Resolving the subtle details of human DNA alkyltransferase lesion search and repair mechanism by single-molecule studies

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Edited by Eric Greene, Columbia University, New York, NY; received September 2, 2021; accepted February 8, 2022 by Editorial Board Member Rodney Rothstein

The O6-alkylguanine DNA alkyltransferase (AGT) is an important DNA repair protein. AGT repairs highly mutagenic and cytotoxic alkylguanine lesions that result from metabolic products but are also deliberately introduced during chemotherapy, making a better understanding of the working mechanism of AGT essential. To investigate lesion interactions by AGT, we present a protocol to insert a single alkylguanine lesion at a well-defined position in long DNA substrates for single-molecule fluorescence microscopy coupled with dual-trap optical tweezers. Our studies address the longstanding enigma in the field of how monomeric AGT complexes at alkyl lesions seen in crystal structures can be reconciled with AGT clusters on DNA at high protein concentrations that have been observed from atomic force microscopy (AFM) and biochemical studies. A role of AGT clusters in enhancing lesion search efficiencies by AGT has previously been proposed. Surprisingly, our data show no enhancement of DNA translocation speed by AGT cluster formation, suggesting that AGT clusters may serve a different role in AGT function. Interestingly, a possible role of these clusters is indicated by preferential cluster formation at alkyl lesions in our studies. From our data, we derive a model for the lesion search and repair mechanism of AGT.

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Significance

We directly visualize DNA translocation and lesion recognition by the O6-alkylguanine DNA alkyltransferase (AGT). Our data show bidirectional movement of AGT monomers and clusters on undamaged DNA that depended on Zn2+ occupancy of AGT. A role of cooperative AGT clusters in enhancing lesion search efficiencies by AGT has previously been proposed. Surprisingly, our data show no enhancement of DNA translocation speed by AGT cluster formation, suggesting that AGT clusters may serve a different role in AGT function. Our data support preferential cluster formation by AGT at alkyl lesions, suggesting a role of these clusters in stabilizing lesion-bound complexes. From our data, we derive a new model for the lesion search and repair mechanism of AGT.
enhancement of alkylguanine repair activity (9) as well as altered protein interactions by the Zn-bound form of AGT compared to the apo protein (10).

To understand the mechanism of lesion search and recognition by AGT and the role of the Zn$^{2+}$ ion, we performed single-molecule imaging studies by fluorescence optical tweezers on AGT with and without a Zn$^{2+}$ ion bound and a long (∼48,500 bp) DNA substrate tethered between two beads held in two optical traps. These data revealed minor effects of Zn occupancy on DNA translocation by AGT during lesion search. AGT with and without a bound Zn$^{2+}$ ion formed clusters on the DNA at high (micromolar) concentrations. Both monomers and clusters of AGT showed similar diffusion behavior on DNA with a rapidly and a slowly diffusing mode. Fast-diffusing species rapidly dissociated from the DNA, while slowly diffusing particles possessed longer lifetimes on DNA of several minutes and displayed diffusional properties that are consistent with a helical movement on the DNA along its minor groove. AGT occasionally converted into a loosely bound conformation that was able to slip on the DNA over large (micrometer) distances. A role of AGT clusters in enhancing lesion search efficiencies by AGT has previously been proposed by us and others (1, 7, 11). Surprisingly, our data show no enhancement of DNA translocation speed by AGT cluster formation, suggesting that AGT clusters on DNA may serve a different role in AGT function. To investigate lesion interaction mechanisms by AGT monomers or clusters, we describe a detailed protocol to insert a single alkylguanine lesion in the DNA within 2 pixels corresponding to 200 nm or ∼60 bp as well as mobile species included monomeric and oligomeric AGT (based on fluorescence intensities, SI Appendix, Figs. S5 and S6). Nonspecific AGT aggregates that were stuck to the DNA tether or attached to the beads typically displayed much higher fluorescence intensities (∼1,000 photon counts, SI Appendix, Fig. S7) and were excluded from our analyses. Lambda phage DNA with biotinylated ends and a fluorophore at 30% of the DNA length is now commercially available (LUMICKS). Using this DNA substrate with a red fluorophore (ATTO647N) at 30% of the DNA length, we confirmed high positional precision (±17 nm) and accuracy (∼165 nm) of our measurements (SI Appendix, Fig. S8). Based on this accuracy, species which showed displacement by more than 200 nm (2 pixels) were counted as mobile. Mean square displacement (MSD) analysis indicated two types of diffusion by AGT on DNA. Complexes either translocated on the DNA with high diffusion constant over short time scales before dissociating rapidly (<10 s green in Fig. 1E) or they remained stably bound over longer times and displayed diffusion constants of ∼0.03 μm$^2$/s (black in Fig. 1E), which is the theoretical limit for rotational diffusion along the DNA double helix (dashed line in Fig. 1E). To investigate the accuracy of our MSD analyses, we again exploited the ATTO647N-containing DNA (SI Appendix, Fig. S9). In our AGT analyses, we included only kymograph traces with positional changes of >200 nm. Interestingly, for the static traces of the ATTO647N fluorescence (at 30% of the DNA length), our analyses also provided diffusion constants, between 0.0036 and 0.0073 μm$^2$/s for traces ≥10 s (SI Appendix, Fig. S9D), representing the limits of our MSD analyses. This resolution limit is likely caused by positional noise in the kymograph traces (accuracy of ∼200 nm, see above) and the accuracy of tracking for MSD analyses (SI Appendix, Fig. S9A). AGT diffusion constants did not depend on the size of complexes, as revealed from separate analyses of high and low fluorescence intensity traces (SI Appendix, Fig. S10 A, B, D, and E) and plots of diffusion constants over fluorescence intensities of complexes (SI Appendix, Fig. S10C). For these analyses, we chose 400 photon counts as the cutoff value based on the intensities of monomeric species for 500 nM QD-AGT (Fig. 1D

Results and Discussion

AGT Monomers and Clusters on DNA. The formation of oligomeric complexes of AGT on DNA with limited stoichiometry at high (micromolar) protein concentrations has previously been demonstrated by AFM imaging and analytical ultracentrifugation (AUC) sedimentation equilibrium analyses (7). Here, we further corroborate the formation of well-defined AGT clusters on DNA by AUC sedimentation velocity (SV) measurements (Fig. 1A). Previously, we had proposed that these clusters may serve to enhance the speed of DNA translocation by AGT and thus the efficiency of AGT lesion search (7). Using single-molecule fluorescence microscopy coupled with a dual-trap optical tweezer system, we investigated here the dynamics of AGT monomers and clusters (at protein concentrations between 500 nM and 17 μM) on a DNA tether spanning between two beads that were fixed in the optical traps (Fig. 1B and C). The proteins were fluorescently labeled with quantum dots (QDs) via an antibody sandwich linker, as previously described (12). QDs are well-established labels in single-molecule experiments due to their strong fluorescence intensities and high photostabilities compared to conventional organic fluorophores, allowing the prolonged observation of labeled molecules over long time scales. We first determined the fluorescence intensity of single AGT monomers. For these experiments, we applied a low (500 nM) concentration of QD-labeled AGT that has been shown to support only monomeric AGT (7, 11). Consistent with the relatively high dissociation constant (K_D of 200 to 1,000 nM) (9, 13, 14) of AGT interactions with nonspecific DNA, these monomers showed only low frequency binding to DNA tethers. The monomeric AGT complexes provided signal intensities of ∼200 fluorescence photon counts (measured per 2 pixels, pixel time of 0.1 ms; Fig. 1D, Left, SI Appendix, Fig. S1). In these experiments, each AGT molecule was labeled by a QD (or 50% labeled in SI Appendix, Fig. S1). For applications of higher (micromolar) concentrations of AGT, we labeled only a fraction of the AGT molecules (125 nM) and added unlabeled AGT to achieve total concentrations of 1.5 to 17 μM without excessive fluorescence background. Although the concentration of fluorescent AGT was kept constant in these measurements, DNA-bound complexes showed increasing fluorescence intensities with increasing AGT concentrations (Fig. 1D), consistent with cluster formation by AGT on the DNA. In these distributions of fluorescence intensities, species with lower intensities than the monomer reference (Fig. 1D, Left) can be seen. These lower signals may be caused by QD blinking (SI Appendix, Fig. S2) or complex positions outside of the focal plane for detection. Importantly, the high protein concentrations did not cause quenching of QDs (SI Appendix, Fig. S3). AGT complexes displayed long lifetimes on the DNA of up to several minutes for monomeric and oligomeric species (SI Appendix, Fig. S4 A and B). We observed both mobile and static complexes (∼20 to 30% static) for all applied concentrations. Importantly, both static species (which may partially represent nonfunctional aggregates or separate complexes bound to DNA within 2 pixels corresponding to 200 nm or ∼60 bp) as well as mobile species included monomeric and oligomeric AGT (based on fluorescence intensities, SI Appendix, Figs. S5 and S6). Nonspecific AGT aggregates that were stuck to the DNA tether or attached to the beads typically displayed much higher fluorescence intensities (≥1,000 photon counts, SI Appendix, Fig. S7) and were excluded from our analyses. Lambda phage DNA with biotinylated ends and a fluorophore at 30% of the DNA length is now commercially available (LUMICKS). Using this DNA substrate with a red fluorophore (ATTO647N) at 30% of the DNA length, we confirmed high positional precision (±17 nm) and accuracy (∼165 nm) of our measurements (SI Appendix, Fig. S8). Based on this accuracy, species which showed displacement by more than 200 nm (2 pixels) were counted as mobile. Mean square displacement (MSD) analysis indicated two types of diffusion by AGT on DNA. Complexes either translocated on the DNA with high diffusion constant over short time scales before dissociating rapidly (<10 s green in Fig. 1E) or they remained stably bound over longer times and displayed diffusion constants of <0.03 μm$^2$/s (black in Fig. 1E), which is the theoretical limit for rotational diffusion along the DNA double helix (dashed line in Fig. 1E). To investigate the accuracy of our MSD analyses, we again exploited the ATTO647N-containing DNA (SI Appendix, Fig. S9). In our AGT analyses, we included only kymograph traces with positional changes of >200 nm. 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AGT complexes were also able to translocate over distances between 200 nm and several micrometers independent of protein concentration (SI Appendix, Fig. S1). These data suggest that AGT monomers and clusters move along the DNA with similar diffusion properties. Importantly, these findings contradict the hypothesis that AGT clusters may serve to enhance the speed of DNA translocation in DNA lesion search by AGT and thus argue for an alternative function of these clusters (see below).
The low diffusion speed species is consistent with a rotational motion of AGT tracing the minor groove of the DNA, in which AGT binds (1). The rapid species of AGT complexes with short lifetimes on the DNA may correspond to loosely bound protein molecules, consistent with the fact that AGT can also slip over large distances on the DNA (see below). A similar diffusion behavior with a slow- and a fast-diffusing species on DNA has previously been reported for other DNA-binding proteins (15–17). These have been interpreted as open (rapid diffusion) and closed (slow diffusion or static complexes) conformations of the proteins (15). Interestingly, for AGT a two-step nucleotide flipping mechanism has previously been revealed from simulations (18) that would allow for rapid sliding on the DNA without base flipping. The authors suggested a gate-keeping, kinetic control mechanism that results in the preferential formation of a stable AGT complex with a lesion (compared to an intact) base inserted into its catalytic pocket. Consistent with a loosely bound state of AGT, we also observed slipping or jumping behavior in 3 to 11% of DNA-bound traces (example traces in Fig. 1 B, Top, and F, Inset, SI Appendix, Fig. S12). The distance on DNA of these jumps was surprisingly high (average of 2.0 μm; Fig. 1f). Similar events have previously been observed for a monomeric restriction enzyme, albeit over slightly shorter distances (<1 μm) and at a much higher frequency (approximately every 20 s) (19). The majority of the slipping/jumping species of AGT showed fluorescence intensities consistent with monomeric complexes (SI Appendix, Fig. S12C). Surprisingly, however, it was also seen for a small number of AGT clusters (high fluorescence intensity species, ~600 photon counts/0.2 ms), which is puzzling considering the presumably tight enclosure of the DNA by AGT clusters in their helical arrangements (6). To investigate if these events represent hopping by AGT over extreme distances, we applied different salt concentrations in our measurements (25 to 100 mM Na+; SI Appendix, Fig. S12A). At high [salt], protein dissociation from the DNA is enhanced, facilitating the microdissociative motion of proteins during hopping. At 100 mM salt, we did not observe any slipping or jumping events over micrometer distances (as observed in 50 mM salt), possibly as a result of the few data that were due to considerably decreased DNA binding at this condition. We also observed a significant reduction in the long-lived, slowly diffusing species compared to lower salt conditions (SI Appendix, Fig. S12A), consistent with complex destabilization by blocking of extensive electrostatic contacts in the DNA interaction interface of AGT, in particular in the proposed model for AGT clusters (6). Importantly, the overall distances that AGT complexes covered on DNA were comparable for the different salt conditions (SI Appendix, Fig. S12A). Due to this lack of distance sensitivity to salt concentration and the fact that we see these extremely rapid, large distance transfer events also for AGT clusters that encircle the DNA, we favor an interpretation of slipping (instead of hopping or jumping) over large distances on the DNA in a loosely bound complex, supported by suppression of strong electrostatic interactions in the cluster–DNA interface. The time resolution of our measurements (~0.04 s, time of scan line along DNA) was not sufficient to fully resolve the proteins during their rapidly slipping motion on the DNA; however, some of the kymographs show traces of fluorescent signals between start and final positions of proteins slipping on the DNA (SI Appendix, Fig. S12C, arrows). Under physiologically conditions (~100 mM monomeric salt), AGT therefore appears to translocate rapidly over micrometer distances with high diffusion constants between 0.1 and 10 μm²/s and short lifetimes on DNA of ~<10 s during lesion search.

**Effects of Zn²⁺ on AGT Activity and Mobility on DNA.** Crystal structures of AGT have revealed a Zn²⁺ ion coordinated by the N-terminal part of the protein (Fig. 2A) (8). Previous studies have reported an enhancement of AGT stability and repair rate under limiting protein conditions (low micromolar with severalfold excess of DNA substrate) by Zn binding, although AGT was shown to be also functional without the cofactor (9). Here, we wanted to investigate effects of the Zn²⁺ ion occupancy on AGT translocation on DNA. We first set out to measure Zn content of three different preparations of AGT using μPIXE (particle induced X-ray emission) (20). One preparation (sample 1) was expressed in the presence of additional Zn²⁺ (100 μM) and purified via amylose affinity chromatography using an N-terminal maltose binding protein (MBP) tag in the protein. The other two were purified via Ni²⁺ NTA His tag affinity chromatography, which has previously been suspected to lead to Zn depletion in the purified proteins (8), and one of them (sample 2) contained an N-terminal MBP tag between the protein and the His tag while the other did not (sample 3). After further size exclusion purification of all three variants, we detected Zn²⁺ ion occupancy of ~0.7/AGT molecule for sample 1 (consistent with approximately one Zn²⁺ ion bound by

![Fig. 2. Effect of Zn²⁺ on AGT mobility on DNA.](image-url)
Table 1. Zinc ion occupancy of different AGT variants used in this study

| Sample* | No. of sulfurs | Zinc/sulfur | LOD | Zinc/protein | Estimated error (%) |
|---------|----------------|-------------|-----|--------------|---------------------|
| Sample 1 | 16             | 0.045       | 0.05 | 0.72         | 19                  |
| Sample 2 | 16             | 0.021       | 0.14 | 0.33         | 32                  |
| Sample 3 | 9              | 0.022       | 0.05 | 0.20         | 30                  |

*Sample 1 is MBP-AGT expressed in the presence of additional zinc and purified via amylose resin. Samples 2 and 3 are MBP-AGT and AGT purified via Ni²⁺-NTA affinity chromatography, respectively. LOD = limit of detection.

Each AGT molecule and with results from previous studies [9]), and 0.2 to 0.3/AGT for the other two (consistent with approximately no Zn²⁺ bound; Table 1). It is also possible that some of the Zn²⁺ ions are bound to the His tag in the proteins as previously suggested (9), which is present in all three variants and is exploited for conjugation to QDs for the fluorescence optical tweezer measurements. However, the Zn²⁺ ion in sample 1 was stably coordinated, as revealed by the fact that it was not removed by incubation in EDTA (1 mM)-containing buffer for 1 h (0.68 Zn²⁺/AGT molecule after incubation with EDTA), arguing for its integration in the AGT structure.

Interestingly, under the conditions of our experiments, the repair activity was comparable for all three AGT preparations (Fig. 2B). Although these results confirmed the functionality of all three protein preparations, they seem in contrast to earlier reports of enhancement of AGT repair rate by Zn binding (9). However, these earlier studies had detected rates of alkyl lesion removal under limiting protein concentrations, while our activity assay measures lesion repair at equilibrium conditions (after a 30- to 60-min incubation) and at higher protein concentrations (10 to 500 nM AGT, 20 nM DNA) and excess of protein (25:1 protein:DNA ratio at maximum [AGT]). Under these conditions, no notable effect of Zn occupancy on AGT activity could thus be observed in our studies.

Fluorescence optical tweezer experiments showed that the diffusive properties of AGT were mildly affected by Zn binding (Fig. 2C). The Zn-bound variant displayed slightly higher diffusion constants and less subdiffusive motion (alpha exponent ~1, consistent with less pausing) compared to Zn-free AGT. It is worth noting, however, that all of the high diffusion coefficients (>0.3 μm²/s) stem from traces with short lifetimes on the DNA (<10 s), which were enhanced for Zn²⁺-containing AGT (SI Appendix, Fig. S10B). Separate lifetime analyses showed enhanced lifetimes of AGT on DNA without Zn²⁺ compared to that with Zn²⁺ bound (mean lifetimes of ~30 s versus ~10 s; SI Appendix, Fig. S4). While the movement of AGT on the DNA was mostly unbiased and bidirectional both with and without Zn²⁺ bound (92 to 95% of mobile traces, see e.g., example traces in Fig. 1B), overall distances translocated by AGT were slightly larger without Zn²⁺ bound (average ~0.9 μm without Zn²⁺ versus ~0.6 μm with Zn²⁺ bound for 4 μM AGT; SI Appendix, Fig. S11). Zn²⁺ coordination has been shown to stabilize AGT structure (9). Furthermore, a recent molecular dynamics study supported enhanced flexibility of AGT lacking a Zn²⁺ ion compared to Zn-bound AGT (10). A possible interpretation of our results is therefore that the slightly increased flexibility of the protein in the absence of a bound Zn²⁺ ligand supports stronger protein–DNA contacts with reduced energetic cost from DNA deformation achieved via subtle conformational adaptation of AGT monomers and clusters to the DNA structure. A more loosely DNA-bound state with less DNA deformation for Zn-free compared to Zn-bound AGT is also supported by a larger number of slipping events for the Zn-free protein (average percentages of slipping events of 3% with Zn²⁺ and 10% without Zn²⁺ from a total of 322 and 213 traces, respectively).

The Zn²⁺ ion is coordinated in the very N-terminal part of AGT (Fig. 2A), which also contributes to the interface of protein–protein interactions in cooperative cluster formation by AGT (6). To interrogate whether different diffusion behaviors may thus derive from different oligomeric states of AGT in the presence and absence of a bound Zn²⁺ ion, we again separated the data based on the fluorescence intensities of the individual traces as above. These analyses showed ratios of high to low fluorescence intensity traces that were slightly larger for AGT with compared to without a bound Zn²⁺ ion (with 18% and 7% of traces with >400 average photon counts for 4 μM data, 37 out of 209 total traces with Zn²⁺ and 9 out of 130 traces without Zn²⁺, respectively) indicating that the Zn²⁺ ion may mildly stabilize AGT clusters.

Lack of Directionality of AGT on ssDNA. The kymographs showed translocation of fluorescently labeled AGT on double-stranded DNA (dsDNA) without any directional bias for the individual AGT–DNA complexes (both with and without bound Zn²⁺). To investigate whether this lack of directionality may be caused by AGT switching DNA strands and each molecule in fact translocated in only one direction on each of the two strands, we next employed a ssDNA tether in our experiments. Previous studies had reported a 3′- to 5′- polarity of AGT clusters on ssDNA (1). This conclusion was derived from elegant experiments that showed lesions being more rapidly repaired when located toward the 5′ end of a DNA strand and that this bias was removed by attaching a streptavidin block approximately in the middle of the oligonucleotide. In contrast, our fluorescence optical tweezer experiments clearly show bidirectional movement of AGT on ssDNA tethers (Fig. 3A). Furthermore, although our data demonstrate AGT cluster assembly at higher AGT concentrations also on ssDNA, these clusters were predominantly static, while mobile species on ssDNA were mostly monomeric (Fig. 3B). These findings can be reconciled when considering that the ssDNA substrate used in the previous study was considerably shorter (~70 nucleotides [nt], compared to ~50,000 nt in our experiments). AGT clusters have been shown to contain ~7 monomer subunits (7), which cover ~30 bp based on the apparent binding site sizes of AGT on dsDNA as well as ssDNA (6, 21, 22). This length spans almost half of the length of the short DNA substrates used in these studies. On the short distance scale, the observed directionality may thus reflect rapid cluster growth by recruitment of additional monomers at their 5′ side at high protein concentrations or protein–DNA ratios and dissociation of subunits from their 3′ side, as also previously suggested (1). In contrast, active ssDNA translocation by AGT, which can cover much longer distances, is not directional, as our data clearly show. MSD analyses of mobile AGT on ssDNA provided a similar profile as for dsDNA, with a rapidly moving and dissociating species and a slowly moving, long-lived species (Fig. 3C).
Overall diffusion constants were only slightly larger for ssDNA than for dsDNA (average for 1.5 μM MBP-AGT with Zn$^{2+}$ bound and 4 μM AGT was 0.8 μm$^2$/s on ssDNA from 20 traces versus 0.5 μm$^2$/s for dsDNA from 121 traces; SI Appendix, Fig. S13D). However, traces on ssDNA showed shorter lifetimes than those on dsDNA of almost exclusively <10 s that covered distances of maximally 2.1 μm (SI Appendix, Fig. S13). These results suggest a stabilization of AGT-DNA complexes by the DNA double helix, consistent with their assembly along its minor groove (6, 7).

**DNA Lesion Interactions by AGT.** To investigate lesion interactions by AGT, we incorporated an alkylguanine lesion into lambda phage DNA at a well-defined position (at 30% of the DNA length; Methods for details). We tested the stability of the resulting lesion substrates by AFM imaging as well as by force extension in optical tweezers (SI Appendix, Fig. S14).

We then followed binding and translocation of AGT on alkyl (methylguanine) lesion-containing DNA tethers in the fluorescence optical tweezer system using a customized Python-based kymotracker tool. Our data show moderate-to-high specificity of AGT for binding to the alkyl lesion (at ~30% of the DNA length) over binding to nonspecific DNA sites for 1.5 μM to 17 μM AGT (Fig. 4A and C and SI Appendix, Figs. S15 and S16). We next analyzed the intensities of DNA-bound species at the lesion and on nonspecific positions (measured as photon counts/2 pixels, i.e., 100 nm and 2 × 0.1 ms). For 1.5 μM AGT, fluorescence intensities at the lesion were again consistent with predominantly monomeric AGT (Fig. 4B and SI Appendix, Figs. S15 and S16). Based on crystal structures, which show monomeric AGT bound to alkyl lesions in DNA, we expected to see also predominantly static, monomeric species at the lesion position for the high protein concentration. Surprisingly, however, our data revealed predominantly clusters of AGT bound mainly at ~30% of the DNA length at 17 μM protein concentration (Fig. 4D, Right). In contrast, intensities at nonspecific DNA sites were consistent with predominantly monomeric species (Fig. 4D, Left). Because of the high prevalence of high-intensity complexes at the lesion position without obvious translocation of these clusters from nonspecific DNA to the lesion site during measurements, these data suggest rapid, preferential assembly of AGT clusters at an alkyl lesion. Interestingly, cluster formation by AGT at alkyl lesions has also previously been suggested from kinetic studies (14). To investigate the possibility that these clusters may be inactive in lesion repair and instead serve to mask or protect the lesion position, we repeated the DNA repair activity assays with the high AGT concentration (17 μM), at which clusters were observed at the lesion in the fluorescence optical tweezer studies. However, repair of alkyl lesions was similarly efficient at these high concentrations compared to lower concentrations (<1 μM) that likely support predominantly monomeric AGT on DNA (SI Appendix, Fig. S17). These findings suggest that alkyl lesion repair by AGT can also occur within AGT clusters. These clusters will be the predominant form of AGT complexes at alkyl lesions under physiological conditions with micromolar AGT concentrations and high AGT:DNA ratios (23), especially under conditions of alkylation stress with upregulated AGT expression (24). The apparent discrepancy between the monomeric AGT complexes on DNA in crystal structures and cooperative clusters of AGT on DNA in biochemical and AFM experiments are likely explained by the excessively different protein:DNA ratios in these experiments and the short DNA substrate length in crystallographic studies. In support of this hypothesis, AUC experiments with high, equimolar concentrations of AGT and a 12-bp DNA substrate (same as used in crystallization [1]) showed sedimentation coefficients consistent with monomeric complexes of AGT on the DNA (SI Appendix, Fig. S18, arrow), compared to oligomeric complexes observed at the same protein concentration but with an ~30-fold lower DNA concentration (SI Appendix, Fig. S18 and Fig. 1A).

![Figure 3](https://doi.org/10.1073/pnas.2116218119)

**Fig. 3.** Bidirectional movement of AGT monomers on ssDNA. (A) Example kymographs of MBP-AGT on ssDNA. (B) Intensities of mobile (Left) and immobile (Right) species on ssDNA (measured as photon counts/0.2 ms). The data were pooled from three experiments at 1.5 to 17 μM AGT and MBP-AGT. Fluorescence intensities of mobile species are predominantly consistent with AGT monomers, although they are smaller than those of the monomeric reference species on dsDNA at 500 nM AGT (Fig. 1D). Lower intensities were also observed for slipping species (SI Appendix, Fig. S12C) and may be caused by detection slightly outside the focal plane, which may be enhanced by the more flexible nature of the ssDNA tether compared to dsDNA. (C) Diffusion constants plotted over lifetimes on DNA for species with high (>400 photon counts) and low (<400 photon counts) intensities. The sensitivity limit of the analyses is indicated as the gray-shaded area.
The DNA tethers in the fluorescence optical tweezer studies contained a single alkyl lesion at 30% of the DNA length. However, because we cannot distinguish between the 30% positions from the two DNA ends, our analyses necessarily contain a nonspecific background from the nonlesion 30% position (see for example the two arrows in the top kymograph in Fig. 4C). Our preparation protocol can also be extended to integrate both a lesion as well as a fluorophore into the DNA, with spacing of 1 to 12 bp depending on the exact position of the lesion within the inserted 13-nt fragment (Methods). These doubly modified DNA tethers will be a powerful asset in these types of experiments by marking the (invisible) lesion with the fluorophore signal and thus indicating the exact position of the lesion. In addition, our preparation did not include ligation of the DNA lesion tethers, resulting in multiply nicked DNA in the experiments (0.1% of DNA sites). Control AFM analyses showed no binding preference by AGT for these nick sites (SI Appendix, Fig. S19). We also confirmed that the presence of nicks in the DNA tether (randomly spaced throughout the substrate) did not majorly affect the binding position distribution of AGT using a customized, ligated DNA substrate (LUMICKS) that contained the same (methylguanine) lesion at the same 30% position of the DNA (SI Appendix, Fig. S20). DNA nicks will, however, present target sites for many DNA-binding proteins, in particular those involved in DNA repair, necessitating the use of a ligated DNA lesion substrate for experiments on those protein systems.

We summarize our findings in the model shown in Fig. 5. Monomeric AGT slides bidirectionally along the DNA tracing the minor groove or alternatively slips rapidly over large distances in a more loosely DNA-bound conformation. At a target lesion, AGT forms a stable complex, from which cooperative cluster formation can nucleate. Using AFM imaging, we have previously shown mild bending of DNA in these clusters by ~30° (7). Clusters may further stabilize the complex, while bending may aid lesion detection by energetically favoring

![Fig. 4. AGT lesion recognition. (A and B) Kymotracker analyses for 1.5 μM AGT and DNA with an alkyl lesion at 30% of its length. (A) Positions of protein traces on DNA (green arrow indicates the lesion position). (B) Fluorescence intensities at the lesion position (25 to 35% of DNA length). SI Appendix, Figs. S15 and S16 show individual intensities over positions on DNA, average intensities at the lesion, and AFM analyses of specificity for lesion binding. (C and D) Kymotracker analyses for 17 μM AGT and DNA with an alkyl lesion at 30% of its length. (C) Positions on DNA (green arrow indicates the lesion position). Example kymographs are shown on the Right with green arrows indicating the 30% positions. (D) Intensity distributions at alkyl lesion positions (25 to 35% of the DNA length, Right) and at nonspecific positions (Left) for 17 μM AGT. Fluorescence intensities were measured within 2 pixels (100 nm and 0.2-ms integration time). SI Appendix, Fig. S15 shows intensities at individual DNA positions and average intensities at the lesion. Because the two ends of the DNA are not distinguishable in our experiments, data for complexes bound at the lesion also contain nonspecific background from the 30% position from the other end of the DNA.](https://doi.org/10.1073/pnas.2116218119)
flipping of the lesion base into the catalytic site pocket of AGT based on enhanced flexibility of DNA at these sites. An additional conceivable role of AGT clusters at an alkyl lesion is the recruitment of further protein factors to the DNA, consistent with the proposed roles of AGT in DNA replication and transcription and the multiple protein interaction partners that have been identified for AGT (25, 26).

A role of AGT clusters (that form at high micromolar concentrations) in the repair of alkylguanine DNA lesions is also consistent with the observation that tumors with low levels of AGT do not profit from AGT inhibition (27). Inhibitors of AGT such as benzylguanine (O6 BG) are exploited during chemotherapy to prevent futile repair of deliberately introduced alkyl damage in DNA. These inhibitors bind to the active site of AGT and block alkyltransferase activity. Previous studies have described a reduction in dsDNA binding affinity by 3 to 4× for alkylated AGT (e.g., with O6 BG) (14). Tumors with high AGT expression levels may thus most severely experience loss in AGT repair activity from reduced cluster formation (at the high protein concentrations) due to reduced DNA binding by AGT. In contrast, for low AGT concentrations, blocking of AGT repair activity would be stoichiometric with applied inhibitor concentration, with reported ED_{50} (median effective dose) values of ~100 nM (for O6 BG) (28). The situation is further complicated by signaling pathways and genomic rearrangements that modulate AGT expression levels in tumors (29, 30); however, this would not change the role of inhibition of AGT, in particular at high protein levels.

Methods

Protein Cloning, Expression, and Purification. The human AGT gene (cDNA from Origene) was cloned into the pETM41 vector by sequence- and ligation-independent cloning (SUC). For this, the primer sets 5′-ctttatcagggatc-3′ (forward) and 5′-ctgtaaatagctgtagctagttc-3′ (reverse) were used for the AGT gene and 5′-cggcttgagtcggcttcgaggc-3′ (forward) and 5′-ggcccctgaaataagattgatcgcctggagagc-3′ (reverse) for the vector. The resulting construct encoded for a (His)_{6}-tag followed by MBP on the N terminus of AGT (MBP-AGT, total molecular mass 63 kDa, 21 kDa + 42 kDa). The construct for AGT (with an N-terminal (His)_{6}-tag but without MBP) was produced by SUC into the pETM14 vector, using the insert-specific primer pair 5′-ctggagacccgccggacctagc-3′ (forward) and 5′-tagctgtagctgtagctagggagc-3′ (reverse) and the vector specific primer pair 5′-TGAGCTATTGATGGACGGCTGCAG-3′ (forward) and 5′-CATGGGCCCCCTGAGAAGACAG-3′ (reverse).

MBP-AGT and AGT were transformed into Escherichia coli BL21 competent cells using the kanamycin resistance of the vector as selection criterion and expressed in kanamycin-containing lysogeny broth (LB) medium overnight at 15 °C after induction with 0.4 mM IPTG. MBP-AGT was also expressed with the addition of 100 μM ZnCl_{2} to the medium.

AGT and MBP-AGT were purified via Ni^{2+}-NTA affinity chromatography. The cell lysates were pelleted in 50 mM Tris (pH 8.0), 300 mM NaCl, 20 mM imidazole, 5% glycerol, and 5 mM DTT and eluted from the Ni-NTA column with 300 mM imidazole. To conserve the bound Zn^{2+} ion in AGT, MBP-AGT that had been expressed in the presence of added ZnCl_{2} was purified in the same buffer as above without imidazole using an amylase bead resin and eluted with 10 mM maltose. Elution fractions from affinity chromatography were concentrated and subjected to size exclusion chromatography using a Superdex 75 16/600 column (GE Healthcare) in 20 mM Tris (pH 8.0), 200 mM NaCl, 1 mM TCEP, 0.1 mM EDTA, and 5% glycerol. Alkylation repair activity was confirmed for all purifications (see below).

μPIXE Analyses of AGT Zn Content. Measurements were carried out at the Ion Beam Centre, University of Surrey (31). Characteristic X-ray emission was induced using a 1.2-μm diameter 2.5-MeV 400-pA proton beam incident on dried protein droplets (volume per droplet approx. 0.1 μL) under vacuum. Emitted X-rays were detected in a silicon drift detector (Rayspec Ltd) with an active area of 80 mm² and an energy resolution of 130 eV at 5.9 keV. Spatial maps were obtained of all elements heavier than neon present, through scanning the proton beam in x and y over the dried sample. Quantitative information, using sulfur (in the protein cysteines and methionines) as an internal standard, was then obtained by collecting 3 or 4 point spectra from each droplet. These spectra were analyzed using GUPIX (32) within DAN32 (20) to extract the relative amount of each element per protein molecule, particularly zinc in the sample.

AGT Activity Assay. To study repair activity by AGT, a 48-bp alkyl lesion containing DNA substrate was used. The DNA was annealed from a 1:1 mixture of the top strand (GCGTACCC GAGTTCAGAAGGCAGA-GAGTCGACC) with a methylguanine base at position 20 and the complementary bottom strand labeled at its 5′ end with a Cy3 fluorophore (Cy3-GGT CGA CTC-0. 1 and 5 mg/mL EDTA, and 5% glycerol. Alkylation repair activity was confirmed for all purifications (see below).

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AUC. SV AUC was carried out using an Optima XL-I analytical ultracentrifuge (Beckman Coulter) with an eight-hole An-50 Ti rotor at 40,000 rpm and 20 °C. The DNA substrate was annealed from the same oligonucleotide sequences as above (AGT activity assay) either with or without the alkylguanine lesion. Samples (~400 μL) of 4 or 17 μM AGT and 500 nM Cy3-labeled DNA, as well as reference buffer solution, were loaded in standard double-sector–charcoal–filled Epon centerpieces equipped with sapphire windows. Data were collected in continuous mode at a step size of 0.003 cm using absorption optical detection at wavelengths of 280 nm and 550 nm (for DNA detection from Cy3 absorption signal). Data were analyzed using the NIH software SEDFIT to determine continuous distributions for solutions to the Lamm equation (21), as previously described (33). An analysis was performed with regularization at confidence levels of 0.68 and floating frictional ratio, time-independent noise, baseline, and meniscus position, to root-mean-square deviation values of between 0.007 and 0.012. Data for lesion containing and undamaged DNA provided comparable separation coefficient spectra, consistent with the rapid repair activity by AGT (on the second to minute time scale compared to hours for the AUC measurements) and similar binding affinities of alkylated and nonalkylated AGT for DNA. Experiments were carried out at least in duplicate for the different AGT concentrations as well as protein-only and DNA-only samples for reference.

Fluorescence Optical Tweezers.

DNA substrate preparation. DNA tethers, on which fluorescently labeled molecules can be observed, need to be sufficiently long and to specifically attach to beads at their ends, which are then held in the optical traps. For this, we biotinylated the ends of lambda phage DNA (48,502 bp, Thermofisher Scientific) by filling up the natural ssDNA overhang ends of the DNA with partially biotinylated deoxynucleoside triphosphates (biotinylated deoxycytidine triphosphate [dCTPs], Sigma-Aldrich) using Klenow polymerase (Promega). This allows for attachment to streptavidin-coated microspheres in the optical traps (Fig. 1C and SI Appendix, Fig. S14A).

For preparation of the methylguanine lesion containing the DNA substrate, the biotinylated lambda phage DNA was incubated with the nicking endonuclease Nt.BsNBI (NEB), which results in particular in two closely located incisions at positions 33,779 and 33,792. All other (ssDNA) incisions are sufficiently distant from each other so that the 13-nt stretch between these nickase sites can be selectively melted out in the presence of an excess of 5′ phosphorylated lesion containing oligo of the same sequence (5′-TCAGA/06 Me dG)CTGAC3′, GeneLink), which is similar but slightly modified from our DNA lesion substrate preparation procedure for AFM studies (12, 34) and similar to a previously published procedure (35). In contrast to the AFM lesion substrate preparation protocol with a circular pUC19N plasmid (~2,800 bp) (12, 34), the long, linear lambda phage DNA (~50,000 bp) is excessively sensitive to shear forces. Centrifugation steps were therefore replaced by dialysis, and the buffer was replaced by a high salt buffer (200 mM NaCl) to protect the DNA. Dialysis also removed excess biotinylated dCTPs from the sample solution. AFM imaging confirmed that this procedure rendered intact, full-length lambda DNA (SI Appendix, Fig. S14B).

In addition, we employed force extension experiments with the optical tweezer system to control successful sample preparation (SI Appendix, Fig. S14C). The gapped lambda DNA (with the 13-nt segment between the 2 nick sites removed, but not yet replaced by the lesion containing insert) was unstable and did not survive stretching to its contour length (16.49 μm) in the optical tweezers. After insertion of the lesion containing the 13-nt oligonucleotide, the DNA substrate could be easily and stably tethered between the beads and stretched to its full extension. Force extension curves showed a slight change in the height of the force plateau for lesion-containing DNA tethers compared to undamaged lambda DNA (n = 8; SI Appendix, Fig. S14C). This surprisingly minor effect is conceivably caused by the slightly different stability of the DNA at the lesion and, more importantly, the presence of unligated nicks (0.1% of DNA sites throughout the DNA substrate). In the final substrate, the lesion is located at 30% of the DNA length (SI Appendix, Fig. S14A).

In addition, for experiments on ssDNA, we used modified lambda DNA (LUMICKS) that can be turned into 48,548-nt ssDNA by overstretching, following the accompanying protocol (LUMICKS).

Protein conjugation. For fluorescence detection, AGT molecules were coupled to QDs with maximum emission at 605 nm (QD605; Thermofisher) via an anti-body sandwich linker, as previously described (12). Briefly, the His6 tag in MBP-AGT or AGT was targeted by a His6 tag-specific mouse monoclonal IgG1 antibody (Dianova), which was then bound by a specific species-specific secondary antibody on the surface of the QDs.

Measurements. Experiments were carried out on a dual optical trap CTrap with three-color laser excitation and three-channel fluorescence detection (LUMICKS). A multichannel flow cell system (LUMICKS) allows us to introduce the streptavidin-coated beads (4.38 μm diameter; LUMICKS) for DNA tethering, the DNA substrate, and the proteins in separate channels (Fig. 1C). Measurements were carried out in a further buffer channel or at the junction between protein and buffer channel. All samples were diluted in measurement buffer (10 mM Tris [pH 7.4], 50 mM NaCl, 1 mM DTT, ± 1 mM EDTA). A total of 1 to 4 pM of end biotinylated DNA substrate (see above) was introduced in the DNA channel. Three different types of DNA substrates were used in the optical tweezers, as follows: 48,502 bp lambda phage DNA (used in experiments on different salt concentrations and for the AGT monomer reference at 500 nM [protein], 48,502 bp lambda DNA with a methylguanine lesion at 30% of DNA length, or 48,548 bp ssDNA tethers. Between 500 nM and 17 μM MBP-AGT or AGT were flowed into the protein channel (see also below). During measurements in the buffer channel, only a single DNA tether spanned between two beads in the optical traps is present, providing high protein:DNA ratios in the experiments. Samples are fluorescently excited by a 488-nm laser, and fluorescence intensities (photons counts/pixel) and positions along the DNA tether are detected by a line scanning photon detector (scanning along the DNA tether) and are visualized in kymographs. Data were recorded at a resolution of 100 nm/pixel and ~0.04 s/scan line (individual pixel time of 0.1 μs).

Measurements were done at a DNA stretching force of 20 pN, which provides fully extended DNA (stretched close to its contour length, 16.49 μm) that displays little flexibility but at the same time no major distortion of the DNA double helical structure. Because AGT likely binds the DNA (1, 7), DNA tension may affect translocation of AGT (monomers or clusters) on the DNA or lesion recognition by AGT. However, control experiments at 5 pN provided comparable results as with 20-pN stretching force (SI Appendix, Fig. S21) indicating minor effects on AGT diffusion constants by a DNA stretching force of 20 pN.

Experiments were carried out for MBP-AGT with and without Zn2+ bound as well as for AGT without Zn2+ and at protein concentrations of 500 nM (MBP-AGT-Zn2+), 1.5 μM (MBP-AGT +Zn2+), 4 μM (MBP-AGT +Zn2+, MBP-AGT -Zn2+, AGT), and 17 μM (MBP-AGT -Zn2+ duplicate). We chose the concentration of 17 μM based on a previous study, in which we had observed a saturation of cluster size for approximately >10 μM (7). The 4 μM AGT condition was selected based on the fact that in these previous analyses a transition from predominantly monomeric to oligomeric complexes had been seen around this AGT concentration.

Fluorescence optical tweezer analyses. Coordinates of QD-labeled AGT along the lesion containing DNA as well as the photon counts (integrated over 2 pixels due to downsampling, pixel time of 0.1 ms) were extracted from kymographs using a custom-written kymotracker Python script (https://harbor.lumicks.com/) (SI Appendix for details). Kymographs were further converted to.tif for further manual analyses in FIJI/ImageJ using a different Python script (LUMICKS). Lifetimes and overall distances moved by AGT were measured manually in FIJI. MSD analyses were carried out using a customized Labview routine on isolated kymograph traces that were Gaussian tracked in ImageJ, as previously described (12, 36) (SI Appendix, Fig. S9).

From our analyses, we obtain protein positions from 0% (at the beads) to 50% (at the center of the DNA tether) because we cannot distinguish between the two DNA ends. Positional analyses with the alkyl lesion containing DNA thus necessarily results in 50% nonspecific background due to two specific positions at 30% from each DNA end (see for example green arrows in Fig. 4C). A DNA substrate containing both a fluorophore and a lesion (Results and Discussion) would have the potential to eliminate this background by indicating the true lesion position.

MSD was determined to obtain diffusion constants and alpha exponents of DNA-bound species from the individual traces in kymographs as previously described (12) (SI Appendix, Fig. S9). Only mobile protein traces (with positional variation of >2 pixel corresponding to 200 nm) were included in MSD analyses. Briefly, diffusion constants (Dm) are derived from MSD via the relationship: $D_m = \frac{1}{\alpha}$ (for procedure, SI Appendix, Fig. S9). For normal, random diffusion, α equals 1. α < 1 indicates subdiffusive movement, for which motion...
is constrained and slower than regular diffusion, whereas $\alpha > 1$ represents faster, superdiffusion (37). Only diffusion constants and alpha values from MSD fits with $R^2 \geq 0.83$ were included in further analyses. In order to evaluate the resulting diffusion constants, the theoretical limit for rotational sliding along the DNA following the groove was approximated using the Bagchi/Blainey/Xie model (38). The Stokes-Einstein equation defines the diffusion constant as follows: $D = k_B T/6\pi R \eta$. For one-dimensional diffusion (along DNA), the friction term contains both translational and rotational friction, as follows: $\xi = 6 \pi R \mu + (2 \pi d/108 \mu )^2 (\eta R \pi^2 \gamma)$ (38). The viscosity $\eta$ of the buffer was 1.0107 x 10^{-3} NIcm^{-2} (Sednterp, T. Laue). $R$ is the hydrodynamic radius of the QD-AGT conjugate, which consists of an antibody-coated QD with a radius of ~9 nm (12), the primary antibody to the His tag on AGT with a radius of ~5.5 nm (39), and AGT with a radius of 2 nm (based on sequence) with or without MBP with a radius of ~3.5 nm (12). The overall radius $R$ of the conjugate is dominated by the large volume of the QD and is ~9.8 nm with MBP and ~9.7 nm without MBP. $BP$ is the distance between two adjacent base pairs (0.34 nm), $R_{OC}$ is the distance between the center of the protein conjugate and the DNA helical axis ($R_{OC}$ on $R + 1$ nm = 10.8 nm or 10.7 nm with and without MBP in the construct). Inserting these values, the limiting diffusion constant for rotational sliding of QD-AGT (both with and without MBP) was calculated as ~0.03 m/s² (indicated as the dashed line in Fig. 1E) versus ~21.6 m/s² for solely translational diffusion. To determine the sensitivity limit of our measurements, we applied MSD analyses also to the ATTO647N fluorochrome at 30% of the DNA tether length (SI Appendix, Fig. S9D). Although these traces were static (movement of <2 pixel), we obtained diffusion constants of 0.004 to 0.007 m/s², indicating the lower limit of sensitivity of our MSD analyses based on positional noise and the accuracy of our MSD tracking procedure. Shorter traces (<10 s) even resulted in diffusion constants of 0 to 0.04 m/s² (average 0.01 m/s²) which is consistent with a lower accuracy of MSD fits of shorter traces (40).

Data Availability. The data supporting the findings of this study are available within the main text and SI Appendix. In addition, all fluorescence kymograph data are available on the Open Science Framework at https://osf.io/nsh3u/.

ACKNOWLEDGMENTS. We thank Michael Fried and Anke Krueger for helpful discussions, Geoff Grime for assistance with the PIXE measurements, and Maxi-milian Dohn and Jonas Kulhavy for contributions to AFM analyses. This work was supported by Deutsche Forschungsgemeinschaft (DFG) Grant TR 671/4-2 to I.T. and publication charges were covered by the Rudolf Virchow Center.

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