D] = 0$ (61). The range reflects 95% confidence limits in stoichiometry ($n$) returned by these analyses.

b The range reflects 95% confidence limits.

Obtained by propagating 95% confidence limits of $K_i$ and $\omega$.

human AGT proteins to the same DNA under the same buffer conditions (14). This supports our conclusion that the presence of the C-terminal His$_6$ tag on the protein used in the current studies has a negligible effect on the affinity of human AGT for this standard DNA template.

Values of $K_i$ and $\omega$ oscillate in phase with each other as DNA length increases (Fig. 5B). The fact that these oscillations are significantly larger than the confidence limits associated with individual parameters and the fact that they in phase argue that this oscillation is not a consequence of fitting correlation between $K_i$ and $\omega$ (as discussed above, $K_i$ and $\omega$ are weakly anticorrelated). Thus, the orientations of AGT proteins and DNA that maximize $K_i$ also position each protein to interact optimally with neighboring AGT molecules. Intriguingly, oscillations in $K_i$ and $\omega$ have similar periods but are of opposite phase to the oscillation of S with DNA length. As a result, the most compact complexes (with smallest S) have relatively stronger protein-DNA and protein-protein interactions than do less compact assemblies with similar stoichiometries. This suggests that AGT complexes formed with single-stranded DNA are somewhat malleable so that when packing density is not constrained at the maximum value (giving $\sim 4$ nt/protein) rearrangements may occur that increase configurational entropy at the expense of slightly suboptimal protein-DNA and/or protein-protein contacts. This interpretation is supported by the decay of the amplitudes of oscillations in $K_i$ and $\omega$ with increasing template length (Fig. 5B). Such decay might be expected if packing interactions become increasingly degenerate with template length.

DISCUSSION

The data presented here are consistent with a cooperative binding model in which AGT molecules bind single-stranded DNA at $\sim$4-nt intervals. Because a single molecule of AGT occupies $\sim$8 bp of DNA duplex (22, 48), it seems likely that AGT molecules overlap along the DNA contour as shown schematically in Fig. 6. Overlapping binding provides opportu-

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**Footnotes:**

13 This interpretation is based on models in which the same protein surface interacts with single-stranded and duplex DNAs. In support of these models, we reason that because AGT repairs both single-stranded and duplex DNAs, the active site cleft must be part of the binding surface for both substrates. In addition, AGT has only one surface with positive electrostatic potential that could accommodate negatively charged DNA (60), and that surface coincides with the one in contact with duplex DNA in currently available crystal structures (22, 39).
AGT Interactions with DNA

AT forms a binding motif with a 4-nt periodicity. A, dependence of statistical binding site size (S) on template length. Data from the entire set of AGT-DNA complexes are shown. Statistical binding site size was calculated using \( S = L/n \) where \( L \) is DNA length in nucleotides and \( n \) is the number of protein molecules bound to a DNA molecule. S values determined from sedimentation equilibrium data are indicated by open squares \( \square \); values obtained from EMSA experiments are indicated by closed circles \( \bullet \). The error bars correspond to 95% confidence limits. B, data for the subset of AGT-DNA complexes formed with DNAs of 30 nt or less. The smooth curve is the least squares fit of the equation \( S = A \cos(BL) + C \) in which \( A \) is the amplitude of the oscillation, \( B \) is the displacement angle in degrees/nt, and \( C \) is an offset equal to the mean value of \( S \). This fit returned \( A = -1.5 \pm 0.3, B = 87.4 \pm 0.4^\circ \), and \( C = 5.23 \pm 0.14 \). Data symbols are defined as in A. C, dependence of the \( \chi^2 \) error function on displacement angle. Data for the dependence of \( S \) on \( L \) were fit with the equation \( S = -1.5 \cos(BL) + 5.2 \) as described for \( B \) except that values of angle \( B \) were fixed. The \( \chi^2 \) values for each fit are graphed as a function of \( B \). The only significant minimum in \( \chi^2 \) is at \( B = 87.4^\circ/\text{nt} \), consistent with models in which AGT forms structures with a fundamental repeat of 360/87.4 = 4.1 nt/protein. The prominent maximum at \( B = 100^\circ \) corresponds to the B angle with the poorest correlation to the data.

nities for protein-protein interactions that may contribute to cooperativity and accounts for the efficient DNA-dependent cross-linking of AGT molecules that we have recently observed. Modestly cooperative binding like that described here (37.9 ± 3.0 ≤ \( \omega \) ≤ 89.8 ± 8.9) is sufficient to account for the range of binding site sizes (4 nt ≤ \( S \) ≤ 9 nt) previously reported for saturated complexes formed with single-stranded DNAs (13, 14, 17). On short DNAs (\( n \leq 40 \) nt), \( \omega \) values in this range are sufficient to produce tight packing that results in oscillation in the binding site size with minima when the number of nucleotides is an integral multiple of 4. On longer DNAs, this cooperativity is not sufficient to suppress binding degeneracy so the statistical binding site size gradually increases and oscillations in \( K_r \) and \( \omega \) decay toward average values (Fig. 5).

AGT is not unique in its reactivity toward single-stranded DNA. Other DNA repair proteins and factors that bind and/or repair single-stranded DNA include the human and vaccinia virus uracil-DNA glycosylases (49, 50), human DNA glycosylases NEIL1 and NEIL2 (51), human apurinic/apyrimidinic endonuclease (APE1) (52), and Xeroderma pigmentosum group A correcting protein (XP-A) (53, 54). In addition, XP-A is associated with the replication protein A single strand-binding protein (55, 56), whereas components of the transcription-coupled nucleotide excision repair complex are associated with XP-B and XP-D DNA helicases (57). It seems reasonable to expect that these interaction partners increase the availability of single-stranded templates for repair. Although no such association has been found to date for AGT, this may be because the interactions are too weak for detection by current methods. Alternatively single-stranded DNA may be sufficiently available for AGT binding, making association with helicases or single strand-binding proteins unnecessary for its function.

The cooperative binding mechanism described here for single-stranded DNA and reported previously for duplex DNA (13, 14) contrasts with the independent binding shown in currently available crystal structures of AGT-DNA complexes (22, 48). At present we cannot account for this difference. To date, we have tested the binding of wild-type human AGT over a range of temperature (4 °C ≤ \( T \) ≤ 30 °C), salt concentrations (0 M ≤ [KCl] ≤ 0.4 M), magnesium ion concentrations (0 mM ≤ [Mg\(^{2+}\)]\(_{\text{free}} \) ≤ 10 mM), zinc availability (Zn\(^{2+}\)-depleted and Zn\(^{2+}\)-saturated proteins and Zn\(^{2+}\)-saturated protein with 0 mM ≤ [Zn\(^{2+}\)]\(_{\text{free}} \) ≤ 10 mM). We have tested Cys\(^{145}\)-methylated and -benzoylated proteins, active site (Cys\(^{145}\)) mutations, both C-terminal and N-terminal His\(_6\)-tagged proteins, and a range of single-stranded and duplex templates. Under all conditions, significant binding cooperativity was observed (14, 26, 58). We conclude that cooperative binding is a bona fide activity of human AGT.

What do these results tell us about the substrate interactions of AGT? Within our sample set, variations in \( K_r \) with changes in substrate identity are small, indicating that binding affinity is not strongly dependent on base composition or sequence. These results are consistent with previous observations based on binding competition assays (13) and the relative sequence independence of DNA repair efficiency (16). The absence of a dependence on base composition or sequence is likely to ensure that repair activity is uniformly available to all sequences.
AGT Interactions with DNA

Cooperative binding may be an important part of the DNA repair mechanism of AGT. In the current results, the most compact complexes have the strongest protein-DNA and protein-protein interactions. Overlapping binding concentrates repair activity at a higher density on the DNA contour than would be available in non-overlapping binding modes. With a new binding site starting every 4 nt, only modest protein displacement is needed for surveillance of every nucleotide in the complex. Together these results suggest that compact, cooperative binding may be part of an efficient lesion search and repair mechanism. Finally although the moderate cooperativity detected here (37.9 ± 3.0 ≤ ω ≤ 89.8 ± 8.9) is insufficient to extend high density binding over hundreds of residues of DNA, it can generate densities approaching one protein every 4 residues for sections of template >50 residues long. Duplex regions of this size are likely to become available between nucleosomes during chromatin remodeling, and single-stranded segments may be transiently available within transcription or DNA replication complexes. The processive movements of these complexes may allow systematic surveillance of large parts of the genome by AGT. In addition, repair just upstream of a replication fork is likely to represent the last opportunity to prevent the conversion of a promutagenic lesion into an actual mutation. Because of its proximity to the replication fork, such last ditch repair may be limited to single-stranded templates.

The results presented here raise a number of questions for future investigation. First, is the mechanism of cooperative binding the same on single-stranded and duplex DNAs? In vitro measurements indicate that both secondary structures are bound with positive cooperativity and remarkably similar affinities and binding densities (13, 14). These results are intriguing because single-stranded and duplex DNAs differ greatly in torsional rigidity. The relative flexibility of single-stranded DNA may allow cooperatively bound AGT proteins to form identical protein-DNA contacts at a binding density of one protein every 4 nucleotides. A comparable density of one protein every 4 base pairs will not result in identical protein contacts with the more rigid B-form duplex unless each protein is rotated approximately (4 bp/protein -1/ 10.4 bp/turn -1 ) × 360°/turn -1 = 138°/protein -1 with respect to its immediate neighbor (similar rotations are shown schematically in Fig. 6). If protein-protein contacts constrain the geometry of the cooperative complex to values other than ~138°/ binding step, the cooperative assembly might cause local DNA unwinding (at rotation angles <138°/binding step) or overwinding (at rotation angles >138°/binding step).

Second, is the mechanism by which damaged bases enter the active site similar for single-stranded and duplex templates? Torsional stress exerted on duplex DNA may influence the extrusion of alkylguanines from the base stack to form the extrahelical conformation seen in crystalline repair complexes (22, 48). Such effects would not be available on single-stranded DNA and may account for the more rapid repair of duplex DNAs than single-stranded DNAs in vitro (19).

Third, how do cooperative interactions influence the rates of AGT binding and dissociation from target DNAs? We have shown previously that AGT is monomeric in the absence of DNA over a wide range of solution conditions (13, 14). This result argues against the maintenance of cooperative protein-protein contacts once AGT proteins have dissociated from DNA and against a role for multimeric protein complexes in DNA binding. Independently of whether AGT binds and dissociates from DNA as a monomer, the portion of a cooperative array of bound proteins that is active in protein binding and/or dissociation is likely to influence the kinetics of these processes. Our current thinking is influenced by the notion that the addition or removal of a protein unit from the middle of a cooperative assembly is likely to be slow compared with the addition or removal of a unit from one end. If this is the case, the rate of

13 For example, S = 4 nt/protein on single-stranded 16-mer DNA and S = 4 bp/protein for the corresponding 16-mer duplex (14).
transfer of AGT molecules between DNA segments may depend more strongly on the concentration of cooperative assemblies (and hence the concentration of end monomers) than on the concentration of DNA-bound AGT monomers themselves. Experiments designed to test these ideas are underway.

Finally, do alkyl transfer rates depend on the length of the cooperative complex? Repair requires the correct juxtaposition of an active AGT monomer and the damaged base. If the correct juxtaposition of AGT and lesion is not achieved during formation of the protein-DNA complex, achieving it may require repositioning of the entire protein array. If this is the case, productive binding and repair might be more rapidly achieved with short protein arrays than with long protein arrays. Similarly do alkyl transfer rates depend on substrate length as seen for the binding parameters $K$ and $\omega$? If so, oligonucleotides with lengths that are multiples of 4 nt will be more efficient substrates than oligonucleotides of intermediate lengths. These factors may contribute to the development of clinically useful oligonucleotide inhibitors of AGT.

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