^{1}H NMR and Optical Spectroscopic Investigation of the Sequence-Dependent Dimerization of a Symmetrical Cyanine Dye in the DNA Minor Groove†

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ABSTRACT: A symmetrical cyanine dye was previously shown to bind as a cofacial dimer to alternating A-T sequences of duplex DNA. Indirect evidence suggested that dimerization of the dye occurred in the minor groove. ^{1}H NMR experiments reported here verify this model based on broadening and shifting of signals due to protons on carbon 2 of adenine and imino protons at the central five A-T pairs of the 11 base pair duplex: 5′-GCGTATATGCG-3′/3′-CGCATATACGC-5′. This binding mode is similar to that of distamycin A, even though the dye lacks the hydrogen-bonding groups used by distamycin for sequence-specific recognition. Surprisingly, the third base pair (G-C) was also implicated in the binding site. UV−vis experiments were used to compare the extent of dimerization of the dye for 11 different sequence variants. These experiments verified the importance of a G-C pair at the third position: replacing this pair with A-T suppressed dimerization. These results indicate that the dye binding site spans six base pairs: 5′-GTATAT-3′. The initial G-C pair seems to be important for widening the minor groove rather than for making important contacts with the dye molecules since inverting its orientation to C-G or replacing it with I-C still led to favorable dimerization of the dye.

Cyanine dyes (*1*) are intensely colored compounds that were widely used initially as photographic sensitizers (*2*). Cyanines have since found many applications as biological stains and fluorescent labels and probes (*3*) due to their high fluorescence quantum yields as well as their sensitivity to environmental factors such as temperature, polarity, and viscosity. The general dye structure consists of two heteroaromatic rings connected by a polymethine linker. Variation of the heterocycle and bridge length allows for tuning of the absorption and emission wavelengths.

Our interest in cyanine dyes originally arose from their potential as visible light sensitive DNA photocleavage agents (*4*). However, we soon discovered that the benzothiazole dye $DiSC_2(5)$ bound to certain sequences of DNA as a cofacial dimer under conditions where the dye is monomeric in the absence of the DNA (*5*). Thus, the DNA templates the assembly of cyanine dye dimers. Moreover, assembly of one dimer greatly facilitated assembly of neighboring dimers, ultimately leading to formation of a helical cyanine dye aggregate. This highly cooperative process is the supramolecular analogue of a chain growth polymerization.

Dimerization/aggregation of $DiSC_2(5)$ on DNA occurred specifically at alternating A/T sequences. It was also found that the length of the aggregate could be controlled by adding alternating A/T tracts in five base pair increments, where one dimer bound to each $(A/T)_5$. The length of the dye approximately spans five DNA base pairs. The strong preference for dimerization at A-T pairs over G-C pairs, however, is not unique to cyanines. This specificity is reminiscent of distamycin A, which dimerizes in the minor groove of DNA at A/T-rich sequences. The binding site size as well as the similarity in the stoichiometry and the sequence-dependence of DNA binding between the cyanine and distamycin led us to propose that dimerization of $DiSC_2(5)$ and its analogues occurs in the minor groove of DNA (*5*,*6*).

This paper describes a combined NMR and UV−vis spectroscopic investigation of the binding of $DiSC_2(5)$ to a short DNA duplex (AT5, Chart 1). NMR[1] spectroscopy has become one of the most popular methods by which to study the structure of biomolecules in solution because of the detailed information it provides, where small molecule or protein binding sites can be pinpointed with atomic resolution. Meanwhile, UV−vis allows for the rapid screening of many different DNA sequences for $DiSC_2(5)$ dimerization based on well-defined spectral shifts. The current work confirms that $DiSC_2(5)$ binds in the minor groove of AT5, with an unexpected binding site size of six base pairs, including not only five alternating A-T pairs but also one flanking G-C pair.

EXPERIMENTAL PROCEDURES

Materials. The iodide salt of $DiSC_2(5)$ was purchased from Molecular Probes (Eugene, OR) and used without further purification. (The dye is no longer available from this supplier but can be purchased from Aldrich Chemical Co.)

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[1] Abbreviations: FID, free induction decay; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy.

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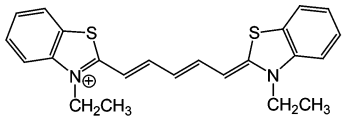
Chart 1



DiSC$_2$(5)

5' - G_1 C_2 G_3 T_4 A_5 T_6 A_7 T_8 G_9 C_{10} G_{11} - 3'
3' - C_{22} G_{21} C_{20} A_{19} T_{18} A_{17} T_{16} A_{15} C_{14} G_{13} C_{12} - 5'

AT5

Stock solutions were prepared in methanol, and concentrations were determined spectrophotometrically in methanol using the manufacturer's extinction coefficient: ϵ_{651} = 260000 M^{-1} cm^{-1}.

Synthetic oligonucleotides were purchased from Integrated DNA Technologies (www.idtdna.com), and DNAs used for NMR experiments were further purified by ethanol extraction to remove glycerol used by the manufacturer in the precipitation process. Stock solutions were prepared in aqueous sodium phosphate buffer (10 mM, pH = 7), and strand concentrations were determined by absorbance at 260 nm using extinction coefficients calculated from DNA nearest-neighbor values. Oligonucleotide duplexes were prepared by mixing equimolar amounts of the two complementary strands, heating to 80 °C for 2 min, and then slowly cooling to room temperature.

Equipment. UV−vis measurements were performed on a Varian Cary3 Bio spectrophotometer equipped with a thermoelectrically controlled multicell holder. NMR experiments were performed on a Bruker Avance DMX-500, using standard Bruker software.

Continuous Variation Experiment. Samples containing variable amounts of DiSC$_2$(5) and AT5 duplex (but constant total concentration = 10 μM) were prepared in 10 mM sodium phosphate and 20% DMSO. UV−vis absorbance spectra were recorded at 20 °C in a cuvette with a 2 mm path length, and the absorbance at 590 nm was plotted versus the mole fraction of dye.

UV−Vis Absorbance Spectra. Samples were prepared containing 10 μM desired duplex with 20 μM DiSC$_2$(5) in 10 mM sodium phosphate and 20% DMSO. UV−vis spectra were recorded at 20 °C in a cuvette with a 2 mm path length.

NMR Sample Preparation. NMR samples contained 10 mM sodium phosphate (pH = 7.0) and 20% DMSO-d_6 (Sigma Aldrich). DNA concentrations were 100 μM (1D experiments) and 1.0 mM (2D experiments).

NMR Experiments and Signal Assignments. 1D ^{1}H NMR experiments were performed using the gradient version of the WATERGATE pulse sequence (*7*, *8*). The spectra (averaged 512 scans) were acquired with 32K complex points over a spectral width of 10000 Hz (D19 = 100 μs, nulls at 14.7, 4.7, and −5.3 ppm).

COSY spectra were recorded in magnitude mode using the standard gradient version from the Bruker software library. The water presaturation version was used (saturation period of 2 s during the recycle delay). A total of 512 FIDs (16 scans each) were recorded, each of 4096 points, with a carrier frequency at the water resonance peak.

Phase-sensitive NOESY experiments (100, 300, and 500 ms mixing times) were acquired in States−TPPI mode and gradient version of WATERGATE for water suppression (same experimental conditions as 1D experiments). A total of 640 t_1 experiments with 64 scans were recorded. The data were processed using XWIN NMR version 3.5.6 on a PC workstation using Windows 2000. Assignments were performed visualizing the processed data in SPARKY (*9*).

Dye Titrations by NMR. NMR titrations were performed using 1D WATERGATE experiments as described above. Samples were prepared containing 100 μM AT5 in 10 mM sodium phosphate (pH = 7.0) and 20% DMSO-d_6. One-dimensional spectra were recorded after successive 50.0 μL additions of 650 μM DiSC$_2$(5) (in 100 μM AT5, 10 mM sodium phosphate, and 20% DMSO). Samples were allowed to equilibrate at 20 °C for 5 min prior to obtaining spectra.

RESULTS

DNA Characterization. The DNA duplex AT5 (Chart 1) was shown previously to effectively promote dimerization of DiSC$_2$(5) (*5*). This can be illustrated by the DNA-induced shift of the dye visible absorption band to shorter wavelength and formation of a 2:1 dye−DNA complex. On the basis of viscometric analysis, molecular modeling, the length of the dye molecule, and the preference for dimerization on alternating A-T versus G-C sequences, we proposed that the dye dimer was assembling in the minor groove of AT5 using the central five A-T base pairs as the binding site (*5*, *10*). The NMR studies described here were undertaken to unambiguously assign the binding site. We use the following terminology to indicate DNA resonances and cross-peaks: numbering of nucleotides starts at the 5′ end, and specific nucleotides are denoted by a subscripted number. The nonexchangeable proton resonances of AT5 were assigned using 2D NMR data, COSY, and NOESY, as previously described assuming a B-form duplex (*11*). COSY and NOESY spectra are shown as Figures S1 and S2 in Supporting Information.

The 1D ^{1}H NMR spectrum collected in H$_2$O/DMSO-d_6 (8:2) showed the characteristic hydrogen-bonded imino proton resonances between 12 and 14 ppm (Figure 1). The NMR data are therefore consistent with a right-handed B-form DNA duplex, in which Watson−Crick base pairing is intact. Eight of the nine expected imino proton resonances for the free 11 base pair duplex were observed, with end base pairs fraying and two resonances overlapping. The G/C imino proton resonances were assigned on the basis of the NOE correlation network C5H-C6H-CNH$_2$-imino proton. The A/T imino protons were assigned on the basis of a series of NOE correlations that involve either a G/C imino proton with an AC2H proton or an A/T imino proton with its associated AC2H proton (Figure S2). The cytosine amino protons were assigned on the basis of correlations with the G/C imino protons.

Dye−DNA Complex. The dipolar coupling network pattern in the NOESY spectrum of AT5 does not change significantly upon addition of DiSC$_2$(5) (Figure S3, Supporting Information), indicating that the B-conformation of AT5 is conserved in the presence of DiSC$_2$(5). This result is consistent with a minor groove binding mode, because intercalation results in the loss of key NOE cross-peaks due to increased distance between the protons on adjacent residues when an intercalator inserts between two base pairs (*12*).

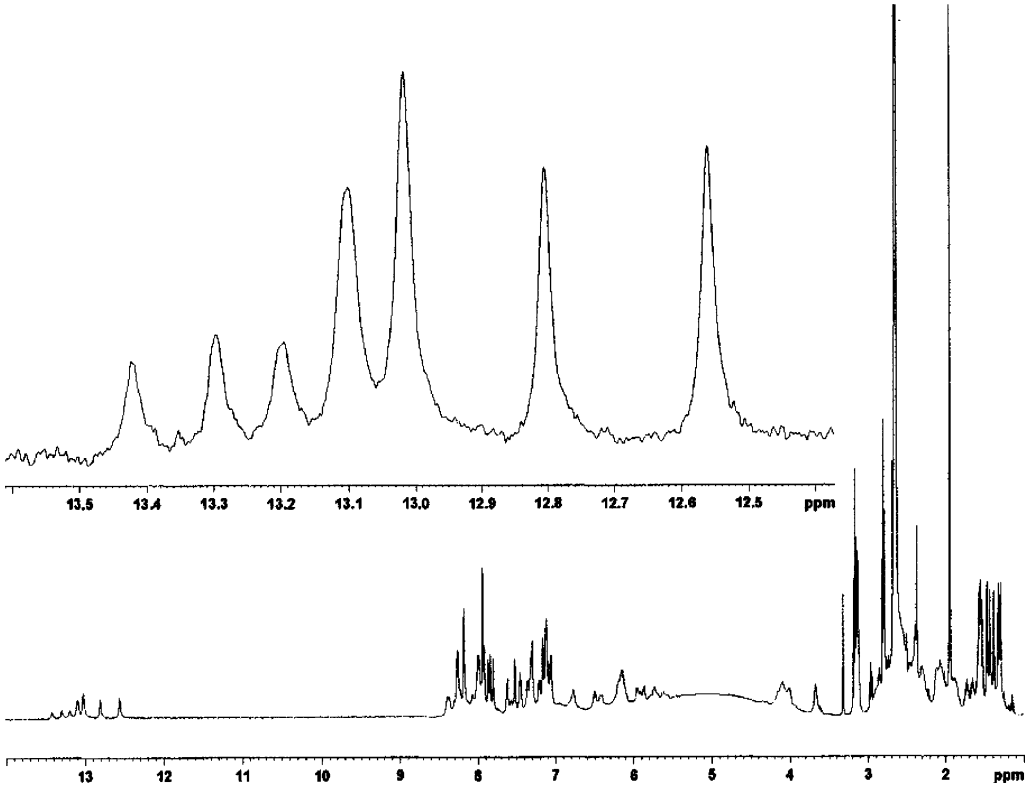


FIGURE 1: 1D ^{1}H NMR spectrum of 100 μM AT5 in 10 mM sodium phosphate buffer (pH = 7.0) and 20% DMSO-d_6. Inset: Expansion of the imino proton region. A total of 512 FIDs were acquired at 20 °C.

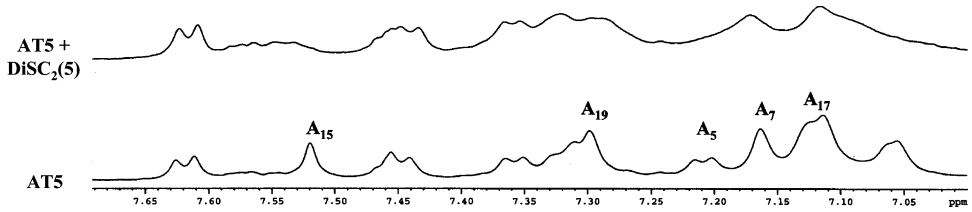


FIGURE 2: Effect of $DiSC_2(5)$ on the AC2H protons of AT5. Peaks for each of the central five A-T pairs are labeled in the bottom spectrum. The DNA concentration was 100 μM, and the dye concentration was either 0 μM (lower spectrum) or 200 μM (upper spectrum).

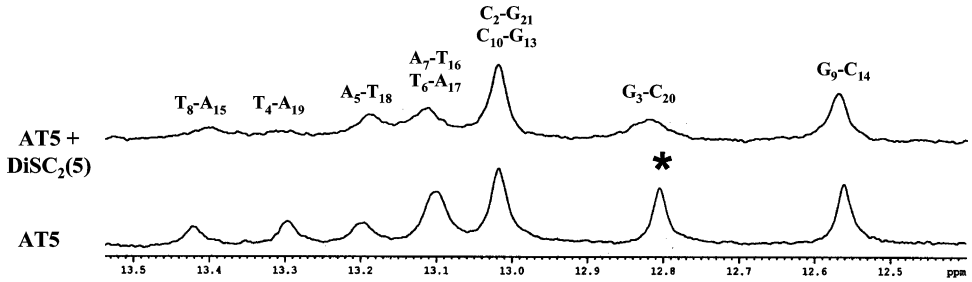


FIGURE 3: Effect of $DiSC_2(5)$ on the imino protons of AT5. The DNA concentration was 100 μM, and the dye concentration was either 0 μM (lower spectrum) or 200 μM (upper spectrum). The imino proton for the G_3-C_{20} base pair is indicated by an asterisk.

Figures 2 and 3 show different regions of the ^{1}H NMR spectra collected during titration of AT5 with $DiSC_2(5)$. Peaks assigned to the protons on carbon 2 of adenine (AC2H) are shown in Figure 2 and are of interest because these protons are located at the floor of the minor groove in an A-T base pair. We observe significant changes, particularly broadening, of some of these peaks as the dye is titrated into the DNA solution, providing strong evidence for minor groove binding by $DiSC_2(5)$ within the central A-T region of the duplex. Moreover, the cross-peaks between the AC2H and imino protons observed in the NOESY spectrum are severely broadened in the presence of $DiSC_2(5)$, except for the T_8-A_{15} pair, which broadens only slightly (data not shown).

The effect of $DiSC_2(5)$ on the hydrogen-bonded imino protons of AT5 is shown in Figure 3. Significant perturbations in chemical shift and peak broadening are observed for imino protons belonging to T_4-A_{19}, A_5-T_{18}, T_6-A_{17}, A_7-T_{16}, and T_8-A_{15}, i.e., the central A-T region designed to bind the dye. Surprisingly, the imino proton for the G_3-C_{20} base pair is also broadened (indicated with an asterisk in Figure 3) while the remaining G/C imino resonances remain unaltered. These results are also consistent with a minor groove binding mode (*13*).

Table 1: Effect of DNA Sequence Changes on $DiSC_2(5)$ Dimerization

DNA	Change	Sequence	A_{593}[a]
AT5	None	5′-GC**GTATAT**GCG-3′ 3′-CG**CATATA**CGC-5′	1.77
1	Delete G_3-C_{20}	5′-GC-TATATGCG-3′ 3′-CG-ATATACGC-5′	1.11
2	Delete T_4-A_{19}	5′-GCG-ATATGCG-3′ 3′-CGC-TATACGC-5′	0.72
3	Delete T_8-A_{15}	5′-GCGTATA-GCG-3′ 3′-CGCATAT-CGC-5′	1.00
4	Delete G_9-C_{14}	5′-GCGTATAT-CG-3′ 3′-CGCATATA-GC-5′	1.68
5	Change G_3→I	5′-GC**I**TATATGCG-3′ 3′-CGCATATACGC-5′	1.56
6	Change G_3→C	5′-GC**C**TATATGCG-3′ 3′-CG**G**ATATACGC-5′	1.43
7	Change G_3→A	5′-GC**A**TATATGCG-3′ 3′-CG**T**ATATACGC-5′	0.92
8	Change G_9→I	5′-GCGTATAT**I**CG-3′ 3′-CGCATATACGC-5′	1.31
9	Add A-T pair	5′-GCGTATAT**A**GCG-3′ 3′-CGCATATA**T**CGC-5′	1.58
10	Change T_6→C	5′-GCGTA**C**ATGCG-3′ 3′-CGCAT**G**TACGC-5′	0.80
11	Change T_8→C	5′-GCGTATA**C**GCG-3′ 3′-CGCATAT**G**CGC-5′	0.60

[a] Spectra were normalized at 650 nm. A_{593} in the absence of DNA is 0.35.

Upon addition of the first aliquot of $DiSC_2(5)$ to AT5 in the 1D NMR titration experiment, we observed broadening and shifting of the proton resonances rather than the appearance of new peaks that would be expected for a dye−DNA complex. This trend continued throughout subsequent additions of dye, and repeating the experiment at 5 °C failed to yield resonances for the complex. These observations indicate that the dye−DNA complex is in intermediate exchange (relative to the NMR time scale) with free dye and unbound DNA, similar to dimerization of distamycin on the 6 bp site 5′-TTTAAA-3′ (*14*).

While we had hoped to use NMR to obtain a high-resolution structure of the dye−DNA complex, the NOESY spectrum of the complex does not contain any contact NOE peaks between the dye and AT5 since all of the dye proton signals significantly broaden, presumably as a result of the intermediate exchange. This effect was observed in a reverse titration experiment, in which AT5 was added to a solution of $DiSC_2(5)$ (data not shown). Cooling to 5 °C did not improve the spectrum, precluding determination of a structure for the complex. Nevertheless, the NMR experiments provide verification that dimerization of $DiSC_2(5)$ occurs within the minor groove of AT5.

Sequence Dependence of Dimerization. A series of duplexes was designed to further study the involvement of G_3-C_{20} in the $DiSC_2(5)$ binding site on AT5 (Table 1). These duplexes feature base pair deletions (**1**−**4**), sequence changes (**5**−**8**, **10**, **11**), or an insertion (**9**) either within or adjacent to the central six base pairs indicated by NMR to be involved in binding of the dye.

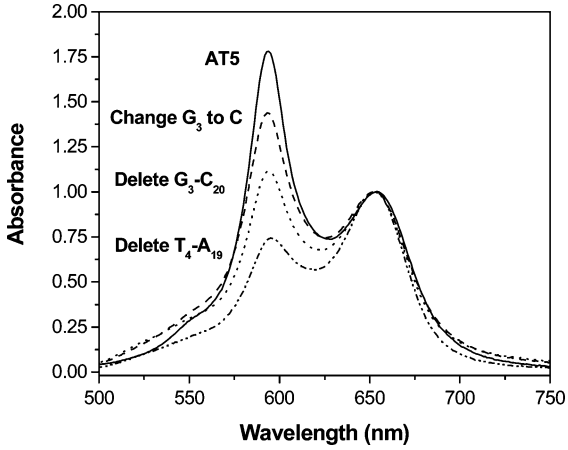


FIGURE 4: UV−vis spectra recorded for 20 μM $DiSC_2(5)$ in the 10 μM DNA duplex. DNA sequence changes are indicated to the left of each spectrum; see Table 1 for full sequences.

Dimerization was investigated by UV−vis absorbance based on the shift in λ_{max} from 650 to 593 nm (representative spectra shown in Figure 4) (*5*). Normalization of the absorbance at 650 nm allows for direct comparison of the amount of dimer formed on each DNA duplex (Table 1). Each of the duplexes promotes dimerization of the dye, but $DiSC_2(5)$ dimerizes to a greater extent on AT5 than on any of the other duplexes. Deletion of G_3-C_{20}, T_4-A_{19}, or T_8-A_{15} (duplexes **1**−**3**) causes significant decreases in dimer formation, emphasizing the importance of these base pairs in promoting dye binding. In contrast, deletion of the G-C base pair at the opposite end of the binding site (i.e., G_9-C_{14}, duplex **4**) has almost no effect on dimerization.

To further probe the role of the G_3-C_{20} base pair in the binding site, sequence changes were made at this position. First, replacing G with I, which lacks the exocyclic amino group at C2, results in only a minor decrease in dimerization (duplex **5**). Similarly, dimerization is favorable on duplex **6**, where the G_3-C_{20} pair was inverted to a C-G pair. In contrast, replacing this base pair with an A-T pair significantly decreases dimerization (duplex **7**).

Alterations at the distal end of the binding site have relatively weaker effects on DNA binding. Replacement of G_9 with I (duplex **8**) and insertion of an A-T pair after T_8-A_{15} (duplex **9**) only slightly decrease the extent of dimerization. Note that both duplexes **7** and **9** contain six contiguous alternating A-T pair sites, but dimerization is only favorable on the latter. Thus, a G-C, C-G, or I-C base pair at the third position effectively promotes dimerization whereas an A-T pair is inhibitory. Finally, replacing either the third or fifth base pair within the central A/T tract with a C-G pair strongly inhibits dimerization (duplexes **10** and **11**).

DISCUSSION

Many natural (*15*, *16*) and synthetic (*17*) polyamides bind sequence specifically in the minor groove of DNA by forming cofacial dimers within the groove. Sequence specificity is achieved through a combination of hydrogen-bonding and van der Waals interactions. The symmetrical cyanine dyes such as $DiSC_2(5)$ are intriguing because they also bind to DNA as dimers, but they lack the H-bond donor and acceptor groups present in the polyamides such as distamy-

cin. The sequence dependence of dimerization and a variety of optical spectroscopic and hydrodynamic experiments performed previously indicated that dimers were forming within the minor groove. Dimerization occurred preferentially at alternating A/T sequences over nonalternating A/T or G/C sequences, and the length of the dye indicated that a five base pair binding site would be sufficient for binding a single dimer, which led to the original design of AT5. Indeed, optical spectroscopy experiments demonstrated formation of a discrete 2:1 complex between $DiSC_2(5)$ and AT5 (*5*).

NMR experiments reported here were designed to verify that the cyanine dye dimer was bound within the minor groove and spanned the central five base pairs of AT5. Several observations are consistent with this model. First, addition of the dye to AT5 has no effect on the NOESY pattern of the cross-correlation peaks corresponding to protons in the major groove. Intercalation results in separation of adjacent base pairs, which is manifested in a loss of these NOE connectivities (*12*). The retention of the NOESY sequential connectivity assignments indicates that only small structural perturbations of the DNA occur and that the dye must be binding nonintercalatively. The same conclusion was reported from NMR for the minor groove binder distamycin A (*18*). The malleable backbones of these small molecules probably account for the lack of DNA distortion, allowing for the ligand to adapt to the DNA structure.

While NOEs between the dye and the DNA are not observed due to broadening of the dye resonances and overlapping signals, significant changes occur in two other sets of DNA protons. First, the protons on carbon 2 of the adenine residues (AC2H) undergo severe broadening in the presence of the dye. Since these protons reside on the floor of the minor groove, these results provide compelling evidence in support of a minor groove binding mode for the dye.

In addition to the effect of the dye on the AC2H protons, the hydrogen-bonded imino protons for all five of the central A-T base pairs are broadened and shifted slightly downfield in the presence of $DiSC_2(5)$. These results provide further support for binding of the dye in the alternating A/T tract, but a surprising result was that the imino proton resonance for the flanking G_3-C_{20} base pair also broadened, indicating that this base pair was also part of the binding site.

It is well-known that the minor groove geometry and flexibility of duplex DNA are greatly affected by sequence (*19*, *20*). Since the dye is unable to make strong hydrogen bonds with groups in the minor groove, we suspected that the G_3-C_{20} base pair might be contributing to the binding site by widening the groove at the 5′ end of the A/T tract (*21*−*23*). We performed UV−vis studies to investigate in greater detail the sequence dependence of $DiSC_2(5)$ binding. Monitoring dimerization by the intensity of the hypsochromically shifted absorbance peak has the advantages of rapidly and economically assessing dimer formation on a relatively large number of sequence variants.

The UV−vis experiments verified the importance of having at least five consecutive alternating A-T pairs in the binding site, with two notable exceptions (Table 1). Dimerization was strongly inhibited for duplexes **1** and **7**, in which the G_3-C_{20} pair was deleted or changed to A-T, respectively. These results illustrate the important role played by this position in defining the dimerization site for the dye. However, note that if the base pair is reversed (i.e., C_3-G_{20}, duplex **5**) or the G is replaced by I (duplex **6**), dimerization is still favorable. Thus, rather than the dye molecules making specific contacts with G_3 and/or C_{20} in AT5, these results indicate that the added width of the groove at a G-C or I-C pair is responsible for promoting dimerization of $DiSC_2(5)$.

While a wide minor groove at the 5′ end of the binding site is important for dimerization, substituting A-T pairs with G-C pairs in the central A/T tract (duplexes **10** and **11**) severely inhibits dimerization. Thus optimal binding of $DiSC_2(5)$ in the minor groove depends on both the groove width and depth. The exocyclic amino group on guanine prevents the dye molecules from penetrating as deeply into the groove as for the unobstructed groove of AT5. Since the dyes must rely on van der Waals interactions and the hydrophobic effect to promote binding to the DNA, G-C replacements are not well tolerated in the A/T tract. This stands in contrast to imidazole-containing polyamides that can form specific hydrogen bonds with the G-NH_2 (*24*, *25*). In addition, distamycin binds as a dimer in the minor groove of 5′-AAATT-3′ as well as variants in which the second, third, or fourth base pair is replaced by G-C (*14*). The cyanine dye appears to be much more selective in this regard.

Finally, the deletion variants indicate an interesting directional preference for dimerization of $DiSC_2(5)$ in the minor groove of AT5. If either G_3-C_{20} or T_4-A_{19} is deleted, dimerization decreases significantly more than when either G_9-C_{14} or T_8-A_{15} at the opposite end of the duplex is deleted. These results are consistent with those obtained by NMR spectroscopy, where the imino protons for the G_3-C_{20} and T_4-A_{19} base pairs were significantly more broadened than those from G_9-C_{14} and T_8-A_{15}.

The distamycin paradigm directed efforts to rationally design minor groove binding ligands for a decade (*12*, *26*, *27*). Compounds that fit in this class have three distinguishing features: (1) their structures are curved and flexible, allowing them to adapt to the curvature of the minor groove; (2) they are monocationic, allowing two molecules to bind in an antiparallel orientation to avoid electrostatic repulsions; and (3) they make specific hydrogen-bonding contacts with groups on the floor of the minor groove. This design ultimately led to the highly successful hairpin polyamides that can bind DNA at subnanomolar concentrations and interfere with transcription. However, the past 5 years has seen the discovery of compounds that bind in the minor groove as dimers yet do not possess one or more of the polyamide characteristics. DB293, reported by Wilson and co-workers, is a dication, yet it clearly binds in the minor groove as a dimer (*28*, *29*). A tricationic analogue of $DiSC_2(5)$ exhibits a similar binding mode (*30*). In these cases, electrostatic repulsions are avoided by offsetting the two molecules such that the positive charges are not aligned. The symmetrical cyanine dyes represented by $DiSC_2(5)$ lack the ability to form H-bonds with the DNA groove, yet they dimerize at similar sequences as distamycin. While these dyes are unlikely to match the affinities and sequence selectivities exhibited by the polyamides, their ability to dimerize in the minor groove expands our understanding of how the DNA accommodates diverse ligand structures.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Three figures showing the 500 MHz 2D COSY spectrum of AT5, 500 MHz NOESY of 1 mM AT5, and two identical regions of the 500 MHz NOESY of 1 mM AT5 and one table listing chemical shifts of protons in AT5. This material is available free of charge via the Internet at http://pubs.acs.org.

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