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Distinguishing Specific and Nonspecific Complexes of Alkyladenine DNA Glycosylase

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Abstract

Human alkyladenine DNA glycosylase (AAG) recognizes many alkylated and deaminated purine lesions and excises them to initiate the base excision DNA repair pathway. AAG employs facilitated diffusion to rapidly scan nonspecific sites and locate rare sites of damage. Nonspecific DNA binding interactions are critical to the efficiency of this search for damage, but little is known about the binding footprint or the affinity of AAG for nonspecific sites. We used biochemical and biophysical approaches to characterize the binding of AAG to both undamaged and damaged DNA. Although fluorescence anisotropy is routinely used to study DNA binding, we found unexpected complexities in the data for binding of AAG to DNA. Systematic comparison of different fluorescent labels and different lengths of DNA allowed binding models to be distinguished, and demonstrated that AAG can bind with high affinity and high density to nonspecific DNA. Fluorescein-labeled DNA gave the most complex behavior, but also showed the greatest potential to distinguish specific and nonspecific binding modes. We suggest a unified model that is expected to apply to many DNA binding proteins that exhibit affinity for nonspecific DNA. Although AAG strongly prefers to excise lesions from duplex DNA, nonspecific binding is comparable for single and double-strand nonspecific sites. The electrostatically driven binding of AAG to small DNA sites (~5 nucleotides of single-stranded and ~6 base pairs of duplex) facilitates the search for DNA damage in chromosomal DNA, which is bound by nucleosomes and other proteins.

Graphical Abstract

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Keywords

fluorescence polarization; fluorescence anisotropy; nonspecific DNA binding; DNA glycosylase; DNA repair

INTRODUCTION

Glycosylases initiate the base excision repair pathway by finding and excising damaged bases from genomic DNA.^{1,2} In humans there are eleven genes encoding DNA repair glycosylases and these belong to four distinct superfamilies. These enzymes differ in their substrate specificity and catalytic mechanisms, but likely share common features that allow them to search DNA and locate rare sites of damage. For example, several of these enzymes have been found to employ facilitated diffusion, whereby nonspecific DNA binding interactions are used by the enzymes to diffuse along DNA in an efficient search for sites of damage.^{3–7} The ability to bind tightly to nonspecific DNA is therefore critical to their biological function. However, it can be very difficult to gain insights into nonspecific DNA binding by enzymes, because affinity is typically weaker than for specific substrate sites and even a short polymer is expected to have many overlapping binding sites. Most structures of DNA glycosylases support monomeric models for damage recognition,⁸ but biochemical evidence has been used in several cases to argue in favor of dimer models.^{9–11} In the current work we examined the stoichiometry and nonspecific DNA binding affinity of human alkyladenine DNA glycosylase (AAG).

AAG excises many *N*-alkylated purines, including 3-methyladenine, 7-methylguanine, and the lipid peroxidation-derived adduct, 1,*N*⁶-ethenoadenine (ϵ A).^{12–14} In addition to these alkylated bases, AAG is responsible for removing hypoxanthine and xanthosine formed from the deamination of adenine and guanine, respectively.^{15,16} The crystal structure of AAG in complex with ϵ A-containing DNA reveals a DNA binding surface that is highly positively charged.¹⁷ The importance of these electrostatic interactions for DNA binding is apparent in the rate of dissociation from DNA which is strongly dependent on the monovalent salt concentration.^{18,19} Mutation of conserved basic residues at the DNA binding surface leads to weakened DNA binding in vitro and reduced DNA repair activity in vivo.^{17,20}

Previous work investigating the searching mechanism of AAG demonstrated that facilitated diffusion is dominated by a rapid hopping mechanism.²¹ Biochemical experiments also demonstrated that AAG is capable of intersegmental transfer, the direct transfer from one DNA segment or molecule to another without fully dissociating from DNA.²² Theoretical treatment of intersegmental transfer has argued that this would be most easily achieved by a multimer with two or more DNA binding sites.²³ DNA binding studies for several different DNA glycosylases have been interpreted with multimeric DNA binding models.^{9–11,24,25} Although AAG is a monomer in solution and in crystal structures of the enzyme bound to lesion-containing DNA,^{17,26} attempts to crystallize AAG in complex with nonspecific DNA have not been successful. Therefore, we used alternate methods to characterize the interactions between AAG and nonspecific DNA.

We used electrophoretic mobility shift assay (EMSA) and fluorescence anisotropy methods to study the binding of AAG to undamaged DNA and compared it to the binding of lesion-containing DNA. Both of these techniques have been widely used to characterize nucleic acid binding proteins. EMSA methods have the advantage of physically separating different complexes via native gels, but the equilibrium can be perturbed by electrophoresis, weak complexes may not be adequately trapped in the gel matrix, and not all complexes can be separated.²⁷ Fluorescence anisotropy measures the tumbling of a fluorescently labeled molecule in solution and can be a versatile method for determining equilibrium binding and binding stoichiometry. Changes in size or shape can result in changes in anisotropy, however, the identity of the fluorophore and the linker that is used to attach it to DNA can impact the anisotropy measurements and gratuitous interactions between the protein and fluorophore are possible.^{28–30} Therefore, it is important to compare binding measurements made with chemically distinct fluorophores, or to compare labeled and unlabeled ligands to verify that the label does not alter protein binding.

We describe a comprehensive study of binding of AAG to specific and nonspecific DNA oligonucleotides. Fluorescence anisotropy titrations could be interpreted with dimerization models, but by employing multiple approaches and systematically varying the length and labeling of the DNA, we provide compelling evidence that interactions between the protein and the label are responsible for many of the complex observations. These experiments confirm that AAG binds as a monomer to specific sites and that it is capable of also binding as a monomer with high affinity to nonspecific DNA. However, we also observed that AAG can coat the DNA at very high density (2 AAG molecules bind to an 11mer duplex). These observations provide new insight into the design and interpretation of fluorescence anisotropy studies of DNA binding proteins.

MATERIALS AND METHODS

Preparation of Proteins and DNA.

The catalytic domain of AAG lacking the first 79 amino acids (80 AAG) was expressed in *Escherichia coli* BL21 (DE3) cells and purified as described.³¹ This well-characterized 80 AAG has an identical rate of *N*-glycosidic bond cleavage on ϵ A lesions as the full-length AAG protein.³¹ Crystallographic¹⁷ and biochemical³² studies with the truncated and full-length proteins, respectively, show analogous DNA binding footprints. The concentration of

AAG was estimated from the UV absorbance, and the active concentration was determined by titration of ϵ A-DNA as described below.³³

DNA oligonucleotides were purchased from Integrated DNA Technologies or the Keck Center at Yale University. Unless otherwise noted, oligonucleotides were labeled on the 5' end of the lesion-containing strand with a 6-fluorescein (FAM), 6-hexachlorofluorescein (HEX) or DyLight 647 (DY647). Oligonucleotides were purified with denaturing polyacrylamide gel electrophoresis (PAGE), extracted and desalted with a C18 column. Concentrations of oligonucleotides were determined from the absorbance at 260 nm and the calculated extinction coefficients. The extinction coefficients for DY647 substrates were estimated using the absorbance of Cyanine 5. The extinction coefficients for ϵ A-containing oligos were estimated by subtracting $9400 \text{ M}^{-1}\text{cm}^{-1}$ from the calculated extinction coefficients for the analogous A-containing oligos and the extinction coefficient of the pyrrolidine-containing substrates assumed no absorbance for the pyrrolidine. Oligonucleotides were mixed in annealing buffer (10 mM NaMES pH 6.5, 50 mM NaCl) with a 1.2-fold excess of the complementary strand and annealed by heating to 95 °C for 3 min, and cooling to 4 °C at a rate of 0.2 °C/s.

Measurement of Fluorescence Anisotropy and Total Fluorescence.

DNA was mixed with varying amounts of AAG in standard binding buffer (50 mM NaHEPES, pH 7.5, 100 mM ionic strength controlled with NaCl, 1 mM EDTA, 1 mM DTT, $\pm 0.1 \text{ mg/mL}$ BSA) at 25 °C. BSA was not necessary and did not affect the anisotropy measurements, but was included in some experiments to stabilize protein for multiple additions and mixings of AAG. All assays are performed at 100 mM Na^+ conditions unless specifically noted to be 200 mM Na^+ (Figures 8, 9 and 10C, Table 1, and Figure S14). All AAG additions were equilibrated for at least 45 s prior to fluorescence detection and all titrations were completed in less than 10 min prior to ϵ A excision. There was no significant difference of ϵ A quenching between 1 min and 10 min reactions indicating that ϵ A was not excised on this time scale. Fluorescence was detected using a T-format PTI QuantaMaster (FeliX software) or L-format Horiba Fluoromax-3 fluorometer (DataMax software). Excitation/emission wavelengths were 493/518 nm for FAM, 531/547 nm for HEX, 643/660 nm for DY647 (6 nm band-pass). Excitation and emission scans were taken using a vertical excitation and magic angle (54.7°) emission. The two emission filters were corrected for differences with the G-factor (Eq 1), and the anisotropy (r) and total fluorescence (F_T) of the fluorescently-labeled DNA were calculated using Equations 2 and 3, where V and H denote the position of the excitation/emission polarization filters ($V=0^\circ$; $H=90^\circ$) and I is intensity of fluorescence.

$$G = \frac{I_{HV}}{I_{HH}} \quad (1)$$

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2 \cdot G \cdot I_{VH}} \quad (2)$$

$$F_T = I_{VV} + 2 \cdot G \cdot I_{VH} \quad (3)$$

Where noted, the anisotropy values were corrected to account for the quenching of the FAM fluorophore that occurred upon AAG binding. Equation 4 describes the ratio of bound to free FAM-DNA where F is the molar concentration of the FAM-DNA, r is the anisotropy, Q is the molar fluorescence of the FAM-DNA, f and b represent the free and bound FAM-DNA states, and r and M represent the measured and corrected anisotropy values, respectively.³⁴ Equation 4 can be rearranged to solve for M (Eq 5).³⁵

$$\frac{F_b}{F_f} = \left[\frac{r - r_f}{r_b - r} \right] \cdot \frac{Q_f}{Q_b} = \frac{M - r_f}{r_b - M} \quad (4)$$

$$M = \frac{\left[\frac{r - r_f}{r_b - r} \right] \cdot \frac{Q_f}{Q_b} \cdot r_b + r_f}{1 + \left[\frac{r - r_f}{r_b - r} \right] \cdot \frac{Q_f}{Q_b}} \quad (5)$$

For binding measurements performed under titration conditions, 100 or 300 nM DNA was mixed with AAG in 250 μ L reaction volume (3 mm path length quartz cuvette). Anisotropy and total fluorescence data were fit with DynaFit (BioKin).³⁶ One, two, and three-site models were used to assess the various binding titrations. DynaFit models and fit parameters are provided in the Supplemental Information. For equilibrium binding conditions, 5 or 15 nM DNA was mixed with AAG in 3 mL reaction volumes for greater sensitivity (10 mm path length quartz cuvette). The change in signal (ΔA) was fit by a binding hyperbola with enzyme (E) in excess over substrate (Eq 6) using KaleidaGraph software.

$$\Delta A = \frac{\Delta A_{\max} [E]}{([E] + K_d)} \quad (6)$$

For competition anisotropy, 150 nM 5'-FAM-labeled 19AAA (undamaged) DNA and 150 nM AAG were premixed at the standard binding conditions (200 mM ionic strength), and then titrated with various inhibitors. The change in anisotropy was normalized (r_N) from 0 (DNA alone) to 1 (AAG:DNA, no inhibitor) and fit using a one-site competitive binding

model where IC_{50} is the concentration of inhibitor that reduces binding of AAG to labeled DNA by half (Eq 7).

$$r_N = \frac{1}{\left(1 + \frac{[I]}{IC_{50}}\right)} \quad (7)$$

The resulting IC_{50} values were converted to a macroscopic K_i value for each inhibitor using Equation 8 in which S is the labeled DNA substrate, I is the DNA inhibitor, and the K_d is the dissociation constant for the substrate (assumed to be 68 nM from the total FAM fluorescence at 200 mM ionic strength measured in Figure 8B).

$$K_i = \frac{IC_{50}}{\left(1 + \frac{[S]}{K_d}\right)} \quad (8)$$

To find the microscopic K_d value for a single undamaged binding site, the macroscopic K_i values for each competitor were multiplied by the estimated number of binding sites (N) on each competitor (Eq 9) in which L is the total length of the oligo, l is the site size or footprint of the enzyme (6 bp for duplex DNA; 5 bp for ssDNA),¹⁷ and the number of sites is doubled to account for both DNA strands. This calculation assumes that AAG binds with equal affinity to all nonspecific sites.

$$N = 2(L - l + 1) \quad (9)$$

Determination of AAG Concentration by eA-Quenching.

The active concentration of AAG was determined by mixing varying amounts of AAG with 200 or 400 nM 19AEA (eA) 5' FAM DNA in binding buffer (100 mM ionic strength) without BSA, because BSA interferes with eA fluorescence. Binding reactions were mixed for 45 s at 25 °C before recording the eA fluorescence at excitation/emission wavelengths of 316/408 nm (both 6 nm band-pass). The eA fluorescence was normalized and fit to a 1:1 binding equation (Eq 10) in which F is normalized fluorescence, A is the amplitude of fluorescence change, E and S are AAG and the substrate, respectively, and the K_d is the dissociation constant of eA binding (estimated to be less than 100 pM). Two protein preparations were used in this study and were 81% and 62% active, respectively (Figure S1). All assays used the corrected active concentrations of AAG.

$$F = 1 - \frac{A \left\{ (K_d + [E] + [S]) - \sqrt{(K_d + [E] + [S])^2 - 4[E][S]} \right\}}{2[S]} \quad (10)$$

Electrophoretic Mobility Shift Assays (EMSA).

Horizontal gels were cast with 1% agarose and 2% acrylamide and run with TGE buffer (25 mM Tris base, 190 mM Glycine, 1 mM EDTA). 1% agarose was melted in 1× TGE and cooled until warm to touch prior to addition of 2% acrylamide (37.5 acrylamide:bis-acrylamide ratio), ammonium persulfate and TEMED. DNA and AAG were incubated for 15 min at 4 °C in standard binding buffer + 10% glycerol (v/v). Ionic strength was adjusted to 100 mM unless noted to be 200 mM (Figure 8C). Longer binding times did not affect the fraction bound. Bromophenol Blue led to altered migration of the AAG•DNA complexes and therefore samples did not include loading dye (data not shown). Lanes were loaded with 50–500 fmol of DNA samples and electrophoresed at 120 V for 30–60 min at 4 °C. Gels were imaged using a Typhoon Trio Fluorescence imager (GE Healthcare) with 488 nm excitation and 520 nm band pass emission filters to detect FAM-labeled DNA species. The fraction shifted (fraction bound) was determined by quantifying the fluorescence intensity of the free (I_f) and bound (I_b) DNA ($F = I_b/(I_f + I_b)$).

RESULTS AND DISCUSSION

Considerable progress has been made in understanding the kinetic mechanism by which AAG searches nonspecific DNA^{6,21} and flips out an alkylated lesion,^{18,33,37,38} but thermodynamic characterization of nonspecific DNA binding has remained elusive. In the current study, we define the stoichiometry and thermodynamics of AAG binding to nonspecific DNA. We employed two common fluorophores that are inexpensive and easily incorporated into DNA using solid phase synthesis, 6-carboxyfluorescein (FAM) and Dylight647 (DY647), and compared their effects on the DNA binding properties of AAG (Figure 1). FAM is a negatively charged dye that is commonly attached to DNA via a flexible linker. Previous studies suggest that FAM does not interact strongly with DNA, leading to weak coupling between the fluorophore and the DNA.³⁹ In contrast, cyanine dyes such as DY647 are positively charged and stack onto the ends of DNA duplexes.^{39–41} This leads to a tighter coupling between the fluorophore and the DNA molecule. By comparing anisotropy and total fluorescence with two independent labels we are able to interpret the spectroscopic signals in terms of DNA binding and stoichiometry, whereas subsets of the data would be consistent with alternative models for AAG binding.

Fluorescence Titrations of AAG Binding to ϵ A-DNA with a 5' FAM Label.

Previous work suggested that a 19-mer oligonucleotide duplex is sufficiently large to satisfy optimal binding of AAG.³² Therefore, we examined binding of AAG to a FAM-labeled 19-mer with a centrally located ϵ A•T lesion site (Figure 1; 19AEA). AAG binds rapidly and tightly to ϵ A-DNA,¹⁸ and hydrolysis of the *N*-glycosidic bond is relatively slow under the conditions employed. This provides the opportunity to use steady-state fluorescence measurements to monitor titration of AAG to its preferred substrate and binding can be measured prior to catalysis.

Titration experiments were performed with a fixed concentration of DNA (100 nM or 300 nM 5' FAM-labeled 19AEA DNA duplexes) and increasing amounts of AAG (Figure 2; DynaFit models described in Figure S2). We observed that the fluorescence anisotropy exhibited a biphasic

behavior (Figure 2A). The binding of the first equivalent of AAG caused a modest change in anisotropy and the second equivalent caused a much larger change in anisotropy. Replotting these data as a function of the ratio of AAG to DNA confirmed that this experiment was conducted under titration conditions ($[DNA] \gg K_d$; Figure 2B). The changes in total FAM fluorescence exhibited a similar biphasic process (Figures 2C and 2D). The first equivalent of AAG gave little or no quenching of the FAM fluorescence and a second equivalent decreased the fluorescence to approximately 70% of the level of the free DNA. This pattern could have been interpreted in terms of a cooperative binding transition in which two molecules of AAG bound to a single ϵ A lesion site. However, we also measured the intrinsic ϵ A fluorescence, which reports on the flipping of the nucleotide into the active site pocket of AAG,^{18,42} and observed a single phase under the same conditions which is consistent with formation of a 1:1 specific complex (Figure 2E; corrected from Figure S1).

The comparison of these three spectroscopic signals (FAM anisotropy, FAM quenching and ϵ A quenching) indicates that the FAM label monitors binding of at least two molecules of AAG (Figure 2F). To account for the 30% quenching of FAM fluorescence, we corrected the anisotropy signal for binding of the second equivalent of AAG (Figure 2F and Figure S3).

Titration of AAG Binding to Undamaged DNA with a 5'FAM Label.

We next performed titrations of undamaged 19-mer DNA duplex with a 5'FAM label in order to compare the anisotropy and total 5'FAM fluorescence with that of the ϵ A-DNA. Titrations performed at 100 nM and 300 nM DNA gave identical dependence on AAG concentration once they are plotted as a function of the number of equivalents (Figure S4). However, AAG induced a very different pattern of changes in anisotropy and FAM fluorescence for the undamaged DNA as compared to the ϵ A-DNA (Figure 3). The overall anisotropy change for the undamaged DNA was the same as for the ϵ A-DNA, but the first equivalent caused a large change in anisotropy (>0.1) followed by a small change, which is the opposite of the behavior on the ϵ A-DNA (Figure 3A). In other words, the first binding event on the undamaged DNA matched the second binding event on the ϵ A-DNA, but at least two molecules of AAG could bind to each DNA molecule under titration conditions. The quenching of total FAM fluorescence occurs upon the binding of the first equivalent of AAG for the undamaged DNA and for the second equivalent of AAG for the ϵ A-DNA (Figure 3B), suggesting that binding of AAG near to the 5'FAM-labeled end causes both quenching of fluorescence and enhanced fluorescence anisotropy.

To further evaluate the unusual behavior of the 19-mer ϵ A-DNA, we performed analogous titration experiments with 25-mer DNA duplexes containing either a 5'FAM or 5'HEX (hexachlorofluorescein) label and either a central ϵ A•T lesion site, undamaged site, or pyrrolidine•T transition state analog (pyr) which is known to bind tightly to AAG,⁴². These experiments showed almost identical changes in anisotropy and total fluorescence as were observed for the 19-mer substrates (Figure S6). Fitting of the anisotropy titrations with DynaFit revealed a slightly better fit of 3 AAG molecules to the 25-mers, as compared to the 2 AAG molecules observed for the 19-mers. As was observed for the 19-mer substrates, the quenching of the total fluorescence followed a single AAG binding event for the undamaged

DNA, and the second AAG binding event for the specific ϵ A or pyr-containing DNA (Figure S6).

We sought an independent method for evaluating the model that multiple AAG molecules were binding to a single DNA, and found EMSA to be a suitable approach. Using mixed agarose/acrylamide native gel conditions we probed the stoichiometry of AAG binding to the same 5' FAM-labeled 19-mer ϵ A-DNA and undamaged DNA as was analyzed in the anisotropy experiments in Figure 3. As predicted by the fluorescence measurements, two sequential binding events could be observed for both DNA samples. For the ϵ A-DNA, there were discrete bands corresponding to 1 and 2 equivalents of AAG (Figure 4A). Although the undamaged DNA also showed two distinct binding events, the bands were not as well defined as with the ϵ A-DNA. It is expected that the nonspecific binding to the undamaged DNA reflects a larger ensemble of binding states and nonspecific complexes could dissociate in the gel (note the smear between bands in the undamaged DNA in Figure 4B and in the second binding event for the ϵ A-DNA in Figure 4A).

Taken together, both EMSA and FAM fluorescence provide strong evidence that two AAG molecules can bind to a 19-mer DNA duplex. The stoichiometric quenching of FAM fluorescence in the first binding event for undamaged DNA and in the second binding event for ϵ A-DNA suggests that AAG has a propensity to bind near to the 5' FAM label. In turn, an interaction between AAG and the FAM moiety, resulting in restriction of the fluorophore mobility, can explain the large change in anisotropy that correlates with the FAM quenching.

Binding Density of AAG on DNA.

To explore the effect of DNA length on the anisotropy signals, we titrated several different length oligonucleotides that were labeled at the 5' end with FAM and contained a single ϵ A•T lesion site. Binding of AAG near the 5' FAM causes a large change in anisotropy that serves as a robust signal for binding, and is presumably attributed to restricted mobility of the fluorophore (Figure 5; DynaFit models in Figure S7). As was observed for the 19-mer substrates, the first AAG binding event is at the lesion site in all substrates tested and there is only a small change in anisotropy. This small change in anisotropy for binding of an ~29 kDa protein has some precedent in the literature as several similar sized DNA binding proteins cause small changes in the range of 0.01 to 0.04 for FAM-labeled oligonucleotides.⁴³ As the length of the oligonucleotide was increased beyond 19 bp, additional molecules of AAG were required to achieve the full anisotropy change. For example, the titration curve for the 25-mer was best fit by a 3-site model. The 49-mer titration curve shows more than three binding events; however, they were not as sharply defined, and for the sake of simplicity, we have employed a three-site DynaFit model. We attributed this more gradual titration of the anisotropy change to competition between nonspecific binding sites away from the 5' FAM and the sites near to it, because the total fluorescence tracks closely with this same stoichiometry and broad response curve. We verified that binding of AAG to the 49-mer was measured under titration conditions as it showed a much different binding density than the 19 and 25-mer oligonucleotides (Figure S8). It should be noted that the 49-mer has a different sequence context at the 5' FAM site, which accounts for the enhancement of fluorescence upon binding to AAG (discussed in Figure 7C).⁴⁴ As expected, differences

in the sequence context at the ϵ A•T lesion site do not change the patterns of binding (Figure S9).

To validate these conclusions, we also performed EMSA experiments for binding of AAG to the 49-mer ϵ A-DNA duplex, and compared the results with those of the shorter duplexes (Figure S10). In contrast to the two binding events on the 19-mer DNA, there were at least 3 binding events with the 25-mer and 49-mer. Therefore, the solution fluorescence measurements and the gel-based EMSA measurements were in good agreement.

We next investigated the possibility that a short hairpin oligonucleotide could be used to limit the number of nonspecific binding sites. The design of an optimal hairpin length was guided by previous systematic analysis of the optimal binding of AAG to abasic and hypoxanthine-containing oligonucleotides.³² The minimal oligonucleotide had an upstream segment of 6 bp and a downstream segment of 2 bp joined by an internal GAAA hairpin (Figure 1; 11TXC DNA). Even on this minimal hairpin, a clear signature for two AAG binding events can be observed in both the anisotropy and total fluorescence (Figure 5; 11-mer DNA). The two distinct binding events were also observed by EMSA (Figure S10). These observations demonstrate that two AAG molecules bind close to each other with an estimated footprint of 6 bp per AAG that is consistent with DNA contacts in the crystal structure of AAG bound to DNA.¹⁷ The hairpin itself does not appear to have a significant impact on binding, because AAG showed very similar binding to an asymmetric 19-mer with an identical upstream segment of 6 bp and a downstream segment of 12 bp (Figure S11). Although it may seem surprising that a 19-mer DNA does not allow an additional molecular of AAG to bind if it requires such a small binding footprint, we suspect that this could be due to problems with end-fraying of terminal base pairs. To achieve the high binding density of two AAG molecules to an 11-mer hairpin, we infer that AAG must bind to opposite faces of the DNA duplex, rather than side-by-side. A crude model suggests that this binding mode could be achieved without obvious steric clash (Figure S12). It is intriguing that an unrelated DNA glycosylase, thymine DNA glycosylase (TDG), is capable of binding in a similar fashion to adjacent sites on DNA.^{9,25}

5'-Fluorophore Identity Affects Anisotropy Signal.

To investigate the role of the 5'-fluorescent label in binding assays, we prepared 19-mer undamaged and ϵ A-DNA with 5' DY647. DY647 is a more chemically stable analog of Cyanine 5 that can be incorporated during solid phase synthesis. Whereas FAM does not stably interact with the end of the DNA,³⁹ NMR studies with Cy5 labeled DNA shows stable stacking of this fluorophore onto the terminal base pair of the DNA.⁴⁰ Stacking of the DY647 label on the end of the duplex is expected to give tight coupling between the tumbling of the fluorophore and of the overall DNA duplex. Consistent with this expectation, the anisotropy of the DY647-labeled DNA is much greater than for the FAM-labeled DNA in the absence of AAG ($r = 0.25$ and 0.05 , respectively for the 19-mer DNA duplexes; Figure 6A).

Titration of DY647-labeled DNA with AAG showed a much smaller increase in anisotropy than was observed for the FAM-labeled DNA, and very little difference was observed between the ϵ A-DNA and undamaged DNA binding titrations (Figure 6B). The total

fluorescence increased slightly for the binding of AAG to the DY647-labeled DNA. Although the change in fluorescence upon binding of AAG to the damaged DNA was fit by a 2-site model, and that of the undamaged DNA was fit by a 1-site model with the DY647-labels, the difference is much less pronounced than was observed for the FAM-labeled samples (Figure 6C). These results suggest that AAG preferentially binds near to the 5'-end and affects the environment of both fluorophores.

The large anisotropy changes observed for binding of AAG to undamaged 5'-FAM-labeled DNA can be attributed to binding near the fluorophore and restricting its movement. To address the question of whether AAG interacts directly with the FAM or to a specific DNA sequence context or structure upstream of the eA site, we measured the anisotropy of AAG binding to undamaged DNA with an alternate sequence or with the FAM on the 5'-end of the complement strand (Figure 7). We found that there is no significant difference in the fluorescence anisotropy for these different DNA, strongly suggesting that AAG is binding preferentially near to the FAM label regardless of the DNA sequence or location of the label (Figure 7A). The total FAM fluorescence for the different DNA duplexes show differences in the amount of fluorescent change, with the alternative sequence quenching to a lesser extent and the 5' FAM on the complement strand exhibiting a small increase in FAM fluorescence (Figure 7B). The different extent of quenching can be at least partially attributed to the different terminal base pair for the oligonucleotides that were tested (Figures 1 and 7C). These relative fluorescence values for the free DNA duplexes are consistent with quenching by a terminal deoxyguanosine nucleotide, and the DNA with a terminal T•A base pair gives the highest fluorescence and leads to the greatest amount of quenching upon AAG binding to DNA (similar results were observed with different length oligonucleotides in Figure 5B).⁴⁴

Affinity of AAG for Undamaged DNA with a 5' FAM Label.

Although the fortuitous interaction of AAG with a 5' FAM dye provided a large signal change that makes it easy to detect binding, it also raised the concern that the DNA binding affinity could be altered due to the presence of this fluorophore. In order to quantify the effect of the fluorescein in DNA binding affinity we first measured the equilibrium binding to undamaged DNA containing a 5' FAM label and then used competition experiments to measure the affinity of unlabeled DNA.

To measure the affinity of AAG for binding to the FAM-labeled undamaged DNA we used a much lower concentration of DNA (5 nM) and varied the concentration of AAG (Figure 8). Under the standard conditions of 100 mM NaCl, both anisotropy and total fluorescence showed high affinity binding that was best fit by a single binding event, presumably because the first protein bound gives rise to the full amount of quenching and most of the change in anisotropy. As the apparent K_d value obtained from the fit is close to the concentration of the labeled-DNA it is likely that these conditions do not reflect equilibrium binding and this value should be considered an upper limit for the true K_d value (Figure S14). Increasing the DNA concentration to 15 nM shifted the apparent K_d value, confirming that binding affinity at 100 mM NaCl was too tight to allow for accurate determination using the signal from the FAM fluorescence ($K_d \approx 5$ nM). We therefore increased the sodium concentration to 200

mM, as this is predicted to weaken DNA binding affinity and facilitate quantitative analysis.⁶ The anisotropy and total FAM fluorescence for AAG binding both showed an increased K_d value at this higher salt concentration (Figure 8). There was no change in the observed K_d value when the DNA concentration was increased to 15 nM, confirming that these measurements were made under equilibrium binding conditions (Figure S14). Hyperbolic fits to a one-site binding model yielded K_d values of 68 nM for the quenching of the FAM fluorescence and 146 nM for the anisotropy value (Figure S14; 5 nM condition). However, since the equilibrium binding shows a one-site binding model, we corrected the anisotropy to account for the 30% quenching in total FAM fluorescence. This correction provides a K_d value of 96 nM, which is in reasonable agreement with the K_d value of 68 nM obtained from the total fluorescence (Figures 8A and 8B).

We next performed EMSA experiments for binding of AAG to the identical undamaged DNA (5 nM) at both 100 mM and 200 mM ionic strength (Figure 8C). Quantification of the EMSA results gave K_d values that are in reasonable agreement with the measurement of FAM quenching in solution (Figure 8D). It is reassuring that two different assays are in agreement, however we consider the solution (fluorescence-based) approach to be more reliable than the EMSA approach, because there was more variability in the EMSA data and some of the AAG•DNA complexes appear to dissociate during electrophoresis.

Affinity of AAG for Undamaged DNA Measured by Competition Assays.

We took advantage of the large change in anisotropy that was observed for binding to the FAM-labeled DNA (Figure 8A), and determined the affinity for both labeled and unlabeled DNA species by competition. We first formed an equimolar complex between AAG and the 5'-FAM labeled 19-mer undamaged DNA, and then mixed this complex with various amounts of undamaged DNA as competitor (Figure 9; Table 1). Unlabeled DNA of the identical sequence exhibited a macroscopic K_i value of 110 nM (Table 1). An analogous 19-mer undamaged DNA with a 5'-DY647 label gave a K_i value of 93 nM that is essentially the same, indicating that AAG does not bind preferentially to DY647 (Figure 9A). We also investigated the ability of different sized undamaged DNA to compete with the FAM-DNA bound AAG (Figure 9B). We expected to observe tighter binding for longer DNA duplexes, because they contain more nonspecific binding sites. However, only a modest correlation between length of the inhibitor and the inhibition constant was observed suggesting that AAG prefers binding near the ends of DNA (Figure 9B and Table 1). An alternate 25-mer sequence was also tested to address the possibility that a particular sequence was important for AAG binding. No sequence specificity was observed, as these different oligonucleotides bind with similar affinity (Table 1). Although it is likely that AAG has a preference for DNA end binding, we observed that the 49-mer is a better inhibitor than either the 19 or the 25-mer, which indicates that AAG is sampling the increased number of nonspecific binding sites in the longer DNA. The preference for end binding is consistent with the DY647 fluorescence changes observed under titration conditions (Figure 6C). A microscopic K_i value of AAG for undamaged DNA can be calculated by multiplying the number of binding sites on each inhibitor by the macroscopic K_i value for the entire oligonucleotide (Table 1). This calculation ignores any preference for end-binding and assumes that AAG binds equally to all sites. Therefore, these values must be treated with caution. Nevertheless, these

data suggest that AAG binds to undamaged DNA in the low micromolar range in the presence of 200 mM Na⁺.

Affinity of AAG for Nonspecific Single-Stranded DNA.

AAG exhibits a preference for excision of lesion bases present in double-stranded (ds) DNA, but it has been shown to catalyze excision of some lesions present on single-stranded (ss) DNA, particularly ϵ A.^{45–47} To compare binding of ssDNA and dsDNA, we used fluorescence anisotropy methods to observe the binding of AAG to undamaged and ϵ A-DNA in a single-stranded context (Figure 10 and Figure S15). As was observed for dsDNA, AAG binding to ssDNA caused a large change in anisotropy. It was surprising to observe no significant difference between titration of ϵ A-containing and undamaged DNA, because AAG is able to recognize and excise ϵ A from ssDNA.^{47,48} This apparent lack of discrimination is likely attributed to the intrinsic flexibility of ssDNA, which could allow interactions with the 5' FAM label even when the protein is bound to a more distant site in the linear sequence. Therefore, some fluorescence quenching and enhanced anisotropy occur regardless of where the protein is bound and continues to change as additional proteins are bound. Comparison of ssDNA and dsDNA titrations shows some notable differences. For ssDNA, two molecules of AAG were required to achieve the full extent of fluorescence quenching (Figure 10B) and more than four molecules of AAG are needed to reach the full change in anisotropy (Figure 10A). This suggests that a greater number of binding sites exist for AAG on ssDNA as compared to dsDNA.

The EMSA approach did not prove to be very informative for characterizing the ssDNA complexes with AAG, because the DNA was retarded into lower mobility complexes without formation of discrete bands (Figure S16). It is likely that an ensemble of states can form in which AAG is bound to many different sites, leading to a wide range of electrophoretic mobility. However, the affinity for ssDNA could be measured with competition assays (Figure 10C). Interestingly, the macroscopic K_d values for the 19-mer ssDNA were only 2-fold larger than the K_d values for 19-mer dsDNAs (Table 1). The difference in affinity for ds and ss DNA can be attributed to both differences in the number of binding sites and in the average affinity for nonspecific ssDNA and dsDNA sites. If we assume a site size of 5 and 6 bp nucleotides for ss and ds substrates, respectively (4 AAG on 19-mer ss; 2 AAG on 11-mer ds) then the microscopic K_d for AAG binding is actually very similar (Table 1). It has been observed that several other DNA repair proteins, exhibit similar energy for nonspecific binding of dsDNA and ssDNA.⁴⁹

CONCLUSIONS

Systematic comparison of anisotropy and fluorescence quenching for nonspecific and specific DNA with FAM and DY647 labeled oligonucleotides has enabled a minimal characterization of AAG binding energy and binding density. Examination of the literature for other DNA binding proteins provides evidence that the behavior that we observe for AAG may be relatively common. Most, if not all DNA repair proteins use nonspecific DNA binding interactions to search DNA for target sites. This affinity for nonspecific DNA can complicate equilibrium measurements. We present a unified model to explain these

observations with different labels and different assays that could be useful for characterization of other DNA repair enzymes (Figure 11). On the one hand it would appear to be advantageous to avoid using fluorescein-based dyes for anisotropy studies, because they are environmentally sensitive and poorly coupled to the tumbling of the nucleic acid to which they are attached. Cyanine and rhodamine dyes exhibit less complex spectroscopic properties.^{39,40,50} On the other hand, these properties can be easily interrogated by measurement of total fluorescence and can give rise to unique signals for the measurement of DNA binding. In the case of AAG, the large change in anisotropy associated with binding near to a FAM label provides a robust assay for competitive binding and allows specific and nonspecific binding events to be readily distinguished.

Stoichiometry and Binding Density for AAG.

Although the sigmoidal anisotropy and total fluorescence measurements that we observe for AAG binding to short oligonucleotides is consistent with a dimerization model for AAG binding, a more complete analysis supports the previously favored monomer model. This model invokes specific binding by an AAG monomer followed by high affinity, but weaker, binding to nonspecific sites. We found that AAG can bind at high density to both duplex and single-strand DNA. For single-strand DNA, we can estimate a binding density of one protein molecule per 5 nucleotides based upon the observation that 4 proteins can bind to a 19-mer oligonucleotide. This is similar to the binding density of AAG on duplex DNA, with 2 AAG molecules binding to a short hairpin corresponding to a density of approximately one protein molecule per 6 bp. This density is very close to the footprint that is observed in the crystal structure of the AAG•DNA complex of 5–6 bp (a 6 bp footprint was assumed for the duplex DNA).¹⁷ The absence of strong interference between closely bound proteins suggests that AAG is capable of searching very small sites of accessible DNA in chromatin. This is fully consistent with observations that AAG can excise damaged bases from some solvent accessible positions of the nucleosome without disrupting the nucleosome structure.⁵¹ Nevertheless, AAG and other glycosylases are inhibited by well positioned nucleosomes and it is expected that DNA breathing and/or repositioning of the nucleosome would be a factor that influences the accessibility of DNA damage to repair proteins.^{51–56}

It is interesting to compare the features of AAG binding to DNA with other DNA repair glycosylases, because these enzymes have independently evolved and use different folds to perform similar biological functions. All known DNA repair glycosylases appear to function as monomers, and many different enzymes have been crystallized as monomers bound to DNA substrate or inhibitor sites.⁸ Although thymine DNA glycosylase is capable of binding as an apparent dimer to DNA,²⁵ biochemical experiments demonstrated that the monomeric form of the enzyme is fully active.⁹ Mismatch uracil DNA glycosylase has been suggested to function as a dimer based on biochemical experiments that included fluorescence anisotropy binding assays.¹⁰ This finding should be viewed with some caution because the pattern of anisotropy is very similar to what we have now observed for AAG, but it is possible that this enzyme is the exception to the monomeric rule. Very recently restriction modification enzymes have been discovered in which dimeric, sequence specific DNA glycosylases induce nicks in both strands of unmethylated DNA via glycosylase mechanisms⁵⁷. In this case, there are obvious advantages to a dimer as a restriction enzyme. In contrast, base

excision repair is most effective when repair is initiated on a single-strand to ensure an intact template strand for repair synthesis.

Binding Affinity of AAG for Nonspecific DNA.

Using anisotropy competition assays, we were able to determine the binding affinity of AAG for ss and ds DNA in the absence of labels. In the presence of 200 mM NaCl and pH 7.5, macroscopic K_d values of 100–200 nM were measured for short oligonucleotides (Table 1). Considering a model of overlapping nonspecific binding sites, we calculate a rough estimate of 3–6 μ M for an average site in dsDNA which is very similar to the estimate of 3 μ M for binding to an average site in ssDNA. The similar affinity of AAG for duplex and ssDNA is consistent with the previous findings that ssDNA contributes to processive searching of DNA,²² and the AAG•ssDNA complex is sufficiently long-lived to allow for DNA annealing without dissociation of AAG.⁴⁷ However, it is surprising that similar binding affinity is observed for ssDNA and dsDNA, because the glycosylase activity of AAG shows a strong preference for duplex DNA. For example, a 6000-fold preference for duplex over single-strand DNA has been reported for AAG-catalyzed excision of hypoxanthine.⁴⁶ This suggests that nonspecific electrostatic interactions are similar with ssDNA and dsDNA, but only in dsDNA is AAG capable of making more extensive specific contacts. This is consistent with the crystal structure of AAG, in which DNA intercalation and contacts with the opposing strand would not be possible in ssDNA. For example, I161 and Y162 intercalate into the DNA and appear to stabilize the extrusion of the flipped out base in the structure of AAG bound to ϵ A-DNA.¹⁷ There are also extensive contacts between the DNA and the side chains of T143 and M164, and electrostatic interactions are formed between R141 and K229 and the phosphodiester backbone. We should note that this current study and the described crystal structure use a truncated form of AAG, which lacks the first 79 N-terminal amino acids that are not conserved among AAG homologs. Full-length AAG has been shown to be more processive than the truncated protein^{6,21} and therefore the affinity of the full-length protein for undamaged DNA is likely to be higher.

A key characteristic of DNA repair glycosylases is that they efficiently search the genome to locate rare sites of damage, but there appear to be significant differences in their biochemical properties. For example, AAG binds much more tightly to nonspecific DNA as compared to OGG1 (8-oxoguanosine DNA glycosylase) and UNG (uracil DNA glycosylase), two other human glycosylases that have been extensively characterized.^{58–60} In line with tighter nonspecific DNA binding, AAG is a more processive enzyme than either OGG1 or UNG under similar solution conditions.^{3,6,59,61} Tighter nonspecific binding, and its associated slower rates of exchange with other sites, carries over into other steps of the multi-step damage recognition process. For example, UNG catalyzes base flipping orders of magnitude faster than AAG, possibly reflecting the narrower window of time a rapidly dissociating enzymes would have to engage target sites as compared to a more slowly dissociating enzyme.^{18,62,63}

Consideration of Fluorescent Labeling Strategies for Characterization of Protein-DNA Complexes.

Fluorescence anisotropy is a versatile technique for characterizing protein-DNA complexes.^{28–30} It is generally advised that multiple labeling strategies be employed to vary the identity and position of the label, but in practice it is rare for systematic comparisons to be performed. In the current study we observed dramatic differences between FAM-labeled and DY647-labeled DNA. These data suggest that AAG interacts with a terminal FAM label and this gives rise to large changes in the fluorescence anisotropy of the protein•DNA complex. Once this is understood, fluorescence anisotropy provides a robust assay for DNA binding and allowed us to distinguish between specific and nonspecific binding modes. Results obtained with many different DNA binding proteins and FAM-labeled DNA suggests that two general patterns are observed. The most simple involves relatively small anisotropy values for binding, suggesting that in these cases there is no interaction between the protein and the FAM-label.^{43,64–67} In other cases, large anisotropy values have been obtained for similar or smaller sized proteins.^{10,58,60} Large changes in anisotropy can occur if mobility of the fluorophore is restricted on the timescale of rotational diffusion due to steric constraints or fluorophore-protein interactions.³⁰ In light of the results that we observe for AAG, we propose that very large observed changes in the anisotropy of FAM-labeled DNA should be treated with caution. Several different DNA glycosylases have been studied with FAM or the closely related HEX labels and binding has been reported to give large changes in anisotropy.^{10,58,60} In these cases it is useful to measure the total fluorescence to ask whether there is an enhancement or reduction in fluorescence.^{30,60} Even if no change is observed, this could reflect similarities between the collisional quenching that can be observed with guanosine nucleotides and with the protein environment that interacts with the dye (see Figure 7C).⁴⁴ Therefore, it is prudent to test a different type of dye. We find that DY647 exhibits different properties from FAM and provides a useful comparison to characterize DNA binding. Similarly Texas Red has been reported as an excellent choice for a fluorescent label that is tightly coupled to DNA and is insensitive to local environment.^{9,39,50} Regardless of the fluorophore employed, we have demonstrated the utility of determining the stoichiometry of DNA repair proteins binding to oligonucleotides, and the importance of comparing changes in both total fluorescence and fluorescence anisotropy in distinguishing different binding modes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

AUTHOR CONTRIBUTIONS and ACKNOWLEDGMENT

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ABBREVIATIONS and KEYWORDS

AAG	human alkyladenine DNA glycosylase (also called MPG, N-methylpurine DNA glycosylase; ANPG, alkyl-N-purine DNA glycosylase)
BER	base excision repair pathway
DY647	DyLight 647
εA	1, <i>N</i> ⁶ -ethenoadenine
EMSA	electrophoretic mobility shift assay
FAM	6-fluorescein
HEX	6-hexachlorofluorescein
Pyr	pyrrolidine

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Figure 1. DNA oligonucleotides.

(A) In each case X reflects A (undamaged), 1,*N*⁶-ethenoadenine (E), or pyrrolidine (Y). The TXC and AXA nomenclature identifies the underlined nucleotides flanking the X. The 11mer TXC oligonucleotide formed a hairpin structure as shown, and all other duplexes were annealed with a 1.2-fold excess of the complementary strand (lowercase letters). Unless otherwise indicated, fluorophores were attached at the 5'-end of the top strand. Structure of 5' FAM (B) and 5' DY647 (C).

Figure 2. Fluorescent measurements for binding of AAG to 5' FAM-labeled ϵ A-DNA under titration conditions.

Fluorescence anisotropy (A, B), normalized total FAM fluorescence (C, D), and normalized ϵ A fluorescence (E) of the FAM-labeled 19AEA DNA measured with increasing amounts of AAG. Two DNA concentrations were used and signals were plotted versus AAG concentration (A, C) or the ratio of AAG to DNA concentration (B, D, E). To facilitate comparison, the data at 100 and 300 nM DNA were averaged together and plotted in panel F. The ϵ A quenching was fit by a 1:1 binding model (Eq 10) and DynaFit was used to fit anisotropy and total fluorescence data with a 2-site binding model (Figure S2). The

anisotropy was corrected to account for FAM quenching (Eq 5 and Figure S3). The average $\pm$ SD is shown (N = 4).

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Figure 3. Comparison of AAG binding to 5' FAM-labeled ϵ A-containing and undamaged DNA under titration conditions.

Anisotropy (A) and normalized FAM fluorescence (B) for AAG binding to 19AAA undamaged (solid circles) or 19AEA ϵ A (open circles) 5' FAM DNA. All data is the average of 100 nM and 300 nM DNA concentrations (Figure 2 and Figure S4). The undamaged DNA data were fit with DynaFit using a 2-site and 1-site model for anisotropy and total fluorescence, respectively (Figure S5). The ϵ A data were fit with 2-site models as described previously. The average $\pm$ SD is shown (N = 4).

Figure 4. Representative EMSA with 5' FAM-labeled ϵ A-containing and undamaged DNA. Equilibrium titration of AAG with 300 nM FAM-labeled 19AEA (A) and 19AAA (B) DNA. Stoichiometry of binding is consistent with the stoichiometry obtained from anisotropy measurements with the same DNA (Figure 3). Quantifications of independent experiments are averaged in C and D (N = 3). The data points are connected by lines to guide the eye.

Figure 5. Binding density of AAG on εA-DNA duplexes.

Anisotropy (A) and normalized total fluorescence (B) of 5'FAM-labeled TEC DNA of different lengths. The 11-mer and 19-mer anisotropy and total fluorescence data were both fit to a 2-site binding model in DynaFit. The 25-mer anisotropy data were fit to a 3-site model and the total fluorescence to a 2-site model. The 49-mer anisotropy and total fluorescence data were both fit to a 3-site binding model. In all cases, the first binding event corresponds to the recognition of εA by AAG. The second binding event corresponds to AAG binding near to the 5'FAM in the case of the shorter oligonucleotides, but is in competition with additional sites in the 49-mer DNA. Parameters from DynaFit models are shown in Figure S7. The average of two different substrate concentrations (100 nM and 300 nM) $\pm$ SD is shown (N = 3–6).

Figure 6. Comparison of binding assays employing 5'DY647-labeled and 5'FAM-labeled DNA. Raw anisotropy (A), change in anisotropy (B) and normalized total fluorescence (C) of 5'-end labeled 19TEC and 19TAC DNA. AAG was incubated with 100 nM or 300 nM DNA under titration conditions for each sample. For the binding of the undamaged DNA the anisotropy was fit to a 2-site model and the total fluorescence was fit by a 1-site model. For the binding of ϵ A-DNA, both anisotropy and total fluorescence were fit to a 2-site model (See Figure S13 for the model and best fit parameters). The average $\pm$ SD is shown (N = 3).

Figure 7. Fluorescence measurements for FAM-labeled undamaged DNA oligonucleotides. Anisotropy (A) and normalized total FAM fluorescence (B) of undamaged 19-mer DNA. The FAM label was located on the 5' end of the indicated strand adjacent to a T•A pair (19-mer = 19AAA; replotted from Figure 3), a C•G pair (19alt), or a G•C pair (19-mer complement = 19AAA). Anisotropy and FAM fluorescence were fit to a 2-site and 1-site model as described for undamaged DNA substrates in Figure S5. (C) Total FAM fluorescence intensity of the different free DNA is shown relative to the 19AAA duplex for experiments with 300 nM DNA. Interaction with guanine in the terminal base pair causes

quenching of FAM fluorescence and accounts for the differences observed for binding of AAG. The average $\pm$ SD is shown (N = 3).

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Figure 8. Equilibrium binding to 5' FAM-labeled undamaged DNA duplex at two concentrations of NaCl.

Corrected anisotropy (A) and normalized total fluorescence (B) of AAG binding to 5 nM 5' FAM-labeled 19AAA DNA. The data were fit to a hyperbolic dependence representing one binding event (Eq 6). The K_d values for the corrected anisotropy/total fluorescence data are 7/5 nM and 96/68 nM at 100 and 200 mM ionic strength, respectively. The average $\pm$ SD is shown (N = 3, 100 mM I; N = 2, 200 mM I). (C) Representative EMSA showing binding of increasing amounts of AAG to 5 nM 5' FAM-labeled 19AAA DNA. (D) Quantification of EMSA experiments fit to a hyperbolic dependence (Eq 6). The K_d values are 6 and 34 nM for the 100 and 200 mM ionic strength conditions, respectively. The average $\pm$ SD is shown (N = 3). Both the anisotropy and EMSA data were corrected to account for the 30% quenching of FAM upon AAG binding.

Figure 9. Competition assay to measure binding of undamaged DNA duplexes.

Anisotropy of 150 nM AAG and 150 nM 5' FAM-labeled 19AAA DNA was determined at 200 mM ionic strength with increasing amounts of inhibitor DNA. (A) Comparison of unlabeled and 5'-DY647 labeled undamaged 19TAC DNA inhibitors. (B) Comparison of different sized undamaged unlabeled TAC inhibitors. All data were fit with a one-site competitive binding model (Eq 7) and results are shown in Table 1. The average $\pm$ SD is shown ($N = 3$).

Figure 10. Fluorescence measurements for binding of AAG to single-stranded 19-mer DNA. (A) Anisotropy and (B) normalized total FAM fluorescence of 5'FAM-labeled single-stranded 19AEA and 19AAA DNA substrates under standard buffer conditions (100 mM ionic strength). Anisotropy and FAM fluorescence data were fit to a 4-site and 2-site model in DynaFit, respectively. The average of two different substrate concentrations (300 nM and 600 nM) $\pm$ SD is shown (N = 3). Double-stranded 19AAA data is replotted from Figure 3 for comparison. (C) Anisotropy of 150 nM AAG and 150 nM 5'FAM-labeled 19AAA DNA incubated with increasing amounts of inhibitor DNA at 200 mM ionic strength conditions.

Inhibitors are double-stranded 19TAC (replotted from Figure 9), single-stranded 19TAC and a single-stranded alternate 19-mer. Data were fit to a one site competition binding model (Eq 7) and results are shown in Table 1. The average $\pm$ SD is shown (N = 2).

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Figure 11. Model for binding of AAG to ϵ A-containing and undamaged DNA.

In the case of 5'FAM-labeled oligonucleotides, AAG binds ϵ A specifically with high affinity (A). If ϵ A is absent (B) or has already been bound, then AAG directly interacts with the FAM, causing a both quenching of the FAM fluorescence and increased anisotropy due to restricted mobility and tighter coupling of the FAM to the protein-DNA complex. In the case of 5'DY647 labeled DNA (C and D), the fluorophore is already tightly coupled to the DNA and little difference is observed between specific and nonspecific binding events, although data suggest a preference of AAG for binding near a DNA end (Figures 6C and 9).

Table 1.Equilibrium constants for binding of AAG to nonspecific DNA^a

Inhibitor	IC_{50} (nM)	K_i , macro (nM)	# Binding sites	K_i , micro (μ M)
19-mer	410 $\pm$ 50	130 $\pm$ 20	28	3.6
19-mer DY647	290 $\pm$ 50	90 $\pm$ 16	28	2.5
25-mer	280 $\pm$ 30	87 $\pm$ 10	40	3.5
25-mer (alt)	400 $\pm$ 20	120 $\pm$ 10	40	5.0
49-mer	230 $\pm$ 20	71 $\pm$ 6	88	6.3
19-mer ss	690 $\pm$ 50	220 $\pm$ 20	15	3.2
19-mer (alt) ss	610 $\pm$ 40	190 $\pm$ 10	15	2.9

^aThe IC_{50} values were determined at 200 mM NaCl for duplex DNA (Figure 9) and for single-strand DNA (Figure 10C). Equations 7–9 were used to calculate the macroscopic K_d and microscopic K_d values. The number of binding sites was estimated with a 6 bp footprint for duplex DNA and a 5 bp footprint for single-strand DNA. The K_d value for the 5'FAM-19AAA competition experiments was taken to be 68 nM based on the FAM quenching data in Figure 8B.