The Kinetic Basis for Sequence Discrimination by Distamycin A

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Distamycin A (Dst), a naturally occurring polyamide antibiotic,[1] is one of the most extensively studied members of a class of molecules that bind in the minor groove of duplex DNA. In the last 10 years, the discovery of the 2:1 drug:DNA complex,[2] recent advances in incorporation of novel heterocycles into the Dst skeleton[3] and large-scale solid-phase synthesis of polyamides[4] have together resulted in molecules based on the Dst scaffold that can bind any desired sequence with subnanomolar affinity and remarkable specificity.[5] While a considerable database of thermodynamic[6] and structural information[7] of their complexes with DNA has been assembled, little is known about the *kinetic* basis for sequence discrimination by these drugs. Clearly, the availability of these drugs in vivo at a desired target site and their ability to compete with various DNA binding proteins will depend critically on their rates of association and dissociation at the abundant competing mismatched sites. Understanding the kinetics of Dst binding to these sites is therefore an important step toward evaluating the potential of polyamide drugs as efficient inhibitors of intracellular processes. In addition, because the commonly used technique for measuring equilibrium binding constants—quantitative DNaseI footprinting titration—is limited to solution conditions optimal for DNaseI cleavage, there is an imperative need for an assay to quickly determine the equilibrium binding constant of any given polyamide at its matched and mismatched sites under physiologically relevant solution conditions. Here we report fluorescence-detected equilibrium and stopped-flow measurements of Dst binding to a set of 10 bp duplexes with different 4 bp core sequences flanked by three GC base pairs on each side (Table 1).

The fluorescence properties of Dst provide a convenient signal for detecting binding to DNA. Fluorescence of Dst is strongly quenched in aqueous solutions and is significantly enhanced upon binding the hydrophobic and rigid environment of the minor groove of DNA.[8] While the difference in fluorescence enhancement upon binding poly dA-dT, calf thymus DNA, and poly dG-dC has been reported before, no systematic study of mismatched sequences has been carried out. Because two distinctive binding modes of Dst are known,[9] to simplify the interpretation of both equilibrium and time-resolved fluorescence studies, we chose a canonical 1:1 binding site and mismatched sequences corresponding to mutations at different positions within it. Five representative sequences and steady-state spectra obtained with each of them are illustrated in Figure 1.

Table 1. Macroscopic Association and Dissociation Rate Constants Measured and the Calculated Equilibrium Association Constants at 25 °C for Sequences GCG(X)CGC, where X is the Indicated 4 bp Core Sequence

site	k_{on} (μM^{-1} s^{-1})	k_{off} (s^{-1})	K_a ($=k_{on}/k_{off}$) (M^{-1})
ATTA	43.8(2.1)	1.75(0.09)	2.5×10^7
TTAG	44.5(3.9)	29.1(0.30)	1.5×10^6
AGTA[a]	16.0(1.9)	1.62(0.04)	(9.8×10^6)
TAGG	25.9(3.1)	51.0(0.26)	5.0×10^5
AGGA[a]	4.1(0.4)	22.2(1.21)	(1.8×10^5)
CAGC	n.m	251(31.0)	
CGGC	n.m	284(57.7)	

[a] Although the association kinetics show a bimolecular process, the values of K_a at these sites are provisional due to some contribution from binding in the 2:1 mode.

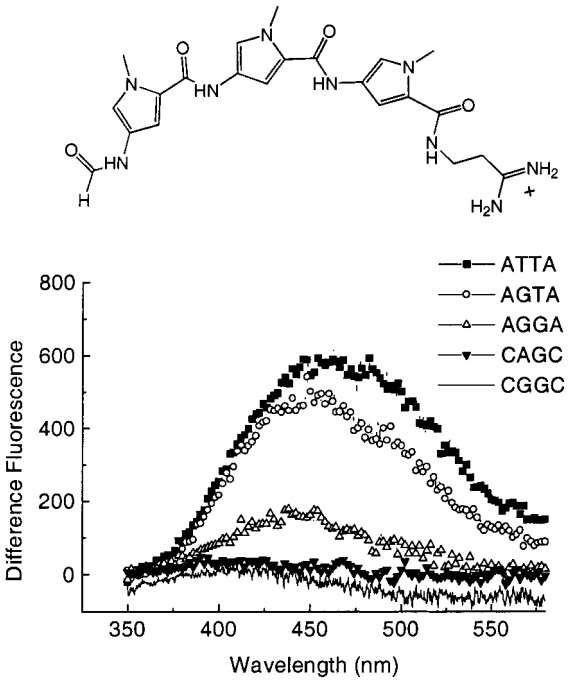

Figure 1. (A) Chemical structure of Distamycin A. (B) Equilibrium difference fluorescence emission spectra of Dst complexed with 10 bp duplexes GCG(X)CGC where X is the indicated 4 bp sequence. Equal volumes of 5 μM solutions of drug and DNA in TKMC buffer[5] were mixed and excited a 300 nm.

As expected, at 2.5 μM drug and DNA concentration, the degree of fluorescence enhancement decreases with increasing number of mismatches. Even a single base pair difference between the target sequence and the canonical Dst binding sequence is sufficient to give an observable difference in fluorescence enhancement. However, we note that at ~10-fold higher drug and DNA concentration, the observed total fluorescence for all sites differs by less than 15% (Supporting Information, Figure 1). This should allow the use of fluorescence signal as a semiquantitative assay for DNA binding in high throughput formats[10] at appropriate drug concentration.

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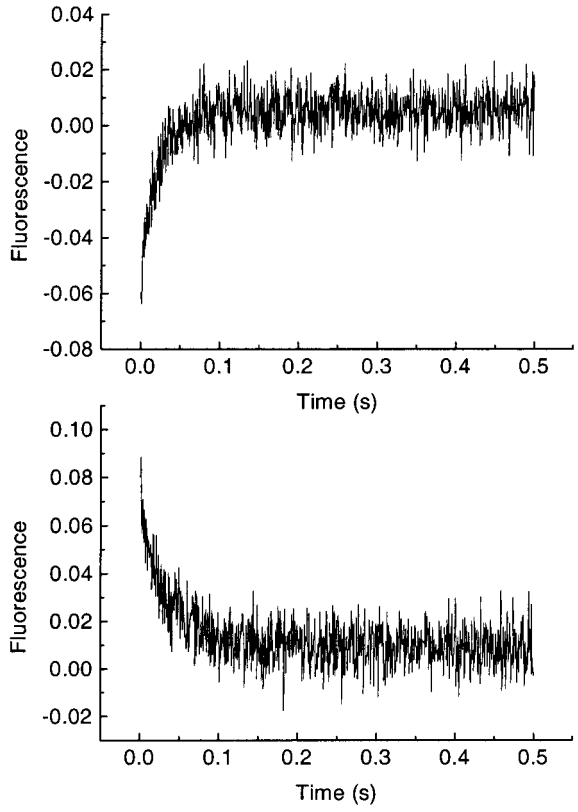

Figure 2. Fluorescence detected stopped flow kinetics of Dst binding to its target sites at a representative site: (A) association signal upon binding the AGGA core sequence and (B) dissociation from complex formed with AGGA.

Next we investigated the kinetic basis for the observed sequence-dependent fluorescence enhancement. In previous studies,[11] we had determined that the association rate constant for Dst binding to its canonical 1:1 site was close to diffusion limited. So it seemed likely that the mechanism for sequence discrimination by Dst might simply involve diffusion-controlled association of Dst with DNA regardless of the sequence, followed by equilibration of the mixture to the most stable complex based on differences in off-rates from various mismatched sequences. To test this proposal we measured the apparent association and dissociation rate constants of Dst binding to each of the sequences described above. Association rate constants were determined from the slopes of plots of apparent association rates vs drug concentration (Figure 2 in Supporting Information) and dissociation rates were determined using SDS sequestration (Table 1).[12] Clearly the differences in equilibrium binding constants cannot be ascribed simply to sequence-dependent variation of off-rates. Both on-rates and off-rates are affected by introduction of mismatches. Moreover, a single base mismatch within the binding site has remarkably different effects depending on its position within the binding site. For example, a GC mismatch at a terminal position causes little change in the association rate and a large increase in the dissociation rate. However, the same mismatch at an internal site causes a decrease in the association rate but a smaller increase in the dissociation rate resulting in a smaller drop in affinity. Thus for any given sequence, the binding of Dst is governed by the interplay of two opposing effects. Inclusion of GC base pairs at internal sites results in a wider minor groove and hence a greater propensity toward binding in the 2:1 mode[13] (see Figure 3A−D in Supporting Information) with the concomitant slower off-rates.[14] At the same time, a GC base pair represents a mismatch and is enthalpically disfavored relative to an AT base pair and causes a slower on-rate. Due to the simplicity and ease of this assay, the above generalization can be tested for more complex polyamides containing monomer residues other than *N*-methyl pyrrole.

In conclusion, our findings reveal that the kinetic basis of sequence discrimination by Dst is not simply the differences in dissociation rates from mismatched sites. Depending on the location of the mismatch within the target site, both association and dissociation rates could be affected. While the pairing rules based on enthalpic discrimination[15] can be used to predict the relative affinity for a given site, the impact of a particular mismatch on binding affinity is also determined by the resulting change in DNA groove width, which governs the extent of 2:1 binding. Therefore, whenever specificity of drugs such as Dst is measured as the ratio of binding constants at a canonical matched site and a site mismatched by a single base pair, the choice of the mismatched site can affect the value of specificity obtained. Finally, we have shown that equilibrium fluorescence studies are a viable alternative to the commonly used DNase I footprinting technique for comparing the affinity of Dst for a family of DNA sequences. We are able to detect measurable changes in fluorescence for as low as 100 nM complex and even a single base pair mismatch gave measurably distinct fluorescence enhancement. This makes fluorescence-detected screening of tight-binding DNA sequences and polyamides in high-throughput formats such as those used for detection of single nucleotide polymorphisms (SNP's) a promising possibility.

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Supporting Information Available: Fluorescence spectra of Dst and DNA at high concentration, difference CD spectra at mutant sites, plots of association rates vs Dst concentration, and job type plot at the ATTA site (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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