Pergamon

Bioorganic & Medicinal Chemistry, Vol. 3, No. 6, pp. 701–711, 1995
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0968-0896/95 $9.50 + .00

0968-0896(95)00061-5

Intercalative Interactions of Ethidium Dyes with Triplex Structures

Eimer Tuite and Bengt Nordén*
Department of Physical Chemistry, Chalmers University of Technology, S-412 96 Gothenburg, Sweden

Abstract—The binding of phenanthridine dyes to triplex poly(dT)*poly(dA)·poly(dT) and its precursor duplex poly(dA)·poly(dT) is characterized using linear dichroism and circular dichroism spectroscopy, and thermal denaturation. The two monomeric dyes ethidium bromide and propidium iodide are shown to behave similarly to each other in intercalating into and stabilizing both the duplex and the triplex structures. However, contrary to expectations, the extra cationic side-chain of propidium iodide provides no significant extra stabilization of triplex compared with ethidium bromide, although propidium does stabilize the duplex more than ethidium. The monomeric dyes appear to have somewhat different binding geometries with the duplex and triplex polymers. The dimeric dye ethidium homodimer is found to bis-intercalate in the triplex as well as the duplex but, in contrast to the monomers, no variation in geometry between duplex and triplex is observed. However, although dimer stabilizes the duplex, it has no effect on the thermal stability of the triplex. This lack of binding preferentiality of the dimer for triplex compared with the monomeric dyes indicates greater constraints on the accommodation of a bis-intercalator in the triplex structure than in the duplex.

Introduction

In recent years there has been a tremendous upsurge of interest in triple helical DNA structures following discoveries that short oligonucleotides can recognise specific sequences of duplex DNA[1] and that an intramolecular triple helical form—H-DNA[2]—can be formed in natural DNA, suggesting a possible regulatory role for this unusual structure. Subsequent research has thus focused on the therapeutic potential of 'antigene' oligonucleotides,[3] i.e. the use of duplex-targeted oligonucleotides to inhibit gene expression just as 'antisense' oligonucleotides are targeted at single-stranded nucleic acids,[4] as well as on the technological possibilities presented by the ability of a oligonucleotide to recognise a specific DNA sequence, such as the design of artificial nucleases.[1,3]

Of the potential DNA triplex structures, it is the pyrimidine*purine·pyrimidine[5] type which has attracted most attention since the dC$^+$*dG·dC and dT*dA·dT triplets are isomorphous and thus relatively stable mixed sequence triplexes can be formed. In these triplexes, recognition results from Hoogsteen base-pairing between the pyrimidine base in the third strand and the purine base in the duplex, as shown in Figure 1B for the T*A·T base-triplet, with the third strand running parallel to the purine strand. However, triplex DNA is not particularly stable, especially under physiological conditions, since the dC$^+$*dG·dC triplex requires acidic ($\leq$ pH 5.5) conditions and the dT*dA·dT triplex needs high ionic strength.[3] Such stability problems as well as other complications with nuclease digestion, cell permeability, etc. have led to the development of many backbone- and base-modified nucleic acid analogues for both technological and therapeutic purposes.[3,4]

However, there is another strategy which has been employed to enhance the stability of triplexes—the covalent attachment of intercalators to the third strand.[3] Several studies have thus examined the binding not only of intercalators[6,7] but also groove binders[8] to triplex, in order to elucidate the factors that give rise to stabilization (or destabilization) of triplex. It has become apparent, for instance, that large crescent-shaped intercalators, perhaps aided by a certain degree of rotational flexibility, can provide strong stabilization,[7a–c,f,g] while minor groove binders may have no effect or may destabilize triplex despite strongly stabilizing its precursor duplex.[8] Ligands which bind to the major groove of the duplex, change their interaction with DNA on binding of the third strand—for instance, methylene blue appears to intercalate[9a] and methyl green has no interaction[9c] with triplex poly(dT*dA·dT), while both appear to bind in the major groove of the duplex.[9b,d]

In this study, we examine the interactions of three related phenanthridine dyes, ethidium bromide (EB), propidium iodide (PrI), and ethidium homodimer (EB$_2$)[10] (Fig. 1A) with triplex poly(dT*dA·dT) and its precursor duplex poly(dA·dT). There have already been both experimental and theoretical studies of the interaction of the archetypal intercalator ethidium bromide with triplex DNA,[6] which has been shown to intercalate and stabilize poly(dT*dA·dT). The main questions that this study addresses are (a) whether adding a long cationic side-chain to the basic phenanthridine chromophore substantially changes

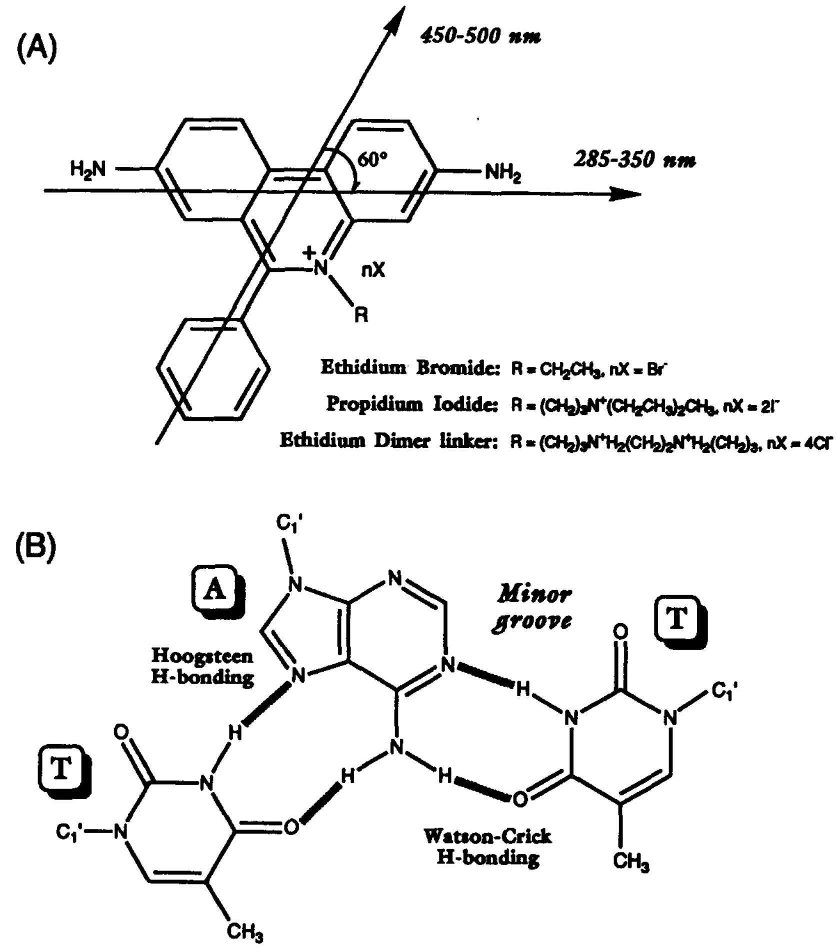

Figure 1. Structures of (A) the phenanthridine dyes and (B) the T*A·T base-triplet.

either the binding of the dye or the stabilization of triplex by the dye, (b) whether a phenanthridine dimer which is known to bind to duplex DNA by bis-intercalation can interact with the triplex in a similar manner, and (c) if this dimer does bis-intercalate, whether it can stabilize the triplex.

Results and Discussion

Absorption spectra

Absorption spectra for EB, PrI, and EB_2, respectively, bound to both duplex poly(dA·dT) and triplex poly(dT*dA·dT) at a nucleotide to chromophore ratio of *ca* 25 are shown in Figures 2A–4A. With both polymers the buffer contains 2 mM Mg^{2+}, which is necessary for the stability of the triplex[3] and was found to have little effect in promoting disproportionation of the duplex to triplex, as judged by circular dichroism measurements.

For each dye upon binding to either polymer, hypochromism and a red-shift are observed in the visible region of the absorption spectrum. The extinction coefficients of these dyes in the visible are quite low so the spectral intensities in this region are weak. Nonetheless, it is clear that for PrI and EB_2 the spectra upon binding to the two polymers are quite similar, while for EB there are some differences with a greater red-shift being observed on binding to triplex. Under the high salt conditions used in these experiments it is probable that these effects arise because of different degrees of binding.

Ethidium is known to have a low affinity for poly(dA·dT) compared to random sequence nucleic acids.[11] Binding parameters have been reported for ethidium bromide with duplex in 100 mM NaCl (K = 3.4 × 10^3 M^{-1} and n = 2.4) and with triplex in 5 mM Mg^{2+} (K = 95 × 10^3 M^{-1} and n = 2.8).[6c] Although these ionic strength conditions are different (and indeed differ from that of this study, 2 mM Mg^{2+}), it is clear that binding to triplex is significantly stronger. Thus, more ethidium is expected to be bound to the triplex (> 60% bound, using the value in 5 mM Mg^{2+} as a lower limit) than the duplex. With both polymers, the LD^r is dependent on wavelength in the visible region, indicating that there are substantial concentrations of free dye in both cases.

The binding of the dicationic PrI and tetracationic EB_2 to duplex poly(dA·dT) and triplex poly(dT*dA·dT) should be stronger than that of the monocationic ethidium. Since, for both dyes, the absorption spectra

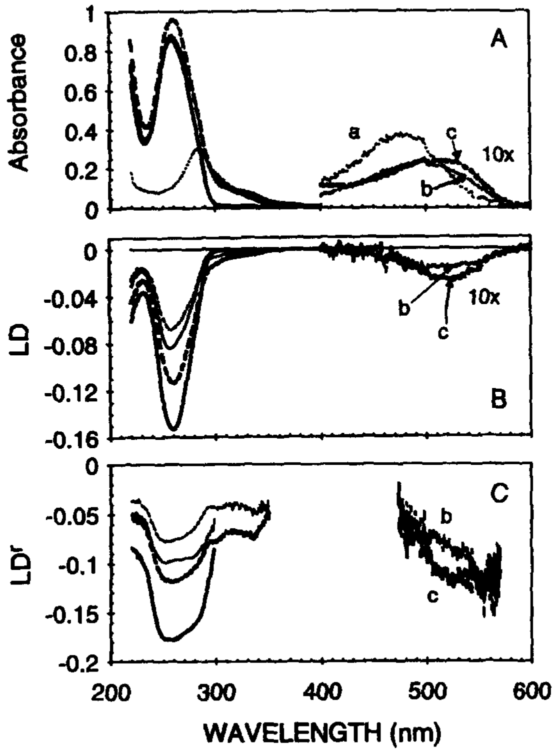

Figure 2. (A) Absorption, (B) LD and (C) LD^r spectra for ethidium bromide bound to duplex and triplex. (—) duplex; (——) triplex; (a) (·········) free dye; (b) (———) EB/duplex; (c) (– – – –) EB/triplex. [EB] = 6 μM, [poly(dA·dT)] = 149 μM, [poly(dT*dA·dT)] = 150 μM, 20 mM phosphate/2 mM Mg^{2+} (pH 7) buffer. The spectra are expanded by a factor of 10 above 400 nm to facilitate comparison.

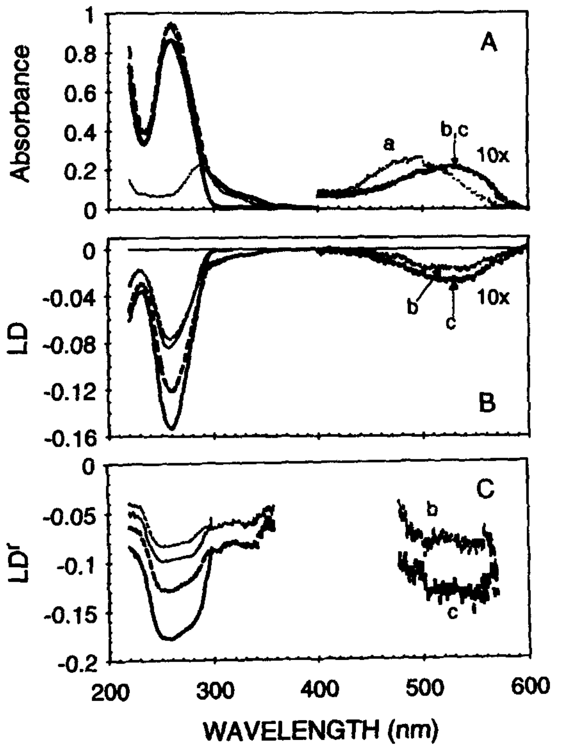

Figure 4. (A) Absorption, (B) LD and (C) LD^r spectra for ethidium homodimer bound to duplex and triplex. (—) duplex; (——) triplex; (a) (·········) free dye; (b) (———) EB_2/duplex; (c) (– – – –) EB_2/triplex. [EB_2] = 3 μM, [poly(dA·dT)] = 149 μM, [poly(dT*dA·dT)] = 150 μM, 20 mM phosphate/2 mM Mg^{2+} (pH 7) buffer. The spectra are expanded by a factor of 10 above 400 nm to facilitate comparison.

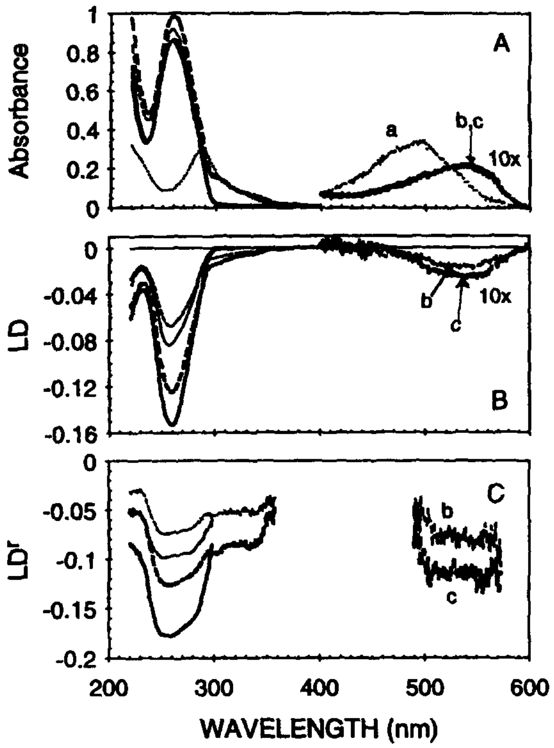

Figure 3. (A) Absorption, (B) LD and (C) LD^r spectra for propidium iodide bound to duplex and triplex. (—) duplex; (——) triplex; (a) (·········) free dye; (b) (———) PrI/duplex; (c) (– – – –) PrI/triplex. [PrI] = 6 μM, [poly(dA·dT)] = 149 μM, [poly(dT*dA·dT)] = 150 μM, 20 mM phosphate/2 mM Mg^{2+} (pH 7) buffer. The spectra are expanded by a factor of 10 above 400 nm to facilitate comparison.

with duplex and triplex are so similar and the LD^r spectra are practically invariant above 500 nm, it appears that PrI and EB_2 are essentially completely bound to both polymers in these experiments.

Linear dichroism spectra

The linear dichroism (LD) spectra of the dyes with duplex and triplex are shown in Figures 2B–4B. In all cases, the LD is entirely negative, both in the UV and the visible bands, a strong indication that binding is intercalative.[12]

It may be noted that each of the dyes causes a reduction of LD at 260 nm for both polymers. Although a reduction in LD suggests a reduction in the orientation of the polymer and is not usually associated with intercalation in normal DNA, it is probably not correct to make such an assumption here since all the available evidence points to intercalation as the binding mode with both duplex and triplex, at least for ethidium.[6c] It is unlikely that this effect is caused by a positive contribution to the LD from an out-of-plane dye transition in the 200–280 nm region since it is not observed with heterogeneous CT-DNA (data not shown). Furthermore, we have noticed similar behaviour of these AT polymers with a number of intercalating dyes, so it appears to be associated with the properties of the polymers. In fact, these polynucleotides are extremely stiff and well-oriented in the absence of dye, much more so than heterogeneous DNA samples. For poly(dA·dT) this may be attributed to its unusual B-form structure which is suggested to involve a stabilizing spine of hydration in the minor groove,[13] high propeller-twisting, and bifurcated H-bonds between adjacent base-pairs, according to crystal structure determinations,[14] molecular dynamics calculations,[15] and linear dichroism measurements.[16] Although this unusual structure might be expected to be relieved on formation of triplex, there have been indications from electrophoretic measurements that the triplex is stiffer than duplex:[17] certainly, it is even more amenable to flow orientation than the duplex.

Interpretation of LD^r

The reduced linear dichroism (LD^r) spectra for the dyes with the AT polynucleotides are shown in Figures 2C–4C. For comparative purposes, LD^r spectra of ethidium and ethidium dimer with calf thymus (CT)-DNA are also shown (Fig. 5), where it is known that the dyes are mono- and bis-intercalated, respectively.[18] The spectra with CT-DNA are typical for these dyes with heterogenous DNA and alternating polynucleotides, and they are somewhat different from those of the dyes with the AT polymers.

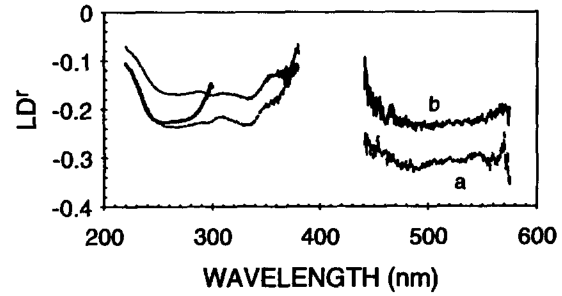

Figure 5. LD^r spectra for ethidium bromide and ethidium homodimer bound to CT-DNA. (—) DNA; (a) (·········) EB/DNA; (b) (—) EB_2/DNA. [EB] = 20 μM, [EB_2] = 10 μM, DNA = 197 μM, 5 mM TBE (pH 8.4).

The LD^r data can be interpreted in terms of orientation angles according to the equation:

$$LD^r(\lambda) = S\,(3/2)\,(3\langle\cos^2\alpha\rangle - 1) \qquad (1)$$

where α represents the angle between the absorbing transition moment and the DNA helix axis. S is an orientation factor describing the degree of orientation of the DNA helix such that $S = 1$ for perfect orientation and $S = 0$ for random orientation. $\langle\cos^2\alpha\rangle$ represents an average over the angular distribution. LD^r is thus zero if α is at the magic angle (54.7°), negative if it is greater than that, and positive if it is smaller.

Using the flow linear dichroism technique, measurements are made on samples that are only partially oriented. Unfortunately, there is no direct means of measuring S, so the data obtained cannot be interpreted in terms of absolute angles. The normal method of analysis involves elimination of S using known transition moment directions:

$$LD^r(\lambda_A)/LD^r(\lambda_B) = (3\langle\cos^2\alpha_A\rangle - 1)/(3\langle\cos^2\alpha_B\rangle - 1) \qquad (2)$$

where A and B refer to two distinct transition moments, most often for the DNA basepair and for a drug transition. This assumes an identical average orientation of the DNA helix at positions with or without bound dye. α_A is then generally fixed at the 86° determined for B-DNA in previous studies,[12] which allows calculation of α_B. Because of the various assumptions made, LD data cannot be used to determine exactly the structure of a DNA–ligand complex, but it is invaluable for placing limits on the range of possible binding geometries. Studies have indicated that the basepairs in B-DNA may, in fact, be inclined by as much as 20° from the helix axis,[16] so this makes determination of angles somewhat uncertain since they will be quite sensitive to the angle used as a reference value.

In Figure 6A we indicate the different terms we use in this study to characterize the inclination of the basepair from perpendicular. The basepair may be both 'tilted' about its short (dyad) axis and 'twisted' about its long axis, giving rise to an overall inclination about an arbitrary 'pivot axis' which remains perpendicular to the helix axis. α thus represents the angle between the helix axis and the effective DNA transition moment (μ_{DNA}), while we use β for the angle in the basepair plane between the pivot axis and μ_{DNA}. We then expand this picture to include an ethidium chromophore intercalated between adjacent basepairs (Fig. 6B). Whereas we do not know the direction of μ_{DNA} in the basepair XY-plane, we have two well defined transitions in the EB chromophore, one parallel to the long axis and the other at 60° from the long axis, directed along the phenyl–phenanthrene bond.

In linear dichroism, the data give angles for transition moments with respect to the helix axis. From Figure 3, it is clear that the closer the direction of a transition moment is to the pivot axis, the more negative the LD^r will be. Most importantly, a dye transition can have a notably more negative LD^r than the DNA basepairs if it is oriented along, or close to, the pivot axis and μ_{DNA} has a significant inclination: this phenomenon is observed for several intercalating dyes.[9a, 19]

LD^r of ethidium bromide with DNA

We begin with an analysis of the EB spectrum with DNA, which is consistent with previously reported LD^r data for EB/DNA.[20-22] Crystal structures of ethidium bromide with dinucleotide mini-helices (UpA and CpG)[23] indicate that the phenyl and ethyl groups both lie in the minor groove with the dye long axis approximately parallel to the base-pair long axis. We attempt to correlate the LD^r data with such a structure, which is possible if the bases have an average inclination with respect to the helix axis.

The EB transition moments have been characterized previously by Houssier and coworkers[20] as shown in

Figure 1A. Absorption in the 285–350 nm region arises from the long-axis transition moment, while at longer wavelengths (450–500 nm) the absorption arises from the transition moment oriented along the phenanthridine-to-phenyl bond at an angle of 60° to the long-axis. Since these two transitions are spectrally well separated and do not significantly overlap the DNA absorption, they can be used to provide information about the orientation of the phenanthridine chromophore with respect to the nucleobases. The transition moments are not expected to be significantly perturbed by modifications of the sidechain and we therefore assume the same transition moment directions for all three dyes. LD^r data at three wavelengths representing the μ_{DNA}, (260 nm), and the two ethidium transitions (330 and 555 nm) are presented in Table 1.

The LD^r values at 330 nm and 260 nm are approximately equal implying that the long axis of the phenanthridine chromophore has the same inclination as the *effective* transition moment for the basepair (μ_{DNA}) with respect to the helix axis, although this does not necessarily imply that they have the same direction. The LD^r at 530 nm is considerably more negative than that at 260 nm (by *ca* 50% for both ethidium and its dimer) indicating that the axis along the phenanthridine-to-phenyl bond is oriented more perpendicular to the helix axis than base-pairs. In order to interpret our data, we must assume a reference angle for one of the transition moments. If the most negative LD^r (that at 555 nm) is set to 90°, implying that this transition lies along the pivot axis, then the other two transitions have an inclination of 74°. However, such a calculation is very sensitive to the reference value which can be seen from Figure 7: if α_{555} is allowed to

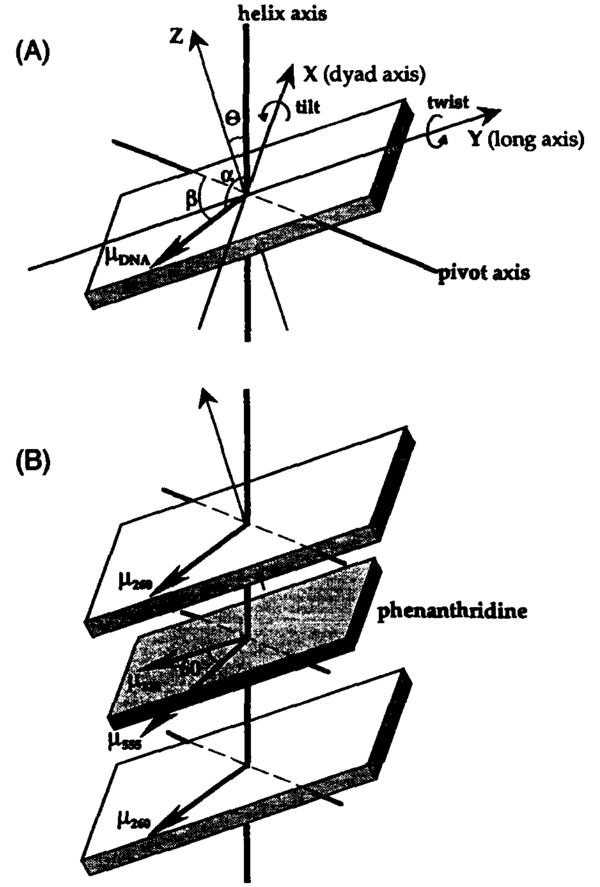

Figure 6. (A) Schematic diagram showing the parameters used to describe the inclination of a base-pair with respect to the helix axis (see text for explanations), and (B) model for intercalation of a phenanthridine chromophore: the dye long axis is parallel to the base-pair long axis, as suggested by crystal structures, and the inclination of the dye reflects that of the adjacent base-pairs.

Table 1. Analysis of LD^r data for phenanthridine dyes bound to duplex and triplex non-alternating A·T polymers and to DNA

Dye	Polymer	No dye LD^r_{260}	+ dye LD^r_{260}	+ dye LD^r_{330}	+ dye LD^r_{555}	α_{260}[a]	α_{330}[b]	α_{555}[c]
EB[d]	(A)·(T)[e]	−0.097	−0.076	−0.045	−0.108	65°	60°	70°
	(T)*(A)·(T)[f]	−0.178	−0.118	−0.072	−0.116	70°	64°	70°
PrI[d]	(A)·(T)	−0.097	−0.074	−0.0521	−0.079	69°	64°	70°
	(T)*(A)·(T)	−0.178	−0.127	−0.086	−0.123	71°	65°	70°
EB_2[g]	(A)·(T)	−0.097	−0.082	−0.063	−0.084	70°	66°	70°
	(T)*(A)·(T)	−0.178	−0.129	−0.084	−0.134	69°	64°	70°
EB[h]	DNA[i]	−0.226	−0.236	−0.234	−0.303	66°	66°	70°
EB_2[j]	DNA[i]	−0.226	−0.168	−0.177	−0.230	65°	66°	70°

[a] α_{260} gives the average orientation of the base-pair long axis.

[b] α_{330} gives the average orientation of the transition moment along the phenanthridine long axis.

[c] α_{530} gives the average orientation of the transition moment at 60° to the phenanthridine long axis. This is fixed at 70° in this analysis.

[d] Ethidium bromide and propidium iodide, 6 μM ⇒ 12.4 base-pairs/dye and 8.3 base-triplets/dye.

[e] Poly(dA)·poly(dT), 149 μM

[f] Poly(dT)*poly(dA)·poly(dT), 150 μM

[g] Ethidium dimer, 3 μM ⇒ 12.4 base-pairs/chromophore and 8.3 base-triplets/chromophore.

[h] Ethidium bromide, 20 μM ⇒ 9.85 base-pairs/dye.

[i] CT-DNA, 5 mM TBE, 197 μM

[j] Ethidium dimer, 10 μM ⇒ 9.85 base-pairs/chromophore.

go to 55°, then the difference between the inclinations for α_{555} and $\alpha_{330}/\alpha_{260}$ diminishes.

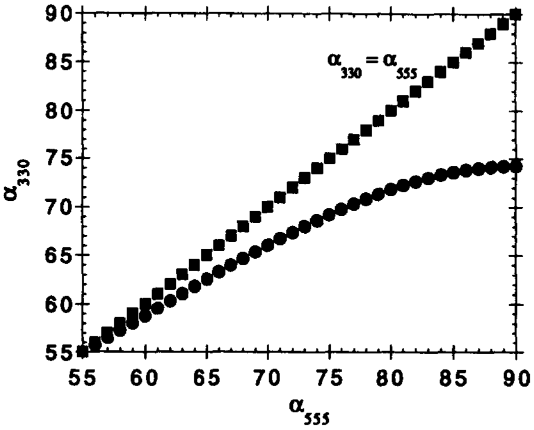

Figure 7. Representation of how α_{330} (derived from the LD^r at 330 nm) changes as the fixed reference angle α_{555} (from the LD^r at 555 nm) is varied between 54.7° and 90° (●●●●). Also shown for comparison is the relation where $\alpha_{330} = \alpha_{555}$ (■■■■■).

We turn now to the results of Hogan *et al.*,[22] obtained using electric dichroism for the same system. By extrapolating to infinite field, which is taken to represent perfect orientation, orientations of $\alpha_{320} = 68 \pm 2°$ and $\alpha_{520} = 69 \pm 2°$ with respect to the CT-DNA helix axis were calculated. These data suggest that to fix one of the EB transition moments at 90°, which results in a 15° difference between the inclination of the two moments is incorrect. If, on the other hand, α_{555} were set to 70°, then $\alpha_{260} = \alpha_{330} = 66°$ which would be consistent with Hogan *et al.*,[22] and also with a base-plane inclination of 15–25° proposed by Johnson and coworkers.[16] Hence, we take these latter values as those representative of ethidium intercalated in B-form DNA, and use them for comparison with the LD^r data for ethidium intercalated in non-standard B-form poly(dA·dT).

LD^r of ethidium homodimer with DNA

The spectrum of the dimer with DNA is very similar to that of the monomer except that it is smaller in magnitude overall: it is likely that this is caused by the binding of the dimer reducing the orientation of the DNA, perhaps by bending it. A recent crystal structure of a dimeric ellipticine derivative having a spermine-like linker (Flexi-Di) with a tetranucleotide d(CGCG)[24] showed a binding of the linker in the major groove with specific H-bond interactions with the groove floor, a de-stacking of the base-pairs, and a bending toward the minor groove. However, we assume, in the first instance, that bound monomeric and dimeric ethidium are similarly oriented, which would place the linker in the DNA minor groove, where there would also be opportunities for H-bonding to stabilize binding. Thus, a similar type of bending could be induced by the ethidium dimer with the result of reducing the orientation of the DNA, an effect not observed with the monomer.

The basis for the similarity of the monomer and dimer spectra can be either that both chromophores in the dimer are bound with the same geometry, which is also that of the monomer, or that the average geometry of the two chromophores in the dimer is the same as that of the monomer. If the dimer does bind in a similar way to the model proposed for Flexi-Di,[24] then the second of these explanations may be most appropriate.

It appears from the LD^r that the dimer is bis-intercalated with DNA since the spectra for EB and EB$_2$ are very similar. If only one chromophore were intercalated and the other were randomly oriented, then it would be anticipated that the dimer LD^r should be half that of the monomer since the second chromophore would contribute to the absorption spectrum but not to the LD. If the second dye were bound along the minor groove, it would be expected to make a positive contribution to the LD^r. Only if the second dye were oriented in, say, the major groove with both transitions close to perpendicular, would an LD^r spectrum such as that observed here result: such a conformation is not likely and the data point to bis-intercalation of EB$_2$.

LD^r of A·T Polynucleotides

It may be noted that there is a strong wavelength dependence of the LD^r in the nucleobase band for both the duplex and the triplex AT polymers, which is not observed with the alternating AT polymer (data not shown). Since the thymine absorption spectrum is at longer wavelengths than that of adenine, this could mean that the adenine and thymine bases have different average orientations with respect to the helix axis. Such an interpretation would be consistent with the model for the poly(dA·dT) duplex proposed by Aymani *et al.*[14c] where, in addition to high propellor twist, the thymine bases are tilted away from perpendicular to a greater extent than the adenine bases: by contrast, in standard B-form DNA, the two bases should be inclined to about the same extent. If this is the cause of the LD^r variation with wavelength for the duplex, then an obvious inference is that both the thymine bases in the triplex have a similar strong tilt from perpendicular since the wavelength dependence is even stronger. Unfortunately, there is little detailed structural information about the triplex with which we can compare. Although there have been several IR[8b,25] and NMR[8c,26] studies on triplex, these have concentrated primarily on the backbone conformation, showing that it corresponds to B-form rather than A-form as originally suggested from fibre diffraction studies;[27] hence, there is no concrete model at the present time for how the bases in triplex are oriented with respect to the helix axis. It is interesting to note that binding of the dyes does not cause this wavelength dependence of LD^r to disappear, although this may simply be due to insufficient dye being bound to induce a detectable effect.

LD^r of phenanthridines with A·T polynucleotides

With the duplex and triplex non-alternating AT polynucleotides, the LD^r spectra are somewhat different from those with DNA. Referring to Table 1, it is clear that the LD^r values in the dye bands are less negative relative to the basepair LD^r than in the complex with CT-DNA; notably the LD^r at 330 nm is less negative than the LD^r at 260 nm. A reduction of the LD^r_{555}/LD^r_{260} ratio for ethidium with poly(dA·dT) compared to CT-DNA has been noted previously.[21a]

It is clear from the sloping LD^r spectra in the visible region for ethidium (Fig. 2C) that substantial quantities of dye remain unbound with both duplex and triplex. The LD^r at 555 nm should be nonetheless approximately representative of the LD^r for the bound dye since the red-shifted absorption at this wavelength should arise mainly from bound dye which is also giving rise to the LD signal. For propidium and ethidium dimer, the LD^r spectra in the visible region show little variation with wavelength, suggesting that essentially all the dye is bound, and the LD^r at 555 nm is representative of that in the entire long wavelength region.

Since several studies have shown that EB and PrI are intercalated with poly(dA·dT),[6c,11,28,29] we interpret the LD^r data in terms of an intercalation model in order to characterize the complexes. Although uncomplexed poly(dA·dT) has a non-standard B-form, the binding of these dyes[6c,29] to poly(dA·dT) is cooperative and this has been interpreted in terms of an allosteric conversion from the nonstandard state to a B-form conformation which can accommodate intercalators more readily (see Thermal Denaturation, below).[29] In such case, we expect the local complex (i.e. intercalator and surrounding basepair) to have a conformation and, thus, an LD^r spectrum similar to that observed in CT-DNA.

We thus set α_{555} to 70° for each of the dyes with poly(dA·dT). For PrI and EB_2, this results in α_{330} lying within 2° of the 66° obtained with CT-DNA. However, the resulting α_{260} is a little larger than with CT-DNA. With EB, the values for α_{330} and α_{260} are lower, but the analysis is rather uncertain here given the prevalence of free dye and the low optical density at 555 nm. We suggest that, rather than reflecting a different orientation of the intercalated dye, the higher values of α_{260} may reflect a different orientation of the basepairs not involved in the intercalation complex and also possibly a different direction in the basepair XY-plane for the effective transition moment (μ_{DNA}) for this polymer compared to heterogeneous DNA. Upon intercalation of a ligand, the high propellor twist should relax and perhaps the reportedly narrow minor groove[14c] widen: the result could potentially be an increased inclination of the base-pair plane from the helix axis to compensate for these changes.

The angles obtained for the dimer in this analysis are very similar to those obtained for PrI, indicating that the compound bis-intercalates with either the orientation of each chromophore or the average orientation of the two chromophores being the same as the monomer orientation.

Surprisingly, when α_{555} for each of the dyes is set to 70° with the triplex, the values for α_{330} and α_{260} thus calculated are close to those obtained with the duplex, suggesting that the dye/triplex complexes may be very similar to the dye/duplex complexes. In this case, the fixing of α_{555} at 70° may appear rather arbitrary. However, the triplex structure does appear to be close to B-DNA[25,26] and the intercalated dye should then have a site similar to that in other B-form polynucleotides. These dyes do not appear large enough to interact with the whole base-triplet and perhaps only involve two bases of each triplet in intercalation so, in fact, their intercalation site could be in the precursor duplex part of the triplex. The orientation of the dye may reflect only the inclination of those parts of the triplex involved in the binding and the value at 260 nm may reflect a more perpendicular orientation of the bases not intimately involved in intercalation. However, without more detailed knowledge of the triplex structure than is presently available, it is difficult to further refine the analysis.

Circular dichroism

Because of the low concentrations of dye used in these experiments, it was not possible to detect with any confidence CD signals in the visible absorption bands where the dye extinction coefficients are low. However, the interaction of the dyes with the polynucleotides can be observed from changes in the CD spectra in the 200–320 nm region (Fig. 8). The duplex and triplex have significantly different CD spectra in the region below 230 nm which allows them to be distinguished in the melting experiments (below). In each case, addition of dye leads to an increase of the CD signal at about 270 nm in a concentration dependent manner. At 217 nm addition of dye to duplex leads to a decrease in the CD signal and addition of dye to triplex gives an increase of the CD signal. This is identical to the behaviour observed by Scaria and Shafer[6c] for ethidium with these polymers, where the binding was characterised as intercalative. At 270 nm, the effects are strongest for ethidium, weaker for propidium, and much weaker for the dimer, indicating that the different dyes have somewhat different couplings of their transitions with those of the surrounding base-pairs and -triplets. The positive contribution to the CD spectra at 270 nm in all cases, however, suggests that the binding geometries might not be substantially different from each other.

Thermal denaturation

The effects of these dyes on the stability of the duplex and triplex polynucleotides were examined by monitoring the temperature dependence of the CD spectra. The triplex, duplex, and single-stranded polymers have significantly different spectra (Fig. 9) so that transitions between them can be detected by monitoring at selected wavelengths. The effects of the phenanthridine dyes on the melting temperatures of the two polymers are collected in Table 2 and there are notable differences between the three dyes. All three dyes stabilize the duplex, as expected,[6c,7f,10d] propidium

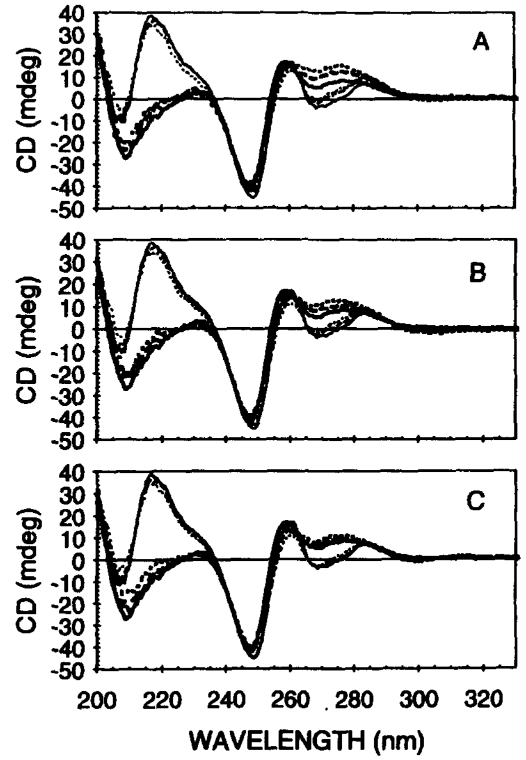

Figure 8. CD spectra of (A) ethidium bromide, (B) propidium iodide, and (C) ethidium homodimer bound to duplex and triplex. In each figure, (—) duplex; (———) P/D = 50, duplex; (·········) P/D = 25, duplex; (—) triplex; (———) P/D = 50, triplex; (·········) P/D = 25, triplex. [poly(dA·dT)] = 149 µM, [poly(dT*dA·dT)] = 150 µM; for P/D = 50, [EB] & [PrI] = 3 µM, [EB$_2$] = 1.5 µM; for P/D = 25, [EB] & [PrI] = 6 µM, [EB$_2$] = 3 µM; 20 mM phosphate/2 mM Mg^{2+} (pH 7) buffer.

and ethidium dimer being much more effective than ethidium itself. By contrast, although the monomeric dyes both provide moderate stabilisation of triplex, as anticipated from previous reports,[6c,7f] the dimer has no significant effect on triplex stability. Ethidium and propidium are known to have low binding constants with the poly(dA·dT) duplex and apparently bind more strongly to triplex, as characterized previously for ethidium.[6c] However, propidium with a long cationic sidechain does not show any significant extra stabilization of triplex compared with ethidium. Clearly, the dimer does not have a preference for binding to triplex although its binding is not inhibited relative to the duplex and it has no particular destabilizing influence on triplex: this may be related to some difficulty in accommodation of the linker even though the intercalating moieties may find the triplex structure a more favourable target for intercalation. Like classical minor groove binding drugs, a linker in the minor groove may have some kind of destabilizing effect on the third strand.[8]

It may be noted that in the 265–300 nm region the CD spectrum changes gradually between that of duplex and single strands, although at wavelengths below 265 nm the transition is quite sharp. This has been noted previously by several authors from CD spectra[30] but also from UV absorption changes with temperature[30c] and has been associated with a conformational change in the polymer. Iso-dichroic points as the CD changes with increasing temperature suggest a two-state transition which probably involves a disruption of the spine of hydration and a concomitant change of conformation of the base-pairs involving a weakening of the stacking interactions.[30] The UV variations have been sucessfully modelled in terms of a disruption of the structured water layers.[13a] It was also found that the intercalating antibiotic daunomycin binds more favourably to poly(dA·dT) as the pre-melting transition occurs, and it

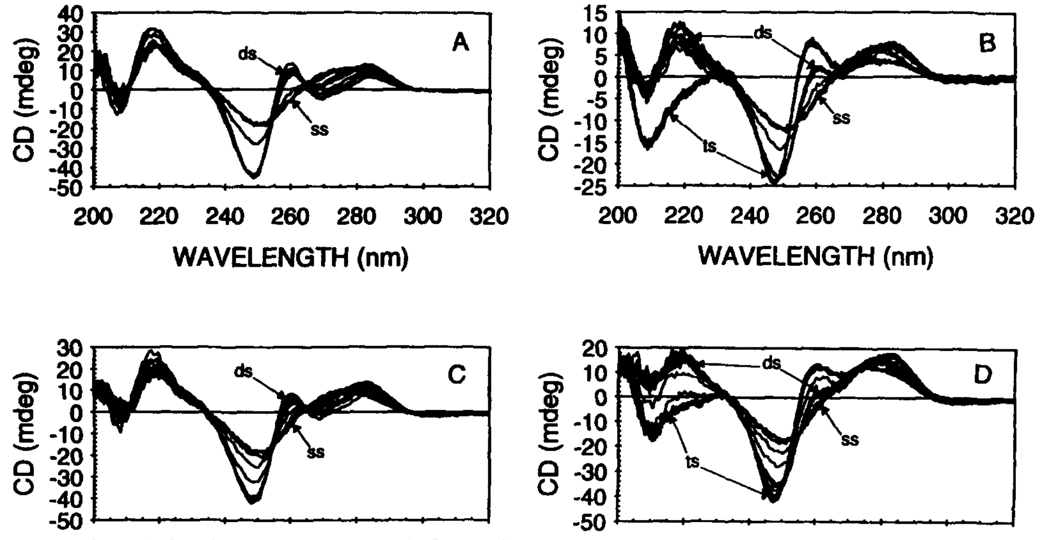

Figure 9. Change of CD as a function of temperature shown for (A) poly(dA·dT), (B) poly(dT*dA·dT), (C) the propidium iodide/poly(dA·dT) complex, and (D) the propidium iodide/poly(dT*dA·dT) complex. [PrI] = 6 µM, [poly(dA·dT)] = 149 µM, [poly(dT*dA·dT)] = 150 µM, 20 mM phosphate/2 mM Mg^{2+} (pH 7) buffer. Spectra corresponding to triplex (ts), duplex (ds), and single-stranded (ss) polynucleotides are indicated by the arrows: temperatures range between 20 and 90 °C and spectra show steps of *ca* 3–6 °C.

Table 2. Effect of phenanthridine dyes on the thermal denaturation of duplex and triplex non-alternating A·T structures

Dye	Duplex A·T[a]	Triplex T*A·T[b]	
	T_m (ds→ss)	T_{m1} (ts→ds)	T_{m2} (ds→ss)
none	63.6 °C	38.8 °C	64.4 °C
EB[c]	64.4 °C	41.9 °C	66.0 °C
PI[c]	71.5 °C	42.1 °C	71.1 °C
EB dimer[d]	68.7 °C	38.9 °C	71.5 °C

[a] Poly(dA)·poly(dT), 149 μM

[b] Poly(dT)*poly(dA)·poly(dT), 150 μM

[c] Ethidium bromide and propidium iodide, 6 μM ⇒ 12.4 base-pairs/dye and 8.3 base-triplets/dye.

[d] Ethidium dimer, 3 μM ⇒ 12.4 base-pairs/chromophore and 8.3 base-triplets/chromophore.

was suggested that the same two-state conformational transition could also be invoked to explain the cooperativity of antibiotic binding.[30c] This was in accord with the interpretation of Wilson and coworkers[29] for the cooperative behaviour and unusual thermodynamics of propidium binding to poly(dA·dT), that a conformational change of poly(dA·dT) was induced by the dye intercalation from a nonstandard to standard B-form which involved a disruption of the polynucleotide hydration layer.

This pre-melting is not observed for the triplex to duplex transition although it is still observed for the duplex to single stranded transition after triplex melting. The binding of the dyes does not cause this transition to disappear completely, at least not above P/D 25, suggesting that the conformational changes induced by dye intercalation occur very locally at the binding sites.

Summary and Conclusions

Monomer binding to A·T polynucleotides

Our LD^r data confirm previous reports that ethidium bromide is intercalated with both duplex poly(dA·dT) and triplex poly(dT*dA·dT). The spectroscopic properties of propidium iodide have been compared with those of ethidium in order to characterize the interactions of propidium with these polymers. The LD^r spectra for propidium with both the duplex and triplex AT polymers were consistent with intercalation, and the two dyes appeared to have very similar binding geometries with these polymers. Although the induced-CD of the dyes made contributions of different signs with duplex (negative) and triplex (positive), this probably reflects differences in the polynucleotide structures rather than different binding geometries.

Both dyes had similar effects on the melting of the triplex even though propidium tended to provide more stabilisation of the duplex than ethidium. This indicates that both dyes have higher affinity for the triplex than the duplex but, surprisingly, propidium does not have a greater stabilizing effect than ethidium. Thus, although the bulky side-chain of propidium does not effect the binding geometry, it does not provide any extra stabilization of the triplex compared to ethidium.

Dimer binding to A·T polynucleotides

LD^r spectra that were as negative as those of ethidium indicated that the dimer was bis-intercalated with both duplex and triplex. As with the monomers, the LD^r analysis suggested that the binding geometry of the dimer with triplex and duplex is similar, and is not greatly different from that with CT-DNA. The orientation of the dimer chromophores with each of these polymers is also similar to that of the monomers, as also observed with CT-DNA.

However, the dimer does not stabilise triplex even though it does stabilise the duplex. This may occur because the linker is not easily accommodated in the triplex structure, even though intercalation of the phenanthridine moieties is favourable. Since the triplex structure seems rather stiff, perhaps it cannot easily accommodate structural deformations which may be associated with dimer binding. Alternatively, a linker in the minor groove may act like classical minor groove binders in destabilising the third strand.

Experimental

Materials

Ethidium bromide, propidium iodide (both Sigma), and ethidium homodimer 1 (Molecular Probes) were used as received. Dye concentrations were determined using their reported extinction coefficients: ε_{480} = 5850 M^{-1} cm^{-1} (ethidium bromide[28]), ε_{493} = 5900 M^{-1} cm^{-1} (propidium iodide[28]), and ε_{492} = 8900 M^{-1} cm^{-1} (ethidium dimer, Molecular Probes). Highly polymerized calf thymus DNA (Type I, sodium salt, Sigma) and the synthetic polynucleotides poly(dA)·poly(dT) (349 base-pairs), poly(dT) (174 bases), and poly(dA) (349 bases) were purchased from Pharmacia and used as received. The concentrations of all nucleic acids are presented in terms of nucleotide concentration. Polynucleotide stock solutions were prepared in 5 mM phosphate (pH 7) and concentrations were determined using the following molar absorptivities: ε_{260} = 6600 M^{-1} cm^{-1} for CT-DNA,[31] ε_{260} = 6000 M^{-1} cm^{-1} for poly(dA)·poly(dT), ε_{264} = 8520 M^{-1} cm^{-1} for poly(dT), and ε_{257} = 8600 M^{-1} cm^{-1} for poly(dA) (Pharmacia P-L Biochemicals Inc., 1993

Catalogue). Measurements with DNA were carried out in 5 mM TBE buffer (2.5 mM Tris, 2.5 mM boric acid, 0.6 mM EDTA, pH 8.4). Other measurements were conducted at room temp. on solutions buffered with 20 mM phosphate (pH 7) containing 2 mM $MgCl_2$. Triplex was prepared by mixing a 2:1 molar ratio of poly(dT) and poly(dA) in this buffer, incubating with stirring at 80° for 30 min and slowly cooling the solution to room temperature. The formation of triplex was confirmed by its characteristic CD spectrum.[6c] Buffers were prepared using chemical grade salts and water purified in a Millipore Milli-Q purification system. Experimental samples were prepared using calibrated micropipettes.

Absorption

UV/vis absorption spectra were recorded on a Varian Cary 2300 spectrophotometer and data were normalized to a 1 cm pathlength.

Circular dichroism (CD)

CD spectra were measured on a Jasco J-720 instrument and data were normalized to a 1 cm pathlength. CD is the differential absorption of left and right circularly polarized light. The data are presented in mdeg as measured—they can be converted to absorbance units through division by the factor 32,980 mdeg. Although the dyes are achiral, they exhibit induced CD signals when bound to nucleic acids as a result of coupling of their electric-dipole allowed transitions with the transitions of the chirally organized nucleobases.[12]

Linear dichroism (LD)

LD spectra were measured on a Jasco J-500A spectropolarimeter, adapted as described previously with an Oxley prism to convert the incident circularly polarized light to linear.[32] Samples were oriented in a flow Couette cell using an outer rotating cylinder with shear gradients of 2500 s^{-1} for duplex and triplex AT samples and 2200 s^{-1} for DNA samples. The experimental pathlength was 1 mm and data were normalized to a 1 cm pathlength. LD is the differential absorption of light plane polarized parallel and perpendicular to the flow direction in the Couette cell. The magnitude of the LD signal depends on the degree of orientation of the sample as well as the molar absorptivity and concentration of the sample.[12a]

Reduced Linear Dichroism (LD^r)

The reduced dichroism (LD^r) is defined as

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

where A_{iso} is the absorption of the sample without orientation. It is related to the orientation of the chromophore as

$$LD^r(\lambda) = S\ (3/2)\ (3\langle\cos^2\alpha\rangle - 1).$$

where α represents the angle between the absorbing transition moment and the DNA helix axis. S is an orientation factor describing the degree of orientation of the DNA helix such that $S = 1$ for perfect orientation and $S = 0$ for random orientation. $\langle\cos^2\alpha\rangle$ represents an average over the angular distribution. S depends on the DNA stiffness and length, the flow rate, and the viscosity of the medium. S can be determined from the dichroism of DNA at 260 nm where the π–π^* transitions are polarized in the plane of the bases; previous studies have indicated an effective angle of 86° between the plane of the nucleobases and the helix axis.[12]

Thermal denaturation

Thermal denaturation of duplex and triplex AT polynucleotides was monitored by CD spectroscopy. Samples were heated in a 1 cm cell with small temperature increments (1–3 °C) and CD spectra recorded in the range 200–350 nm after 20 min equilibration. Melting profiles were obtained at several different wavelengths.

Acknowledgement

This work has been supported by the Swedish Natural Science Research Council (NFR).

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5. Abbreviations: CT-DNA, calf-thymus DNA; EB, ethidium bromide; EB_2, ethidium homodimer; PrI, propidium iodide; (*) represents Hoogsteen H-bonding between bases and (·) represents Watson–Crick H-bonding. Thus, poly(dA·dT) represents the Watson–Crick anti-parallel non-alternating duplex [poly(dA)]·[poly(dT)], and poly(dT*dA·dT) represents the triplex [poly(dT)]*[poly(dA)]·[poly(dT)] formed when the third strand binds in the duplex major groove parallel to the purine strand.

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(*Received in U.S.A.* 18 *October* 1994; *accepted* 19 *December* 1994)