Fluorescence Anisotropy of DNA/DAPI Complex: Torsional Dynamics and Geometry of the Complex

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ABSTRACT Fluorescence depolarization of synthetic polydeoxynucleotide/4′-6-diamidino-2-phenylindole dihydrochloride complexes has been investigated as a function of dye/polymer coverage. At low coverage, fluorescence depolarization is due to local torsional motions of the DNA segment where the dye resides. At relatively high coverage, fluorescence depolarization is dominated by energy transfer to other dye molecules along the DNA. The extent of the observed depolarization due to torsional motion depends on the angle the dye molecule forms with the DNA helical axis. A large torsional motion and a small angle produce the same depolarization as a small torsional motion and a large projection angle. Furthermore, the extent of transfer critically depends on the relative orientation of dye molecules along the DNA. The effect of multiple transfer is examined using a Monte Carlo approach. The measurement of depolarization with transfer, at high coverage, allows determination of the dye orientation about the DNA helical axis. The value of the torsional spring constant is then determined, at very low coverage, for few selected polydeoxynucleotides.

INTRODUCTION

[1]Polydeoxynucleotide/4′,6-diamino-2-phenylindole dihydrochloride (DAPI) binds in the DNA minor groove, preferentially to AT base pairs (Schweizer, 1976; Kapuscinski and Szer, 1979; Masotti et al., 1982; Manzini et al., 1983). The binding modalities and stoichiometry have been investigated extensively (Kapuscinski and Szer, 1979; Manzini et al., 1983; Kapuscinski and Skoczylas, 1978; Manzini et al., 1985a; Kubista et al., 1987; Wilson et al., 1989, 1990). It is well established that DAPI binds to AT-rich regions with high affinity. However, a second binding modality has been proposed in which DAPI also binds to GC pairs, although with reduced affinity (Manzini et al., 1983; Wilson et al., 1989, 1990; Cavatorta et al., 1985; Barcellona and Gratton, 1990; Tanious et al., 1994). DAPI is strongly fluorescent in the complex with AT pairs, yet is virtually quenched when associated with GC pairs (Kapuscinski and Szer, 1979; Masotti et al., 1982; Manzini et al., 1983, 1985b). Furthermore, electrostatic interactions of the positively charged DAPI polar expansions with the nearby negative phosphate groups are possible. Several questions still remain regarding DAPI binding in the minor groove. For example, it is still debated whether the DAPI-DNA complex spans three of four AT base pairs (Eriksson et al., 1993). In addition, the dynamical properties of the DNA in the DNA AT-rich regions are a matter of renewed interest (Loontiens et al., 1991; Mohan and Yathindra, 1991; Clegg et al., 1993; Eriksson et al., 1993; Jansen et al., 1993).

In the present study we report a steady-state fluorescence anisotropy investigation of the complex polyd(A) · polyd(T)/DAPI. The basic experimental observation is that the fluorescence anisotropy of DAPI is a function of the P/D ratio. The qualitative and quantitative explanation of this observation requires consideration of the several processes that can cause depolarization (Paoletti and Le Pecq, 1971; Wu et al., 1991). The model used to explain the experimental observation provides the orientation and torsional dynamics of the DAPI/DNA complex. The two main physical effects that cause depolarization are the torsional motion of the DNA segment where DAPI is bound and, at high DNA coverage, energy transfer to other DAPI molecules in different orientations. In the limit of low DNA coverage, only torsional motions of the DNA region where the dye is bound produce depolarization. However, we can only observe the total amount of depolarization. The same depolarization can be obtained by two factors: a large torsional motion combined with a small projection angle of the DAPI transition dipole moment on the plane perpendicular to the helical axis (angle ϕ), or a small torsional motion combined with a relatively large ϕ. To remove this ambiguity, we need to assume a value of the torsional constant or of the projection angle. We note that the contribution to steady-state anisotropy due to torsional motion is relatively small for the DAPI/DNA complex because of the relatively short DAPI fluorescence lifetime (4 ns) and the relatively small angle ϕ (about 45–50°). Most of the studies reported in the literature refer to the DNA/ethidium intercalation complex. Ethidium has a much longer lifetime (about 25 ns) and a larger angle ϕ, of about 70°. If we consider the other process that causes depolarization, i.e., energy transfer between DAPI molecules, in the limit of full coverage, we can think of the DNA/DAPI complex as an ordered system, due to the constant angle of DAPI molecules with respect to the helical axis. The effect of this order is to provide a limiting value of the anisotropy at long times. Given the fast depolarization due to transfer to next neighbor at full coverage,

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the steady-state anisotropy is essentially determined by the limiting anisotropy, which in turn is determined by the angle ϕ. Therefore, the observation of the limiting anisotropy at full coverage allows the determination of the angle ϕ, which in turn allows the determination of the spring constant. Following the above reasoning, we can, in principle, determine the ϕ angle and the spring constant only from steady-state anisotropy measurements. However, time-resolved measurements are necessary to determine if the limiting anisotropy has been reached, and to rule out other possible interpretations. We note that a crucial assumption of the model is that the geometry of the complex is independent of the DNA coverage, i.e., that the angle ϕ determined at high coverage has the same value at low coverage. In the discussion section we critically analyze this assumption of the model. We note that our model cannot be simply applied to the DNA/ethidium complex at high coverage, because of the intercalation type of binding, which produces unwinding of the double-stranded helix. However, if unwinding only modifies the angle in the plane perpendicular to the helical axis, then our model should be applicable "in principle."

DEPOLARIZATION DUE TO TORSIONAL MOTION

The theory describing torsional motions of a long polymer chain has been presented by the group of Schurr (Allison and Schurr, 1979; Thomas et al., 1980; Schurr and Fujimoto, 1988). In the so-called intermediate regime in which a fast transient and the overall motion of the polymer are neglected, an analytical form for the time-resolved anisotropy decay has been derived. In this theory, the DNA molecule is modeled as a string of beads connected by torsional springs. The size of the bead is considered to be one base pair. The extent of torsional motion about the local torsional axis is determined by the torsional spring constant, by the temperature, and by the frictional coefficient. The basic equation for the anisotropy decay in the time domain is reported below:

$$r_b = r_o(A_1 + A_2 e^{-c\sqrt{t}} + A_3 e^{-4c\sqrt{t}}) \quad (1)$$

$$c = kT(\pi\gamma K_s)^{-0.5} \quad (2)$$

$$A_1 = (1.5\cos^2\phi - 0.5)^2 \quad (3)$$

$$A_2 = 3.0\sin^2\phi\cos^2\phi \quad (4)$$

$$A_3 = 0.75\sin^4\phi. \quad (5)$$

In these expressions, k is the Boltzmann constant (1.38×10^{-16} erg/K), T is the absolute temperature, K_s is the spring constant in units of dyne · cm, $\gamma = 4\pi\eta a^2 h = 6.15 \times 10^{-}$ dyne cm s (Schurr et al., 1992), η is the medium viscosity (1 cp), a is the hydrodynamic radius of the bead (12 Å), and h is the height of the bead (3.4 Å). We note that Eq. 2 contains the product of the spring constant and the size of the bead. To extract the value of K_s from the fitting parameter c (Eq. 2), we must make an assumption about the volume of the rotating element. For the DNA/DAPI complex, the rotation element can be larger than a single base. The angle ϕ is the angle of the transition dipole moment of the fluorophore with the helical axis. These expressions assume that the excitation and emission transition dipole moments are parallel, i.e., $r_0 = 0.4$, and that the torsional axis of rotation is coincident with the DNA helical axis. For DAPI, the value of the initial anisotropy is about 0.39 at 400 nm, and it decreases at lower wavelengths with a value of 0.360 at 340 nm. The anisotropy decay expression has been incorporated into the expression for the parallel and perpendicular polarized intensity decay:

$$I(t)_{par} = \frac{I_0}{3}(1 + 2r_b(t))e^{-\Gamma_f t} \quad (6)$$

$$I(t)_{per} = \frac{I_0}{3}(1 - r_b(t))e^{-\Gamma_f t}, \quad (7)$$

where I_o is the excitation intensity and Γ_f is the fluorescence decay rate. The above equations were used to calculate the steady-state anisotropy in the limit of low DNA coverage.

The extent to which the torsional motion can be observed in a fluorescence anisotropy measurement is determined by the orientation of the emission transition moment of DAPI about the helical axis. If the orientation of the DAPI emission transition moment is parallel to the helical axis, the torsional motion has no depolarization effect. Instead, if the transition moment is perpendicular to the helical axis, a small torsional motion can cause a large depolarization. As a consequence, it is impossible to determine separately the torsional spring constant, unless the angle of the DAPI molecule about the helical axis is independently determined. Fig. 1 shows the correlation between the angle ϕ and

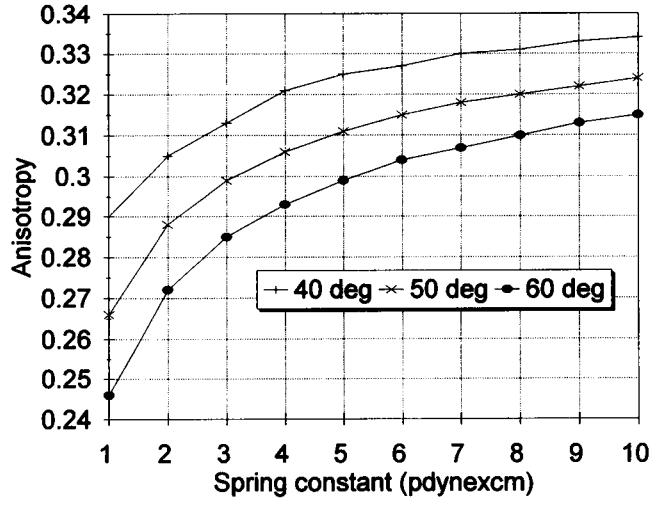

FIGURE 1 Steady-state anisotropy as a function of the spring constant K_s for different values of the angle ϕ. This plot is derived from a simulation of the anisotropy decay using the Monte Carlo method described in the text, for ϕ equal to 40° (+), 50° (×), 60° (●).

the spring constant K_s. The same value of the steady-state anisotropy can originate from different values of the spring constant, depending on the value of the angle ϕ. We note that this correlation is not only in the steady-state value, but the entire anisotropy decay curve is virtually identical as we move along the correlation line (Barcellona and Gratton, 1995).

DEPOLARIZATION DUE TO ENERGY TRANSFER

Energy transfer to identical molecules can occur via the Förster mechanism if there is overlap between the absorption and the emission spectrum of the dye. Fig. 2 shows that there is a significant overlap for DAPI. We calculated the characteristic Förster distance for this homo pair and we found $r_0 = 21.4$ Å, assuming a κ^2 factor of 1 and an index of refraction of 1.7. In the regime of low P/D, in which there is a high probability that two DAPI molecules are within the transfer distance, energy transfer can be very efficient. However, the transfer rate depends not only on the distance between donor and acceptor, but also on the κ^2 orientation factor.

Förster (1951) has derived the basic formulas that describe the rate $\Gamma_{d,a}$ of nonradiative energy transfer in the weak coupling approximation:

$$\Gamma_{d,a} = \Gamma_d\left(\frac{R_0}{d}\right)^6 \tag{8}$$

$$R_0^6 = \frac{\kappa^2 K_T}{\Gamma_d} \tag{9}$$

$$\kappa^2 = (\cos\theta_{12} - 3\cos\theta_1\cos\theta_2)^2 \tag{10}$$

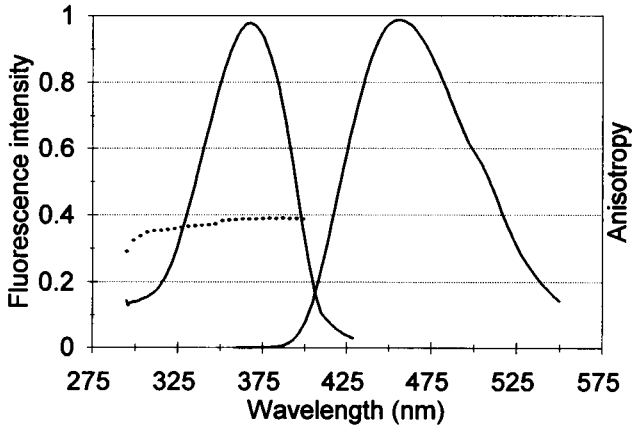

FIGURE 2 Normalized excitation and emission spectrum of DAPI bound to polyd(A) · polyd(T). The overlap integral was calculated using the spectra in the figure. A value of the extinction coefficient of 23,000 $M^{-1}cm^{-1}$ at 342 nm, fluorescence lifetime of 4 ns, and using 1.7 for the refractive index of the medium. The dashed curve is the excitation anisotropy spectrum at −20°C in glycerol.

Γ_d is the decay rate of the donor without acceptor and d is the distance between donor and acceptor. The κ^2 factor depends on the relative orientation of the donor-acceptor transition dipole moments, where θ_{12} is the angle between the direction of the two dipole moments and θ_1 and θ_2 are the angles of the two dipoles with the line joining the centers of the dipoles. The rate K_T depends on the overlap integral and on the medium refractive index (Förster, 1951). The distance d and the κ^2 factor have been calculated for the relative position of the donor-acceptor pair along the DNA molecule and the distance of the center of the dye molecule from the helical axis (8 Å):

$$d = \sqrt{2r^2(1 - \cos\theta) + l^2} \tag{11}$$

$$\cos\theta_{12} = \sin^2\phi\cos\theta + \cos^2\phi \tag{12}$$

$$\cos\theta_1 = \frac{r}{d}\sin\theta\cos\phi + \frac{l}{d}\cos\phi \tag{13}$$

$$\cos\theta_2 = \frac{r}{d}\sin\theta(\cos\theta - 1) + \frac{r}{d}\sin\theta\cos\theta\sin\phi + \frac{l}{d}\cos\phi, \tag{14}$$

where the angle ϕ is the angle with the helical axis already defined, θ is the rotation angle for n bases movement (multiple of 36°), and l is the distance between n bases along the helical axis (multiple of 3.4 Å). The distance of the dye molecule in the minor groove from the DNA helical axis (8 Å) is different from the hydrodynamics radius of the DNA molecule that was assumed to be 12 Å.

The expressions of the decay of the intensity parallel and perpendicular to the excitation direction in the presence of energy transfer between an isolated pair donor-acceptor are as follows:

$$I(t)_{per} = \frac{I_0}{3}\left(1 + \frac{r_{01}}{2}(1 + e^{-K_T t}) + \frac{r_{02}}{2}(1 - e^{-K_T t})\right)e^{-\Gamma_f t} \tag{15}$$

$$I(t)_{per} = \frac{I_0}{3}\left(1 - \frac{r_{01}}{2}(1 + e^{-K_T t}) - \frac{r_{02}}{2}(1 - e^{-K_T t})\right)e^{-\Gamma_f t}, \tag{16}$$

where r_{01} is the anisotropy of the donor only, and r_{02} is that of the acceptor only. These equations are valid in the limit in which there is no additional motion of the donor-acceptor pair. In the presence of an additional torsional motion, these equations must be modified (Wu et al., 1991), as we will discuss later. In this section we discuss depolarization due to energy transfer alone, independently of other depolarization mechanisms, to better understand which parameters affect transfer and what are the limiting values of the anisotropy due to transfer only as a function of DNA coverage. We have assumed that the anisotropy of the acceptor is equal to that of the donor multiplied by a factor that accounts for the different orientation of the transition dipole moment of the acceptor. This factor depends on the location of the acceptor

relative to the donor along the DNA molecule, and it must be evaluated for each donor-acceptor pair:

$$r_{02} = r_{01} \frac{3\cos^2\theta_{12} - 1}{2}, \quad (17)$$

where θ_{12} is the angle between donor and acceptor previously defined. The equation of the depolarization due to energy transfer contains only two variables, i.e., the angle between donor and acceptor and the rate of transfer. The angle between donor and acceptor can only take discrete values, depending on the location of the acceptor relative to the donor on the DNA molecule. The distance distribution between DAPI molecules along the DNA is dependent on both geometrical constraints and the stoichiometry of binding. In addition, we assumed that each DAPI molecule occupies three consecutive bases. Therefore the minimum distance between the centers of two DAPI molecules is about 15 Å. It is unclear whether at the shortest distances Förster theory is still applicable. If Förster theory is not applicable, the values of the transfer rate can be larger than we have estimated. In our derivation, we have assumed that the DAPI molecule is placed tangent to a cylinder coaxial with the polymer at a distance of 8 Å from the helical axis (Kubista et al., 1987). Under these assumptions, only one angle, the angle ϕ, is sufficient to determine the orientation of the DAPI molecule, i.e., the angle with the cylinder axis. Furthermore, we have assumed that the transition dipole moment is coincident with the long molecular axis of DAPI, which includes the indole fluorescent moiety (Clegg et al., 1993; Eriksson et al., 1993; Jansen et al., 1993; Kubista et al., 1987). Given the above geometry, we can now sequentially place DAPI molecules along the DNA double helix and calculate the relative contribution to transfer of an acceptor DAPI molecule 1,2,. . .n bases distant from the donor DAPI molecule. Because DAPI molecules can only be placed at discrete positions along the DNA minor groove, we cannot average over the angular distribution, but we must calculate the transfer rate for each possible dye location. As a result of this calculation, transfer to some specific locations is more probable than to others (Fig. 3). Note that if a dye molecule is located four bases away, transfer is essentially inhibited. This observation has a notable consequence at very high dye coverage. In the limit of full coverage, if dye molecules can be placed three bases apart, then the transfer rate can be very large (see Fig. 3). Instead, if dye molecules can be placed at a distance of four bases, the transfer efficiency is very small. If we assume random coverage of the DNA, a peculiar effect arises as we increase the dye concentration. The Monte Carlo method described in the next section was used for the analysis of the effect of transfer at different DNA coverages. If dye molecules can be placed three bases apart, the increase of the dye concentration is always accompanied by an increase in the overall transfer efficiency and a consequent decrease in the energy emitted at the excitation site (Fig. 4 *a*). Instead, if dye molecules can be placed every four bases, as the dye concentration increases, we should reach a minimum of the emission from the site of excitation. As the DNA coverage increases, then the transfer efficiency decreases and the emission occurs from the same site where the excitation occurred (Fig. 4 *b*). This effect arises because the probability of configurations with dye molecules 5 and 6 bases apart decreases (where transfer efficiency is larger; see Fig. 3) as the DNA coverage increases. As we will see later, only transfer to dye molecules that can be placed once every three bases is consistent with the experimental results.

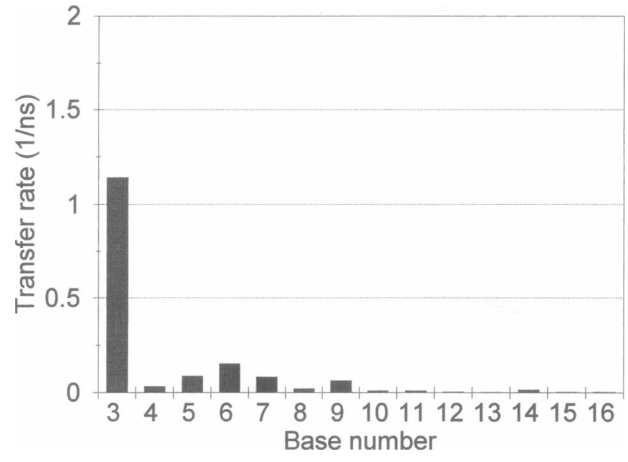

FIGURE 3 Transfer rate to DAPI molecules located at different distances from the donor. Förster theory was used to calculate the transfer rate. The overlap integral was evaluated from the spectra of Fig. 2. It was assumed that there are 10 bases per helix turn and that the DAPI molecule is located tangent to a cylinder 8 Å from the helix axis. The κ^2 orientation factor was evaluated at each location.

Another important effect associated with energy transfer must be considered. The dye molecules occupy definite locations along the DNA helix, and the depolarization motion due to the torsional movement is relatively small (for a similar discussion, see Wu et al., 1991). The case of transfer to dye molecules along the DNA molecule must be treated in the framework of the theory of transfer to fixed acceptors. If transfer can occur only among isolated pairs of dye molecules, then the contribution to depolarization due to the emission from the acceptor is always relatively small. In the limit in which the transfer rate is very large, the energy is equally divided between the donor and the acceptor, because mutual transfer occurs until either the donor or the acceptor emits. At least half of the energy is emitted from the same dye that was originally excited. Instead, if multiple transfer can occur, then the energy can migrate to distant locations and the depolarization due to transfer can be large. The equations reported above for the calculation of the parallel and perpendicular intensities are no longer valid. We have specifically calculated the probability for multiple transfer using a Monte Carlo code and we have found that, at full DNA coverage, transfer to distant dye molecules is quite efficient (Fig. 4 *a*).

The angle of the transition dipole moment of DAPI about the helical axis not only plays a role in the depolarization

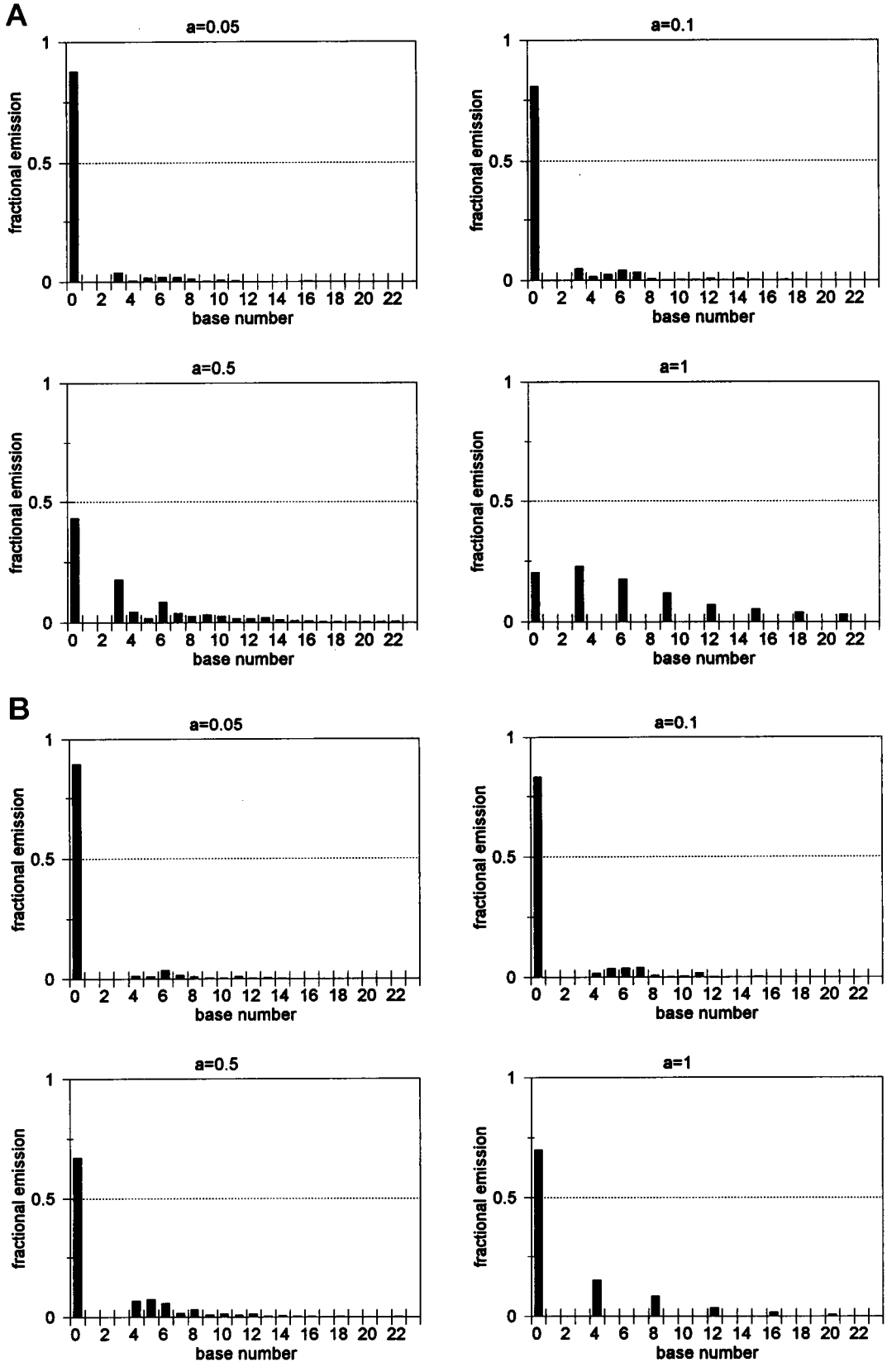

FIGURE 4 Fraction of energy emitted at a base location for different values of DNA fractional coverage. The set of curves are calculated for (*a*) dye molecules located at each of three bases and (*b*) each of four bases. The Monte Carlo code was used to calculate the number of excitations that lead to transfer at each base location.

due to torsional motion; it determines the limiting anisotropy (time-infinite anisotropy) due to energy transfer. If the transition dipole moment is parallel to the helical axis, energy transfer does not contribute to depolarization, because the acceptor has the same orientation as the donor. If the transition dipole moment forms an angle ϕ about the helical axis different from zero but less than 55°, there is an "order" in the system. The dye molecules appear to be located in a cone around the DNA helical axis. In the case of multiple sequential transfer, it is the cone aperture that determines the value of the steady-state anisotropy and of the limiting anisotropy. Because transfer at full DNA coverage can be very efficient, the value of the steady-state anisotropy should be close to the limiting anisotropy. The measurement of the steady-state anisotropy as a function of DNA coverage, in the limit of full coverage, can provide an accurate measurement of the dye transition moment orientation about the helical axis. The effect of energy transfer on the steady-state anisotropy is shown in Fig. 5, where the value of the anisotropy is reported at different DNA coverage. Two cases were analyzed for dye molecules located every three bases or every four bases. The results show that depolarization due to energy transfer causes a relatively small effect in the anisotropy value if dye molecules occupy four consecutive sites, but energy transfer causes a dramatic effect on the anisotropy if each dye molecule occupies three consecutive sites (Fig. 5).

MONTE CARLO SIMULATION

A Monte Carlo simulation was performed to calculate the time-resolved emission anisotropy of the DNA-DAPI complex. A similar model was originally proposed by Paoletti and Le Pecq (1971) and later refined (Genest and Wahl, 1978). In our simulation, a stretch of DNA molecule 10,000 bases long was randomly covered with DAPI molecules according to a selected P/D ratio. The 10,000-base length of the DNA stretch was chosen for calculation purposes. When the length of the molecule is chosen to be much larger than the maximum distance for efficient transfer (which is about twice the value of r_0), the results of the simulation of the depolarization due to energy transfer are largely independent of the molecule length. For each dye molecule, considered to be the donor, a region 20 bases long in both directions along the DNA molecule was analyzed. The particular configuration of acceptors around the donor was stored and used to calculate the total transfer efficiency, which is a function of the overall configuration of acceptor around the donor. Then the transfer probability to one particular acceptor of the configuration was computed. The excitation was then transferred to the acceptor, according to a probability that depends on the transfer rate to the particular acceptor location and orientation. The acceptor that has received the energy is then used as a new center for further transfer. The transfer continues until the energy is emitted. Of course, the overall emission probability is independent of the number of transfers. The result of the Monte Carlo simulation is a table of the location of the emitter with respect to the excitation and the time of emission at that particular acceptor location. This table is then used to calculate the time-resolved decay of the emission intensity in the parallel and perpendicular polarization directions and then the values of the steady-state and time infinite anisotropy. In the final anisotropy calculation, the additional depolarization due to torsional motion is included using the equation of Schurr (Schurr and Fujimoto, 1988). The combination of the change of orientation of the emission dipole due to energy transfer and that due to torsional motion was treated according to the method of Wu et al. (1991) using the probability table of emitting from a given base at a given time obtained from the Monte Carlo simulations at each dye coverage and at different values of the angle ϕ. We note that the limiting values of the emission anisotropy due to torsional motion and those due to energy transfer are similar (apart from the depolarization due to bending of the DNA molecule that will be discussed later). In fact, at very long times, the depolarization due to torsional motion and that due to transfer have the effect of populating all directions on a cone of aperture ϕ. Of course, the rate of transfer and the rate of rotation determine the time at which the limiting value is obtained.

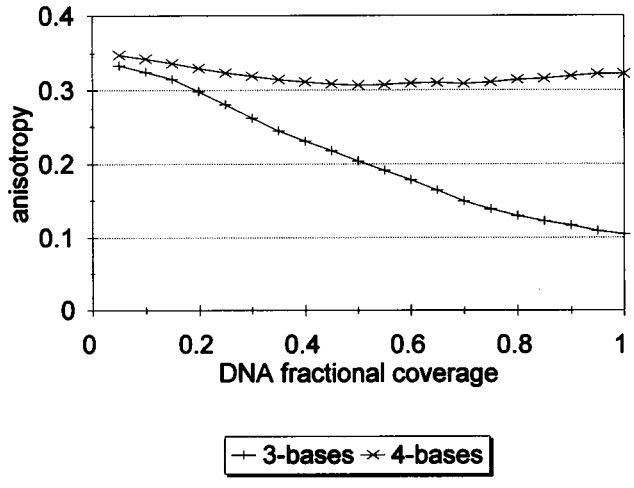

FIGURE 5 Steady-state anisotropy as a function of DNA fractional coverage. The Monte Carlo code was used to evaluate the effect of multiple transfer. (Upper curve) Each dye molecule occupies four consecutive bases. (Lower curve) Each dye molecule occupies three consecutive bases.

MATERIALS AND METHODS

4′-6-Diamidino-2-phenylindole dihydrochloride (DAPI) was obtained from Boehringer Mannheim and checked for purity by thin-layer chromatography. Before each measurement, solutions were filtered with a 0.45-μm Millipore filter to avoid scattering particles. The same treatment for the doubly distilled water was used throughout. Polyd(A) · polyd(T) double-stranded polynucleotide with complementary strands and polyd(A-T) · polyd(A-T) polynucleotide alternating copolymer were from Pharmacia Biotech. The polymers were checked for contaminants, such as proteins and phenol, by the ratio of the absorbance at 260/280 nm, which was about

1.8. The polymers were also checked for the average molecular weight and for the homogeneity of the polymer length by density gradient centrifugation and by electrophoresis at low voltage, in a neutral 6% polyacrylamide gel. The sedimentation coefficients at neutral pH were $S_{20,w}$ = 9S, corresponding to an average length of about 1000 base pairs and a molecular weight of 0.64×10^6, and $S_{20,w}$ = 13S, corresponding to an average length of about 2800 base pairs and a molecular weight of 1.8×10^6, for the homopolymer and the alternating one, respectively. Ultrapure calf thymus DNA was obtained from Sigma Chemical Co., molecular biology division. The DNA molecule has been purified by equilibrium buoyant density gradient centrifugation in cesium chloride and dialyzed against 1 mM NaCl, 1 mM Tris HCl, pH 7.4. The size determination was performed by field inversion gel electrophoresis on a 0.8% agarose gel in TBE buffer and yielded an average length of approximately 23 kb. Inorganic chemicals were reagent grade. All polynucleotides were initially dissolved in aqueous buffered solutions containing 0.1 M NaCl, 0.1 M Tris, pH 7.4.

The concentrations of the polymer solutions were determined using the following extinction coefficients: polyd(A) · polyd(T) (6000 at 260 nm), polyd(A-T) · polyd(A-T) (6600 at 262 nm), calf thymus DNA (6600 at 262 nm). A molar extinction coefficient of 23,000 $M^{-1}cm^{-1}$ at 342 nm was used to calculate the concentration of DAPI solutions. Twelve sets of samples at different dye saturations were examined, for each of which we prepared two (or more) different samples. The saturation was achieved, depending on the final concentration of polymer, by adding increasing amounts of polymer to give the desired P/D ratio, as moles of phosphate (P) to dye (D). DAPI concentration was kept constant, by adding a polymer solution containing DAPI at the same molarity as that of the initial, polymer-free solution. Steady-state polarization measurements were performed at the Laboratory for Fluorescence Dynamics at the University of Illinois at Urbana-Champaign, on the ISS (ISS, Inc., Champaign, IL) microprocessor-controlled photon counting spectrofluorometer, with "L" optics and an automatic polarizer's holder. Details of the instrumentation and the acquisition unit can be found in the literature (Gratton and Limkeman, 1983). Analysis of the polarization values was achieved with the ISS software package. All the measurements were performed using blank subtraction and correction for the instrument g-factors. Polarization measurements for obtaining the r_0 value were performed on DAPI in glycerol at −20°C. All other measurements were carried out at 20°C, using a circulating water bath for temperature control.

RESULTS

The value of the steady-state anisotropy of the polyd(A) · polyd(T)/DAPI complex, at different P/D ratios, is reported in Fig. 6. The anisotropy has been measured at four different excitation wavelengths in the range 340 nm to 400 nm. For clarity, only the value at 340 nm is shown in Fig. 6. There is a wavelength dependence of the time zero anisotropy r_0, due to the combination of different electronic transitions that contribute in the shorter wavelength range. As a consequence, there is a decrease in r_0 from about 0.39 at 400 nm to about 0.34 at 325 nm (Fig. 2).

Our results show that the steady-state anisotropy depends on the P/D ratio. At large P/D ratios, the anisotropy assumes a limiting value (wavelength dependent), extrapolated at a "zero salt" concentration of about 0.313 at 340 nm. This limiting value at high P/D ratio only depends on the depolarization due to torsional motion. We can determine the value of the spring constant from Fig. 1 if we know the value of the angle ϕ. At full DNA coverage (low P/D ratio), the steady-state anisotropy reaches a plateau at around 0.075 for excitation at 340 nm (Fig. 6). In principle, from the value of the anisotropy at the plateau, at low P/D ratio, we can

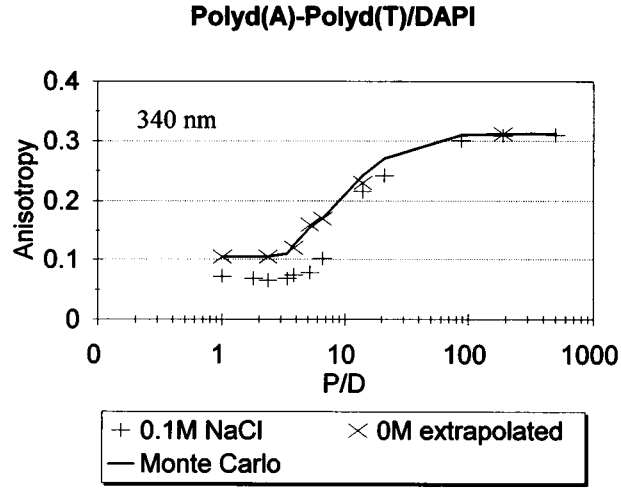

FIGURE 6 Steady-state anisotropy as a function of P/D ratio. Experiments were performed at 340 nm. The solid line corresponds to the Monte Carlo simulation using the following set of parameters: $\phi = 50°$, $R_o = 21.2$ Å, and $K_s = 5 \times 10^{-12}$ dyne cm.

determine the value of the angle ϕ by inverting Eq. 3 (Wu et al., 1991), because efficient transfer at high coverage causes the anisotropy to rapidly drop to the limiting value. However, DAPI binds to DNA in at least two ways (Manzini et al., 1983; Wilson et al., 1989, 1990). In the so-called weak binding, or type II binding, DAPI is interacting with the charged phosphate groups. We then studied the effect of ionic strength on the steady-state anisotropy at low P/D ratio because at high salt concentration binding to the phosphate should be inhibited. As the ionic strength increases, by addition of NaCl, the anisotropy increases first, and then decreases at high salt concentration (Fig. 7). The increase in the steady-state anisotropy value in the 1 to 2 M NaCl is due to the detachment of DAPI from the phosphate backbone. At higher salt concentration, the effect of NaCl on DNA

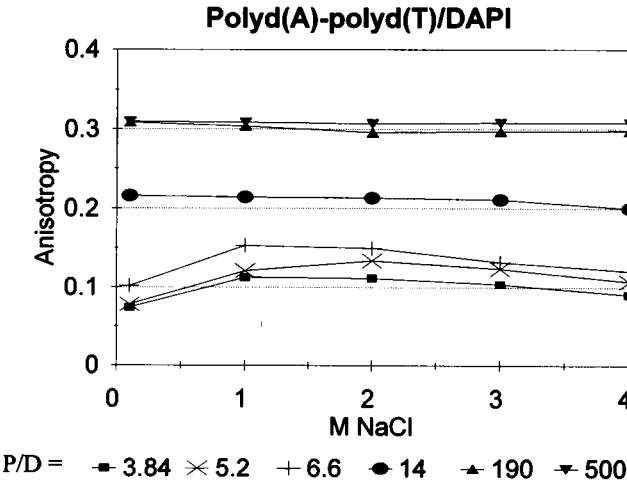

FIGURE 7 Steady-state anisotropy as a function of salt concentration for different P/D ratios.

conformation causes the steady-state anisotropy to decrease. This effect is quite noticeable at low P/D ratio, but it almost disappears at high P/D. This finding is further discussed in the next section. By extrapolating to "zero salt" using the plots of Fig. 7, we have determined the extrapolated values of the steady-state anisotropy shown in Fig. 6, using the value at high salt concentration, where only type I binding is present. We have used the asymptotic values of the anisotropy at low/P/D (i.e., 0.105) and at "zero salt" (Fig. 6) to determine the value of the angle ϕ to be about 42°, using Eq. 3. If we use the raw values of the anisotropy at the plateau (i.e., 0.075), instead of the values extrapolated at "zero salt," we obtain a value for the angle ϕ of about 45°. We note that the method of extrapolation has only a minor effect on the value of the angle ϕ.

DISCUSSION

The asymptotic P/D values

For the evaluation of the methodology we propose, we first discuss those factors that can influence the asymptotic values of the anisotropy at low and high P/D ratio, because it is from these values that we determine the orientation of the DAPI molecule with respect to the helical axis and the spring constant. We note that a wrong estimation of the rate of transfer will not modify the asymptotic value at high P/D ratios, but only the P/D range at which the asymptotic value is reached. Instead, in the low P/D range, a series of factors can influence the asymptotic value of the steady-state anisotropy. The important observation is the plateau in the steady-state anisotropy curve from a P/D of about 1 to a P/D of 5.5. Of course, the stoichiometry of binding is such that, at a nominal P/D ratio of 3, the homopolymer might be fully covered, so that a plateau between P/D 1 and 3 might imply binding saturation. However, the anisotropy plateau continues up to a P/D of about 5.5. The major additional effect at very high coverage (in the P/D range from 1 to 5.5) is the establishment of the regime of multiple transfer, which causes emission from points far from the point of excitation. Because of DNA bending, emission from a site far from the excitation site can additionally contribute to the observed depolarization. The effect of bending on decreasing the steady-state anisotropy should be larger with larger DNA coverage, because the efficiency of multiple transfer increases dramatically at very low P/D ratio. The plateau in the anisotropy below P/D of 5.5 excludes the possibility that multiple transfer strongly enhances depolarization. We calculated the average transfer distance as a function of DNA coverage, and it increases very much only when the fractional coverage is above 0.8 (nominal P/D of about 3.8). Using the Monte Carlo simulation that gives the distribution of emitting positions as a function of time and DNA coverage, we have evaluated the effect of (static) bending using the corrections to the anisotropy decay given by Wu et al. (1991), which accounts for the DNA persistence length. We have found that bending has a negligible effect on the steady-state anisotropy. In fact, at full coverage, a region of about 20 bases is affected by multiple transfer (Fig. 4). For a persistence length of about 500 Å, this portion of 20 bases should correspond to an additional angle of about 7°, which should have a very small additional effect on the steady-state anisotropy at low P/D ratio, because the emission is already depolarized. Under the assumption that there are no other depolarization mechanisms, we can estimate the value of the angle ϕ from the asymptotic value of the steady-state anisotropy at low P/D. Using the full time-resolved Monte Carlo simulation we have estimated an angle between 42° (value corresponding to the correction for the salt effect) and 45° (the raw value). Note that 45° is a maximum value for the angle ϕ. If the angle is lower than 42°, the plateau at about 0.1 (salt-corrected data) in the steady-state anisotropy cannot be reached, unless other mechanisms for depolarization, which must be independent on DNA coverage, have been neglected.

Once the angle ϕ has been determined at high coverage, we can obtain the value of the torsional spring constant by interpolating the value at the extreme low coverage (about 0.313) using the plots of Fig. 1. The value obtained is about 4×10^{-12} dyne cm (for an angle ϕ of about 42°), which is in the lower range of previously reported values for the DNA/ethidium complex (Schurr et al., 1988; Wu et al., 1991; Collini et al., 1992). However, we stress that the value of the torsional constant cannot be obtained unambiguously from the steady-state anisotropy measurement, because of the correlation with the angle ϕ shown in Fig. 1.

The large increase in depolarization observed by increasing the DNA coverage is a strong indication that DAPI occupies three consecutive bases on the DNA (Fig. 5). One should note that the rate of transfer determines the P/D value at which a plateau in the anisotropy is reached, but has relatively small influence on the anisotropy value of the plateau, which depends on the angle ϕ. Inspection of the experimental anisotropy curve shows that there is a very small increase of the steady-state anisotropy at low P/D values (Fig. 6). This increase is due in part to the contribution of the free dye, that has a relatively large anisotropy (of about 0.2) because of its short lifetime (150 ps). Time-resolved measurements (Barcellona and Gratton, 1995) confirm that it is the contribution of the free dye (or of a short lifetime species, such as that arising from type II binding) that causes the small rise of the anisotropy below a P/D of 2.

The intermediate P/D region

We can use the values of the angle ϕ, the torsional spring constant, and the results of the Monte Carlo simulation to predict the behavior of the steady-state anisotropy in the P/D range from about 6 to 200. We have found that a good agreement with the experimental data (Fig. 6) can be obtained with a value of the transfer rate of 2.4×10^7 ns, which is the value calculated using the spectral superposi-

tion (Fig. 2). This observation points out that the Förster mechanism quantitatively accounts for our results. A 20% deviation from this value causes a relatively large deviation of the experimental curve from the simulated curve. We also note that a direct fit of the steady-state anisotropy curve cannot be performed with the method presented in this article, because the values of the anisotropy are simulated using a Monte Carlo method. Other factors can also affect the calculations in this intermediate P/D range. For example, binding cooperativity can invalidate the Monte Carlo simulations in this range, but not at the extreme values that we have used for our estimation and that are the basis of the application of our methodology.

Critical evaluation of the model

Cooperativity of binding

It has been reported, by binding equilibrium experiments, that the interaction of DAPI with poly[d(A-T)]$_2$ displays a pronounced positive cooperativity, and the results cannot fit the model of McGhee and von Hippel (Wilson et al., 1990). Instead, such results are well fitted with the allosteric-transition model of Dattagupta et al. (1980), which is consistent with a transition between two DNA forms, induced by the ligand binding. The same conclusion has been reached by Nordèn and co-workers (Eriksson et al., 1993), who applied optical spectroscopic techniques to DAPI/polynucleotides complexes. In particular, the characteristic two types of CD spectra, ascribed to two modes of binding, might be also explained with an allosteric binding, i.e., DAPI molecules contiguously bound to AT clusters should change their local conformation. Competition between the two types of DAPI binding for adjacent sites was also previously proposed. In fact, when a cluster of three or four AT base pairs has bound a DAPI molecule by the type I mode, the adjacent site in the polymer prefers to bind a new DAPI molecule with a type II mode (Manzini et al., 1983). In the case of (dAT)$_3$, both dye molecules are bound with mode I, but the curvature of the Scatchard plot indicates that the second DAPI molecule is bound with lower strength. The binding, in this case, can be facilitated by repulsion or by the presence of end effects, because of the shortness of the chain. By taking into account such reported evidence, the possibility arises that the ϕ angle obtained at a low P/D ratio can be different from the angle at a high P/D ratio, because of DNA (DAPI-induced) conformational transition. To further analyze this possibility we "calibrated" the value of the "spring constant" using ethidium bromide, which intercalates into DNA. We assumed that at very low coverage we are measuring local hydrodynamic properties rather than binding properties of the fluorescent probe. At very high P/D ratios the angle between the ethidium and the helix axis is known to be 70.5° (Schurr and Fujimoto, 1988), and we measured the lifetime of the complex to be 25 ns. By using the same analytical procedure, i.e., simulating curves such as those of Fig. 1, but for the ethidium bromide molecule, we obtained a value of the spring constant of 5×10^{-12} dyne cm for the homopolymer/ethidium complex. We used this value of the spring constant to "calibrate" the angle for the DAPI, and we found that an angle of 50° is necessary to give the value of 5×10^{-12} dyne cm for the spring constant for an anisotropy of 0.313. Therefore, we have indirect evidence that, at high coverage, the angle ϕ depends somewhat on the amount of DAPI bound to DNA. This effect only occurs at high coverage and brings the angle ϕ from about 50° at high P/D to about 45–42° at low P/D. Although this change appears to be very small, it seems that the anisotropy measurements are able to pick up this subtle difference. This change in the dye's orientation is consistent with the increase in DAPI tilt observed by linear dichroism in the same P/D range (Kubista et al., 1987).

Salt effects

The addition of NaCl to polyd(A) · polyd(T)/DAPI complex, up to about 4 M, has a relatively small effect on the anisotropy at a high P/D ratio. At a P/D of 500 the anisotropy decreases from about 0.313 extrapolated at "zero salt" concentration to about 0.308 at 4 M. This effect is seen at all wavelengths in the range 340 nm to 400 nm. If the decrease of the anisotropy at high salt concentration is entirely due to a salt-induced change of the torsional constant, it should correspond to a decrease in the torsional constant from about 5×10^{-12} dyne cm (at low salt) to about 4×10^{-12} dyne cm (at 4 M NaCl), as estimated from the graph of Fig. 1 using an angle of 50°. A similar decrease in the torsional constant in the same salt concentration range has previously been reported (Schurr et al., 1992).

At low P/D, increasing the salt concentration produces a large effect on the steady-state anisotropy. In this P/D range, at least two salt-dependent processes coincide to change the value of the steady-state anisotropy. DAPI, weakly bound to phosphate groups, is detached as the salt concentration reaches about 2 M NaCl, causing an increase in the steady-state anisotropy. It appears that the DAPI species that is affected by salt either has a small anisotropy due to large rotational freedom or is differently oriented with respect to DAPI in the minor groove. We note that at low P/D, energy transfer is the major depolarization mechanism. If there is a DAPI species that is oriented differently from the dye molecules in the minor groove, the depolarization due to transfer can be very large. As a consequence, the reduction of this dye species, obtained by increasing the salt concentration, results in an increase of the anisotropy. However, the addition of NaCl over about 2 M causes a relatively large decrease in the anisotropy in the P/D range up to about 20 (Fig. 7). This decrease is larger at a low P/D ratio and decreases as the P/D increases. We have estimated, using the Monte Carlo simulation, the expected decrease in the anisotropy in the P/D region below 20, due to the salt-induced changes of the torsional constant observed at a high P/D ratio. We have found that the measured decrease in the

TABLE 1 Torsional spring constants at 20°C

DNA	K_s (10^{-12} dyne × cm)
Polyd(A) · polyd(T) (low salt)	5.0 ± 0.2
Polyd(A) · polyd(T) 4 M NaCl	4.0 ± 0.2
Calf thymus DNA (low salt)	3.5 ± 0.2
Poly(dA-dT) (low salt)	2.5 ± 0.2

steady-state anisotropy is much larger than that expected from a change in the torsional constant from 5×10^{-12} dyne cm to 4×10^{-12} dyne cm. Therefore, either there is a polymer conformational transition that changes the angle ϕ, which is DAPI and salt dependent, or the additional decrease of the anisotropy at high salt is due to energy transfer to distant parts of the polymer molecule, which are randomly oriented, thereby producing additional depolarization. At high salt, polydeoxynucleotides could assume a more compact form (presumably with a different ϕ angle) with pronounced tertiary structure components, already postulated for natural DNA (Reinert et al., 1981). A local apparent solenoid-related DNA tertiary structure component, suggested to form at high salt concentration and at high dye coverage (Reinert, 1993), can explain the pronounced decrease of the steady-state anisotropy observed at salt concentration in the 3–4 M range.

Measurement of the torsional spring constant of selected DNAs

One prediction of our model is that the steady-state anisotropy of DAPI bound to natural DNA should always be relatively large. The occurrence of long stretches of AT base pairs is very unlikely, and depolarization due to energy transfer should play a modest role in natural DNAs. In fact, our previous results of steady-state anisotropy of DNA/DAPI complexes, using calf thymus DNA, show a relatively high value of the anisotropy (about 0.303 with excitation at 340 nm) and a relatively small dependence of value of the anisotropy upon the P/D ratio (Barcellona and Gratton, 1993). Once we have established the value of the angle ϕ to be about 50°, we can use the graph of Fig. 1 to determine from the steady-state anisotropy the value of the torsional spring constant at a high P/D ratio. Table 1 summarizes our findings.

The orientation of the DAPI in the complex with DNA that arises from our fluorescence anisotropy measurements can be compared with the crystal structure of the DNA/DAPI complex in a dodecamer determined by Dickerson's group (Larsen et al., 1989). In particular, from their published structure, the ϕ angle is about 42°, in the same range of as determination (42° to 45°) using steady-state fluorescence anisotropy data.

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