Fluorescent \( d(CGCGAATTCGCG) \): characterization of major groove polarity and study of minor groove interactions through a major groove semantophore conjugate

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ABSTRACT
The major and minor groove in duplex DNA are sites of specific molecular recognition by DNA-binding agents such as proteins, drugs and metal complexes and have functional significance. In view of this, understanding of the inherent differences in their environment and the allosteric information transfer between them induced by DNA-binding agents assumes importance. Site-specific incorporation of 5-aminodansyl-dU, (U\(*\)) in oligonucleotides \( d(CGCGAAU'TCGCG) \) and \( d(CGCGAATU'CGCG) \) leads to fluorogenic nucleic acids, in which the reporter group resides in the major groove. The fluorescent observables from such a probe are used to estimate the dielectric constant of the major groove to be \(~55\)D, in comparison to the reported non polar environment of the minor groove \(~20\)D in poly d[AT]-poly d[AT]. An exclusive minor groove event such as DNA-netropsin association can be quantitatively monitored by fluorescence of the dansyl moiety located in the major groove. This suggests existence of an information network among the two grooves. The fluorescent DNA probes as reported here may have potential applications in the study of structural polymorphisms in DNA, DNA-ligand interactions and triple helix structure.

INTRODUCTION
An understanding of the molecular basis of DNA recognition by proteins, drugs and various ligands is crucial to discern the chemistry underlying the basic cellular processes, their regulation and rational design of drugs. The major and minor grooves in duplex DNA act as conduits of molecular information required for DNA association with other molecules since hydrogen bonding centers in bases are pointed into these grooves \((1)\). Large molecules such as proteins binding to nucleic acids, recognize DNA via specific interactions in the major groove \((2)\); smaller DNA binders such as antibiotics interact with DNA either by intercalating the base pairs or by association in the minor groove or both \((3)\). Extensive X-ray crystallographic studies have indicated specific structural changes induced in DNA upon complexation with other molecules \((4)\) and are well supported in many cases by spectroscopic studies in solution \((3–5)\). The expression of molecular forces that dictate and control affinities/ specificities of DNA binding agents (proteins/drugs) are modulated by local micro-environments. Hence characterization of the environment in the grooves of DNA complexes, assumes importance in delineating the relative contributions of various molecular interactions in stabilizing DNA complexes. In view of the functional importance of major and minor grooves in DNA recognition, it would be appropriate to study the inherent differences in their environments and the information exchange/transfer that is possible among them upon DNA binding with other molecules. In this paper it is demonstrated that an exclusive minor groove event such as DNA-netropsin association can be quantitatively monitored by changes in dansyl fluorescence as observed from the major groove. The major groove polarity of DNA in oligonucleotides \(2–5\) has been characterized using an environment sensitive fluoroprobe, dansyl group rigidly linked to C-5 of dU and directed in the major groove (Figure 1) as a 'semantophore'.

MATERIALS AND METHODS
All chemicals used were of the highest purity available. Netropsin was procured from Boehringer Mannheim. The oligonucleotide sequences \(2–5\) were synthesized by phosphoramidite chemistry on a Pharmacia GA Plus DNA synthesizer using 5-aminodansyl-5'-O-dimethoxytrityl-2'-dU-3'-O-phosphoramidite in place of standard T amidite for \(2–5\), as reported earlier \((6)\) and were purified by FPLC and rechecked by RP HPLC. FPLC: (PepRPC HR 5/5, Pharmacia) Buffer A: 5% \(CH_3CN\) in 0.1 M TEAA; Buffer B: 30% \(CH_3CN\) in 0.1 M TEAA. Gradient 0%B, 3 min; 0–15%B, 5 min; 15–75%B, 35 min; 75–100%B, 1 min. HPLC: Buffers A and B, same as FPLC. Gradient: A to B 20 min.

5-aminodansyl-2'-dU \(6\) was prepared by detritylation of 5'-O-dimethoxytrityl-5-aminodansyl-2'-dU using p-toluensulfonic acid in dichloromethane at 0°C for 15 min. The reaction mixture was washed with aqueous \(NaHCO_3\), concentrated to

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dryness followed by column purification over silica gel (100–200 mesh) using dichloromethane:methanol as eluant. 1H NMR (DMSO-d6): 8 11.4 (s, 1H, NHSO2-), 9.7 (s, 1H, N3H), 8.45, 8.3 and 8.1 (3xd, 3H, dansyl Ar-H), 7.75 (s, 1H, H6), 7.6 (m, 2H, dansyl Ar-H), 7.25 (d, 1H, dansyl Ar-H), 6.1 (t, 1H, H1'), 5.3 (d, 1H, 3'OH), 4.9 (t, 1H, 5'-OH), 4.2 (m, 1H, H4'), 3.75 (m, 1H, H3'), 3.4 (m, 2H, H5'and H5' '), 2.8 (s, 6H, N(CH3)2), 2.1 and 1.9 (m, 2H, H2' and H2''). UV melting experiments on DNA duplexes 2–3 were performed with or without netropsin in 10 mM Tris buffer, pH 7.0, containing 100 mM NaCl and 20 mM MgCl2 using Perkin Elmer Lambda 15 UV/VIS spectrophotometer, fitted with a temperature programmer and heating at a rate of 0.5°C/min. Fluorescence measurements were done on a Perkin Elmer model LS-50 B spectrometer attached to a Julabo programmable water circulator for variable temperature experiments (Tm). The fluorescent DNA samples dissolved in the above buffer were excited at 323 nm and the emission monitored at 500 nm using a spectral bandwidth of 2.5 nm. CD spectra were recorded on a Jobin Yvon instrument at pH 7.0. Association constants were calculated (7) from the I/a Vs 1/L plots, where a is the fraction of oligonucleotide bound to netropsin and L is the effective netropsin concentration.

For characterization of the major groove polarity, fluorescence spectra of 6 were recorded in mixed organic/aqueous solvent systems prepared by stirring distilled water with appropriate volume percent of 1,4-dioxane (spectroscopic grade). Emission and excitation wavelength maxima were obtained after smoothening each spectrum by using a quadratic polynomial and are accurate to ± 0.5 nm.

**RESULTS AND DISCUSSION**

We chose dansyl as fluoroprobe because it has large Stoke's shift and further, it responds to perturbations in local environments such as changes in solvation, ligand binding, etc. by undergoing spectral shifts (8). Such alterations in the spectral properties of dansyl fluorophore have been previously used to detect substrate binding to protein (9) and to study interaction of DNA with the Klenow fragment of DNA polymerase I (10).

**Synthesis, characterization and stability of 5-aminodansyl-dU-oligonucleotide**

We have previously reported successful synthesis of DNA containing 5-amino-dU using trifluoroacetyl as a NH2 protector (6). Fluorescent analogues of these oligonucleotides containing 5-amino-dansyl residue (2–5) were synthesized by an identical procedure except that trifluoroacetyl was replaced by dansyl group which serves as both a protector and a fluorescent label. The coupling efficiency of dansyl amidite was similar to the commercial amidites of normal nucleosides. The sulphonamide group is stable to oligonucleotide synthesis and deprotection conditions and hence the final products are fluorescent. The oligonucleotides after purification by FPLC and rechecking by HPLC showed high purity desirable for biophysical studies. The successful incorporation and retention of 5-aminodansyl-2'-dU (U*) in the final oligonucleotides was confirmed by (i) stability to EcoR1 restriction digest since the modification is in recognition site, (ii) base composition analysis by snake venom phosphodiesterase followed by alkaline phosphatase and (iii) UV absorbance at 335 nm (broad) and fluorescence with emission at 500 nm upon excitation at 335 nm due to presence of dansyl group.

Dickerson's dodecamer d(CGCGAATTCGCG) (11) is one of the most well studied oligonucleotides, both in free form and as a complex with a variety of ligands. The two T residues in the above sequence were replaced one at a time by 5-aminodansyl-dU (U*) to yield the oligonucleotides 2 and 3. Substitution at a C-5 of pyrimidine residue does not significantly affect the standard Watson–Crick mode of hydrogen bonding in dA–dT base pair (12,13) since the modification is in recognition site, (ii) base composition analysis by snake venom phosphodiesterase followed by alkaline phosphatase and (iii) UV absorbance at 253 nm (negative Cotton effect). The CD spectra of modified oligonucleotides 2 and 3 (not shown) were similar to that of Dickerson's dodecamer d(CGCGAATTCGCG) in the region 220–320 nm (negative Cotton effect at 253 nm, positive Cotton effect at 280 nm with cross over at 270 nm) indicating no major alterations of base stacking in 2 and 3. The CD profile overall corresponds to the B-form. As seen by temperature dependent UV changes (Figure 2), the dansylated oligonucleotides 2 and 3 gave a lower Tm (48°C) compared to the unmodified dodecamer (60°C) suggesting a slight destabilising effect on duplex, locally induced by amidodansyl group in dU.

The oligonucleotides 2–5 are self complementary and hence their duplexes contain two dansyl groups, one in each strand. Although these differ in the position of dansyl groups, the fluorophores are symmetrically located in the major groove. The observed fluorescence properties are therefore a sum of contribution from both dansyl groups of duplex. In duplexes 2 and 3, the two dansyls are within the binding site of netropsin (AATT) while in duplex 4 and 5 they are outside the binding site, separated by 10 base pairs in case of 4 and 6 base pairs in 5. The λem of 4 and
5 were blue shifted by 8–10 nm compared to that of 2 and 3. The 
$T_m$ of 2 and 3 were determined from temperature dependent
fluorescence studies. The melting studies showed an enhance-
ment in fluorescence intensity with increasing temperature, the
intensity attaining a maximum (Figure 3) just around the melting
temperature of oligonucleotide. This rise in fluorescence intensity
as a function of temperature is invariant with the oligonucleotide
concentration in the range 0.1 μM to 10 μM. From this fact and
that of an earlier report (14), the observed temperature of
maximum fluorescence intensity was taken as an approximate
measure of melting of duplex. This $T_m$ value was in close
agreement with that obtained from UV melting studies. The fact
that DNS–DNA exists in B-form at room temperature (as seen by
CD profile and UV/fluorescence $T_m$) substantially supports the
use of DNS–DNA 2 and 3 as suitable models for studying DNA
interactions.

Interaction of netropsin with DNS–DNA

Netropsin is an antiviral minor groove binding drug (3a, 15) and
its complexation with B-DNA has been studied by using a
combination of spectroscopic and calorimetric techniques. The
molecular details of its interaction in minor groove has been
established by NMR spectroscopy (5) and X-ray crystallographic
studies (11). We sought to examine the effect of netropsin binding
to DNA in the minor groove using the fluorescent dansyl probe
located in the major groove. Upon stoichiometric addition of
netropsin into DNS–DNA 2, an increase in intensity was obtained
in the dansyl fluorescence emission at 500 nm as a function of
ligand concentration. The fluorescence enhancement reached
near saturation at 1:1 stoichiometry (Figure 4). The stoichiometry
of binding as computed from a plot of 1/a vs 1/[L] was also close
to 1. The modified oligonucleotide 3 behaved in a similar way and
in both cases, no appreciable shifts in λex and λem were seen upon
netropsin addition. The association constants (Table 1) calculated
from the binding isotherm (25°C) were in the range 107–108
M$^{-1}$, corresponding to a binding free energy, $ΔG$ of $-10.8$ to $-12.1$
kcal M$^{-1}$ which is in close agreement with the literature reported
value (15) for netropsin binding to decamer d(GCGAATTCGC)
1. Thus dansyl fluorescent probe present in major groove
efficiently monitors netropsin binding in the minor groove. The
binding of netropsin with the oligonucleotides 2 and 3 was also
established by UV and fluorescence (Figure 3) melting experi-
ments. Compared to DNS–DNA alone, in 1:1 complex of
DNS–DNA:netropsin, the $T_m$ as measured by both UV and
fluorescence, was enhanced by similar extents ($ΔT_m \sim 31 ^\circ C$) due
to thermal stabilization of DNS–DNA by netropsin binding.

The association of netropsin to DNA involves displacement of
the spine of hydration in the minor groove corresponding to
AATT stretch (11,15). Further, the binding widens the minor
groove by 0.5–2.5 Å, accompanied by a bending of helix axis by
8°, but without unwinding or elongation of the double helix (13).
The observed increase in fluorescence intensity of DNS located
in the major groove of 2 is a result of local structural changes
induced in DNA upon minor groove binding by netropsin. The oligonucleotide 3 has the fluorophore located on a adjacent base to that in 2 and the fluorescence titration of 3 with netropsin also gave similar results. In both 2 and 3, dansyl is linked from the major groove side to a base pair that is directly involved in hydrogen bonding with netropsin in the minor groove. It is possible that the observed changes in fluorescence properties is a direct consequence of the electronic changes induced in base pairs by netropsin binding. To examine this, the oligonucleotides 4 and 5 which have the fluorophore located outside the region of netropsin binding (AAAT) were synthesized. Upon netropsin titration, both 4 and 5 gave a higher percent enhancement (−30%) in intensity compared to 2 and 3 (−10%) and there was no shift in  in due to complexation (Figure 5). The computed binding constants for oligonucleotides 2−5 are shown in Table 1.

**Table 1. Association constants and free energy changes for netropsin binding with fluorescent DNA**

<table>
<thead>
<tr>
<th>Compound No</th>
<th>Oligonucleotide</th>
<th>Ka (M⁻¹)</th>
<th>ΔG (kcal M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GCGAATTCCG</td>
<td>2.8 x 10⁸</td>
<td>−11.5</td>
</tr>
<tr>
<td>2</td>
<td>CGCGAu*TGCGG</td>
<td>5.9 x 10⁷</td>
<td>−10.8</td>
</tr>
<tr>
<td>3</td>
<td>CGCGAu*TCCCGG</td>
<td>7.5 x 10⁷</td>
<td>−10.9</td>
</tr>
<tr>
<td>4</td>
<td>GCu*GTGAATTCCAGC</td>
<td>1.5 x 10⁸</td>
<td>−11.1</td>
</tr>
<tr>
<td>5</td>
<td>GCTGu*GAATTCCAGC</td>
<td>7.8 x 10⁸</td>
<td>−12.1</td>
</tr>
</tbody>
</table>

*All binding experiments were performed at 25°C.
*Taken from ref. 15.

Figure 5. Overlay of fluorescence spectra of DNS-DNA 4, without netropsin (A) and increasing netropsin concentration.

**Characterization of major groove polarity**

The fluorescence emission spectra of many fluorophores are sensitive to polarity of their surrounding environment (17). This polarity dependence arises from (i) interaction of dipole moment of the fluorophore in the excited state with the electrical field induced by the surrounding solvent dipoles and (ii) specific chemical interactions such as hydrogen bonding, charge-transfer interactions etc. between the fluorophore and the solvent molecules. The former is a general solvent effect governed by the orientation polarity (f) which is derived from the refractive index (n) and dielectric constant (ε)

\[ f = \frac{(ε-1)/(2ε+1)}{[(n^2-1)/(2n^2+1)]} \]

of the medium. The orientation polarity of the solvent reflects the local polarity changes due to reorientation of solvent molecules around the excited fluorophore. In the absence of solvent−fluorophore specific interactions, orientation polarity effects provide a major contribution to the emission spectral shifts. The physicochemical interactions between fluorophore and the solvent molecules causing the general solvent effects is described by Lippert equation (17).

\[ Δν = (2Δμ^2hca^2) × f \]

where Δν is the Stoke’s shift (the difference between excitation and emission wavelength, expressed as energy difference in cm⁻¹), Δμ is the difference in dipole moment between the excited and the ground states of the fluorophore, h is Planck’s constant, c is the speed of light and a is the radius of the cavity in which the fluorophore resides. Since for a given fluorophore, a and Δμ remain constant, the above equation implies that the Stoke’s shift is directly related to solvent polarity (f) changes. A calibration curve (Lippert plot) may be generated for variation of Stoke’s shift (Δν) with respect to orientational polarity (f) and this exhibits a linear behavior if general solvent effects predominate over specific solvent effects. When the fluorophore is in an unknown environment, from the measured Stoke’s shift the orientation polarity (f) can be obtained which is interpolated to yield the dielectric constant (ε) of the medium.

It has been reported that HOECHST 33258 which is a highly fluorescent environment-sensitive drug selectively binds to AT
regions in the minor groove and induces sequence specific structural changes in the resulting DNA complex (18). A comparison of the fluorescent observables of this complex with the corresponding properties of free ligand in different solvent systems of variable polarity (neat organic and mixed organic/aqueous solvents) has enabled the determination of the polarity of the minor groove which is around 20D.

In order to estimate the dielectric constant of the major groove in DNS–DNA, the fluorescence parameters ($\lambda_{\text{ex}}$ and $\lambda_{\text{em}}$) of the monomer 5-aminodansyl-2'-dU 6 were first measured in media of different dielectric constants generated by varying ratios of dioxane-water (Table 2). The Stoke's shift $\Delta V$ calculated from the above parameters, exhibited a linear correlation with the orientation polarity (Figure 6) suggesting the dominant influence of general solvent effect in the observed fluorescence properties of 6. Assuming that such a correlation of orientation polarity and dielectric constant for 5-aminodansyl-2'-dU 6 is also valid on its incorporation into the major groove of Dickerson's dodecamer, the observed Stoke's shift of DNS–DNA 2–5 can be used to estimate the polarity exhibited by conjugated dansyl in the major groove. The Stoke's shift among 2–5 is spread over a range of 100 cm$^{-1}$, with an average at 10900 cm$^{-1}$. Upon interpolation this gave an orientation polarity of $\lambda_{\text{ex}} - \lambda_{\text{em}}$ = 320 (Figure 6, arrow a). This implies that the major groove in 2–5 is non-polar compared to the bulk water (80D), but considerably more polar than the minor groove (20D). Although slight variation was seen in Stoke's shift among 2–5, the magnitude of difference ($\Delta V$– 100 cm$^{-1}$) is much less to effect significant changes in $\lambda_{\text{ex}}$ and hence the value of the interpolated $\lambda_{\text{ex}}$. It may be added that, upon netropsin complexation, no changes were seen in $\lambda_{\text{ex}}$ and $\lambda_{\text{em}}$ of DNS–DNA 2–5 and extension of the above principle suggest no appreciable alternation of the polarity of the major groove upon netropsin complexation in the minor groove. This method of polarity estimation using dansyl moiety in 2–5 is valid since the fluorophore is regiospecifically and rigidly conjugated to DNA by a sulphonamide bond, without much freedom for flexible averaging over different environments.

<table>
<thead>
<tr>
<th>% Dioxane</th>
<th>$\epsilon$</th>
<th>$n$</th>
<th>$f$</th>
<th>$\lambda_{\text{ex}}$</th>
<th>$\lambda_{\text{em}}$</th>
<th>$\Delta V$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-Dioxane</td>
<td>0.307</td>
<td>323</td>
<td>500</td>
<td>10959</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNS–DNA 2</td>
<td>0.307</td>
<td>323</td>
<td>499</td>
<td>10919</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNS–DNA 3</td>
<td>0.307</td>
<td>320</td>
<td>492</td>
<td>10925</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNS–DNA 4</td>
<td>0.307</td>
<td>490</td>
<td>10842</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNS–DNA 5</td>
<td>0.307</td>
<td>490</td>
<td>10842</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values for $\epsilon$, $n$, $f$ corresponding to various Dioxane:H$_2$O compositions are taken from ref 18.

It is well known that fluorescence properties are also influenced by ionic strength and pH of the medium and the exact values of the latter are difficult to obtain for organic/aqueous mixed solvents. In order to examine such effects on dansyl fluorescence, the dependence of Stoke's shift of dansyl in 6 was measured over a range of ionic strength (0.1 M to 5 M) and pH (3 to 7.5). It was observed that the Stoke's shift was invariant under these conditions and hence the induced differences in fluorescence properties in various solvent systems is due to bulk solvent properties. It may be pointed out that the minor groove polarity estimated for DNA complexes with a non-conjugated fluorophore may have significant contribution from the free, uncomplexed ligand and so may lead to substantial errors in polarity estimation. The presently described method employing site-specific covalent conjugation exhibits negligible effect on the Stoke's shift of a fluorophore since it has been shown that there is little, if any effects on the optical properties of a free fluorescent ligand upon conjugation to a synthetic polymer (18).

**CONCLUSION**

The molecular recognition of nucleic acids by proteins and drugs mainly occurs from the major and minor grooves, and it is therefore important to know the molecular environment in these regions. We have employed a environment sensitive fluorophore, dansyl covalently linked to dU at C5 to quantitate the major groove dielectric constant which turns out to be $\sim$55D in contrast
to the more non-polar minor groove (20D). It is also found that significant 'groove cross talk' exists between the major and minor groove as indicated by fluorescence changes in dansyl fluoroprobe in the major groove upon binding of netropsin in the minor groove. Further potential applications of such fluorescent DNA probes include study of structural polymorphism in DNA, DNA–peptide interactions and investigation of triple helix formation by using fluorescent DNA as the Hoogsteen strand and studies in these directions are in progress.

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