Unusual Binding of Ethidium to a Deoxyoligonucleotide Containing a B–Z Junction†

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ABSTRACT: Equilibrium binding studies are reported that describe the interaction of the simple intercalator ethidium with a hexadecadeoxynucleotide (BZ-1) that contains a stable B-Z junction. The transition of BZ-1 from its low-salt right-handed form to its high-salt hybrid form containing the junction between left- and right-handed DNA was found to greatly facilitate ethidium binding. Ethidium binding to all conformational forms of BZ-1 was found to be complex and highly cooperative, a result that indicates an unusually robust conformational mobility in the molecule. Binding results were quantitatively analyzed by using an allosteric model that provides a description of energetics of the conformational transitions in BZ-1, in addition to estimates of the binding constants for ethidium binding to the different conformations. These results provide a quantitative description of a structurally specific intercalator–DNA interaction.

Sequence-specific antibiotic binding to DNA is now well documented (Pullman, 1989), and in a few cases, the molecular determinants of such interactions are beginning to be understood (Pullman, 1989; Wang, 1990; Chaires et al., 1990). While some antibiotics certainly do bind to specific DNA sequences, the absolute specificity of such interactions is, in comparison to protein–DNA interactions, low. This fact is a direct consequence of the relatively small DNA binding site sizes (2–5 bp) typically found for antibiotics (Dervan, 1986). Nonetheless, certain antibiotics may bind to specific DNA sequences with binding constants of $\geq 10^7$ M^{-1} (Chaires et al., 1990), suggesting that antibiotics might effectively compete with specific protein–DNA interactions and possibly, by this mechanism, interfere with gene expression. Experimental support for such a notion has recently appeared (Bartkowiak et al., 1989).

Another possible specific, high-affinity, antibiotic binding mode might arise from the interaction of drug molecules with unique DNA structures. Such binding might be termed "structural specificity". DNA can adopt radically different geometries, which may coexist within the same DNA molecule (Wells, 1988). These structures might be unique pharmacological targets. For an intercalating antibiotic, any DNA structure that would facilitate the insertion of the drug between adjacent base pairs might be a high-affinity binding site. Junction regions between different helical conformations (e.g., B and Z, A and B), bends, bulges, and base-pair mismatches are possible examples of such structures. These structures might facilitate intercalation by partially unwinding or unstacking the DNA helix and thereby reducing the unfavorable free energy barrier that must accompany the disruption of stacked base pairs at the intercalation site during the course of the binding reaction. The possibility of structurally specific antibiotic binding to DNA has received little systematic attention. Previous studies that have quantitatively studied the effect of differing overall helical geometry on intercalator binding have shown that standard B-form DNA is preferred as an intercalation site over Z-form DNA (Chaires, 1986a,b; Walker et al., 1985a,b; Lamos et al., 1986) or over the nonstandard right-handed B′ form adopted by poly(dA)-poly(dT) in solution (Herrera & Chaires, 1989). There are few experimental strudies that have unambiguously identified an unusual DNA structure that significantly favors intercalator binding over normal B-form DNA.

We report here initial studies designed to explore the possibility of structurally specific intercalator binding, using as a model system the interaction of the simple intercalator ethidium with a deoxyoligonucleotide containing a unique structure, a B–Z junction. The hexadecanucleotide (henceforth "BZ-1")

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     *   *   *   *
5'C-G-C-G-C-G-C-G-A-C-T-G-A-C-T-G

3'G-C-G-C-G-C-G-C-T-G-A-C-T-G-A-C
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where the asterisk indicates 5-methylcytosine, was used in our study. BZ-1 has been demonstrated, by CD and NMR spectroscopy, to form a hybrid structure at high NaCl concentrations, in which the G-C end of the molecule adopts the left-handed Z structure, the central 3 bp form a stable junction region, and the remainder of the molecule adopts the right-handed B conformation (Sheardy, 1988; Sheardy & Winkle, 1989). The energetics of the NaCl-induced conformational transition of BZ-1 to its hybrid form are well characterized (Doktycz, et al., 1990). Our hypothesis is that the B–Z junction will be a high-affinity intercalation site, since the junction contains at least one base pair that is dramatically distorted (Sheardy & Winkle, 1989). A similar suggestion has been previously made (Walker et al., 1985a).

Results obtained with this model system show that the binding of ethidium to all conformational forms of BZ-1 is surprisingly complex, and all binding isotherms obtained show pronounced positive cooperativity. Unambiguously, however, ethidium binds most tightly to BZ-1 under ionic conditions that initially favor the hybrid form of the molecule, which therefore contains a B–Z junction. Our results provide a unique example of a DNA conformational transition to an unusual structure that facilitates the binding of a simple intercalator.

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Materials and Methods

Materials. BZ-1 was synthesized by the phosphoramidite method and purified by trityl-selective reverse-phase HPLC, as previously described (Sheardy, 1988). The stoichiometry of the duplex formed was verified by the method of continuous variations. The T_m of BZ-1 in BPE buffer (consisting of 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, and 1 mM Na_2EDTA, pH 7.0) was found to be 59 °C, a value in excellent agreement with previous determinations (Sheardy, 1988). The concentration of BZ-1 solutions was determined by UV absorbance, with a calculated extinction coefficient of E^m_{260nm} = 13000 M^{-1} (bp) cm^{-1} for the duplex form of BZ-1. Ethidium (Sigma Chemical Co., St. Louis, MO) concentrations were determined by absorbance, assuming E^m_{480nm} = 5600 M^{-1} cm^{-1}.

Circular Dichroism Measurements. CD spectra were recorded with a Jasco J-500A spectropolarimeter, interfaced to and controlled by an IBM PS/2 computer. At least four spectra were recorded and averaged for each determination. Molar ellipticity was calculated from the relation $[\theta] = 100\theta/Cl$, where θ is the measured ellipticity, C is the molar concentration of base pairs, and l is the path length.

Fluorescence Titrations. Ethidium binding was monitored by fluorescence measurements on a Perkin-Elmer 650-40 spectrofluorometer, with λ_{ex} = 525 nm and λ_{em} = 605 nm. Titrations were conducted at 20 °C with a 2-mL sample at a concentration of 36.5 μM bp. The concentration of bound ethidium (C_b) was calculated from the relation $C_b = C_t (I - I_0)/(V - 1)I_0$, where C_t is the known total ethidium concentration, I is the observed fluorescence intensity, I_0 is the fluorescence intensity of the identical concentration of ethidium in the absence of DNA, and V is the experimentally determined ratio of the fluorescence intensity of totally bound ethidium to that of free ethidium. V was experimentally determined at each salt concentration used by titration of a fixed amount of ethidium with DNA until a constant emission intensity was obtained. Free ethidium concentrations (C_f) were obtained from the conservation relationship $C_t = C_b + C_f$. The binding ratio r is defined as $r = C_b/[\text{DNA bp}]_{total}$.

Results and Analysis

In solutions containing high NaCl concentrations, BZ-1 undergoes a conformational transition from a right-handed B form to a hybrid form containing both left-handed and right-handed regions. Figure S1 (in the supplementary material) shows difference CD spectra obtained at several NaCl concentrations over the range of this transition. The NaCl concentration at the transition midpoint was found to be 3.5 M, a value in excellent agreement with the previously published value (Sheardy, 1988).

The binding of ethidium to BZ-1 at several NaCl concentrations, and consequently at several points along its transition to the hybrid B–Z form, was examined by fluorescence titration. The primary data from fluorescence titration experiments are shown in Figure S2 (in the supplementary material). Ethidium fluorescence is strongly enhanced upon binding to DNA. As the NaCl concentration was increased, we observed a shift in the titration curves indicative of saturation of ethidium binding sites at lower total ethidium concentrations. Such behavior would arise if ethidium binding to BZ-1 becomes tighter with increasing salt. Alternatively, such behavior could arise if the optical properties of bound ethidium vary with NaCl concentration. To rule out the latter possibility, the limiting ratio of the fluoresence of bound and free ethidium was determined to be constant over the range of 1.5–4.5 M NaCl, with $V = F_b/F_0$ = 16 at the excitation and emission wavelengths used. Assuming that the observed fluorescence enhancement is linearly related to the fraction of ethidium bound, the concentration of free and bound ethidium may be calculated from the primary fluorescence titration data of Figure S2, yielding the Scatchard plots shown in Figure 1. These plots immediately show several unusual qualitative features of the interaction of ethidium with BZ-1. First, over the entire range of NaCl concentrations, ethidium binding to BZ-1 is complex and displays positive cooperativity, as indicated by the initial positive slope of the Scatchard plots. Second, with increasing NaCl concentration, the ethidium binding affinity initially decreases slightly but then dramatically increases. Indeed, a 10-fold change in the ordinate axis is required to accommodate the data obtained in 4.5 M NaCl (Figure 1C).

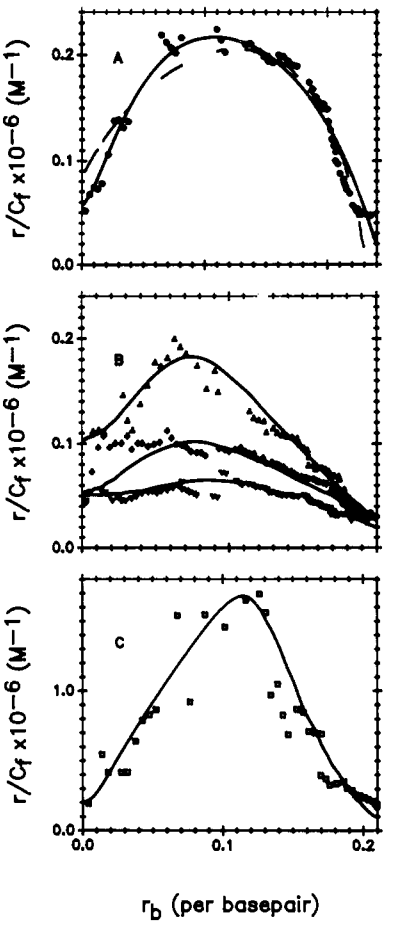


FIGURE 1: Binding of ethidium to BZ-1 as a function of increasing NaCl concentration. Data are presented in the form of Scatchard plots of the binding ratio r_b (mol of bound ethidium/mol of bp) versus r_b/C_f, where C_f is the free ethidium concentration. (A) In BPE buffer; (B) in BPE buffer plus (from top to bottom) 3.5, 2.4, and 1.5 M NaCl; (C) in BPE buffer plus 4.5 M NaCl. In all cases, data are presented as symbols, and the solid lines are calculated by using the allosteric model described in the text and the parameters listed in Table I.

The analysis of primary fluorescence titration data described above assumes a linear relationship between the observed fluorescence signal and the amount of bound ethidium. While this is the usual assumption made and one that has been verified repeatedly in other studies, the assumption must be tested by independent means, especially in the case of the apparent cooperative binding isotherms seen in Figure 1. We have used two independent approaches to verify the assumption. First, Ward (1985) suggests that a plot of $\Delta F/\Delta F_{max}$ versus the apparent fractional occupancy may be used to verify the assumed linear response of optical signal to bound ethidium. Such a plot is shown in Figure S3 (supplementary

Table I: Summary of the Parameter Estimates for the Allosteric Interaction of Ethidium with the Hexadecanucleotide BZ-1 as a Function of NaCl Concentration[a]

NaCl (M)	K_1 ($\times 10^{-6}$ M^{-1})	K_2 ($\times 10^{-6}$ M^{-1})	ω_2	K_2/K_1	s
0	0.05	0.16	10.0	3.1	0.97
1.5	0.05	0.1	3.5	2.0	0.96
2.4	0.05	0.15	3.2	3.0	0.96
3.5	0.1	0.34	2.0	3.4	0.95
4.5	0.2	7.0	1.0	35.0	0.83

[a] The parameters are defined for the allosteric model of Crothers and co-workers (Dattagupta et al., 1980). K_1 and K_2 are the binding constants for the interaction of ethidium with form 1 and form 2 DNA, respectively. ω_2 is the cooperativity constant referring to ethidium binding to form 2. The parameter s is the propagation constant for the conversion of a base pair at a preexisting interface from form 1 to form 2. The remaining parameters for the allosteric model were found to be constant, as follows: $\omega_1 = 1.0$, $n_1 = 2$, $n_2 = 4$, and $\sigma = 0.01$.

material), whose linearity supports the assumption made in obtaining the data of Figure 1. Second, methods of "model-free" analysis of spectral titration data have appeared (Schütz et al., 1979; Fritzsche & Walter, 1990), a version of which has been independently presented for fluorescence titration data (Bujalowski & Lohman, 1987). We have implemented the latter method to verify the cooperative binding isotherms seen in Figure 1. Following the method outlined by Bujalowsi and Lohman (1987), several fixed concentrations of ethidium were titrated with increasing amounts of BZ-1 in separate experiments. These data may be combined to obtain estimates of the average binding density and free ligand concentration without making any assumptions about the relationship between the fractional signal change and the fraction of bound ligand [see Schütz et al. (1979), Fritzsche and Walter (1990), and Bujalowski and Lohman (1987)]. Figure S4 (supplementary material) shows that data obtained by this method agree within experimental error with the binding data shown in Figure 1 in the critical region of low binding densities. The agreement of the data obtained by this independent approach confirms the apparent cooperativity in the binding isotherms and suggests that it does not result from an erroneous assumption concerning the linearity of the spectral response.

Attempts to fit the binding isotherms shown in Figure 1 to the extended model of McGhee and von Hippel that includes cooperative ligand interactions (McGhee & von Hippel, 1974) were unsuccessful. That model cannot account for the shapes of the Scatchard plots shown in Figure 1, and fitted curves show systematic deviations from the experimental data, particularly in the region of low r values. An illustration of a fit of this type is shown as the dashed line in the Scatchard plot shown in Figure 1A. These data may, however, be quantitatively analyzed by the allosteric binding model of Crothers (Dattagupta et al., 1980), by using the approach described fully by Chaires (1986a). Briefly, the model postulates that the DNA exists in two conformational forms in equilibrium. Conversion of DNA from form 1 to form 2 is described by a mechanism requiring a nucleation step (with an equilibrium constant $\sigma^2 s$) followed by a propagation step (with an equilibrium constant s). Antibiotic may bind to either DNA form, with binding described by the neighbor exclusion parameters K_i, n_i, and ω_i, where $i = 1$ or 2, referring to binding to each DNA form. Eight parameters thus are required to generate binding isotherms for the model. As discussed by Chaires (1986a), rigorous least-squares methods may be used to fit portions of the binding isotherms to obtain the statistical best estimates for K_1, K_2, and n_2, which may then be constrained in the subsequent analysis. Parameter estimates found to

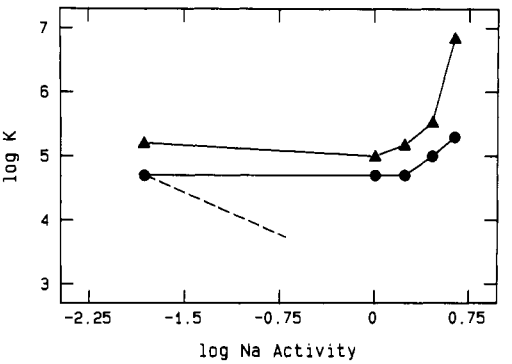


FIGURE 2: Dependence of the equilibrium binding constants upon Na^+ activity. The logarithms of K_1 (closed circles) and K_2 (closed triangles) are shown as a function of the logarithm of Na^+ activity. The dashed line is the calculated decrease in log K expected from polyelectrolyte theory for the interaction of a singly charged ligand with DNA. Both K_1 and K_2 clearly deviate from this expected behavior.

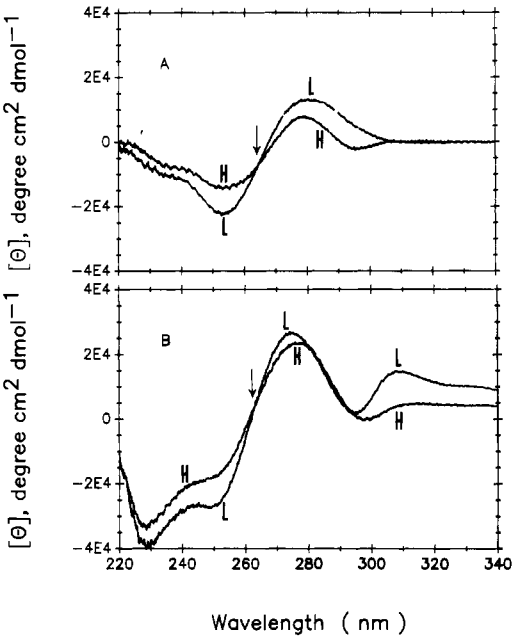
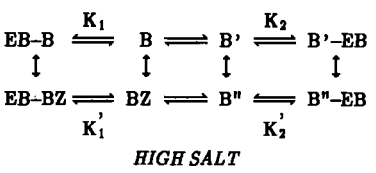


FIGURE 3: CD spectra of hexadecanucleotide BZ-1 in the absence (A) or presence (B) of saturating amounts of ethidium. In each panel, the curve labeled L refers to low salt (BPE buffer), while the curve labeled H refers to high salt (BPE buffer plus 4.5 M NaCl). The arrows indicate the points at which the curves cross over.

provide isotherms that match the binding data of Figure 1 are collected in Table I. Their meaning will be discussed more fully below, but it should be emphasized here that both K_1 and K_2, the ethidium binding constants, increase with increasing NaCl, over the range in which BZ-1 undergoes the conformational transition to the hybrid form. Figure 2 shows the dependence of K_1 and K_2 on Na^+ activity as a double logarithmic plot. Such behavior is inconsistent with the expected behavior predicted from modern polyelectrolyte theory (Record et al., 1978), which predicts that increased NaCl concentration should *decrease* the affinity of a positively charged ligand toward DNA. The dashed line in Figure 2 shows the slope expected for the decrease in log K for the binding of ethidium to B-form DNA, calculated by Record's theory (Record et al., 1978).

The conformation of BZ-1 in high and low salt, in the absence and presence of ethidium, was examined by CD spectroscopy (Figure 3). These spectra show that, at the start of the ethidium titration, BZ-1 is in the right-handed B form in low salt and is in the hybrid form in 4.5 M NaCl (Figure

Scheme I: Minimal Set of Equilibria Required To Account for the Observed Binding Data for the Interaction of Ethidium with BZ-1[a]



[a] The reactant ethidium ("EB") is not written to reduce the complexity.

3A). With saturating amounts of bound ethidium, the spectra shown in Figure 3B are obtained. These spectra correspond to the end point of the ethidium titration and reflect the structure of the fully saturated oligonucleotide following the allosteric conversion to form 2. While the high and low salt solutions both show similar spectra, distinct differences are observed. The most prominent difference is in the range 300–340 nm, which shows a distinct spectral band in low salt but not in high salt.

Discussion

The binding of ethidium to the oligonucleotide BZ-1 is surprisingly complex and displays pronounced positive cooperativity over a wide range of ionic conditions. These cooperative interactions are most readily explained by an allosteric model in which ethidium binding is coupled to conformational transitions in BZ-1. Scheme I shows a minimal set of conformational and binding equilibria required to account for the observed binding data. The most striking result to emerge from these binding studies is that high salt concentrations facilitate ethidium binding, in contrast to the predictions of polyelectrolyte theory. Tighter ethidium binding must arise from a NaCl-induced conformational transition in BZ-1 to a form that facilitates intercalation.

Two Conformational Forms of BZ-1 in Low Salt. The positive cooperativity observed for ethidium binding to BZ-1 in low salt is most readily explained by the coupling of ligand binding to a conformational transition in the oligonucleotide. CD spectra indicate that in low salt BZ-1 is in a right-handed B conformation (Figure 3). Ethidium binds initially to this form of BZ-1 with the binding constant K_1. Further ethidium binding evidently shifts BZ-1 to a second right-handed conformation, indicated as B′ in Scheme I, to which ethidium binds with a binding constant K_2. The B′ form of BZ-1 binds ethidium 3 times more tightly than does the initial B form (Table I). The B and the B′ forms are nearly isoenergetic, with the free energy for the conversion of a base pair at a preexisting interface from one form to another being $\Delta G^0 = -RT \ln (s) = +18$ cal (mol of bp)$^{-1}$ at 20 °C. The detailed structural differences between the B and B′ conformations are unknown. Previous ^{31}P and ^{1}H NMR measurements have, however, indicated subtle, temperature-dependent conformational fluctuations in the junction region in BZ-1 at temperatures below the T_m (Sheardy & Winkle, 1989). Interestingly, the sequence 5′GTG, which is a common feature in a number of DNA sites where proteins bind, was found by NMR to have a higher imino proton exchange rates than normal B-form DNA at temperatres well below the melting temperature (Lu et al., 1983). A similar sequence (5′GTC) is found in the BZ-1 junction region. Further, the triplet sequence of 5′TCG was found by two-dimensional NMR spectroscopy to show unique structural properties (Chary et al., 1987) and in particular to be partially unwound. Such a triplet sequence is found in the center of BZ-1. It is possible that the B to B′ transition involves either or both of these sequence elements.

Ethidium Molecules Are Clustered When Bound to BZ-1 in Low Salt but Not in High Salt. A unique aspect of ethidium binding to the low-salt form of BZ-1 is reflected by the magnitude by ω_2. The parameter ω may be thought of as the equilibrium constant for moving ethidium molecules bound at isolated sites into proximity such that they occupy contiguous sites (McGhee & von Hippel, 1974). The value $\omega_2 = 10$ indicates that occupancy of contiguous sites is strongly favored and therefore that ethidium molecules will be clustered. Independent support for ethidium clustering is provided by the CD spectra of Figure 3B. The induced CD in the region of 300–350 nm has been shown to arise from ethidium clustering (Lamos et al., 1986). The presence of this induced CD band in low salt and its absence in high salt suggests clustering of ethidium molecules in low salt but not in high salt. More rigorous proof of this interpretation of these CD spectra will require more detailed CD titration studies and evaluation of the molar ellipticities in the region 300 - 350 nm as a function of bound ethidium. Recent affinity cleavage studies on a BZ-junction-containing oligonucleotide using methidium-propyl-EDTA show a nonrandom distribution of cleavage sites in the low-salt, right-handed form of the molecule but not in the high-salt, junction form, an observation consistent with our inference of ethidium clustering (Guo et al., 1991). Upon increasing NaCl concentration, the clustering phenomenon is abolished. Table I shows that ω_2 decreases to 1.0 in high-salt solutions.

Ethidium Binds Very Tightly to the High-Salt Forms of BZ-1. Under high-salt conditions (4.5 M NaCl), BZ-1 undergoes a conformational transition to a form containing both left- and right-handed regions, which therefore contains a B–Z junction region. This conformer is denoted BZ in Scheme I. The hybrid BZ form is converted to a right-handed intercalated form (B″) upon binding of ethidium, as judged by its CD spectrum (Figure 3B). The free energy for the conversion of the BZ to the B″ form is modest, with $\Delta G^0 = -RT \ln (s) = +108$ cal (mol of bp)$^{-1}$ at 20 °C. K_1' and K_2' denote the ethidium binding constants to the two forms of BZ-1 under these conditions, where the prime is used to emphasize the high salt conditions and the distinction between the initial conformational states when compared to the right-handed low-salt conformation. The high-salt forms of BZ-1 bind ethidium very tightly. A value of $K_1' = 0.2 \times 10^6$ M^{-1} is found, referring to the binding of ethidium to the junction-containing form of BZ-1. The value of K_1' is 4 times greater than the K_1 value observed in low salt, which refers to binding to the right-handed form of BZ-1. If the conformation of BZ-1 were to remain entirely in the right-handed form in 4.5 M NaCl, a reduction in the value of K_1 to 360 M^{-1} would be expected, assuming that $(\delta \ln K)/(\delta \ln M^+) = -0.88$ in accord with polyelectrolyte theory (Record et al., 1978). The observed value in 4.5 M NaCl is over 500 times greater than this calculated expected value. Conversion of BZ-1 to the form containing the B–Z junction therefore greatly facilitates ethidium binding. The junction-containing form is not, however, the highest affinity form of BZ-1. A value of $K_2' = 7.0 \times 10^6$ M^{-1} is observed, a value that is 40 times greater than any other binding constant measured in this study (Table I). This binding constant refers to the B″ form of BZ-1 (Scheme I). The CD spectra shown in Figure 3B indicate that the B″ form is an intercalated, right-handed form, but it must be distinct from the B′ form observed in low salt, since there are discernible differences in the CD spectra of the two forms. It is important to stress that the dramatic increase in the ethidium

binding constants in 4.5 M NaCl is highly unusual behavior that sharply contrasts with that observed in studies of intercalator binding to left-handed Z DNA (Chaires, 1986a,b; Walker et al., 1985a,b). In these studies, intercalator binding was strongly *reduced* by the high-salt conversion of DNA to the left-handed form.

Does Ethidium Bind to the B–Z Junction with High Affinity? Our hypothesis entering these studies was that the B–Z junction would be a high-affinity intercalation site. We find in fact that the junction-containing molecule in high salt binds ethidium with greater affinity than does the low-salt right-handed form. However, the junction-containing molecule is apparently *not* the form with the highest affinity for ethidium. Indeed, if this were the case, we would expect ethidium to allosterically convert BZ-1 to the hybrid form, even under low-salt conditions. Instead, in high salt, ethidium converts the junction-containing molecule to what appears to be an intercalated right-handed form. This right-handed form must be in a different conformation than the low-salt form, since its affinity for ethidium is dramatically enhanced and its CD spectrum is discernably different.

Conclusions

The hexadecadeoxynucleotide BZ-1 has an unusually robust conformational flexibility. The ethidium binding studies reported here require that at least four distinct conformations of BZ-1 exist, each with distinctive ethidium-binding properties. NaCl induces a structural transition to forms of BZ-1 that bind ethidium with substantially higher affinity. Instead of the decrease in the ethidium binding constants expected from polyelectrolyte theory, these high salt BZ-1 structures bind ethidium with binding constants orders of magnitude greater than those observed in low salt. Such behavior is one of the few examples of a DNA conformational transition away from the standard B form to a form that facilitates intercalator binding. Exactly what these alternate conformational forms are, and why intercalation is facilitated, awaits clarification by more detailed structural studies. These studies show that BZ-1 is a useful and intriguing model system for the study of structurally specific intercalator binding.

Acknowledgments

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Supplementary Material Available

Figures S1–S4, showing difference CD spectra, fluorescence titration experiments, a plot of $\Delta F/\Delta F_{max}$ versus fractional occupancy, and a comparison of binding data (4 pages). Ordering information is given on any current masthead page.

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