DNA Binding Dynamics

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Single-Molecule Approach to DNA Minor-Groove Association Dynamics**

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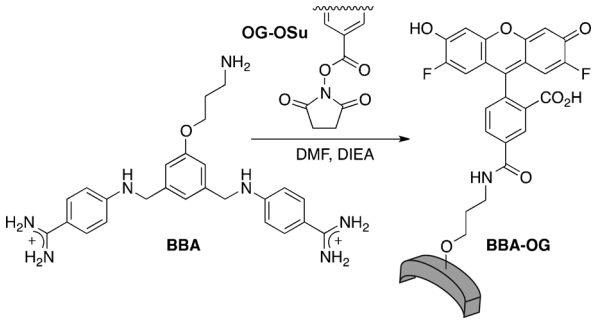
Chemists have long pursued the design and preparation of small molecules that can recognize specific DNA sequences. Deciphering the human genome and on-going efforts to sequence the genomes of many other organisms have provided a wealth of information about DNA targets of both therapeutic and diagnostic interest, and therefore there is a renewed interest in the development of smart DNA minor-groove binders.[1,2] Despite the detailed structural and thermodynamic information available regarding the interaction of a number of minor-groove binders with DNA, kinetic data are much more scarce. In addition, the very low dissociation rates typical of these agents result in very slow binding dynamics, which are hard to measure experimentally.[3] Typically, stopped-flow methods are used, but this technique can yield complex kinetics and artifacts arise from the relatively high binder concentrations required. Moreover, the dissociation rates are usually obtained with indirect SDS-sequestering techniques that only give apparent rates. Dynamic data of archetypical minor-groove binders such as distamycin or Hoechst 33258 indicate that the association process is very fast and nearly diffusion limited, which is surprising given the severe geometric constraints imposed upon the inclusion of a binder into the narrow minor groove.[4–7]

Cationic bisbenzamidines are able to target AT-rich sequences preferentially over those containing GC pairs. Prominent examples of this family of molecules, such as pentamidine or furamidine, have found clinical applications and are used for the treatment of several major tropical diseases.[8] Nevertheless, the toxic side effects of these bisbenzamidines[9] have encouraged the search for new derivatives with improved efficacy, improved pharmacological properties, and reduced adverse effects.[10] Bisbenzamidines bind short AT-rich DNA sequences by insertion in the narrow minor groove. NMR spectroscopy and X-ray crystallographic studies agree on the general model of the interaction, wherein the positively charged amidinium groups are situated deep in the minor groove, making both direct and indirect hydrogen bonds with the DNA bases and electrostatic contacts with the bottom of the groove.[11]

Recently, we reported several rapid and practical approaches to synthesizing bisbenzamidine DNA binders, some of which can be easily conjugated to functional groups or fluorophores.[12] Using these methods, we made the conjugate **BBA-OG** (Scheme 1), which features an Oregon Green (OG) fluorophore attached to the DNA binder, bisbenzamidine (BBA). We envisioned that the presence of a negatively charged carboxylate in the fluorophore would increase the DNA dissociation rate, and thereby allow us to extract kinetic information using fluorescence correlation spectroscopy (FCS). This technique provides a sensitive single-molecule approach to study the fast kinetics of biomolecular interactions,[13] yielding accurate values for the binding equilibrium constant, the association and dissociation rate constants, and the diffusion coefficients,[14,15] from which the diffusion-controlled rate constants can be estimated.[16] To the best of our knowledge, this technique has not been previously used to analyze the dynamics of minor-groove binders. The wide dynamic range of FCS makes it possible to identify the rate-limiting step in the association event by comparison of the dynamics of the association to DNA with different sequences and chain lengths, and thus to evaluate processes such as sliding or two-dimensional diffusion, which



Scheme 1. Synthesis of Oregon Green-labeled bisbenzamidine **BBA-OG**. DMF = dimethylformamide; DIEA = *N*,*N*-diisopropylethylamine.

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have been proposed as likely mechanisms for the binding of proteins to DNA.[16,17]

FCS titrations of **BBA-OG** with three short hairpin (**hp**) oligonucleotides (12 bp + loop) containing as key target sequences AAATTT, AATTT, and a non-target control GGCCC and with a longer dsDNA (50 bp) containing one AAATTT site, were performed. For the **AAATTT-hp**, an additional FCS titration at a higher salt concentration was measured (see Supporting Information for full DNA sequences). Figure 1 shows normalized fluorescence correlation

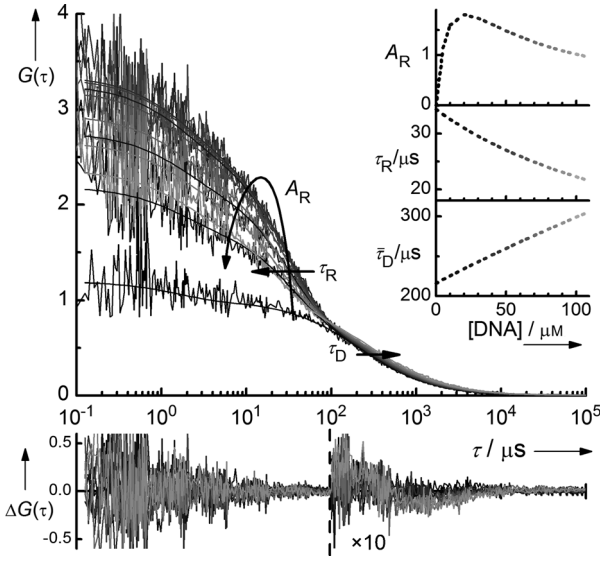


Figure 1. FCS curves of the titration of **BBA-OG** with **AAATTT-ds** and results of a global fit of the dynamic-equilibrium model (Supporting Information, Equation S1–S4). Insets: variation of the mean diffusion time $\bar{\tau}_D$, the relaxation time τ_R, and the relaxation amplitude A_R with the [DNA] calculated from the fit parameters. Lower panel: unweighted fit residuals. The small deviations at high DNA concentrations are from minor fluorescent impurities in the DNA samples. Above 100 μs a factor of 10 was applied.

curves of the binding of **BBA-OG** to the longer dsDNA. Without DNA, only a translational diffusion term of the binder with correlation time τ_D is observed. Addition of DNA increases τ_D, reflecting the slower diffusion of the dye when bound to DNA. Additionally, a new term appears with a shorter correlation time τ_R, which decreases as the concentration of DNA is increased (see insets in Figure 1). τ_R is the relaxation time of the reversible binding process: $\tau_R = (k_+[\mathrm{DNA}] + k_-)^{-1}$ with k_+ and k_- being the association and dissociation rate constants, respectively. The large amplitude A_R of this term is because of the large increase in the brightness of bound **BBA-OG**.[18] The diffusion and relaxation terms show the expected concentration dependence and are independent of the irradiance.[18,19] To reduce parameter correlation, the FCS titration series were analyzed by global target fits[14,15] with a common dynamic-equilibrium model of the diffusion, triplet, and relaxation terms (Figure 1 and Figure S1–S4). The parameters that best fit the whole data set are given in Table 1 and Figure 2.

Table 1: Association (k_+) and dissociation (k_-) rate constants and binding equilibrium constant (K) of **BBA-OG** with dsDNA. The values of k_+ are calculated from the fit parameters k_- and K ($K = k_+/k_-$).

DNA[a]	k_+ [$\times 10^8$ M^{-1} s^{-1}]	k_- [$\times 10^4$ s^{-1}]	K [$\times 10^3$ M^{-1}]
AAATTT-hp[a]	1.36 ± 0.01	3.19 ± 0.02	4.27 ± 0.03
AATTT-hp[a]	0.95 ± 0.01	4.89 ± 0.05	1.95 ± 0.02
GGCCC-hp[a]	0.083 ± 0.003	14.0 ± 0.4	0.059 ± 0.001
AAATTT-ds[a]	1.56 ± 0.02	2.84 ± 0.02	5.47 ± 0.05
AAATTT-hp[b]	0.65 ± 0.03	4.63 ± 0.01	1.41 ± 0.03

[a] [NaCl] = 0.1 M, [b] [NaCl] = 1.0 M. See the Supporting Information for complete oligonucleotide sequences.

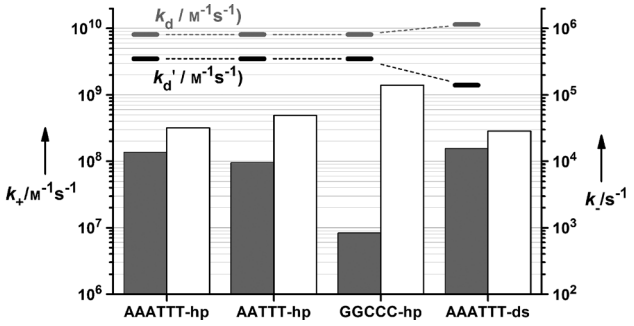


Figure 2. Association (gray, left scale) and dissociation (white, right scale) rate constants of **BBA-OG** with DNA, calculated from FCS measurements. Estimates of the diffusion-controlled association rate contants k_d and k_d', defined in Scheme 2 (see Supporting Information).

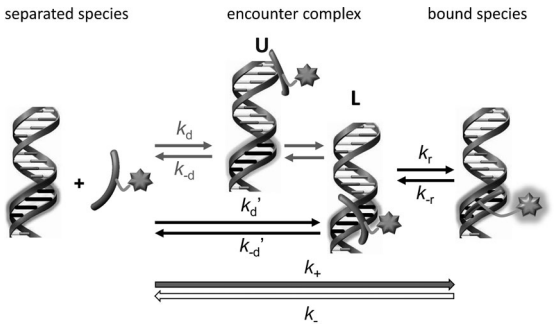
Figure 2 shows that the association rate constant decreases by more than one order of magnitude from the AT-rich to the GC-containing sequences, whereas the dissociation rate constant increases three to five times. Both association and dissociation contribute to the specificity for the AT-rich tracts, but the association has a higher effect. This is in agreement with reported results for the binding of the antibiotic distamycin to sequences with different numbers and positions of mismatched sites.[4] Moreover, the absolute values of k_+ for the AT sequences coincide well with those reported for other minor-groove binders[4–7] and show the small decrease with increasing Na^+ ion concentration expected for groove binders,[20] suggesting they might have analogous association mechanisms. Nevertheless, the dissociation rate constants are about four orders of magnitude higher than those of typical minor-groove binders, resulting in much lower binding constants.

The above results indicate much weaker specific interactions between **BBA-OG** and the DNA minor groove, which is probably owing to the electrostatic repulsion between the negatively charged OG moiety and the DNA phosphate backbone. The higher binding constants of unlabeled bisbenzamidines supports this explanation.[12] On this basis, we can consider **BBA-OG** as a valid model for the study of the association process of small molecules binding to the minor groove of DNA.

To determine the rate-limiting step for the association process, we compared the dynamics of binding to the short DNA **AAATTT-hp** with that to the much longer **AAATTT-ds**, which has been designed to avoid additional AT-rich sites.

Curiously, we did not find significant differences in the binding dynamics of these two duplexes (Table 1, Figure 2). The similar dissociation (k_-) is readily explained in terms of similar specific interactions with the AAATTT sequence. However, if diffusion plays an important role, the association dynamics should be affected by the increase of the DNA length.

For a more detailed analysis, we propose a two-step mechanism like that previously used to describe other supramolecular binding processes (Scheme 2).[14,15] The first step is the formation of an encounter complex between DNA and binder, with a diffusion-controlled association rate which can be estimated based on the geometry of the DNA and binder. The second step is the insertion of the binder into the minor-groove with a rate constant k_r, a process which conveys structural rearrangements or the breakdown and reconstitution of the network of water molecules surrounding the interacting species. The experimentally determined k_+ corresponds to the overall reaction described by a single relaxation time and is determined by the rate-limiting step. This mechanism is therefore different to the sequential model proposed for the binding of Hoechst 33258 to DNA[7] which involves two reactions, with their corresponding two observed reaction times.



Scheme 2. Two-step mechanism proposed for the binding of **BBA-OG** to DNA involving the formation of an encounter complex, with the binder localized at the DNA reactive site (L) or unlocalized (U).

Assuming a rod-like structure of the DNA with a localized reactive site, we estimate lower bounds (see Supporting Information) of the diffusion-controlled association rate constants k_d and k_d' for the formation of unlocalized (U) and localized (L) encounter complexes, respectively, where the binder is either located arbitrarily at any position of the DNA (U) or already localized near to the reactive site (L) (Scheme 2).[16]

The estimated values of k_d' for the formation of localized complexes L are of course significantly lower than those of k_d corresponding to the formation of U, especially in the case of the long dsDNA (Figure 2). Nevertheless, even for the most specific sequences, both k_d and k_d' are at least one order of magnitude higher than the observed overall rate constant k_+. This shows that the diffusion-controlled formation of the encounter complex is not the rate-limiting step, even if the binder is required to be located near the reactive sequence (L complex). The encounter complexes U and L are in rapid pre-equilibrium with the separated species so that the overall rate of association between **BBA-OG** and DNA is not diffusion limited but determined by the next step of the process, namely the unimolecular inclusion into the minor groove. This step is probably controlled by geometric and orientational requirements, which depend on the critical dimensions of both the binder and the minor groove. The presence of an exocyclic 2-amino group in the minor groove of GC tracts[21] may explain the much lower value of k_+ obtained for **GGCCC-hp**.

We can also conclude that processes that increase the rate of formation of localized encounter complexes, such as sliding or two-dimensional diffusion along the DNA strand,[16,17] do not play a key role in the association rate of **BBA-OG**, and probably also do not for other minor-groove binders.

In summary, our results indicate that the association process of **BBA-OG** to dsDNA is not controlled by diffusion, but by the rate-limiting insertion of the binder into the minor-groove. Moreover, we find that the association process has an important effect on the specificity to AT-rich sites, whereas the differences in the binding affinity are mainly determined by the dissociation rate. This mechanism might constitute a general mechanism for small minor-groove binders, but this should be confirmed with further studies on more typical minor-groove agents. This information should be useful for the design of new DNA binders with optimized properties and for the future understanding of the behavior of these molecules in more complex cellular environments. This work has also shown the potential of FCS for the study DNA binding dynamics of minor-groove binders using labeled derivatives.

Experimental Section

Oligonucleotides were purchased from Thermo Fisher Scientific Inc. Nucleotide sequences and the procedure for reconstitution and annealing are provided in the Supporting Information. Details of the synthesis of the **BBA-OG** can also be found in the Supporting Information. Experimental setup and conditions, as well as the details of the data analysis for the FCS measurements have been published elsewhere[18,22] and are specified for these experiments in the Supporting Information. The uncertainties given in Table 1 represent one standard deviation as obtained by the fits.

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