Kinetic Analysis of Steps in the Repair of Damaged DNA by Human O°-Alkylguanine-DNA Alkyltransferase*§

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Rates of individual steps in the removal of alkyl groups from O°-methyl (Me) and -benzyl (Bz) guanine in oligonucleotides by human O°-alkeylguanine DNA alkyltransferase (AGT) were estimated using rapid reaction kinetic methods. The overall reaction yields hyperbolic plots of rate versus AGT concentration for O°-MeG but linear plots for the O°-BzG reaction, which is ~100-fold faster. The binding of AGT and DNA (double-stranded 30-mer/36-mer complex) appears to be diffusion-limited. The rate of dissociation of the complex is ~25-fold slower (~1 s⁻¹) for DNA containing O°-MeG or O°-BzG than unmodified DNA. The fluorescent dC-analog 6-methylpyrrolo[2,3-d]pyrimidine-2(3H)one deoxyribonucleoside (pyrrolo dC), which pairs with G, was positioned opposite G, O°-MeG, or O°-BzG and used as a probe of the rate of base flipping. A rapid increase of fluorescence (k ~ 200 s⁻¹) was observed with O°-MeG and O°-BzG and AGT but not with a Gly mutation at Arg¹¹, which has been implicated in base flipping with crystal structures. Only weak and slower fluorescence changes were observed with G-pyrrolo dC or T2-aminopurine pairs. These rate estimates were used in a kinetic model in which AGT binds and scans DNA rapidly, flips O°-alkylG residues, transfers the alkyl group in a chemical step that is rate-limiting in the case of O°-MeG but not O°-BzG, and releases the dealkylated DNA. The results explain the overall pattern of rates of alkyl group removal versus AGT concentration and the effects of the mutations, as well as the greater affinity of AGT for DNA with O°-alkylG lesions.

DNA is subject to damage by endogenous and exogenous chemicals, which generate DNA adducts or otherwise altered DNA bases. The DNA repair process is essential to protect genome integrity (1, 2). The DNA repair protein O°-alkylguanine-DNA transferase (AGT) directly removes an alkyl group from the O° position of guanine. AGT also repairs O¹-methyl-thymine, but the rate of this repair is species-specific, and human AGT repairs this adduct very poorly (3). AGT represents an important protective mechanism for protecting DNA from environmental alkylating agents but its presence can have some adverse effects. The presence of AGT in tumor cells is a major factor in resistance to certain therapeutic alkylating agents such as temozolomide and 1,3-bis(2-chloroethyl)nitrosourea. Also, AGT enhances the toxicity of 1,2-dibromomethane and some related bifunctional electrophiles (4). The mechanistic details of how AGT repairs O°-alkylguanine adducts have been of great interest (3). A crystal structure of an AGT mutant (C145S) bound to an O°-MeG-containing oligonucleotide provided valuable structural insights for the reaction mechanism (5). The alkylated guanine must be flipped out of the DNA double helix for the subsequent transfer of the alkyl residue. In the case of another DNA repair enzyme, UDG, this base flipping or damaged base recognition process may depend on both the binding properties of the protein and the intrinsic DNA strand flexibility (6).

AGT is able to repair O°-MeG in any sequence context. Some reports have suggested that AGT is not sequence-dependent in its activity (7), but other work shows that is clearly not the case and that some sequences are repaired more efficiently than others (8, 9). Spratt et al. (10) reported binding interactions between AGT and the O°-MeG and suggested that the AGT active site interaction requires the N1 and O6 atoms of a guanine adduct or a thymine adduct. Escherichia coli AGT (AdaC), which has a smaller substrate binding pocket, prefers an O°-MeG adduct over a bulky O°-BzG adduct (11). The larger adduct O°-pyridoxylobutyl G was repaired by human AGT, although not very efficiently, compared with a O°-BzG adduct (12). These results imply that the size of the AGT active site is crucial for substrate recognition. O°-BzG is a favored substrate for human AGT compared with the O°-MeG adduct (12–14).

The literature varies considerably in reports of the rates and kinetics of human and other AGT reactions. In several cases the reaction is regarded to be second-order, with rate constants varying from 1.4 × 10⁶ to 1 × 10⁷ M⁻¹ s⁻¹ (7, 10, 15–17). However, AGT does have affinity for DNA (9, 18–20) and, in consideration of this, the mechanism might better described as one with a Kᵢ for the reversible binding of AGT and DNA, followed by a first-order chemical step (9). However, the rates of base flipping and any scanning processes are unknown.

In this report, we studied the rates of alkyl transfer, dissociation, and base flipping for O°-MeG and O°-BzG adducts in a double-stranded oligonucleotide with AGT. Several mutants

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1 The abbreviations used are: AGT, O°-alkylguanine-DNA transferase; Bz, benzyl; DTT, dithiothreitol; Me, methyl; pol T7, bacteriophage T7 exonuclease-deficient DNA polymerase; UDG, uracil DNA glycosylase; WT, wild type.
involving the site-directed mutations in the AGT active site were examined. The measured kinetic parameters were evaluated in a more complex reaction model, which involves the four steps of DNA-AGT association, base flipping, alkyl transfer, and DNA-AGT dissociation (Scheme 1). The implications of this model are discussed.

**EXPERIMENTAL PROCEDURES**

Chemicals—UDG, piperidine, and O'-BzG were obtained from Sigma-Aldrich; [γ-32P]ATP was purchased from PerkinElmer Life Sciences. All oligonucleotides (Table I) were purchased from Midland Certified Reagents (Midland, TX) or Operon Technologies (Alameda, CA), except the O'-BzG-containing oligonucleotide, which was synthesized in the Vanderbilt facility using general phosphoramidite technology (Scheme 2) (21). The oligonucleotides were purified by either HPLC or denaturing gel electrophoresis before use (21). For the measurement of reaction rates, the [32P]labeled strand contained a U instead of a T to allow for UDG/piperidine cleavage to generate shorter products that could be readily separated and analyzed (Scheme 2).

Enzymes—AGTs (all human) were expressed in E. coli and purified as previously described (5, 22, 23). Pol T7 ma-Aldrich; [30874/H9262 could be readily separated and analyzed (Scheme 2). Enzymes—AGTs (all human) were expressed in E. coli and purified as previously described (5, 22, 23). Pol T7 ma-Aldrich; [30874/H9262 could be readily separated and analyzed (Scheme 2).

**Presteady-state Rapid-quench Measurements of Reaction Rates—** Rapid-quench experiments were carried out in a KinTek Quench-Flow Apparatus (Model RQF-3, KinTek Corp., Austin, TX). Reactions were started by rapid mixing of AGT (12.5 μM) with DNA substrate (10.9 μM) (Scheme 2). Final concentrations were 50 μM Tris, pH 7.8, 1 mM DTT, 25 μM DNA, and varying concentrations of AGT proteins. The sample drive syringes contained deionized H2O. The reactions were quenched with a modified procedure (24, 25) using a plasmid encoding pol T7 -pG5X (Amp); protein concentrations were estimated using a 1,000 kDa molecular weight standard. The mixtures were incubated at 37 °C for 30 min, followed by piperidine treatment (0.2M piperidine, 95 °C, 30 min) and analyzed using the Applied Photophysics software. Reactions were recorded over the range of 400–500 nm. The bandpass was 5 nm for both the emission and the excitation slits.

**Kinetics of O6-Alkylguanine Alkyl Transfer**

**Scheme 1. Kinetic mechanism for transfer of an alkyl group from DNA (O6-alkylG) to AGT.**

![Kinetic mechanism diagram](http://www.jbc.org/content/3874/38/30874.large.jpg)
initiated by rapid mixing of equal volumes of primer/template complex (400 nM in 50 mM Tris buffer, pH 7.8) with AGT proteins (1.6 μM in 50 mM Tris buffer, pH 7.8, containing 2 mM DTT). All reactions were done at 25 °C. Traces are presented as averages of several individual reactions.

Kinetic Simulations—Fitting and simulation were done using the program DynaFit (BioKin, Ltd., Pullman, WA) (28), used with a Macintosh G5 computer (Apple Computer, Cupertino, CA), with x,y files (see Supplementary Data).

RESULTS

Measurement of Reaction Rates—Relatively long nucleotide pairs (Table I) were synthesized and used to ensure coverage of the protein active site, and the rates for AGT and some mutants were compared. One issue with these longer substrates was the small difference in mobility between alkylated and the non-alkylated (product) DNA in analytical gel electrophoresis. A dT residue was replaced by a uracil residue in the DNA strand two bases 3′ of the alkylated dG, and (after the reactions were quenched) the DNA samples were treated with UDG/piperidine to generate the 5′-32P-labeled 13-mer fragments, which were readily resolved (Scheme 1 and Fig. 1). The rates of

<table>
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<tr>
<th>AGT</th>
<th>Substrate</th>
<th>k_r</th>
<th>K_d</th>
<th>k_r/K_d (Me) or second-order rate (Bz)</th>
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<tr>
<td>WT</td>
<td>O6-MeG</td>
<td>0.30 ± 0.03</td>
<td>98 ± 32</td>
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<td>R128L</td>
<td>O6-MeG</td>
<td>0.15 ± 0.03</td>
<td>1800 ± 360</td>
<td>0.079</td>
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<td>Y114F</td>
<td>O6-MeG</td>
<td>0.13 ± 0.03</td>
<td>2720 ± 1350</td>
<td>0.048</td>
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<tr>
<td>R128G</td>
<td>O6-MeG</td>
<td>0.12 ± 0.03</td>
<td>&gt;4000</td>
<td>&lt;0.03</td>
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<tr>
<td>WT</td>
<td>O6-BzG</td>
<td>320</td>
<td></td>
<td></td>
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<tr>
<td>R128L</td>
<td>O6-BzG</td>
<td>110</td>
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<td></td>
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<td>Y114F</td>
<td>O6-BzG</td>
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<td>R128G</td>
<td>O6-BzG</td>
<td>3.2</td>
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</table>

Kinetics of O6-Alkylguanine Alkyl Transfer
alkyl transfer from O^6-MeG and O^6-BzG to AGT were measured using rapid quench conditions, because of the rapid reaction rates. O^6-MeG oligonucleotides (25 nM) were incubated with varying concentrations of AGT (Fig. 2). Reaction rates (k_{\text{obs}}) were estimated by fits to a single-exponential equation, and the k_{\text{obs}} values were plotted against AGT concentration to generate the k_{\text{reaction}} (k_r) and K_r values (Table II). Mutations at Arg\(^{128}\) and Tyr\(^{114}\); two residues implicated from site-directed mutagenesis and crystallography work (5, 22, 23), significantly reduced the AGT repair efficiency.

The mutations had smaller effects with the O^6-BzG substrate than the O^6-MeG, except in the case of R128G (Table II). The repair rates were much higher for the O^6-BzG substrate, and the reactions were complete in ~200 ms with the range of different AGT concentrations used (Fig. 3A). The k_{\text{obs}} versus [AGT] traces produced linear fits for both WT AGT and the mutants (Fig. 3B), and the slope (k_{\text{off}}/[AGT]) was used as the parameter reflecting the overall DNA repair efficiency (Table II).

Estimation of AGT-DNA Dissociation Rates Using Presteady-state Rapid-quench Experiments—AGT-DNA dissociation rates were measured using the design shown in Scheme 3. (Attempts to directly observe binding and dissociation rates by fluorescence measurements were unsuccessful.) \(^{32}\)P-labeled DNA was premixed with AGT to form a complex and then rapidly mixed with pol T7/"thioredoxin. After the AGT-DNA complex dissociation, free DNA is trapped by the excess pol T7 to form a complex (K_{d}~15 nM (26)). With the addition of dNTPs, the pol T7 reactions proceeded for 0.25 s before quenching and, thus, the new pol T7-DNA complexes are detected as one-base incorporation products, monitored by gel electrophoresis. The pol T7-DNA complex only accounts for a portion of the total DNA; the amplitude does not affect the estimates of the dissociation rates.

Dissociation rates for different AGT-DNA combinations are listed in Tables III and IV, based on single exponential fits of the data (Fig. 4). The rates of dissociation with the O^6-alkyl-modified DNA could not be measured with catalytically active AGT, and therefore either the C145A and C145S mutants (Table III) or the alkylated AGT (Table IV) was used. The results with unmodified DNA (Table IV) indicate that mutation or alkylation of Cys\(^{145}\) did not alter the rates of dissociation. In general, all AGTs quickly dissociate from unmodified DNAs (k_{\text{off}}~15–39 s^{-1}) but dissociate more slowly from the alkylated DNAs (0.6–1.4 s^{-1}). The k_{\text{off}} rates were measured for two benzylated AGTs, the WT, and the R128L mutant (47 s^{-1}).

\(^{2}\) After the benzylation treatment, some modified AGT was lost during Sephadex G-10 column filtration because of inherent instability (29, 30). Freshly benzylated AGTs were used in these assays. Benzylation of the C145 residue of AGT is readily done with O^6-Bz guanine (27), but complete methylation is not straightforward and was not done here.

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### TABLE III

<table>
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<tr>
<th>Assay</th>
<th>AGT:DNA</th>
<th>DNA</th>
<th>k_{\text{off}}</th>
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<td>AGT (C145A) + O^6-MeG DNA</td>
<td>8:1</td>
<td>100</td>
<td>1.2 ± 0.3</td>
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<tr>
<td>AGT (C145S) + O^6-MeG DNA</td>
<td>8:1</td>
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<td>0.6 ± 0.1</td>
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<tr>
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<td>25</td>
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### TABLE IV

<table>
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<th>Assay</th>
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<tr>
<td>AGT (WT)</td>
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</tr>
<tr>
<td>AGT (C145A)</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>AGT (R128L)</td>
<td>15 ± 6</td>
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<tr>
<td>WT AGT-Bz</td>
<td>47 ± 11</td>
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<tr>
<td>AGT (R128L-Bz)</td>
<td>47 ± 8</td>
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</tbody>
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Measurement of Base Flipping by Fluorescence—A pyrrolo dC fluorescent tag was paired with an alkylated dG adduct in the DNA duplex (Scheme 4). If the modified dG is flipped out, the fluorescence signal of pyrrolo dC should increase, because the quenching effect from base pairing is diminished, as in the case of 2-aminopurine work done with UDG (31, 32). AGT-C145A, mutant totally devoid of activity, was added to a pyrrolo dC DNA duplex, and the fluorescence changes were recorded (using buffer for background subtraction) (Fig. 5). The fluorescence change because of the AGT itself was non-detectable. With O^6-BzG-containing DNA, the fluorescence signal was increased >2-fold upon the addition of AGT. The amplitude of the fluorescence change was smaller for the O^6-MeG adducts, in part because of the higher fluorescence background from the DNA alone. Unmodified DNA showed a weak fluorescence change, probably due in part to the looser binding of the unmodified guanine by AGTs (Table IV). These titration experiments were also done with the AGT-C145S mutant, which generated similar results (not shown).

Presteady-state Stopped-flow Fluorescence Measurement of Base Flipping—Base flipping rates were measured using the change in fluorescence measured with the pyrrolo dC-containing oligonucleotides (Fig. 6A and Table V). The lower amplitude and slower flipping rate for the unmodified DNA reflects the weak interactions of AGT with non-alkylated DNA. The base
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pairing between pyrrolo dC and an unmodified G is stronger than with an O^6-alkylG, and AGT should be less likely to flip the G than an O^6-alkyl G, based on work with UDG mechanisms (6). Unmodified G may not fit the AGT binding pocket as well as G O^6-alkyl if the guanine residue is flipped out, although this possibility does not find support in the analysis of the crystal structure (5). Overall, the low fluorescence amplitude and slow rate of change suggest only weak and nonspecific interactions between the unmodified DNA and the AGT. The reactions with O^6-BzG substrates were further investigated using AGT mutants (Fig. 6B). Arg^{128} is a key residue thought to be involved in base flipping (5) and intrudes the DNA duplex, based on the x-ray crystal structures. With WT AGT, two reaction phases were observed, with the faster having a rate of 240 s^{-1}. There was also a slow phase (14 s^{-1}), which may be attributed to the DNA-AGT dissociation step (Table IV). One interpretation of this result is that pyrrolo dC can pair with the repaired G only after the total release by the AGT. Consistently, when Arg^{128} was replaced by Gly to abolish the base flipping “arm” (5), there was only a very limited fluorescence change.

The rates presented in Table V are fits of the data to single exponential plots and do not reflect the contributions of rates of association of AGT and DNA and the reverse reactions. The limited fluorescence changes and very rapid reactions did not allow a systematic analysis by varying reactant concentrations. However, some of the available data were fit in the context of Scheme 1. Using a k_1 value of 10^9 M^{-1} s^{-1} (see below) and k_{-1} of 1 s^{-1} for modified DNA (Table III), an optimal fit was found with k_{obs} = 350 s^{-1} (slightly faster than the k_{obs} 200 s^{-1} (Fig. 7A). The weaker fluorescence change seen with unmodified G (Fig. 6A) was also analyzed. The trace clearly cannot be fit to a system with k_{2} = 240–350 s^{-1}. The data were fit to a burst equation with k = 20 s^{-1} and a second, linear phase of 0.02 s^{-1}. The rate suggested for unmodified G (20 s^{-1}) might be fast enough to be part of the O^6-MeG reaction (Fig. 2B) but not the O^6-BzG reaction (Fig. 2B).

In other experiments (results not presented), the kinetics of fluorescence changes were examined after mixing a similar oligonucleotide containing a 2-aminopurine:T pair. The intrinsic fluorescence of 2-aminopurine is greater than that of pyrrolo dC, but the magnitude of the observed fluorescence changes was less than that observed for the pyrrolo dC:G pair (Figs. 6A and 7B), leading to the conclusion that base flipping does not readily occur with the 2-aminopurine:T pair and, by inference, the A:T pair.

DISCUSSION

AGT is involved in repair of O^6-alkylG modifications. This protein is not an enzyme in the usual sense because multiple turnovers do not occur, but the reaction mechanism can be considered in the context of catalysis, in that the process should involve sequential association of AGT with DNA, scanning, flipping, alkyl transfer, and dissociation (Scheme 1). Evidence for base flipping comes from examination of the crystal structure of an AGT-DNA complex (5). We placed a fluorescent pyrrolo dC residue opposite O^6-alkylG and measured what we believe to be rates of base flipping using this approach (Scheme 4, Figs. 6 and 7). Rates of dissociation of AGT from DNA complexes were measured, and the rate of dissociation is reduced considerably by the presence of an alkylated G (Tables III and IV).

Previous studies on the catalytic mechanism of AGTs have focused on O^6-MeG transfer. Some reports interpret the results in terms of a second-order reaction for alkyl transfer (7, 10, 15, 17) although this view might not seem reasonable in light of known binding of AGT to DNA (20). Another study with a 21-mer duplex yielded data that indicated Michaelis-Menten-like kinetics, i.e. a hyperbolic plots of reaction rates versus [AGT] (9), and provided evidence for the existence of an ES complex (i.e. AGT-DNA). Another issue raised in that work was that gel mobility assays, despite their thermodynamic deficiencies, suggest that AGT has ~10-fold greater affinity for O^6-MeG-containing DNA than unmodified DNA (9). Several points can be made about these earlier studies: (i) The reactions are fast enough that rapid mixing methods are required to study the kinetics, as shown in the report of Meyer et al. (9) and in the present work (Figs. 2 and 3). (ii) Reaching saturation of the reaction with increasing amounts of AGT is not trivial, even after taking the

FIG. 4. Estimation of dissociation rates of DNA-AGT complexes. A, mixture of 32P-labeled 30-mer/O^6-MeG 36-mer (200 nM) and AGT-C145A (1.6 μM) was mixed with pol T7 (450 nM) (with 9 μM thioredoxin) at time intervals ranging from 0.05 to 30 s at 25 °C. Polymerization was initiated by the addition of 200 μM dNTP and 5 mM MgCl_2, with a constant reaction time of 0.25 s. B, mixture of 32P-labeled 30-mer/O^6-MeG 36-mer (200 nM) and AGT-C145S (1.6 μM) was mixed with pol T7 (450 nM) (with 9 μM thioredoxin) at time intervals ranging from 0.05 to 30 s at 25 °C. C, mixture of 32P-labeled 30-mer/O^6-BzG 36-mer (50 nM) and AGT-C145A (200 nM) was mixed with pol T7 (100 nM) (and 2 μM thioredoxin) at time intervals ranging from 0.05 to 30 s at 25 °C. Graphs were fit to a single-exponential equation in the program GraphPad Prism using the equation y = E_f + E_o(1 – e^{-k_{obs}t}) where E_f is the free DNA concentration, E_o is the AGT-bound DNA concentration, k_{obs} is the dissociation rate of DNA from AGT-DNA complex, and t is time. See Tables III and IV for estimates of k_{obs}.
oligomerization of AGT into consideration (20), and limited data sets can have the appearance of linearity (9) (Fig. 2). (iii) The structural work strongly implicates a base flipping step (5), which needs to be included in any kinetic model.

The 30-mer/36-mer double-stranded oligonucleotide pair (Scheme 2) was used to ensure that the AGT was sufficiently filled with the substrate, based on the footprinting (33) and crystallography results (5) and to allow for scanning, if this would be a kinetic issue. The ratio \( k_r/K_d \) (Table II) for WT human AGT of \( 3 \times 10^6 \) M\(^{-1}\) s\(^{-1}\) with \( O^6\)-MeG DNA can be compared with the “\( k_{\text{inactivation}}/K_d \)” values of \( 4.8 \times 10^6 \) and \( 3.8 \times 10^5 \) M\(^{-1}\) s\(^{-1}\) for two different sequences (9) and the so-called second-order rates in the literature, several of which our value is similar to (16, 17). The value of \( 10^9 \) M\(^{-1}\) s\(^{-1}\) reported by Chan et al. (15) is clearly too high, and the model used is not viable. Those authors concluded that binding to DNA is rate-limiting in the \( O^6\)-MeG reaction, which is certainly not the case. Clearly, rapid kinetic methods provide an advantage in analysis of the kinetics of this system. Only a few studies have been done with \( O^6\)-BzG in DNA, and these results are in qualitative agreement that repair of \( O^6\)-BzG is more efficient than \( O^6\)-MeG (12, 13). We found that the repair of \( O^6\)-BzG in DNA is extremely fast and probably nearly diffusion limited (Table II). Terashima et al. (34) considered the removal of several substituted benzyl derivatives and reported no differences among the substituted benzyl compounds. However, the analysis was very limited, and no kinetic analysis was used.

**SCHEME 4.** Measurement of base flipping using pyrrolo dC fluorescence changes. The base pairing scheme is speculative.

**FIG. 5.** Fluorescence changes of duplex DNA (pyrrolo dC) upon addition of AGT-C145A. Pyrrolo dC was placed opposite the adduct position and fluorescence spectra were recorded (\( \lambda_{\text{ex}} \)= 360 nm). A, titration of unmodified DNA (200 nM) with AGT-C145A. AGT-C145A final concentrations were 0, 0.16, 1.6, 3.2, and 4.8 \( \mu \)M. B, titration of \( O^6\)-MeG DNA (200 nM) with AGT-C145A. AGT-C145A final concentrations were 0, 0.16, 1.6, and 3.2 \( \mu \)M. C, titration of \( O^6\)-BzG DNA (200 nM) with AGT-C145A. AGT-C145A final concentrations were 0, 0.16, 1.6, 3.2, and 4.8 \( \mu \)M. D, E, and F show plots of the results from A, B, and C, respectively.
The mechanism shown in Scheme 1 was fit with as many experimentally determined rate constants as possible for the O6-MeG transfer reaction with WT AGT and two mutants (Fig. 8) (see Supplemental Data for sample DynaFit files). For the fitting, $k_{-1}$ was 1 s$^{-1}$ (Fig. 4 and Table III), $k_{4}$ was 50 s$^{-1}$ (Fig. 4 and Table IV), and $k_{9}$ and $k_{-2}$ were 350 and 35 s$^{-1}$, respectively (Fig. 7 and Table V). $k_{1}$ and $k_{-4}$ should be equal, although $k_{-4}$ is not an issue in that the approach used the sum of AGT-R-DNA plus free DNA in the analysis of the rate of product formation (Fig. 8). A reasonable fit was obtained with the upper parameters and $k_{1} = k_{-4} = 5 \times 10^{9}$ M$^{-1}$ s$^{-1}$, which yielded $k_{1} = 25$ s$^{-1}$ in a reiterative analysis. Slower association rates ($k_{1}$) failed to provide an appropriate initial slope for the fit. Another issue is that the O6-BzG results (Fig. 3) require a very rapid association rate, because the rate constant for the overall reaction is $3 \times 10^{8}$ M$^{-1}$ s$^{-1}$ (Table II). Association rate constants this fast (>10$^{9}$ M$^{-1}$ s$^{-1}$) are known for protein-protein binding and considered to reflect the interaction of complementary charge surfaces at low ionic strength (35). Our attempts to monitor binding of AGT and fluorescently labeled oligonucleotides were unsuccessful, possibly because of the rates of the association, which must be even faster than the pyrrolo dC base flipping that we observed (Fig. 6).

The reaction rate with O6-BzG was $3 \times 10^{8}$ M$^{-1}$ s$^{-1}$ for WT AGT (Table II). Attempts to fit these reactions to the model (Scheme 1 and Fig. 8) were not very useful in that distinguish-}

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### Table V

<table>
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<td>AGT-R128L + O6-BzG</td>
<td>81 ± 15</td>
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<td>AGT-R128G + O6-BzG</td>
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<td>AGT-Y114F + O6-BzG</td>
<td>145 ± 14</td>
<td>15.8 ± 0.8</td>
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<td>AGT-G160W + O6-BzG</td>
<td>39 ± 4</td>
<td>1.8 ± 0.1</td>
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<tr>
<td>AGT-C145S + O6-BzG</td>
<td>190 ± 20</td>
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<tr>
<td>AGT-C145S + O6-MeG</td>
<td>200 ± 22</td>
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</tr>
</tbody>
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*Very low signal change (Fig. 6B).*

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The reaction rate with O6-BzG is $3 \times 10^{8}$ M$^{-1}$ s$^{-1}$ for WT AGT (Table II). Attempts to fit these reactions to the model (Scheme 1 and Fig. 8) were not very useful in that distinguishing rate of base flipping ($k_{1}$) and alkyl transfer ($k_{2}$) is not feasible. This appears to be a nearly diffusion-limited reaction.
unmodified DNA), consistent with earlier work with gel shift assays suggesting higher affinity for modified DNA (9). If we assume that the association rates are identical, then the difference should be about 25-fold (Tables III and IV). (ii) The mechanism (Scheme 1 and Fig. 8) explains the saturation observed in the O6-MeG reaction compared with O6-BzG (Figs. 2B and 3B). The existence of a low rate for the chemical step \( k_3 \) in the O6-MeG reaction will produce saturation but with O6-BzG this step is never rate-limiting. Exactly what this rate is with O6-BzG in double-stranded DNA is unknown (with the free base O6-Bz guanine the rate is only 600 \( \text{m}^{-1} \text{s}^{-1} \) (27)). (iii) In the O6-MeG reaction, the alkyl transfer step \( k_3 \) is much faster than the overall rate of the reaction, even though \( k_3 \) is the slowest forward step in the reaction (Fig. 8). This result is related to the presence of multiple forward steps and the contribution of reverse reactions. (iv) If the proposed mechanism (Fig. 8) is valid, then the methyl transfer reaction with AGT can be considered a relatively processive process, in that binding of modified DNA to AGT should proceed until repair occurs, rather than dissociation and reassociation before transfer.

When AGT binds to DNA, a scanning process must occur before an alkylated G is detected. Measurements of DNA scanning rates can be made in some cases (36), but in the case of AGT this process must be extremely fast because it precedes base flipping. Thus, the rate must be greater than the high rates observed for base flipping (Table V and Fig. 8) and for O6-BzG repair at high AGT concentrations (Fig. 3B and Table II), i.e., \( >200 \text{ s}^{-1} \). This fast scanning rate cannot be a rate limitation in the case of the O6-methylguanine reaction and is probably not even in the case of O6-benzylguanine, in that it appears to be faster than the base flipping.

Base flipping appears to be a very real process for AGT, as judged by the results of previous crystallography work and the present studies with pyrrolo dC (Figs. 5 and 6). The rate estimated here \( (k_2 = 200–350 \text{ s}^{-1}) \) can be compared with forward and reverse rates of 1260 \( \text{s}^{-1} \) and 40 \( \text{s}^{-1} \) estimated for base flipping by UDG (31), although the work on overall rates of dealkylation of O6-BzG suggest that the flipping may be even faster than 350 \( \text{s}^{-1} \) (Fig. 3B). One question is whether AGT flips only O6-alkylG or every base (37). Duguid et al. (37) have suggested that AGT does not have to flip every base. We did detect some increased fluorescence of pyrrolo dC paired with G when AGT was added (Fig. 5). However, the change in fluorescence was weaker than with O6-alkylG (Fig. 5), and the rate of change was too slow (Figs. 6 and 7) to be consistent with a role in the mechanism, particularly with O6-BzG (Fig. 3). If flipping of every G is an obligatory feature of AGT, then such flipping should be observed in the case of the oligonucleotide with O6-BzG as well as the one containing O6-MeG. We saw weak changes in the fluorescence of 2-aminopurine (paired with T) upon mixing with AGT (results not shown) but in light of the rates and the expected higher fluorescence of 2-aminopurine relative to pyrrolo dC, we do not consider these changes to mean that AGT flips T bases.

In summary, we have used rapid reaction methods to provide more insight into the mechanism of repair of alkylated DNA. O6-BzG damage is repaired with an efficiency ~100-fold greater than O6-MeG; in a reaction that may be approaching a diffusion-controlled process. The rate of dissociation of DNA is much slower when an O6-alkylG is present. Base flipping appears to be a fast process that can be attenuated by mutation of Arg128, suggested from other work to be involved in the phenomenon. Estimated rates of individual reaction steps can be used with a kinetic model that includes reversible association of AGT with damaged DNA, reversible base flipping, alkyl transfer, and dissociation of the AGT-DNA complex (Fig. 8). The model can be used to rationalize the effects of AGT mutants proposed to be involved in base flipping and the difference in the rates of repair of O6-MeG and O6-BzG moieties in DNA.

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Kinetic Analysis of Steps in the Repair of Damaged DNA by Human \( \text{O}^6 \) -Alkylguanine-DNA Alkyltransferase

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