Investigation of DNA dynamics and drug–DNA interaction by steady state fluorescence anisotropy

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ABSTRACT

We have used steady-state fluorescence polarization anisotropy (<FPA>) of ethidium probe molecules bound to DNA to investigate DNA–DNA interactions and the effect of high densities of intercalating drugs on the internal motions of DNA responsible for depolarization of the ethidium fluorescence. To calibrate the method, we examined the effect of DNA length on <FPA> using DNA varying in size from 10–150 base pair. The association of ~30 base pair DNA at high concentrations was then detected by its effect on <FPA>. With sample concentrations approaching those commonly used in various physical experiments (NMR, Raman) significant DNA–DNA interactions are observed. With high molecular weight DNA (>500 base pair), the limiting value of the <FPA> (0.23) is due to internal motions of the DNA (and bound chromophores). The <FPA> of ethidium probe molecules (1 drug/200 base pair) is unaffected by the addition of high levels (1 drug/2 base pair) proflavine. This indicates that either the elastic properties of DNA are unaffected by high densities of intercalated drug or that the depolarization of the ethidium fluorescence is due to highly localized motions of the base pairs that are unperturbed by binding of drugs at neighboring sites.

INTRODUCTION

Fluorescence polarization techniques have been used extensively to probe the properties of DNA and DNA–drug interactions [1–6] and protein–DNA complexes [7,8]. Time-resolved fluorescence depolarization measurements have been especially valuable in probing fast (~50 ps–100 ns) dynamic processes in DNA and at least three different processes contribute to the depolarization in high molecular weight DNA [1,3,5]. Rapid initial decay has been attributed to wobbling of the drug in the intercalation site [2,3,5]. Further decay of the fluorescence polarization anisotropy in the 1–50 ns region results primarily from torsional motions of the DNA, and finally, the long time decay arises from bending of the DNA helix [1,3,9,10]. With short DNA (<50 base pair), end-over-end tumbling and spinning of DNA about its long axis also contributes to the depolarization. In this paper, we use the steady-state fluorescence polarization anisotropy, <FPA>, to study (i) DNA–DNA interactions and (ii) the effect of intercalating drugs on local internal motions in DNA and the elastic
properties of DNA. To apply \(<\text{FPA}\>) measurements to these problems, we first examine the effect of helix length and solvent viscosity on the \(<\text{FPA}\>). Once these "calibration" measurements were made, \(<\text{FPA}\>) measurements were used to study the intermolecular association of DNA molecules in concentrated solutions and the effect of high concentrations of a second intercalating drug (proflavine) on the \(<\text{FPA}\>) of ethidium probe molecules. As a practical matter, we have also used \(<\text{FPA}\>) to monitor the DNase I digestion of DNA for preparing large quantities of short (10-100 base pair) DNA for these and other physical studies.

MATERIALS AND METHODS

**Materials.** High molecular weight calf thymus DNA (Type I), DNase I, ethidium bromide, proflavine, and buffer reagents were obtained from Sigma Chemicals Co., and the bis(acridine) spermine was a gift from Dr. William A. Denny. The AcA44 gel was obtained from LBK and the 12 base pair restriction fragment was the gift of Dr. Robert D. Wells. The 150 base pair fragments were obtained from digestion of nucleosomal core particles and were a gift of Dr. Juli Feigon. The \(d(AT)\) sample was prepared by Drs. W.A. Denny and W. Leupin in this lab.

**Fluorescence Measurements.** Steady state fluorescence anisotropy was measured on an Aminco-Bowman SPF-500 spectrofluorometer equipped with rotating sheet polarizers placed in the excitation and emission light path. The four intensity components \(I_{hv}, I_{vh}, I_{hh},\) and \(I_{vv}\), where the subscripts refer to the horizontal or vertical positioning of the polarizers in the excitation or emission light path, respectively, were used to calculate the ratio [11]:

\[
\alpha = \frac{I_{hh} - I_{vv}}{I_{hv} - I_{vh}}
\]

(1)

The steady-state fluorescence anisotropy is given by [2]:

\[
<\text{FPA}> = \frac{\alpha - 1}{2 + \alpha}
\]

(2)

Some measurements were also made with a sample holder that allowed direct examination of samples in NMR microcells.

Unless otherwise stated, measurements were performed at 20°C in a buffer containing 0.1 M NaCl, 0.01 M sodium cacodylate at pH 7. For measurements on the ethidium fluorescence, the excitation and emission wavelengths were 520 and 610 nm and the slit widths were set at 5 nm. Typical experiments were performed using 1.5x10^{-4} M in DNA phosphates.
DNA Digestion. A typical reaction mixture consisted of 50 mL of the EB-DNA complex with a DNA concentration of 3.5 mM (in phosphates) and a phosphate-to-dye (P/D) ratio of about 180 in 0.1 M sodium acetate and 5 mM MgCl$_2$ at pH 5. Before adding enzyme (2 units/mL), the fluorescence anisotropy was measured. Once the enzyme was added, 2 mL of the reaction mixture was transferred into a fluorescence cell and the anisotropy was measured every 5 min. When the appropriate anisotropy was reached, the digestion was quenched by the addition of 12.5 mL of 0.25 M EDTA and cooling. The sample was extracted twice with a buffer saturated phenol to remove the EB and enzyme and then six times with cold ether to remove the phenol. Following overnight dialysis vs. buffer (0.1 M NaCl, 0.01 M EDTA, containing 0.01 M sodium cacodylate at pH 7.0), the samples were ethanol precipitated twice, dried, and dissolved in 1 mL of 0.01 M NaCl, 0.01 M sodium cacodylate buffer at pH 7 and fractionated on a 1.5x80 cm AcA44 column. The size of the DNA in the various fractions was determined by electrophoresis on 7% polyacrylamide gels vs. a Hae III digest of pBR322.

THEORY

In analyzing the <FPA>, we assumed that as a first approximation the EB-DNA complex behaved as a rigid ellipsoid with the fluorophore bound perpendicular to the long axis of the double helix. The limiting value of the anisotropy was assumed to be 0.37 [3,5]. The decay of FPA is biexponential [6] and depends on the molecular rotational correlation times $\theta_1$ and $\theta_2$ and the fluorescence lifetime $\tau$ of the intercalated EB. The rotational correlation times are related to the diffusional constants $D_1$ and $D_2$ for rotation about the short and long axis by [2]

$$\theta_1^{-1} = 6D_1$$
$$\theta_2^{-1} = 2D_1 + 4D_2$$

and the steady-state <FPA> is given by [2,9]

$$<\text{FPA}> = 0.37 \left( \frac{0.25}{1+\tau/\theta_1} + \frac{0.75}{1+\tau/\theta_2} \right).$$

The values for $D_1$ and $D_2$ are calculated for different length helices using the Broersma equations [12]:

$$D_1 = \frac{3kT \ln(L/L_b)}{nL^3} \left( 1.57 + 7(\ln^{-1}(L/L_b) - 0.28)^2 \right)$$

\[2805\]
\[ D_2 = \frac{kT}{n \eta L b^2} \]  

where \( k \) is Boltzmann's constant, \( T \) is the absolute temperature, \( \eta \) is the solvent viscosity, \( L \) is the helix length (3.4 Å times the number of base pairs), and \( b \) is the helix radius (11 Å) [13]. This formula is not valid for short DNA (<15 base pair) where \( D_1 + D_2 \). In this case, the \( <\text{FPA}> \) is given by [14]:

\[ <\text{FPA}> = 0.37(1+\frac{\tau k T}{\eta V})^{-1} \]

where the DNA volume \( V = \frac{\pi L^3}{6} \).

RESULTS

Anisotropy as a Function of Helix Length. To evaluate the effect of helix length on the anisotropy of EB bound to DNA, we measured the fluorescence anisotropy of 15 DNA fractions ranging from 10 to 150 base pair and obtained the results shown in Fig. 1. Except for the two smallest fragments (a synthetic DNA decamer and a 12 base pair restriction fragment) and the 150 base pair fragment (isolated from chicken erythrocyte nucleosomes), the DNA was fractionated by gel filtration and sized on 7% polyacrylamide gels vs. Hae III fragments of pBR322. This procedure typically

![Fig. 1. The effect of helix length on the \(<\text{FPA}>\) of EB-DNA complexes. The samples were obtained by DNase digestion and fractionation and were analyzed by gel electrophoresis. The solid line connects the experimental data, the dotted line shows the \(<\text{FPA}>\) expected if the depolarization were due only to helix tumbling, and the broken line shows the \(<\text{FPA}>\) observed for high molecular weight DNA at 20°C.](image-url)
gave DNA with a polydispersity of ±10%. A solid line connects the experimental values and the broken line at <FPA>=0.23 shows the limiting value measured for high (>500 base pair) molecular weight DNA. These data clearly demonstrate that for the short DNA the <FPA> depends on helix length. There are only small differences between the 150 base pair long complex and the high molecular weight DNA. The dotted line in Fig. 1 is the <FPA> expected if depolarization were due solely to helix tumbling. This theoretical line was calculated from Eqs. (4) or (7) using correlation times \( \theta_1 \) and \( \theta_2 \) calculated for the various helix lengths (Eqs. (3), (5) and (6)). There is good agreement between the calculated and observed <FPA> only for the shortest (10-12 base pair) fragments, and the disagreement becomes larger as the length increases. These results demonstrate that internal motions (local motions of the bases, elastic torsional motions) contribute significantly for the larger fragments. Studies on the decay of FPA suggest that the dye motions are due to the torsional motions of the DNA [1,3,4,6,9,10,15]. When the amplitudes of the local base motions and elastic torsional deformations are large and fast, and the overall tumbling is slow, the theoretical and experimental curves are expected to deviate. The results in Fig. 1 show that the deviation becomes apparent for ~20 base pair long DNA, which has correlation times of 17.5 and 6.6 ns for \( \theta_1 \) and \( \theta_2 \), respectively. This is to be expected since transient FPA measurements on high molecular weight DNA indicate substantial depolarization occurs within 20-30 ns [1,5,10].

**Viscosity Effects on <FPA>**. Figure 2 shows the effect of viscosity on

![Fig. 2. The effect of solvent viscosity on the <FPA> for the 31 (●) and 97 (○) base pair EB-DNA complexes. The broken lines show the expected viscosity dependence for the two complexes, assuming rigid rod behavior for DNA.](image-url)
Fig. 3. The digestion of high molecular weight DNA by DNase I as monitored by the steady-state fluorescence polarization of ethidium bromide. The sample contained 3.5 mM DNA, 20 μM EB, and 2 units/mL DNase I. See Materials and Methods for details.

the $\langle FPA \rangle$ of 31 and 97 base pair long EB-DNA complexes and the broken lines show the dependence expected on the basis of Eq. (4) alone. Increasing the solvent viscosity could affect the $\langle FPA \rangle$ by increasing the rotational correlation times and by reducing the amplitudes of internal motion.

These experiments, performed at constant temperature by adding sucrose to increase the solvent viscosity, show that at the highest viscosities ($\langle n^* \rangle$), the $\langle FPA \rangle$ approaches the limiting value of 0.37±0.005 for both samples. This limiting value for $\langle FPA \rangle$ is less than the theoretical value of 0.4 presumably due to rapid motions (100 ps) of the EB in the intercalation site [3,5]. The $\langle FPA \rangle$ of the high molecular weight complexes, where depolarization is largely due to internal motions, is less affected by the increased solvent viscosity [2,6]. In the 31 base pair complex, there is a substantial contribution to depolarization from helix tumbling and therefore the $\langle FPA \rangle$ is strongly affected when the tumbling is slowed by the increased solvent viscosity.

Preparation of Short DNA Fragments. The digestion of high molecular weight calf thymus DNA by DNase I was followed by monitoring the steady-state $\langle FPA \rangle$ of bound EB, and the reaction was quenched when the appropriate anisotropy was reached. Fig. 3 shows a digestion that was allowed to go to completion at 30°C. In the experiments where monodisperse DNA fragments were required, the digestion was quenched when the anisotropy dropped to 0.17. Gel filtration showed DNA ranging from 30-100 base pairs, so the observed $\langle FPA \rangle$ (0.17) was some average of the helix lengths. More extensively digested samples were less polydisperse, but still required fractionation. In general,
the yield of DNA after phenol and ether extraction and ethanol precipitation was 50%.

**Effect of DNA Concentration on FPA.** Figure 4 shows the effect of DNA concentration on the \( <\text{FPA}> \) of an ~30 base pair EB-DNA complex. The anisotropy of the concentrated samples was measured in 5 mm diameter NMR microcells (volume = 0.12 mL) with the P/D ratio was kept constant at 200/1. Note that for DNA concentrations greater than 10 mM base pair the anisotropy increases noticeably. This increase may arise from a combination of intermolecular associations and increases in solvent viscosity that reduce the molecular tumbling rates. By comparing the data in Figs. 1 and 4, we conclude that in the concentrated (~20 mM base pair) solution of 30 base pair DNA, the effective tumbling time has been increased to that expected for a ~45 base pair fragment in dilute solution (i.e. from ~60 to ~120 ns).

**Effect of Drug Binding Density on FPA.** If the binding density of the ethidium probe molecules were increased to high levels, fluorescent energy transfer between neighboring dyes on the double helix could cause a decrease in the \( <\text{FPA}> \) [16]. Therefore, to evaluate the effect of intercalator binding density on the internal motions of the bound EB probe molecules without such complications, we studied the effects of adding a second intercalator, proflavine, to the EB-DNA complex (P/D=200). Proflavine was chosen because it has been often used as a probe of DNA and its spectroscopic properties do not interfere with the measurement of EB fluorescence anisotropy [17]. We also examined the effects of a bisintercalator bis(acridine) spermine since we have already made NMR studies of its complexes with DNA [18,19]. Figure 5 shows the effect of increasing proflavine concentrations on the steady-state \( <\text{FPA}> \) of EB-DNA complexes ranging from 33 to 150 base pair, and the effect of
Fig. 5. The effect of intercalating drugs on the $\langle FPA \rangle$ for a number of EB-DNA complexes. $\langle FPA \rangle$ is plotted vs. the number of added proflavine molecules for the 33 (△), 60 (○), and 150 (□) base pair complexes. The results obtained with a high molecular weight EB-DNA complex in the presence of increasing concentrations of bis(acridine) spermine (•) are also presented. For proflavine the abcissa values correspond to drug per base pair and for bis(acridine) to drug per two base pairs.

Increasing concentrations of the bisintercalator on the $\langle FPA \rangle$ of the EB complex with high molecular weight DNA. There is a small effect of the added intercalator on the 33 base pair fragment ($\langle FPA \rangle$ increases from 0.12 to 0.15) but almost no effect on the $\langle FPA \rangle$ of the 150 base pair DNA fragment. One possible interpretation is that the increase in $\langle FPA \rangle$ of the 33 base pair complex results from the increase in DNA contour length that accompanies intercalation of the drug. If all the added proflavine intercalates, the helix length would be equivalent to that of a 49 base pair fragment, and a value for $\langle FPA \rangle$ of =0.155 would be expected (Fig. 1). For the 60 base pair fragment, where the reduced value of $\langle FPA \rangle$ (depolarization) is due partly to tumbling and partly to internal motions, the effect is reduced, but corresponds again to the lengthening of the DNA fragment.

For the 150 base pair and the higher molecular weight DNA in the presence of bis(acridine), the $\langle FPA \rangle$ was independent of the drug binding density. Since the tumbling of these larger molecules is too slow to cause depolarization, this experiment demonstrates that the binding of an intercalator just a few base pairs away from the fluorescent probe does not affect the internal motions that lead to depolarization of the probe fluorescence.

**DISCUSSION**

In this paper, simple measurements of the steady-state fluorescence
polarization anisotropy, \( \langle \text{FPA} \rangle \), of ethidium probe molecules bound to DNA were used to examine the effect of DNA concentration on the rotational tumbling of short (~30 base pair) DNA and the effect of high concentrations of a second drug molecule (proflavine or bis(acridine) spermine) on the internal motions of the DNA sensed by an ethidium probe molecule. In the course of preparing the DNA used in these studies, we also found it convenient to use \( \langle \text{FPA} \rangle \) measurements to monitor the course of an enzymatic digestion of the DNA.

**Calibration of the \( \langle \text{FPA} \rangle \) Measurements.** For DNA fragments shorter than 150 base pairs, the \( \langle \text{FPA} \rangle \) depends on molecular tumbling as well as DNA torsional motions and local motion of the probe molecule. Therefore, the observed variation of \( \langle \text{FPA} \rangle \) with helix length provides a convenient way to monitor the average size of DNA fragments. When the DNA fragments have tumbling times comparable to or shorter than the fluorescence lifetime (23±2 ns) [2,20], the depolarization is from tumbling of the helix. The results in Fig. 1 show that as DNA length increases from 10 to 75 base pairs, the observed \( \langle \text{FPA} \rangle \) increases by more than a factor of three and therefore within this size range the \( \langle \text{FPA} \rangle \) is sensitive to any factors that affect the overall tumbling time of the molecule. Consequently, the \( \langle \text{FPA} \rangle \) of a 31 base pair fragment is very strongly affected by increasing the solvent viscosity as shown in Fig. 2. The \( \langle \text{FPA} \rangle \) of a 97 base pair fragment is less affected because molecular tumbling makes a smaller contribution than internal motions to the fluorescence depolarization. The \( \langle \text{FPA} \rangle \) of the longer DNA is independent of helix length because the molecular tumbling is so slow that the \( \langle \text{FPA} \rangle \) is due to internal motion of the DNA. These motions are independent of the length, above a certain size.

**DNA Digestion Monitored by \( \langle \text{FPA} \rangle \).** Because of the pronounced length dependence of the \( \langle \text{FPA} \rangle \) for shorter DNA, the \( \langle \text{FPA} \rangle \) provides a simple method for following the enzymatic digestion of DNA. In the experiments shown in Fig. 3, high molecular weight DNA was subjected to DNA DNase I digestion and aliquots were removed during the digestion and analyzed by \( \langle \text{FPA} \rangle \) and gel electrophoresis. When the \( \langle \text{FPA} \rangle \) dropped from 0.23 to about 0.17, the sample consisted of collection of fragments ranging from 30-100 base pair. Because \( \langle \text{FPA} \rangle \) can be measured rapidly, this method may be useful in deciding when to stop a digestion to obtain a desired size range. This technique should also be useful in monitoring preparation of short DNA fragments (~50 base pair) by sonication techniques rather than awaiting the results of a gel electrophoresis analysis.

**\( \langle \text{FPA} \rangle \) Studies of DNA-DNA Association.** Two recent studies show that
low molecular weight DNA molecules associate (gel) at high concentrations [21,22]. Since many physical measurements are made on concentrated DNA samples, this naturally raises questions about the effect DNA-DNA association on the interpretation of the results. This problem is particularly important in NMR relaxation measurements on DNA where analysis of the relaxation rates requires information on molecular tumbling times [23-27]. To provide this information, we have studied the effects of DNA concentration on ⟨FPA⟩ and the results of this experiment are shown in Fig. 4. Over a large concentration range (extending from $10^{-4}$ to $10^{-2}$ M base pair), the ⟨FPA⟩ of a 31 base pair fragment of DNA remain constant at 0.12. However between 10-20 mM base pair, the ⟨FPA⟩ increased from 0.12 to 0.14. By comparing these results with those shown in Fig. 1, we conclude that at 20 mM base pair the effective tumbling time has increased from 60 ns characteristic of a 30 base pair fragment to that characteristic of a 45 base pair fragment or a dimer (i.e. 120 ns) [12,28-30]. A similar effect was observed with the oligonucleotide poly(G-C) in an equivalent buffer containing 1 mM EDTA (T. Hard, unpublished results). By making time-resolved measurements at several wavelengths it might be possible to determine whether the high concentration reduces axial spinning and overall tumbling to the same extent [11]. Moreover, it would be interesting to have results obtained over a range of ionic strengths and temperatures. Since the ethidium bromide probe molecule can be detected at a low binding density (1 drug/200 base pair), this method can be used to directly probe molecular tumbling in NMR samples without removing the samples from the NMR tube and without affecting the NMR spectra of the DNA. We also note that by addition of suitable probe molecules at low binding density (~200 P/D) to samples containing high concentrations of a second drug, we could study drug induced intermolecular association effects that have complicated the interpretation of previous NMR studies of (DNA-drug) interaction [18,31,32]. The results of the examination of the effect of high drug binding density on the ⟨FPA⟩ of ethidium probe molecule in dilute DNA solutions is discussed below.

Effect of High Drug Binding Densities on the ⟨FPA⟩ of an Ethidium Probe. Previous studies of intercalating drugs have demonstrated that the binding site size per drug is typically 2-2.5 base pair; this leads to the notion that once a drug is bound to the DNA, binding of a second drug at an adjacent site is prohibited [33,34]. The structural origin of this "site exclusion principle" [35] might also imply that the local conformational dynamics of DNA are affected at the drug binding site. To explore the effect of high
concentrations of intercalators on the internal motions of DNA, we have examined the variation of the \(<\text{FPA}\rangle\) of ethidium probe molecules as a function of added amounts of a second drug and these results are shown in Fig. 5. When proflavine was added to 33 base pair DNA, the \(<\text{FPA}\rangle\) of ethidium increased slightly from 0.125 to 0.15. This increase can be explained by an increase in length (and decrease in tumbling rate) resulting from intercalating one proflavine per 2 base pair (compare with Fig. 1). With the higher molecular weight DNA, most of the depolarization of the fluorescence \(<\text{FPA}\rangle=0.23, \text{vs.} 0.37\rangle\) is due to internal motions (torsional elastic deformations or localized motions of the individual base pairs and adjacent bound dye). Consequently, an increase in the length of high molecular weight DNA due to intercalation of additional dye would not be expected to increase the \(<\text{FPA}\rangle\). For 150 base pair DNA, high levels of proflavine have virtually no effect on the ethidium \(<\text{FPA}\rangle\). With high molecular weight DNA, the ethidium \(<\text{FPA}\rangle\) is also unaffected by the addition of 0.5 bis(acridine) spermine per base pair. These observations could have one of several possible interpretations. If we accept the current interpretation of the fluorescence depolarization data that the depolarization of the ethidium probe fluorescence is due to elastic torsional deformations of the DNA, we would then conclude that the torsional force constant of DNA is unaffected by high levels of proflavine. If, on the other hand, local motions of the base pairs and adjacent probe molecule are responsible for depolarization, then it is these motions that are unaffected by neighboring proflavine molecules. Regardless of the exact interpretation we have the unexpected observation that high binding densities of a second intercalating drug (proflavine or bis(acridine) spermine) do not appreciably affect the local high frequency motions responsible for depolarizing the ethidium fluorescence.

While the simple \(<\text{FPA}\rangle\) measurements provide limited information about the dynamics of bound drugs and DNA dynamics, they are clearly valuable in certain studies including DNA-DNA associations and the effect of drugs on internal motions of DNA, and even the enzymatic digestion of the DNA. Because \(<\text{FPA}\rangle\) measurements are easily performed with standard fluorescence spectrometers, a wide range of experimental conditions can be rapidly scanned. Once especially interesting experimental conditions have been found, time resolved measurements that require much more sophisticated instrumentation and are more time consuming, could be carried out.
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