Characterization of the Binding of the Fluorescent Dyes YO and YOYO to DNA by Polarized Light Spectroscopy

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Abstract: The interaction between double-stranded (ds) T2 DNA and the fluorescent dyes YO (oxazole yellow) and YOYO (dimer of oxazole yellow) has been studied with optical spectroscopic methods. Flow linear dichroism (LD) spectra of YO–DNA and YOYO–DNA complexes show that, at mixing ratios dye:DNA base below 0.20 and 0.125 for YO–DNA and YOYO–DNA, respectively, the long axis in the YO chromophore is approximately perpendicular to the DNA helix axis, an orientation consistent with intercalation. This conclusion is supported by the induced negative circular dichroism (CD), the transfer of energy from the DNA bases to the bound YOYO, and the unwinding of supercoiled DNA by YOYO. At higher mixing ratios, a CD exciton appears and the steady increase in the reduced linear dichroism amplitude during the intercalation phase is changed to a decrease, suggesting that a second binding mode starts to contribute. For YO, the exciton has the expected pattern for dimeric interaction between chromophores bound to the surface of the DNA. For YOYO, the new binding mode appears at a mixing ratio where, if the dye is assumed to follow the nearest neighbor exclusion principle, all intercalation sites are filled up. Thus, this second binding mode is proposed to be an external binding mode. Fluorescence anisotropy measurements show that the limiting anisotropy value (mixing ratio dye:base → 0) for YO–DNA is about twice that of YOYO–DNA. In addition, the decrease in fluorescence anisotropy with the mixing ratio is much stronger for YO–DNA compared to YOYO–DNA. These observations have been explained in terms of depolarization of the emission due to Förster energy transfer between the intercalated chromophores.

Introduction

In 1992, Rye *et al.*[1] reported development of two new dyes designated YOYO (Figure 1) and TOTO for use as fluorescent probes in DNA analysis. The dyes are homodimers of oxazole yellow (YO) and thiazole orange (TO), which are asymmetric cyanine dyes with a chain between the quinoline and the oxazole and thiazole rings, respectively. The monomers are bridged with a bis-cationic linker similar to the link between the monomers in the ethidium homodimer.[1-3]

YOYO and TOTO were found to have excellent properties for detection and quantitation of DNA fragments separated on electrophoresis gels.[1,4] They have large molar absorptivities (>84 000 M^{-1} cm^{-1})[5] and are virtually nonfluorescent in free form but show very strong fluorescence when complexed with double-stranded DNA (the fluorescence enhancement factor for YOYO has been reported as high as 3200).[3] The detection of DNA in the gels is as sensitive as that attainable by radioisotopes and much more sensitive than that by the often used dye ethidium bromide.[3,1] The dyes were also found to bind so strongly to DNA (the binding constants have been estimated to be in the range 10^{10}–10^{12} M^{-1})[3] that the complexes remain stable during electrophoresis and the DNA samples can therefore be prestained and run in absence of free dye in the gel.[1] YO and YOYO are assumed to intercalate and bisintercalate, respectively,[1,3] but to our knowledge, no experimental evidence has been presented verifying this assumption.

We are studying the electrophoretic behavior of ds DNA in gels by fluorescence video microscopy, a technique which was developed a few years ago[6-8] and provides a possibility for *in situ* observations of individual, fluorescently stained large DNA molecules while undergoing electrophoresis. The excellent fluorescent properties of YOYO and its high binding affinity to double-stranded DNA make the dye suitable as a probe in this technique. However, the electrophoretic behavior of probed DNA molecules should not differ significantly from that of unstained DNA, and the binding mode of the probe and how the binding affects the electrophoretic charge, the length, and the flexibility of DNA are thus important to map out. We are currently testing YOYO and some other fluorescent probes as staining agents for visualization of large DNA molecules and the work presented in this paper is a part of these studies.

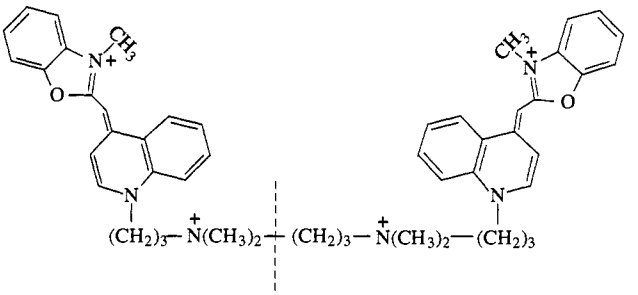

Figure 1. Structure of oxazole yellow homodimer YOYO [YOYO-1]. The left part of the figure up to the dashed line through the chain linking the two monomers in the dimer constitutes, with an additional methyl group on the end nitrogen, the structure of YO (YO-PRO-1).

Materials and Methods

Chemicals and Sample Preparation. The double-stranded DNAs used in this study are linear coliphage T2 DNA (164 kbp) and the supercoiled

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and the relaxed forms of circular PhiX 174 RFI DNA (5386 bp). The T2 DNA was obtained from Sigma and the PhiX 174 RFI DNA from New England Biolab. YO and YOYO (for the structure, see Figure 1) were purchased from Molecular Probes (trade names YO-PRO-1 and YOYO-1).

All experiments have been performed at room temperature (ca. 20 °C) in a buffer containing 50 mM Tris, 50 mM boric acid, and 1.25 mM EDTA. Concentrations were determined spectrophotometrically using the following molar absorptivities: $\epsilon_{260} = 6600\ M^{-1}\ cm^{-1}$ for DNA bases, $\epsilon_{457} = 96\ 100\ M^{-1}\ cm^{-1}$ for YOYO,[5] and $\epsilon_{481} = 66\ 000\ M^{-1}\ cm^{-1}$ for YO.[5] Working solutions of the dyes were prepared fresh immediately prior to use.

In a titration experiment on the supercoiled PhiX 174 RFI DNA, YOYO was successively added to the same DNA sample, and after each addition, the absorbance and LD were measured. In all other studies, the linear T2 DNA was used and the dye–DNA complex was prepared by adding the DNA solution (with a concentration of about twice the final concentration) to the dye solution in order to avoid precipitation. The solution was then equilibrated for at least 48 h in order to secure that the binding of the dye to the DNA was homogeneous before measurements were started. The mixing ratio, d/b, is defined as the concentration ratio between dye and DNA base.

Linear Dichroism and Circular Dichroism. Linear dichroism (LD) spectroscopy on oriented DNA–ligand complexes can provide information about the binding geometry of the ligands.[9] LD is defined as the difference in absorption of light polarized parallel and perpendicular to the macroscopic axis of orientation (the orienting field direction):

$$LD(\lambda) = A_{\parallel}(\lambda) - A_{\perp}(\lambda) \quad (1)$$

The LD is normalized to be independent of concentration and path length through division by the isotropic absorbance, A_{iso}, to form the reduced linear dichroism, LD^r:

$$LD^r(\lambda) = LD(\lambda)/A_{iso}(\lambda) \quad (2)$$

For molecules with uniaxial orientational symmetry like DNA, LD^r can be described as a product between an orientation factor S and an optical factor O. The optical factor is related to the angle α between the light-absorbing transition moment of the chromophore, which could be a DNA base or the bound dye, and the principal orientation axis of the molecules. The orientation factor describes the average orientation of this axis relative to the orienting field:[10]

$$LD^r = SO = {}^3/_2 S(3\langle\cos^2\alpha\rangle - 1) \quad (3)$$

S is 1 for a perfect oriented sample and 0 for a randomly oriented sample.

With the conditions prevailing in our experiments, the DNA is in its B-conformation. After the LD of the DNA bases has been measured at 260 nm of the linear DNA, where the principal orientation axis of the molecule is the helix axis, the angle α in eq 3 can then be assigned equal to 86° (the average tilt angle of the DNA bases relative to the helix axis), which gives the linear DNA an optical factor $O = -1.48$.[11,12] The corresponding optical factors of the supercoiled and the relaxed forms, respectively, of the circular DNA are unknown. However, we have used these DNAs only for a qualitative LD study where the purpose has been to find out whether or not YOYO unwinds the supercoils, and in such a study, it is sufficient to know that the total LD^r amplitude of the supercoiled form is smaller than that of the relaxed form.[13]

DNA was oriented in a shear flow field generated in a Couette cell.[14,15] LD was measured on a Jasco J500 spectropolarimeter equipped with a one-quarter wave plate as described elsewhere,[16-18] and the isotropic absorbance was measured on a Cary 2300 spectrophotometer.

Circular dichroism (CD) is defined as the difference in absorption of left and right circularly polarized light:

$$CD(\lambda) = A_L(\lambda) - A_R(\lambda) \quad (4)$$

Achiral molecules, like YO and YOYO, do not show CD, but when they are bound to a chiral molecule such as DNA, they may exhibit CD (induced CD), measurement of which can provide information about the binding complementing that is obtained from linear dichroism.[19-22] The induced CD may result either from coupling between the electronic transition moment of the adduct and moments of the DNA bases or from coupling between electronic transition moments with the same oscillation frequencies of adducts close to each other and with a favorable geometry (exciton coupling).[23] CD spectra were measured on a Jasco J720 spectropolarimeter using a 1 cm quartz cell.

Fluorescence and Fluorescence Anisotropy. Fluorescence and fluorescence excitation anisotropy (FA) spectra were measured on a SPEX Fluorolog-τ2 or an AMINCO SPF-500 "corrected spectra" spectrofluorimeter.

In the FA measurements, the fluorimeter was equipped with Polaroid film polarizers and the fluorescence intensities were measured with the polarizers set either vertically (v) or horizontally (h) in the excitation and emission light beams. The degree of anisotropy, r, was calculated as

$$r = \frac{I_{vv} - I_{vh}G}{I_{vv} + 2I_{vh}G} \quad (5)$$

where G is the ratio I_{hv}/I_{hh} used for instrumental correction. In the subscript of I, the first letter refers to the polarization direction of the excitation polarizer and the second letter to the polarization direction of the emission polarizer.

Results

A. Absorption and Dichroism Spectra. Figures 2 and 3 show normal (isotropic) absorption, linear dichroism, and reduced linear dichroism spectra of solutions containing YO and YOYO, respectively, of varying concentrations and T2 DNA of constant concentration (45 μM base). In Figures 2 (top) and 3 (top), absorption spectra of the pure DNA at this concentration and of the free dye at a concentration corresponding to that used at the highest mixing ratio are also included.

Absorbance. There are, as shown in Figure 2 (top), two absorption bands in the near UV/vis absorption spectrum of free YO; one strong band in the visible region with a peak at 481 nm and a shoulder at about 460 nm and one weak band in the UV region with peaks at 278 and 299 nm. In the presence of DNA, the visible band, where DNA shows no absorption, mainly retains its shape but the absorption intensity decreases (hypochromicity) and the band is also red-shifted (effects evidencing binding to DNA) so that the absorption maximum now occurs at 490 nm.

The absorption spectrum of free YOYO (Figure 3 (top)) differs from that of free YO only at the high-energy side of the visible absorption band, where YOYO shows a very strong peak (absorption maximum at 457 nm) and where YO only shows a shoulder, a spectral difference that indicates that the two YO chromophores in free YOYO are close enough in space to interact electronically. In presence of DNA, the band is red-shifted and shows hypochromicity but it also changes shape so that the whole spectrum becomes similar to that displayed for YO and the YO–DNA complex. These changes indicate that the two YO chromophores in YOYO become more separated when the dye binds to DNA and that they interact with the DNA in much the same way as YO.

Linear Dichroism. The LDs of both the YO– and the YOYO–DNA solutions are negative at all mixing ratios both in the UV

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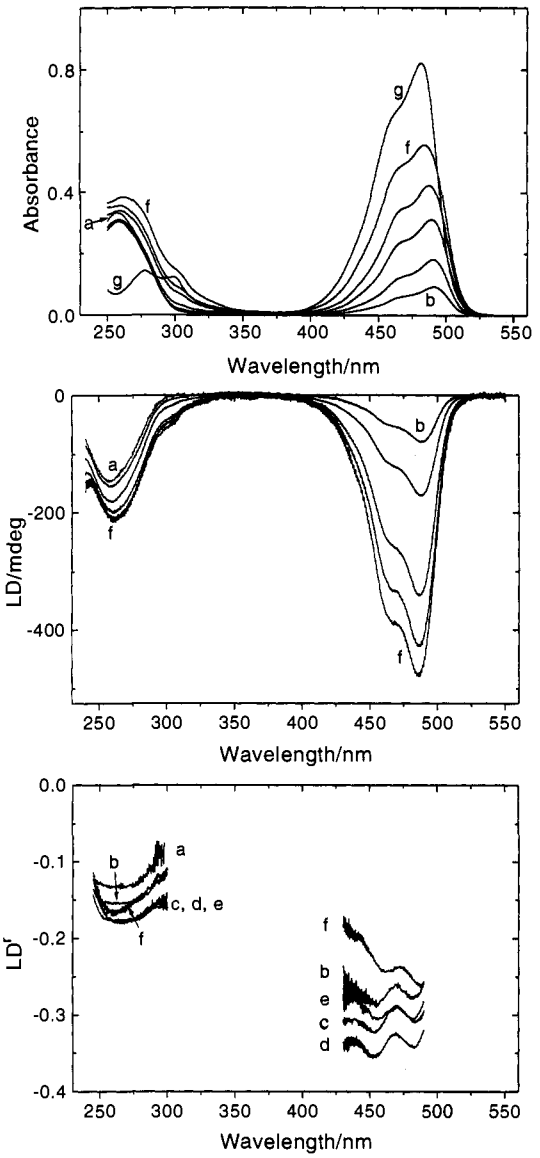

Figure 2. Absorption, linear dichroism (LD), and reduced linear dichroism (LD^r) spectra of T2 DNA, 45 μM bases in buffer, in the presence of varying amounts of YO. The mixing ratios (YO:DNA base) of the samples are, from a–f, 0 (pure DNA), 0.05, 0.10, 0.20, 0.25, and 0.40. Sample g is free dye at the same concentration as in sample f. The optical pathlength is 1 cm in the absorbance measurements and 0.1 cm in the linear dichroism measurements. Shear gradient 310 s^{-1}.

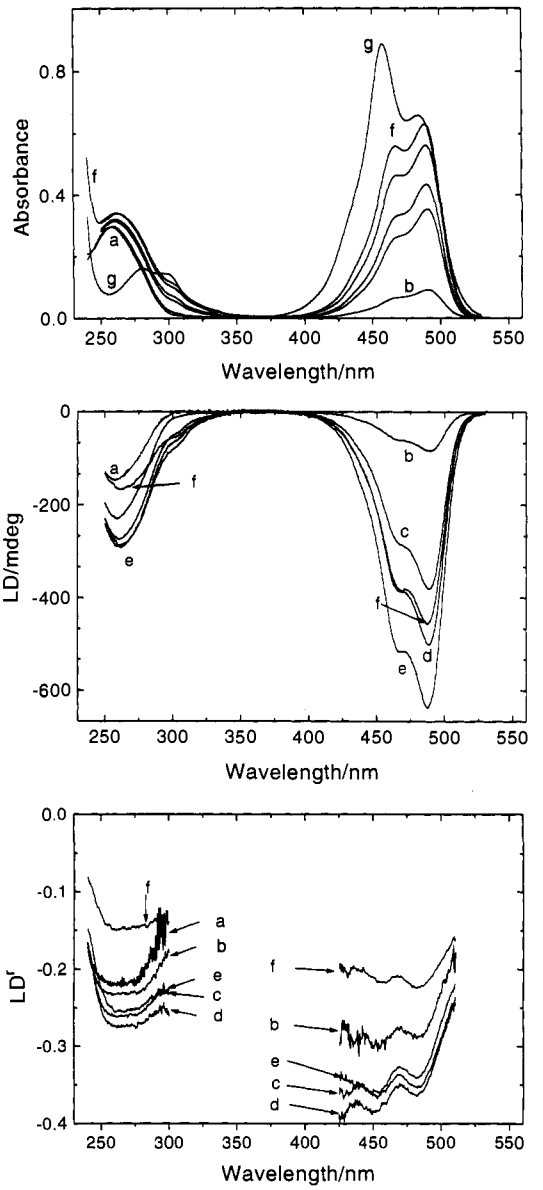

Figure 3. Absorption, linear dichroism (LD), and reduced linear dichroism (LD^r) spectra of T2 DNA, 45 μM bases in buffer, in the presence of varying amounts of YOYO. The mixing ratios (YOYO:DNA base) of the samples are, from a–f, 0 (pure DNA), 0.02, 0.10, 0.12, 0.17, and 0.20. Sample g is free dye at the same concentration as in sample f. The optical pathlength is 1 cm in the absorbance measurements and 0.1 cm in the linear dichroism measurements. Shear gradient 310 s^{-1}.

region, where DNA as well as the dyes contributes to the absorbance, and in the visible region, where only the dyes absorb (Figures 2 (center) and 3 (center)). In both cases, the LD profile in the visible band is very similar to the corresponding isotropic absorbance profile (Figures 2 (top) and 3 (top)), which is also reflected in the nearly constant LD^r over this band (Figures 2 bottom) and 3 (bottom)). For both complexes, the LD^r in the visible band is slightly more negative than the LD^r in the UV band. In the YOYO–DNA case, the LD^r amplitude in the visible band increases with increased d/b ratio up to $d/b = 0.125$, whereupon it decreases. A similar rise and fall in the LD^r amplitude, with a maximum at $d/b = 0.20$, is observed for YO–DNA.

Circular Dichroism. Figure 4, top and bottom, shows CD spectra of the YO– and YOYO–DNA complexes, respectively, at some representative mixing ratios. The CD in the visible region originates from the dyes and is induced by their binding to DNA. YO–DNA shows at d/b ratios up to 0.20 a weak negative band in this region with a shape that corresponds to the absorption band of YO in DNA and an amplitude that increases approximately linearly with the dye concentration, characteristics that are consistent with a CD due to interaction between the dye and the DNA bases. With further increase in d/b, a superimposed CD consisting of two bands of different sign appears. Such a CD couplet is typical for an exciton CD in a DNA complex, *i.e.* evidences dipole–dipole interaction between the bound dyes. For d/b ratios up to 0.125, YOYO–DNA also displays a negative CD band in the same wavelength region and with the same behavior as that shown by YO–DNA up to $d/b = 0.20$. At mixing ratios above 0.125, an exciton CD superimposed on this CD band is observed also in this case but with a pattern that is inverse to that of YO–DNA; the positive band is now at shorter wavelengths than the negative band.

B. LD Titration of Supercoiled DNA. The supercoiled form of DNA shows a less negative flow LD^r than the corresponding relaxed form.[13] We have exploited this fact to find out if YOYO induces such structural changes in the DNA molecule that the helix unwinds when titrated with YOYO. The LD^r spectra (not

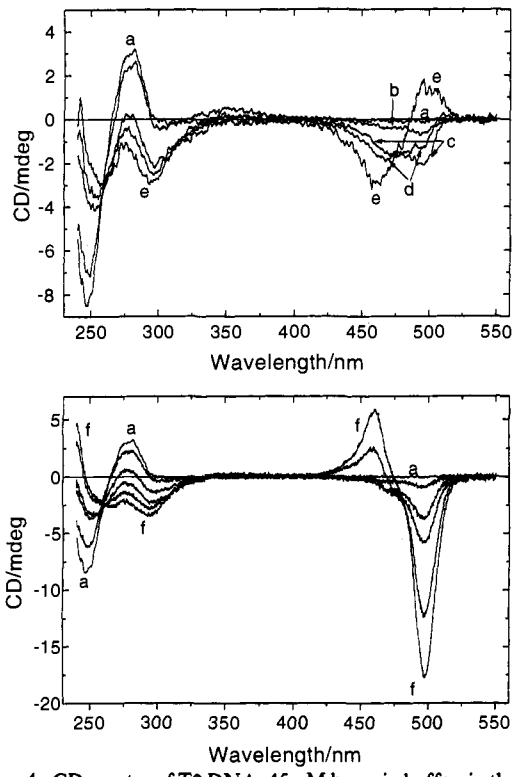

Figure 4. CD spectra of T2 DNA, 45 μM bases in buffer, in the presence of varying amounts of (top) YO and (bottom) YOYO. The mixing ratios (dye:DNA base) of the samples are (top), from a–e, 0 (pure DNA), 0.05, 0.25, 0.40, and 0.50 and (bottom), from a–f, 0 (pure DNA), 0.02, 0.10, 0.12, 0.17, and 0.20. Optical path length is 1 cm.

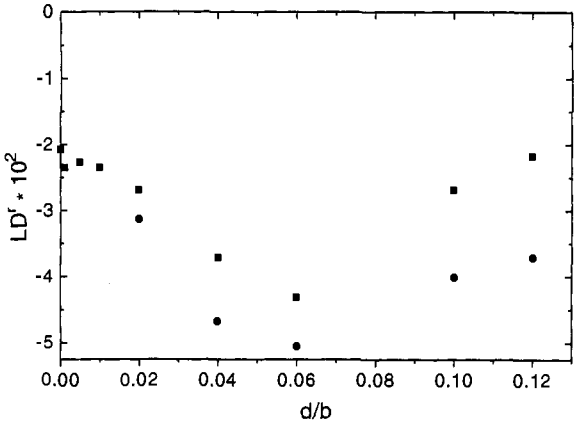

Figure 5. LD^r of YOYO–PhiX 174 RFI DNA complexes at 260 nm (■, DNA absorption band) and at 482 nm (●, YOYO absorption band) versus mixing ratio d/b (YOYO:DNA base). DNA concentration between 15 and 47 μM. Shear gradient 3720 s^{-1}. Optical pathlength 1 mm.

shown) were found to be very similar to those of YOYO in the presence of the linear T2 DNA (Figure 3), indicating that the dye binds in the same way to both DNAs. In a plot of LD^r at 260 nm (contributions predominantly from the DNA bases), and at 482 nm (contribution only from the bound dye), versus the mixing ratio (Figure 5), the LD^r in both cases first decreases and reaches a minimum at $d/b = 0.06$, whereupon it increases again, a behavior indicating removal and reversal of supercoils due to unwinding.

C. Fluorescence Measurements. Absorption and excitation spectra are similar for the YOYO–DNA (Figure 6) and the YO–DNAsolutions (not shown). The emission spectrum, also similar for YOYO–DNA and YO–DNA, is almost a mirror image of the low-energy absorption band, and the excitation spectrum is similar to the absorption spectrum and its shape is independent of emission wavelength. These results indicate that there is only one emitting

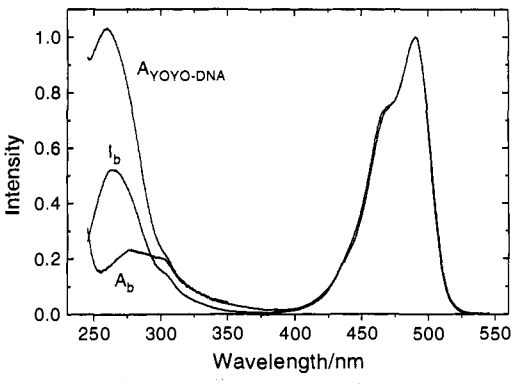

Figure 6. Absorption spectrum, A_b, of YOYO bound to T2 DNA (at a mixing ratio YOYO:DNA base of 0.10) normalized at 490 nm to the fluorescence excitation spectrum, I_b, of the YOYO–DNA solution. A_b was calculated as the difference between the absorption spectrum of YOYO complexed with DNA, $A_{YOYO-DNA}$, and the absorption spectrum of a pure DNA solution at the same DNA concentration. YOYO concentration 1.3 μM. Emission wavelength 550 nm (bandpass 4 nm).

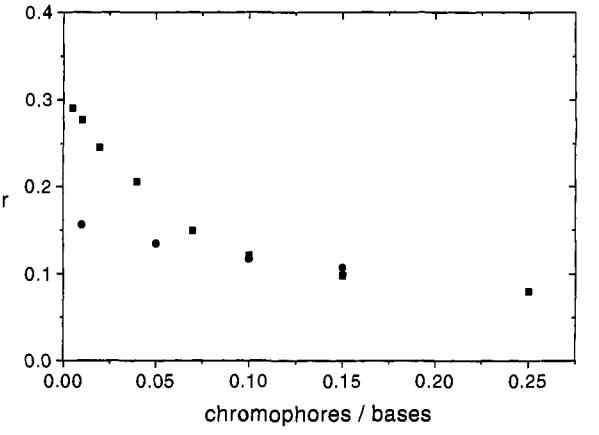

Figure 7. Fluorescence anisotropy (r) of YO– (■) and YOYO–DNA (●) at various ratios of YO chromophore:DNA base. The polarization components are averages of 200 points. Excitation wavelength 460 nm (bandpass 0.5 nm). Emission wavelength 540 nm (bandpass 40 nm). Temperature 21 °C. The optical density at 490 nm was below 0.05 in order to avoid reabsorption.

species, the YO chromophore, in the solution and that a single electronic transition in the chromophore is responsible for the absorption and fluorescence in the visible wavelength region. The Stokes shift for both YO and YOYO is approximately 750 cm^{-1}.

D. Fluorescence Anisotropy (FA). The FA spectra of YOYO–DNA and YO–DNA, respectively, over the visible absorption band are wavelength-independent (not shown), also indicating that only one electronic transition in the YO chromophore is responsible for the absorption.

Figure 7 shows how the degree of anisotropy, r (at $\lambda_{em} = 540$ nm), for YO– and YOYO–DNA depends on the mixing ratio (note that in this figure the mixing ratio is expressed as the ratio of chromophores:bases, which is the same as the d/b ratio for YO but twice the d/b value for YOYO). The limiting anisotropy value for YOYO at low binding ratios is about one-half of that for YO. The most plausible explanation for this difference, as well as for the strong decrease in anisotropy with increasing d/b observed for YO, is depolarization of the emission due to energy transfer between the chromophores. At low mixing ratios, the average distance between two chromophores in YO–DNA is too large to give any appreciable energy transfer, the depolarization is thus small, and the result will be a high anisotropy value. When the mixing ratio is increased, the energy transfer becomes more efficient and, therefore, a decrease in the anisotropy is observed. In the YOYO–DNA complex, the two chromophores of the YOYO molecule are, independent of the binding ratio, close to

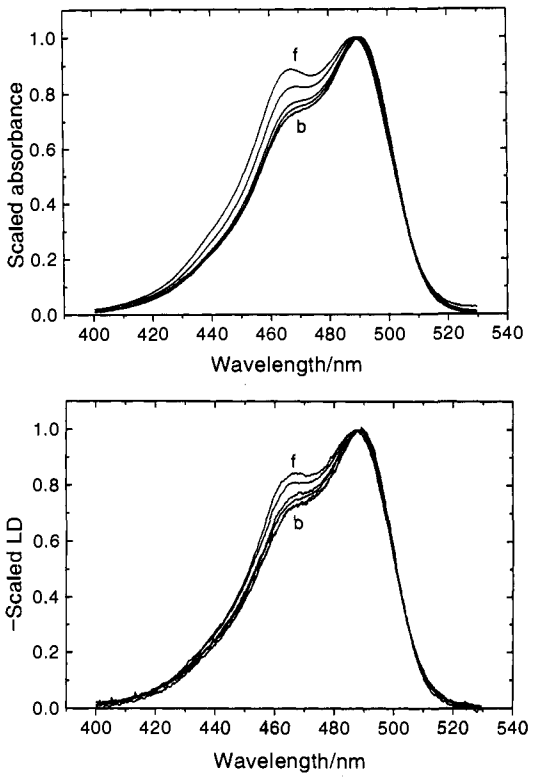

Figure 8. (top) Absorption spectra from Figure 3 (top) and (bottom) linear dichroism spectra from Figure 3 (center) normalized to the same peak height. For experimental conditions, see Figure 3.

each other, resulting in a significant energy transfer even at very low binding ratios. This picture is supported by results obtained from a computer simulation based on a theoretical model of depolarization due to Förster energy transfer between intercalated YO chromophores.[24] Furthermore, this simulation shows that the intercalation is non-cooperative, obeys the nearest neighbor exclusion principle, and unwinds DNA an angle of about 20° per chromophore.

Discussion

A. Two Binding Modes. YOYO. In Figure 8 (top), where the visible absorption spectra of all the YOYO–DNA solutions are normalized to make comparison easier, it can be seen that with increasing d/b the 490 nm peak is displaced toward shorter wavelength (blue shift) at the same time that the shoulder grows in intensity. The changes are very small for d/b ratios below 0.125, then become more pronounced, so that at the highest mixing ratio ($d/b = 0.20$) the peak has moved to 488 nm and the shoulder has grown to a clearly visible peak. These changes could be explained by an increased amount of free dye in the solution. However, comparison with the corresponding, normalized LD spectra (Figure 8 (bottom)) shows that this explanation can be dismissed. The LD spectra, which are due *totally* to *bound* dye, are very similar to the absorption spectra, thus indicating that practically all the dye present in the solutions is bound to the DNA due to the high binding constant.[3] In our case, where a single electronic transition is responsible for the absorption, the reduced dichroism shall then be constant over the absorption band. Due to a small blue shift of the LD spectra relative to the absorption spectra, the reduced dichroism displays local variations over the visible absorption band. This blue shift indicates the existence of more than one binding environment with different spectral shifts and different binding angles for the dye, for example, due to local motion of the chromophore within its binding site. The small changes in the shapes of the absorption, the LD and the LD^r spectra, up to $d/b = 0.125$, indicate, however, that one type of binding mode dominates up to this mixing ratio, whereafter a second binding mode starts to contribute. Two binding modes are also suggested by the changes seen in the LD^r amplitude, where a steady increase changes to a decrease after $d/b = 0.125$, and in the CD spectra, where a DNA-induced CD grows steadily but without change in the profile up to $d/b = 0.125$, whereupon an exciton type CD appears which then dominates the spectrum.

YO. The spectra of the YO–DNA solutions (Figure 2) are very similar to those of the YOYO–DNA solutions (Figure 3), indicating large similarities in the binding of the two dyes to DNA. There are, however, differences. From the normalized absorption and LD spectra (not shown), it can be seen that the change of the profiles, which follows each increase in mixing ratio, is stronger in the absorption spectrum than in the LD spectrum, indicating that the amount of free dye in the solution in this case increases substantially with increasing mixing ratio. The affinity for DNA binding is thus weaker for YO than for YOYO. Another difference between YO and YOYO is that for YO the change in shape of the LD spectrum occurs more gradually over the whole mixing ratio region than for YOYO, suggesting a more gradual transition between the two binding types. That the binding constants for the two binding modes of the YO chromophore are more equal in YO–DNA than in YOYO–DNA can be seen also when one compares the mixing ratios where the weaker binding mode begins to give a clear contribution in the spectra of the two dyes. For YO, this occurs after $d/b = 0.20$ (one YO chromophore per five bases), as judged from the increase followed by the decrease in the LD^r amplitude (Figure 2 (bottom)) and from the change in the profile of the DNA induced CD (Figure 4 (top)), whereas for YOYO (see above) this occurs after $d/b = 0.125$ (one YO chromophore per four bases).

B. First Binding Mode—Intercalation. Linear Dichroism. The LD^r of the visible absorption band is strongly negative in both the YO– and the YOYO–DNA complexes (Figures 2 (bottom) and 3 (bottom)) indicating that the transition moment of the bound YO chromophores is almost perpendicular to the DNA helix axis. Since spectroscopic studies of the free dyes have confirmed that there is only one electronic transition in the visible band and that this transition is long axis polarized,[25] the long axis of the bound YO chromophores is also oriented perpendicular to the helix axis, which is consistent with an intercalative binding geometry. The LD^r amplitude increases with increasing binding ratio, which is an additional indication of intercalation since intercalators extend the DNA chain and make it stiffer, which should result in an increase in the degree of orientation of the DNA.

An interesting observation, though not unusual,[26-28] is the larger LD^r amplitude in the dye absorption band compared to the LD^r in the UV absorption band where the DNA bases dominate the absorption. This suggests that the long axis of the YO chromophore is, on the average, closer to perpendicular to the helix axis than are the DNA bases. If a perpendicular geometry is assumed for the YO chromophore, an average base inclination from perpendicular of 17° at $d/b = 0.10$ can be calculated (eq 3). This is a surprisingly high value since most dichroism measurements indicate that any tilt from perpendicular orientation of the bases in B-form DNA should be less than 10°.[9] However, it has also been inferred, from LD measurements in the vacuum UV region, that the individual bases could be tilted by as much as 16–25°.[29] Furthermore, intercalation needs space and extends

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the distance between the base pairs and it cannot be ruled out that this process in itself leads to a change in the average base inclination.

The larger LD^r amplitude in the dye absorption band could also be due to the fact that the binding of the dye leads to a local stiffening of the DNA chain at the binding site and thus a better orientation of these segments compared to the orientation of the unperturbed ones. However, in that case, one expects that the difference between the LD^r amplitudes shall decrease with increasing mixing ratio, but a close inspection of Figure 3 (bottom) instead shows that the LD^r amplitude in the dye absorption band increases faster with increasing binding ratio than the LD^r amplitude in the DNA absorption band. The latter effect indicates that there may be some positive LD contribution from UV transitions of the dye which are oriented nonperpendicular to the helix axis. Resolution of the LD contributions from the dye and the DNA in the UV region (260 nm) was performed by forming linear combinations of the LD spectra of pure DNA and dye–DNA complexes at two different mixing ratios. This shows that the average angle between the helix axis and the UV transitions in both YO and YOYO is about 68° (optical factor $O = -0.88$). Since the dye absorption in the UV band is low compared to the DNA absorption, the calculated angle is too large to be able to explain the whole effect. Therefore, we suggest that significant inclination of the bases from perpendicular orientation is the major reason for the larger LD^r amplitude in the dye absorption band.

Circular Dichroism. At low mixing ratios, the induced CD of YO bound to the DNA increases with increasing mixing ratio and the CD band has a shape that is nearly independent of the mixing ratio, confirming the conclusion that a single binding geometry dominates at these mixing ratios (Figure 4 (top)). A very similar induced CD in YOYO (Figure 4 (bottom)) indicates that its two YO chromophores have the same orientation in DNA as the YO-monomer. In both cases, the induced CD is negative, which suggests that the YO chromophore is intercalated and that its transition moment, and thus the long axis of the chromophore, is parallel to the long axis of the base-pair pocket.[21,30]

Contact Energy Transfer. It has been shown that excitation energy due to light absorbed by the DNA bases may be transferred to dye molecules bound to DNA.[31-34] The transfer has been considered as evidence for intercalation since it can only occur over very short distances.[33] To investigate if energy is transferred from DNA to the bound YOYO, the spectra obtained at the mixing ratio of 0.10 were utilized as follows. The absorption spectrum of the bound dye was calculated as the difference between the absorption spectrum of the YOYO–DNA solution (the amount of free YOYO is negligible, see above) and the absorption spectrum of a pure DNA solution at the same DNA concentration, assuming that the DNA absorption characteristics are not changed in the complex. This spectrum was then normalized to the excitation spectrum of the YOYO–DNA solution at the wavelength 490 nm, where DNA shows no absorption. In the absence of energy transfer, the two normalized spectra shall be identical over the whole wavelength region provided the fluorescence quantum yield is independent of excitation wavelength, but if energy is transferred from the DNA bases to the bound dye, the intensity of the excitation spectrum shall be larger than the intensity of the absorption spectrum in the part of the UV region where DNA absorbs. As can be seen in Figure 6, the two spectra (denoted A_b and I_b) coincide well at all wavelengths except where DNA absorbs where the excitation spectrum (I_b) is significantly larger than the absorption spectrum (A_b), showing that energy transfer occurs in the complex, thus suggesting that the binding of YOYO to DNA is intercalative.

LD Titration Experiment on Supercoiled DNA. Unwinding of supercoiled DNA, expected for intercalating compounds,[35] has been studied by methods such as gel electrophoresis,[36,37] sedimentation velocity,[38,39] and viscosimetry.[40] Recently, Swenberg *et al.*[13] have shown that unwinding and rewinding of supercoiled DNA caused by intercalating dyes can be followed by the flow LD technique. They found that LD^r first decreased to a minimum with increasing binding ratio of the dyes and then increased as the binding ratio increased further. The decrease was attributed to unwinding, the minimum to the point where all supercoils were removed, and the increase to introduction of left-handed supercoils. Our titration of the supercoiled PhiX 174 RFI DNA with YOYO gives a similar LD^r behavior in the d/b region between 0 and 0.12 (Figure 5), which suggests that YOYO removes and reverses supercoiling by intercalation. The LD^r titration curve obtained at 482 nm has almost the same shape as that at 260 nm, with in both cases a minimum at a d/b ratio of 0.06 (the equivalence point where all supercoils are removed). The quantitative difference between the curves, with LD^r at 482 nm more negative than LD^r at 260 nm, is about the same as that observed in the LD^r for the linear T2 DNA at the corresponding mixing ratios and wavelengths (Figure 3 (bottom)), which evidences that the mode of binding to the two DNAs is very similar.

It is in principle possible to calculate the unwinding angle per bound YOYO from our titration experiment. However, in contrast to the procedure used to prepare the samples for the other experiments in this work (see Materials and Methods), the titration experiment was performed, for practical reasons, by addition of a dye solution to the same DNA solution. This procedure gives heterogeneous binding as revealed by electrophoretic analysis of the samples (not shown) and thus an unreliable equivalence point. The titration experiment shall therefore be seen as a qualitative study made to answer the question whether or not YOYO unwinds DNA.

Fluorescence Anisotropy. The anisotropy, r, plotted in Figure 7 originates solely from the bound dyes since the fluorescence from free YO and YOYO is negligible. It can be seen that the anisotropy of YO in the limit of a zero binding ratio, where depolarization of the emission due to energy transfer between the chromophores can be neglected, has a value of 0.31. For a totally rigid system of immobile chromophores, the anisotropy attains a maximum value of 0.40, provided the absorbing and emitting transition moments are parallel.[23] The limiting value obtained here mostly reflects the mobility of the dye within its binding site in the DNA macromolecule since tumbling and bending of this molecule occur on time scales that are much longer than the fluorescence lifetime of YO in DNA, which has been found to be 3 ns.[25] The high anisotropy thus indicates a binding mode with restricted motion.

Taken together the results of the LD, CD, contact energy transfer, and LD titration on supercoiled DNA provide clear evidence that the binding of YO and YOYO to ds DNA at low mixing ratios is by mono- and bisintercalation, respectively. YOYO is a cationic heterocyclic compound, and it is not unusual that such compounds, for example ethidium, proflavine, and psoralen, bind to double-stranded DNA by intercalation.[9] The corresponding dimeric compounds may intercalate either with one or two of its moieties. One restriction for bisintercalation is, if the nearest neighbor exclusion principle is followed, that the

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length of the chain linking the two monomeric units must be long enough to leave two base pairs between their moieties. The ethidium dimer (chain length 11.4 Å) has been shown to intercalate with only one of its chromophores[41] whereas YOYO, with a similar length of the linking chain, is bisintercalating. Further, a diacridine with a chain length of 9.9 Å is found to monointercalate,[42] but *N*-[3-(9-acridinylamino)propyl]amine, which has the same linking chain, is supposed to bisintercalate.[43] These examples show that, in addition to the chain length, there are other factors, for example binding affinity to DNA, that influence if the dye mono- or bisintercalates.

C. Second Binding Mode—Groove Binding? An exciton CD shown by bound dyes is generally taken as evidence for formation of dimers of chiral disposition on the surface of the DNA chain.[9] The CD of the YO–DNA complex indicates that an exciton is present from $d/b = 0.20$ even though it becomes clearly visible first from $d/b = 0.5$ due to overlap with the monomeric CD from the intercalated YOs (Figure 4 (top)). The exciton has the form of a −/+ pattern with increasing wavelength, indicating that the interacting YO chromophores are oriented in a right-handed way relative to each other.[44] For a dimer formed by monomers that are externally bound to the DNA, this is also the expected screw sense since their orientation should be directed by the pitch of the DNA helix.

In YOYO–DNA, the exciton is observed when the mixing ratio, which in this case is almost equal to the binding ratio due to the high affinity of YOYO to DNA, is just above 0.125 (Figure 4 (bottom)). The number of surface-bound dye molecules should then be small if, as our results indicate, bisintercalation with nearest neighbor exclusion is the predominant mode of binding until all intercalation pockets are filled up. This gives an indication that the exciton is not due to dimeric interaction between YO chromophores from different YOYO molecules close to each other on the surface but that it is more likely to be an effect of interaction between the two chromophores in the same molecule. An indication that these can come close enough to each other to interact electronically is found in the isotropic absorption spectrum of free YOYO, which has been interpreted as the result of exciton splitting due to dimeric interaction between the two YO chromophores in YOYO, stacked together with their long axes parallel.[25] Binding to the surface of DNA, for instance to one of the grooves where the space is limited, may favor such a conformation and also increase the interaction since the environment at the surface is more polar than in the bulk of the solution, thus allowing the charged YO chromophores to come even closer to each other. A further indication for intramolecular interaction is the +/− pattern of the CD exciton of YOYO–DNA, which shows that the bound dimers have left-handed chirality, *i.e.* opposite to that expected for intermolecular interaction between externally bound molecules, the orientation of which should be directed by the pitch of the DNA helix.

The increase of the LD^r amplitude of both YO– and YOYO–DNA during the intercalation phase is changed to a decrease when the dyes start to bind to the surface of the DNA (Figures 2 (bottom) and 3 (bottom)). This indicates that the optical factor of the externally bound dyes is quite different from that of the intercalated ones. The decrease shows that the factor is larger, *i.e.* the externally bound dyes are oriented with angles between their long axes (the directions of their transition moments) and the DNA helix axis which are less than the corresponding angle for the intercalated molecules, which should be nearly 90°. If, for instance, the dyes bind inside one of the grooves of the DNA, and are aligned along the groove, the pitch of the groove determines an effective angle of approximately 40–50°,[11] or an optical factor in the range +0.4 to +1.1, which shall be compared with the value −1.5 for an angle of 90°. However, the decrease in the LD^r amplitude may also in part be due to a decrease in the degree of orientation of the complexes. The dyes are positively charged, and the external binding may cause a decrease of the surface charge of the DNA and thereby an increase in its flexibility.

Conclusions

The main conclusions of the present study of the binding of YO and YOYO to DNA can be summarized as follows.

(1) Both dyes interact with the DNA double helix in much the same way, but the affinity for binding is higher for YOYO than for YO as is clear from a comparison between the LD and absorption spectra of the dye-DNA solutions.

(2) At mixing ratios dye:DNA base up to 0.20 for YO and 0.125 for YOYO, the predominant mode of binding is monointercalation for YO and bisintercalation for YOYO, where in both cases the long axis of the YO chromophore orients parallelly to the long axis of the base-pair pocket.

(3) For mixing ratios dye:DNA base above 0.20 for YO and 0.125 for YOYO, external binding to the DNA begins to contribute noticeably. In the YO case, this is concluded from the appearance of an exciton with the expected pattern for intermolecular dimeric interaction between molecules bound to the surface of the DNA. In the YOYO case, an exciton also appears but with the inverse pattern compared to that of YO, suggesting that the interaction is between YO chromophores on the same molecule, which is folded at the chain linking the chromophores so that these are close to each other.

(4) A higher limiting fluorescence anisotropy value as well as a much stronger decrease in fluorescence anisotropy with mixing ratio for YO–DNA compared to YOYO–DNA may be explained by depolarization of the emission due to Förster energy transfer between the intercalated chromophores.

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