Interactions of Aristololactam β-D-Glucoside with Right-Handed and Left-Handed Forms of Synthetic Deoxyribonucleic Acid: Spectroscopic and Thermodynamic Study†

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ABSTRACT: The interaction of aristololactam β-D-glucoside (ADG) with different polymorphic structures of poly(dG-me^{5}dC)·poly(dG-me^{5}dC), poly(dG-dC)·poly(dG-dC), and poly(dI-dC)·poly(dI-dC) has been studied by spectrophotometric, spectrofluorimetric, circular dichroism, UV melting profiles, and thermodynamic analysis. The binding of ADG to B-form duplexes is characterized by the typical bathochromic and hypochromic effects in the absorption spectrum, quenching of steady-state fluorescence intensity, a decrease in fluorescence quantum yield of ADG, an increase in fluorescence polarization anisotropy, an increase of thermal transition temperature, and perturbation in circular dichroic spectrum. Scatchard analysis indicates that ADG binds to the right-handed form of each polymer in a noncooperative manner. Comparative binding parameters determined from absorbance titration by Scatchard analysis, employing the excluded site model, indicate a stronger binding of ADG to the B-form of poly(dG-me^5-dC)·poly(dG-me^{5}dC) than to the B-form of poly(dG-dC)·poly(dG-dC) or poly(dI-dC)·poly(dI-dC). Thermodynamic parameters ($\Delta G°$, $\Delta H°$, and $\Delta S°$) obtained by van't Hoff analysis of the data show that the process of binding to all B-form duplexes is exothermic and enthalpy driven as characterized by a favorable negative enthalpy change ($\Delta H°$). The binding is opposed by a negative entropy change ($\Delta S°$) contribution. Conformational changes indicate that the alkaloid converts the left-handed form of poly-(dG-dC)·poly(dG-dC), and its methylated analogue and high salt form of poly(dI-dC)·poly(dI-dC) to a bound right-handed form, while it inhibits both the rate and extent of the B to Z transition. These studies reveal that ADG binds strongly to B-form polymers while it does not bind to polymers of Z-form.

In recent years there has been considerable interest in the elucidation of nonconventional double-helical conformations and their biological roles (Larsen & Weintraub, 1982; Rich et al., 1984; Saenger, 1984; Jovin et al., 1987; Mirkin & Frank-Kamenetskii, 1994). It is now a clearly established fact that DNA[1] can adopt a variety of unusual conformations in response to changes in solution conditions. In addition to well known global changes in DNA conformation like A-, C-, D-, or Z-DNA, properties of certain nonconventional structures like parallel-stranded DNA, triple-helical form of DNA, etc., have been reinvestigated by several workers (Wang et al., 1979; Rippe et al., 1992; Singleton & Dervan, 1992; Raghunathan et al., 1993).

The conformational polymorphism and the flexibility of the double helix are also revealed on binding of small molecules to the polymer. In recent years there have been a number of reports elucidating the factors that govern the affinity and specificity of binding of many naturally occurring compounds to DNA (Waring, 1981a,b; Neidle & Waring, 1983; Scaria & Shafer, 1991; Durand et al., 1992). One important class of these compounds binds to DNA by the mechanism of intercalation and is an important tool in molecular biology and in clinical applications (Waring, 1981a,b; Neidle & Waring, 1983, Wilson, 1989; Wang, 1992).

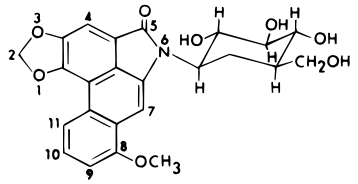

FIGURE 1: Chemical structure of aristololactam β-D-glucoside.

Aristololactam β-D-glucoside or 6-β-D-glucopyranosyl-8-methoxybenzo(f)-1,3-benzodioxolo[6,5,4-cd]indol-5(6H)-one (Figure 1) is one of the aristolochia group of alkaloids that have attracted recent attention for their prospective clinical and pharmacological uses (Chen & Zhu, 1987; Cassady et al., 1990). Recently, it has been shown that the alkaloid binds to DNA by the mechanism of intercalation with a considerable specificity toward GC-rich DNA, especially alternating GC polymer (Chakravorty et al., 1989a,b; 1990; Nandi et al., 1991).

Alternating polymers like poly(dG-dC)·poly(dG-dC) or its methylated analogue undergo conformational changes in response to different local environmental changes (Pohl & Jovin, 1972; Behe & Felsenfeld, 1981). Poly(dI-dC)·poly-(dI-dC) contains a strictly alternating self-complementary

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[1] Abbreviations: ADG, aristololactam β-D-glucoside; B, right-handed; Z, left-handed; DNA, deoxyribonucleic acid; BPE, (4 mM Na^+) buffer, pH 7.0, containing 1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, and 0.25 mM EDTA; BPES, (20 mM Na^+) buffer, pH 7.0, containing 1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, 0.25 mM EDTA, and 16 mM NaCl; DMSO, dimethyl sulfoxide; CD, circular dichroism; T_m, thermal melting temperature; P/D, DNA nucleotide phosphate/drug molar ratio; *D*/*P*, drug/DNA nucleotide phosphate molar ratio.

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sequence of deoxyinosine and deoxycytosine. Its structure as revealed from the unusual X-ray diffraction pattern and inverted CD spectrum was earlier interpreted in terms of left-handed helices (Mitsui et al., 1970; Ramaswamy et al., 1982; Drew & Dickerson, 1982). However, recent vacuum CD (Sutherland & Griffin, 1983) and two-dimensional NMR studies provided considerable evidence to substantiate the right-handed B-conformation of poly(dI-dC)·poly(dI-dC) (Mirau & Kearns, 1984) in solution.

In order to elucidate the characteristics of binding of ADG with right-handed and left-handed forms of DNA and to gain more insight into the molecular nature of its recognition to these conformational variants, we have investigated the interaction of ADG with right-handed and left-handed forms of three synthetic polynucleotides poly(dG-me^{5}dC)·poly(dG-me^{5}dC), poly(dG-dC)·poly(dG-dC), and poly(dI-dC)·poly(dI-dC) using various spectroscopic and thermodynamic techniques. Our study gives comparative information on how the alkaloid responds to the various conformations of these alternating polymers.

EXPERIMENTAL PROCEDURES

Poly(dG-me^{5}dC)·poly(dG-me^{5}dC) [hereafter poly(dG-m^{5}dC)], poly(dI- dC)·poly(dI-dC) [hereafter poly(dI-dC)], and poly(dG-dC)·poly(dG-dC) [hereafter poly(dG-dC)] were purchased from Pharmacia BioTech Inc., Piscatway, NJ. These are of highest grade commercially available and were used without further purification. Each polynucleotide was sonicated in a Labsonic 2000 sonicator (B. Braun Swiss) by using a needle probe of 4 mm diameter. After sonication, the polymer was extensively dialyzed under sterile conditions. The average length of sonicated polynucleotide was found to be 270 ± 60 base pairs determined from viscometric measurements as described by Maiti et al. (1982). Their nativeness and purity were tested by using UV spectral characteristics and circular dichroic and T_m analysis (Nandi et al., 1991; Kumar & Maiti, 1994). Their concentrations in terms of nucleotide phosphate were estimated spectrophotometrically using known molar extinction coefficients (ϵ) 7000 M^{-1} cm^{-1} at 255 nm for poly(dG-m^{5}dC), 8400 M^{-1} cm^{-1} at 255 nm for poly(dG-dC), and 5900 M^{-1} cm^{-1} at 252 nm for poly(dI-dC) as reported (Chen, 1986; Nandi et al., 1991; Vorlic'kova & Sagi, 1991).

ADG was extracted from *Aristolochia indica* and crystallized from ethanol. Its purity was checked by thin layer chromatography, melting point determination, and mass and NMR spectral analysis as described (Chakraborty et al., 1989a,b, 1990). The concentration of ADG was obtained spectrophotometrically by using a molar extinction coefficient (ϵ) of 10 930 M^{-1} cm^{-1} at 398 nm in dimethyl sulfoxide (DMSO). All B-form DNA binding experiments were performed in citrate phosphate (CP) buffer (pH 7.0) as described (Kumar & Maiti, 1994) at constant sodium molarity of 10 mM with 240 mM DMSO. pH measurements were done on an electronic pH meter (Electronic Corp., Hyderabad, India) with an accuracy of ±0.01 units. T_m measurements for B-form of the polymers were performed in the presence of BPE-DMSO (4 mM Na^+), pH 7.0, containing 1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, 0.25 mM EDTA, and 240 mM DMSO. Thermodynamic measurements were performed in BPES-DMSO (20 mM Na^+), pH 7.0, containing 1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, 0.25 mM EDTA, 16 mM NaCl, and 240 mM DMSO. Glass-distilled deionized water and analytical grade reagents were used throughout.

Formation of Left-Handed Structure. The B to Z transition was measured using an individual sample for each sodium chloride concentration. Usually, a sample of 2.5 mL of buffer containing 60−70 μM polymer was brought to the desired sodium chloride concentration by the addition of solid sodium chloride and then dialyzed against a large volume of buffered sodium chloride solution of the desired concentration for at least 16 h at room temperature. Following dialysis, the complete UV and CD absorption spectra were recorded. The ratio of absorbance at 260 nm to that at 295 nm was used as a measure of B to Z transition (Pohl & Jovin, 1972). In some experiments the Z-form poly(dG-m^{5}dC) was prepared in low salt as described (Feuerstein et al., 1985). The formation of Z-form was routinely monitored by UV−visible spectral analysis as well as by CD measurements. Under the present experimental condition we obtained the Z-form structure of poly(dG-m^{5}dC) and poly(dG-dC) at sodium molarity of 3.0 and 4.5 M, respectively. The low salt Z-form of poly(dG-m^{5}dC) was obtained in buffer of sodium molarity less than 0.8 mM at neutral pH. The high salt induced structure of poly(dI-dC) was prepared in solution of 4.5 M sodium ion which resembles the altered structure obtained by Vorlic'kova and Sagi (1991) at a sodium molarity of 4.6 M. This structure is predominantly a right-handed form as evidenced from vibrational circular dichroism (VCD) study of Wang and Keiderling (1993).

Kinetics of the Right-Handed to Left-Handed Transition. The B to Z kinetics were measured by monitoring the change in the poly(dG-dC) absorbance at 295 nm which accompanies the transition. Experiments were performed following the general method described by Mirau and Kearns (1983). Absorbance spectra were recorded on a Shimadzu UV 260 spectrophotometer equipped with a temperature controller (SPR 5) at 25 °C. To examine the effect of the alkaloid on the B−Z transition, the B-form poly(dG-dC) was added to a high salt solution of the alkaloid, and the absorbance at 295 nm was recorded with constant stirring.

UV−Visible Spectroscopy. The UV−visible absorption spectra of ADG mixed with or without polynucleotides were obtained using a Shimadzu UV-260 spectrophotometer (Shimadzu Corp., Kyoto, Japan) in a thermostatically controlled cell holder equipped with a thermoprogrammer (model KPC-5) and a temperature controller (SPR 5) in matched quartz cell of 1 cm path length as described earlier (Nandi et al., 1991). The decrease in absorbance of ADG at 398 nm upon binding to the polynucleotide was used to calculate the equilibrium concentration of free and bound alkaloid. Binding studies for the B- and Z-conformations were carried out at 25 °C.

Data Analysis. Binding data obtained from spectrophotometric titration were cast into the form of Scatchard plot of r/C_f vs r, where r is the number of alkaloid molecules bound per mole of nucleotide and C_f is the molar concentration of the free alkaloid. Nonlinear binding isotherms were observed in each case, and the data were fitted to a theoretical curve which is drawn according to the excluded site model (Crothers, 1968) developed by McGhee and von Hippel (1974) for a nonlinear noncooperative ligand binding system.

$$r/C_f = K'(1 - nr)[(1 - nr)/(1 - (n - 1)r)]^{(n-1)} \quad (1)$$

where K' is the binding constant to an isolated DNA binding site, and n is the exclusion parameter in nucleotides.

In practice, the parameters r and C_f are determined from the change in absorbance at a particular wavelength (i.e., the absorption maximum of the free alkaloid). If A_F, A_B, and A represent the absorbance of the initially, finally, and partially titrated alkaloids, respectively, then the fraction of the bound ligand molecules, α_b, would be given by

$$\alpha_b = (A_F - A)/(A_F - A_B) \quad (2)$$

The molar concentration of free (C_f) and bound (C_b) alkaloid molecules and r could be evaluated from the following equation, where D and P represent the total input ligand and the DNA phosphate concentration, respectively.

$$C_f = (1 - \alpha_b)D$$

$$C_b = \alpha_b D$$

$$r = C_b/P = \alpha_b D/P \quad (3)$$

Binding data were analyzed by using the programme SCATPLOT, version 1.2 (Ray et al., unpublished work) which works on an algorithm as described in Nandy et al. (1993) to determine the best fit parameters to eq 1. The best fit parameters correspond to the minimum least-squares variance between the theoretical and experimental values (Nandy et al., 1993).

Thermodynamic Study. Temperature-dependent absorption studies of ADG binding to the B-form polymers were carried out at 15, 25, and 40 °C for poly(dG-m^{5}dC) and poly(dG-dC) and 15, 25, and 35 °C for poly(dI-dC) either by a complete titration at a given temperature or by increasing the temperature of a sample containing a fixed ratio of D/P as described (Chakaborty et al., 1990) allowing an equilibrium period of 5 min before each spectrum was recorded. Thermodynamic parameters were estimated according to the following equations. The changes in Gibbs free energy ($\Delta G°$) were calculated from the binding constant at a particular temperature using

$$\Delta G° = -RT \ln K' \quad (4)$$

The binding enthalpy change ($\Delta H°$) was determined from the plots of ln K' vs $1/T$ following the van't Hoff equation

$$[\delta \ln K'/\delta(1/T)] = -\Delta H°/R \quad (5)$$

The entropy change ($\Delta S°$) was estimated from the relationship

$$\Delta S° = -(\Delta G° - \Delta H°)/T \quad (6)$$

UV Melting Study. Absorbance versus temperature profiles for polynucleotide duplexes and ADG-duplex complexes in appropriate solution conditions were measured at a particular wavelength with a thermostatically controlled Shimadzu UV-260 spectrophotometer. The temperature was scanned with a heating rate of either 0.5 or 1 °C/min.

Spectrofluorimetric Study. Fluorescence measurements were recorded at 25 °C on a Hitachi F-4010 spectrofluorimeter (Hitachi Ltd., Tokyo, Japan) to which was attached a EYELA UNICOOL UC-55 (Tokyo Rikakikai Co. Ltd., Tokyo, Japan) temperature controller. Measurements were made in fluorescence free quartz cells of 1 cm path length as described (Kumar et al., 1992). Uncorrected fluorescence spectra are reported.

Fluorescence Quantum Yield. Steady-state fluorescence quantum yield was calculated using the following equation as described by Chakraborty et al., (1989a).

$$\Phi_s = (F_s \epsilon_q C_q / F_q \epsilon_s C_s) 0.55 \quad (7)$$

where s and q denote sample and quinine sulfate in 0.1 N H_2SO_4, respectively, F denotes the integral area of the fluorescence with excitation at the same wavelength, ϵ is at the wavelength of excitation, and C represents the corresponding concentration.

Fluorescence Polarization Anisotropy. Fluorescence anisotropy measurements were carried out as described (Larsson et al., 1994) using

$$A = \frac{I_{vv} - I_{vh}G}{I_{vv} + 2I_{vh}G} \quad (8)$$

where G is the ratio I_{hv}/I_{hh} used for instrumental correction. I_{vv}, I_{vh}, I_{hv}, and I_{hh} represent the fluorescence signal for excitation and emission with the polarizer set at (0°,0°), (0°,90°), (90°,0°), and (90°,90°), respectively (Larsson et al., 1994).

Circular Dichroism. CD spectra were recorded on a JASCO J20A spectropolarimeter (JASCO Corp., Tokyo, Japan) with a thermoelectrically controlled cell holder connected to a EHC 363 temperature controller or JASCO J720 CD spectropolarimeter attached with a temperature controller and thermal programmer model PTC 343 interfaced with a COMPAQ PC 486 in a rectangular quartz cuvette of 1 cm path length as reported earlier (Nandi et al., 1991; Kumar & Maiti, 1994). Spectral measurements were carried out at 25 °C for both B- and Z-form DNA.

RESULTS

Absorption Spectral Analysis. The visible absorption spectrum of ADG (Figure 2) has two bands with absorption maxima centered at around 330 and 398 nm. The effect of increasing the concentration of B-form polynucleotides on the absorption spectrum of ADG shows hypochromism and bathochromism in both of these bands. The typical changes observed with B-form of poly(dG-m^{5}dC) are shown in Figure 2A. A clear isosbestic point is observed at about 420 nm in these spectra and is indicative of equilibrium between bound and free alkaloid molecules. On the other hand, the spectral characteristics of ADG did not show any significant change at a high Z-DNA/ADG ratio (Figure 2B). This result suggests that the ADG−Z-DNA complex is not an intercalative complex, and that the observed small changes in the absorption spectrum of the alkaloid arise from its intercalation into B-form DNA which would have been formed at that P/D. Similar observations were reported by Chaires (1986) for the daunomycin−Z-DNA complex.

Evaluation of Binding Parameters. The Scatchard plots derived from measurements of equilibrium concentrations of free alkaloid and alkaloid bound to all B-form polymers were concave upward and indicate more than one type of binding mode.

We found that the binding isotherms were concave upward in all cases, indicating a noncooperative binding process. Since we found no sign of sigmoidal behavior for the

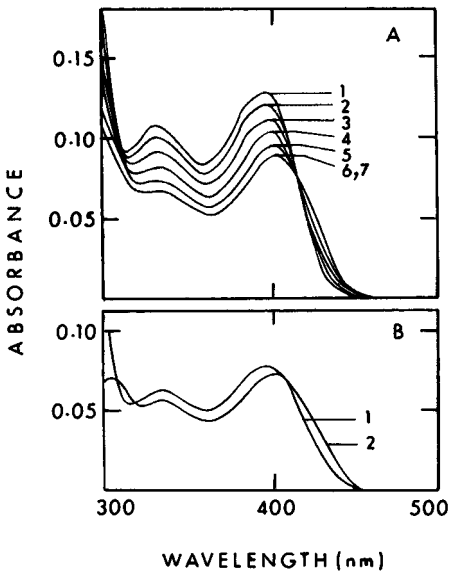

FIGURE 2: Representative absorption spectrum of ADG (10.72 μM) treated with (A) right-handed B-form of poly(dG-m^{5}dC) in 10 mM citrate phosphate buffer (pH 7.0) at 25 °C. Curves 1−7 denote the absorption spectrum of ADG treated with 0, 7.12, 17.71, 28.17, 41.93, 55.48, and 62.17 μM poly(dG-m^{5}dC), respectively, and (B) left-handed form of poly(dG-m^{5}dC) in 3.0 M NaCl at 25 °C. Curves 1 and 2 denote the absorption spectrum of ADG treated with 0 and 101.84 μM poly(dG-m^{5}dC), respectively. DNA concentration is expressed in terms of nucleotide phosphate.

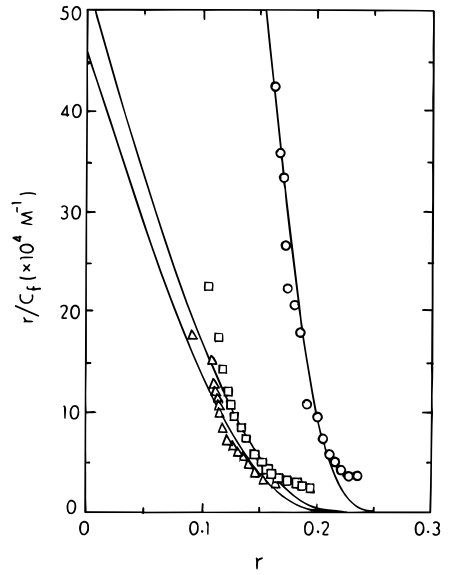

FIGURE 3: Representative Scatchard plots of ADG binding to B-form of poly(dG-m^{5}dC) (○), poly(dI-dC) (□), and poly(dG-dC) (△) in 10 mM citrate phosphate buffer (pH 7.0) at 25 °C. Solid lines were drawn by analyzing the data according to the neighbor exclusion model using the program SCATPLOT. *K′* and *n* values are presented in Table 1. Binding data are limited to DNA/ADG ratios corresponding to percentages of bound ADG ranging from 30% (lower limit) and 95% (upper limit).

occurrence of cooperativity in our system, we adopted the neighbor exclusion model (Crothers, 1968; McGhee & von Hippel, 1974) for noncooperative binding phenomena to fit our experimental data. We have also found that such a model adequately fits the data within the regions of Scatchard plot corresponding to the range 30% (lower) to 95% (upper) of each polynucleotide bound alkaloid. Representative binding isotherms for B-form polymers are illustrated in Figure 3. The quantitative binding parameters presented in Table 1 indicate that the value of *K′* of poly(dG-m^{5}dC) (B-form) is about 7 times higher than that of the B-form of poly(dI-dC) and poly(dG-dC). However, the binding sites are not changing appreciably as evidence from the *n* values (Table 1).

Thermodynamics of the ADG−DNA Complex. The influence of temperature on alkaloid−DNA complexation has been used to derive the thermodynamic parameters of ADG binding to three B-form polynucleotides. In all the cases the Scatchard plots (not shown) derived from equilibrium between bound and free alkaloid at different temperatures were concave upward and represent more than one type of binding mode. The binding isotherm data were fit to eq 1, and the data for binding parameters are presented in Table 1. Table 1 shows that the binding affinity decreases with increasing temperature for each polymer but the *n* value appears to be almost invariant. A van't Hoff plot of ADG complexation with each of the three B-form polynucleotides (figure not shown) shows a linear relationship indicating a small value of heat capacity change analogous with that observed for other intercalators. The negative change in enthalpy and entropy is maximum for poly(dG-m^{5}dC) and minimum for poly(dG-dC) among the polymers studied. The binding process is exothermic and enthalpy driven. A cumulative plot of negative enthalpy change versus negative entropy change for three polymers studied (data not shown) shows a linear correlation with a slope of 459.5 K and a correlation coefficient of 0.9896. Changes in enthalpy were compensated by changes in entropy to produce a relatively small change in free energy in all the cases.

Thermal Melting Study. To gain a further insight into the intercalation phenomenon, we have studied the thermal denaturation of the B-form of each the polymers in the presence and the absence of ADG. The thermal melting profiles (data not shown) show enhancement of thermal melting stability in all the three B-form polymers in the presence of ADG. Melting profiles indicate a cooperative melting transition with percentage hyperchromicity changes within the normal range for canonical B-form DNA at pH 7.0. At saturation ($D/P \sim 0.37$) the T_m's of the B-form of poly(dG-m^{5}dC) and of poly(dG-dC) were increased by 3.5 and 3 °C, respectively, while for B-form poly(dI-dC) about 14.3 °C enhancement was observed (Table 2). It was observed that melting profile of each polymer was identical for a heating rate of either 1.0 or 0.5 °C/min, indicating that enough time was allowed for thermal equilibration.

Fluorescence Study. The characteristic steady-state emission spectrum of ADG in the region of 420−620 nm has an emission maximum at 490 nm when excited at 400 nm. The effect of addition of various forms of polynucleotides to ADG in different buffer systems was monitored through spectrofluorimetric studies. A progressive quenching effect of the fluorescence emission spectrum of ADG by the B-form of the polynucleotides studied was observed (data not shown). The steady-state fluorescence intensity of ADG was quenched maximally by the B-form of poly(dG-m^{5}dC). The quantitative data on fluorescence quantum yield (Figure 4) of various ADG−DNA complexes show that the relative quantum yield decreases with increasing *P*/*D* ratio and level off at saturation. It was observed that the interaction of ADG with poly(dG-m^{5}dC) was strongest among the three B-form polymers. The effect of salt concentration on the steady-state fluorescence intensity of ADG was also studied over a wide range of

Table 1: Binding and Thermodynamic Parameters for Interaction of ADG with B-Form Polynucleotides in 20 mM BPES Buffer (pH 7.0) Obtained from Spectrophotometric Study[a]

DNA	temp (°C)	K' ($\times 10^5$ M^{-1})	n	$-\Delta G°$ (25 °C) (kcal/mol)	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (25 °C) (cal/K·mol)
poly(dG-m^{5}dC)	15	85.00 ± 4.0	4.00 ± 0.10			
	25	35.00 ± 4.0	4.00 ± 0.10	8.98 ± 0.05	13.86 ± 0.06	16.38 ± 0.05
	40	13.00 ± 2.0	4.00 ± 0.10			
poly(dI-dC)	15	10.00 ± 0.5	4.10 ± 0.20			
	25	5.10 ± 0.3	4.40 ± 0.20	7.83 ± 0.04	10.87 ± 0.05	10.19 ± 0.04
	35	2.85 ± 0.3	4.80 ± 0.10			
poly(dG-dC)	15	8.10 ± 0.3	4.40 ± 0.20			
	25	4.50 ± 0.4	4.60 ± 0.30	7.76 ± 0.02	10.35 ± 0.10	8.70 ± 0.05
	40	2.00 ± 0.1	4.75 ± 0.30			

[a] Three determinations each.

Table 2: Binding Data for the ADG Complexation with the B-Form of Different Polynucleotides Obtained from Relative Quantum Yield Values, Fluorescence Polarization Anisotropy Values, and Thermal Melting Profiles[a]

polymer	Φ/Φ_o[b]	anisotropy[c]	ΔT_m[d]
poly(dG-m^{5}dC)	0.10 ± 0.01	0.21 ± 0.01	3.5 ± 0.3
poly(dI-dC)	0.70 ± 0.04	0.20 ± 0.01	14.3 ± 0.5
poly(dG-dC)	0.21 ± 0.01	0.140 ± 0.005	3.0 ± 0.3

[a] Three determinations each. [b] *P*/*D* values at saturation for poly(dG-m5dC), poly(dI-dC), and poly(dG-dC) are 41.0, 93.0, and 90.0, respectively. [c] *P*/*D* values for poly(dG-m5dC), poly(dI-dC), and poly(dG-dC) are 35.0, 100.0, and 105.36, respectively. Average anisotropy value for the free alkaloid in 20 mM BPES is $(8.58 \pm 0.10) \times 10^{-3}$. [d] ΔT_m is the [T_m of bound − T_m of native polymer]. T_m of B-form of poly(dG-m5dC), poly(dI-dC), and poly(dG-dC) in 4 mM BPE buffer are 91.5, 24.7, and 75 °C, respectively. Melting studies in presence of ADG were performed for a *D*/*P* ratio of 0.372, 0.368, and 0.357 for poly(dG-m5dC), poly(dI-dC), and poly(dG-dC), respectively.

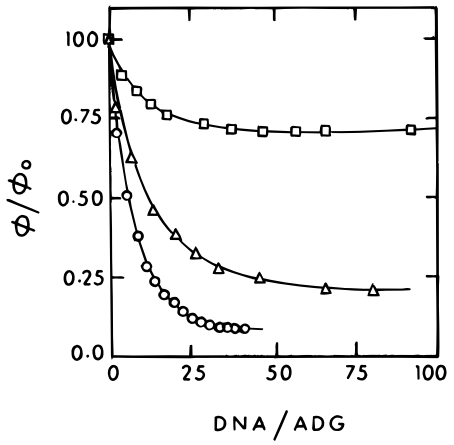

FIGURE 4: Steady-state fluorescence quenching of ADG on binding to different B-form polynucleotides. Plots denote the relative quantum yield (Φ/Φ_o) versus [DNA/ADG] ratio in 10 mM citrate phosphate buffer (pH 7.0) upon interaction with poly(dG-m^{5}dC) (○), poly(dG-dC) (△), and poly(dI-dC) (□) at 25 °C.

sodium molarities, and it was observed that Na^+ molarity greater than 1 M effectively quenches the intensity of the ADG emission band. It was also observed that neither the high salt induced forms of poly(dG-m^{5}dC) and poly(dG-dC) nor the low salt induced form of poly(dG-m^{5}dC) appreciably influence the fluorescence characteristics of ADG, indicating that ADG does not bind to the Z form structure of polymers.

Fluorescence Polarization Anisotropy. Fluorescence anisotropy measurements were also done with B-form polymers. Further evidence for the intercalation of ADG was obtained from the fluorescence polarization measurements. It was found that the fluorescence polarization increases considerably upon binding of ADG to the B-form of all the polymers studied (Table 2). Table 2 shows a large increase in polarization value upon binding of alkaloid to the B-form of poly(dG-m^{5}dC), suggesting that intercalation of ADG into this structure is more favorable than to the other two B-form polymers.

Circular Dichroic Study. Conformational aspects of the binding have been deduced from the CD studies. The interaction of ADG to various polymorphic structures was followed in the ultraviolet region where each polynucleotide assumes a right-handed conformation in low salt buffer. In the presence of ADG the CD spectrum of the B-form of poly(dG-m^{5}dC) and poly(dG-dC) was perturbed significantly. The changes in the CD spectrum of both of the polymers with increasing concentration of ADG are depicted in Figure 5, panels A and C. The molar ellipticity of the positive band of each B-form polymer increased progressively with increasing concentration of ADG, while the negative bands increased with a gradual shift of the band maximum toward the lower wavelength side until saturation at a wavelength of about 233−234 nm. The presence of a weak extrinsic CD band, predominantly positive in character, was noticed in the poly(dG-dC) spectrum at 315 nm (Figure 5C), while no such band appeared for poly(dG-m^{5}dC) (Figure 5A). However, a clear isoelliptic point at a wavelength around 245 nm appeared in the CD titration. Figure 5B shows that the native B-form spectrum of poly(dI-dC) was perturbed significantly in the presence of the alkaloid. Initially the 280−285 nm band decreased in ellipticity as ADG concentration was increased with a concomitant enhancement of the 260−265 nm band ellipticity. Again the small negative band in the 240−245 nm region is increasing significantly with a gradual peak shift toward the lower wavelength side with saturation (*D*/*P* around 0.6) at 235 nm. Here again a positive extrinsic CD band appeared with increasing ADG concentration at 306 nm. The increase in the 306 nm band ellipticity reached saturation at *D*/*P* around 0.6. An isoelliptic point is also apparent near the 245 nm region.

Figure 6A−C illustrates that curve number 1 is the representative salt induced structures which resembles the Z-form of poly(dG-m^{5}dC) (Figure 6A), the high salt induced form of poly(dI-dC) (Figure 6B), and the Z-form of poly(dG-dC) (Figure 6C). The CD spectra obtained from titration of Z-form poly(dG-m^{5}dC) or poly(dG-dC) with ADG are displayed in Figure 6, panels A and C, respectively. All undergo a large conformational change when the polymer

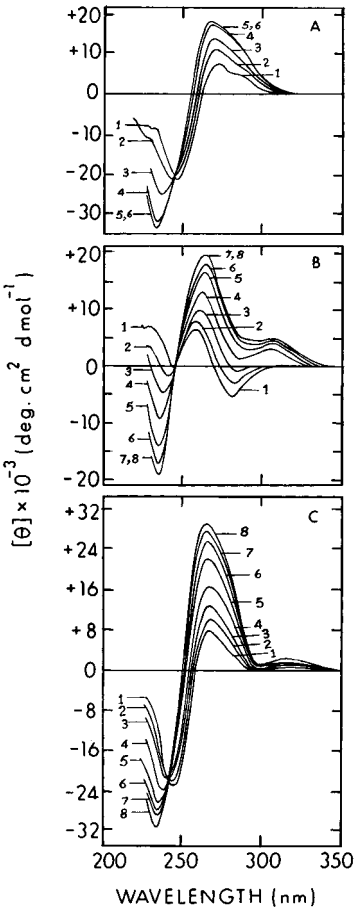

FIGURE 5: Representative circular dichroic spectra resulting from the interaction of ADG with B-form of different polynucleotides in 10 mM citrate phosphate buffer (pH 7.0) at 25 °C. (A) Curves 1−6 denote 60.10 μM poly(dG-m^{5}dC) treated with 0, 3.35, 6.69, 13.33, 18.85, and 25.42 μM ADG, respectively. (B) Curves 1−8 denote 70.29 μM poly(dI-dC) treated with 0, 3.61, 7.20, 11.97, 17.88, 23.74, 29.57, and 35.34 μM ADG, respectively. (C) Curves 1−8 denote 70.71 μM poly(dG-dC) treated with 0, 3.72, 7.43, 14.07, 21.41, 28.70, 35.94, and 43.13 μM ADG, respectively. The expressed molar ellipticity is based on polynucleotide concentration.

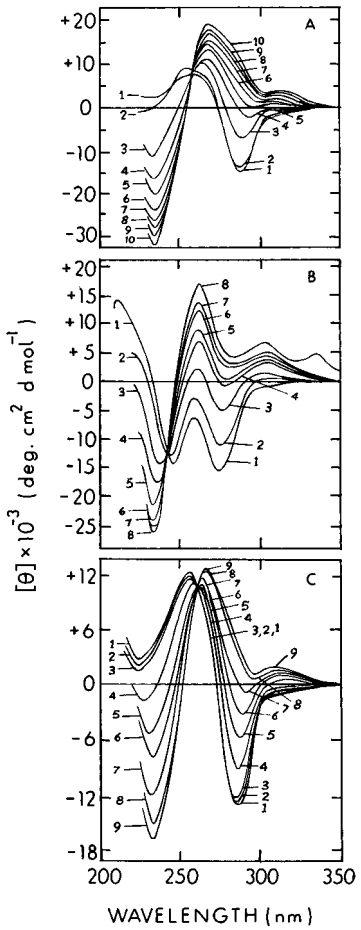

FIGURE 6: Representative circular dichroic spectra resulting from the interaction of ADG with different polynucleotides in buffer under high salt conditions at 25 °C. (A) Curves 1−10 denote 60.35 μM poly(dG-m^{5}dC) treated with 0, 3.57, 8.02, 10.69, 13.33, 15.98, 18.62, 22.13, 26.51, and 30.87 μM ADG, respectively, in the presence of 3.0 M NaCl. (B) Curves 1−8 denote 76.47 μM poly(dI-dC) treated with 0, 3.77, 9.40, 14.07, 18.72, 23.135, 27.97, and 37.15 μM ADG, respectively, in the presence of 4.5 M NaCl. (C) Curves 1−9 denote 70.71 μM poly(dG-dC) treated with 0, 2.23, 5.95, 13.33, 20.67, 27.97, 35.21, 42.41, and 50.98 μM ADG, respectively, in the presence of 4.5 M NaCl.

switches from Z-form to the bound right-handed form. When ADG was added to the Z-form of poly(dG-m^{5}C) at low salt molarity (<0.8 mM), a similar Z to B conformational transformation was observed (data not shown). Figure 5A,C and Figure 6A,C show the similarity in the CD spectral characteristic of alkaloid-bound polynucleotide at saturation regardless of the initial conformation of the individual polymer used.

In the case of the high salt induced form of the poly(dI-dC) polymer, the CD spectral characteristics were greatly affected with increasing concentrations of ADG, and the results are presented in Figure 6B. The salt induced poly(dI-dC), however, interacted in a remarkably different manner. The molar ellipticity value of the negative CD band at 260 nm increased significantly and acquired almost 4 times the initial value at saturation ($D/P \sim 0.6$). Figure 6B shows the total reversal from the altered structure to the B-form bound structure when D/P reached 0.6 (spectrum 7) giving a spectrum which is analogous to the B conformation of the polymer in the presence of the alkaloid (Figure 6B). However, at a D/P ratio of 0.6−0.7, two peaks appear in the wavelength region of 300−350 nm. This may be a direct consequence of the structural variation of the polymer in low and high salt buffer.

In a separate experiment we have measured the kinetics of the B to Z transition of poly(dG-dC) by monitoring the change in absorbance of the polymer at 295 nm in the absence and presence of ADG in 4.5 M NaCl concentration. This salt concentration is high enough to convert poly(dG-dC) from the B to Z conformation.

Figure 7 shows the absorbance traces for a poly(dG-dC) solution following a sudden jump in salt concentration in the absence and in the presence of the alkaloid with constant stirring. Figure 7A clearly illustrates that ADG is significantly inhibiting the rate of the B to Z transition. The extent of inhibition was further confirmed from the CD spectral characteristics presented in Figure 7B. These show that ADG appears to bind more tightly to the B-form. DMSO does not affect the B−Z transition of polymers used which as shown by experiments carried out in presence of 240 mM DMSO.

DISCUSSION

Results obtained previously (Chakraborty et al., 1989a,b, 1990) have indicated that ADG intercalates to DNA as evidenced from hypochromism and bathochromism of the

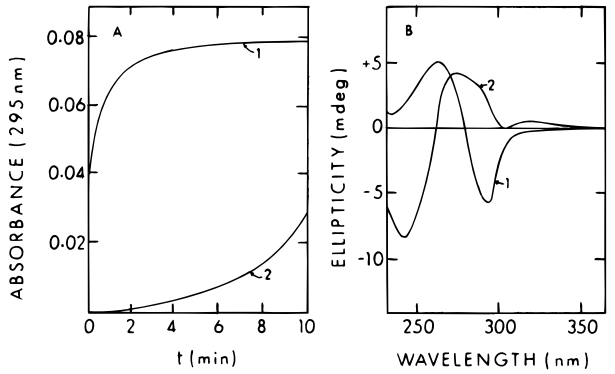

FIGURE 7: Effect of ADG (27.34 μM) on the kinetics of B to Z transition of 45.65 μM poly(dG-dC) in buffer containing 4.5 M NaCl. (A) Absorbance traces at 295 nm as a function of time in the absence (curve 1) and presence (curve 2) of ADG with constant stirring. (B) Characteristic CD spectrum of curve 1 and curve 2 of panel A after 10 min of treatment.

absorption band, quenching of the fluorescence intensity, stabilization of DNA against thermal denaturation for the melting transition, the sign and magnitude of the thermodynamic parameters, the increase in the contour length of sonicated rod-like DNA, and induced unwinding and rewinding process of covalently closed superhelical DNA. Further studies (Nandi et al., 1991) on the molecular nature of the specificity of ADG toward DNAs of varying base composition and sequence indicated that the binding affinity of ADG is higher for GC-rich DNA than for AT-rich DNA with considerable specificity toward alternating GC polymer. The intercalation of ADG to natural DNA is characterized by a favorable negative enthalpy change opposed by a negative entropic contribution to the binding. To investigate the molecular nature of the interaction with two polymorphic forms of alternating heteropolymers of GC and their structural analogue, we have carried out a series of physicochemical measurements.

Right-Handed Form DNA-ADG Complexation. The results presented here show that ADG has strong affinity toward the alternating methylated GC polymer when compared with the alternating GC as well as IC polymer. Binding analysis from the spectrophotometric data indicates that the number of occluded sites (*n*) are more or less the same as presented in Table 1. This high binding affinity is further evident from the steady-state fluorescence studies where the binding to the B-form poly(dG-m^{5}dC) remarkably quenches the intrinsic fluorescence intensity and decreases the fluorescence quantum yield of ADG. These data support our previous results obtained for the complexation of ADG with alternating GC polymer (Nandi et al., 1991). Despite a higher binding constant for poly(dI-dC) than poly(dG-dC), the extent of decrease in relative quantum yield (Figure 4) is lower for the former. This discrepancy could be correlated with the overall structure of the polynucleotide and the relative orientation of the drug after forming complex with poly(dI-dC). Our data on fluorescence polarization measurements clearly indicate that upon intercalation into the helix the rotational motion of the benzophenanthrine chromophore moiety is significantly restricted in all the three B-form polymers. Accordingly, the fluorescence from the bound chromophore in each alkaloid−polynucleotide complex is polarized. In the absence of the polymer, the fluorescence from the benzophenanthrine chromophore is weakly polarized (Table 2) due to the rapid tumbling motion of the chromophore in the aqueous medium. The large increase in the polarization upon binding to poly(dG-m^{5}dC) suggests strong favorable intercalation of ADG to this polymer. Again, the lesser polarization value in ADG-poly(dG-dC) compared to poly(dI-dC) may be due to the different orientation of the ADG molecule at the intercalation site in the two polymers as the glucoside ring in ADG being noncoplanar with the rest of the molecules produces significant effect in the alkaloid fluorescence intensity (Charaborty et al., 1989a). In this context it is interesting to note that several groups have studied the fluorescence anisotropy properties of intercalative drugs (Farmer et al., 1991) with DNA, and their values are in the vicinity of the 0.20−0.30 where the maximum theoretical fluorescence anisotropy value is 0.40 (Farmer et al., 1991). The results of thermal stabilization of B-form poly(dG-dC) and poly(dG-m^{5}dC) by ADG (Table 2) show only about 3−4 °C stabilization, while that of poly(dI-dC) is about 14.3 °C. The small stabilization with poly(dG-dC) is consistent with our earlier observation (Nandi et al., 1991). Several factors are known to contribute to the thermal stabilizing ability of the ligands on the DNA duplex: (a) molecular shape of the complex, (b) van der Waals interaction between the ligand and DNA base pairs, (c) the formation of hydrogen bonds by the ligand with the base pairs or to the groove of the helix, and (d) the existence of the structure of water in the form of spine of hydration in the DNA helical grooves (Nandi et al., 1991; Liepinsh et al., 1992; Kubinec & Wemmer, 1992; Maltseva et al., 1993; Berman, 1994). The higher degree of stabilization in B-form of poly(dI-dC) and ADG complex relative to two other complexes may reflect the differences in the molecular orientation of ADG at the intercalation site including the water structure around the polymer and also the temperature at which the complex is melted.

Our CD data provide an independent measure of the binding affinity of ADG to the three B-form structures and highlight the extent of conformational change associated with the binding of ADG to each polymer. The spectral characteristics are similar to those reported for other intercalators (Waring, 1981a). However, the perturbation of the CD bands of poly(dG-m^{5}dC) is different from that of poly(dG-dC) where a small extrinsic band appeared around 310 nm. This band indicates the asymmetric arrangements of ADG upon binding with poly(dG-dC), while the saturation value attended by poly(dG-dC) is higher than that of poly(dG-m^5-dC). The appearance of the extrinsic CD band may be explained in terms of the higher *D*/*P* value at saturation in the case of poly(dG-dC). The spectral features of the unwinding of the poly(dG-dC) duplex (B-form) upon intercalation and consequent appearance of extrinsic CD is again in conformity with our earlier observations (Nandi et al., 1991). The spectral changes associated with the B-form of poly(dI-dC) are distinct from the other two polynucleotides. The initial native spectrum of poly(dI-dC) have been transformed to the bound B-form CD spectrum which is similar to the poly(dG-dC) with the association of an extrinsic CD band. In this context it has been noticed that in the presence of intercalators CD spectral patterns differ from system to system. Equilibrium studies of the ethidium binding to poly(dI-dC) (Bresloff & Crothers, 1981) indicate conformational switching. In fact, conformational alteration of poly(dI-dC) from B-form structure to A-form structure has been observed under the influence of berberine chloride

(Kumar et al., 1992) and trifluoroethanol (Vorlic'kova & Sagi, 1991) using CD spectroscopy.

The CD spectral data suggest the possibility of a helix to helix conformational transition from a noncanonical B-form structure to B-form upon binding of ADG to poly(dI-dC). The difference in appearance of the extrinsic CD band for the three polymers can be accounted for by the difference in asymmetry induced in these structures.

The temperature dependence of binding of ADG to these three B-form polynucleotides has been used to derive thermodynamic parameters (enthalpy, entropy, and free energy). Conceptually, the thermodynamic parameters describing the binding reactions may be divided into three contributions. First is the contribution from the molecular interactions between the bound ligand and the DNA binding site as result of hydrogen bonding, hydrophobic interactions, van der Waals interactions, and electrostatic interactions (Zieba et al., 1991; Nandi et al., 1991). Next are contributions arising from the conformational changes in either the DNA or the drug upon binding. Finally there are contributions which may be coupled processes like ion release, proton transfer, or changes in the water of hydration (Chalikian et al., 1994). Inspection of thermodynamic data (Table 1) for all three polynucleotides studied reveals that the DNA binding of the alkaloid is an exothermic process and binding to poly(dG-m^{5}dC) produced largest negative change in enthalpy and entropy among the three polynucleotide studied at the intercalation site. However, for all the three polynucleotides the binding is favored by a negative enthalpy change and opposed by a decrease in entropy. A negative value of entropy change may result from a postbinding decrease in flexibility of the double helix which is a characteristic of the intercalation phenomenon (Nandi et al., 1991). It is interesting to observe from Table 1 that the decrease in enthalpy and entropy is of the order poly(dG-dC) < poly(dI-dC) < poly(dG-m^{5}dC) and also that the change in enthalpy and entropy compensated one another to produce a relatively small Gibbs free energy change. Table 1 clearly demonstrates that the binding process is enthalpy driven. The observed compensation temperature (the slope of a plot of the negative enthalpy change versus the negative entropy change, data not shown), 459.5 K, is much higher than the harmonic mean temperature 299 K (i.e., the reciprocal of the average of the reciprocal temperatures expressed in Kelvin). All of these strongly suggest binding events where water plays an important role. Recent physicochemical studies have revealed some intriguing hydration patterns for the nucleic acid that appear to depend on base composition, sequence, and DNA conformation, all of which represent interrelated properties (Westhof, 1988; Kubinec & Wemmer, 1991; Liepinsh et al., 1992; Buckin et al., 1994). In drug−nucleic acid complexes water molecules add stability by participating in the intermolecular interaction (Westhof, 1988; Chalikian et al., 1994; Berman, 1994), and binding of a ligand to a double helix can change the state of hydration of a duplex (Berman, 1994). In our study the thermodynamic characteristics of ADG−polynucleotide complexes show that the enthalpy−entropy change is different for different polymers perhaps reflecting the differential pattern of hydration in the B-form architecture of each polymer (Heinemann & Hahn, 1992) upon binding of the alkaloid.

Interaction of ADG with Left-Handed Form DNA. Our spectrophotometric and spectrofluorimetric titration data show that ADG does not bind to the Z-form of poly(dG-m^{5}dC) and poly(dG-dC). Again it has been found that ADG does not bind to the high salt induced form of poly(dI-dC) as revealed from spectrophotometric and spectrofluorimetric studies. This ligand binding phenomenon of poly(dI-dC) at the high salt clearly indicates the difference between the high salt structure and low salt form of poly(dI-dC). Results presented in Figure 7 demonstrate that ADG inhibits both the rate and extent of the B to Z transition. Drugs from the various classes of intercalators (acridines, phenanthridines, actinomycins, anthracyclines, etc.) alter the kinetics of the B to Z transition suggesting that inhibition of the B to Z transition is a general property of intercalators. The present study demonstrates that ADG shows extraordinary effectiveness toward the inhibition of the B to Z transition which is comparable to actinomycin D (Mirau & Kearns, 1983). ADG can convert Z DNA back to a bound right-handed form under all conditions which otherwise favor the Z-form.

Mirau and Kearns (1983) and Gilbert et al. (1991) reported the relative efficiency of actinomycin D and ethidium bromide as an inhibitor of the B to Z transition. Allosteric conversion of Z DNA to a bound right-handed conformation by daunomycin has been reported (Chaires, 1986) under conditions where the energetically unfavorable Z to B transition was found to be driven by coupling to the energetically favorable interaction of the drug with B-form DNA. Recently, another group reported the elsamicin A induced conversion of Z form of poly(dG-dC) and poly(dG-m^{5}dC) to bound right-handed form (Jimenez-Garcia & Portugal, 1992). We have found that our results are in agreement with the above observations and show that ADG is an effector of the Z to B conformational change. The local difference in the CD spectral changes associated with these two polynucleotides is due in part to the fact that there may be differences in the three-dimensional structure of the bound right-handed forms of these two duplexes owing to different stacking arrangements of the alkaloid after binding to these two duplexes.

Our spectral data for poly(dI-dC) at 4.5 M NaCl concentration matches with that found by Vorlic'kova and Sagi (1991) which they have pointed out as similar to the X-form of the alternating heteropolymer of AT. Vibrational circular dichroism results of Wang and Keiderling (1993) for poly(dI-dC) at high salt conditions showed little change in band shape from the low salt conditions. Their results show little indication of the formation of B−Z intermediate state at high NaCl concentration. On the other hand, they could not conclusively reject the possibility either (Wang & Keiderling, 1993). Our CD spectral data of ADG binding to low salt and high salt forms of poly(dI-dC) (Figures 5B and 6B) suggest the presence of a B−Z intermediate state, whatever minor the difference from the B-form is. This is clear from the presence of an isoelliptic point and induced bands on the CD spectra. CD spectral data in Figure 5B can be considered as an example of B−Z intermediate to bound right-handed conformational transition. The asymmetry induced in both of the cases is distinct and different from one another which can be correlated to the differences in their drug free state.

CONCLUSIONS

In this report we have demonstrated that ADG interacts differentially with various polymorphic forms of poly(dG-

dC), poly(dG-m^{5}dC), and poly(dI-dC) as revealed from various spectroscopic and thermodynamic measurements. The following features emerge out of our interpretation of the data.

(1) Spectrophotometric, thermal melting stabilization, steady-state fluorescence polarization anisotropy, and CD data clearly support that the mode of binding of ADG to B-form polymers is intercalative in nature and strongest for B-form methylated DNA. Binding is of the order poly(dG-m^{5}dC) > poly(dI-dC) > poly(dG-dC).

(2) Thermodynamic study revealed that the interaction is exothermic, and the binding process is enthalpy driven. The intermolecular interaction is characterized by a favorable negative enthalpy change and opposed by a negative entropy change in each B-form polymer.

(3) CD spectral data clearly demonstrated that ADG binds to the B-form of all the three polymers resulting in the spectrum of bound canonical right-handed complex. It inhibits B to Z transition as reported for other intercalators. The alkaloid induces a conformational switching from left-handed induced form to bound right-handed form upon binding to left-handed poly(dG-m^{5}dC) and poly(dG-dC).

(4) ADG does not bind to Z-form of poly(dG-m^{5}dC) and poly(dG-dC) polymers or to high salt induced form of poly-(dI-dC), but it converts these structures to bound B-form structures.

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