Role of Magnesium Ion in Mithramycin−DNA Interaction: Binding of Mithramycin−Mg^{2+} Complexes with DNA

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ABSTRACT: Mithramycin is an anticancer drug that blocks macromolecular synthesis via reversible interaction with the DNA template in the presence of bivalent metal ions such as Mg^{2+}. The role of Mg^{2+} in this antibiotic−DNA interaction is not clear. We approached the problem in two steps via studies on the interactions between (i) mithramycin and Mg^{2+} and (ii) mithramycin−Mg^{2+} complex(es) and DNA. Spectroscopic techniques such as absorption, fluorescence, and CD were employed for the purpose. From equilibrium and kinetic studies, we earlier reported that MTR forms two different types of complexes with Mg^{2+} [Aich, P., & Dasgupta, D. (1990) *Biochem. Biophys. Res. Commun. 173,* 689]. The two complexes are referred to as complex I (with 1:1 stoichiometry in terms of mithramycin:Mg^{2+}) and complex II (with 2:1 stoichiometry in terms of mithramycin:Mg^{2+}). In this report, we have further characterized these complexes by fluorescence spectroscopy. Interactions of these complexes with calf thymus DNA were examined to elucidate their binding. Evaluation of binding parameters (intrinsic binding constant and stoichiometry) from spectrophotometric and fluorimetric titrations suggests that the complexes bind differently to the same DNA. Measurement of van't Hoff enthalpies for the interaction of the two ligands and DNA shows that the complex I−DNA interaction is exothermic, in contrast to the endothermic nature of the complex II−DNA interaction. This could originate from a difference in the molecular nature of the interactions between the complexes and calf thymus DNA. Our studies to detect the nature of the groove via which these complexes bind to DNA suggest that both complexes approach via the minor groove of the DNA. These studies help us to understand the role of Mg^{2+} in the DNA-binding properties of mithramycin. A comparative study is also made among the Mg^{2+}- and DNA-binding properties of mithramycin and another aureolic acid class of antibiotic, chromomycin A_3.

Mithramycin (MTR,[1] also known as plicamycin), produced from *Streptomyces plicatus*, is an anticancer antibiotic clinically employed for the treatment of testicular carcinoma and Paget's disease (Calabresi & Parks, 1985; Calabresi & Chabner, 1991). It belongs to the aureolic acid group that includes the structurally related antibiotics chromomycin A_3 ($CHRA_3$) and olivomycin. Its structure (Figure 1) consists of a chromomycinone moiety (aglycon ring), either side of which is linked to six-membered sugar residues such as D-mycarose, olivose, and oliose via *O*-glycoside linkages (Gause, 1975; Thiem & Meyer, 1979, 1981). The antitumor property of the drug observed in experimental tumors is ascribed to its inhibitory effect on the replication and transcription processes during macromolecular biosynthesis (Gause et al., 1965; Keniry et al., 1987). It inhibits expression of the c-myc protooncogene that plays an important role in the regulation of cellular proliferation and differentiation. It also prevents the binding of SP1 to SV40 early promoters. Prevention of the formation of these DNA−protein complexes results in selective inhibition of *in vitro* promoter function (Ray et al., 1989a,b; Snyder et al., 1991). This is acribed to a reversible interaction of the antibiotic with its prime cellular target, DNA, and the subsequent blockade of its template activity for DNA and RNA dependent polymerases (Gause, 1975; Kersten et al., 1966; Hartman et al., 1968). An interesting feature of the MTR−DNA interaction is the requirement of a bivalent metal ion, such as Mg^{2+} (Goldberg & Friedman, 1971), at and above physiological pH.

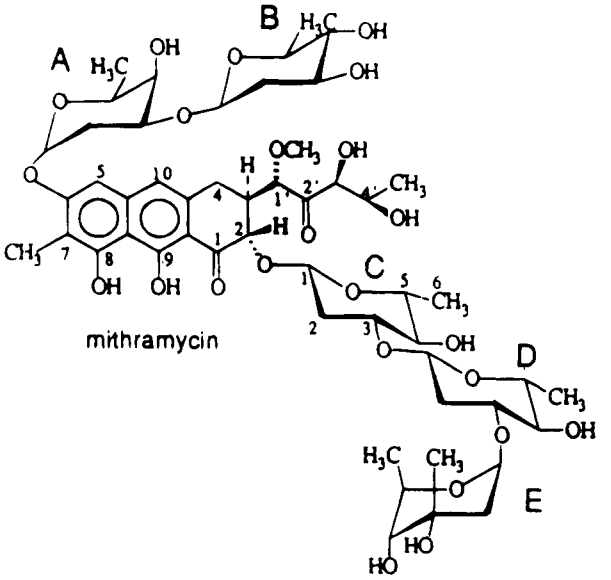

FIGURE 1: Structure of mithramycin (Thiem & Meyer, 1981).

Optical (Nayak et al., 1973, 1975; Prasad & Nayak, 1976; Dasgupta et al., 1978; Sarker & Chen, 1989) and NMR

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[1] Abbreviations: MTR, mithramycin; $CHRA_3$, chromomycin A_3; CD, circular dichroism; AUFS, absorbance unit full scale; *F* (a.u.), fluorescence (arbitrary units); CT DNA, calf thymus DNA; poly(dG−dC), poly(dG−dC)·poly(dG−dC).

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spectroscopic studies (Berman et al., 1985; Keniry et al., 1987, 1993; Shafer et al., 1988; Gao & Patel, 1989; Sastry & Patel, 1993), enzymatic and chemical footprinting (van Dyke & Dervan, 1983; Fox & Howarth 1985; Cons & Fox, 1989; Stankus et al., 1992), and hydrodynamic (Illrionova et al., 1970; Waring, 1971; Dasgupta et al., 1978) studies were carried out to understand the molecular basis of the MTR−DNA interaction in the presence of Mg^{2+}. Optical spectroscopic studies indicated a guanine base selectivity originating from hydrogen bonding between a potential site in the drug and the amino group of guanine base (Cerami et al., 1967). Footprinting studies (van Dyke & Dervan, 1983; Fox & Howarth, 1985) further confirmed the base selectivity and suggested a consensus sequence of recognition by the drug. It also indicated that a flanking sequence, not containing G-base, influences the nature of the consensus sequences (van Dyke & Dervan, 1983; Cons & Fox, 1989: Stankus et al., 1992).

NMR studies on the drug−oligonucleotide(s) complexes led to the proposition that MTR binds to DNA via the minor groove (Keniry et al., 1985, 1993; Sastry & Patel, 1993). Results from these studies are as follows. A [drug−dimer]−Mg^{2+} complex, instead of free drug, binds to the oligonucleotide at millimolar concentrations of Mg^{2+}. Saccharide chains have a role in determining the binding site on nucleotides. Base specificity occurs as a sequel to H-bonding between the phenolic group of MTR and the amino group of the guanine base. Measurements of sedimentation profiles of circular DNA in the presence of increasing concentrations of the drug favored an external binding mode (Waring, 1971). In contrast, viscosity measurements of DNA in the presence of MTR indicated an intercalative mode of interaction that was dependent on the concentration of Mg^{2+} (Dasgupta et al., 1978). Results from association and dissociation kinetic studies of the MTR−DNA interaction also favored an intercalative mode of binding (Prasad & Nayak, 1976; Dasgupta et al., 1978).

In spite of all these reports, a molecular mechanism of the interaction with DNA and the mode of binding (external or intercalative) for this drug are not yet unequivocally established. Neither is the role of Mg^{2+} in the interaction well understood. Obviously, a knowledge of the latter is necessary to have a clear picture of the former, especially the molecular basis of the interaction.

MTR is anionic at and above physiological pH; therefore, Mg^{2+} was proposed as a counterion to bring about the binding of this anionic drug to polyanionic DNA (Waring, 1981). The following observations provide evidence against this proposition. The binding parameters for MTR−DNA interactions were also found to be dependent on the relative concentrations of Mg^{2+} and MTR, even when Mg^{2+} is present at a concentration higher than that required for 1:1 stoichiometry (Dasgupta et al., 1978; Sarker & Chen, 1989). Kinetic studies of MTR−DNA interactions were also reported to be dependent on the concentration of Mg^{2+} (Prasad & Nayak, 1976; Dasgupta et al., 1978). A lacuna in these studies was the proper identification of the DNA-binding ligand when the drug interacts with DNA in the presence of Mg^{2+}. That is why often contradictory conclusions were reported for these studies over different ranges of concentrations (micromolar to millimolar) of Mg^{2+}. Recent reports from this laboratory on the role of Mg^{2+} in the $CHRA_3$−DNA interaction (Aich et al., 1992a,b) and the NMR studies (Sastry & Patel, 1993; Keniry et al., 1993) were the exceptions, where identification of the nature of the ligand was given appropriate consideration.

These results emphasize a reevaluation of the role of Mg^{2+} in the MTR−DNA interaction. We are attempting this via a two-step approach to studies on the interaction between (i) the drug and Mg^{2+} and (ii) drug−Mg^{2+} complex(es) and DNA. As a result of our studies on the binding of MTR with Mg^{2+}, we demonstrated, for the first time, that MTR could bind to Mg^{2+} in the absence of DNA, leading to stable complexes between the drug and Mg^{2+} (Aich & Dasgupta, 1990). It implies that *in vivo* the MTR−Mg^{2+} complex, instead of free MTR, is the biologically active species with DNA-binding potential. These complexes have different stoichiometries (drug:Mg^{2+}) and formation constants, respectively (Aich & Dasgupta, 1990). In the subsequent discussion we refer to them as complex I (1:1 and 1.8×10^4 M^{-1}) and complex II (2:1 and 1.6×10^3 M^{-1}). These results help to identify the ligand that binds to DNA in the reports mentioned here.

There are no other studies at present to characterize the interaction of these ligands with DNA. A comparison with our results (Aich & Dasgupta, 1990) suggests that conditions chosen in NMR studies would lead to the formation of complex II (Sastry & Patel, 1993; Keniry et al., 1993). Therefore, in the present paper, we report the results of optical spectroscopic studies, such as absorption, fluorescence, and CD, aimed at (i) the further elucidation of the molecular nature of complex II and (ii) the evaluation of binding potentials of the two complexes with DNA. As a continuation of the second part, thermodynamic parameters for interactions between these complexes and DNA are also determined, because they provide knowledge about the macroscopic characterization of the forces responsible for the sequence specific interaction between a drug and DNA (Marky et al., 1983; Zimmer & Wähnert, 1986; Remeta et al., 1991; Gilbert et al., 1991; Guo et al., 1992; Breslauer, 1992; Bailey et al., 1993).

For these studies, DNA from CT was chosen as the natural DNA, because it consists of comparable percentages of AT and GC base pairs. Because the size and nature of complexes I and II are different, we therefore probed the nature of the groove via which they bind to DNA by two methods (Baguley, 1982; Dasgupta & Goldberg, 1985). In the first method, the ability of the complexes to substitute the well-known intercalator ethidium bromide (EtBr), which binds to DNA approaching via the minor groove, is examined (Waring, 1981). In the second method, affinities of the ligands are compared for two DNAs with comparable G-C contents, but in one [T4 *bacteriophage* (T4)] of these the major groove is blocked by bulky residues such as glucose (Watson, 1987). DNAs from *Clostridium perfringens* (CP, 32% G-C content) and T4 (34% G-C content) are the polymers chosen for the purpose. An earlier report indicated that the pK_a of MTR (Illrionova et al., 1970) is 5.0; therefore, we carried out the present investigation at pH 8.0. This ensures a homogeneous population of negatively charged antibiotic molecules. Most of the earlier studies were also done at or near pH 8.0 (e.g., Nayak et al., 1973, 1975; van Dyke & Dervan, 1983; Keniry et al., 1987, 1993; Banville et al., 1990a,b; Shafer et al., 1988; Sarker & Chen, 1989; Stankus et al., 1992; Sastry & Patel, 1993).

MATERIALS AND METHODS

Mithramycin, Tris, magnesium chloride solution (4.9 M), and calf thymus (CT), coliphage T4 (T4), and *Clostridium perfringens* (CP) DNAs were purchased from Sigma Chemical Company (St. Louis, MO). Poly[d(G−C)] was from Pharmacia Biotech Ltd. (Sweden). Unless mentioned otherwise, all studies were carried out in 20 mM Tris-HCl buffer (pH 8.0). The buffer was prepared in triple-distilled (in an all-quartz apparatus), deionized water. The concentration of MTR was estimated from its extinction coefficient value of 10.0 mM^{-1} cm^{-1} at 400 nm (Bakhara et al., 1968). CT DNA was deproteinized by the chloroform/phenol extraction method and precipitated with ethanol. It was then redissolved in 20 mM Tris-HCl buffer (pH 8.0), dialyzed extensively against the same buffer containing 5 mM EDTA to remove divalent metal ions, and finally dialyzed against the buffer only to remove EDTA. The purity of the DNA was checked and its concentration was determined spectrophotometrically.

Absorption, fluoroescence, and CD spectra were recorded with a Hitachi U-2000 spectrophotometer, a Hitachi F-4010 fluorimeter, and a Jasco J-500 spectropolarimeter, respectively. All fluorescence measurements for MTR and its complexes with Mg^{2+} were carried out at an excitation wavelength of 470 nm, instead of 405 nm (Sarker & Chen, 1989), because we observed a time dependent decrease in the fluorescence emission (by more than 50%) of the antibiotic (and its complexes with Mg^{2+}) upon excitation at 400 nm. On the other hand, no significant change (less than 2%) in the emission intensity could be detected upon excitation at 470 nm, a wavelength away from the peak (at 400 nm) in the absorption spectrum of the free antibiotic (data not shown). The possibility of any red-edge effect is eliminated because we did not notice any change in the emission peak (at 540 nm) with the shift in the excitation wavelength from 400 to 470 nm. Background emission (<5% at maximum) was corrected for by the subtraction of signals from blank buffer or DNA plus buffer samples. No correction was made for optical filtering effects, because the absorbance of the samples (at 470 nm) never exceeded 0.02. The CAT mode, with the number of repeat scans between 4 and 8, was employed to record the fluorescence spectra. CD values were expressed as molar ellipticity: $[\theta] = (\theta_{obs}100)/(cl)$, where θ_{obs}, l, and c denote the observed ellipticity (in millidegrees), the pathlength of the cuvette (in centimeters), and the molar concentration of the absorbing species, respectively. All spectra reported here are averages of two runs.

Measurement of Steady-State Fluorescence Polarization Anisotropy, $\langle r \rangle$ (FPA). This was calculated from $\langle r \rangle = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$ (Cantor & Schimmel, 1980), where I denotes the intensity and the subscripts refer to vertical (V) or horizontal (H) positionings of the excitation and emission polarizers. $G = I_{HH}/I_{HV}$ was used to correct for polarizing effects in the emission monochromator and detector. The excitation and emission wavelengths were 470 and 540 nm, respectively.

Analysis of Binding Data. The results from spectrophotometric titration to study the ligand (antibiotic−Mg^{2+}−DNA interaction were analyzed in the following ways. (i) Scatchard plot:

$$r/c_f = K_0(n - r) \quad (1)$$

where $r = c_b/c_p$ (c_b is the concentration of bound ligand and c_p is the concentration of DNA), n is the binding stoichiometry in terms of bound ligand per nucleotide, and K_0 is the intrinsic binding constant (Scatchard, 1949). (ii) Li and Crothers type plot:

$$1/\Delta\epsilon = 1/\Delta\epsilon' + 1/[K_{ap}\Delta\epsilon'(c_p - c_d)] \quad (2)$$

where $\Delta\epsilon = \epsilon_{obs} - \epsilon_f$($\epsilon_{obs}$ is the apparent molar extinction coefficient of the bound ligand measured from the observed absorbance during titration; ϵ_f is the molar extinction coefficient of the free ligand), $\Delta\epsilon' = \epsilon_b - \epsilon_f$($\epsilon_b$ is the molar extinction of the bound ligand), K_{ap} is the apparent binding constant ($K_{ap} = K_0 n$), and c_d is the concentration of ligand (Li & Crothers, 1969; Dasgupta & Goldberg, 1985). This equation is valid under the condition $c_p \gg c_d$, which is followed by keeping at least an 8-fold excess of DNA. For construction of the Scatchard plot, the concentration of the bound ligand, c_b, was evaluated from the relation

$$c_b = \Delta A/(\epsilon_b - \epsilon_f) \quad (3)$$

where ΔA denotes an increase in the absorbance of the ligand upon addition of DNA. An absorbance change at 440 nm was employed for the construction of these plots.

The increase in the fluorescence of the ligand(s) as a function of the added concentration of DNA was also analyzed to construct the binding isotherm according to the Scatchard method mentioned earlier. The concentration of the bound ligand (c_b) was calculated as follows: $c_b = (Q/Q_{max})c_{tot}$, where Q is the fractional dequenching during titration, Q_{max} is the fractional dequenching when the ligand is totally bound to DNA, and c_{tot} is the initial imput concentration of the drug (Hard et al., 1989). Q is determined from the relation, $Q = (I - I_0)/I_0$, where I_0 and I are the emission intensities of the free ligand and the DNA-bound ligand (during titration), respectively. Q_{max} is defined as $Q_{max} = (I_{max} - I_0)/I_0$, where I_{max} is the emission intensity of the DNA-bound ligand. I_{max} is obtained from a plot of $1/I$ against $1/c_p$ (c_p is the concentration of DNA) (Wang & Edelman, 1971); the resulting straight line is extrapolated to the y-axis, and the intercept on the y-axis gives the value of $1/I_{max}$. Thus, known values of c_b, c_{tot}, and c_p allow us to construct the Scatchard plot according to eq 1. This approach is based on the assumption of a linear relation between emission intensity and the concentration of the ligand, which we found to be valid up to [MTR] = 50 μM, corresponding to an excitation wavelength of 470 nm (unpublished observation). There are reports suggesting the aggregation of $CHRA_3$, a related antibiotic, at lower concentrations (Stankus et al., 1992). However, the conditions for those studies, done in the presence of 25% ethanol, are different from the conditions in the present study, thus not permitting any comparison among them.

In all of these cases, experimental points in the binding isotherm were subjected to least-squares analysis with a view to obtaining the best-fit straight line representing the binding isotherm. For the determination of binding parameters for the interactions between antibiotic−Mg^{2+} complexes and DNA, MTR was preincubated with Mg^{2+} for 1 h at the desired temperature to ensure the complete formation of the antibiotic−Mg^{2+} complex. Small aliquots of DNA were then

added to the antibiotic−Mg^{2+} complex, and the equilibrium spectrum, corresponding to the addition of each aliquot, was noted when there was no further change in the spectrum. During the titration, the total dilution due to the addition of DNA was restricted to 5% of the initial volume, and the correction due to dilution was incorporated in the calculation of the binding parameters.

Determination of Thermodynamic Parameters. The thermodynamic parameter, ΔH_{vH} (van't Hoff enthalpy) was evaluated from a variation of K_{ap} with temperature:

$$d \ln K_{ap}/dT = \Delta H_{vH}/RT^2 \quad (4)$$

where R and T are the universal gas constant and absolute temperature, respectively (Castellan, 1989). For the determination of ΔH_{vH}, K_{ap} values were measured by means of eq 2 at four different temperatures: 15, 25, 30, and 35 °C, respectively. The slope of the best-fit line from the plot of ln K_{ap} against $1/T$ gave the value of ΔH. Other thermodynamic parameters, ΔG (free energy) and ΔS (entropy), were determined as follows. ΔG was determined from the following relations:

$$\Delta G = -RT \ln K_{ap}$$

$$\Delta G = -RT \ln(K_0 n) \quad (5)$$

where K_{ap} was evaluated from the Li and Crother type of plot (eq 2) and $K_0 n$ was evaluated from the Scatchard plot (eq 1). This is done because it helps us to compare two methods of analysis for the results obtained from spectrophotometric titrations. Known values of ΔG and ΔH_{vH} gave the value of ΔS from the relation

$$\Delta G = \Delta H_{vH} - T\Delta S \quad (6)$$

RESULTS

MTR−Mg^{2+} Interactions. It is mentioned elsewhere in this paper that equilibrium spectroscopic (absorption and CD) and kinetic studies showed that MTR forms two different types of complexes at and above physiological pH with Mg^{2+} in the absence of DNA (Aich & Dasgupta, 1990).

The fluorescence property of MTR is employed for the additional characterization of complex II. As reported in the case of $CHRA_3$ (Aich et al., 1992a,b), formation of the complexes between MTR and Mg^{2+} is associated with a decrease in the emission intensity of free MTR, with a greater extent of quenching for complex II. A red shift of the peak in the emission spectrum of free MTR is another distinctive feature marking the formation of this cmoplex. Experimental values from the fluorescence titration of MTR by Mg^{2+}, in the range of concentrations of Mg^{2+} corresponding to the formation of complex II, could be well fit by the Stern−Volmer equation with the quenching constant, $K_q = 1.5 \times 10^3$ M^{-1}. This corroborates well with the value of the association constant, 1.6×10^3 M^{-1}, estimated from the spectrophotometric titration of MTR with Mg^{2+} (Aich & Dasgupta, 1990). This suggests that the quenching associated with the formation of complex II is the ground-state feature of this complex. FPA values for free MTR and complexes I and II are 0.023, 0.038, and 0.23, respectively. They reflect the state of aglycon moieties in the two MTR−Mg^{2+} complexes. There is a large increase in the FPA value for complex II; comparatively, little change in the value of the anisotropy is associated with the formation of complex I. This may originate from differences in the Brownian motion of the two complexes.

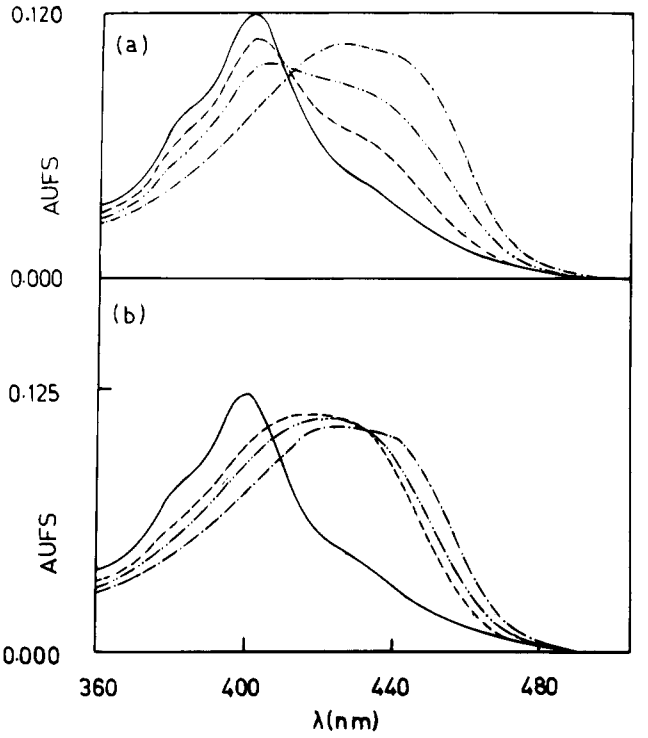

FIGURE 2: (a) Absorption spectra (360−520 nm) of complex I [MTR (1.2×10^{-5} M) plus Mg^{2+} (2.3×10^{-4} M)] in the absence (− −) and presence of DNA at concentrations of 4.3×10^{-5} (−•−) and 2.0×10^{-4} M (−•−), respectively, in 20 mM Tris-HCl buffer (pH 8.0) at 30 °C. The spectrum of free MTR (1.2×10^{-5} M, —) is also shown for comparison. (b) Absorption spectra (360−520 nm) of complex II [MTR (1.2×10^{-5} M) plus Mg^{2+} (7.3×10^{-3} M)] in the absence (− −) and presence of DNA at concentrations of 4.3×10^{-5} (−•−) and 2.0×10^{-4} M (−•−), respectively, in 20 mM Tris-HCl buffer (pH 8.0) at 30 °C. The spectrum of free MTR (1.2×10^{-5} M, —) is also shown for comparison.

Interaction of MTR−Mg^{2+} Complexes with DNA. One would expect that two MTR−Mg^{2+} complexes would bind differently to the same DNA. The following experiments were done with a view to verifying this. In all experiments related to this part of the results, concentrations of MTR and Mg^{2+} were so chosen that they led to the formation of the desired single type of MTR−Mg^{2+} complex (i.e., either complex I or complex II).

Progressive changes in the absorption spectra of complexes I and II upon the addition of different concentrations of DNA (Figure 2a,b) indicate the association between them. The following characteristic features of the changes, namely, (i) the red shift and broadening of the peaks and (ii) the increase in the absorption beyond 410 nm of the spectrum of free MTR, are consistent with earlier observations for this (Nayak et al., 1973) and the related drug, $CHRA_3$ (Aich et al., 1992a). These changes result from DNA-induced alteration in the electronic environment of chromomycinone moiety in MTR−Mg^{2+} complex. Similar types of changes in the absorption spectra of the ligands ethidium bromide (EtBr), an intercalator, and distamycin, a groove binder, mark their association with DNA (Li & Crothers, 1969, Zimmer & Wahnert, 1986). Close scrutiny of the spectra of complexes I and II at near-saturating concentrations of DNA (2.0×10^{-4} and $1.94 \times$

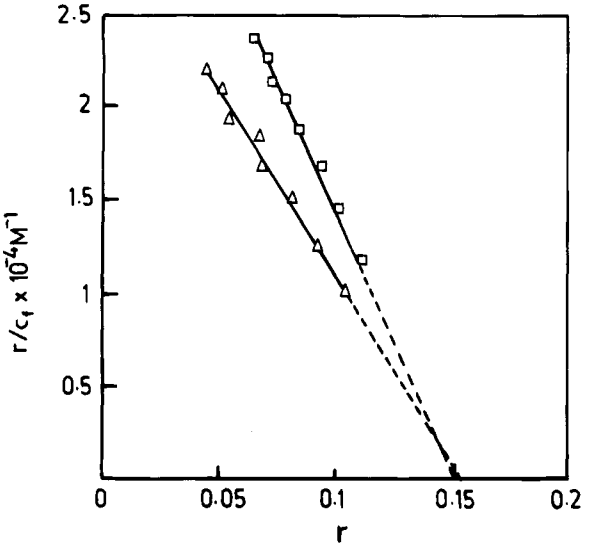

FIGURE 3: Scatchard plots for the complex I and DNA interaction of MTR from absorption (△) and fluorescence studies (□) under the conditions mentioned in Figures 4a and 5a.

10^{-4} M, respectively; Figure 2a,b) shows that they do not overlap. It suggests differences in the electronic environment of the chromophores for the two complexes when they bind to DNA.

In addition to changes in the absorption spectra, the fluorescence spectra of complexes I and II exhibit an increase in emission intensity upon binding to DNA. This may be ascribed to a change either in the local electronic environment of the chromophore(s) or in the overall conformation of the DNA-bound ligand(s). Both factors may also act in a concerted fashion. The increase in fluorescence of MTR–Mg^{2+} complexes at 540 nm (at an excitation wavelength of 400 nm) was also reported previously (Sarkar & Chen, 1989). The evaluation of binding parameters from these data is not appropriate. However, quantitation of their results is suspect, because the antibiotic is photosensitive at 400 nm (see Materials and Methods).

The nature of the plots of the apparent extinction coefficients (at 450 nm) and the fluorescence emission intensities (at 550 nm) of complexes I and II, as a function of DNA concentration, indicates non-cooperative binding. A similar trend was also noted for the interaction between two types of $CHRA_3$–Mg^{2+} complexes and DNA (Aich et al., 1992a,b).

Binding parameters, the intrinsic binding constant (K_0), and the stoichiometry (n, number of nucleotides per drug molecule) of the interactions between MTR–Mg^{2+} complexes and DNA were evaluated using the preceding data by means of Scatchard plots (eq 4). Figure 3 shows the collinearity of the experimental points (obtained from spectrophotometry and fluorescence) and, therefore, further indicates the non-cooperative nature of the interaction. Table 1 gives the corresponding binding parameters evaluated from such plots. The following points are noted from the table: (i) Binding parameters are different for the interactions between complexes I and II and DNA at the same concentration of MTR. This is valid for both concentrations (11 and 28 μM) of MTR. It supports the proposition that complexes I and II bind differently to the same DNA. (ii) On comparison of the values of the binding parameters (listed in Table 1), it could be stated that the binding parameters are independent of the absolute concentrations of MTR and

Table 1: Binding Parameters for MTR–Mg^{2+} Complexes with CT DNA[a]

method	MTR (μM)	Mg^{2+} (mM)	complex	K_0 ($\times 10^5$ M^{-1})	n (drug/nucleotide)
spectro-	11.0	0.24	I	2.1 ± 0.2[b]	0.15[b]
photometry	[9.0][c]	[0.14]	I	[3.0]	[0.14]
	11.0	7.3	II	1.1 ± 0.2	0.21
	[9.0]	[8.5]	II	[1.0]	[0.18]
fluorimetry	28.0	0.48	I	2.8 ± 0.3	0.15
	[26.0][c]	0.21	I	[2.7]	[0.13]
	28.0	10.7	II	1.15 ± 0.1	0.19
	[26.0]	21.0	II	[1.8]	[0.18]

[a] The values are in 20 mM Tris-HCl buffer (pH 8.0) at 25 °C, obtained by means of a Scatchard plot described under Materials and Methods. Concentrations of Mg^{2+} are chosen so that they would lead to the formation of complex I (at lower concentrations of Mg^{2+}) or complex II (at higher concentrations of Mg^{2+}). [b] Average of two values from two sets of determinations with different batches of MTR. [c] Numbers in square brackets denote corresponding values for $CHRA_3$. They are quoted from our report on the interaction of $CHRA_3$–Mg^{2+} complexes with CT DNA (Aich et al., 1992).

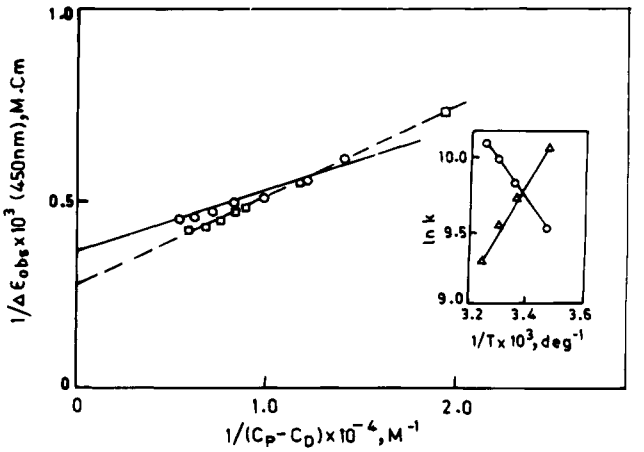

FIGURE 4: Li and Crothers type of plot (see eq 2 in the text) for the interaction of DNA with complex II [MTR (1.2×10^{-5} M) plus Mg^{2+} (7.3×10^{-3} M)] at 15 (□) and 35 °C (○), respectively, in 20 mM Tris-HCl buffer (pH 8.0) at 30 °C. The inset to this figure shows the change in equilibrium constants (ln K) for the interaction between DNA and complexes I (△) and II (○) with respect to temperature ($1/T$), according to method of van't Hoff analysis.

Mg^{2+}. This means that any effect of the aggregation of MTR, if there is any at all, does not manifest itself during binding of the MTR–Mg^{2+} complexes with DNA. (iii) The values of the binding parameters from spectrophotometry and fluorimetry agree well within the limits of experimental error, thereby supporting the validity of the proposition.

Apparent binding constants ($K_{ap} = K_0 n$) for the interactions between complexes I and II and DNA were determined from changes in the absorbances of complexes I and II in presence of excess DNA (eq 2). Values of K_{ap} were evaluated at four different temperatures, 15, 25, 30, and 35 °C, for each complex. Representative plots of $1/\Delta\epsilon$ against $1/(c_p - c_d)$ to estimate K_{ap} for complex II–DNA interaction at 15 and 35 °C are shown in Figure 4. The inset to the figure demonstrates a plot of ln K_{ap} against $1/T$ for the interactions of DNA with complexes I and II. The ΔH_{vH} values were determined from the slope of the best-fit line in the preceding plot. Since there is no significant change in n (stoichiometry, drug:nucleotide) with temperatures from 15 to 35 °C, it is justifiable to plot ln K_{ap} as a function of $1/T$ to obtain the ΔH_{vH} values.

Table 2: Thermodynamic Parameters for the Interaction between MTR−Mg^{2+} Complexes and DNA[a]

complex	ΔG^b (kcal/mol)	ΔH_{vH} (kcal/mol)	ΔS (eu)
I ([MTR][c] = 11.0 μM, [Mg^{2+}] = 0.23 mM)	−5.8 (−6.2)[d]	−7.2	−4.7
II ([MTR] = 11.0 μM, [Mg^{2+}] = 7.3 mM)	−5.8 (−5.9)	5.1	36.6

[a] The values are in 20 mM Tris-HCl buffer (pH 8.0). The methods to evaluate them are described in the text. [b] Values of ΔG were determined at 25 °C from the relation $\Delta G = -RT \ln K_{ap}$. [c] Terms in the square brackets denote concentrations. [d] Values of ΔG in the first bracket were determined at 25 °C from the relation $\Delta G = -RT \ln(K_0 n)$.

Other thermodynamic parameters (ΔG and ΔS) were calculated with the help of eqs 5 and 6. Table 2 summarizes the values. The determination of ΔG from apparent binding constant values obtained by the Scatchard plot (eq 1) and by the Li and Crothers type of plot (eq 2) leads to comparable values. Binding of complex I with DNA is exothermic, while the complex II−DNA interaction is endothermic and thereby entropy-driven.

Differences in the thermodynamic parameters for the interactions of the two complexes with DNA prompted us to check the nature of their binding groove (major or minor) in DNA. The larger size of complex II containing two molecules of MTR raises the possibility of its binding via the major groove of DNA. The ability of the complexes to substitute EtBr, which intercalates via the minor groove of DNA, was examined from the reduction in fluorescence intensity of bound EtBr. Figure 5 shows that the addition of increasing concentrations of complex II leads to a progressive decrease in the fluorescence intensity of EtBr. Dissociation of EtBr from poly[d(G−C)] leads to the observed reduction in fluorescence intensity. Such a decrease is ascribed to the formation of free EtBr, which has a lower quantum yield than DNA-bound EtBr (Baguley, 1982). This experiment suggests the substitution of EtBr by MTR. The inset to Figure 5 shows a decrease in the fluorescence emission intensity of EtBr at 592 nm upon the addition of MTR at two representative concentrations (4.7×10^{-4} and 4.7×10^{-2} M, respectively) of Mg^{2+}. Complexes I and II are formed at these concentrations, respectively.

The second approach to the identification of the nature of the binding groove is a comparison of the affinity constants for the interaction of both ligands, complexes I and II, with T4 and CP DNA. Binding isotherms obtained from the plots of the relative change in fluorescence intensity of the ligand as a function of the input concentration of these DNAs are also typical of the non-cooperative type. Values of K_{ap}, measured from the concentration of DNA corresponding to 50% of the total change in fluorescence intensity of the ligands, are 0.4×10^3 and 0.5×10^3 M^{-1} for the binding of complex I with CP and T4 DNA, respectively, and 1.05×10^3 and 0.8×10^3 M^{-1} for the binding of complex II with CP and T4 DNA, respectively. It is noted that the K_{ap} values for the interaction of a ligand for two DNAs are comparable within the limits of experimental error.

DISCUSSION

Major conclusions emerging from the preceding studies are summarized as follows. Complexes I and II have different fluorescence spectroscopic properties. This trend

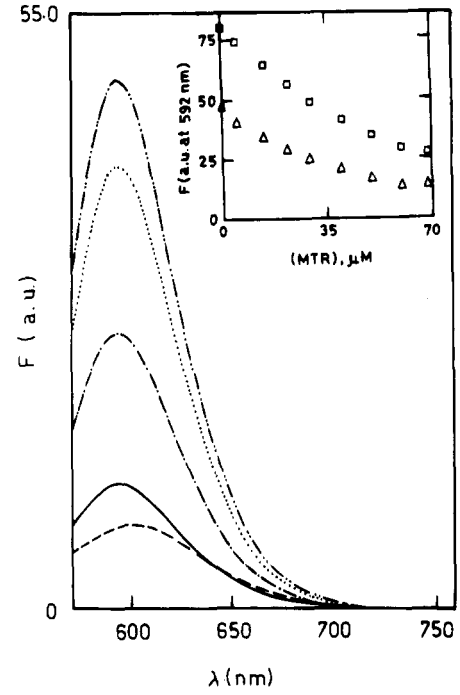

FIGURE 5: Changes in the fluorescence emission spectrum of the EtBr(4.0×10^{-6} M)−poly(dG−dC)(3.0×10^{-5} M) complex (—•—) upon the addition of MTR at concentrations of 5.2×10^{-6} (•••), 2.95×10^{-5} (—•—), and 6.9×10^{-5} M (—), respectively, in the presence of [Mg^{2+}] = 4.7×10^{-2} M in 20 mM Tris-HCl buffer (pH 8.0) at 30 °C. The spectrum of EtBr in the presence of Mg^{2+} (— —) is also shown. The inset to this figure shows changes in the fluorescence emission intensity of ethidium bromide as a function of the input concentration of MTR in the presence of Mg^{2+} at concentrations of 4.7×10^{-4} (□) and 4.7×10^{-2} M (△). Before the addition, MTR was incubated in Mg^{2+} at the concentrations mentioned here for an hour.

is in accordance with the other spectroscopic features, such as absorbance and CD, reported earlier (Aich & Dasgupta, 1990) and further supports the proposition that they are two molecular entities with distinctive three-dimensional structures. Therefore, the recognition of the same DNA by either of these complexes is different in nature and magnitude. However, preliminary experiments suggest that both complexes bind via the minor groove of DNA. This is especially remarkable in view of the relatively larger size of complex II. Minor groove binding of complex II is also proposed from NMR experiments (Sastry & Patel, 1993). These points are discussed in detail, along with a comparative study of the features of the interaction between a structurally related antitumor antibiotic, $CHRA_3$, and CT DNA [reported from our laboratory: Aich et al. (1992)].

MTR−Mg^{2+} Interaction. Earlier studies have shown that complex formation between MTR and Mg^{2+} is associated with a change in the optical properties of the free drug. We have employed the fluorescence of the drug as an additional probe for the structural features of complex II because of the relevance to understanding its DNA-binding properties. The reduction in the quantum yield for this complex is due to a radical change in the environment of the fluorophore, namely, the chromomycinone moiety (Figure 1) in the complex. A Mg^{2+}-coordinated dimer model (for complex II) consisting of two aromatic rings, in principle, leads to a favorable situation resulting in the deactivation of the singlet excited state and, hence, the reduced quantum yield. Interactions between chromomycinone rings in the dimer might

contribute in part to its free energy of formation. The relatively high value of FPA, characteristic of complex II, is also consistent with this model, which suggests hindered Brownian motion for the molecule (Lakowicz, 1983). However, the present data are not sufficient to eliminate the possibility of the observed increase in FPA for complex II due to a change in the lifetime of the excited state of the MTR upon complex formation with Mg^{2+} (Lakowicz, 1983).

Similar spectroscopic features for $CHRA_3-Mg^{2+}$ complexes (Aich et al., 1992) provide additional support for our present results and indicate that this class of drugs adopts the Mg^{2+}-coordinated dimer type of structure for the interaction with its prime cellular target, DNA, at physiological concentrations (millimolar) of Mg^{2+}. Our proposition also gains support from NMR studies carried out by other groups (Keniry et al., 1993; Sastry & Patel, 1993). A similar model for the structure of complex II was assumed to explain the observed changes in chemical shifts and NOEs in the NMR spectra of MTR(and $CHRA_3$)−oligonucleotide complexes at millimolar conentrations of Mg^{2+} (Sastry & Patel, 1993; Keniry et al., 1993). On the other hand, complex I may not involve a radical change in the environment of the chromophore, as is evident from the relatively lower perturbation in the spectroscopic features for MTR when complex I is formed [present fluorescence data and results from our previous report: Aich and Dasgupta (1990)]. Electrostatic and/or H-bonding interactions probably play important roles in its formation. The electrostatic contribution to the formation of complex I is also suggested from the ionic strength dependence of the affinity constant ($9.0 \times 10^3\ M^{-1}$ in 200 mM Tris-HCl buffer, pH 8.0) for its formation.

Interaction between MTR−Mg^{2+} Complexes and DNA. Concentrations of Mg^{2+} to make MTR−Mg^{2+} complex(es) were chosen so that the DNA-binding ligand is a single population of either complex I or complex II. As shown earlier, the titration of MTR with Mg^{2+} is biphasic, where [Mg^{2+}] spans from 0 to 235 μM for the first phase, and that for second phase ranges from 235 μM to 10 mM (Aich & Dasgupta, 1990). The concentration of Mg^{2+} (235 μM) chosen to form complex I is low enough not to lead to the formation of complex II, as indicated from the value of its affinity constant. We also noted that, at [Mg^{2+}] = 10 mM, there is no progressive conversion of complex I to complex II in the absence of DNA. In case of complex II, we chose the concentration of Mg^{2+} corresponding to the plateau of the second phase titration curve of MTR with Mg^{2+} (Aich & Dasgupta, 1990). Furthermore, independent spectroscopic experiments showed that, at such high concentrations of Mg^{2+}, only the $(MTR)_2-Mg^{2+}$ complex could form. Binding of the two complexes with CT DNA is indicated from progressive changes in the absorption and fluorescence spectra of the ligands in the presence of increasing concentrations of DNA. If there were two populations of drug−Mg^{2+} complex, this would be reflected in the nature of the resulting binding isotherm for their interactions with DNA. From our data, we do not notice any significant deviation from a single monophasic type of binding isotherm that could be attributed to a single type of population of DNA-binding ligand at a particular representative concentration of Mg^{2+}. The addition of 1 M NaCl does not have any effect on the spectrum of an equilibrium mixture of the complexes and DNA, thus indicating the specific nature of the interaction.

Characteristic red shifts of absorption peaks in the spectra of free ligand(s) in the presence of DNA originate from a perturbation of the $\pi \rightarrow \pi^*$ transition in the chromomycinone ring bound to DNA. Such red shifts were reported for a classical intercalator, such as EtBr, or a typical groove binder, such as distamycin (Zimmer & Wähnert, 1986). Therefore, we disagree with an earlier suggestion (Sarkar & Chen, 1989) and do not mark it as a diagnostic feature to propose the intercalative mode of binding for these ligands. It further implies that complexes I and II could bind via different modes, yet apparently one may notice the same trend in changes in absorption. More important is the observation that the spectra of the complexes of the two ligands in the presence of saturating concentrations of DNA do not overlap (compare relevant spectra in Figure 2a,b). These results are consistent with the proposition that DNA-bound complexes, complexes I and II, respectively, have different spectroscopic properties. Nonidentical structures of the ligands could be a potential cause of this difference.

Changes in the electronic environment of the fluorophore, the chromomycinone ring, as a result of the ligand(s)−DNA interaction also led to the increase in its fluorescence. Such an increase could be ascribed to the following factors: (i) a change in conformation of the ligand(s) involving sugar residues; (ii) an alteration of microenvironments, such as local viscosity or hydrophobicity; and (iii) an enhancement in the extent of excited-state proton transfer (Lepecq & Paoletti, 1967), which otherwise was not facile in free MTR−Mg^{2+} complexes. The present data are not sufficient to comment on their relative contributions.

Differences in the recognition of CT DNA by the two complexes, I and II, clearly are borne out by the results in Table 1. A scrutiny of the earliest footprinting studies by a chemical method (van Dyke & Dervan, 1983), taking into account the formation of two types of MTR−Mg^{2+} complexes, further supports our observation. That study demonstrated an increase in the antibiotic-protected regions at higher concentrations of MTR (100 μM) and Mg^{2+} (200 μM), which would favor the formation of complex II. In contrast, a lesser number of sites is protected at lower concentrations of MTR (25 μM) and Mg^{2+} (50 μM), which would lead to complex I. Our data also show an increase in the binding stoichiometry as we go from complex I to complex II. Footprinting with hydroxyl radical in the presence of a millimolar concentration of Mg^{2+} does not indicate any such dependence on the concentration of the drug, because only one type of complex (II) is formed under these conditions (Cons & Fox, 1989, 1990).

The difference in thermodynamic parameters associated with the interactions of the ligands with the same DNA is the most significant feature that provides evidence in favor of the different nature of the molecular recognition of DNA by two complexes, I and II. The possibility of differences in the conformation of DNA at different concentrations of Mg^{2+}, as a source for the opposite nature of ΔH_{vH} values, appears remote because so far there has been no report of a gross change in the B-DNA type conformation of CT DNA in the presence of millimolar concentrations of Mg^{2+} (Zimmerman, 1982; Duguid et al., 1993). We also did not notice any significant change in the absorption or CD spectra of CT DNA over the range of concentrations of Mg^{2+} used in the present study (data not shown). The drug−Mg^{2+} complex follows a linearity in absorption and fluorescence

as a function of the concentration of ligand in the range 0−50 μM MTR. Therefore, one might suggest that there is no self-association of the ligands under our experimental conditions. This eliminates any possible contribution to ΔH_{vH} values reported here from the enthalpy of self-association of the ligand(s) (Chaires et al., 1982; Remeta et al., 1991).

In order to account for the opposite nature of the change in heat content, we also looked into the possibility that the two complexes bind via different DNA grooves (major or minor). Results obtained from studies on (i) the potential of the ligand to substitute a minor groove-binding agent and (ii) the relative binding affinity of the ligands for natural DNAs with the major groove accessible or blocked by bulky groups suggest that both ligands, complexes I and II, approach via the minor groove of DNA. The minor groove-binding mode for MTR in the presence of millimolar concentrations of Mg^{2+} is also supported by NMR studies. The studies from Patel's group have shown that, in addition to hydrogen bonding and van der Waals interaction of MTR aglycon with the DNA minor groove, trisaccharide segments of each monomer in the MTR dimer are positioned inside the minor groove and are directed toward either end of the duplex DNA (Sastry & Patel, 1993). Our suggestion of minor groove binding is also consistent with the proposition that H-bonding involving the 2-amino group of the guanine base determines its G-C base specificity, because this group is accessible to the ligand approaching via the minor groove only. However, it is not supported by the study showing that the reaction of dimethyl sulfate at the N7 of guanine located in the major groove of DNA is modified in the presence of mithramycin (Cons & Fox, 1990). We may speculate that a reason for this contradictory result may be the possibility of covalent modification of the aglycon moiety of MTR by dimethyl sulfate. Presently, we are attempting to further characterize the mode of binding of each type of complex with DNA and compare their binding sites in a restriction fragment by footprinting analysis.

The negative change in van't Hoff enthalpy for the complex I−DNA interaction is comparable to that for intercalators like ethidium bromide and daunomycin (Wilson & Jones, 1982; Chaires et al., 1990; Remeta et al., 1991) or groove-binding antibiotics such as distamycin [Zimmer and Wahnert (1986) and related references in the review article on calorimetric evaluation of thermodynamic parameters for DNA−ligand interactions by Breslauer et al. (1992)]. On the other hand, the positive change in van't Hoff enthalpy for complex II−DNA interaction might arise from the non-intercalative mode of binding, because a survey of the literature shows that positive values of ΔH_{vH} so far have been reported to be characteristic for the external binding mode of the ligands to DNA (Dasgupta & Goldberg, 1985; Breslauer et al., 1992). The external binding mode involving aglycon−DNA minor groove interactions and saccharide−DNA minor groove interactions was also proposed from NMR studies of the MTR−oligonucleotide(s) interaction in the presence of millimolar concentrations of Mg^{2+} (Gao & Patel, 1990; Sastry & Patel, 1993; Keniry et al., 1993). The positive value of ΔH_{vH} for complex II−DNA interaction could be ascribed to three plausible factors: (i) there is a change in conformation of the Mg^{2+}-coordinated dimer (i.e., complex II) upon complexation with DNA (the altered conformation energetically is not favorable, but it is stabilized by the interaction with DNA); (ii) binding of the dimer to DNA induces a structural distortion of DNA from the B-form at or near the binding site; and (iii) there is an endothermic contribution of the removal of electrostricted water molecules from DNA, resulting from the dehydration effect of Mg^{2+}. The conformational change of complex II could be one of the factors for the increase in its fluorescence upon binding to DNA.

The second proposition is supported by NMR studies that indicate a transition to non-B-DNA structure at the binding loci of MTR in the presence of millimolar concentrations of Mg^{2+} (complex II) to oliognucleotide (Gao & Patel, 1990; Sastry & Patel, 1993; Keniry et al., 1993). One such study (Sastry & Patel, 1993) has shown that the central cytidine in a hexamer duplex adopts an A-DNA sugar pucker and a glycosidic torsion angle in the MTR dimer−DNA complex. It was also supported by footprinting studies that demonstrated that MTR alters the structure of $(AT)_n$ sequences flanking the G-C binding sites of the ligand (Cons & Fox, 1990). The higher stoichiometry of complex II (0.18 or 1 antibiotic molecule per 2.5 base pairs; Table 1), in spite of its larger size, provides indirect evidence for a structure distorted from normal B-DNA at the binding site. A widening of the minor groove probably occurs to accommodate the Mg^{2+}-coordinated dimer. The free energy change required for the interaction between complex II and DNA is derived largely from the entropy change ($\Delta S = 37$ eu). Such a positive change in entropy results from the disordering of water molecules, which were in an orderly placement along the grooves, especially minor, of B-DNA (Chuprina et al., 1991). As a result, B-DNA conformations at the ligand-binding site become disrupted. In contrast, the binding of the relatively smaller ligand, complex I, to DNA possibly does not perturb the hydration spine of DNA to a major degree, so that the change in entropy is low.

Comparison of Binding Potentials of MTR and $CHRA_3$ for Mg^{2+} and DNA. The difference in constituent sugar moieties of MTR and $CHRA_3$ potentially could influence interactions of these drugs with Mg^{2+} and DNA in the presence of Mg^{2+}. A comparison of spectroscopic features of their complexes with Mg^{2+} [present data and Aich and Dasgupta (1990)] reveals that they do not differ significantly in terms of the formation of complex I. Binding parameters [affinity constants and stoichiometries (drug:Mg^{2+})] are also identical (Aich & Dasgupta, 1990; Aich et al., 1992). On the contrary, dissimilarities in some of these properties were observed in the case of complex II. Although the stoichiometry (drug:Mg^{2+} = 2:1) is same for complex II with MTR and $CHRA_3$, the affinity constant for its formation is about 3-fold higher in the case of MTR (1.6×10^3 M^{-1}) than that of CHRA, (5.6×10^2 M^{-1}). We also noted a marked difference in the CD spectra of MTR and $CHRA_3$ during the formation of complex II. Comparative study indicates that the nature of Cotton effects in the CD spectra (350−220 nm) of complex II is dependent on the drugs. This could occur as a result of differences in the extent of exciton coupling of the two aglycons in dimer−Mg^{2+} complexes for MTR and $CHRA_3$ (Harada & Nakanishi, 1983). The origin of this chirality dependent spectroscopic property could be due to differences in either the nature of sugar residues on their disposition with respect to the aglycons in the drug−Mg^{2+} complex. These are mutually related, and the present data are not sufficient to distinguish between these possibilities. The pK_a's of MTR and $CHRA_3$ are 5.0 and 7.0,

respectively (Illrionova et al., 1970). At pH 8.0, both are predominantly anionic. Therefore, the higher value of the association constant of MTR toward Mg^{2+} for the formation of complex II could not be ascribed to the difference in pK_a values.

We also compared the influence of different sugar residues present in two drugs upon the recognition of DNA by drug–Mg^{2+} complexes. Scrutiny of the results with complex I could not suggest any remarkable differences between them. Comparison of the stoichiometries (moles of ligand bound per nucleotide) and thermodynamic parameters [present results and reports from Aich et al. (1992a)] for complex I–DNA interaction leads to this conclusion. This is further justified by their comparable spectroscopic properties. On the other hand, one could assume *a priori* from the preceding discussion that differences in their saccharide chains would be more pronounced in the case of complex II–DNA interactions for these two drugs. Comparison of thermodynamic parameters for the complex II–DNA interaction suggests an almost 1.6-fold higher ΔH_{vH} value for $CHRA_3$ (8.1 kcal/mol) than for MTR (5.1 kcal/mol). Without overemphasizing this difference, it could be proposed that this may originate from different sugar residues and their three-dimensional structures of the drug–Mg^{2+} complex. We also observed from binding stoichiometry that 1 molecule of complex II spans 2.5 base pairs in the case of MTR, while for $CHRA_3$, 1 molecule of complex II spans about 3.0 base pairs.

There are some NMR reports (Gao et al., 1992; Sastry & Patel, 1993; Keniry et al., 1993) that support our proposition that the solution structures of drug dimer–oligonucleotide complexes of MTR and $CHRA_3$ have global similarities as well as local differences. Other NMR studies demonstrated that the saccharide chains of these drugs play important role in determining the binding site on nucleotides, and as a consequence the trisaccharide moiety may be conformationally altered (Keniry et al., 1993). The patterns of cross peaks for the interaction of aglycons with DNA bases and the nature of the NOE contacts between sugars and the minor groove of same oligonucleotide duplex also depend on the drug. Therefore, this group suggested that saccharide chains have a role in sequence specific interactions with DNA. However, NMR results were obtained under conditions where the self-aggregation of drug molecules might occur to such an extent that it could compete with its ability to form the dimer–Mg^{2+} complex. The present results are not sufficient to comment upon the role of sugar moieties in determining sequence specificity, since the effect, if any, will average out over random sequences of bases of natural DNA used for our study. Further studies of oligonucleotides with defined sequences containing G-C bases are being performed to establish this effect.

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