Biochemistry

 Volume 37, Number 32 August 11, 1998

Articles

Circular Dichroic and Kinetic Differentiation of DNA Binding Modes of Distamycin†

Fu-Ming Chen* and Feng Sha

Department of Chemistry, Tennessee State University, Nashville, Tennessee 37209-1561

Received April 27, 1998; Revised Manuscript Received June 12, 1998

ABSTRACT: DNA binding modes of distamycin (DST) were investigated via comparative binding studies with oligomeric duplexes of the form d(GCG-X-GCG)·d(CGC-Y-CGC), where Y is complementary to X and X = 4- or 5-base binding site. It was found that 1:1 and 2:1 drug−duplex complexes exhibit distinctly different circular dichroic (CD) spectral characteristics and can, thus, serve as diagnostic tools for binding mode differentiation. CD intensity profiles at 265 or 275 nm as a function of drug to DNA ratios can reveal the extent of binding cooperativity for 2:1 complex formation (i.e., the relative binding affinities of 2:1 vs 1:1) at a 5-base-paired binding site. Comparison of these profiles leads to the following qualitative ranking for the binding cooperativity for the studied sites: AAGTT, ATATA ≥ AAACT > AATAA, AAATA, AAAGT > AATAT > TAAAA ≥ AAATT ≥ AAAAA ≥ ATAAA, AAAAT. The plausibility of this ordering is strengthened by its agreement with the ranking established by earlier NMR studies on some of the sequences. The significantly slower DST dissociation kinetics of the 2:1 complexes as compared to those of 1:1 made the kinetic measurements of SDS-induced dissociation by the stopped-flow technique possible. The results indicate that the AAGTT site exhibits the slowest DST dissociation rate, with a characteristic time of 35 s. The rates of dissociation in general correlate reasonably well with the cooperativity order found via equilibrium CD measurements (the higher the binding cooperativity, the slower the rate of dissociation). Base sequence specific binding of DST was also found for the 1:1 complex formation at the 4-base-paired sites, with AAAA, TTTT, ATTT, and AAAT sequences exhibiting the highest binding affinities.

Sequence-specific recognition of DNA by proteins and small molecules is a central element in the regulation of many biological processes. Understanding the structure, sequence specificity, and forces responsible for the binding of antibiotics to DNA is an important first step in the design of new drugs and sequence-specific probes. Two closely related antibiotics, netropsin (NET) and distamycin A (DST), have received considerable attention as models of sequence-specific nonintercalative DNA binding molecules. These two oligopeptides are potent antibacterial, antiviral, and antineoplastic agents whose pharmacological activity has been correlated to their abilities to bind to DNA (*1*). They form noncovalent complexes with duplex DNA in the minor groove and exhibit considerable preference for AT-rich domains such as the promoter regions. These antibiotics, thus, act as template poisons and inhibit DNA-dependent polymerase activities. Both DST and NET show a DNA

† Research supported by Army Medical Research Grant DAMD17-94-J-4474 and a subproject of Minority Biomedical Research Support Grant S06GM0892.

* Corresponding author. Telephone: (615)963-5325. FAX: (615)963-5434. E-Mail: chenf@harpo.tnstate.edu.

S0006-2960(98)00950-7 CCC: $15.00
Published on Web 07/21/1998

binding affinity which is in some cases significantly larger than that of typical intercalating drugs (*2*). Until recently, physical studies have focused mainly on the smaller NET molecule, and the results from such studies were then extended by analogy to the larger DST molecules. Recent years, however, have seen the upsurge of interest in using DST as a model for the DNA minor groove binding with A·T preference.

There have been numerous structural studies characterizing the DNA complexes of these antibiotics. In most of the crystallographic complexes studied thus far, a single ligand is bound per binding site of several successive A·T base pairs. The ligand is bound deep in the minor groove, making van der Waals contacts with both sides of the groove and forming hydrogen bonds between amides of the ligand and the acceptor groups of the bases (*3*). The positively charged end groups of the ligands are positioned to favorably interact with the electrostatic potential of the DNA. All of these factors contribute to the high affinity of these molecules for DNA. The preference of NET and DST binding to A·T sequences was further rationalized in terms of the protrusion of the amino group of guanosine into the minor groove which interferes sterically with binding at sites containing G·C pairs (*4*). This assertion, however, was being challenged by a study with poly(d2NH_2A-dT) indicating that despite the presence of the 2-amino group this polynucleotide exhibits significant binding to NET and DST (*5*).

Several NMR studies of 1:1 DST−DNA complexes have provided further insights into both the specificity and the forces responsible for the tight binding of this drug. The structure of the DST−d(CGCGAATTCGCG)$_2$ complex was determined by a combination of 2D NMR experiments and molecular mechanics calculations (*6*). It was found that the minimal binding site consists of just four A·T base pairs and that DST fits snugly into the 5′-AATT-3′ minor-groove binding site. This complex is characterized by van der Waals contacts between adenine C2H and drug H3 protons, potential three-center hydrogen bonds between drug amide and adenine N3 and thymine O2 atoms that protrude from the minor groove, and stacking of DNA O1′ atoms over each of the three pyrrole rings. The first two rings of the drugs are approximately parallel, while the third ring is turned to conform to the rotation of the helix. No large structural changes were observed for the DNA. It was suggested that the dipole-induced dipole interactions of the sugar O1′ atoms and the three *N*-methylpyrrole rings contribute to the stability of the complex and provide a molecular basis for the fact that binding is enhanced by the addition of *N*-methylpyrrole rings.

Calorimetric studies (*7*) have shown that DST binds tightly to the same four-base-paired site within the sequence d(GCGAATTCGC)$_2$, with a binding constant of 2.7×10^8 M^{-1}. This is in striking contrast to the binding constant of 2×10^5 M^{-1} reported for the 5′-TATA-3′ site within the sequence d(GGTATACC)$_2$, obtained by quantitative analysis of footprinting data (*8*). Although the discrepancies may largely be attributed to the sequence length and experimental techniques, sequence specificity of this drug may also be the culprit. The possible sequence specificity of this drug is further underscored by a recent crystallographic study of DST with d(CGCAAATTTGCG)$_2$ (*9*) revealing that the drug binds to the 5′-ATTT-3′ sequence, although other sites with four A·T base pairs were available in this oligomer.

Most interestingly, NMR studies have further indicated that binding sites of at least five base pairs in length can accommodate two DST molecules side-by-side in an antiparallel orientation (*10, 11*). In this 2:1 complex, each ligand preserves all the molecular recognition elements of minor groove binders. The extent of binding cooperativity (i.e., the ease of forming a complex of 2:1 vs 1:1 DST−duplex) depends strongly on the DNA sequence. Recently, Geierstanger et al. (*12*) have observed that DST can also bind to 5-base-paired sequences which contain G·C as well as A·T base pairs. When DST is titrated into a sample containing the binding site AAGTT·AACTT, a single set of new resonances is observed, saturating at a final stoichiometry of two DST molecules per DNA duplex. In addition, a crystal structure of the side-by-side complex of DST with [d(IC)$_4$]$_2$ has been reported (*13*). While DST binds exclusively 2:1 to AAGTT·AACTT and ATATA·TATAT even at low [ligand]/[duplex] ratios, only 1:1 DST complexes are observed with AAAAA·TTTTT at [ligand]/[duplex] ratios of up to 1. At higher ratios, DST also forms 2:1 complexes with AAAAA·TTTTT (*14*). The AAATT site was found to be intermediate in its cooperativity for 2:1 DST binding (*10*). These results indicate that the DST binding modes are strongly dependent on the base sequence of the binding site. In contrast to DST, the dication NET binds only as a single molecule per binding site, suggesting that charge interactions inhibit the side-by-side arrangement of two NET molecules in the minor groove.

These studies reinforce the importance of base sequence on the formation of both 1:1 and 2:1 complexes. Understanding the factors which govern the formation of these two types of complexes will be of value in discovering new ligands, which can be optimized for binding in specific modes to specific sequence targets. Therefore, a systematic sequence−specific binding study of DST was carried out, utilizing oligonucleotides containing binding sites of 4 and 5 base pairs. Spectral titrations and comparisons of spectral characteristics using these two types of oligomers were made to provide binding parameters and to find a possible means of differentiating these two modes of binding. CD spectral measurements were found to be most promising since a dimer formation devoid of plane or center of symmetry can lead to the formation of an exciton-type of CD couplet. This spectral feature is very distinct from that of monomer binding. The results of this study were then compared with those of NMR works already carried out on some of the sequences. This report describes our efforts and findings.

MATERIALS AND METHODS

Synthetic oligonucleotides were purchased from Research Genetics, Inc., Huntsville, AL, and used without further purification. These oligomers were purified by the vendor via reverse-phase oligonucleotide purification cartridges and exhibited single-band electrophoretic mobilities in denaturing polyacrylamide gel electrophoresis with stated purities of ≥95%. Concentrations of these oligomers (per nucleotide) were determined by measuring the absorbances at 260 nm after melting, with the use of extinction coefficients obtained via nearest-neighbor approximation using mono- and di-

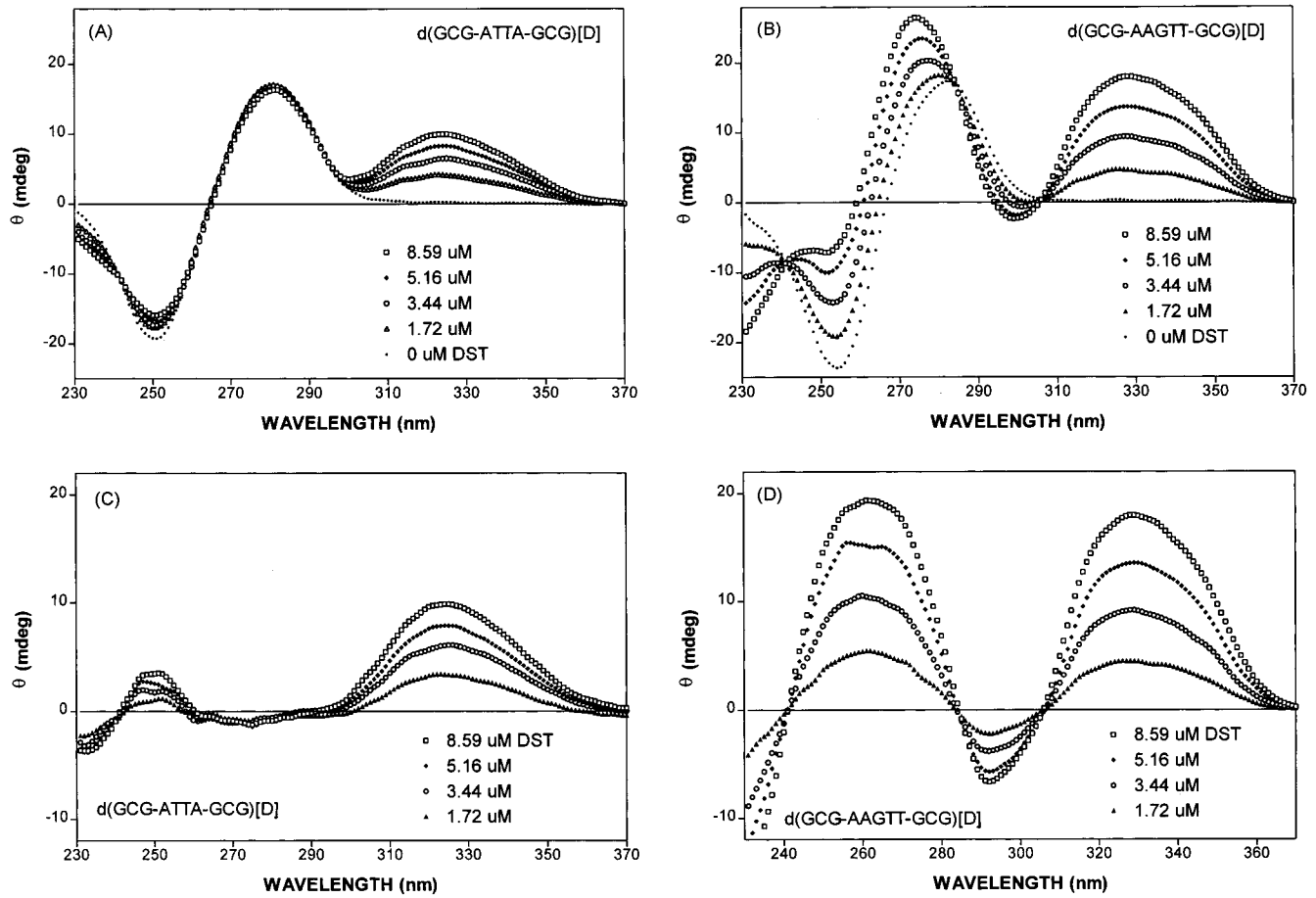

FIGURE 1: CD spectral comparison at room temperature for 4 μM (in duplex) d(GCG-ATTA-GCG)·d(CGC-TAAT-CGC) (panel A) and d(GCG-AAGTT-GCG)·d(CGC-AACTT-CGC) (panel B) in the presence of varying amounts of DST. The corresponding difference spectra, having the DNA spectral contributions subtracted off, are shown in panels C and D, respectively.

nucleotide values tabulated in Fasman (*15*). DNA heteroduplexes were formed by annealing complementary oligomers via heating equal molar mixtures to 95 °C for 5 min and slowly cooling back to ambient temperature. DST was purchased from Sigma and used without further purification. The extinction coefficient of 34 000 M^{-1} cm^{-1} at 303 nm was used for the DST concentration determination. Absorption spectral measurements were made with a Cary 1E spectrophotometric system. Thermal denaturation experiments were carried out with 1 cm semimicro cells by monitoring absorbances at 275 nm and maintaining a heating rate of 0.5 °C/min by the temperature controller accessory. Absorption spectral titrations of Job-type were carried out at 25 °C by making spectral measurements of two separate cells originally containing 5 μM DST and DNA (in duplex), respectively. Simultaneous transfer of a given volume from one cell to the other is then made, and the spectra were again taken. The process continued with progressive volume increases at strategic points. Absorbance changes at 350 nm were used to obtain equilibrium binding isotherms. Since DST is not very stable in aqueous solutions, the drug solution was prepared the same day the measurements were made. All experiments were carried out in 10 mM HEPPS [*N*-(2-hydroxyethyl)piperazine-*N*′-propanesulfonic acid] buffer solution of pH 8 containing 0.1 M NaCl and 1 mM $MgCl_2$.

Circular dichroic (CD) spectra were measured at room temperature with a Jasco J-500A recording spectropolarimeter using water-jacketed cylindrical cells of 2 cm path length. CD spectral titrations were made by starting with an oligomer of 80 μM (in nucleotide) which is followed by subsequent progressive additions of aliquots of DST stock. Ellipticities at 330 nm were used to construct equilibrium binding isotherms. Stopped-flow kinetic measurements were made with an Olis RSM-1000 system.

RESULTS

CD Spectral Characterization of 1:1 and 2:1 DST−Duplex Complexes. As mentioned earlier, a 4-base-pair site can only bind one DST molecule whereas the AAGTT site can bind two molecules to form an exclusively 2:1 complex. Oligomers containing ATTA and AAGTT were selected as prototypes for the 1:1 and 2:1 binding modes to investigate their possible spectral differences and to obtain binding parameters. DST is not optically active when free in solutions. In the presence of DNA, however, a sizable CD spectrum can be induced upon complex formation. CD spectral characteristics are compared in Figure 1 for d(GCG-ATTA-GCG)[d] (panel A) and d(GCG-AAGTT-GCG)[d] (panel B) in the absence and in the presence of increasing amounts of DST, where [d] designates the DNA heteroduplex. It is apparent that the two modes of binding induce distinctly different CD spectral characteristics. DST binding to the ATTA-containing decameric duplex results in a CD intensity enhancement of a band centering around 325 nm, with surprisingly little spectral alterations below 300 nm, except for some changes near 250 and 230 nm (panel A).

The maintenance of an isoelliptic point near 240 nm suggests a two-component process, with the complex being of 1:1 drug:duplex ratio. In contrast, binding of DST to -AAGTT- results in a more dramatic spectral change. In addition to the maintenance of the isoelliptic point at 241 nm and a somewhat larger intensity enhancement with a slight red shift of the 325 nm band, gross spectral alterations were seen in the spectral region below 300 nm. The appearance of a negative indentation near 291 nm and the presence of additional isoelliptic points around 284 and 306 nm are quite noteworthy (see panel B). The presence of these isoelliptic points again signifies a two-component binding process, with the bound species now being the 2:1 complexes as determined by an earlier NMR experiment (*12*). The general CD spectral features appear to be similar to those observed for the DST−d(CGC-AAATT-GGC)•d(GCC-AATTT-GCG) system (*16*).

The CD spectral alterations of these two binding modes can be more clearly seen if presented as difference spectra, where the DNA spectral contributions have been subtracted out. The results are shown in panels C and D for the -ATTA- and -AAGTT-containing oligomers, respectively. In addition to a stronger 330 nm band, a large positive CD band is induced near 265 nm upon DST binding to the -AAGTT- site with a magnitude slightly larger than that of the 330 nm band. The presence of three isoelliptic points (at 306, 284, and 241 nm) and the induction of a negative CD maximum near 291 nm are much more transparent in the difference CD spectra (panel D).

The observation of distinctly different CD spectral characteristics induced by the binding of DST at the -AAGTT- site when compared to the -ATTA- site is consistent with 2:1 complex formation at this 5-base binding site. An exciton-type CD couplet is to be expected for some absorbance bands if the drug dimer bound to the DNA minor groove is devoid of a plane or center of symmetry. Indeed, the dramatic positive and negative CD intensity enhancements near 265 and 230 nm, respectively, and the appearance of a negative maximum near 291 nm are consistent with the presence of exciton couplets centering near 240 and 285 nm, respectively.

CD Spectral Characteristics and Sequence-Specific Binding of DST at the 4-Base-Paired Binding Sites. To further investigate the feasibility of using CD spectral features to characterize the DST binding mode and to elucidate its possible sequence specificity, a series of oligomers were studied. As a first step in elucidating the tetranucleotide sequence specificity of DST, binding studies were carried out on decameric duplexes of the form d(GCG-X-GCG)• d(CGC-Y-CGC), where Y is complementary to X and X = AAAA, AAAT, AATA, ATAA, TAAA, ATTT, TATT, TTAT, TTTA, and TTTT. Representative CD difference spectral characteristics are shown in Figure 2. The general [DST]-dependent spectral features (especially those of TAAA, see panel D) are similar to those observed for the -ATTA-containing oligomer presented earlier (see Figure 1C). These are a sizable positive CD intensity enhancement near 325 nm and the presence of an isoelliptic point near 240 nm flanked by weak positive and negative intensity enhancements at 250 and 230 nm, respectively. Some sequence-dependent spectral differences, however, are also evident. For example, the progressive induction of a negative band near 275 nm resulted in the appearance of an additional isoelliptic point at 265 nm for the -AAAT-, -AAAA-, and -TTTT-containing decameric duplexes (see panels A, E, and F, respectively). Notice also the significantly smaller induced CD intensities of the 325 nm band for the -AATA- and -ATAA- sites (panels B and C, respectively), evidence of less favorable DST binding preference at these sequences. Similar studies were also made with oligomers containing the sequence isomers ATTT, TATT, TTAT, and TTTA. Again some sequence-dependent spectral features were observed (results not shown).

Ellipticities at 330 nm were used to construct Scatchard plots, and the binding parameters extracted via linear least-squares fits are shown in Table 1. Binding constants ranging from 1.5×10^7 to less than 2×10^5 M^{-1} were obtained, underscoring some sequence specificity of this drug. Consistent with the 1:1 complex formation, the extracted binding densities at strong binding sites are found to be slightly larger than 1 drug molecule bound per duplex. Comparison of the extracted binding constants led to a qualitative ranking on the DST binding affinities to the 4-base-paired sites studied. The results are (in descending binding affinity) as follows: TTTT, AAAA, ATTT ≥ AAAT >TAAA, TTTA, TTAT ≥ TATT > ATAA > AATA.

DST binding also enhances DNA duplex stability. Melting temperature increases upon DST binding for the oligomers studied are included in Table 1 for comparison. Although the extent of melting temperature increase does not correspond exactly to the established binding order, the general trend of larger and smaller melting temperature increases for higher and lower DST binding affinities appears to be in order.

Sequence Specificity and Binding Mode Characterization of 5-Base-Paired Sites via CD Titration. Sequence-specific binding and CD spectral characterization of DST at the 5-base-paired sites were investigated by comparative studies with undecameric duplexes of the form d(GCG-X-GCG)-[d], where X = AAAAA, AAAAT, AAATA, AATAA, ATAAA, TAAAA, AAATT, AATAT, ATATA, AAACT, and AAAGT. Difference CD spectra of some representative titrations are shown in Figure 3. The gross spectral features of DST binding at these 5-base-paired binding sites are quite distinct from those of the 4-base-paired counterparts. The appearance of a negative difference CD spectral maximum near 291 nm and somewhat larger positive CD intensity enhancements around 330 and 265 nm are the outstanding features of DST binding at the 5-base-paired binding sites.

For the -AAAAA- and -AAAAT-containing oligomers (panels A and B, respectively), the initial progressive intensity increase of the weak negative CD band around 275 nm and the maintenance of an isoelliptic point near 265 nm are clearly evident for [DST]/[duplex] ≤ 1. These spectral features are very similar to those observed for the 4-base-paired sites -AAAA- and -AAAT- (see Figures 2E and 2A, respectively.) For [DST]/[duplex] ratios greater than 1, however, the 265 nm isoelliptic point is no longer maintained. Instead, CD intensities near this wavelength become progressively more positive to result in the appearance of a new isoelliptic point in the 285−290 nm region. These spectral alterations differ significantly from those of -AAGTT-, where the maintenance of the 285-nm isoellptic point is evident throughout the titration (see Figure 1D). These results

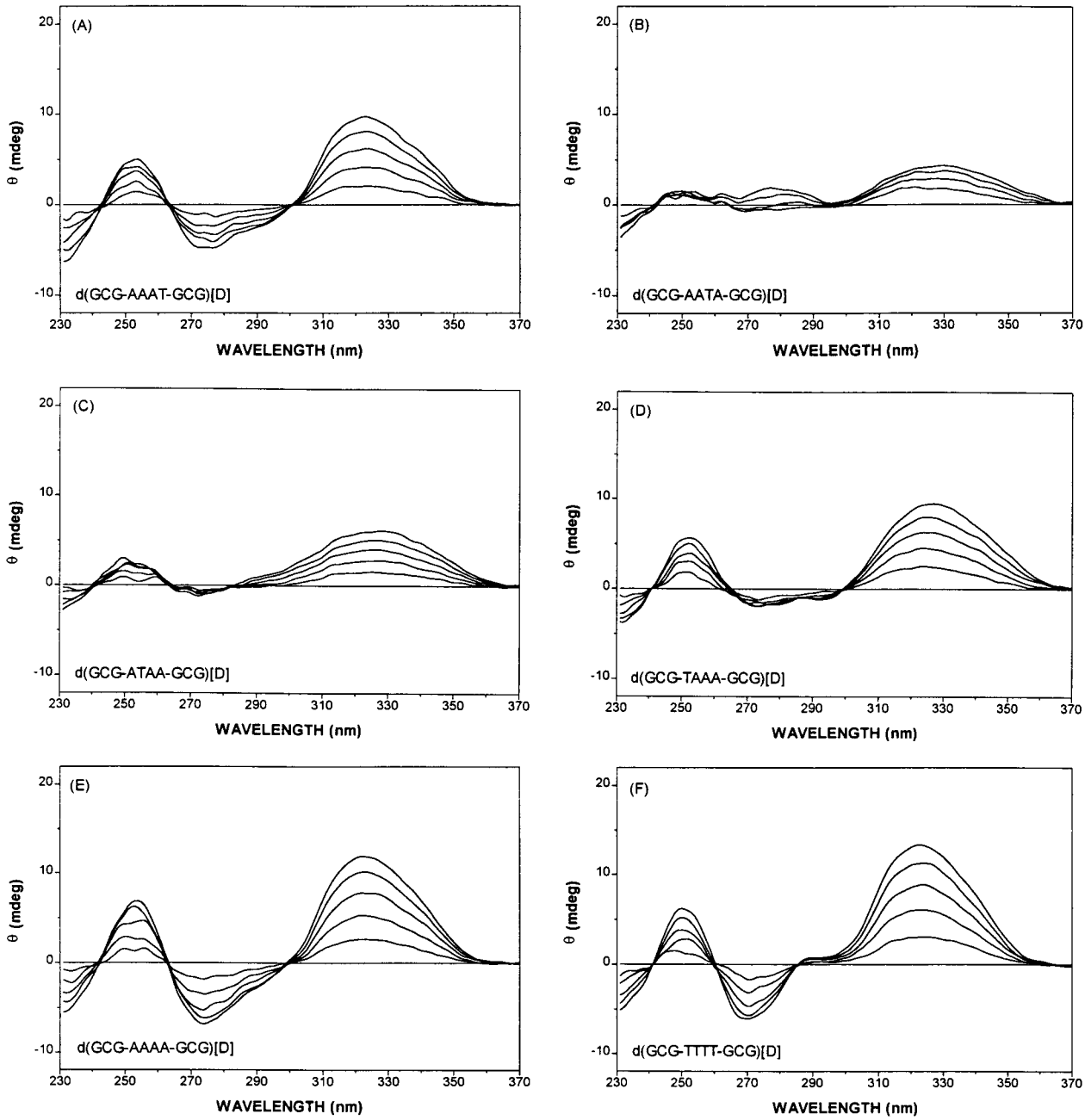

FIGURE 2: Comparison of induced CD difference spectra of DST at representative 4-base-paired binding sites. The spectra shown correspond to 4 μM DNA duplex in the presence of 0.86, 1.72, 2.58, 3.44, and 4.30 μM DST. Oligomers shown are d(GCG-AAAT-GCG)·d(CGC-ATTT-CGC) (panel A), d(GCG-AATA-GCG)·d(CGC-TATT-CGC) (panel B), d(GCG-ATAA-GCG)·d(CGC-TTAT-CGC) (panel C), d(GCG-TAAA-GCG)·d(CGC-TTTA-CGC) (panel D), d(GCG-AAAA-GCG)·d(CGC-TTTT-CGC) (panel E), and d(GCG-TTTT-GCG)·d(CGC-AAAA-CGC) (panel F).

suggest the formation of a 1:1 complex when [DST]/[duplex] is ≤1 and is subsequently followed by 2:1 complex formation when the ratios are greater than 1. This interpretation is consistent with earlier NMR titrations of the AAAAA·TTTTT site showing that 1:1 complexes are formed up to a [DST]/[duplex] ratio of 1 with subsequent 2:1 complex formation only after saturation of the 1:1 binding mode.

Although no clear-cut presence of an isoelliptic point near 265 nm is evident during the [DST]/[duplex] ≤ 1 phase of titration for the -AAATT-containing oligomeric duplex (panel C), the induction of a significantly larger CD intensity near 265 nm during the [DST]/[duplex] ≥ 1 phase of titration is clearly apparent. These results suggest the presence of a 2-step binding process with a higher cooperativity for 2:1 binding than those of AAAAA- and AAAAT-containing oligomers, a result consistent with the earlier NMR finding (*14*). As for the titrations of the AAACT-containing oligomeric duplex (panel D), the spectral changes are characterized by an immediate progressive positive intensity enhancement near 265 nm (instead of the presence of an initial isoelliptic point) and the maintenance of an approximate isoelliptic point near 285 nm. These spectral features are reminiscent of those of the AAGTT site, suggesting nearly exclusive 2:1 complex formation at the AAACT site, bypassing the 1:1 complexation stage.

Single-Wavelength CD Intensity Profiles and Cooperativity of 2:1 Complex Formation. As noted earlier, 1:1 complex formation at most sequences is accompanied by little or no

Table 1: Comparison of Distamycin A Binding Parameters and Melting Temperatures

oligomer	K (μM^{-1})	n (/duplex)	t^0_m (°C)[a]	t_m (°C)[a]	Δt_m (°C)
d(GCG-AAAA-GCG)·d(CGC-TTTT-CGC)	14.9	1.1	38.0	53.1	15.1
d(GCG-AAAT-GCG)·d(CGC-ATTT-CGC)	10.9	1.4	45.0	53.5	8.5
d(GCG-AATA-GCG)·d(CGC-TATT-CGC)	<0.2		43.5	47.5	4.0
d(GCG-ATAA-GCG)·d(CGC-TTAT-CGC)	0.33		39.8	45.0	5.2
d(GCG-TAAA-GCG)·d(CGC-TTTA-CGC)	1.5		37.5	45.0	8.5
d(GCG-ATTT-GCG)·d(CGC-AAAT-CGC)	14.4	1.1	46.0	55.5	9.5
d(GCG-TATT-GCG)·d(CGC-AATA-CGC)	1.0	1.1	43.7	52.0	8.3
d(GCG-TTAT-GCG)·d(CGC-ATAA-CGC)	1.2		41.3	48.1	6.8
d(GCG-TTTA-GCG)·d(CGC-TAAA-CGC)	1.3	1.2	43.5	47.5	4.0
d(GCG-TTTT-GCG)·d(CGC-AAAA-CGC)	15.5	1.1	45.4	53.3	7.9

[a] t^0_m and t_m are melting temperatures of 40 μM (base) oligomeric duplexes in pH 8 buffer containing 0.1 M NaCl and in the absence and in the presence of 7 μM DST, respectively.

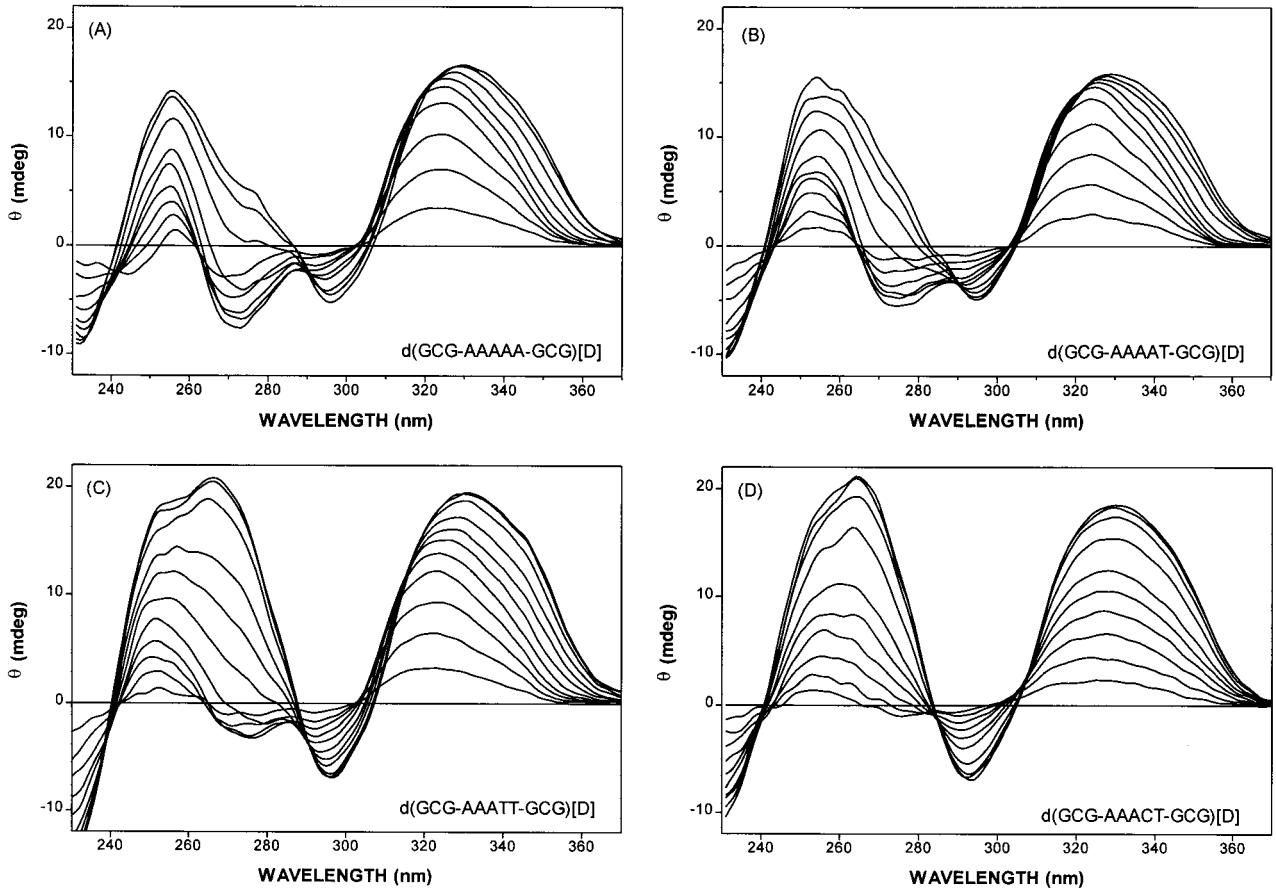

FIGURE 3: Comparison of induced CD difference spectra of DST at representative 5-base-paired binding sites. The spectra shown correspond to 4 μM DNA duplex in the presence of 0.86, 1.72, 2.58, 3.44, 4.30, 5.16, 6.88, 8.60, 10.3, 12.1, and 13.8 μM DST. Oligonucleotides represented are d(GCG-AAAAA-GCG)·d(CGC-TTTTT-CGC) (panel A), d(GCG-AAAAT-GCG)·d(CGC-ATTTT-CGC) (panel B), d(GCG-AAATT-GCG)·d(CGC-AATTT-CGC) (panel C), d(GCG-AAACT-GCG)·d(CGC-AGTTT-CGC) (panel D).

change in the CD intensity near 265 nm whereas a dramatic intensity enhancement is induced at this same wavelength upon formation of 2:1 complexes. This suggests that monitoring of the CD intensity at this wavelength may provide useful information in differentiating binding modes at different sites. CD intensities at 265 nm were plotted against [DST]/[duplex], and the results for some representative oligomers are shown in Figure 4. For the oligomer containing the -AAAAT- site, the intensity remains near zero until the [DST]/[duplex] ratios surpass 1, wherein the 265 nm intensity becomes more enhanced. These results are consistent with the formation of 1:1 complexes when the [DST]/[duplex] ratios are below 1 and 2:1 complex formation when they exceed 1. The [DST]/[duplex] ratio at which the intensity starts to take off becomes progressively smaller for the AAATT, AATAT, and AAATA sites at approximate values of 0.8, 0.6, and 0.4, respectively. These results suggest that the 2:1 complex formation becomes more cooperative and begins at a lower and lower drug/duplex ratio. For the ATATA and AAACT sites, the intensity enhancement begins immediately at the start of the titration. The intensity profile for the prototypical AAGTT sequence (not shown) is very similar to those exhibited by these two sites. These results are consistent with their nearly exclusive formation of the 2:1 complexes even at low drug/duplex ratios. Intensity monitoring at 275 nm may in fact be more informative since its intensity becomes slightly more negative during 1:1 complex formation for some sites whereas its

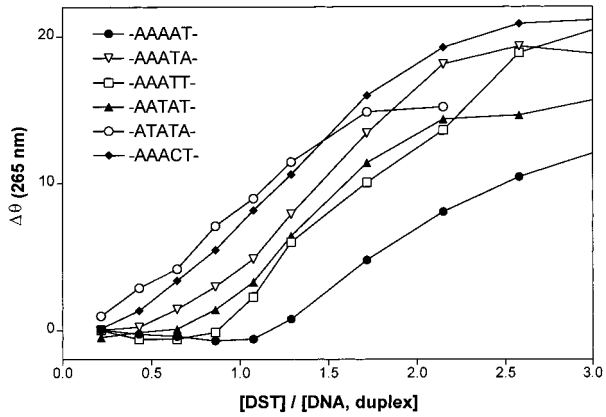

FIGURE 4: CD intensity profiles for representative oligomers as a function of [DST]/[duplex] at 265 nm for representative 5-base-paired binding sites.

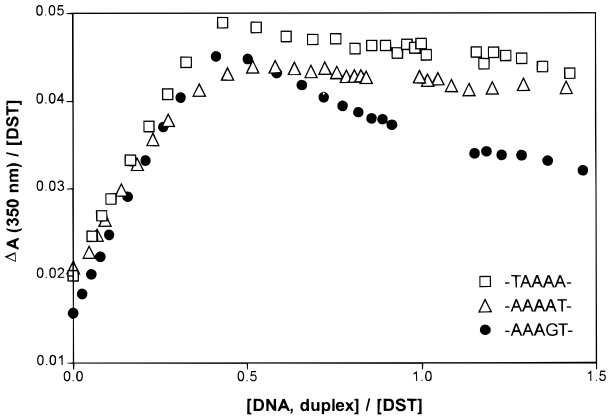

FIGURE 5: Representative equilibrium binding isotherms plotted as apparent extinction coefficients at 350 nm vs [duplex]/[DST]. Absorption titrations were carried out at 25 °C. The discontinuities exhibited in the binding isotherms are the consequence of not being able to reach equal drug concentrations in our two-cell Job-type titrations.

intensity turns more positive with subsequent 2:1 complex formation. Therefore, the distinction of these two modes of binding can more readily be made with the intensity monitoring at this wavelength by noting the slope change from negative to positive (results not shown). Based on the comparison of [drug]/[duplex] ratios at the intensity takeoff of these profiles, the extent of binding cooperativity for the formation of 2:1 complexes can be ranked approximately as follows: AAGTT, ATATA ≥ AAACT > AATAA, AAATA, AAAGT > AATAT > TAAAA ≥ AAATT ≥ AAAAA ≥ ATAAA, AAAAT.

Evidence of 2:1 Complex Formation via Absorption Spectral Titrations for the 5-Base-Paired Sites. DST exhibits an absorbance maximum at 303 nm when free in solutions. Successive additions of DNA lead to slight bathochromic shifts and intensity enhancements near 330 nm. Absorbance changes at 350 nm (to avoid interference from the residual DNA absorbance) were used to construct binding isotherms and to obtain binding parameters. Binding isotherms via absorbance monitoring were constructed to provide evidence for the formation of 2:1 complexes for oligomers containing 5-base-paired binding sites. Representative plots of ϵ (apparent) vs [duplex]/[DST] are shown in Figure 5. It is apparent that these binding isotherms (and indeed for all the sequences studied) show breaks near [duplex]/[DST] = 0.5, suggesting the formation of complexes having 2 drug molecules bound per duplex.

Table 2: Comparison of SDS-Induced DST Dissociation Kinetic Parameters at 20 °C for the 5-Base-Paired Sites[a]

oligomer	K (s^{-1})	τ (s)
d(GCG-AAAAA-GCG)·d(CGC-TTTTT-CGC)	2.46 ± 0.04	0.41
d(GCG-AAAAT-GCG)·d(CGC-ATTTT-CGC)	2.53 ± 0.01	0.40
d(GCG-AAATA-GCG)·d(CGC-TATTT-CGC)	0.28 ± 0.003	3.57
d(GCG-AATAA-GCG)·d(CGC-TTATT-CGC)	0.61 ± 0.01	1.63
d(GCG-ATAAA-GCG)·d(CGC-TTTAT-CGC)	2.16 ± 0.04	0.46
d(GCG-TAAAA-GCG)·d(CGC-TTTTA-CGC)	1.04 ± 0.02	0.96
d(GCG-AAATT-GCG)·d(CGC-AATTT-CGC)	0.69 ± 0.004	1.45
d(GCG-AATAT-GCG)·d(CGC-ATATT-CGC)	2.23 ± 0.05	0.45
d(GCG-ATATA-GCG)·d(CGC-TATAT-CGC)	0.144 ± 0.001	6.94
d(GCG-AAGTT-GCG)·d(CGC-AACTT-CGC)	0.0284 ± 0.0003	35.2
d(GCG-AAAGT-GCG)·d(CGC-ACTTT-CGC)	1.98 ± 0.05	0.51
d(GCG-AAACT-GCG)·d(CGC-AGTTT-CGC)	2.02 ± 0.07	0.50

[a] Measurements were made by mixing a solution containing 7.5 μM DST/75 μM DNA (in nucleotide) with an equal volume of 2% SDS solution. The time-dependent absorbance profile at the 341−347 nm region was used for curve-fit.

Binding Mode Differentiation via Kinetic Measurements. Due to the ability of the DST dimer to make extensive hydrogen bonding and van der Waal contacts with both walls of the minor groove, it is anticipated that both their association and their dissociation kinetics will be considerably slower than the corresponding 1:1 complexes. Thus, stopped-flow kinetic measurements were made. The rates of association kinetics appear to be too fast to give meaningful rate information, although there are indications that the rates of DST binding at the 5-base-pair sites are indeed slower than at the 4-base-pair sites. Although the rates of dissociation from the 4-base-paired sites are too fast to be measured by the stopped-flow technique, those of 5-base sites are amenable for such measurements. The extracted SDS-induced DST dissociation rate parameters via single-exponential fits are summarized in Table 2. It is apparent that the measurable rates of DST dissociation vary greatly among these 5-base-paired sites, ranging from a characteristic time of 35.2 s for the AAGTT site to 0.4 s for the AAAAT site. The ranking of the dissociation rates is (slowest at the left): AAGTT > ATATA > AAATA > AATAA ≥ AAATT > TAAAA > AAAGT, AAACT, ATAAA, AATAT, AAAAA, AAAAT. Except for AAACT and AAAGT, the ordering appears to conform in general to that of 2:1 binding cooperativity as suggested via equilibrium CD titrations. Our observed SDS-induced DST dissociation rates of the 2:1 complexes appear reasonable since NMR studies of DST binding with $d(CGCAAATTTGCG)_2$ revealed that the off-rate for the drug from the 2:1 mode was found to be slow on the NMR time scales, with a characteristic time of 5 s at 30 °C (*11*).

DISCUSSION

Of all A·T specific minor groove binders studied thus far, DST and its analogues appear to be the only molecules found to form both 1:1 and 2:1 complexes. Based on X-ray (*17*) and NMR (*6*) data, this drug spans 4 base pairs in forming a 1:1 complex. However, a 5-base-paired site can be occupied simultaneously by two DST molecules side-by-side with the positively charged end groups pointing in opposite directions (*10*, *11*). The degree of cooperativity,

i.e., the relative binding affinities of the 2:1 vs 1:1 modes, depends strongly on the DNA sequence. Our results indicate that these two binding modes exhibit distinctly different CD spectral characteristics. Thus, CD measurements can be employed to identify and differentiate the DNA binding modes and to obtain meaningful binding parameters for this drug.

Sequence specificity of DST for the formation of the 1:1 complex is observed, and binding parameters extracted via Scatchard plots suggest equilibrium binding constants ranging from 1.5×10^7 to less than 2×10^5 M^{-1}. Sequences containing a 3 or 4 homo-base stretch appear to provide stronger 1:1 bindings whereas the presence of alternating sequences at the binding sites seems to significantly reduce their DST binding affinities. Our finding of strong DST binding at the -ATTT- site appears to be consistent with the recent crystallographic study of DST+d$(CGCAAATTTGCG)_2$ revealing that the drug bound to only one site, namely, 5′-ATTT-3′, although other 4-base sites were also available (*9*). In addition, it was shown by NOESY experiments that DST binds to the AAATT site as a 1:1 complex at low [DST]/[duplex] ratios and the AAAT/ATTT site is preferred by 2.2 to 1 over the AATT/AATT site (*10, 11*).

Sequence-dependent spectral variations were also observed for the 5-base-paired binding sites studied. CD intensity profiles at 275 and 265 nm vs [DST]/[duplex] provide indications on the cooperativity of the 2:1 binding mode at these sites to result in a qualitative ranking. In contrast to 1:1 complex formation, sequences containing 3 or more homo-base stretches appear to provide a less favorable environment while sequences with alternating bases provide a more favorable environment for 2:1 complex formation.

Binding isotherms via absorbance titration further confirm the formation of 2:1 complexes at these 5-base-paired binding sites. In addition, SDS-induced drug dissociation experiments indicate considerable variation in their rates of drug dissociation, with AAGTT and AAAAT sites exhibiting the slowest and the fastest dissociation rates, respectively. Except for some G·C-containing sequences, the ranking via dissociation rates appears to generally conform to that of binding cooperativity of the 2:1 complexes, with the highest cooperative binding exhibiting the slowest DST dissociation rate.

The validity of our cooperativity ordering on 2:1 complex formation appears to be supported by earlier NMR studies on oligomers containing some of the same sequences (*14*). NMR titrations of the AAAAA·TTTTT site have shown that 1:1 complexes are formed up to a [DST]/[duplex] ratio of 1, which is then followed by 2:1 complex formation. On the other hand, the opposite behavior is seen with the alternating site ATATA·TATAT. Only a 2:1 complex is observed for all [DST]/[duplex] ratios, even as low as 0.25 equiv of DST per DNA duplex. An intermediate case is found for the sequence AAATT·AATTT in which NMR results (*11*) indicate that at a [DST]/[duplex] ratio of 0.75:1 about 10% of the population of the bound DNA is already in the 2:1 complex form. These results, thus, suggest that the 2:1 binding cooperativity follows the order ATATA > AAATT > AAAAA (*14*), in agreement with our ranking. Furthermore, their studies also suggested that in oligomers containing a AAGTT, AAACT, or AAAGT site, only 2:1 complexes were observed. Indeed, all three of these sites are high on our ranking order.

Based on their NMR results, Wemmer et al. (*14*) had speculated that the binding behavior of DST reflects the local variations in DNA groove geometry and flexibility, especially the groove width. DST forms tight complexes with DNA only if it can provide close van der Waals contacts with the walls of the minor groove, and the DNA minor groove must expand to accommodate two DST molecules. A comparison of crystal structures suggests that the minor groove is narrowest in poly(dA)-type sequences (*18*−*21*) and such sequences are also particularly stable against base-pair opening (*22*). The AAAAA·TTTTT and sites containing 3 or 4 homo-base, therefore, represent preferred 1:1 DST binding sites because of the narrowness of their grooves. The slight extra width and greater flexibility may have contributed to the observed weaker DST affinities for 1:1 complex formation at sites containing ATA or TAT and enhanced cooperativity of 2:1 binding at ATATA·TATAT. NMR studies have further indicated that 2:1 complexes of DST and its analogues are able to form close contacts and tight complexes with the minor groove of mixed G·C/A·T sequences. This seems somewhat puzzling in view of the fact that the A·T specificity of NET and DST has been attributed to the steric interference with binding at sites containing G·C pairs (*4*), a consequence of the protruding amino group of guanosine at the minor groove. However, it has been seen in the crystal structure of B-form DNA that the minor groove in regions containing G·C pairs is decidedly wider (*20, 23, 24*). It was thus suggested that the loss of shape complementarity and contact surface between ligand and groove is more important than the depth of the groove (*14*). A wider groove easily accommodates a 2:1 complex, making AAGTT the most cooperative 2:1 binding site. This same binding mode is observed for the sequences AAAGT·ACTTT and AAACT·AGTTT as well, indicating that the G·C pair need not be in the center of the site. It should be pointed out, however, that our results indicate they exhibit distinctly different dissociation kinetic behaviors. The two sites with off-centered G·C exhibit 7-fold faster dissociation rates than that with a central G·C base pair.

The notion that the introduction of a G·C base pair into an A·T stretch of DNA widens the minor groove locally and results in DST binding exclusively in the 2:1 mode appears plausible. This may also explain why the IIICC·IICCC site only exhibits one type of complex with a 2:1 stoichiometry (*25*). It was shown that this complex is a 2:1 side-by-side complex exactly as that observed with AAATT·AATTT. As base pairs in the AAATT·AATTT site are successively replaced with I·C base pairs, DST binding becomes progressively more cooperative in proportion to the number of substituted base pairs, apparently without any preference for location in the sequence. Since I·C base pairs contain the same minor groove functional groups as A·T base pairs, their binding differences most likely are the consequence of different groove geometry due to differences in base pair stacking, propeller twist, and other structural parameters. It would be reasonabe to suspect that the structural attributes of I·C are quite similar to those of G·C.

Rentzeperis and Marky (*16*) had carried out thermodynamic characterization of NET and DST binding to an AAATT-containing oligomer and found that binding of NET

to this sequence occurs with favorable enthalpy and small but also favorable entropy. The first DST binding yielded similar affinities ($\sim 10^7$ M^{-1}) and more favorable enthalpy contribution to that of NET binding. However, the binding of the second molecule gives a much more favorable enthalpy, which was interpreted as arising from increased van der Waals interactions, a heat capacity effect, and much less favorable entropy, arising from a combination of ion and water interactions. These contributions lead to a favorable free energy of binding slightly less than that for the first DST molecule.

It should be noted in passing that DST complexation with a nucleic acid triple helix has also been investigated recently (*26*). CD spectral analysis indicates that DST can bind to the triple-stranded form but its binding affinity is weaker than the corresponding duplex form. The bound DST appears to exhibit a conformation and an environment slightly different from those bound to the corresponding double-stranded form. Thermal denaturation experiments demonstrate that DST binding destabilizes the triplex whereas it stabilizes the duplex. Furthermore, the 2:1 complex formation is not observed with the triple-stranded host. The inability of the triplex to form 2:1 complex was rationalized in terms of its failure to expand the minor groove to bind two DST molecules due to the presence of the third strand bound at the major groove of DNA.

These results suggest that there are subtle differences in the minor groove width for different DNA sequences, and that DST is a sensitive probe for these differences in the DNA structure. Information obtained from such studies should aid in the design of new drugs and should also provide insight into both DNA conformational flexibility and protein−DNA interactions.

REFERENCES

1. Hahn, F. E. (1977) *Pharmacol. Ther., Part A 1*, 475−485.
2. Luck, G., Zimmer, C., Reinert, K., and Arcamone, F. (1977) *Nucleic Acids Res. 4*, 2655−2670.
3. Zimmer, C., and Wahnert, U. (1986) *Prog. Biophys. Mol. Biol. 47*, 31−112.
4. Kopka, M. L., Yoon, C., Goodsell, D., Pjura, P., and Dickerson, R. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1376−1380.
5. Dasgupta, D., Howard, F. B., Sasisekharan, V., and Miles, T. (1990) *Biopolymers 30*, 223−227.
6. Pelton, J. G., and Wemmer, D. E. (1988) *Biochemistry 27*, 8088−8096.
7. Breslauer, K. J., Remeta, D. P., Chou, W.-Y., Ferrante, R., Curry, J., Zaunczkowski, D., Synder, J. G., and Marky, L. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8922−8926.
8. Fish, E. L., Lane, M. J., and Vournakis, J. N. (1988) *Biochemistry 27*, 6026−6032.
9. Coll, M., Frederick, C. A., Wang, A. H.-J., and Rich, A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8385−8389.
10. Pelton, J. G., and Wemmer, D. E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5723−5727.
11. Pelton, J. G., and Wemmer, D. E. (1990) *J. Am. Chem. Soc. 112*, 1393−1399.
12. Geierstanger, B. H., Dwyer, T. J., Bathini, Y., Lown, J. W., and Wemmer, D. E. (1993) *J. Am. Chem. Soc. 115*, 4474−4482.
13. Chen, X., Ramakrishnan, B., Rao, S. T., and Sundaralingan, M. (1994) *Nat. Struct. Biol. 1*, 169−174.
14. Wemmer, D. E., Geierstanger, B. H., Fagan, P. A., Dwyer, T. J., Jacobsen, J. P., Pelton, J. G., Ball, G. E., Leheny, A. R., Chang, W.-H., Bathini, Y., Lown, J. W., Rentzeperis, D., Marky, L. A., Singh, S., and Kollman, P. (1994) in *Structural Biology: The State of the Art. Vol 2. Proceedings of the 8th Conversation in the Discipline Biomolecular Stereodynamics* (Sarma, H., and Sarma, M. H., Eds.) pp 301−323, Adenine Press, New York.
15. Fasman, G. D., Ed. (1975) *CRC Handbook of Biochemistry and Molecular Biology*, 3rd ed., Vol. I, p 589, Chemical Rubber Company Publishing, Cleveland, OH.
16. Rentzeperis, D., and Marky, L. A. (1995) *Biochemistry 34*, 2937−2945.
17. Coll, M., Aymami, J., van der Marel, G. A., van Boom, J. H., Rich, A., and Wang, A. H.-J. (1989) *Biochemistry 28*, 310−320.
18. Wing, R., Drew, H., Takano, T., Broka, C., Tanaka, S., Itakura, K., and Dickerson, R. E. (1980) *Nature 287*, 755−758.
19. Nelson, H. C. M., Finch, J. T., Bonaventura, L. F., and Klug, A. (1987) *Nature (London) 330*, 221−226.
20. Yoon, C., Prive, G. G., Goodsell, D. S., and Dickerson, R. E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6332−6336.
21. DiGabriele, A. D., Sanderson, M. R., and Steiz, T. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1816−1820.
22. LeRoy, J. L., Charretier, E., Kochoyan, M., and Gueron (1988) *Biochemistry 27*, 8894−8898.
23. Heinemann, U., and Alings, C. (1989) *J. Mol. Biol. 210*, 369−381.
24. Goodsell, D. S., Kopka, M. L., Cascio, D., and Dickerson, R. E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2930−2934.
25. Fagan, P., and Wemmer, D. E. (1992) *J. Am. Chem. Soc.114*, 1080−1081.
26. Durand, M., and Maurizot, J. C. (1996) *Biochemistry 35*, 9133−9139.

BI980950L