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Ethidium Binding to Left-Handed (Z) DNAs Results in Regions of Right-Handed DNA at the Intercalation Site†

G. Terrance Walker, Michael P. Stone,‡ and Thomas R. Krugh*
Department of Chemistry, University of Rochester, Rochester, New York 14627
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ABSTRACT: The equilibrium binding of ethidium to the right-handed (B) and left-handed (Z) forms of poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) was investigated by optical and phase partition techniques. Ethidium binds to the polynucleotides in a noncooperative manner under B-form conditions, in sharp contrast to highly cooperative binding under Z-form conditions. Correlation of binding isotherms with circular dichroism (CD) data indicates that the cooperative binding of ethidium under Z-form conditions is associated with a sequential conversion of the polymer from a left-handed to a right-handed conformation. Determination of bound drug concentrations by various titration techniques and the measurement of circular dichroism spectra have enabled us to calculate the number of base pairs of left-handed DNA that adopt a right-handed conformation for each bound drug; 3-4 base pairs of left-handed poly(dG-dC)·poly(dG-dC) in 4.4 M NaCl switch to the right-handed form for each bound ethidium, while approximately 25 and 7 base pairs switch conformations for each bound ethidium in complexes with poly(dG-dC)·poly(dG-dC) in 40 μM $[Co(NH_3)_6]Cl_3$ and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 2 mM $MgCl_2$, respectively. The induced ellipticity at 320 nm for the ethidium–poly(dG-dC)·poly(dG-dC) complex in 4.4 M NaCl indicates that the right-handed regions are nearly saturated with ethidium even though the overall level of saturation is very low. The circular dichroism data indicate that ethidium intercalates to form a right-handed-bound drug region, even at low r values where the CD spectra show that the majority of the polymer is in a left-handed conformation.

The striking observation of the salt-induced cooperative conformational change of poly(dG-dC)·poly(dG-dC) from a right-handed helix to the left-handed (Z) helix (Pohl & Jovin, 1972; Wang et al., 1979) is a clear illustration that deoxyribonucleic acid (DNA)[1] can exist in a variety of conformations and that the structure has a pronounced effect on the function of DNA [for reviews, see Rich et al. (1984) and Wells et al. (1980), and references therein]. Poly(dG-dC)·poly(dG-dC) assumes a left-handed (Z-form) conformation in 4.4 M NaCl, to which ethidium does not bind efficiently until the ethidium concentration reaches approximately 20 μM (Pohl et al., 1972). The intercalation of ethidium is accompanied by a highly cooperative left- to right-handed conformational transition of the polynucleotide, as evidenced by circular dichroism spectroscopy. In subsequent experiments, van de Sande & Jovin (1982) studied the binding of ethidium, actinomycin D, and mithramycin to a condensed form of poly(dG-dC)·poly(dG-dC) in $MgCl_2$–ethanol which was designated as Z*-DNA. All three drugs were found to reverse the sedimentability of Z*-DNA, an observation consistent with the alteration of the conformation of poly(dG-dC)·poly(dG-dC) from a left-handed helix to a right-handed helix (but not a "B" form since the duplex is distorted to accommodate the ligands). Ethidium binding to left-handed poly(br^{8}dG-br^{5}dC)·poly(br^{8}dG-br^{5}dC) and poly(dG-br^{5}dC)·poly(dG-br^{5}dC) also is accompanied by a left- to right-handed transition (Moller et al., 1984; Rio & Leng, 1984). The binding of netropsin induces a left- to right-handed reversal of poly(dG-dC)·poly(dG-dC), whereas distamycin-3 is ineffective (Zimmer et al., 1983). Kinetic studies involving the inhibition of the salt-induced B to Z transition of poly(dG-dC)·poly(dG-dC) by proflavin, ethidium, actinomycin D, and bis(methidium)spermine have been interpreted in terms of inhibition of the nucleation and propagation of the Z form (Mirau & Kearns, 1983). Adriamycin and daunomycin also have been reported to effectively inhibit

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‡ Present address: Department of Chemistry, Vanderbilt University, Nashville, TN 37235.

[1] Abbreviations: DNA, deoxyribonucleic acid; Me_2SO, dimethyl sulfoxide; CD, circular dichroism.

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the B to Z transition of poly(dG-dC)·poly(dG-dC) (van Helden, 1983; Chaires, 1983) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (Chen et al., 1983).

The carcinogen *N*-acetoxy-2-(acetylamino)fluorene binds to poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) at the C(8) position of guanine and facilitates the formation of Z-DNA (Sage & Leng, 1980, 1981; Santella et al., 1981, 1982; Spodheim-Maurzot et al., 1982; Rio & Leng, 1984). Conversely, low levels of the carcinogen aflatoxin B_1 inhibit the formation of Z-form poly(dG-dC)·poly(dG-dC) under high-salt conditions (Nordheim et al., 1983). Analysis of the circular dichroism spectrum of an *N*-acetoxy-2-(acetylamino)fluorene-modified strand of d(CCACGCACC) in a 1:1 mixture with the complementary strand d(GGTGCGTGG) indicates that the carcinogen-modified duplex adopts a left-handed conformation even in low-salt buffers (Sanford & Krugh, 1985).

The goal of the present experiments is to provide a detailed analysis of the interaction of ethidium with poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) under conditions in which the polymers adopt a left-handed conformation in the absence of drug. An important feature of the present approach is the analysis of CD data in combination with equilibrium binding isotherms, which provide the bound drug to DNA base pair ratio (r), enabling us to characterize the binding of ethidium to poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) under B- and Z-form conditions. The equilibrium binding data and the CD spectra are considered in terms of the distribution of ethidium along the helix, the helical distance over which conformational alterations are propagated from the binding site, and the local conformation of the binding site. A subsequent paper will report on the interaction of actinomycin D and actinomine with these polynucleotides (Walker et al., 1985).

Materials and Methods

Ethidium bromide was purchased from Sigma and recrystallized from methanol. Poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) were purchased from P-L Biochemicals/Pharmacia and used without further purification. Similar results were obtained in selected experiments which were repeated with poly(dG-dC)·poly(dG-dC) that was pretreated with S1 nuclease (Bethesda Research Laboratories). A buffer consisting of 50 mM NaCl and 5 mM $H_2NC(CH_2OH)_3$ [tris(hydroxymethyl)aminomethane], pH 8, will be referred to as the 50 mM sodium buffer. For certain experiments, this buffer was used with the addition of varying amounts of either $MgCl_2$ or $[Co(NH_3)_6]Cl_3$; these buffers will be correspondingly referred to as the 0.2 mM magnesium buffer, the 2 mM magnesium buffer, and the 40 μM cobalt hexaammine buffer. The buffer used for the high-salt experiments consisted of 4.4 M NaCl, 10 mM Na_2HPO_4, and 10 mM Na_2EDTA, pH 7, and will be referred to as the 4.4 M sodium buffer. All buffers were passed through either a 0.22- or 0.45-μm Millipore filter to remove particulate matter. The molar extinction coefficients used for the compounds are listed in Table I [in the supplementary material (see paragraph at end of paper regarding supplementary material)]. Unless otherwise noted, all molar extinction coefficients for free ethidium and the polynucleotides were determined from a plot of absorbance vs. concentration for data obtained from dilution of a concentrated stock solution (dissolved in 50 mM sodium buffer) into the indicated solution. All polymer solutions are expressed in terms of base pair concentration.

The left-handed (Z) form of poly(dG-dC)·poly(dG-dC) in 4.4 M sodium buffer was established after a 1-h equilibration at room temperature. Poly(dG-dC)·poly(dG-dC) in 40 μM cobalt hexaammine buffer and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 2 mM magnesium buffer were heated at 37 °C for 20 min to induce the left-handed forms of the polynucleotides, although the B to Z conversion will occur at room temperature over several hours. The left-handed conformations of the polynucleotides were confirmed by CD spectroscopy. Poly(dG-dC)·poly(dG-dC) is known to aggregate in the presence of cobalt hexaammine under certain conditions (Widom & Baldwin, 1980); however, the polynucleotide does not aggregate in 40 μM cobalt hexaammine buffer in the base pair concentration range of 10–25 μM (Behe & Felsenfeld, 1981). Our comparison of CD spectra before and after centrifugation (16000g for 10 min) and the absence of any appreciable light scattering at wavelengths greater than 350 nm are consistent with the lack of aggregation of Z-form poly(dG-dC)·poly(dG-dC) under the prescribed cobalt hexaammine conditions. All DNA solutions were routinely inspected for aggregation by checking for anomalous absorbance and CD effects. Calf thymus DNA was prepared by the method of Muller & Crothers (1975) with the inclusion of a T1 RNase (Sigma) digestion step (100 units/mg of DNA for 30 min at 37 °C) and a proteinase K (Beckman) digestion step (25 μg/mg of DNA for 2 h at 37 °C).

Optical Titrations. Optical titrations were performed on a Varian Cary 219 UV/visible spectrophotometer with digital display at the instrument's ambient temperature (≈30 °C). Confirmation of either the B or Z form of the polynucleotide was obtained from CD spectra recorded prior to equilibration with drug. Successive aliquots from an ethidium solution were pipetted at 15-min intervals into a DNA solution contained in either a 5- or 10-cm path-length cell. A detailed description of the methods used to analyze the optical titration data is available in the supplementary material. The data from optical titrations involving a conformational transition may give rise to unusual shapes in the plots of the apparent extinction coefficient, ϵ_{app}, vs. the [drug]/[base pair] ratio which were used to estimate the extinction coefficients of the bound ligand (e.g., see Figure 1 in the supplementary material).

Ethidium concentrations were kept well below the concentration at which aggregation is observable in all experiments performed in the low-salt tris(hydroxymethyl)aminomethane buffers. Ethidium dimerization in 4.4 M sodium buffer was not readily apparent in the absorption measurements and is not thought to significantly affect the results because the free ethidium concentration remains nearly constant at 20 μM until ethidium approaches saturating binding levels.

Single-Cell Partition Analysis. The interaction of ethidium at low r values with the B and Z forms of poly(dG-dC)·poly(dG-dC) and with the B form of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) was examined by single-cell partition analysis [e.g., see Graves & Krugh (1983a)]. The organic phase was 1-nonanol (Kodak), while the aqueous phase consisted of the indicated buffer. The phases were equilibrated by vigorous shaking on a wrist-action shaker for 2 h at 22 °C. The phases were separated by low-speed centrifugation, and the fluorescence intensities of the aqueous and organic phases were measured (Perkin-Elmer/MPF44A). The organic phase was removed, and the aqueous phase was diluted with an equal volume of Me_2SO to dissociate the bound drug. The fluorescence of the aqueous/Me_2SO solution was then obtained. Values of r were determined from the fluorescence intensities of the aqueous (or aqueous/Me_2SO) phase, while the C_f value was determined from the organic phase fluorescence. Under the given conditions, there was no evidence that

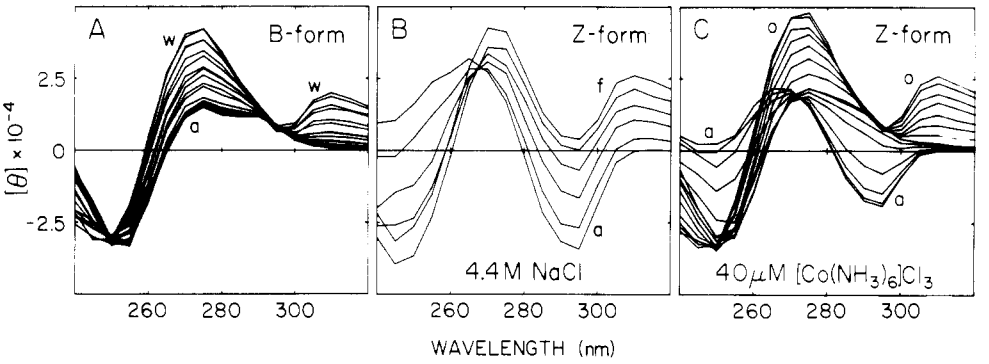

FIGURE 2: Circular dichroism spectra of ethidium–poly(dG-dC)·poly(dG-dC) solutions under B-form (A) and Z-form (B and C) conditions. In each panel, the initial spectrum of poly(dG-dC)·poly(dG-dC) in the absence of ethidium has been denoted with an "a", while the final spectrum of the titration, which corresponds to the largest [ethidium]/[base pair] ratio, has been denoted alphabetically corresponding to its ordinal position in the titration. The buffer conditions were 50 mM sodium buffer (A), 4.4 M sodium buffer (B), and 40 μM cobalt hexaammine (C). The initial base pair concentrations were 12.2 (A), 49.4 (B), and 11.2 μM (C). The [θ] values take into consideration dilution of the polynucleotide (<10%) during the titration.

Me_2SO might stabilize the Z form. Error bars have been included in the binding isotherms obtained by this method as an estimate of the random error associated with single cell analysis.

Circular Dichroism Measurements. All circular dichroism (CD) spectra were recorded in 1-, 2-, or 5-cm path-length cells at room temperature (≈23 °C) on a JASCO J-40 spectropolarimeter interfaced to a Digital PDP 11/34 computer. The spectra were recorded from 320 to 240 nm at 5-nm intervals with the data signal averaged, base line corrected, and transferred to a Tektronix 4051 graphics terminal and Tektronix 4662 plotter for analysis and plotting. Molar ellipticity, [θ], values were calculated in terms of the polynucleotide base pair concentration.

Δε values at 320 nm for ethidium bound to poly(dG-dC)·poly(dG-dC) were calculated according to eq 1 where θ

$$\Delta\epsilon_b^{320} = \epsilon_L - \epsilon_R = \theta/(32.98 C_b l) \qquad (1)$$

is ellipticity (degrees), C_b is the molar concentration of bound ethidium, and l is the path length (centimeters). The bound ethidium concentration was determined directly from absorption spectroscopy.

In all experiments except those performed in 4.4 M sodium buffer, CD titrations were performed concurrently with optical titrations, from which the r value of each sample was obtained directly. In the case of the 4.4 M sodium buffer experiments, independent samples for each ethidium to DNA ratio were prepared and allowed to equilibrate for 24 h at 22 °C prior to recording the CD spectra. Values of r were calculated from the input drug to DNA ratio in conjuction with binding isotherm data with an estimated error of less than 10%.

Results

Circular Dichroism Spectra Monitor the Conformation of the Polymers. The CD spectra of poly(dG-dC)·poly(dG-dC) and poly(dG-m^5dC)·poly(dG-m^5dC) undergo large changes when the polynucleotides switch from right-handed (B) to left-handed (Z) helices. For example, the positive ellipticity observed in the 290–300 nm region under B-form conditions changes to negative ellipticity under Z-form conditions (Pohl & Jovin, 1972). The exact shape of the initial Z-form CD spectra in the absence of ethidium depends upon the polynucleotide and the particular buffer conditions [i.e., 4.4 M NaCl, 40 μM $[Co(NH_3)_6]Cl_3$, or 2 mM $MgCl_2$]. The CD spectra of poly(dG-dC)·poly(dG-dC) and poly(dG-m^5dC)·poly(dG-m^5dC) have been previously reported; the corresponding spectra in Figures 2 and 3 agree with the previous

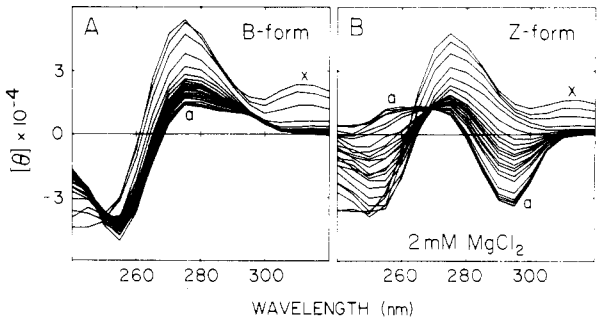

FIGURE 3: Circular dichroism spectra of ethidium–poly(dG-m^5dC)·poly(dG-m^5dC) solutions under B-form (A) and Z-form (B) conditions. In each panel, the initial spectrum of poly(dG-m^5dC)·poly(dG-m^5dC) in the absence of ethidium has been denoted with an "a" while the final spectrum of the titration, which corresponds to the largest [ethidium]/[base pair] ratio, has been denoted with an "x". The buffer conditions were 0.2 mM magnesium buffer (A) and 2 mM magnesium buffer (B). The initial base pair concentrations were 11.3 (A) and 12.0 μM (B). The [θ] values take into consideration dilution of the polynucleotide (<8%) during the titration.

data [e.g., see Pohl & Jovin (1972), Pohl et al. (1972), and Behe & Felsenfeld (1981)].

Circular Dichroism Spectra of Ethidium–Polynucleotide Complexes. The CD spectra from ethidium titrations with poly(dG-dC)·poly(dG-dC) and poly(dG-m^5dC)·poly(dG-m^5dC) under both B-form and Z-form conditions are shown in Figures 2 and 3. Upon the addition of ethidium to a solution of left-handed polynucleotide, the CD spectrum characteristic of Z-form DNA switches toward an ethidium–polynucleotide CD spectrum which is similar to that observed for a solution of the respective ethidium–polynucleotide complex under B-form conditions. The similarity of the ethidium-saturated polynucleotide CD spectra, regardless of the initial polynucleotide conformation, suggests a similar conformation for the complexes. Comparison of curves w (Figure 2A), f (Figure 2B), and o (Figure 2C) illustrates the similarities of the CD spectra of ethidium-saturated poly(dG-dC)·poly(dG-dC) in the three solutions. Correspondingly, the final CD spectra associated with poly(dG-m^5dC)·poly(dG-m^5dC) saturated with ethidium under both B-form and Z-form conditions agree quite closely (curves x in panels A and B of Figure 3, respectively). Although similar in shape and magnitude, the spectra of the corresponding drug-saturated polynucleotides are not identical, which presumably reflects slight variations in the conformations of the complexes in the different solutions. Variations in the shape of CD spectra of ethidium–DNA complexes due to differences in buffer conditions are precedent in the liter-

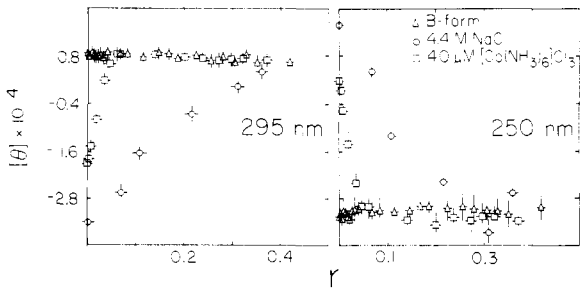

FIGURE 4: Molar ellipticity values as a function of r for ethidium titrations with poly(dG-dC)·poly(dG-dC) under B- and Z-form conditions. The data from Figure 3 are plotted at 295 and 250 nm. The buffer conditions were 50 mM sodium buffer (B form) (△), 4.4 M sodium buffer (Z form) (○), and 40 μM cobalt hexaammine buffer (Z form) (□).

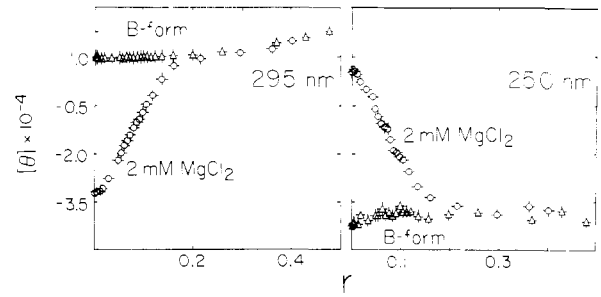

FIGURE 5: Molar ellipticity as a function of r for ethidium titrations with poly(dG-m^{5}dC)·poly(dG-m^{5}dC) under B- and Z-form conditions. The data from Figure 4 are plotted at 295 and 250 nm. The buffer conditions were 0.2 mM magnesium buffer (B form) (△) and 2 mM magnesium buffer (Z form) (○).

ature. For example, Dahl et al. (1982) have noted that the magnitude of the 307-nm band of ethidium–calf thymus DNA complexes is a function of the counterion concentration (0.45–86 mM Na^+).

Addition of ethidium to Z-form poly(dG-dC)·poly(dG-dC) in 4.4 M sodium buffer does not alter the CD spectrum of the polynucleotide until the total ethidium concentration exceeds the ~20 μM concentration required for efficient binding, as previously reported by Pohl et al. (1972). [The total ethidium concentration in curve b (Figure 2B) was 20.3 μM.] This behavior stands in sharp contrast to that observed for the polymers under B-form conditions where the fraction of bound drug reaches a maximum at low ratios of ethidium/base pairs. An experiment was performed with calf thymus DNA in 4.4 M NaCl where changes in the visible absorption spectrum and fluorescence intensity of ethidium were apparent for a 0.85 μM sample of ethidium in 4.4 M sodium buffer after addition of calf thymus DNA (data not shown), which confirms that the difference in the B- and Z-form experiments is the conformational transition of the polynucleotide. The CD spectrum of B-form poly(dG-dC)·poly(dG-dC) exhibits isoelliptic points at 250 and 295 nm upon ethidium binding (Figure 2A). These wavelengths display large changes in ellipticity when the polynucleotide undergoes a B to Z transition (Figure 2) and provide convenient wavelengths to monitor the left- to right-handed transition of the polynucleotide as ethidium binds, as will be shown below. In addition, the fluorescence-detected circular dichroism spectra of these systems (Lamos et al., 1986) and ethidium–dinucleotide systems (Dahl et al., 1982) show that the ellipticity of bound ethidium goes through a minimum at ~250 and ~290 nm. The CD data at other wavelengths support the conclusions presented in this paper, but the contribution to the observed ellipticity from bound ethidium makes the data analysis less straightforward than at 250 and 295 nm.

Only 3 or 4 Base Pairs of Left-Handed Poly(dG-dC)·Poly(dG-dC) in 4.4 M NaCl Switch to a Right-Handed Form per Bound Ethidium. Figure 4 contains plots of molar ellipticity, $[\theta]$, at 250 and 295 nm as a function of r for the ethidium titrations of poly(dG-dC)·poly(dG-dC) under B- and Z-form conditions. Error bars have been included in Figures 4 and 5 as an estimate of the random error associated with the values of $[\theta]$ and r. An ethidium titration of Z-form poly(dG-dC)·poly(dG-dC) in 4.4 M sodium buffer produces an approximately linear change in $[\theta]$ at 250 and 295 nm as a function of r, converging with B-form data at $r \approx 0.35$. From the reciprocal of this r value, and allowing that the line is slightly curved, we estimate that 3 or 4 base pairs of Z-form poly(dG-dC)·poly(dG-dC) in 4.4 M sodium buffer switch to a right-handed form per bound drug. The 250- and 295-nm data (Figure 4) provide convincing evidence that the binding of ethidium to poly(dG-dC)·poly(dG-dC) in 4.4 M sodium buffer distorts only the immediate region of the binding site. Low level binding of ethidium does not induce extensive alterations in the CD spectrum of the left-handed polynucleotide as might be expected from long-range allosteric effects. Nearly saturating levels of bound ethidium are required before the ellipticity at 295 and 250 nm assumes the values of right-handed DNA.

Approximately 25 Base Pairs of Left-Handed Poly(dG-dC)·Poly(dG-dC) in 40 μM Cobalt Hexaammine Buffer Switch to a Right-Handed Form per Bound Ethidium. Addition of ethidium to Z-form poly(dG-dC)·poly(dG-dC) in 40 μM cobalt hexaammine buffer results in very steep $[\theta]$ vs. r plots which converge with the B-form data at $r \approx 0.04$, indicating that the left- to right-handed conversion is complete at a level of one ethidium bound per 25 ± 5 base pairs (Figure 4). The CD data clearly indicates that the effect of the bound ethidium goes beyond the immediate binding site in cobalt hexaammine buffer. The linearity of the data at 295 and 255 nm suggests that the polymer consists of right-handed and left-handed regions without indication of intermediates with alternate conformations. For example, we find no evidence to suggest a B–Z junction of 10–12 base pairs in length with a gradual transition between the two conformations.

Approximately 7 Base Pairs of Left-handed Poly(dG-m^{5}dC)·Poly(dG-m^{5}dC) in 2 mM $MgCl_2$ Buffer Switch to a Right-Handed Form per Bound Ethidium. $[\theta]$ vs. r plots are presented in Figure 5 for ethidium binding to poly(dG-m^{5}dC)·poly(dG-m^{5}dC). For the titration of Z-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (2 mM magnesium buffer), the change in molar ellipticity, $[\theta]$, at 250 and 295 nm indicates that the left- to right-handed conversion is complete when ethidium is bound at a level of one drug per ~7 base pairs. The linear conversion of the CD data is indicative of a progressive conversion of left-handed poly(dG-m^{5}dC)·poly(dG-m^{5}dC) to a right-handed structure; there is no indication of the formation of intermediate forms of alternate conformation.

$\Delta\epsilon_b^{320}$ Values Reflect Local Saturation. The observed ellipticity above 315 nm arises solely from ethidium bound to the polynucleotide. The ellipticity at 320 nm exhibits a markedly different behavior in the B-form titration when compared to the titration in 4.4 M NaCl. Note the negligible ellipticity in the 315–320-nm region during the early part of the titration under B-form conditions (Figure 2A), in contrast to the significant ellipticity observed in the titration under Z-form conditions (Figure 2B). A plot of $\epsilon_L - \epsilon_R$ at 320 nm expressed in terms of the concentration of bound ligand (i.e., $\Delta\epsilon_b^{320}$, Figure 6) graphically illustrates the difference between the two sets of data at 320 nm. Previous experiments have

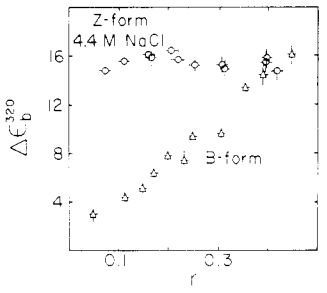

FIGURE 6: Plot of $\epsilon_L - \epsilon_R$ at 320 nm as a function of r for ethidium titrations with poly(dG-dC)·poly(dG-dC) under B-form (Δ) and Z-form (O) conditions. The $\Delta\epsilon_b^{320}$ values were calculated from the concentration of bound ethidium. The B-form buffer was 50 mM sodium while the Z-form buffer was 4.4 M sodium. The initial base pair concentrations were 89.7 and 116.4 μM, respectively, under the B- and Z-form conditions while the respective total ethidium concentration ranges were 4.84–60.6 and 26.1–83.7 μM. The base pair concentrations decreased less than 8% during the experiment as a result of dilution.

shown that the magnitude of $\Delta\epsilon_b^{320}$ for ethidium titrations of various DNAs is a function of the degree of helical saturation [e.g., see Dalgeish et al. (1971), Aktipis & Martz (1974), Houssier et al. (1974), and Dahl et al. (1982), and references therein]. It has been suggested that the r value dependence of $\Delta\epsilon_b$ in the 310–350-nm region results from either dye–dye interactions or a change in the structure (asymmetry) of the binding site [e.g., see Dahl et al. (1982), and references therein). Although we favor the interpretation in which the structure of the site is a function of the degree of saturation, either interpretation of the r value dependence of $\Delta\epsilon_b^{320}$ leads to the conclusion that the magnitude of $\Delta\epsilon_b^{320}$ reaches a maximum value as the helix is saturated.

For the B-form titration data, the values of $\Delta\epsilon_b^{320}$ increase as a function of r, reaching a maximum value of ~16 as the helix is saturated (Figure 6). This observation is consistent with data for ethidium binding to a number of native DNAs [e.g., see Aktipis & Martz (1974) and Dalgleish et al. (1971)]. In contrast, data from the high-salt (4.4 M NaCl) titration exhibit $\Delta\epsilon_b^{320}$ values of ~16 at all values of r (Figure 6). It should be noted that the 310–340-nm ellipticity associated with ethidium binding to calf thymus DNA in 5 M NaCl (Aktipis & Kindelis, 1973) displays an r value dependence analogous to the B-form data presented in Figure 6. We therefore assert that the unique behavior of the Z-form titration data in Figure 6 indicates that ethidium intercalates in clusters to form regions of ethidium-saturated poly(dG-dC)·poly(dG-dC) in 4.4 M NaCl. We propose that, at all r values, the clustered regions of bound ethidium adopt a conformation equivalent to a fully saturated helix, even though the overall degree of helix saturation may be quite low.

The experiments in 40 μM cobalt hexaammine buffer were limited to maximum DNA base pair concentration of ~25 μM (as used for the experiments in Figure 2C) to avoid condensation or precipitation of the polynucleotide. Consequently, we were unable to obtain values of $\Delta\epsilon_b^{320}$ for the titration in 40 μM cobalt hexaammine buffer that were as reliable as those obtained in 4.4 M sodium buffer or 50 mM sodium buffer where high DNA concentrations are allowable. The CD spectra associated with the ethidium titration of left-handed poly(dG-dC)·poly(dG-dC) in 40 μM cobalt hexaammine buffer do not display any appreciable ellipticity at 320 nm, from which we conclude that there is no appreciable clustering of the ethidium at low levels of ethidium saturation of the polymer ($r < 0.1$), which is consistent with the observation that 25 ± 5 base pairs of the polymer adopt a right-handed conformation for each bound ethidium.

Isoelliptical Points. The ethidium-induced changes in the CD spectra proceed in a systematic manner as a function of the bound ethidium. The CD spectra from an ethidium titration of right-handed poly(dG-dC)·poly(dG-dC) (Figure 2A) display isoelliptical points at ~250 and ~295 nm. (To facilitate the time averaging used to record these spectra, the data were collected at 5-nm intervals; the uncertainty in the wavelength of an isoelliptical point is estimated to be ±3 nm.) Isoelliptical points at ~250 and ~295 nm are observed also for ethidium binding to poly(dG-dC)·poly(dG-dC) in 40 μM cobalt hexaammine buffer (Z-form conditions) for $r > 0.04$, which is the minimum r value for which the conversion of the polymer to a right-handed form is complete [curves g–o (Figure 2C)]. The ellipticity in the ethidium titration of Z-form poly(dG-dC)·poly(dG-dC) in 4.4 M sodium buffer does not approach the values observed for the isoelliptical points at ~250 and ~295 nm until saturating limits of drug are obtained (Figure 2B). In the case of B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 0.2 mM magnesium buffer, an isoelliptical point at ~250 nm is observed over the entire titration [curves a–x (Figure 3A)] while an isoelliptical point is observed at ~295 nm for $r < 0.15$ [curves a–t (Figure 3A)]. Under Z-form conditions (2 mM magnesium buffer) an isoelliptical point at ~250 nm is observed for $r > 0.15$, the minimum r value for which the left-handed poly(dG-m^{5}dC)·poly(dG-m^{5}dC) has switched to a right-handed conformation [curves r–x (Figure 3B)]; the remainder of the titration consists of ethidium binding to the right-handed form of the polymer which gives rise to the same isoelliptic point as observed for the titration under B-form conditions.

Isoelliptical points are also observed in the 260–280-nm region in the spectra associated with ethidium titrations of left-handed polynucleotides. For the experiments with ethidium binding to poly(dG-dC)·poly(dG-dC) in 4.4 M sodium buffer, an isoelliptical point at ~268 nm is observed throughout the titration except for the final spectrum which corresponds to nearly saturating levels of ethidium (Figure 2B). The CD spectra associated with the reversal of left-handed poly(dG-dC)·poly(dG-dC) in 40 μM cobalt hexaammine buffer ($r < 0.04$) display an isoelliptical point at ~272 nm [curves a–h (Figure 2C)]. Likewise, an isoelliptical point at ~269 nm is observed for the left- to right-handed conversion of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 2 mM magnesium buffer [curves a–r (Figure 3B)]. The appearance of these isoelliptical points is consistent with a systematic left- to right-handed conversion of the polynucleotides without formation of intermediate structures of alternate conformation, although we note that contributions from minor species would go undetected.

Scatchard Plots. Scatchard (1949) analysis of drug binding data consists of a plot of r/C_f vs. r, where r is the ratio of the bound drug to DNA base pair concentration and C_f is the free drug concentration. Scatchard (1949) analysis of ligand binding to a polynucleotide substrate has been extensively reviewed by Crothers (1968) and McGhee & von Hippel (1974). A positive slope of the data in a Scatchard plot is indicative of positive cooperative binding. The reciprocal of the intercept of the r axis provides an estimation of the site-exclusion parameter, which is defined as the number of DNA base pairs per bound ligand at saturating levels of drug.

The magnitudes of the r/C_f values observed in any binding isotherm are particularly sensitive to accurate determination of the free drug concentration, C_f. The majority of the uncertainty in C_f is associated with determination of the dif-

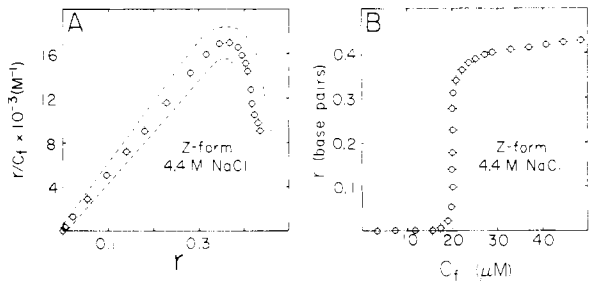

FIGURE 7: Equilibrium binding isotherm for the interaction of ethidium with poly(dG-dC)·poly(dG-dC) under Z-form conditions (4.4 M sodium buffer). A Scatchard plot is shown in panel A, and a plot of r vs. the free drug concentration, C_f, is shown in panel B. The dashed lines project the limits of the Scatchard plot corresponding to a ±5% uncertainty in the molar extinction coefficient of bound ethidium. The initial base pair concentration of 49.9 μM decreased less than 5% as a result of dilution.

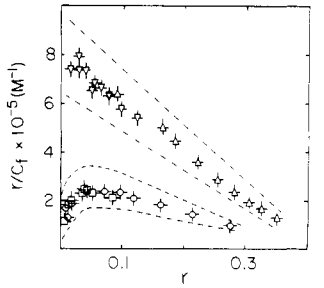

FIGURE 8: Scatchard analyses of the binding of ethidium to poly(dG-dC)·poly(dG-dC) under B-form (▽, △) and Z-form (□, ○) conditions. The B-form buffer was 50 mM sodium while the Z-form buffer was 40 μM cobalt hexaammine. The dot–dash lines (- · -) and the dashed lines (- - -) project the limits of the binding isotherms corresponding to a ±5% uncertainty in the molar extinction coefficient of bound ethidium for the B- and Z-form titrations, respectively. Data obtained by the single-cell partition analysis are represented by the symbols (▽) and (□), for which the base pair concentration range was 10.1–11.4 μM. Data obtained by optical titration methods are represented by the symbols (△) and (○), for which the initial base pair concentrations were 11.2 and 12.0 μM, respectively, under B- and Z-form conditions. The base pair concentrations decreased by less than 6% during the optical titrations as a result of dilution.

ference between the molar extinction coefficients of free and bound drug ($\epsilon_f - \epsilon_b$). Error associated with $\epsilon_f - \epsilon_b$ changes the magnitude of the r/C_f values but does not significantly change the overall shape of the binding isotherm in the present experiments. For each Scatchard plot obtained by optical titration methods, we have traced out two limiting binding isotherms based upon the estimated error in $\epsilon_f - \epsilon_b$. The general shape of the binding isotherm is represented by the actual data points while the absolute magnitude of the binding isotherm is projected to lie within the limits of the two traced lines. The uncertainty in the value of $\epsilon_f - \epsilon_b$ acts, to a first approximation, as a scaling factor in the sense that the data points shift in concert as the value of $\epsilon_f - \epsilon_b$ is changed. Errors associated with pipetting and instrument readings contribute random error to the binding isotherms as represented by error bars.

Equilibrium Binding of Ethidium to Poly(dG-dC)·Poly(dG-dC) in 4.4 M Sodium Buffer. A Scatchard plot of the optical titration data for the interaction of ethidium with left-handed poly(dG-dC)·poly(dG-dC) in 4.4 M sodium buffer is shown in Figure 7. The r/C_f values extrapolate to the origin because only a small amount of ethidium binds before the concentration of free drug reaches 20 μM. A plot of r vs. C_f (Figure 7B) illustrates that the free drug concentration remains effectively constant at 20 μM until the polynucleotide is more than 80% saturated [as previously reported by Pohl et al. (1972)]. This nearly constant free drug concentration for $0.02 < r < 0.35$ results in the unusual shape of the Scatchard plot. At r values greater than 0.35, a steep negative slope is observed with an apparent intercept on the r axis at $r \approx 0.50$ corresponding to a site-exclusion parameter of 2 base pairs per bound drug, which is identical with the values obtained under B-form conditions. The ellipticity data at 250 and 295 nm, the constant value for $\Delta\epsilon_b^{320}$ as a function of r, and the shape of the binding isotherm are in agreement with the interpretation that only limited regions (3–4 base pairs) of the Z-form polymer adopt a right-handed conformation per bound ethidium, that the ligands are bound in clusters, and that there are no long-range allosteric transitions occurring.

Equilibrium Binding of Ethidium to Poly(dG-dC)·Poly(dG-dC) in 40 μM Cobalt Hexaammine Buffer. Ethidium binding to poly(dG-dC)·poly(dG-dC) in 40 μM cobalt hexaammine buffer was examined by single-cell partition analysis and optical titration methods. As shown in Figure 8, excellent agreement was obtained between the two techniques. At low r values, ethidium binds in a positive cooperative manner to poly(dG-dC)·poly(dG-dC) in 40 μM cobalt hexaammine buffer, as evidenced by the positive slope in the Scatchard plot for $0 < r < 0.05$. At r values greater than 0.05, the isotherm may be represented as a neighbor exclusion type binding isotherm with a site-exclusion parameter of 2 base pairs per bound drug. The CD data indicate that the left-handed polynucleotide has switched to a right-handed form by $r \approx 0.05$. At this point in the binding isotherm, the free ethidium concentration is 0.2 μM, which is 2 orders of magnitude lower than the 20 μM required for complete reversal of Z-form poly(dG-dC)·poly(dG-dC) in 4.4 M sodium buffer.

The equilibrium binding data may be compared to the CD data. Recall that the ellipticity data at 295 nm change rapidly from values characteristic of the left-handed Z form to values similar to the right-handed form (Figure 4) and that above $r = 0.04$ the CD data were equivalent to the data from a B-form titration. Likewise, the Scatchard plot reaches a maximum at $r \approx 0.04$ and takes on a shape characteristic of ethidium binding to B-form polymer. Thus, all data indicate that ethidium binding results in an allosteric transition of left-handed poly(dG-dC)·poly(dG-dC) in 40 μM cobalt hexaammine buffer.

Equilibrium Binding of Ethidium to Poly(dG-dC)·Poly(dG-dC) in 50 mM Sodium Buffer. Ethidium binding to B-form poly(dG-dC)·poly(dG-dC) in 50 mM sodium buffer was examined by single-cell partition analysis and optical titration methods as shown in Figure 8. Excellent agreement was obtained between the two techniques. Ethidium binding to poly(dG-dC)·poly(dG-dC) under these B-form conditions results in a Scatchard plot which is indicative of noncooperative neighbor-exclusion binding. The apparent binding constant, K_{app}, and site-exclusion parameter, n, are approximately 8.3×10^5 M^{-1} and 2 base pairs per bound drug, respectively, in agreement with previously published data [e.g., see Winkle et al. (1982) and Bresloff & Crothers (1981), and references cited therein].

Ethidium Binding to Poly(dG-m^{5}dC)·Poly(dG-m^{5}dC) in 2 mM Magnesium Buffer. The binding of ethidium to poly(dG-m^{5}dC)·poly(dG-m^{5}dC) under Z-form conditions (2 mM magnesium buffer) is characterized by a binding isotherm with a positive slope for $0 < r < 0.15$ (Figure 9). A plot of r vs. C_f (inset of Figure 9) shows that the free drug concentration remains relatively constant during the portion of the experiment in which the conformation of the left-handed polynucleotide is changing. At r values greater than 0.15, which

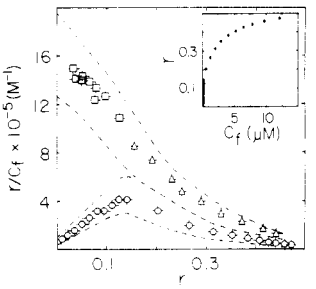

FIGURE 9: Scatchard analyses of ethidium binding to poly(dG-m^{5}dC)·poly(dG-m^{5}dC) under B-form (□, △) and Z-form (○) conditions. The B-form buffer was 0.2 mM mangesium while the Z-form buffer was 2 mM magnesium. The dot–dash lines (·-·) and the dashed lines (- - -) project the limits of the binding isotherms corresponding to a ±5% uncertainty in the molar extinction coefficient of bound ethidium for the B- and Z-form titrations, respectively. The inset represents a plot of r vs. the free ethidium concentration (C_f) for the Z-form titration. Data obtained by the single-cell partition analysis are represented by the symbol (□), for which the base pair concentration was 12.0 μM. The data represented by the symbols (△) and (○) were obtained by optical titration methods for which the initial base pair concentrations were 25.9 and 19.9 μM, respectively, for the B- and Z-form titrations. The base pair concentrations decreased by less than 20% during the optical titrations as a result of dilution.

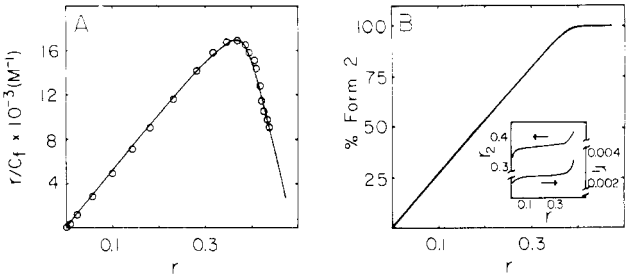

FIGURE 11: Calculated binding isotherm. (A) Comparison of the experimental data for ethidium binding to poly(dG-dC)·poly(dG-dC) in 4.4 M sodium buffer (○) and a binding isotherm (—) calculated by using the theory for induced allosteric changes in the conformation of DNA (Bresloff & Crothers, 1981). The parameters used in the calculation were $n_1 = 2$, $n_2 = 2$, $K_2/K_1 = 300$, $\tau_1 = 1$, $\tau_2 = 3$, $S = 0.56$, and $\sigma = 0.013$. (B) Calculated percent of the DNA base pairs in form 2, indicating the structural transition inferred from the shape of the experimental binding isotherm. The inset shows the r values for form 2, r_2, and form 1, r_1, as a function of the overall r value for the polymer. Note the separate scales for r_2 and r_1.

represents the point at which the left- to right-handed conversion is complete as judged by the ellipticity and isoelliptic points in the CD spectra, the binding isotherm is indicative of neighbor-exclusion binding with a site-exclusion parameter of 2 base pairs per bound drug.

Ethidium Binding to Poly(dG-m^{5}dC)·Poly(dG-m^{5}dC) in 0.2 mM Magnesium Buffer. The binding of ethidium to the B form of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 0.2 mM magnesium buffer was examined by single-cell partition analysis and optical titration methods, as shown in Figure 9. Ethidium binds to B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in a noncooperative neighbor-exclusion manner with an apparent binding constant, K_{app}, and site-exclusion parameter of approximately 16×10^5 M^{-1} and 2 base pairs per drug, respectively. A comparison of the two binding isotherms in Figure 9 illustrates the effect of the B to Z transition of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) on the ethidium binding isotherm.

Effect of Cobalt Hexaammine on the Binding of Ethidium to Calf Thymus DNA. The binding isotherms from ethidium titrations of calf thymus DNA in 50 mM sodium buffer, both in the absence and in the presence of 40 μM $[Co(NH_3)_6]Cl_3$, are shown in Figure 10 (in the supplementary material). Both binding isotherms may be represented, within experimental error, as nearest-neighbor exclusion isotherms. The K_{app} values are 4.5 (±0.5) × 10^5 M^{-1} and 2.0 (±0.5) × 10^5 M^{-1} in the absence and presence of 40 μM $[Co(NH_3)_6]Cl_3$, respectively, which corresponds to a 2–3-fold decrease in binding affinity to calf thymus DNA upon addition of 40 μM $[Co(NH_3)_6]Cl_3$ to the 50 mM sodium buffer. The effect of cobalt hexaammine is not surprising since it exhibits site-specific binding and plays an important role in the structure and condensation of DNA [e.g., see Widom & Baldwin (1980), Behe & Felsenfeld (1981), Chen et al. (1983), and Gessner et al. (1985)].

Analysis of the Binding Isotherms by an Allosteric Binding Model. We have analyzed the equilibrium binding isotherm for the interaction of ethidium with poly(dG-dC)·poly(dG-dC) in 4.4 M NaCl using the model for induced allosteric changes in DNA which was published by Crothers and co-workers (Dattagupta et al., 1980; Bresloff & Crothers, 1981). The readers are referred to these papers for the development of the model. Dr. Donald M. Crothers kindly provided a copy of the computer program used for these analyses. In brief, the model allows two structures for DNA, called form 1 and form 2. The binding characteristics of the ligand to the two forms are respectively described by K_1 and K_2, the intrinsic binding constants, n_1 and n_2, the neighbor-exclusion ranges, and τ_1 and τ_2, the cooperativity parameters, where $K\tau$ is the binding constant for a drug bound the minimum distance from another drug molecule. Form 1 represents left-handed DNA while form 2 represents right-handed DNA. The unusual shape of the binding isotherm for the 4.4 M NaCl titration of poly(dG-dC)·poly(dG-dC) with ethidium is well reproduced by the calculated curve (Figure 11A); the slight differences between the experimental and calculated curves at any point are well within experimental uncertainties. The calculated binding isotherm was computed wth $\tau_2 = 3$. The data also may be reproduced, to within experimental error, without invoking cooperative binding of ethidium to form 2 DNA, although the match between the experimental and calculated data is not as good as the calculated curve shown in Figure 11A. No attempt was made to perform a detailed nonlinear regression to obtain the "best-fit" parameters because the eight independent parameters preclude the usefulness of this effort. Independent experiments must be designed to determine whether ethidium binds to form 2 (right-handed) DNA with positive cooperativity. The conclusions are the same from the analysis of either calculated isotherm and provide support for the conclusions from the analysis of the experimental data presented above. For example, the experimental r/C_f values reach a maximum at $r \approx 0.36$ (Figure 11A), and at this r value the calculated binding isotherm indicates that 94% of the polymer is the right-handed form 2 DNA (Figure 11B). Note that by $r \approx 0.36$ the ellipticity at 295 nm and/or 250 nm coincides with the B-form titration data (Figure 4). Thus, the analysis of the binding data in terms of an induced allosteric transition leads to the same conclusions as the interpretation of the CD data.

The calculated data provide another basis for confirming the interpretation of the $\Delta\epsilon_b^{320}$ data (Figure 6) in terms of the formation of regions of right-handed polymer to which one ethidium is intercalated for every three base pairs, even at the lowest levels of binding (i.e., ethidium binds in clusters). For each point on the binding isotherm, the computer program calculates the r value for form 1 and form 2 DNA, r_1 and r_2, as well as the r value for the entire polymer [$r = r_1$(fraction of form 1) + r_2(fraction of form 2)]. From the inset in Figure 11B, we note that r_2 equals 0.35 even though the overall r value

is only 0.02, illustrating that the form 2 (right-handed) DNA is nearly saturated with ethidium, even at low levels of overall binding of ethidium to the polymer.

The computer program also calculates the average length of the two forms of the polymer at each point in the binding isotherm. As expected, the average length of form 1 decreases while the average length of form 2 increases. At $r = 0.1$ the average length of form 1 is 195 base pairs while the form 2 regions have an average length of 70 base pairs. Both regions have an average length of 115 base pairs at $r = 0.19$. At $r = 0.36$ the average length of form 1 is 30 base pairs while form 2 has an average length of 480 base pairs. The theoretical binding isotherm is consistent with the analysis of the experimental data in terms of the sequential conversion of the polymer from a left-handed (Z) form to a right-handed (B) form. The observation that Z* DNA supports the binding of ethidium (van de Sande & Jovin, 1982) may be interpreted as an indication of the sequential conversion of the polymer conformation, as opposed to the all-or-none model. The NMR data of Shafer et al. (1984) also are consistent with a sequential conversion model.

We have not extended the calculation of the binding isotherms to the interaction of ethidium with the left-handed forms stabilized by $[Co(NH_3)_6]^{3+}$ or Mg^{2+} because the possibility of site binding to the polymers of the Z-stabilizing cations is not a part of the allosteric transition model of Bresloff & Crothers (1981).

Discussion

The analysis of the present data suggests that left-handed and right-handed conformations of the polymer may coexist on the same strand. It is not surprising that sodium counterions at a concentration of 4.4 M are able to dampen over a very short distance the electrostatic interactions that accompany the reversal of the helix. However, when 40 μM $[Co(NH_3)_6]^{3+}$ is used to stabilize the left-handed conformation of poly(dG-dC)·poly(dG-dC), between 20 and 30 base pairs switch to a right-handed form for each bound ethidium; we suggest that this longer range effect results from the lower intrinsic stability of the $[Co(NH_3)_6]^{3+}$ stabilized Z form, the low counterion concentration in this solution, and the nature of the $[Co(NH_3)_6]^{3+}$ interaction with DNA. For the 2 mM magnesium-stabilized Z form of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) approximately 7 base pairs switch from a left-handed to a right-handed conformation for each bound ethidium; this value is intermediate between the high-salt and cobalt hexaammine values and presumably reflects the presence of millimolar concentrations of the divalent cation. The ability of the polymers to support both left-handed and right-handed (bound drug) conformations on the same duplex suggests that the activity of enzymes on left-handed templates [e.g., see Butzow et al. (1984), Durand et al. (1983), and van de Sande & Jovin (1982)] may be associated with the induction of local right-handed regions of the polymer.

The CD spectra suggest that the local environment of the polynucleotide at the binding site is a right-handed helix (vide infra), which implies that the intercalation of ethidium requires the nucleation of two B′–Z interfaces (B′ is used here to refer to right-handed regions of the polynucleotide containing bound drug). The 20 μM concentration required for efficient binding in 4.4 M sodium buffer may be conceptualized as the concentration required to nucleate a region of the polynucleotide into a right-handed (drug-bound) conformation. Since the binding affinity of ethidium decreases with increasing cation concentration [see, for example, LePecq & Paoletti (1967), Aktipis & Kindelis (1973), Houssier et al. (1974), Record et al. (1978), Manning (1978), and Wilson & Lopp (1979)], and since the relative stability of the B and Z forms of poly(dG-dC)·poly(dG-dC) is salt dependent, the shape of the r vs. C_f curve is expected to depend strongly on the concentration of Na^+ ions; this has been observed in recent experiments (T. R. Krugh et al., unpublished data). The concentration of free ethidium remains constant at 20 μM over the $0 < r < 0.4$ range of the poly(dG-dC)·poly(dG-dC) titration in 4.4 M sodium buffer which may reflect that the number of B′–Z interfaces remains relatively constant during the left- to right-handed conversion of the helix.

Ethidium binds to most native DNAs with nearest-neighbor exclusion and with very little cooperativity (Bresloff & Crothers, 1981); *Escherichia coli* DNA is an exception in that cooperative binding of ethidium is observed at low r values (Winkle et al., 1982). Ethidium has been shown to stabilize oligonucleotides containing a bulged uracil base (Lee & Tinoco, 1978) and to bind preferentially to an A·I mismatch in poly(I)·poly(A,C) (Helfgott & Kallenbach, 1979). The cooperative binding of ethidium to the right-handed form of poly(dG-dC)·poly(dG-dC) in 4.4 M sodium buffer ($\tau_2 = 3$ in the calculated isotherm) may result from the favorable binding of ethidium to B–Z junctions, since these junctions are local regions of structurally distorted DNA. The structural dynamics of nucleic acids are dependent upon the local sequence (Englander & Kallenbach, 1984), and locally unwound regions have been suggested as an important component in the pathway for drug intercalation (Banerjee & Sobell, 1983; H. M. Sobell, personal communication, 1985). It has been observed that B–Z interfaces in bacterial plasmids containing left-handed $(dC\text{-}dG)_n$ inserts are receptive to S1 nuclease digestion (Singleton et al., 1982), thereby suggesting the presence of an unwound conformation at the junction between B-DNA and Z-DNA. Interfaces consisting of unbound DNA may provide a natural binding site since ethidium could intercalate without prior unwinding of base pairs, propagating the B′ region without increasing the number of interfaces.

In a report that appeared as this paper was in preparation, Shafer et al. (1984) observed binding to poly(dG-dC)·poly(dG-dC) in 4.4 M NaCl at high base pair/drug ratios and interpreted their data as differing from the results of Pohl et al. (1972). However, the equilibrium binding isotherm and the r vs. C_f graph (Figure 7) explain the apparent discrepancy. The important aspect of the experiment is the concentration of ethidium and poly(dG-dC)·poly(dG-dC) and not the base pair/drug ratio as suggested by Schafer et al. (1984). Using the present results, we expect the 30 μM solution of ethidium used by Shafer et al. (1984) to exhibit binding to poly(dG-dC)·poly(dG-dC) at all base pair/ethidium ratios, since this concentration is larger than the 20 μM required for efficient binding.

Does Ethidium Intercalate into Z-DNA? Gupta et al. (1983) and Shafer et al. (1984) have suggested that ethidium may intercalate into left-handed poly(dG-dC)·poly(dG-dC). The CD spectra of the left-handed polynucleotides change directly toward that characteristic of a right-handed drug–DNA complex, without evidence for intercalation into left-handed polymers. The fluorescence-detected circular dichroism spectra obtained to date also support this conclusion (Lamos et al., 1986). Furthermore, the apparent lack of binding of ethidium to left-handed poly(dG-dC)·poly(dG-dC) in 4.4 M NaCl until the concentration of free ethidium reaches 20 μM is not consistent with ethidium intercalating into a left-handed helix with an affinity constant any larger than 10^3 M^{-1}. Thus, we conclude that ethidium has little affinity for

left-handed poly(dG-dC)·poly(dG-dC) in 4.4 M sodium buffer ($\leq$500 M^{-1} is estimated from the calculated isotherms). The interpretation of the data in terms of the sequential conversion of the polymer from a left-handed to a right-handed duplex, as opposed to an all-or-none process, removes the need to propose the intercalation of ethidium into Z-DNA and allows all the data to be interpreted by a single model. Note, however, that these statements reflect equilibrium binding and are not meant to discount the possibility of transient intercalation into either Z-form DNA or β-DNA (H. M. Sobell, personal communication).

Acknowledgments

Appreciation is extended to Dr. David E. Graves for his assistance with the single cell analysis and to John M. Castle whose current research in this laboratory has contributed to the interpretation of the present material.

Supplementary Material Available

Molar extinction coefficients for the compounds (Table I), a description of the approach used to determine the extinction coefficients of bound ethidium, the analysis of the optical titration data, and the equilibrium binding isotherm for ethidium binding to calf thymus DNA in 50 mM sodium buffer and in 40 μM cobalt hexaammine buffer (Figure 10) (6 pages). Ordering information is given on any current masthead page.

Registry No. Poly(dG-dC), 36786-90-0; poly(dG-m^{5}dC), 51853-63-5; ethidium, 3546-21-2.

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Interaction of Drugs with Z-DNA: Cooperative Binding of Actinomycin D or Actinomine to the Left-Handed Forms of Poly(dG-dC)·Poly(dG-dC) and Poly(dG-m^{5}dC)·Poly(dG-m^{5}dC) Reverses the Conformation of the Helix†

G. Terrance Walker, Michael P. Stone,‡ and Thomas R. Krugh*
Department of Chemistry, University of Rochester, Rochester, New York 14627
Received January 2, 1985; Revised Manuscript Received June 26, 1985

ABSTRACT: The interaction of actinomycin D and actinomine with poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) under B- and Z-form conditions has been investigated by optical and phase partition techniques. Circular dichroism data show that the conformation at the binding site is right-handed, even though adjacent regions of the polymer have a left-handed conformation. Actinomycin D binds in a cooperative manner to poly(dG-dC)·poly(dG-dC) under both B-form and Z-form conditions. Analysis of the circular dichroism data shows that 5 ± 1 base pairs of left-handed poly(dG-dC)·poly(dG-dC) in 4.4 M NaCl switch to a right-handed conformation for each bound actinomycin D. When the left-handed form of poly(dG-dC)·poly(dG-dC) is stabilized by the presence of 40 μM $[Co(NH_3)_6]Cl_3$, 25 ± 5 base pairs switch from a left-handed to a right-handed conformation for each bound actinomycin D. Actinomine binds cooperatively to left-handed poly(dG-dC)·poly(dG-dC) in 40 μM $[Co(NH_3)_6]Cl_3$ and to left-handed poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 2 mM $MgCl_2$. Actinomine does not bind to left-handed poly(dG-dC)·poly(dG-dC) in 4.4 M NaCl at concentrations as high as 100 μM. Each bound actinomine converts 11 ± 3 base pairs of left-handed poly(dG-dC)·poly(dG-dC) in 40 μM $[Co(NH_3)_6]Cl_3$ and 7 ± 2 base pairs of left-handed poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 2 mM $MgCl_2$. The binding isotherm data also indicate that the binding site has a right-handed conformation. The actinomycin D–poly(dG-dC)·poly(dG-dC) binding isotherm in 4.4 M NaCl may be fit by an allosteric binding model, which also indicates that the conformation of poly(dG-dC)·poly(dG-dC) is altered only in the vicinity of the bound ligands and that intercalation into a right-handed binding site is much more favorable than intercalation into a left-handed helix. The conversion of the polymer in 4.4 M NaCl from a left-handed to a right-handed conformation occurs in a sequential manner, forming regions of right-handed poly(dG-dC)·poly(dG-dC) that are nearly saturated with actinomycin D. The important factors controlling the binding are the charge of the drug and the forces stabilizing the left-handed conformation. The biological implications of the simultaneous existence of B and Z forms by these polymers are discussed.

In the preceding paper (Walker et al., 1985) we presented results on the interaction of ethidium with left-handed (Z) forms of poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in various buffers [see the preceding paper (Walker et al., 1985) and Rich et al. (1984) for a review of left-handed (Z) DNA]. Experiments also were performed with actinomycin D, an important antitumor drug, and actinomine in order to discern whether the highly cooperative binding of ethidium to left-handed poly(dG-dC)·poly(dG-dC) in 4.4 M NaCl (Pohl et al., 1972; Walker et al., 1985) is unique or is observed with other drugs as well.

The unusually slow dissociation of actinomycin D from DNA correlates directly with the ability to inhibit DNA-dependent RNA polymerase (Muller & Crothers, 1968; Rosenberg et al., 1982). Mirau & Kearns (1983) have shown that actinomycin D is an effective inhibitor of the salt-induced B–Z transition of poly(dG-dC)·poly(dG-dC) and have correlated this with the slow association and dissociation kinetics, while van de Sande & Jovin (1982) have demonstrated that actinomycin D will bind to a condensed form of Z-DNA in a $MgCl_2$–ethanol solution. The pentapeptide side chains of actinomycin D are responsible in part for its high DNA binding affinity and site-exclusion parameter of approximately four base pairs per drug (Muller & Crothers, 1968; Wells & Larson, 1970; Sobell et al., 1971). Actinomine lacks the pentapeptide side chains of actinomycin D, has a 2+ charge at neutral pH (Muller & Crothers, 1968) (Figure 1), and presents the opportunity of studying the influence of the pentapeptide side chains on the reversal of left-handed DNA, although the 2+ charge of actinomine plays an important role in its binding characteristics.

In this report, we correlate circular dichroism (CD) results with equilibrium binding isotherms obtained from optical titration and phase partition methods to characterize the binding of actinomycin D and actinomine to poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) under B- and Z-form conditions. The equilibrium binding and CD data are con-

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‡ Present address: Department of Chemistry, Vanderbilt University, Nashville, TN 37235.

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