The Sugars in Chromomycin A_3 Stabilize the Mg^{2+}–Dimer Complex†

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ABSTRACT: Chromomycin A_3 (CRA_3) is a glycosylated antitumor antibiotic that binds as a dimer to the minor groove of DNA, with a Mg^{2+} cation (or another divalent cation with a radius less than 0.85 Å) forming the center of the dimer. It has been shown that the chromose sugars are necessary for DNA binding [Kaziro & Kamiyama (1967) *J. Biochem.* (*Tokyo*) *62*, 424–429; Kamiyama (1968) *J. Biochem.* (*Tokyo*) *63*, 566–572], although the reason for this has not been addressed. We have investigated the role that the chromose sugars play in metal complexation in solution (methanol) by comparing the optical behavior of CRA_3 and its aglycon, CRN, in the presence of various divalent metals (Mg^{2+}, Ni^{2+}, and Ca^{2+}). The results show that CRA_3 forms a dimeric complex [i.e., $(CRA_3)_2M$, where M is a metal ion] in the presence of 1 mol equiv of either Ni^{2+} or Mg^{2+} but a 1:1 complex in the presence of the much larger Ca^{2+}. In contrast, CRN forms a 1:1 complex $(CRN{\cdot}M)^+$ with all three metals under identical conditions (1:1 mole ratio of drug to metal). Thus, for the smaller metal ions the sugars stabilize the 2:1 CRA_3–metal complex in solution. NMR data on the 2:1 CRA_3–Mg^{2+} complex show that the trisaccharide of one CRA_3 molecule lies in close proximity to the chromophore of the other CRA_3 molecule. This interaction, which is also present in the Mg^{2+}–CRA_3–DNA complex [Gao & Patel (1989) *Biochemistry 28*, 751–762], appears to be related to the stability of the dimer in solution. Larger ions such as Ca^{2+} presumably hinder this interaction, interfering with dimer formation. It is known that Ni^{2+} and Mg^{2+} promote the binding of CRA_3 to DNA but Ca^{2+} does not [Itzhaki et al. (1990) *Biopolymers 29*, 481–489; Gao & Patel (1990) *Biochemistry 29*, 10940–10956]. We have therefore concluded that the chromose sugars are necessary for DNA binding in large part because they help organize and stabilize the 2:1 drug–metal complex required for binding.

Chromomycin A_3 (CRA_3,[1] Figure 1) is a member of the aureolic acid group of antitumor antibiotics (Skarbek & Speedie, 1981). It was first isolated from fermentation broths of *Streptomices griseus* in 1960 (Shibata et al., 1960), but its structure was fully established only in 1982 (Miyamoto et al., 1967; Harada et al., 1969; Thiem & Meyer, 1979; Riccio & Nakanishi, 1982; Franck et al., 1986). CRA_3 has been shown to bind to DNA, terminating RNA and DNA synthesis (Wakisaka et al., 1963; Ward et al., 1965). Mg^{2+} or another divalent cation with a radius less than 0.85 Å is necessary for DNA binding (Ward et al., 1965; Kamiyama, 1968; Itzhaki et al., 1990; Gao & Patel, 1990; Gao et al., 1992). DNA footprinting and spectroscopic experiments have shown that CRA_3 in the presence of Mg^{2+} is selective for GC-rich regions of DNA and appears to recognize dGdG dinucleotide steps with a binding constant around 10^7 M^{-1} (Ward et al., 1965; Kersten et al., 1966; van Dyke & Dervan, 1983; Fox & Howarth, 1985; Stankus et al., 1992). Gao and Patel have recently studied the solution structure of the CRA_3–Mg^{2+}–DNA complex by NMR and have concluded that CRA_3 binds as a dimer to the minor groove of DNA, with a Mg^{2+} cation forming the center of the dimer (Gao & Patel, 1989; Banville et al., 1990). Using NMR data in conjunction with molecular modeling, Patel has developed a model for the dimer bound to DNA (Gao et al., 1992). The structural work suggested

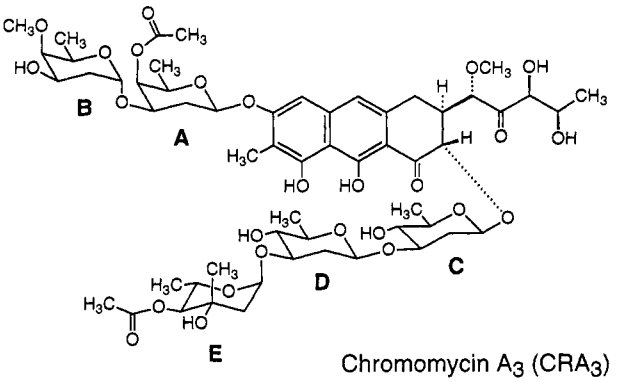

FIGURE 1: Structures of chromomycin A_3 (CRA_3) and its aglycon, chromomycinone (CRN).

that the primary role of the Mg^{2+} is to bring two CRA_3 molecules together in a particular orientation for binding to DNA.

We are interested in the role that the CRA_3 sugars play in dimer formation and DNA binding. In 1967 it was shown that the aglycon of CRA_3, chromomycinone (CRN, Figure 1), does not inhibit RNA polymerase, presumably because it does not bind to DNA (Kaziro & Kamiyama, 1967). This is consistent with the observation that CRN, unlike CRA_3, does not precipitate with calf thymus DNA in the presence of Mg^{2+} (Kamiyama, 1968). Furthermore, analogs of CRA_3

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[1] Abbreviations: ^1H NMR, proton nuclear magnetic resonance; [θ], molar ellipticity; CD, circular dichroism; CRA_3, chromomycin A_3; CRN, chromomycinone; DQF-COSY, double-quantum filtered correlation spectroscopy; FAB-MS, fast atom bombardment mass spectrometry; k, total concentration of the drug and the metal; ROESY, two-dimensional rotational frame nuclear Overhauser enhancement spectroscopy; TLC, thin-layer chromatography; x_{drug}, mole fraction of the drug; y_{max}, maximum of the y scale curve.

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lacking some of the sugars are less active than CRA_3 in binding to DNA (Koschel et al., 1966; Behr et al., 1969). On the basis of such findings it was suggested that the sugars in CRA_3 are required for DNA binding (Kayasaka et al., 1969). However, in the NMR studies few NOEs were observed between the CRA_3 sugars and the DNA, raising questions as to how the sugars contribute to DNA binding (Gao et al., 1992).

We have studied the ability of CRA_3 and CRN to form complexes in methanol with several divalent cations (Ni^{2+}, Mg^{2+}, and Ca^{2+}). Our results show that CRA_3 forms a stable 2:1 CRA_3–metal complex with both Ni^{2+} and Mg^{2+}, two metals known to promote DNA binding, but a 1:1 CRA_3–metal complex with Ca^{2+}, a metal which does not promote DNA binding. In contrast, CRN forms a 1:1 complex with all three metals under identical conditions (i.e., a 1:1 mole ratio of drug to metal). These results imply that for smaller metal ions the sugars on CRA_3 somehow stabilize the dimeric complex in solution. NMR data on the 2:1 CRA_3–Mg^{2+} complex in CD_3OD show that there is a close association between the C-D-E trisaccharide of one CRA_3 molecule and the chromophore of the other CRA_3 molecule. This interaction may be related to the stability of the dimer in solution. Larger ions such as Ca^{2+} presumably hinder the interaction, preventing the formation of a stable dimer. Since this interaction is also observed in the CRA_3–Mg^{2+}–DNA complex (Gao et al., 1989), the complex that forms in methanol and the one that binds to DNA are structurally similar. This is good evidence that the solution studies on metal complexation in methanol are relevant to understanding the drug–metal–DNA interaction. We have therefore concluded that the chromomycin sugars are necessary for DNA binding in large part because they help organize and stabilize the 2:1 drug–metal dimer required for DNA binding.

EXPERIMENTAL PROCEDURES

Crude chromomycin A_3 (CRA_3), received as a gift from Professor K. Nakanishi, was purified by flash column chromatography on Silica gel 60 (230–400 mesh, EM Science) using CH_3OH–CH_3COOH–$CHCl_3$ (3:1:96 v/v/v). Its purity was confirmed by TLC, ^1H NMR, and FAB-MS. Chromomycinone (CRN) was prepared by acidic degradation of chromomycin A_3, as reported elsewhere (Miyamoto et al., 1967). Stock solutions of the drugs were prepared by weighing the solids and dissolving them in a known volume of solvent.

All other chemicals were purchased from Aldrich. Metal stock solutions were prepared from the respective chlorides. Methanol was deaerated prior to use by purging with argon for 10 min.

UV Titrations. Ultraviolet–visible spectra from 220 to 500 nm were recorded on a 8452A diode array Hewlett-Packard spectrophotometer at room temperature (25 °C). Signal averaging was done at 4.0 s. Each spectrum was corrected by background subtraction.

Fresh stock solutions of the drugs and the metal salts in methanol were prepared prior to each titration. The stock solutions were diluted with methanol to the desired concentration. For each titration drug concentrations were kept constant and metal concentrations were varied. Titration curves were overlaid in order to detect isosbestic points.

Job Titrations. Job plots were generated following the procedure described by Angelici and others (Angelici, 1977; Cantor & Schimmel, 1980). Separate equimolar stock solutions of drug and metal were prepared. A series of solutions was prepared by mixing different volumes of the equimolar solutions of the two components to give solutions having identical total molar concentrations (defined as k M) but different mole fractions. The absorbance of each working solution was read at the appropriate working wavelengths, against a reference solution containing only the drug at the same nominal concentration. The difference between each pair of readings was plotted against the mole fraction of the drug in the mixtures, generating an absorbance difference graph.

For CRA_3 a variation of the method of continuous variation plots (Likussar & Boltz, 1971) was used to calculate the formation constant of the dimer. A tangent for the absorbance difference graph at $x_{drug} = 1.00$ was drawn and its intercept at $x_{drug} = 0.67$ was determined. This value, A_{theor}, corresponds to the theoretical absorbance difference for a 2:1 complex of infinite stability (i.e., nondissociating) at a concentration of $k/3$ M. The absorbance differences were divided by A_{theor}, generating a normalized absorbance scale (y scale). The maximum value of the normalized Job plot (y_{max}, at $x_{drug} = 0.67$, as expected) was used to calculate the formation constant of the dimer (K_f) by the formula derived for a 2:1 complex: $\log K_f = 0.3522 - 2 \log k + \log y_{max} - 3 \log (1 - y_{max})$.

CD Titrations. CD spectra from 220 to 500 nm were measured at 25 °C using a 1.0-mm solution cell with an Aviv CD spectrometer (Model 62 DS) with a Lauda K-4/R water bath. The wavelength increment was set at 1.0 nm and the signal averaging was 1.0 s. Each spectrum was corrected by background subtraction and treated with a curve-smoothing program. CD values were expressed in molar ellipticity $[\theta]$ (Cantor & Schimmel, 1980): $[\theta] = (\theta_{obs} \times 100)/(cl)$, where θ_{obs}, l, and c represent the observed ellipticity (in millidegrees), the path length of the cuvette (in centimeters), and the molar concentration of the absorbing species, respectively.

CD titrations were performed in the same way as UV titrations. The total amplitude of the split CD between 250 and 300 nm was monitored as a function of metal concentration.

^1H NMR Spectroscopy. Samples of vacuum-dried CRA_3 and its magnesium complex were prepared in CD_3OD (2–5 mM). One- and two-dimensional ^1H NMR experiments were recorded in a JEOL GSX 500-MHz spectrometer at 45 °C.

DQF-COSY data sets were acquired in the phase-sensitive mode with 2048 data points in the t_2 dimension and 400 data points in the t_1 dimension. The sweep width was 3460 Hz and the recycle delay was 2.5 s.

ROESY data sets were acquired with 2048 points in the t_2 dimension and 400 data points in the t_1 dimension. The same channel was used for the hard $\pi/2$ pulse and the spin-lock field, which was set at 2.75 KHz to minimize Hartmann–Hahn artifacts. The carrier frequency was set at 6.08 ppm and the sweep width was 5700 Hz. Mixing times ranged from 40 to 80 ms. The repetition delay was 2.5 s.

Following acquisition, the data were transferred to a Silicon Graphics 310VGX computer and processed using Felix (Hare Software, Inc.). In general, data were apodized with a skewed sine bell squared function shifted by 90° in both dimensions and the t_1 dimension was zero-filled to produce a 1024 × 1024 complex matrix. After Fourier transformation, both dimensions were baseline corrected.

Mass Spectrometry. Mass spectra measurements were performed at the Princeton University Department of Chemistry Mass Spectra Facility on a Kratos MS50TC operated in FAB mode. Samples were immobilized in a thioglycerol matrix.

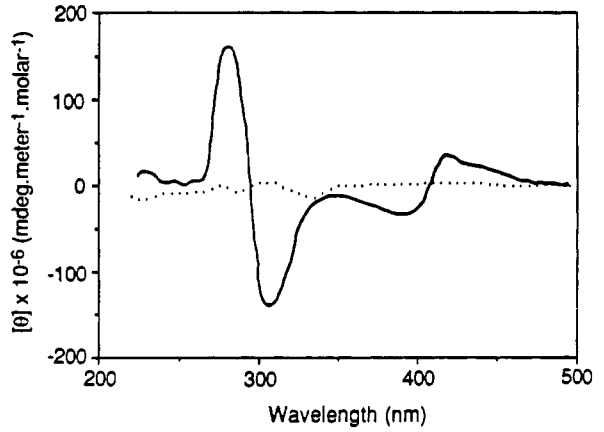

FIGURE 2: CD spectra of CRA_3 (200 μM) at 25 °C in 20 mM Tris buffer at pH 8.4 (continuous line) and in methanol (broken line).

RESULTS

On the Use of Methanol as the Solvent for Studying Complex Formation. The interaction of CRA_3 with various metals in aqueous buffer has been studied previously (Ward et al., 1965; Nayak et al., 1975; Itzhaki et al., 1990; Gao & Patel, 1990; Aich et al., 1992). We originally intended to carry out the following studies in aqueous buffer as well. However, CRA_3 aggregates even at micromolar concentrations in water (Hayasaki et al., 1969; Berman et al., 1985; Weinberger et al., 1988), and we have found that this aggregation precludes analysis of the data on complex formation.

For example, Figure 2 shows the CD spectrum of CRA_3 in aqueous buffer at pH 8.4. Under these conditions CRA_3 has an exciton coupling-type CD band with a negative Cotton effect centered at 280 nm (Harada & Nakanishi, 1983). As we and others (Aich et al., 1992) have observed, this split CD does not change dramatically when low amounts (up to 12 mol equiv) of Mg^{2+} are added. In part on the basis of this observation, it was recently argued that the stoichiometry of the complex formed at moderate concentrations of Mg^{2+} is 1:1 (Aich et al., 1992). As shown in Figure 2, however, CRA_3 does *not* have a split CD in methanol, a solvent in which we know it does not aggregate (data not shown). This result (as well as theoretical considerations; see Discussion) makes it unlikely that the CD pattern observed for CRA_3 in water in the absence of Mg^{2+} is due to an unassociated monomer. We have therefore questioned the interpretation that CRA_3 forms a 1:1 complex as Mg^{2+} is added in moderate excess.

We have studied the interaction of CRA_3 and CRN with three divalent cations of different sizes in methanol. The studies were carried out in methanol instead of water to avoid aggregation effects and circumvent the difficulties it can cause in data analysis. Two of the metals studied, Ni^{2+} and Mg^{2+}, have been shown to promote CRA_3–DNA binding, while the other, Ca^{2+}, does not (Itzhaki et al., 1990; Gao & Patel, 1990). Our results help explain both the role of the chromose sugars in DNA binding and why certain metals promote DNA binding while others do not.

Binding Assays with Ni^{2+} and Mg^{2+}

Studies with Chromomycin A_3: (a) UV Titration of CRA_3 in Methanol. UV–vis spectra of CRA_3 (65 μM for Mg^{2+}, 68 μM for Ni^{2+}) were recorded at different concentrations of added metal. Overlays of the absorbance spectra of CRA_3 in the presence of Ni^{2+} and Mg^{2+} are shown in Figure 3. Isosbestic points can be seen in the overlays,

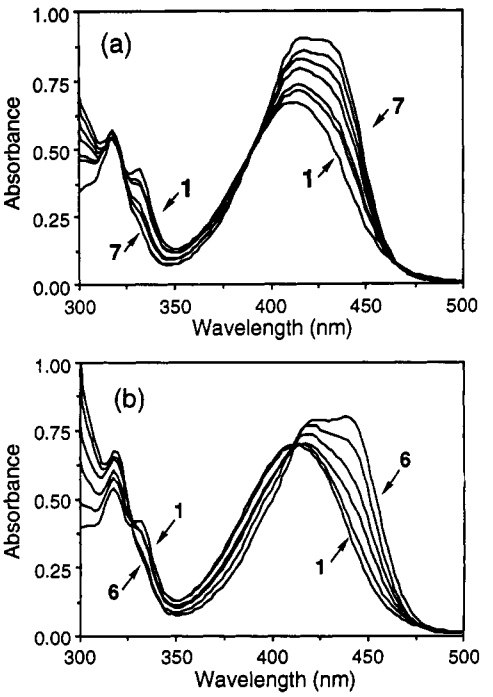

FIGURE 3: (a) Absorption spectra of CRA_3 (65 μM) in the presence of Mg^{2+} concentrations ranging from 0 (graph 1) to 41.3 μM (0.64 mol equiv, graph 7) in methanol at 25 °C. Notice the presence of isosbestic points at 322, 390, and 466 nm. Further addition of Mg^{2+} led to loss of isosbestic points. (b) Absorption spectra of CRA_3 (68 μM) in the presence of Ni^{2+} concentrations ranging from 0 (graph 1) to 45 μM (0.60 mol equiv, graph 6) in methanol at 25 °C. Notice the presence of isosbestic points at 328 and 412 nm. Upon further addition of Ni^{2+} these isosbestic points were lost and new isosbestic points at 330, 350, and 426 nm were observed (data not shown).

indicating a two-state transition for formation of both metal complexes (Tinoco et al., 1978). Absorbances at 440 nm (for Mg^{2+}) and at 460 nm (for Ni^{2+}) were monitored at metal concentrations up to 450 mol equiv and plotted to give the titration curves shown in Figure 4. These titration curves present two distinct regions, region I and region II, which will be considered separately below.

Region I: Mg^{2+}. Region I corresponds to the early part of the titration, up to around 1 mol equiv of metal (75 μM). The absorbance at 440 nm follows a sigmoidal curve (Figure 4a). It levels off at 0.50 mol equiv of added metal, indicating that the transition from CRA_3 monomer to its Mg^{2+} complex is complete. These facts suggest that region I corresponds to the conversion of CRA_3 to its Mg^{2+} dimer, $(CRA_3)_2Mg$.

A Job titration for Mg^{2+} was performed at 440 nm at 25 °C. The mole fraction of Mg^{2+} was varied between 0.00 and 0.50 (i.e., 0.0–1.0 mol equiv), corresponding to the Mg^{2+} mole fractions present in region I of the absorbance titration. A normalized plot (Figure 5) of the data showed a distinctive maximum at $x_{drug} = 0.68 \pm 0.02$. The theoretical maximum for a 2:1 complex is 0.67 (Likussar & Boltz, 1971). The Job titration therefore confirms that the stoichiometry of the CRA_3–Mg^{2+} complex formed in region I is 2:1. From the normalized plot y_{max} was found to be 0.765 ± 0.016, corresponding to a formation constant of $(5.9 \pm 2.9) \times 10^9$ M^{-2}.

Ni^{2+}. For Ni^{2+}, region I corresponds to metal concentrations from 0 to 52.5 μM (0.77 mol equiv). In region I Ni^{2+} shows very similar behavior to Mg^{2+}. The titration curve for absorbance at 460 nm is sigmoidal (Figure 4b). The absorbance plateaus at 0.50 mol equiv of metal, indicating that the transition from CRA_3 monomer to its Ni^{2+} complex

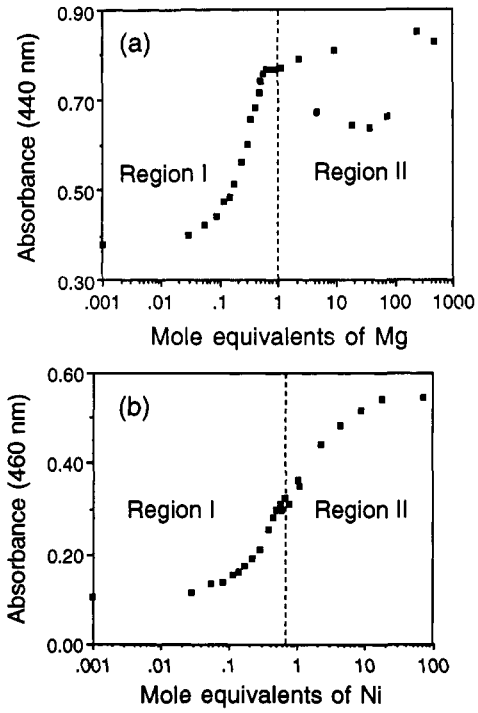

FIGURE 4: (a) Absorbance at 440 nm monitored for the Mg^{2+} titration of CRA_3 (65 μM) in methanol. Region I corresponds to Mg^{2+} concentrations from 0 to 75 μM (0–1.2 mol equiv), and region II corresponds to Mg^{2+} concentrations from 75 μM to 30 mM (1.2–460 mol equiv). (b) Absorbance at 460 nm monitored for the Ni^{2+} titration of CRA_3 (68 μM) in methanol. Region I corresponds to Ni^{2+} concentrations from 0 to 52.5 μM (0–0.8 mol equiv), and region II corresponds to Ni^{2+} concentrations from 52.5 μM to 4.8 mM (0.8–70 mol equiv).

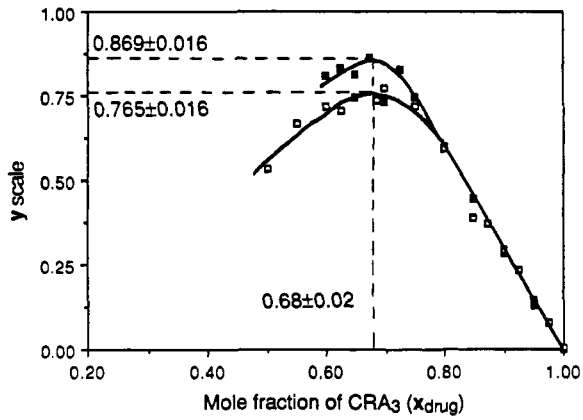

FIGURE 5: Normalized Job plots for Mg^{2+}–CRA_3 (□) and Ni^{2+}–CRA_3 (■) systems in methanol at 25 °C (see Experimental Procedures). The total concentration of drug and metal was set at 150 μM. For both systems the maximum of the plots occurred at $x_{drug} = 0.68 \pm 0.02$, indicating the formation of a 2:1 CRA_3–metal complex. For Mg^{2+} the value of y_{max} was 0.765 ± 0.016, resulting in a formation constant of $(5.9 \pm 2.9) \times 10^9$ for the $(CRA_3)_2Mg$ complex. For Ni^{2+} the value of y_{max} was 0.869 ± 0.016, resulting in a formation constant of $(3.9 \pm 3.3) \times 10^{10}$ for the $(CRA_3)_2Ni$ complex.

is complete. These facts suggest that region I corresponds to the conversion of CRA_3 to its Ni^{2+} dimer, $(CRA_3)_2Ni$.

A Job titration for Ni^{2+} in region I was also performed at 460 nm, at 25 °C. The mole fraction of Ni^{2+} was varied between 0.00 and 0.60, as shown in Figure 5. The plot showed a maximum at $x_{drug} = 0.70 \pm 0.02$, confirming the 2:1 stoichiometry of the complex. In the normalized Job plot y_{max} was found to be 0.869 ± 0.016, resulting in a formation constant of $(3.9 \pm 3.3) \times 10^{10}$ M^{-2}.

Region II: Mg^{2+}. For Mg^{2+}, region II corresponds to metal concentrations from 75 μM to 30 mM (1.2–460 mol equiv).

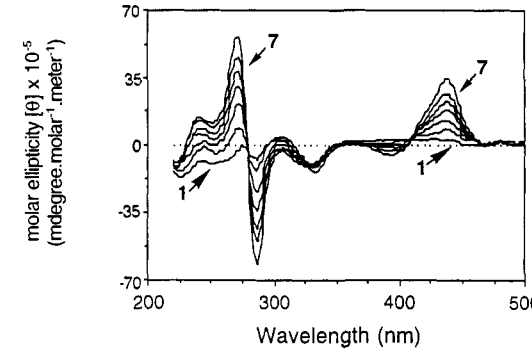

FIGURE 6: CD spectra for CRA_3 (200 μM) at concentrations of Mg^{2+} varying from 0 (graph 1) to 100 μM (0.50 mol equiv, graph 7). Notice the isosbestic points at 282, 328, and 408 nm, indicating a two-state transition. Further addition of Mg^{2+} led to the decrease of the amplitude of the exciton coupling-type band without discernible isosbestic points.

In this region there were no isosbestic points in the UV–vis spectra of CRA_3 (data not shown). Hyperchromicity effects were observed at 270–285 nm. The readings at 440 nm varied in a haphazard way with the Mg^{2+} concentration. These facts suggest that nonspecific association (aggregation) of CRA_3 takes place when excess Mg^{2+} is added. We believe that other processes are also occurring, specifically, the gradual conversion of the 2:1 complex to the 1:1 complex. This belief, however, is based on other results, including the CD data for Mg^{2+} (vide infra).

Ni^{2+}. For Ni^{2+} region II corresponds to metal concentrations from 52.5 μM to 4.8 mM (0.77–70 mol equiv). Upon addition of more than 0.80 mol equiv of Ni^{2+}, the UV–vis spectra presented further changes (data not shown). There was no hyperchromocity at 280 nm, indicating that aggregation is not a serious problem in methanol at high concentrations of Ni^{2+}. The isosbestic points at 328 and 412 nm disappeared, indicating the presence of another species in solution. However, new isosbestic points were identified at 330, 350, and 426 nm, indicating a two-state transition from the dimer to this new species. The transition was followed by the absorbance at 460 nm, and the titration data showed that this second transition was complete after the addition of 30 mol equiv of Ni^{2+} (Figure 4b).

(b) CD Studies of CRA_3 in Methanol. Preliminary studies done in our lab showed that CRA_3 has a relatively flat CD in methanol (Figure 2). The addition of Mg^{2+} or Ni^{2+} led to the formation of two exciton coupling-type CD bands, one with a negative Cotton effect centered around 280 nm and the other with a positive Cotton effect centered around 435 nm.

CD transition curves were recorded at a fixed CRA_3 concentration and varying Mg^{2+} or Ni^{2+} concentrations (shown for Mg^{2+} in Figure 6). For both metals, the exciton coupling-type CD bands increased in amplitude as the metal concentration was increased up to 0.6 mol equiv (Figure 6). Isosbestic points were present, again consistent with a two-state process. Plots of the molar ellipticity differences between 272 and 287 nm as a function of Ni^{2+} or Mg^{2+} concentration (for amounts of metal up to 0.6 mol equiv) showed a sigmoidal curve (data not shown). The similarity between the UV and CD titration curves for region I suggests that both techniques monitor the same process in solution.

In the presence of greater concentrations of Ni^{2+} or Mg^{2+}, the exciton coupling-type CD band slowly decreased (data not shown). For Mg^{2+}, which promotes nonspecific aggregation at high concentrations, there were no isosbestic points for this region. For Ni^{2+}, an isosbestic point was present at

Table I: Proton Chemical Shifts of CRA_3 (δ mon) and the 2:1 CRA_3–Mg^{2+} Complex (δ dim) in CD_3OD at 45 °C[a]

	H1			H2(a)			H2(e)			H3			H4		
	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm
A	5.38	5.48	–0.10	2.03	2.07	–0.04	2.10	2.10	0.00	4.13	4.04	0.09	5.18	5.16	0.02
B	5.10	5.08	0.02	1.87	1.87	0.00	1.61	1.63	–0.02	3.97	3.96	0.01	3.23	3.22	0.01
C	5.12	5.09	0.03	1.60	1.31	**0.29**	2.60	2.05	**0.55**	3.74	2.60	**1.14**	3.02	2.75	**0.27**
D	4.74	3.12	**1.62**	1.46	1.15	**0.31**	2.35	1.71	**0.64**	3.67	3.60	0.07	3.05	2.90	0.15
E	5.05	5.05	0.00	1.93	2.08	–0.15	1.93	1.95	–0.02	1.44	1.45	–0.01	4.68	4.75	–0.07

	H5			H6			Ac			OMe		
	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm
A	3.93	3.95	–0.02	1.20	1.24	–0.04	2.14	2.16	–0.02			
B	3.93	3.89	0.04	1.24	1.27	–0.03				3.56	3.56	0.00
C	3.33	3.04	**0.29**	1.33	1.24	0.09						
D	3.41	2.93	**0.48**	1.33	1.19	0.14						
E	4.11	4.16	–0.05	1.12	1.28	–0.16	2.09	2.14	–0.05			

	H2			H3			H4(a)			H4(e)			H5			H7		
	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm
CHR	4.73	4.44	**0.29**	2.80	2.42	**0.38**	2.96	2.89	0.07	2.64	2.52	0.12	6.72	6.57	0.15	2.15	2.10	0.05

	H10			H1′			OMe 1′			H3′			H4′			H5′		
	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm	δ mon	δ dim	Δ ppm
CHR	6.80	6.61	0.19	4.88	4.76	0.12	3.42	3.43	–0.01	4.16	4.15	0.01	4.23	4.21	0.02	1.25	1.25	0.00

[a] CRA_3 (monomer) shifts are listed as δ mon; 2:1 $CRA_3 - Mg^{+2}$ complex (dimer) shifts are listed as δ dim. The changes in chemical shift of the drug upon complexation are reported as Δ ppm = (δ mon – δ dim) in similar experimental conditions. Positive values of Δ ppm represent upfield shifts upon formation of dimer, and negative values represent downfield shifts upon formation of dimer. Values of |Δ ppm| ≥ 0.20 ppm are shown in boldface type. Large shielding effects are observed primarily in the C and D rings.

282 nm, indicating a two-state transition from the $(CRA_3)_2$-Ni dimer to another species. In the presence of 30 mol equiv of either Mg^{2+} or Ni^{2+} (which coincides with the end point of the second transition according to the UV titration of Ni^{2+}) the CD spectrum is almost flat, like the spectrum of pure CRA_3 in methanol. Thus, the disappearance of the exciton coupling-type CD bands appears to be associated with the conversion of the dimer to the monomer complex. It is not surprising that the 2:1 complex is converted to the 1:1 complex in the presence of excess metal ion; this is consistent with the mass-action principle.

(*c*) *Mass Spectrometry.* We have found that a CRA_3–Mg^{2+} complex can be reproducibly prepared by forming a suspension of Florisil in a methanol–chloroform solution of CRA_3, filtering the unreacted solid, and removing the solvent under reduced pressure (Skarbek & Speedie, 1981). FAB-MS analysis of this complex showed a molecular ion cluster consistent with the dimer $(CRA_3)_2Mg$. When this complex was redissolved in methanol, it presented an exciton coupling-type CD spectrum identical to that obtained at the end point of region I (i.e., the end point of the transition from uncomplexed monomer to dimer complex). Adding aqueous 1 M EDTA to this solution regenerated the spectrum characteristic of the unassociated CRA_3 monomer. Taken together, the results provide additional confirmation that the exciton coupling-type CD spectrum corresponds to the $(CRA_3)_2Mg$ dimer.

The mass spectroscopy results also provide additional (albeit indirect) evidence that $(CRA_3)_2Mg$ complex is more stable than the monomeric complex. The dimer forms preferentially when CRA_3 is exposed to Florisil, an insoluble magnesium silicate.

(*d*) *^1H NMR Spectroscopy.* The UV and CD titration data show that in methanol CRA_3 forms a 2:1 complex with Mg^{2+}. In order to shed light on the solution structure of the 2:1 CRA_3–Mg complex, its ^1H NMR spectrum was analyzed. The proton resonances of CRA_3 and its magnesium dimer in CD_3OD were assigned with a combination of 1D and 2D (DQF-COSY and ROESY) experiments, and the assignments are reported in Table I.

There are two major differences in the proton spectrum of the monomer and the magnesium dimer. First, several resonances of the dimer are shifted upfield relative to the monomer. As an extreme example, D1 (the anomeric proton of the D ring) is shifted 1.62 ppm upfield upon complexation. In general, such large shielding effects can only be caused by the proximity of the proton in question to an aromatic system (Wüthrich, 1986). Second, the dimer presents ROEs between protons in the D ring and protons in the A and chromophore rings. These contacts are more likely to be intermolecular than intramolecular because the latter case would require a serious and unreasonable distortion of the CRA_3 molecule. Taken together, these observations imply that in the dimer the CDE trisaccharide of one CRA_3 lies in close proximity to the chromophore of the other CRA_3, in a stacking interaction. A similar interaction is observed in the CRA_3–Mg^{2+}–DNA complex studied by Gao and Patel (1989, 1992). Indeed, the degree of chemical shift changes observed upon the dimerization of CRA_3 in methanol is comparable with the ones observed upon formation of the CRA_3–Mg^{2+}–DNA complex, as indicated in Figure 7. These results indicate that the structure of the $(CRA_3)_2Mg$ dimer in CD_3OD is very similar to the structure of the dimer in the DNA-bound form.

Studies with Chromomycinone: (*a*) *UV Titration of CRN in Methanol.* The UV–vis spectrum of CRN in methanol changes upon addition of Mg^{2+} or Ni^{2+}, indicating complex formation. UV–vis spectra of CRN were recorded at various concentrations of metals (shown for Mg^{2+} in Figure 8). Absorbances were monitored at 430 nm (for Mg^{2+}) and 440 nm (for Ni^{2+}) and plotted as a function of metal concentration (Figure 9).

As shown in Figure 9, the transition was complete after the addition of 1 mol equiv of either metal. The transition curves

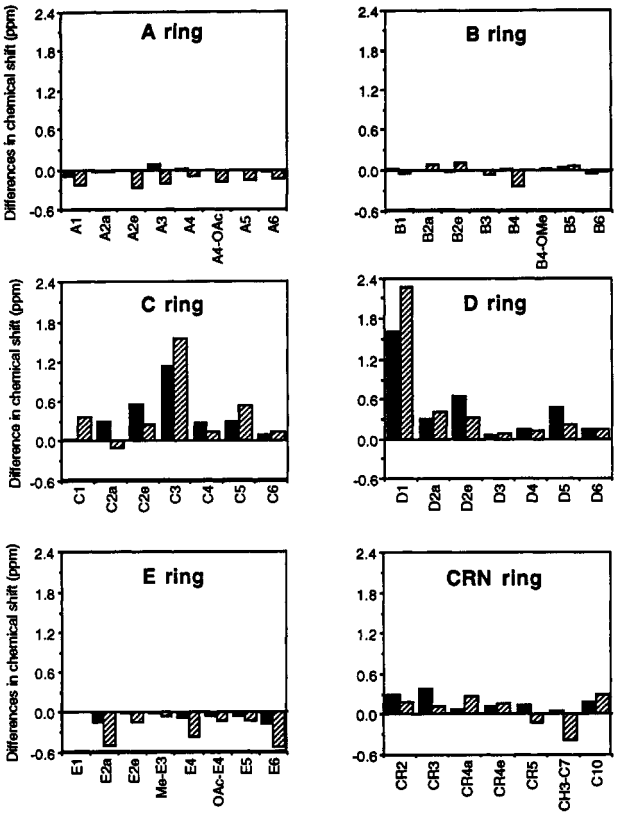

FIGURE 7: Differences in chemical shift of CRA_3 upon formation of the drug–Mg^{2+} 2:1 complex in methanol (solid bars) and a CRA_3–Mg^{2+}–DNA octamer complex in water (hatched bars). The differences are defined as δ(free) – δ(complex), where δ(free) correspond to CRA_3 in CD_3OD at 45 °C. The values of δ(complex) for the drug–DNA complex are from Gao and Patel (1989).

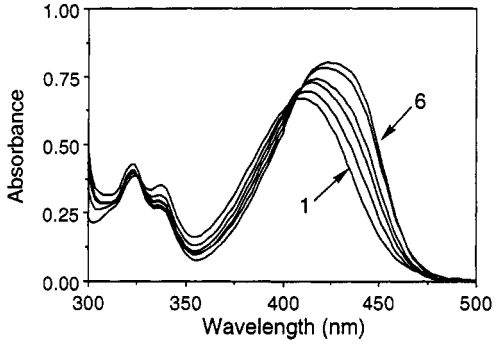

FIGURE 8: Absorption spectra of CRN (75 μM) in the presence of Mg^{2+} concentrations ranging from 0 (graph 1) to 3 mM (40 mol equiv, graph 6). No isosbestic points were valid throughout the titration.

are sigmoidal but no isosbestic points were conserved throughout the transition, suggesting that the transition is *not* a two-state process from CRN to a single metal complex.

Job titrations with both Mg^{2+} and Ni^{2+} were performed in methanol and the results are shown in Figure 10. Instead of the distinct maximum present for the CRA_3 case, there is a plateau in both Job plots from x_{drug} = 0.70 to 0.50. The evidence supports a model where CRN is converted to both $M(CRN)_2$ and $[M{\cdot}CRN]^+$ upon addition of the metal M^{2+} (where M^{2+} is Ni^{2+} and Mg^{2+}). The Job titration suggests that the 1:1 complex predominates as the metal concentration is increased to 1 mol equiv. The UV–vis titration curve indicates that the transition to the 1:1 complex is complete after 1 mol equiv has been added.

(b) CD Titrations of CRN in Methanol. The CD spectrum of CRN in methanol is almost flat. Addition of Mg^{2+} or Ni^{2+}

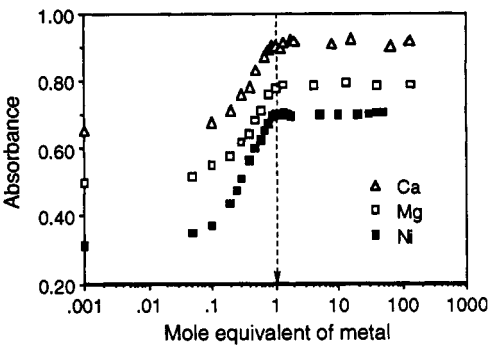

FIGURE 9: Absorbance monitored for the titration of CRN with Ni^{2+} (75 μM CRN, readings at 440 nm, ■), Mg^{2+} (75 μM CRN, readings at 430 nm, □), and Ca^{2+} (68 μM CRN, readings at 430 nm, △) in methanol at 25 °C. The transitions were effectively complete after the addition of 1.0 mol equiv of any of the three metals. No further changes were observed even in the presence of 100 mol equiv of any of the metals.

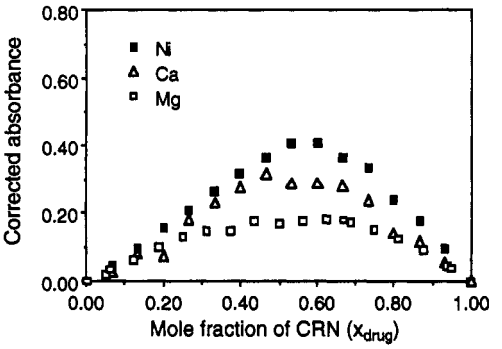

FIGURE 10: Nonnormalized Job plots for Mg^{2+}–CRN (□), Ni^{2+}–CRN (■), and Ca^{2+}–CRN (△) systems in methanol. For the three studied metals a plateau from x_{drug} = 0.70 to 0.50 is obtained, indicating that 2:1 and 1:1 complexes are formed. The plots also establish that the 1:1 complex predominates in solution once 1.0 mol equiv of each metal is added.

to solution did not produce major changes in the CD spectrum (data not shown).

Binding Assays with Ca^{2+}

(a) UV and CD Titrations of CRA_3 in Methanol. The UV–vis spectrum of CRA_3 was recorded at different concentrations of added Ca^{2+}. Overlays of the absorbance spectra at different metal concentrations were obtained (data not shown). The absorbance at 424 nm was monitored and plotted as a function of metal concentration.

Upon addition of Ca^{2+} the spectrum of CRA_3 presented changes, indicating interaction with the metal. After addition of 0.10 mol equiv of metal, hyperchromicity effects were observed at 280 and 430 nm, and the readings at 424 nm varied in a nonsystematic way as the Ca^{2+} concentration was further increased. The individual spectra in the Ca^{2+} titration were similar in this respect to the spectra obtained for region II of the Mg^{2+} titration. These facts suggest that CRA_3 aggregates in the presence of even small amounts of Ca^{2+}. Aggregation obscures any other processes that may be occurring.

CD transition curves were recorded for the same solutions used in the UV titration. Increasing Ca^{2+} concentrations did not produce the exciton coupling bands present in the Mg^{2+}–CRA_3 case, suggesting that the 2:1 CRA_3–Ca^{2+} complex is not formed in appreciable amounts during the titration. The CD spectra are similar to the CD spectra for the monomeric complex of CRA_3 and Ni^{2+} or Mg^{2+} (which are similar to the original spectrum of CRA_3). This observation suggests that

Ca^{2+} forms only a 1:1 complex with CRA_3. However, extensive aggregation, indicated by UV, prevents a definitive analysis of the Ca^{2+}–CRA_3 interaction.

(*b*) *UV and CD Titrations of CRN in Methanol.* The UV–vis spectrum of CRN in methanol changes upon addition of Ca^{2+}, suggesting complex formation. UV–vis spectra of CRN were recorded at various metal concentrations. Absorbances were monitored at 430 nm and plotted as a function of metal concentration (Figure 9).

The titration curves resemble the ones for the Mg^{2+}–CRN and Ni^{2+}–CRN studies. The full range of the transition was complete after the addition of 1 mol equiv of metal. Some isosbestic points were not conserved throughout the titration, suggesting that more than two species took part in the process. Excess metal did not alter the UV curves further.

A Job titration was done in methanol for Ca^{2+} at 424 nm (Figure 10). The curve presented a distorted shape with a plateau between $x_{drug} = 0.70$ and 0.50. This suggests a model similar to the Mg^{2+}–CRN case, with formation of both 1:1 and 2:1 complexes between CRN and Ca^{2+} at low concentrations of Ca^{2+}. The 1:1 complex predominates at 1 mol equiv of Ca^{2+}.

CD transition curves from 210 to 500 nm were recorded for the same solutions used in the UV studies. Throughout the titration the CD signal remained flat and similar to the original CRN spectrum (data not shown).

DISCUSSION

CRA_3 Forms a 2:1 Complex with Mg^{2+} and Ni^{2+}. Over the years CRA_3 and its DNA complex have been studied by many different physical techniques, including UV–vis (Kamiyama, 1968; Hayasaka et al., 1969; Behr et al., 1969; Itzhaki et al., 1990), CD (Weinberger et al., 1988; Aich et al., 1992), and NMR spectroscopies (Gao & Patel, 1989, 1990; Gao et al., 1992). Most of the studies have been done in aqueous buffer in order to approximate intracellular conditions. It has been shown, however, that CRA_3 aggregates in aqueous solutions at micromolar concentrations (Hayasaka et al., 1969; Weinberger et al., 1988). This observation raises concerns about the validity of some conclusions drawn from studies on the behavior of CRA_3 in aqueous buffer.

Recently, Aich et al. reported that CRA_3 forms two types of complexes with Mg^{2+} in aqueous buffer (Aich et al., 1992). A plot of the absorbance of CRA_3 at 440 nm as a function of the Mg^{2+} concentration is biphasic, suggesting the formation of two distinct complexes. Complex I reportedly forms in the presence of less than 12 mol equiv of Mg^{2+}. This complex was identified as the 1:1 complex partly on the basis of its CD spectrum, which has an exciton coupling-type CD spectrum similar to the one of CRA_3 in water in the absence of Mg^{2+}. Complex II forms as the Mg^{2+} concentration is increased further (up to several hundred mole equivalents of Mg^{2+}). This complex, which has an almost flat CD spectrum, was identified as the 2:1 complex. Data points for both phases of the titration were successfully fit to the proposed model of complex formation. It was also shown that the thermodynamic parameters for CRA_3 binding to DNA depend significantly on the Mg^{2+} concentration. This result was taken both as further proof for the existence of the two complexes and as evidence that they have different binding modes.

Unfortunately, Aich et al. did not take aggregation effects into consideration in analyzing their data. Since aggregation in water is known to be a problem even at low concentrations of CRA_3 and pH 8.0 (Weinberger et al., 1988), their conclusions are open to question. Moreover, it seems counterintuitive that complex II should present a *lower* metal mole fraction than complex I and yet form only in the presence of a much larger excess of metal. We have reinvestigated the interaction of CRA_3 with Mg^{2+} in methanol, a solvent in which CRA_3 does not aggregate up to concentrations of at least 2 mM (data not shown). We found that the CD spectrum of CRA_3 in methanol does not have an exciton coupling-type band like it does in aqueous buffer. It is therefore likely that the CD spectrum of CRA_3 in basic aqueous buffer represents some sort of aggregate (vide infra). In any case, the data in methanol indicate that CRA_3 forms a stable 2:1 complex with Mg^{2+} at low concentrations. As one might expect, it is possible to drive the equilibrium to the 1:1 complex by adding a sufficient excess of Mg^{2+} (up to 20 mol equiv).

CRN Forms a 1:1 Complex with Mg^{2+}. In contrast to CRA_3, CRN forms a 1:1 complex in the presence of 1 mol equiv of Mg^{2+}. Although there is some 2:1 complex in solution in the presence of less than 0.5 mol equiv of Mg^{2+}, the 2:1 complex can be converted entirely to the 1:1 complex by adding only 1 mol equiv of Mg^{2+}.

The Carbohydrates in CRA_3 Stabilize the 2:1 Complex. The fact that CRA_3 forms a 2:1 complex with 1 equiv of Mg^{2+} while CRN forms a 1:1 complex under the same conditions indicates that the carbohydrates in CRA_3 stabilize the dimeric complex. The implication of this result can be observed in the following scheme:

reaction I

$$\text{drug} + Mg^{2+} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [(\text{drug})Mg]^+ \qquad K_1 = k_1/k_{-1}$$

reaction II

$$[(\text{drug})Mg]^+ + \text{drug} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} [(\text{drug})_2Mg] \qquad K_2 = k_2/k_{-2}$$

where K_1 and K_2 represent equilibrium constants for reactions I and II, respectively. Both drugs should present similar values of K_1, since a 1:1 metal–drug complex does not present extreme steric congestion around the metal center and the carbohydrates are not likely to modify the complexation affinity of *individual* chromophores for Mg^{2+}. In the case of CRN the 2:1 complex that forms at low mole ratios of Mg^{2+} is converted to the 1:1 complex by the addition of a stoichiometric amount of Mg^{2+}, implying that $K_2 \ll K_1$. In the case of CRA_3, however, it is necessary to add a large excess of metal to convert the 2:1 complex to a 1:1 complex. Therefore, K_2 is more favorable for CRA_3 than for CRN. Once the first CRA_3 molecule binds to Mg^{2+}, the sugars somehow favor the binding of a second CRA_3 molecule.

The NMR data on the $(CRA_3)_2Mg$ complex in methanol shows an interaction between the chromophore of one CRA_3 and the trisaccharide of the other CRA_3. This interaction may be related to the stability of the 2:1 complex. The energetic basis for the stabilization is not clear, but it may be due in part to hydrophobic contacts between the chromophore and the trisaccharide. Alternatively, there may be a direct interaction between the sugars and the metal. It should be possible to distinguish between these mechanisms by studying other derivatives of CRA_3.

CRA_3 Does Not Form a 2:1 Complex in Solution with Large Divalent Cations. Studies by others have shown that some divalent cations facilitate CRA_3–DNA binding, while others do not (Itzhaki et al., 1990; Gao & Patel, 1990). We

have investigated the interaction of CRA_3 with Ni^{2+} and Mg^{2+}, two cations known to promote CRA_3–DNA binding, and with Ca^{2+}, a cation that does not promote DNA binding. We found that CRA_3 forms a dimeric complex with Ni^{2+} and Mg^{2+} but not with Ca^{2+}. UV–vis spectroscopy indicates that CRA_3 *does* interact with Ca^{2+}, but CD spectroscopy shows that the complex formed does not have the exciton coupling-type CD characteristic of the dimer. It seems likely that CRA_3 forms a 1:1 complex with Ca^{2+}, although nonspecific aggregation (indicated by hyperchromicity) of CRA_3 in the presence of this metal prevents an unequivocal analysis of the UV titration data. It is possible that a large ion, such as Ca^{2+}, hinders the interaction between the sugars of one CRA_3 molecule and some part of the other CRA_3 molecule. As discussed above, this interaction appears to play the primary role in stabilizing the dimer relative to the monomer. Therefore, the CRA_3 sugars play an important role in determining the size of the metal that can be accommodated in the dimer.

The titration data for CRN show that it interacts in a similar manner with all three metals. At very low concentrations of the metals (less than 0.50 mol equiv), there is some 2:1 CRN–metal complex formed. As the metal concentration increases, the 1:1 CRN–metal complex begins to predominate. Once 1 mol equiv is added, the 1:1 CRN–metal complex is the major species in solution.

Are These Results Relevant to the Behavior of CRA_3 in Water? One question that needs to be addressed is whether the results on metal complex formation in methanol are relevant to the behavior of CRA_3 in water, particularly since Aich et al. have proposed that CRA_3 forms a 1:1 complex with Mg^{2+} in water at concentrations where we observe a 2:1 complex in methanol (Aich et al., 1992). Although it is not possible to determine conclusively whether the trend we observe in methanol also holds for water, there are some facts which suggest that it does. In fact, we believe the methanol results help explain some of the data obtained in water.

Fact I. An exciton-type CD band with a negative Cotton effect centered at 280 nm can be observed both for the CRA_3 dimer in methanol (Figure 6) and for CRA_3 in aqueous buffer at moderate concentrations of Mg^{2+} (Aich et al., 1992). Exciton-type CD bands derive from the interaction of two chromophores in close proximity (Harada & Nakanishi, 1983); since CRA_3 presents a single chromophore in its structure, the presence of an exciton-type CD band implies that two or more drug molecules are held together. The exciton pattern can be used to shed light on nature of the association between chromophores (Bosnich, 1969; McCaffery et al., 1969). The UV band at 280 nm has been assigned as a ^1B_b transition along the long axis of the chromophore (Harada et al., 1969). There are two possible relative orientations of the chromophores that would lead to the observed exciton-type CD band with a negative Cotton effect: facially stacked with a left-hand twist (Goto & Kondo, 1991) or edge-on with a left-hand twist. In the case of the dimer in methanol, the NMR data (see above) support the edge-on model to explain the exciton-type CD band.

In the case of CRA_3 in water, the CD band at 280 nm in the absence of Mg^{2+} is probably due to facial stacking. This kind of stacking interaction has been observed for various anthrocyanins in water (Goto & Kondo, 1991; Kondo et al., 1992) and is supported by the decreasing molar absorptivity of CRA_3 in the UV–vis region with increasing concentration (Hayasaka & Inoue, 1969). Upon addition of Mg^{2+} (up to 10 mol equiv of metal) there are small but reproducible changes in the CD pattern, suggesting at least some conversion of the stacked form to a Mg^{2+} dimer form. However, once again aggregation in water prevents detailed analysis of the data.

Fact II. When a large excess of Ni^{2+} or Mg^{2+} is added to CRA_3 in methanol, the CD spectrum characteristic of the dimer disappears. Aich et al. observe a similar trend in water: the exciton-type CD band centered at 280 nm disappears as a large excess of Mg^{2+} is added. They attribute this to formation of the 2:1 CRA_3–Mg^{2+} complex from the 1:1 complex as excess Mg^{2+} is added. However, our CD and UV–vis titration data for Ni^{2+} (a metal which behaves in a manner very similar to Mg^{2+} but does not promote nonspecific aggregation of CRA_3 species at high concentration) clearly indicate that the 2:1 complex is converted to the 1:1 complex in the presence of high concentrations of metals and that *this process is associated with the disappearance of the split CD characteristic of the dimer.* The monomer would not be expected to present an exciton-type CD band since its structure does not present organized chromophores in close contact.

Fact III. The strongest evidence for the relevance of the methanol results comes from NMR studies on the CRA_3–Mg^{2+} 2:1 complex. The combination of unusual chemical shift changes and intermolecular ROEs indicates that this complex in methanol has a structure that is very similar to the CRA_3–Mg^{2+} complex bound in the minor groove of DNA (Gao & Patel, 1989). Although there may be some differences in the equilibrium constants for metal chelation of the first and second CRA_3 molecules in water and methanol, the dimer that forms in both solvents is essentially identical. If one accepts the evidence that the trends in water and methanol are similar, then our studies on metal complex formation in methanol shed light on phenomena that cannot readily be studied in water because of aggregation effects.

CONCLUSIONS

We have shown that in methanol CRA_3 forms a dimeric complex with Mg^{2+} and Ni^{2+} but a monomeric complex with Ca^{2+}. In contrast, CRN forms a monomeric complex with all three metals. Therefore, for divalent cations of small enough size, the CRA_3 sugars stabilize the dimer. We have also shown that the C-D-E sugars on one molecule interact with the chromophore of the other molecule in the dimer. We have proposed that this interaction is related to the stability of the complex and that it cannot occur if the metal ion is too large. Since this interaction is also observed in the CRA_3–Mg^{2+}–DNA complex, the complex that forms in methanol and the one that binds to DNA are structurally similar. On the basis of this and other evidence, we have concluded (a) that these methanol results are relevant to understanding the behavior of CRA_3 in water and (b) that they therefore imply that the chromomycin carbohydrates are necessary for DNA binding in large part because they help organize and stabilize the metal–drug dimer.

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SUPPLEMENTARY MATERIAL AVAILABLE

Figures A–H, showing binding data for CRA_3 and CRN (8 pages). Ordering information is given on any current masthead page.

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