Differential Interactions of the Mg^{2+} Complexes of Chromomycin A_3 and Mithramycin with Poly(dG-dC)·Poly(dC-dG) and Poly(dG)·Poly(dC)

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ABSTRACT: The interaction of the two anticancer antibiotics, chromomycin A_3 and mithramycin, with the polynucleotides poly(dG-dC).poly(dC-dG), representative of B-DNA, and poly(dG)·poly(dC), representative of A-DNA, in the presence of Mg^{2+} is studied by spectroscopic techniques such as absorbance, fluorescence, and dircular dichroism (CD). The studies were done with both drug·Mg^{2+} complexes, I and II, having 1:1 and 2:1 stoichiometries with respect to drug and Mg^{2+}, respectively [Aich, P., Sen, R., & Dasgupta, D. (1992) *Biochemistry 31,* 2988−2997]. The objective of the present work is 2-fold. First, an attempt is made to understand the structural basis of the ligand−DNA interaction, particularly the role of DNA backbone conformation with its groove size and the accessibility of the 2-amino group in the minor groove of guanosine. Second, the role of the antibiotic saccharide moieties in the association with DNA was studied. For this purpose, the spectroscopic characterization of the binding was done followed by the evaluation of binding parameters and associated thermodynamics. Analysis of the observed thermodynamics for the ligand−DNA interactions in terms of the different structures of the polynucleotides was done. The salient results are as follows. Complex I does not discriminate significantly among the A- and B-forms of DNA when it binds to them in an entropy-driven process. On the other hand, complex II for both drugs recognizes B- and A-forms of DNA in different ways. This observation implies that the sequence specificity shown by this complex is a sequel to the difference in the parameters such as groove size and accessibility of the guanosine amino group. Another important finding is that binding with the same polynucleotide is not comparable for the complex II of the two drugs. It emphasizes the involvement of the sugar moieties, when the drug·Mg^{2+} complex binds to DNA. The presence of an acetoxy group in the sugars of chromomycin A_3 imparts some distinctive specific features of the association of the chromomycin dimer·Mg^{2+} complex with DNA. Finally, the results are compared with those available from NMR studies of different drug−oligonucleotide complexes under conditions where complex II is the ligand.

Mithramycin (MTR)[1] and chromomycin A_3 (CHR) are two naturally occurring antibiotics produced from *Streptomyces plicatus* and *Streptomyces griseus,* respectively (Figure 1). They belong structurally to the aureolic acid group, having the same chromophore (aglycon ring). The difference is in the nature of the sugar rings connected to either side of the aglycon ring via O-glycosidic bonds (Figure 1; Thiem & Meyer, 1979, 1981). The observed antitumor properties of these drugs in experimental tumors are ascribed to their inhibitory effects on replication and transcription processes during macromolecular biosynthesis (Gause, 1975; Calabresi & Chabner, 1991). They are (G-C) base-specific (Goldberg & Friedman, 1971; Waring, 1981) and require Mg^{2+} for binding to DNA at and above neutral pH (Waring, 1981). It was shown from our laboratory that two different types of drug·Mg^{2+} complexes are formed when the drug binds to Mg^{2+} in the absence of DNA (Aich & Dasgupta, 1990; Aich et al., 1992). The stoichiometries of these complexes are 1:1 (complex I) and 2:1 (complex II) in terms of drug:Mg^{2+} ratio. These complexes, which are different molecular entities, are the DNA binding ligands at and above physiological pH. It was established from spectroscopic and thermodynamic studies that the modes of binding of the two drug·Mg^{2+} complexes to calf thymus DNA are different (Aich & Dasgupta, 1995).

The selectivity for guanine base was proposed from a comparison of the binding affinities of the antibiotics with polynucleotides and natural DNA containing different percentages of G-C bases (Goldberg & Friedmann, 1971). Subsequent footprinting studies with restriction fragments revealed that the antibiotic(s) prefers a GpG or GpC dinucleotide step (Van Dyke & Dervan, 1983). However, all GpG or GpC/CpG sites are not equally protected and the flanking sequences, not containing guanine bases play an important role in the degree of protection (Fox & Howarth, 1985; Cons & Fox, 1989; Stankus et al., 1992). Sarker and Chen (1989) carried out a study on the effect of different GC sequences at the polymer level upon the MTR−DNA interaction in the presence of Mg^{2+}. Recently, Liu and Chen (1994) have reported sequence-specific binding of CHR to DNA decamers, d(GTA-XGCY-TAC) where X, Y = A, G, C, or T, in the presence of millimolar concentrations of Mg^{2+}. NMR studies were also done to elaborate the mode of

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[1] Abbreviations: MTR, mithramycin; CHR, chromomycin A_3; poly(dG-dC), double-stranded alternating copolymer, poly(dG-dC)·poly(dC-dG); *F* (a.u.), fluorescence (arbitrary units); CD, circular dichroism.

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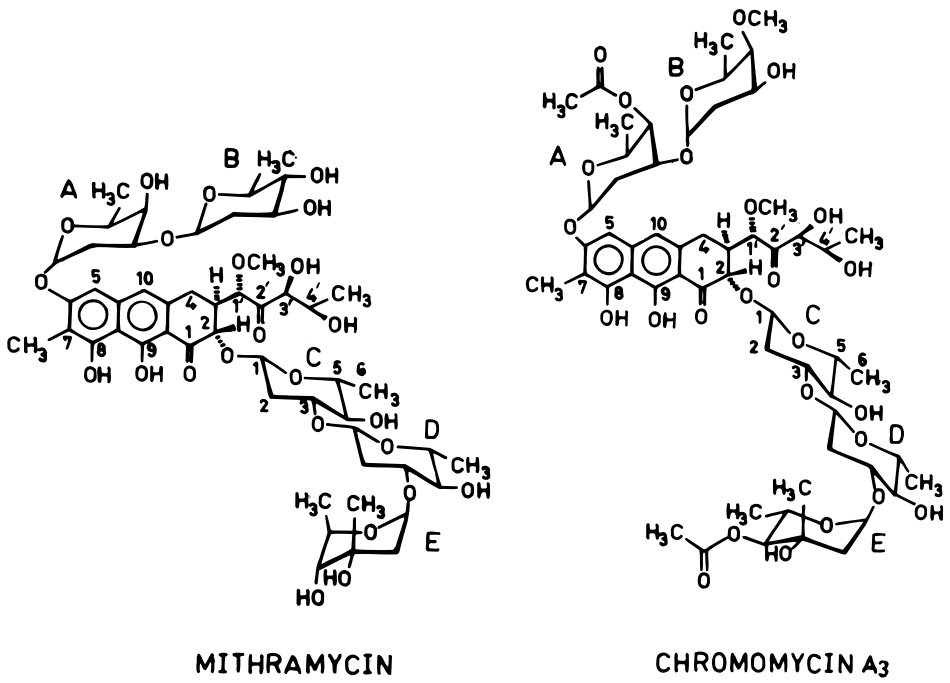

FIGURE 1: Structure of chromomycin A_3 and mithramycin (Thiem & Meyer, 1979).

binding of these drugs at millimolar concentration of Mg^{2+} to oligonucleotides containing (GpC) sequences flanked by A and T bases. Drug dimer·Mg^{2+} complex binds to oligonucleotide at millimolar concentration of Mg^{2+}. These studies provided useful information about the geometry of the complexes formed between complex II and oligomers. According to these studies, base specificity occurs as a sequel to H-bonding between the phenolic group of the antibiotic and the amino group in the minor groove side of guanine base in a Watson−Crick base-paired DNA duplex (Banville et al., 1990; Keniry et al., 1987, 1993; Gao & Patel,1989; Sastry & Patel, 1993; Sastry et al., 1995). NMR results and molecular dynamics simulation studies were interpreted in terms of a B to A structural transition of the oligonucleotides at the binding site induced by the bulky dimer·Mg^{2+} complex (Gao & Patel, 1989; Sastry & Patel, 1993; Sastry et al., 1995). Positive change of the enthalpy during complex II−DNA association was also ascribed to a change in conformation from the low-salt B-form of DNA at the binding site of the drug dimer·Mg^{2+} complex (Aich et al., 1992; Aich & Dasgupta, 1995). Other studies from our laboratory indicated that this complex could discriminate between right- and left-handed forms of poly(dG-dC) (Dasgupta et al., 1992). We showed that calf thymus DNA binding properties of complex II for the two antibiotics are different, implying that their saccharide chains are involved in the interaction (Aich & Dasgupta, 1995). NMR studies also proposed the same (Keniry et al., 1993; Sastry & Patel, 1993). However, a lacuna in the above studies is the absence of information on the effect of DNA conformation upon the accessibility of the 2-amino group of guanine to the drug·Mg^{2+} complex, which finally leads to the sequence-specific recognition. Moreover, measurement of thermodynamic parameters in conjunction with NMR and molecular dynamics simulation is very useful in such cases. Also, NMR studies do not tell anything about the molecular basis of the sequence-specific recognition of DNA by complex I.

Therefore, as a continuation of our studies to understand the molecular basis of the interactions of these antibiotics with the prime cellular target, DNA, we report here a comparative analysis of the interactions of the drug (CHR and MTR)·Mg^{2+} complexes with two polynucleotides, poly(dG-dC) and poly(dG)·poly(dC), using several spectroscopic techniques. These polynucleotides bind to the drugs via hydrogen-bond formation involving the 2-amino groups of guanine base, yet they may show differences in binding characteristics due to intrinsic conformational differences of the polynucleotides. At low ionic strength poly(dG-dC) remains in the B-form (Saenger, 1983) and poly(dG)·poly(dC) has an A conformation (Wells et al., 1970; Sarma et al., 1986) characterized by a C3′-*endo* furanose ring and a wider and shallower minor groove. We chose the polynucleotides to examine how minor groove dimensions and local helical parameters of DNA influence the mode of interaction of these ligands with DNA. The study would help us to understand the effects of (i) the different sequences, GpG and GpC/CpG, (ii) sequence-dependent structural polymorphism of DNA, and (iii) different saccharide moieties of the antibiotics upon the sequence specificity of drug−DNA interaction. The polynucleotides were preferred to short oligomers, because in the latter the "end effect" due to flanking unstacked base pairs might induce alteration of the drug-binding specificity (Dickerson, 1992). This might constrain extrapolation of the results to natural DNA, particularly in the case of a bulky ligand like drug dimer·Mg^{2+} complex II. Spectroscopic techniques such as absorption, fluorescence, and CD were used because they permit us to work at micromolar ranges (1−50 μM) of concentration of the drug, where aggregation of the free drug is absent as detected by absorption, fluorescence, and CD spectroscopy and there is a single population of the drug to start with. The interactions were further characterized from the evaluation of the associated thermodynamic parameters, free energy, enthalpy, and entropy. Analysis of these data has helped us to understand the structural basis of the recognition, such as the role of backbone conformation with its groove size and accessibility of the amino group in guanosine. Finally, the results are discussed taking into view the

available structural information from NMR spectroscopic studies and subsequent molecular dynamics simulations.

MATERIALS AND METHODS

Mithramycin, chromomycin A_3, Tris, and magnesium chloride solution (4.9 M) were from Sigma Chemical Co. The polynucleotides, poly(dG-dC) and poly(dG)·poly(dC), were from Pharmacia Biotech Ltd., Sweden. Unless mentioned otherwise, all studies were done in 20 mM Tris-HCl buffer, pH 8.0. The buffer was prepared in quartz-distilled deionized water from a Milli-Q source (Millipore Corp). The concentrations of the antibiotics and polynucleotides were determined from the known molar extinction coefficients (Wells et al., 1970; Aich et al., 1992; Aich & Dasgupta, 1995).

Absorption, fluorescence, and CD spectra were recorded with a Hitachi U-2000 spectrophotometer, a Shimadzu RF-540 spectrofluorometer, and a Jasco J-720 spectropolarimeter, respectively. Fluorescence measurements for the drugs and their complexes with Mg^{2+} were carried out at an excitation wavelength of 470 nm instead of the absorption maximum to avoid photodegradation (Aich et al., 1992). Absorbance of the samples did not exceed 0.02. Therefore, we did not correct the emission intensity for optical filtering effect. The CD spectra were recorded in a cuvette of 1-cm path length. They were expressed as observed value in millidegrees. All spectra are average of two runs. They were smoothed within the permissible limits by the inbuilt software of the instrument.

The results from the spectrofluorometric titration to evaluate the binding parameters for the polynucleotide−ligand interactions were analyzed in the following ways. Apparent binding constant (K_{app}) was determined by means of the following equation (Wang & Edelman, 1971):

$$1/\Delta F = 1/\Delta F_{max} + 1/[K_{app}\cdot\Delta F_{max}(c_p - c_0)] \quad (1)$$

where ΔF is the change in fluorescence emission intensity at 540 nm ($\lambda_{ex} = 470$ nm) upon addition of each aliquot of polynucleotide and ΔF_{max} is the same parameter when the ligand is totally bound to DNA. c_p is the concentration of the polynucleotide and c_0 is the initial concentration of the ligand. A linear plot of $1/\Delta F$ against $1/(c_p - c_0)$ is extrapolated to the ordinate. The reciprocal of the intercept on the ordinate gives the value of ΔF_{max}. K_{app} was obtained from the ratio of intercept and slope of the plot. This approach is based on the assumption that the emission intensity is linearly proportional to the concentration of the ligand. It was checked and found to be valid for the concentration range (1−50 μM) of the ligand employed here. The second method used the Scatchard equation (Scatchard, 1949) to estimate the intrinsic binding constant (K_0) and binding stoichiometry (n):

$$r/c_f = K_0(n - r) \quad (2)$$

r/c_f is plotted against r and the best-fit straight line of the experimental points was drawn. In the above equation, $r = c_b/c_p$, where c_b is the concentration of the bound ligand and c_p is the input concentration of DNA. n is the binding stoichiometry expressed as the number of bound drug molecules per nucleotide and K_0 is the intrinsic binding constant ($K_{app} = K_0 n$). The concentration of the bound ligand (c_b) was calculated from $c_b = (\Delta F/\Delta F_{max})c_0$.

Similarly, the results from the spectrophotometric titration were analyzed as follows (Dasgupta & Goldberg, 1985):

$$1/\Delta\epsilon = 1/\Delta\epsilon' + 1/[K_{app}\Delta\epsilon'(c_p - c_0)] \quad (3)$$

where $\Delta\epsilon = \epsilon_{obs} - \epsilon_f$ (ϵ_{obs} is the apparent molar extinction coefficient of the bound ligand measured from the observed absorbance for each point of titration curve, and ϵ_f is the molar extinction coefficient of the free ligand), $\Delta\epsilon' = \epsilon_b - \epsilon_f$ (ϵ_b is the molar extinction coefficient of the bound ligand) (Li & Crothers, 1969; Dasgupta & Goldberg, 1985). This equation is valid under the condition $c_p \gg c_0$, which was followed by keeping at least an 8-fold excess of DNA. For the Scatchard plot, concentration of the bound ligand (c_b) was evaluated from the relation $c_b = \Delta A/(\epsilon_b - \epsilon_f)$, where ΔA is the increase in the absorbance (at 440 nm) of the ligand upon addition of the polynucleotide.

Binding stoichiometry of the polynucleotide−ligand complex was also estimated from the intersection of two straight lines of the plot of normalized increase in fluorescence against the ratio of the input concentrations of polynucleotide and ligand. It is obtained as the number of bound nucleotides per drug molecule, and therefore, it is the reciprocal of the binding stoichiometry (n) determined from the Scatchard plot.

Thermodynamic parameters, ΔG (free energy), ΔH (heat content), and ΔS (entropy), were evaluated from:

$$\ln K_{app} = -(\Delta H/RT) + (\Delta S/R) \quad (4)$$

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

where R and T are the universal gas constant and absolute temperature, respectively (Castellan, 1989). For determination of ΔH, the apparent (K_{app}) or intrinsic (K_0) binding constant was measured at three different temperatures. ΔH and ΔS values are obtained from the slope and intercept of the plot of ln K_{app} (or ln K_0) against $1/T$.

RESULTS

Binding of Complexes I and II to the Polynucleotides. Concentrations of the drugs and Mg^{2+} were so chosen in these experiments with the help of the results from spectrophotometric titration of drug with Mg^{2+} (Aich et al., 1992; Aich & Dasgupta, 1995) that they led to formation of a single type of complex (either complex I or complex II) and we do not get a mixed population of the two complexes. Association of the ligands, drug·Mg^{2+} complexes I and II, with the polynucleotides was indicated from changes in the absorption, fluorescence, and CD spectra of the ligands and the polynucleotide concentration-dependent nature of the changes.

A red shift and broadening of the peak in the visible absorption spectrum of the ligand and an increase in the absorbance at longer wavelength were the features of the polynucleotide-induced change in absorption spectra. Panels a and b of Figure 2 represent the absorption spectra of complex I for MTR in the presence of poly(dG)·poly(dC) and that of complex II for MTR in the presence of poly(dG-dC), respectively. There is an isosbestic point for a particular set of ligand−polynucleotide interactions which remains the same over the range of input concentrations of

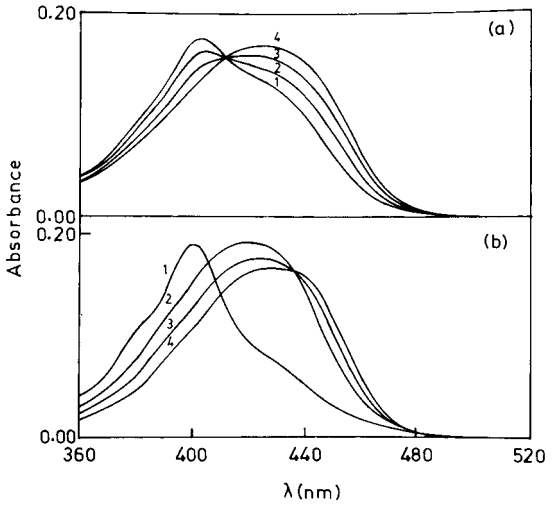

FIGURE 2: Absorption spectra of MTR and its Mg^{2+} complex in 20 mM Tris-HCl buffer, pH 8.0, at 25 °C. (a) Change in the absorbance spectra of MTR (18.8 μM) plus Mg^{2+} (0.21 mM) mixture (complex I) in the absence (1) and in the presence of (2) 22.2 μM, (3) 64.6 μM and (4) 84.9 μM poly(dG)·poly(dC). (b) Change in the absorbance spectra of MTR (18.8 μM) plus Mg^{2+} (9.9 mM) mixture (complex II) in the absence (2) and presence of (3) 54.2 μM and (4) 153.0 μM polydG-dC. The spectrum of free MTR (18.8 μM) is also shown for comparison in panel b (spectrum 1).

the polynucleotide (Figure 2). The observation of a good isosbestic point supports analysis in terms of free ligand and one type of bound ligand. In general, the extent of red shift and increase in the absorbance of the complexes upon addition of a saturating concentration of polynucleotide were dependent on the nature of both the complex and the polynucleotide. This is consistent with our earlier reports on the interactions of the complexes with calf thymus DNA (Aich et al., 1992; Aich & Dasgupta, 1995).

Increase in fluorescence of the ligands occurred as a result of binding to the polynucleotide. A blue shift of the peak accompanied the increase in fluorescence. The extent of blue shift and increase in the fluorescence intensity of the ligand depend on the nature of the complex and the polynucleotide. These features are apparent from the representative fluorescence spectra shown in the four panels of Figure 3. Comparison of the spectra in terms of intensity of the peaks and their positions (summarized in Table 1) brings out more specific differences. Among the polynucleotides, poly(dG)·poly(dC) induces a larger blue shift for both complexes of the same drug, thereby showing that the two drug·Mg^{2+} complexes have different electronic environments in the two polynucleotides. Among the same complexes of the two drugs with poly(dG-dC), complex I for CHR has the largest blue shift. It appears that the discriminating features among the two drugs are better reflected when their Mg^{2+} complexes bind to the alternating copolymer. On the other hand, the relative increase in the fluorescence of the ligand upon binding to DNA is maximal when poly(dG)·poly(dC) binds to complex II of CHR.

We compared CD spectroscopic properties of the complexes in the presence of the two polynucleotides. CD spectra of both complexes changed when they bound to the polynucleotides (Figure 4). Changes in the spectrum of complex I for both drugs in the presence of the polynucleotides are grossly similar to each other and to those reported for natural DNA (Aich et al., 1992; Aich & Dasgupta, 1995).

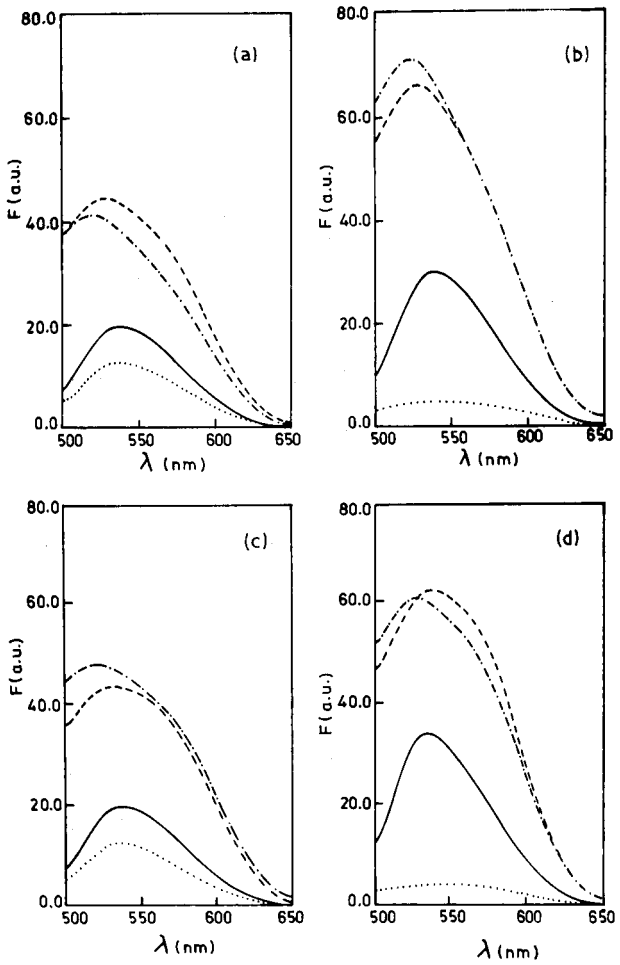

FIGURE 3: Fluorescence emission spectra of complexes I and II for CHR and MTR under different conditions in 20 mM Tris-HCl buffer, pH 8.0, at 37 °C. (a) CHR (17 μM) alone (—); CHR plus 0.33 mM Mg^{2+} (complex I) (···); complex I in the presence of 150 μM poly(dG)·poly(dC) (— · —); complex I in the presence of 200 μM poly(dG-dC) (---). (b) CHR (18 μM) alone (—); CHR plus 11.3 mM Mg^{2+} (complex II) (···); complex II in the presence of 172 μM poly(dG)·poly(dC) (— · —); complex II in the presence of 97 μM poly(dG-dC) (---). (c) MTR (18 μM) alone (—) MTR plus 0.21 mM Mg^{2+} (complex I) (···); complex I in the presence of 148 μM poly(dG)·poly(dC) (— · —); complex I in the presence of 175 μM poly(dG-dC) (---). (d) MTR (17 μM) alone (—); MTR plus 9.9 mM Mg^{2+} (complex II) (···); complex II in the presence of 176 μM poly(dG)·poly(dC) (— · —); complex II in the presence of 93 μM poly(dG-dC) (— — —). Concentrations of the polynucleotides are saturating. The fluorescence spectra shown here are unsubtracted for the contribution from the buffer base line (values less than 2%).

However, there are fine differences between the spectra in the presence of the polynucleotides, e.g., the cross-over points and intensity of the negative band in the region 350−400 nm are different (Figure 4a). Features of the CD spectra of complex II for MTR are comparable in the presence of the two polynucleotides (not shown). There is a difference only in the band intensity of the bound ligand. However, CD spectra of complex II for CHR in the presence of two polynucleotides have markedly different features. A positive peak at 467 nm of poly(dG-dC)-bound complex II shifts to 416 nm for poly(dG)·poly(dC)-bound complex II (Figure 4b).

Evaluation of the Affinity Parameters, Intrinsic Binding Constant (K_0) and Binding Stoichiometry (n), for Polynucleotide−Ligand Interaction. We estimated the affinity parameters, *viz.*, binding constant and stoichiometry, for the polynucleotide−ligand interactions from the absorption and

Table 1: Peak Shift in Fluorescence Emission Spectrum of Complexes I and II upon Interaction with Polynucleotides in 20 mM Tris-HCl Buffer, pH 8.0, at 37 °C

drug	[Mg^{2+}] (mM)	complex	DNA	$\Delta\lambda^a$ (nm)
CHR	0.33	**I**	poly(dG-dC)	10
			poly(dG)·poly(dC)	15
CHR	11.3	**II**	poly(dG-dC)	7
			poly(dG)·poly(dC)	13
MTR	0.21	**I**	poly(dG-dC)	2
			poly(dG)·poly(dC)	16
MTR	9.9	**II**	poly(dG-dC)	3
			poly(dG)·poly(dC)	12

[a] The values are measured with reference to the peak in the fluourescence spectrum of the drugs in the absence of Mg^{2+}. Excitation wavelength was 470 nm; the excitation and emission slit-widths were 5 and 10 nm, respectively. The methodology to prepare the complexes is described in Materials and Methods.

fluorescence titration of the ligand in the presence of varying concentrations of the polynucleotide. Since a knowledge of the binding stoichiometry would help to compare the geometry of the different ligand−polynucleotide complexes, we employed it as a parameter to characterize the complexes. Representative binding isotherms (Figure 5) for complex II of CHR with the two polynucleotides appears to suggest noncooperative interaction. As reported earlier in the case of calf thymus DNA (Aich & Dasgupta, 1995), representative Scatchard plots (Figure 6) for complex II of MTR with the polynucleotides are linear within the limit of experimental error and do not show significant deviation characteristic of neighbor exclusion-type binding near the abscissa (Mcghee & von Hippel, 1974). The double-reciprocal plots (Li & Crothers, 1969; Wang & Edelman, 1971) are also linear (not shown). We also evaluated the binding stoichiometry from the intersection of the two straight lines representing binding isotherms as shown in Figure 5. Table 2 summarizes the values of binding stoichiometry and affinity constant characterizing the association. The internal consistency of these values determined by different analytical approaches justifies the validity of the methods for analysis of the binding. Furthermore, the two methodologies, absorbance and fluorescence titration of the complexes, give comparable results. The binding stoichiometry characterizing complex II−polynucleotide(s) interactions in the case of MTR also agrees grossly with the value reported earlier for similar systems (Sarker & Chen, 1989). However, the concentration of Mg^{2+} (0.5 mM) used in their study might lead to a mixed population of the ligands, complex I and II (Aich & Dasgupta, 1990). Therefore, we refrain from any detailed comparison. Difference in the molecular nature of the two complexes, I and II, is clearly indicated from a comparison of the two binding parameters characterizing their associations with the same polynucleotide. This is consistent with our earlier results reported from the studies on the interaction of these complexes with calf thymus DNA (Aich et al., 1992a). In the case of complex I for both drugs, poly(dG-dC) shows a higher affinity for MTR as indicated from a higher value for K_{app} (K_0n). Poly(dG)·poly(dC) does not show such preference in binding to complex I of both drugs. On the other hand, poly(dG)·poly(dC) has a higher affinity for complex I of CHR relative to poly(dG-dC), and the reverse is the trend for complex I of MTR. Summing up, we propose that complex I−polynucleotide interaction depends upon the nature of the drug and ploynucleotide, though it is not so pronounced as in the case of complex II−polynucleotide interaction. The drug-dependent nature of the association of either poly(dG-dC) or poly(dG)·poly(dC) with complex II is apparent from the binding stoichiometry of the complexes. The two polynucleotides bind to complex II of the same drug with different binding stoichiometries. The dependence of DNA binding parameters of the same type of complex upon the nature of the antibiotic reflects the role of sugar residues in the ligand−DNA recognition. Though each ligand exhibits a different affinity constant (K_0) and stoichiometry (n) for the polynucleotides, it is interesting to note that the apparent affinity constants do not vary widely. This could explain the observation that both GpG and GpC steps are protected by complex II during footprinting studies (Van Dyke & Dervan, 1983).

The above features are better understood if we examine the thermodynamic parameters of the association instead of comparison of the affinity constants at a single temperature, because enthalpy−entropy compensation may give comparable free energy changes for the interaction ($\Delta G = \Delta H - T\Delta S$).

Evaluation of Thermodynamic Parameters. van't Hoff thermodynamic parameters were evaluated from the variation of apparent binding constant ($K_{app} = K_0n$), estimated by means of the fluorescence titration (Aich et al., 1992), with temperature. Panels a and b of Figure 7 contain the representative plots of ln K_{app} against $1/T$ for the interactions of both polynucleotides with complexes I and II of CHR. The opposite slopes in the van't Hoff plots for the interaction between complex II of CHR and the two polynucleotides is a notable feature. Table 3 summarizes the values for eight different systems. Binding of complex I for MTR and CHR with both polynucleotides were endothermic with small ΔH values. It indicates that these were entropy-driven processes. Interactions of complex II for MTR with the two polynucleotides were exothermic in nature with a significant difference in the values for the associated enthalpy changes. On the other hand, the interaction of complex II for CHR with poly(dG-dC) was endothermic and, therefore, an entropy-driven process. In contrast, its association with poly(dG)·poly(dC) was exothermic.

DISCUSSION

We have reported here the interaction of four ligands with two polynucleotides, poly(dG-dC) and poly(dG)·poly(dC). The important conclusions emerging from our study are as follows. Each ligand shows a difference in the binding and thermodynamic parameters of association with poly(dG-dC) and poly(dG)·poly(dC); this is direct evidence for the base-sequence-specific nature of the ligand−DNA interaction. It may be ascribed to the difference in the structures of the two polynucleotides in terms of the minor groove size and accessibility of the guanosine amino groups. The base sequence specificity in the recognition of DNA was reported earlier in the footprinting studies of the association of the drug·Mg^{2+} complexes with restriction fragments of defined sequences (Van Dyke & Dervan, 1983; Stankus et al., 1992). However, proper characterization of the ligands formed at different ranges of concentrations of Mg^{2+} was lacking in these studies. In addition, the conformations of the restriction fragments were also not defined in terms of the plausible sequence-dependent structural heterogeneity. The present

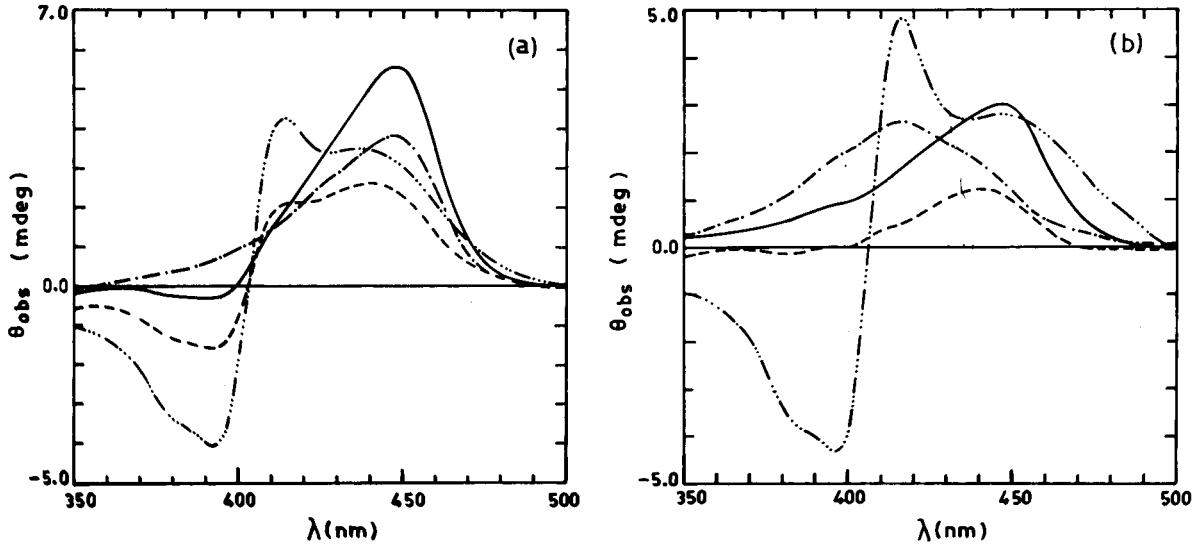

FIGURE 4: CD spectra (visible region) of MTR and CHR and their Mg^{2+} complexes under different condition in 20 mM Tris-HCl buffer, pH 8.0, at 25 °C. (a) MTR (20 μM) alone (− ··· −); MTR·Mg^{2+} complex I ([MTR] = 20 μM and [Mg^{2+}] = 0.2 mM) (---); complex I in the presence of 100 μM poly(dG)·poly(dC) (− · −); complex I in the presence of 98.5 μM poly(dG-dC) (—). (b) CHR (20 μM) alone (− ··· −); CHR·Mg^{2+} complex II ([CHR] = 20 μM and [Mg^{2+}] = 11.7 mM) (---); complex II in the presence of 118 μM poly(dG)·poly(dC) (− · −); complex II in the presence of 121 μM poly(dG-dC) (—).

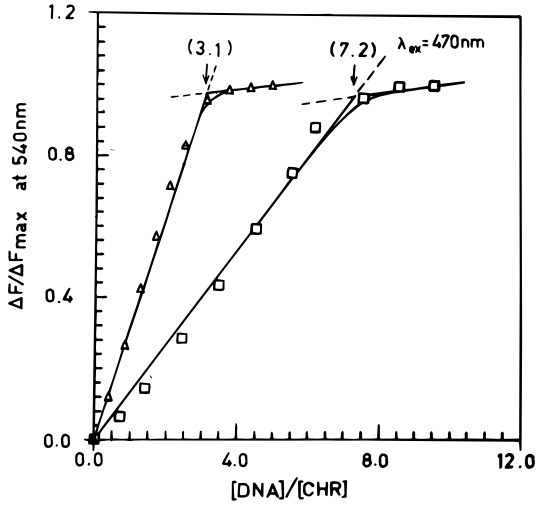

FIGURE 5: Binding isotherms for the association of CHR·Mg^{2+} complex II ([CHR] = 18 μM and [Mg^{2+}] = 11.3 mM) with polynucleotides in 20 mM Tris-HCl buffer, pH 8.0, at 37 °C: poly(dG)·poly(dC) (○) and poly(dG-dC) (△). The normalized increase in fluorescence ($\Delta F/\Delta F_{max}$) is plotted against the ratio of molar concentrations of the polynucleotides to drug. The binding stoichiometry in terms of number of nucleotide bases/drug molecule is the value at the intersection of the two broken lines as shown in the figure.

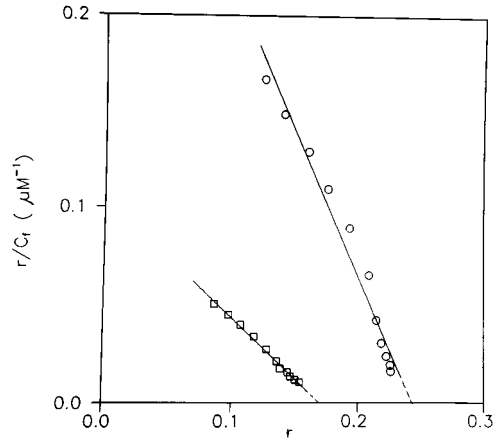

FIGURE 6: Scatchard plots for the interaction of MTR·Mg^{2+} complex II ([MTR] = 17 μM and [Mg^{2+}] = 9.9 mM) with polynucleotides in 20 mM Tris-HCl buffer, pH 8.0, at 15 °C: poly(dG-dC) (○) and poly(dG)·poly(dC) (□).

data show that complex II recognizes B- and A-forms of DNA in different ways. As an extension of this observation, it may be suggested that the sequence specificity is a consequence of the difference in the sequence-dependent structural heterogeneity. Another important finding is that binding with the same polynucleotide is not comparable for complex II of the two drugs, thereby clearly emphasizing the involvement of the sugar moieties when the drug·Mg^{2+} complex binds to DNA.

Results from the present studies also provide further support to our earlier proposition that the two complexes, I and II, for both MTR and CHR recognize the same DNA with different binding and thermodymamic parameters. It justifies our previous characterization of these complexes as two different DNA binding ligands (Aich et al., 1992a; Aich & Dasgupta, 1995). Under *in vivo* conditions, the intracellular concentration of Mg^{2+} would lead to the formation of complex II.

Binding Potentials of the Two Complexes with the Same Polynucleotide Are Different. Complexes I and II of CHR and MTR are separate molecular entities. The results unequivocally indicate that the mode of interaction as well as the conformation of each type of complex bound to the polynucleotides is different. Comparison of the spectroscopic properties of the complexes when they are bound to the polynucleotides further supports this conclusion. The DNA binding properties of these two species are summed up as follows.

Both complexes I and II recognize GpG and GpC dinucleotide steps with comparable apparent binding constant (K_{app}, Table 2). But the binding site size is different. Complex II of both drugs protects about 6−7 bases of poly(dG)·poly(dC) and about 3−4 bases of poly(dG-dC). On the other hand, with the exception in the case of complex I of MTR−poly(dG-dC) interaction, this ligand has a comparable site size of 4−5 bases for both polynucleotides. It

Table 2: Binding Parameters for the Interaction of Drug·Mg^{2+} Complexes I and II with Polynucleotides in 20 mM Tris-HCl Buffer, pH 8.0, at 37 °C[a]

drug	complex	DNA	K_0[b] ($\times 10^5$ M^{-1})	n[b]
CHR	**I** ([Mg2+] = 0.33 mM)	poly(dG-dC)	2.7 ± 0.7	0.23 (0.23)[c]
		poly(dG)·poly(dC)	7.8	0.21 (0.21)
CHR	**II** ([Mg^{2+}] = 11.3 mM)	poly(dG-dC)	3.0	0.32 (0.31)
		poly(dG)·poly(dC)	4.6	0.15 (0.14)
MTR	**I** ([Mg^{2+}] = 0.21 mM)	poly(dG-dC)	13.0 ± 0.7	0.15 (0.15)
		poly(dG)·poly(dC)[d]	5.0	0.20 (0.21)
		poly(dG)·poly(dC)	6.3	0.21 (0.21)
MTR	**II** ([Mg^{2+}] = 9.9 mM)	poly(dG-dC)	7.5	0.24 (0.24)
		poly(dG-dC)[d]	5.2	0.26 (0.26)
		poly(dG)·poly(dC)	4.9	0.18 (0.17)

[a] Complexes I and II were prepared by mixing the drug (17−20 μM) and specified concentration of Mg^{2+} in the buffer and incubating for 1 h. The values were determined from spectrofluorometric titrations such as that shown in Figure 3. [b] Intrinsic binding constant (K_0) and binding stoichiometry (n in drug bound per nucleotide base) were determined as stated under Materials and Methods. [c] Shown in parentheses is the binding stoichiometry from the binding isotherm shown in Figure 5. [d] Determined from spectrophotometric titrations, a representative example of which is shown in Figure 2.

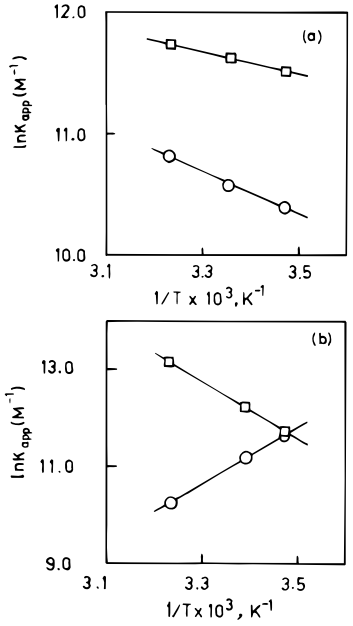

FIGURE 7: van't Hoff plots for the interaction of CHR·Mg^{2+} complexes and polynucleotides in 20 mM Tris-HCl buffer, pH 8.0. (a) Complex I ([CHR] = 17 μM, [Mg^{2+}] = 0.33 mM) with poly(dG)·poly(dC) (□) and poly(dG-dC) (○). (b) Complex II ([CHR] = 18 μM, [Mg^{2+}] = 11.3 mM) with poly(dG)·poly(dC) (○) and poly(dG-dC) (□).

indicates that the variation in the secondary structure of the DNA does not affect grossly the binding geometry of complex I. Complex I of the drugs consists of one drug molecule and one Mg^{2+}; it is relatively smaller in size and could position itself in the deep minor groove of B-DNA as well as in the shallow and wide minor groove of A-DNA. It does not discriminate among the two forms significantly. However, the effect of different sugar residues is noticed when we compare the association of complex I for CHR and MTR with B-form DNA, i.e., poly(dG-dC) (Table 2). The difference in the minor groove dimension of the two polynucleotides leads to dissimilar binding geometries for complex II of the two drugs. It becomes apperent if we compare the binding stoichiometry of complex II for the same drug to the homo- and heteropolynucleotide. The minor groove dimension and helical rise of poly(dG)·poly(dC), with an intrinsic A-type structure, will not be the same as the minor groove dimension and helical rise of poly(dG-dC) after it undergoes complex II-induced conformational transition from B- to A-form at the binding site. Hence, the similar area blocked by complex II would cover more base pairs in A-type structure than in B-type or partially A-type structure. This suggestion is also supported from the considerable blue shift in the CD bands of complex II upon complexation with poly(dG)·poly(dC) compared to that obtained for complex I (Figure 4).

That the modes of interaction of both complexes with the two polynucleotides are different is further indicated from the values of the thermodynamic parameters, though the enthalpy−entropy compensation occurs in such a way that both complexes I and II recognize the GpG and GpC dinucleotide steps with a comparable free energy of about 6−7 kcal/mol. A low positive ΔH value for the interaction between complex I and the two polynucleotides indicates that the nature of the interaction is predominantly electrostatic and the major source of binding free energy comes from the release of electrostricted counterions and water molecules from the minor groove. Present results of the thermodynamics of complex I−polynucleotide interaction do not, however, agree with the trend reported earlier for calf thymus DNA. This disagreement could be ascribed to the difference in the nucleotide sequence flanking the GC or GG dinucleotide steps, since the width of the minor groove that accommodates the drug·Mg^{2+} complex depends on the flanking sequences (Bhattacharyya & Bansal, 1992). Effect of the flanking sequence upon the interaction mode of the drug with DNA in the presence of millimolar concentration of Mg^{2+} was also reported earlier (Cons & Fox, 1989). We also observe a sequence-dependent variation of the thermodynamic parameters characterizing the association of these ligands with oligonucleotides of different sequences (unpublished observations).

Complex II of CHR and MTR Discriminates between A- and B-Forms of DNA. Complex II of both drugs binds differently to the two polynucleotides. A significant change in enthalpy is also a distinctive feature of the complex II−polynucleotide interactions. It implies that the dimeric nature of complex II could decipher the variation in the secondary structure more efficiently than complex I containing one molecule of the drug.

The above feature for the binding of complex II of the drugs to the two polynucleotides can be explained on the basis of the conformational preferences of the two sequences. Poly(dG)·poly(dC) adopts an A-DNA conformation under

Table 3: Thermodynamic Parameters for the Interaction between Drug·Mg^{2+} Complexes I and II with Polynucleotides in 20 mM Tris-HCl Buffer, pH 8.0[a]

drug	complex	DNA	ΔH (kcal/mol)	ΔS (e.u.)	ΔG^b (kcal mol^{-1} K^{-1})
CHR	**I** ($[Mg^{2+}] = 0.33$ mM)	poly(dG-dC)	2.9 ± 0.2 (3.1)[c]	30.9	−6.3
		poly(dG)·poly(dC)	1.8 ± 0.4 (0.8)	29.1	−6.9
CHR	**II** ($[Mg^{2+}] = 11.3$ mM)	poly(dG-dC)	11.8	64.5	−7.4
		poly(dG)·poly(dC)	−11.6	−17.0	−7.0
MTR	**I** ($[Mg^{2+}] = 0.21$ mM)	poly(dG-dC)	3.1 ± 0.3 (3.6)	33.2	−6.9
		poly(dG)·poly(dC)	4.1 ± 0.5 (4.4)	36.6	−6.7
MTR	**II** ($[Mg^{2+}] = 9.9$ mM)	poly(dG-dC)	−5.7	4.5	−7.0
		poly(dG-dC)[d]	−5.3	3.2	−6.3
		poly(dG)·poly(dC)	−10.2	−11.2	−6.8

[a] Complexes I and II were prepared by mixing the drug (17−20 μM) and specified concentration of Mg^{2+} in the buffer and incubating for 1 h. The binding constants for calculation of thermodynamic parameters were determined from spectrofluorometric titrations such as that shown in Figure 3. [b] Values of ΔG were determined at 25 °C. [c] Shown in parentheses are values of ΔH estimated from the variation of K_0 with temperature. K_0 was obtained by means of eq 2. [d] Determined from spectrophotometric titrations, a representative example of which is shown in Figure 2.

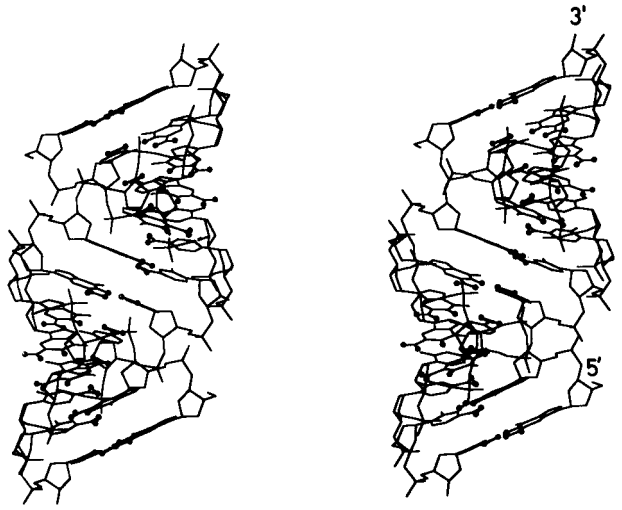

FIGURE 8: Stereo diagram of poly(dG)·poly(dC) in regular A-form.

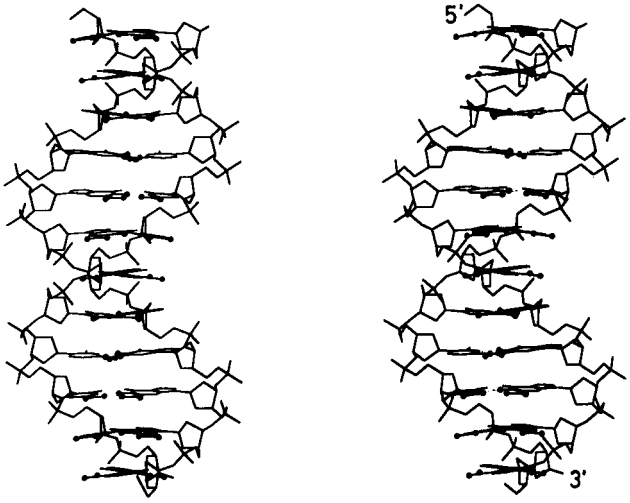

FIGURE 9: Stereo diagram of poly(dG-dC) in regular B-form.

the experimental conditions, while poly(dG-dC) has a B-DNA geometry. Figures 8 and 9 show the stereo diagrams of the two polynucleotides in regular A- and B-forms (Chandrasekaran et al., 1989; Chandrasekaran & Arnott, 1989). The amino groups are circled, because different studies have indicated that the hydrogen bond between the amino group and a potential site such as a hydroxyl group in the aglycon ring is responsible for the G-base specificity of the antibiotics (Keniry et al., 1987; Gao & Patel, 1989). It is apparent from the figure that a greater number of closely spaced amino groups are available in the minor groove of A-DNA. The number of such accessible amino groups in both A- and B-DNA structures does not change upon changing polynucleotide sequence (not shown).

The A-form of poly(dG)·poly(dC) has a wide (about 11 Å) and shallow minor groove and exposed 2-amino groups of G. Thus the dimer·Mg^{2+} complexes of both drugs could easily place themselves into the minor groove, making the potential H-bond more feasible. This binding does not require any distortion in the structure of DNA, which is reflected in the exothermic nature of association with negative enthalpy change. It is characterized by low ΔS. On the other hand, the drug dimer·Mg^{2+} complex cannot reach the bases in the minor groove of B-DNA (width ∼6 Å) to form a strong H-bond. Therefore, in the case of binding of the drug dimer·Mg^{2+} complex with poly(dG-dC), the polynucleotide may alter its conformation from standard B-DNA geometry, with a narrow and deep minor groove, to an A-DNA-like conformation with a wider and shallower minor groove, possibly with a smaller number of bound water molecules across the grooves. NMR studies with oligonucleotide d(T-T-G-G-C-C-A-A) have revealed that complex II of CHR binds to DNA with a wide minor groove (Gao & Patel, 1989). Four contiguous sequences including the central guanine residues have an A-like conformation in the complex.

Thermodynamic Parameters for Complex II−Polynucleotide Interactions Indicate Involvement of the Sugar Moieties. The notable feature like the opposite nature of enthalpy change associated with complex II−poly(dG-dC) interactions originates from the different sugars in the two drugs. The net change in enthalpy (ΔH_{total}) associated with the complex II−polynucleotide interaction could be expressed as the sum of the following contributions:

$$\Delta H_{total} = \Delta H_{bind} + \Delta H_{B-A} + \Delta H_{dimer}$$

The right-hand side of the above equation represents three contributions, namely, DNA binding enthalpy (ΔH_{bind}), conformational enthalpy change of DNA at the ligand binding site (ΔH_{B-A}), and conformational enthalpy change of the drug dimer·Mg^{2+} complex upon binding (ΔH_{dimer}).

In the case of poly(dG)·poly(dC), the second term is either absent or insignificant as explained above. The first term is negative because the drug dimer could be snugly fitted into the wide and shallow minor groove of A-DNA leading to favorable van der Waals and H-bonding interactions. Here,

the observed difference in the enthalpy values for the two drugs (Table 3) originates from the chemical structures of their sugar rings. The main difference between the antibiotics is the presence of two acetoxy groups in the A and E sugars of CHR. A simple quantum chemical charge calculation using CNDO approximation shows accumulation of high negative charge at the C═O group of the acetoxy moiety in CHR. Thus, this moiety is important in additional hydrogen-bond formation with the 2-amino groups of G bases.

The above scenario changes when complex II of CHR binds to poly(dG-dC). B → A transition in poly(dG-dC) at low ionic strength in the presence of the drug dimer·Mg^{2+} complex leads to a positive value of ΔH_{B-A}. This overcomes the negative enthalpy change (ΔH_{bind}) arising from hydrogen bonding and van der Waals interaction. The net result is an increase in enthalpy. The positive entropic contribution, from the release of minor-groove-bound water molecules due to the B−A transition of poly(dG-dC) at the binding site of complex II of CHR, compensates the unfavorable enthalpy change. This trend is reversed when complex II of MTR binds to poly(dG-dC) in an enthalpy-driven association process. It brings out the role of the different chemical structures of the two drug dimer·Mg^{2+} complexes in recognition of the same DNA. Unlike CHR, the absence of a significant change in the CD spectrum of the MTR dimer·Mg^{2+} complex upon interaction with poly(dG-dC) also suggests that the extent of B → A transition to accommodate the dimer is not so radical in the case of MTR. It has been shown by NMR and restrained molecular dynamics studies (Sastry et al., 1995) of a complex between MTR and a self-complementary decamer duplex that the E sugars make specific contacts in the DNA minor groove. The two drug dimer·Mg^{2+} complexes have different conformations as indicated from different spectroscopic studies including NMR. Therefore, the contributions of ΔH_{B-A} and ΔH_{dimer} are markedly different for the two drugs when they bind to poly(dG-dC). The difference in the conformation of the dimer·Mg^{2+} complex for the two drugs leads to different values of ΔH_{bind}. This is further supported from the visual comparison of the two final structures of the drug−oligomer complexes as reported in NMR and molecular dynamics simulations (Gao & Patel, 1989; Sastry and Patel, 1993). Two possibilities exist in the case of interaction of MTR dimer·Mg^{2+} complex with poly(dG-dC). Either the complex binds to the polynucleotide without perturbation of the B-DNA conformation or it widens the minor groove without any radical change to the A-DNA conformation. The net result is the opposite nature of the enthalpy changes (Table 3).

Thus it appears from the above studies that complexes II of CHR and MTR bind similarly to poly(dG)·poly(dC) in the A-DNA conformation. However, complex II of CHR is more specific due to presence of acetoxy groups and hence converts poly(dG-dC) from B-form to A-form. Complex II of MTR is less specific and probably binds to poly(dG-dC) in its intrinsic B-DNA conformation also. Further model-building studies and thermodynamic analysis of different oligonucleotides with designed nucleotide sequences are in progress in our laboratory in order to understand the importance of groove geometry in the drug−DNA interaction.

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