Studies of the 2:1 Chromomycin A_3–Mg^{2+} Complex in Methanol: Role of the Carbohydrates in Complex Formation

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Abstract: Chromomycin A_3 (CRA_3) is a glycosylated antitumor antibiotic that binds to DNA as a 2:1 drug–Mg^{2+} complex. We have previously shown that the carbohydrates of CRA_3 stabilize the 2:1 drug–Mg^{2+} complex in methanol. In the present study we investigate the structure of the 2:1 drug–Mg^{2+} complex in methanol. The 2:1 CRA_3–Mg^{2+} complex is octahedral in methanol, with the possibility of forming eight diastereoisomers. A combination of conformational analysis and DNMR indicates that only two of these isomers are formed. In both isomers the CDE trisaccharide of one CRA_3 molecule contacts the chromophore of the other CRA_3 molecule, stabilizing the 2:1 complex relative to the 1:1 complex. The major isomer in methanol is identical with the 2:1 complex that binds to DNA, reported by Patel and co-workers (*J. Mol. Biol.* **1992**, *223*, 259–279). In addition, studies of degradation products of CRA_3 show that the entire CDE trisaccharide is critical for stable dimer formation. The AB disaccharide and the acyclic side chain do not appear to play major roles in dimer formation. Taken together, our results show that the ability to form stable dimers is critical for DNA binding and the CDE trisaccharide specifically stabilizes the dimeric complex with the appropriate shape to bind to the right-handed DNA duplex. The results reported here provide a starting point for designing synthetic model compounds based on CRA_3. Studies on such model compounds will establish minimal structural requirements for dimer formation and DNA binding and may lead to the development of new antitumor agents.

Introduction

In recent years there has been much interest in glycosylated antitumor antibiotics such as the calicheamicins,[1] the esperamicins,[2] the anthracyclines,[3] and the aureolic acids.[4] The carbohydrates in these drugs are known to be important for DNA binding and site selectivity. However, their actual role in drug–DNA interactions has not been fully established.[5] A better understanding of the role of the carbohydrates in DNA binding could allow the rational design of DNA binders and antitumor agents.

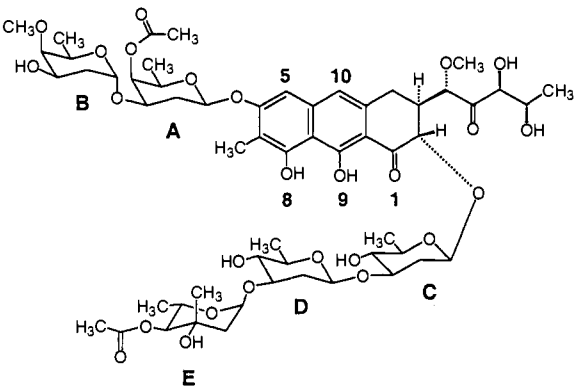

Figure 1. Structure of chromomycin A_3 (CRA_3).

Chromomycin A_3 (CRA_3, Figure 1) is a member of the aureolic acid group[3a] and was first isolated in the 1960's.[6] Studies have shown that CRA_3 binds to DNA at GC-rich regions[7] and inhibits replication and transcription processes.[8] Mg^{2+} was found to be necessary for binding,[9] and in 1989 NMR studies by Gao and Patel established that CRA_3 binds as a 2:1 drug–Mg^{2+} complex in the minor groove of DNA.[10a] In the complex the drug acts as a bidentate ligand, coordinating the metal through an ionized phenolate (O_9) and the neighboring ketone oxygen (O_1). A crystal structure of a CRA_3–Mg^{2+}–DNA complex shows that Mg^{2+}

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(1) (a) Zein, N.; Sinha, A. M.; McGahren, W. J.; Ellestad, G. A. *Science* **1988**, *240*, 1198. (b) Lee, M. D.; Ellestad, G. A.; Borders, D. B. *Acc. Chem. Res.* **1991**, *24*, 235.

(2) (a) Long, B. H.; Golik, J.; Forenza, S.; Ward, B.; Rehfuss, R.; Dabrowiak, J. C.; Catino, J. J.; Musial, S. T.; Brookshire, K. W.; Doyle, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2. (b) Sugiura, Y.; Shiraki, T.; Konishi, M.; Oki, T. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3831.

(3) (a) Kersten, H.; Kersten, W. *Inhibitors of Nucleic Acid Synthesis: Biophysical and Biochemical Aspects*; Springer-Verlag: New York, 1974. (b) Lown, J. W. Anthracycline and Anthracenedione-Based Anticancer Agents, 1988, Section 2. *Bioact. Mol.* **1988**, *6*.

(4) (a) Skarbek, J. D.; Speedie, M. K. In *Antitumor Compounds of Natural Origin: Chemistry and Biochemistry*; Aszalos, A., Ed.; CRC Press: FL, 1981; Vol. I, pp 191–235. (b) *Molecular Basis of Specificity in Nucleic Acid–Drug Interactions*; Pullman, B., Jortner, J., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1990; Vol. X, pp 414–422. (c) Gause, G. F. In *Antibiotics*; Vol. III, *Mechanism of Action of Antimicrobial and Antitumor Agents*, Corcoran, J. W., Hahn, F. E., Eds.; Springer-Verlag: New York, 1975; pp 197–202.

(5) For an insight on the role of sugars of calicheamicin in DNA binding and site selectivity, see: (a) Drak, J.; Iwasawa, N.; Danishefsky, S.; Crothers, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7464. (b) Walker, S.; Landovitz, R.; Ding, W.-D.; Ellestad, G. A.; Kahne, D. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4608. (c) Walker, S.; Murnick, J.; Kahne, D. *J. Am. Chem. Soc.*, preceding paper in this issue.

(6) Chromomycin A_3 was isolated from fermentation broths of *Streptomyces griseus*: (a) Shibata, M.; Tanabe, K.; Hamada, Y.; Nakazawa, K.; Miyabe, A.; Hitomi, H.; Miyamoto, M.; Mizuno, K. *J. Antiobiot., Ser. B* **1960**, *13*, 1. However, its structure was fully established only in 1982: (b) Miyamoto, M.; Kawashima, K.; Kawamatsu, Y.; Shinohara, M.; Tanaka, K.; Tatsuoka, S.; Nakanishi, K. *Tetrahedron* **1967**, *23*, 421–437. (c) Harada, N.; Nakanishi, K.; Tatsuoka, S. *J. Am. Chem. Soc.* **1969**, *91*, 5896–5898. (d) Thiem, J.; Meyer, B. *J. Chem. Soc., Perkin Trans. 2* **1979**, 1331–1336. (e) Riccio, R.; Nakanishi, K. *J. Org. Chem.* **1982**, *47*, 4589–4592. (f) Franck, R. W.; Bhat, V.; Subramanian, C. S. *J. Am. Chem. Soc.* **1986**, *108*, 2455–2457.

(7) (a) Ward, D. C.; Reich, E.; Goldberg, I. H. *Science* **1965**, *149*, 1259–1263. (b) Kersten, W.; Kersten, H.; Szybalski, W. *Biochemistry* **1966**, *5*, 236–244. (c) van Dyke, M. W.; Dervan, P. B. *Biochemistry* **1983**, *22*, 2373–2377. (d) Fox, K. R.; Howarth, M. R. *Nucl. Acids. Res.* **1985**, *13*, 8695–8714. (e) Stankus, A.; Goodisman, J.; Dabrowiak, J. *Biochemistry* **1992**, *31*, 9310–9318. In this work it was argued, based on quantitative footprinting results, that one CRA_3 molecule binds in the DNA minor groove first and that a second CRA_3 molecule then binds to form the 2:1 complex with Mg^{2+}. The implication is that the DNA somehow facilitates formation of the 2:1 CRA_3–Mg^{2+} complex. However, analysis of the results is complicated by aggregation effects—see ref 12c.

(8) Wakisaka, G.; Uchino, H.; Nakamura, T.; Sotobayashi, H.; Shirakawa, S.; Adachi, A.; Sakurai, M. *Nature* **1963**, *198*, 385. See also ref 7a.

(9) (a) Kamiyama, M. *J. Biochem. (Tokyo)* **1968**, *63*, 566–572. (b) Itzhaki, L.; Weinberger, S.; Livnah, N.; Berman, E. *Biopolymers* **1990**, *29*, 481–489.

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occupies an octahedral site, coordinated by two water molecules and two CRA_3 molecules.[11a]

We are interested in the role that the carbohydrates play in the activity of CRA_3. Transcription inhibition studies showed that degradation products of CRA_3 lacking some of the sugars bind less effectively to DNA and are less active than the intact drug.[12] The aglycon of CRA_3, chromomycinone (CRN), does not bind to DNA at all.[13] These studies established that the sugars are critical for effective binding but did not indicate why. NMR studies performed by Gao and Patel provided a detailed picture of a CRA_3–Mg^{2+}–DNA complex.[10d] However, it remained unclear if the sugars influence dimer formation or if the DNA duplex is responsible for the assembly and stabilization of the dimeric metal complex. We recently showed that in methanol CRA_3 forms a 2:1 drug–metal complex [*i.e.*, $(CRA_3)_2Mg$] in the presence of 1 mol equiv of Mg^{2+}; in contrast, CRN forms a 1:1 complex under identical conditions (1 mol equiv of metal).[14] Since CRA_3 binds to DNA as a 2:1 drug–Mg^{2+} complex, this result suggests that one role of the CRA_3 sugars is to stabilize the drug dimer that binds to DNA. This result also suggests that the DNA duplex is not required for dimer formation.[15]

In the present work we have taken a closer look at how the carbohydrates influence the structure of the dimeric metal complex in methanol. CRA_3 forms an octahedral 2:1 drug–Mg^{2+} complex in methanol, with water molecules presumably completing the coordination sphere of the metal. There are eight *possible* octahedral diastereoisomers with the formula $Mg(CRA_3)_2(H_2O)_2$. A conformational analysis indicates that unfavorable steric interactions involving the CDE trisaccharide prevent the formation of six of these diastereoisomers. Of the other two complexes, dynamic NMR shows that one diastereoisomer is heavily favored. Further NMR studies indicate that the CDE trisaccharide of one CRA_3 molecule and the aromatic chromophore of the other CRA_3 molecule are juxtaposed in both the major and minor diastereoisomers. This interaction apparently stabilizes the dimer complex relative to the monomer in solution. NMR data suggest that the predominant diastereoisomer is structurally identical to the 2:1 drug–Mg^{2+} complex that binds to DNA. Thus, the sugars in CRA_3 specifically organize the dimeric complex with the appropriate shape to bind to the right-handed DNA duplex.

By studying degradation products of CRA_3 we have shown that all three sugars of the CDE trisaccharide are critical for the stabilizing interaction. The B sugar does not play a significant role in dimer stability. (Since the A ring cannot be removed selectively, we do not yet know whether it is necessary for dimer formation.) Moreover, indirect evidence suggests that the acyclic side chain is not involved in dimer formation. It remains to be established whether the ability to form a stable dimer is enough to ensure effective DNA binding.[41] Nevertheless, the results reported here provide a starting point for designing synthetic model compounds based on CRA_3. Studies on such model compounds will shed further light on the structural requirements for DNA binding and may lead to the development of new antitumor agents or other DNA binders.

Experimental Procedures

CRA_3 for NMR experiments was purchased from Sigma and used without further purification. The 2:1 CRA_3–Mg^{2+} complex was prepared by forming a suspension of Florisil (Aldrich) in a methanol–chloroform solution of CRA_3, filtering the unreacted solid, and removing the solvent under reduced pressure. The degradation products of CRA_3 were prepared by acidic digestion of CRA_3, as reported previously.[16] Their identity was confirmed by FAB-MS and ^1H NMR. Stock solutions of the drugs were prepared by weighing the solids and dissolving them in a known volume of solvent. Methanol was deaerated prior to use by purging with argon for 10 min. Metal stock solutions were prepared from the respective chlorides.

^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 proton NMR experiments were recorded in a JEOL GSX 500-MHz spectrometer.

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 repetition delay was 2.5 s. For each t_1 increment 48 scans were collected.

ROESY data sets were acquired in the phase-sensitive mode, with 2048 points in the t_2 dimension and 400 data points in the t_1 dimension. For each t_1 increment 48 scans were collected. 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. Mixing times ranged from 40 to 80 ms. The sweep width was 5700 Hz and the repetition delay was 2.5 s.

Exchange spectra (NOESY) data sets were acquired at −25 °C, with 2048 points in the t_2 dimension and 256 data points in the t_1 dimension. For each t_1 increment 48 scans were collected. The mixing time was set at 25 ms. The sweep width was 3500 Hz and the recycle time was 2 s.

Following acquisition, the data were transferred to a Silicon Graphics 310VGX computer and processed with the Felix program (Hare Software, Inc.). In general, data sets were apodized with a skewed sine bell squared function by 90° in both dimensions and zero-filled in the t_1 dimension to 1024 points prior to Fourier transformation. Both dimensions were then baseline corrected.

Analysis of Chemical Exchange by DNMR. DNMR allows the calculation of the activation free energy for an interconversion process which is slow in the NMR time scale. For an uncoupled AB system with unequal populations (such as the aromatic resonances CR5 and CR10 in the dimer), the free energies of activation are given as[17] $\Delta G^{\ddagger}_{A\rightarrow B} = 4.57T_c\{10.62 + \log x/[2\pi(1 - \Delta P)] + \log T_c/\Delta\nu_{AB}\}$ and $\Delta G^{\ddagger}_{B\rightarrow A} = 4.57T_c\{10.62 + \log x/[2\pi(1 + \Delta P)] + \log T_c/\Delta\nu_{AB}\}$, where T_c is the decoalescence temperature, defined as the temperature (in Kelvin) at which the peaks undergo maximum broadening before decoalescing; ΔP is the difference in fractional populations of the exchanging states, estimated by integration of decoalesced resonances at low temperatures; and $\Delta\nu$ is the chemical shift difference (in Hz) at the slow exchange limit. It has been shown[17] that at the coalescence temperature $\Delta P = [(x^2 - 2)/3]^{3/2}(1/x)$. Numeric solutions of x as a function of ΔP have been calculated by Egan and Mislow.[18]

UV Titrations. UV–vis spectra from 220 to 800 nm were recorded on a 8452A diode array Hewlett-Packard spectrophotometer at room temperature (25 °C). Signal averaging was done at 5.0 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 (65 μM for CRA_3; 75 μM for A–CRN–CDE; 100 μM for BA–CRN–CD, A–CRN–C, and A–CRN; 75 μM for CRN) and metal concentrations were varied. Titration curves were overlaid in order to detect isosbestic points. Absorbances at 440 nm (for A–CRN–CDE and BA–CRN–CD) and at 430 nm (for A–CRN–C and A–CRN)

(10) (a) Gao, X.; Patel, D. J. *Biochemistry* **1989**, *28*, 751–762. (b) Banville, D.; Keniry, M.; Kam, M.; Shafer, R. *Biochemistry* **1990**, *29*, 6521–6534. (c) Gao, X.; Patel, D. J. *Biochemistry* **1990**, *29*, 10940–10956. (d) Gao, X.; Mirau, P.; Patel, D. J. *J. Mol. Biol.* **1992**, *223*, 259–279.

(11) (a) Hendrickson, W. A. 10th International Biophysics Congress, Vancouver, British Columbia, 1990. For reviews on other DNA binding metal complexes see: (b) Barton, J. K. *Comments Inorg. Chem.* **1985**, *3* (*6*), 321–348. (c) Barton, J. K. *Science* **1986**, *233*, 727–734. (d) Sigman, D. S. *Biochemistry* **1990**, *29*, 9097–9105. (e) Sigman, D. S.; Chen, C-h. B. *Ann. Rev. Biochem.* **1990**, *59*, 207–236.

(12) (a) Koschel, K.; Hartmann, G.; Kersten, W.; Kersten, H. *Biochem. Z.* **1966**, *344*, 76–86. (b) Behr, D.; Honikel, K.; Hartmann, G. *Eur. J. Biochem.* **1969**, *9*, 82–92. (c) Hayasaka, T.; Inoue, Y. *Biochemistry* **1969**, *8*, 2342–2347.

(13) Kaziro, Y.; Kamiyama, M. *J. Biochem.* (*Tokyo*) **1967**, *62*, 424–429. See also ref 7a.

(14) Silva, D. J.; Goodnow, R.; Kahne, D. *Biochemistry* **1993**, *32*, 463–471.

(15) However, the DNA duplex may further stabilize the drug–metal complex. Evidence for this comes from binding experiments with Cd^{2+}: this rather large metal does not form a stable 2:1 complex in methanol (unpublished results from this lab) but promotes weak drug–DNA interaction—see ref 10c.

(16) (a) Miyamoto, M.; Kawashima, K.; Kawamatsu, Y.; Shinohara, M.; Tanaka, K.; Tatsuoka, S.; Nakanishi, K. *Tetrahedron* **1967**, *23*, 421–437. (b) Yoshimura, Y.; Koenuma, M.; Matsumoto, K.; Tori, K.; Terui, Y. *J. Antibiot.* **1988**, *41*, 53–72.

(17) Shanan-Atidi, H.; Bar-Eli, K. H. *J. Phys. Chem.* **1970**, *74*, 961.

(18) Egan, W. Ph.D. Dissertation, Princeton University, 1971, pp 30–31.

were monitored at Mg^{2+} concentrations up to 50 mol equiv and plotted to give the titration curves shown in Figure 6.

Job Titrations. Job plots were generated following the procedure described by Angelici and others.[19] 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 drugs that form stable 2:1 drug–Mg^{2+} complexes, a variation of the method of continuous variation plots[20] was used to calculate the formation constant of the complex. 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: $\log K_f = 0.3522 - 2 \log k + \log y_{max} - 3 \log (1 - y_{max})$.

Results

The following studies were carried out in methanol because CRA_3 aggregates even at micromolar concentrations in water,[12c] preventing clear analysis of data on complex formation. Previous results indicate that studies of the Mg^{2+} complex formation in methanol are relevant to understanding the binding of CRA_3 to DNA.[14]

a. The 2:1 CRA_3–Mg^{2+} Complex Is Octahedral in Methanol. Using UV binding assays we previously showed that CRA_3 forms a stable 2:1 drug–Mg^{2+} complex in methanol.[14] A FAB-MS analysis of the 2:1 complex (methanol matrix) shows a molecular cluster consistent with the formula $Mg(CRA_3)_2(H_2O)_2$.[21] The two water molecules are most likely coordinated to the Mg^{2+} ion, suggesting that the cation is octahedral in the complex. Further evidence for the octahedral state of the metal comes from NMR studies[14] showing that the structure of the 2:1 drug–Mg^{2+} complex in methanol is very similar to the 2:1 complex that binds to DNA.[10d,11a] Since the 2:1 CRA_3–Mg^{2+} complex that binds to DNA is octahedral, it is likely that the 2:1 complex in methanol is also octahedral.

Nevertheless, we were not able to study the coordination state of the Mg^{2+} ion in solution because Mg^{2+} is a d^0 metal with no appreciable absorption in the UV–vis region. However, Co^{2+} is a d^7 ion that is considered to be a good model for d^0 metals of comparable size such as Mg^{2+}.[22] Co^{2+} presents characteristic absorption bands in the UV–vis region according to its coordination geometry and has been extensively used to study metal coordination in proteins and similar systems.[23] Furthermore, Co^{2+} forms a stable 2:1 CRA_3–metal complex in methanol[24] and, like Mg^{2+}, has been shown to promote CRA_3–DNA binding.[9b,10c]

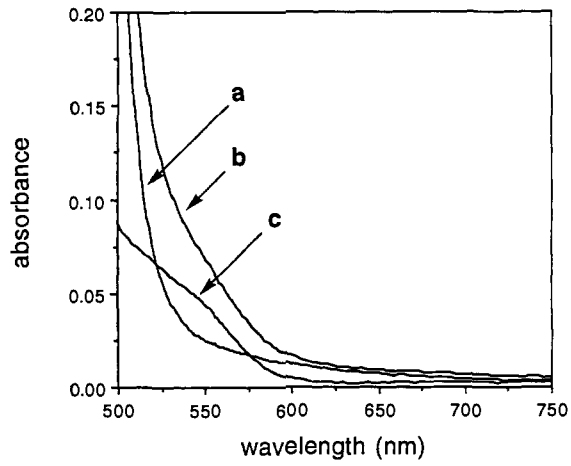

Figure 2. Detail of the UV–vis spectra of CRA_3 (a) and the 2:1 CRA_3–Co^{2+} complex (b), both 2.5 mM in methanol at 25 °C. Curve c corresponds to the spectral difference between curves b and a. The low-intensity shoulder around 500 nm in curve c suggests that the 2:1 CRA_3–Co^{2+} complex is octahedral in solution. Since Co^{2+} is considered to be a good model for Mg^{2+}, the data suggest that the 2:1 CRA_3–Mg^{2+} complex is also octahedral in solution.

Upon addition of Co^{2+} to a methanol solution of CRA_3, a broad shoulder shows up around 500 nm (Figure 2). The position of the shoulder and its low extinction coefficient (≈ 30 M^{-1} cm^{-1}) are characteristic of an octahedral or slightly distorted octahedral complex in solution.[22] (The absence of intense bands in the 600–700-nm range indicates that the complex is not tetrahedral.) We assign the two extra ligands in the Co^{2+} octahedral complex as solvent molecules (probably water).

b. There Are Eight Possible Octahedral Diastereoisomers of the 2:1 CRA_3–Mg^{2+} Complex. The formula $Mg(CRA_3)_2(H_2O)_2$ can give rise to eight possible octahedral complexes,[25] which are diastereoisomeric because CRA_3 is a chiral ligand. The complexes are represented by structures **I**–**VIII** in Figure 3. In the complexes the CRA_3 molecules act as acetylacetonate (acac) ligands, chelating the metal through the O_1 and O_9 oxygen atoms. In complexes **I**–**VI** the water molecules are *cis*, and in complexes **VII** and **VIII** they are *trans*.

Analysis of CPK models of the eight possible isomers indicates that some of them can be excluded on steric grounds. First, the two CRA_3 chelating rings cannot come close enough to form the complex if they are located in the same plane. This fact leads to the exclusion of isomers **VII** and **VIII**. Furthermore, isomers **I** and **V** present unfavorable steric interactions between the chromophore of one CRA_3 molecule and the trisaccharide of the other CRA_3 molecule, which should prevent their formation. Finally, in the isomers **II** and **VI** the CDE trisaccharides create steric congestion around the metal center, preventing the simultaneous coordination of the two water and two CRA_3 molecules to the metal.

(19) (a) Angelici, R. *Synthesis and Technique in Inorganic Chemistry*, 2nd ed.; W. B. Saunders Co.: Philadelphia, PA, 1977. (b) Cantor, C.; Schimmel, P. *Biophysical Chemistry*, Part III; Freeman: New York, 1980.

(20) Likussar, W.; Boltz, D. F. *Anal. Chem.* **1971**, *43*, 1265–1272.

(21) Water (rather than methanol) is present in the complex. Water may be preferred as a ligand by Mg^{2+} because it is smaller and harder than methanol. Interestingly, MS-FAB analysis of concentrated solutions of the 2:1 complex shows a molecular ion corresponding to the formula $Mg(CRA_3)_2$, with no water molecules coordinated to the metal. Such behavior has been observed before in ML_2 complexes, where L is an acac (acetylacetonate) ligand. These complexes form adducts with nucleophilic solvents (such as H_2O, DMF, acetone, or alcohols). Upon removal of the solvent, the complexes lose these neutral ligands (forming ML_2) and become strong Lewis acids. Redissolution of ML_2 regenerates the adducts: Graddon, D. P. *Coord. Chem. Rev.* **1969**, *4*, 1–28. See also ref 22.

(22) Cotton, F. A.; Wilkinson, G. *Advanced Inorganic Chemistry*; John Wiley & Sons: New York, 1988.

(23) (a) Bertini, I.; Viezzoli, M. S.; Luchinat, C.; Stafford, E.; Cardin, A. D.; Behnke, W. D.; Bhattacharyya, L., Brewer, C. F. *J. Biol. Chem.* **1987**, *262*, 16985–16994. (b) Fraústo da Silva, J. J. R.; Williams, R. J. P. *The Biological Chemistry of the Elements: The Inorganic Chemistry of Life*; Clarendan Press: Oxford, 1991.

(24) UV–vis binding assays were performed with CRA_3 and Co^{2+}. The results are similar to the ones observed for CRA_3 and Mg^{2+}: a 2:1 CRA_3–Co^{2+} complex is formed in the presence of 1 mol equiv of Co^{2+}. In the normalized Job plot for the drug–Co^{2+} system y_{max} was found to be 0.507 ± 0.016 (with $k = 225$ μM), resulting in a formation constant of $(1.88 \pm 0.44) \times 10^8$ M^{-2} for the 2:1 complex.

(25) Fortman, J. J.; Sievers, R. E. *Coord. Chem. Rev.* **1971**, *6*, 331–375.

(26) Similar orientation of ligands was observed in the X-ray structure of a 2:1 complex between a bulky acac ligand and Mg^{2+}: bis(1,3-diphenyl 1,3-propanedione)–bis(DMF) magnesium(II): Hollander, F. J.; Templeton, D. H.; Zalken, A. *Acta Crystallogr.* **1973**, *B29*, 1289.

(27) Notice also that this analysis does not take into account conformation variations in the oligosaccharides of CRA_3 in the complex. However, NMR studies on calicheamicin suggest that oligosaccharides behave as fairly rigid structural units—ref 4c.

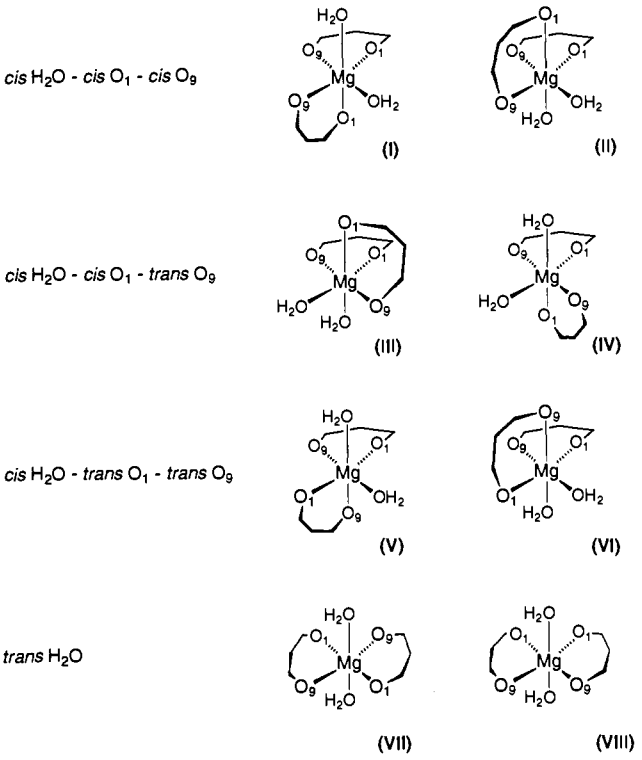

Figure 3. Schematic representation of the eight possible diastereoisomeric complexes with the formula $Mg(CRA_3)_2(H_2O)_2$. CRA_3 is a bidentate ligand, coordinating to the metal ion through the O_1 and O_9 oxygen atoms. Conformational analysis indicates that unfavorable steric interactions in isomers **I**, **II**, **V**, **VI**, **VII**, and **VIII** prevent their formation in solution.

The only isomers that do not present visible steric problems are **III** and **IV**, in which the O_9 groups are *trans* and the CRA_3 chelate rings occupy different planes.[26] It is not possible to assess which is more stable based simply on the CPK model analysis.[27] However, as shown below, NMR studies on the complex in solution shed more light on the relative energies of the two isomers.

c. Dynamic DNMR of the 2:1 CRA_3–Mg^{2+} Complex in CD_3OD Shows Two Isomers. In an earlier paper we reported the chemical shifts of the 2:1 CRA_3–Mg^{2+} complex in CD_3OD at 45 °C.[14] The assignments were performed at 45 °C because the resonances are broadened at room temperature. The broadness suggested some kind of chemical exchange process in solution.[28]

We have investigated the exchange process using dynamic NMR. We monitored the aromatic CR5 and CR10 resonances in the 2:1 drug–Mg^{2+} complex because they are well resolved and occur in the least crowded region in the spectrum (Figure 4). At 45 °C the two aromatic singlets are sharp. As the temperature is lowered, the resonances become increasingly broad, reaching a maximum at 5 °C; below this temperature the peaks decoalesce into *two* sets of resonances, both of them distinct from uncomplexed CRA_3. The peaks do not present further changes upon cooling to temperatures as low as −30 °C.

A similar DNMR experiment performed with CRA_3 did not show decoalescence of the aromatic or any other proton resonances. This result implies that the decoalescence observed with the dimer derives not from any high barrier process in CRA_3 itself, but from some exchange process in the complex. The most reasonable explanation is that the dimer exists as two interconverting metal complexes in solution. At high temperatures the exchange process is fast and the resonances are averaged. As the temperature decreases the interconversion process becomes slow on the NMR time scale and two sets of peaks are observed. Decoalescence is only observed for protons located in the vicinity of the chelating center, providing support for the idea that the interconversion process is exchange around the metal center.

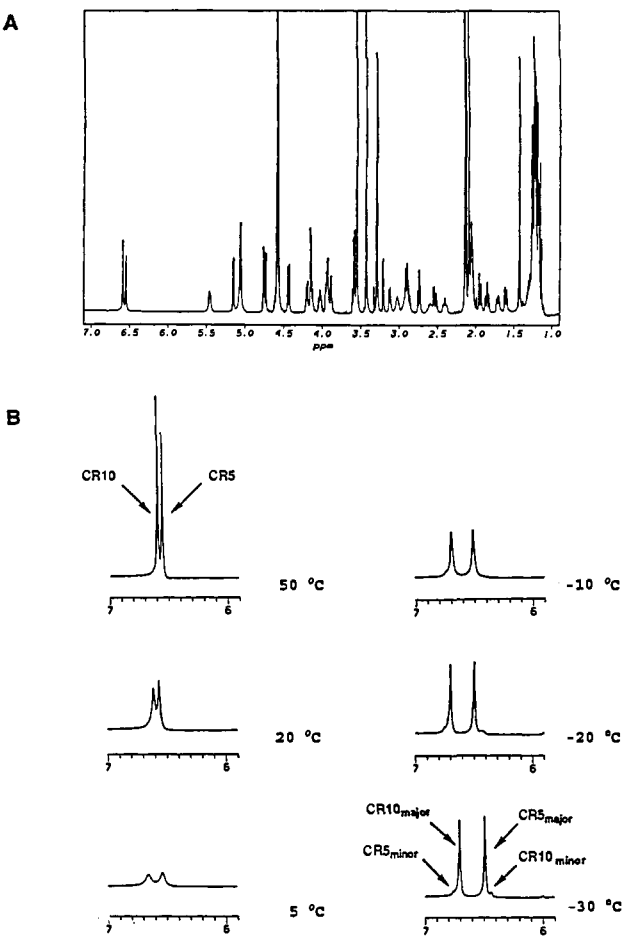

Figure 4. (A) 1D ^1H NMR of the 2:1 CRA_3–Mg^{2+} complex in CD_3OD at 50 °C. The resonances are sharp at this temperature. Notice that the aromatic singlets (CR5 and CR10, located between 6 and 7 ppm) are well-resolved and located in the region of lowest overlap in the spectrum. (b) Aromatic region (6–7 ppm) of the 2:1 complex at various temperatures. At 5 °C the original set of peaks decoalesces at two distinct sets of peaks, indicating the existence of two interconverting complexes in solution. Peak integration at −25 °C shows that the ratio between the complexes is 19.5:1, corresponding to ΔG = 1.6 kcal/mol at that temperature.

The two complexes exist in a ratio of 19.5:1 at −25 °C—a difference of 1.6 kcal/mol between the structures. The free energies of activation for major–minor and minor–major conversions were calculated to be 14.7 and 13.1 kcal/mol respectively.

d. The Structure of the Major Complex. The proton resonances of CRA_3[29] and the 2:1 CRA_3–Mg complexes (major and minor

(28) The broad peaks of the dimer spectrum in CD_3OD cannot be attributed to aggregation processes. In UV studies the dimer obeys Beer's law in the concentration range (2–5 mM) used in NMR experiments, indicating that the complex does not aggregate under these conditions. For the use of NMR techniques in conformational studies, see: (a) *Application of NMR Spectroscopy to Problems in Stereochemistry and Conformation Analysis*; Vol. 6. *Methods in Stereochemical Analysis*; Takeuchi, Y., Marchand, A. P., Eds.; VCH Publishers: New York, 1986. (b) Sandström, J. *Dynamic NMR Spectroscopy*; Academic Press: London, 1982.

(29) The reduced number of interresidue ROE's for the drug monomer in CD_2Cl_2, CD_3OD, and DMSO-d_6 suggests that the sugars assume an extended conformation in the monomer. Kam *et al.*, using NOESY experiments with CRA_3 in CD_2Cl_2, observed two NOE's between the AB and CDE oligosaccharides and proposed that in CD_2Cl_2 the drug monomer adopts a highly compact conformation, with the two carbohydrate chains parallel to each other on the side of the aglycone. This result is not confirmed by our work. We did not observe the mentioned peaks in the ROESY spectrum of the monomer in CD_2Cl_2, which in principle is much more sensitive than a NOESY for a molecule in the weight range of CRA_3: Kam, M.; Shafer, R. H.; Berman, E. *Biochemistry* **1988**, *27*, 3581–3588.

(30) In the ROESY experiment at −25 °C (where the complexes are in slow exchange on the NMR time scale) the ROEs for the major isomer are easily identified because they have opposite sign from the diagonal peaks.

Table I. Proton Chemical Shifts of CRA_3 (δ_{CRA_3}) and of the 2:1 CRA_3–Mg^{+2} Complexes (Major (δ_{major}) and Minor (δ_{minor})) in CD_3OD at −25 °C[a]

	H1					H2(a)					H2(e)				
	δ CRA_3	δ major	Δ major	δ minor	Δ minor	δ CRA_3	δ major	Δ major	δ minor	Δ minor	δ CRA_3	δ major	Δ major	δ minor	Δ minor
A	5.38	5.64	**−0.26**	5.25	0.13	2.03	2.02	0.01	*	*	2.10	2.07	0.03	*	*
B	5.10	5.11	−0.01	5.08	0.02	1.87	1.83	0.04	1.77	0.10	1.61	1.58	0.03	1.53	0.08
C	5.12	5.22	−0.10	5.18	−0.06	1.60	1.30	**0.30**	*	*	2.60	2.04	**0.56**	*	*
D	4.74	2.84	**1.90**	3.19	**1.55**	1.46	1.08	**0.38**	*	*	2.35	1.69	**0.66**	1.65	**0.70**
E	5.05	4.98	0.07	5.27	**−0.22**	1.93	2.12	−0.19	*	*	1.93	1.92	0.01	*	*

	H3					H4					H5				
	δ CRA_3	δ major	Δ major	δ minor	Δ minor	δ CRA_3	δ major	Δ major	δ minor	Δ minor	δ CRA_3	δ major	Δ major	δ minor	Δ minor
A	4.13	4.07	0.06	*	*	5.18	5.23	−0.05	*	*	3.93	3.97	−0.04	*	*
B	3.97	3.94	0.03	*	*	3.23	3.23	0.00	*	*	3.93	3.91	0.02	*	*
C	3.74	2.78	**0.96**	*	*	3.02	2.76	**0.26**	*	*	3.33	3.16	0.17	2.68	**0.65**
D	3.67	3.65	0.02	3.70	−0.03	3.05	2.91	0.14	2.74	**0.31**	3.41	2.73	**0.68**	3.45	−0.04
E	1.44	1.45	−0.01	*	*	4.68	4.80	−0.12	4.73	−0.05	4.11	4.21	−0.10	4.11	0.00

	H6					Ac					OMe				
	δ CRA_3	δ major	Δ major	δ minor	Δ minor	δ CRA_3	δ major	Δ major	δ minor	Δ minor	δ CRA_3	δ major	Δ major	δ minor	Δ minor
A	1.20	1.21	−0.01	*	*	2.14	2.16	−0.02	*	*					
B	1.24	1.21	0.03	*	*						3.56	3.55	0.01	*	*
C	1.33	1.23	0.10	*	*										
D	1.33	1.15	0.18	*	*										
E	1.12	1.30	−0.18	*	*	2.09	2.15	−0.06	*	*					

	2					3					4(a)				
	δ_{CRA_3}	δ_{major}	Δ_{major}	δ_{minor}	Δ_{minor}	δ_{CRA_3}	δ_{major}	Δ_{major}	δ_{minor}	Δ_{minor}	δ_{CRA_3}	δ_{major}	Δ_{major}	δ_{minor}	Δ_{minor}
CHR	4.73	4.49	**0.24**	4.37	**0.36**	2.80	2.35	**0.45**	*	*	2.96	2.85	0.11	*	*

	4(e)					5					7				
	δ_{CRA_3}	δ_{major}	Δ_{major}	δ_{minor}	Δ_{minor}	δ_{CRA_3}	δ_{major}	Δ_{major}	δ_{minor}	Δ_{minor}	δ_{CRA_3}	δ_{major}	Δ_{major}	δ_{minor}	Δ_{minor}
CHR	2.64	2.47	0.17	*	*	6.72	6.50	**0.22**	6.77	−0.05	2.15	2.10	0.05	*	*

	10					1′					OMe 1′				
	δ_{CRA_3}	δ_{major}	Δ_{major}	δ_{minor}	Δ_{minor}	δ_{CRA_3}	δ_{major}	Δ_{major}	δ_{minor}	Δ_{minor}	δ_{CRA_3}	δ_{major}	Δ_{major}	δ_{minor}	Δ_{minor}
CHR	6.80	6.71	0.09	6.44	**0.36**	4.88	4.85	0.03	4.77	0.11	3.42	3.39	0.03	*	*

	3′					4′					5′				
	δ_{CRA_3}	δ_{major}	Δ_{major}	δ_{minor}	Δ_{minor}	δ_{CRA_3}	δ_{major}	Δ_{major}	δ_{minor}	Δ_{minor}	δ_{CRA_3}	δ_{major}	Δ_{major}	δ_{minor}	Δ_{minor}
CHR	4.16	4.21	−0.05	*	*	4.23	4.21	0.02	*	*	1.25	1.25	0.00	*	*

[a] CRA_3 (monomer) shifts are listed as δ CRA_3; the major 2:1 CRA_3–Mg^{2+} complex shifts are listed as δ major; the minor 2:1 CRA_3–Mg^{2+} complex shifts are listed as δ minor. For the major and minor complexes the changes in chemical shift of the drug upon complexation are reported as Δ ppm = (δ mon − δ major) and Δ ppm = (δ mon − δ minor), respectively. Positive values of Δ ppm represent upfield shifts upon formation of dimer, and negative values represent downfield shifts upon formation of dimer. Values of $|\Delta \text{ ppm}| \geq 0.20$ ppm are shown in boldface type. Notice that for both dimers they are observed primarily in the C and D rings. The minor complex resonances marked with an asterisk have not been unambiguously assigned by 2D NMR exchange spectroscopy because the corresponding major complex resonances occur in crowded spectral regions. However, they are expected to be within 0.15 ppm from the corresponding major resonances.

complexes) in CD_3OD at −25 °C were assigned with a combination of 1D and 2D (DQF-COSY, NOESY, and ROESY[30]) experiments. The assignments of CRA_3 and the major[31] complex are reported in Table I.

There are two striking differences in the proton spectrum of the monomer and the major complex. First, some resonances of the major complex are shifted upfield relative to the monomer. For example, the D1 proton is shifted 1.90 ppm upfield upon complexation. Such large shielding effects can be caused by proximity of the proton in question to an aromatic system.[32] Second, the major complex presents intermolecular ROEs between protons of the CDE trisaccharide and protons in the chromophore and A sugar (Table II).[33] Taken together, these results indicate that in the major complex the CDE trisaccharide of one CRA_3 molecule is stacked over the chromophore of the other CRA_3 molecule.

The NMR information (coupling constants, ROEs, and chemical shifts) was used to generate a model for the major complex. The proposed structure for the major isomer is given in Figure 5. In this model the ligand orientation around the Mg^{2+} is identical with that in complex **III**, with the O_9 phenoxide groups *trans* and the O_1 keto groups *cis*. The trisaccharides of the two CRA_3 molecules point in opposite directions, whereas the non-ionized O_8 phenols are held in close proximity. In the major complex several protons (C2a, C3, D1, D2e, and D5) are located above the aromatic ring, which explains their unusual upfield shifts (see Table I). On the other hand, the protons E2a, E3, and E5 are located near the edge of the aromatic ring. This is consistent with the small deshielding effect observed for these protons.

(31) The chemical shifts of resonances of the major complex at −25 °C match closely the corresponding ones of the complex at 45 °C. This is consistent with the high ratio between major and minor complexes in solution: since the chemical shifts at 45 °C correspond to a weighted average of corresponding chemical shifts for the major and minor isomers, they should be similar to the ones for the major isomer.

(32) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley & Sons: New York, 1986.

(33) These observed ROE's are absent from CRA_3 itself. These contacts are not likely to be intramolecular since in this case extreme distortion of the molecule would be required. Similar reasoning has been applied previously to the analysis of CRA_3–Mg^{2+}–DNA complex; see ref 10a.

Table II. Intramolecular ROE's in the Major and Minor 2:1 CRA_3–Mg^{2+} Complex in CD_3OD at −25 °C

major isomer[a]			minor isomer[b]		
resonances		ROE[c]	resonances		ROE[c]
C3	CR5	w	C3	CR3	w
C5	CR2	m	D1	CR10	s
D1	CR5	w	D5	CR10	w
D3	CR7(Me)	w	D6	A1	mw
D3	CR5	m			
D3	CR10	ms			
D5	A1	m			
D5	CR7(Me)	w			
E1	CR7(Me)	ms			

[a] Determined as ROEs (opposite sign of the diagonal peaks) in the ROESY spectrum of the 2:1 complex at −25 °C. [b] Determined as exchange transfer ROEs (same sign as the diagonal peaks) in the ROESY spectrum of the 2:1 complex at −25 °C.[36] [c] s, strong; m, medium; w, weak.

Further support for the structure of the major complex derives from CD studies. CRA_3 in methanol has a nearly flat CD spectrum. However, the 2:1 CRA_3–Mg^{2+} complex in methanol presents a strong exciton coupling type CD band with a negative Cotton effect around 280 nm.[14] (The UV band at 280 nm has been assigned as a ^1B_b transition along the long axis of the chromophore.[34a]) Exciton-type CD bands derive from the interaction of two chromophores in close proximity.[34b] Assuming the edge-on orientation proposed for the two CRA_3 molecules in the dimer, the negative Cotton effect indicates that at least in the major isomer the two CRA_3 chromophores are arranged in an anticlockwise rotation mode. This orientation is indeed present in the proposed major isomer structure (see structure **III** in Figure 3).

The major isomer presents a curved shape with a right-hand twist, which appears to be complementary to the right-handed DNA twist. Moreover, the chemical shifts of the protons in the major complex in solution are very close to the chemical shifts reported by Patel and co-workers for the drug–DNA complex.[10a,14] We have thus concluded that the major complex in solution is identical with the dimer bound to DNA duplex.

e. The Structure of the Minor Complex. The low intensity of the minor complex resonances precludes their assignment by 2D techniques such as DQF-COSY and ROESY. However, since the major and minor complexes interconvert on the NMR time scale, the minor complex could be assigned by a 2D exchange experiment at −25 °C.[35] The resonances of the minor complex were identified from exchange cross peaks to previously assigned resonances of the major complex. The assignments of the minor complex are reported in Table I, and intermolecular ROEs for the minor complex are reported in Table II.[36]

Like the major complex, the minor complex presents unusual chemical shifts relative to CRA_3. The intermolecular ROEs and the high shielding observed for several resonances in the C and D rings (*e.g.*, 1.55 ppm for D1 and 0.96 ppm for C3) suggest that the minor complex also contains an interaction between the trisaccharide of one molecule and the chromophore of the other molecule. However, there are differences in the degree to which different protons are shielded in the two complexes (compare, for example, D5 in Table I), suggesting that the relative orientation of the CRA_3 molecules in the minor complex is different from the one in the major complex. On the basis of the NMR information, we propose a model for the minor isomer (Figure 5). In this model the ligand orientation around the metal is identical to structure **IV** (Figure 3). The trisaccharides of the two CRA_3 molecules point in opposite directions and the non-ionized O_8 phenols also point in opposite directions. The protons C2e, C3, C5, D1, and D2e are located above the aromatic ring, which explains their significant shielding with respect to the monomer. The proton D5 in the minor complex is located on the outside edge of the chromophore, which accounts for the small difference in chemical shift from the monomer. The minor complex presents an extended structure, very different from the right-hand twisted structure of the major complex.

f. The CDE Trisaccharide Stabilizes the Dimer. We have previously shown that the carbohydrates in CRA_3 stabilize the dimeric complex in solution.[14] However, the initial studies did not provide information on which individual sugars are necessary for dimer formation. To elucidate this point, we have compared the ability of a series of degradation products of CRA_3 (represented as BA–CRN–CDE) to form dimeric complexes in methanol. The degradation products used were A–CRN–CDE, BA–CRN–CD, A–CRN–C, and A–CRN.[37] UV–vis spectra of the drugs under study were recorded at different concentrations of added Mg^{2+}. Absorbances of the drugs were monitored at varying concentrations of metal and plotted to give the titration curves shown in Figure 6.

The behavior of CRA_3 and CRN in the presence of increasing concentrations of Mg^{2+} has been described previously.[14] The salient points are summarized for the purpose of comparing the behavior of degradation products. As shown in Figure 6, the CRA_3 titration presents two distinct regions. In region I the transition, complete upon the addition of 0.50 mol equiv of metal, corresponds to the two-state conversion of the CRA_3 monomer to the 2:1 drug–Mg^{2+} complex. In region II the binding analysis is complicated by nonspecific association (aggregation). However, other evidence shows that region II corresponds to conversion of the 2:1 to the 1:1 complex in the presence of a large excess of Mg^{2+}, consistent with the mass-action principle.

The CRN titration presents a single transition, complete upon the addition of 1 mol equiv of metal, which corresponds to formation of the 1:1 complex. The transition does not present isosbestic points, indicating that multiple complex species are present during the titration, possibly several 2:1 complexes or a mixture of 1:1 and 2:1 complexes. The transition to the 1:1 complex is complete after 1 mol equiv has been added.

A–CRN–CDE. The degradation product A–CRN–CDE presents a very similar behavior to CRA_3 upon titration with Mg^{2+}. There are two distinct regions in the absorbance plot (Figure 6), from 0 to 1.0 mol equiv of metal (region I) and from 1.0 to 50 mol equiv of metal (region II), corresponding to two transitions.

The transition in region I is effectively complete upon addition of 0.50 mol equiv of Mg^{2+}. Isosbestic points can be observed in the overlays of the UV spectra for region I, indicating a two-state transition. These facts suggest that region I corresponds to the conversion of A–CRN–CDE to its 2:1 drug–Mg^{2+} complex. A Job titration for the Mg^{2+}–drug system confirms the stoichiometry of the complex in region I as 2:1 (Figure 7). From the normalized Job plot y_{max} was found to be 0.535 ± 0.016, corresponding to

(34) (a) Harada, N.; Nakanishi, K.; Tatsuoda, S. *J. Am. Chem. Soc.* **1969**, *91*, 5896–5898. (b) Harada, N.; Nakanishi, K. *Circular Dichroic Spectroscopy—Exciton Coupling in Organic Stereochemistry*; University Science Books: Mill Valley, CA, 1983.

(35) Exchange cross peaks are observed between a given resonance of the major complex and the corresponding resonance of the minor isomer, provided that the two resonances differ significantly in chemical shift. The exchange peaks have the same sign as the diagonal peaks in a ROESY experiment. (a) Derome, A. E. *Modern NMR Techniques for Chemical Research*; Pergamon Press: Oxford, 1987. (b) *Two-Dimensional NMR Spectroscopy—Application for Chemists and Biochemists*; Vol. 9, *Methods in Stereochemical Analysis*; Croasmum, W. R., Carlson, R. M. K., Eds.; VCH Publishers: New York, 1987.

(36) In the ROESY experiment it is possible to observe transfer ROEs, which arise from a close contact that is present in the minor complex but is absent in the major complex. These transfer ROEs have the same sign as the diagonal peaks and are observed between the corresponding resonances of the *major* complex. For further information see ref 35.

(37) In principle, the importance of the D ring in dimer formation should be studied by using the degradation products BA–CRN–CD and BA–CRN–C. The latter degradation product could not be isolated from the acidic digestion of CRA_3. However, UV binding and NMR evidence suggest that the B ring does not interact with other sugars in the dimer and is not important in dimer stabilization. Thus, the comparison of BA–CRN–CD and A–CRN–C may be used to estimate the importance of the D ring for dimer stabilization.

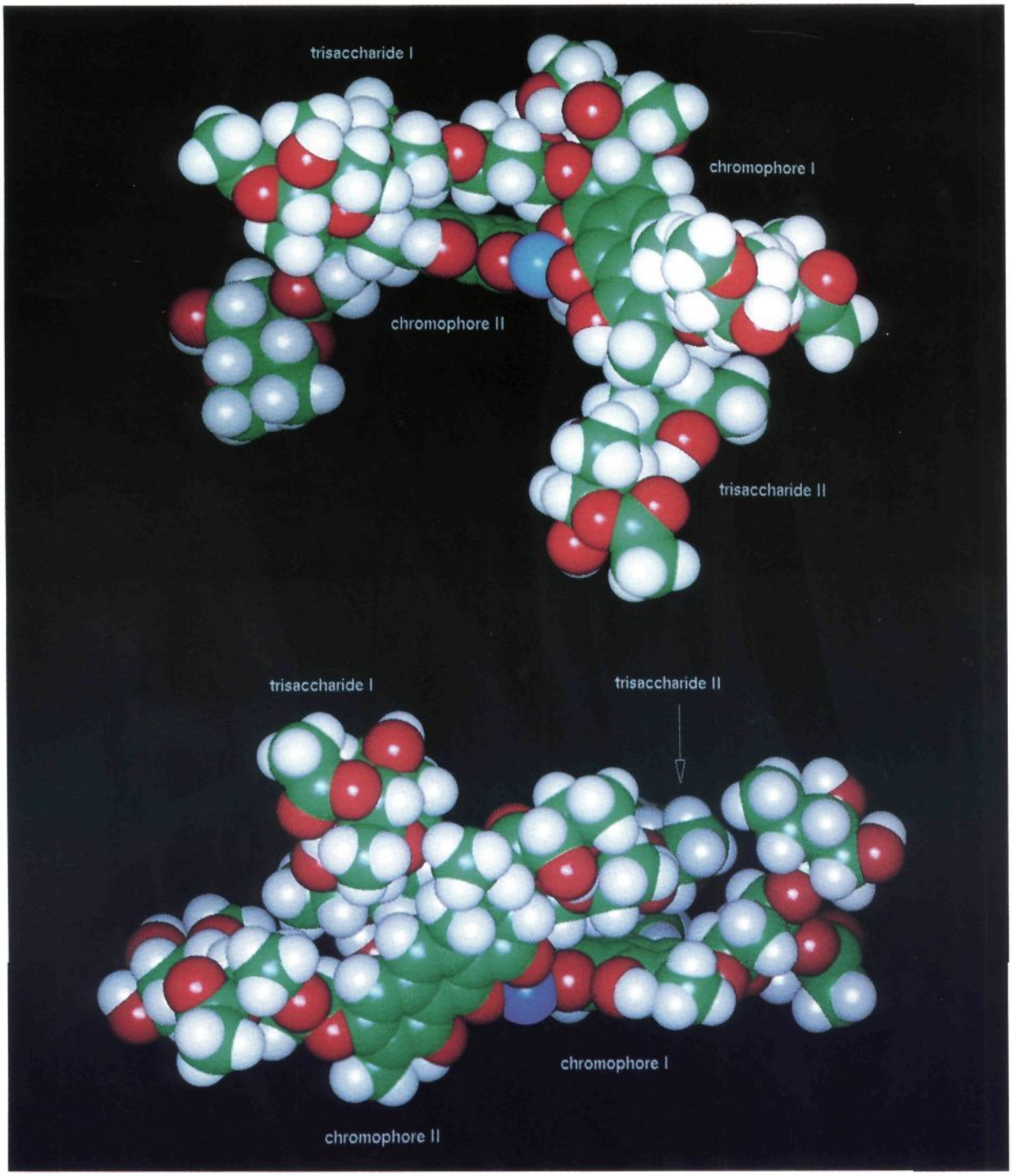

Figure 5. CPK models of the major (a, top) and minor (b, bottom) 2:1 CRA_3–Mg^{2+} complexes $[Mg(CRA_3)_2(H_2O)_2]$. The drug molecules are docked in a manner that satisfied the NMR constrains derived from chemical shifts and ROEs. (The two water molecules coordinated to Mg^{2+} are omitted for clarity.) The structures have not been energy minimized. In both isomers the CDE trisaccharide of one CRA_3 molecule is juxtaposed to the chromophore of the other CRA_3 molecule. This interaction appears to be responsible for dimer stability. The major isomer presents a curved shape with a right-hand twist (complementary to the right-handed DNA twist) and is identical with the 2:1 complex that binds to DNA.[10d] The minor isomer presents an extended structure.

a formation constant of $(1.20 \pm 0.16) \times 10^9\ M^{-2}$. The formation constant derived for the 2:1 CRA_3–Mg^{2+} complex in methanol was $(5.9 \pm 2.9) \times 10^9\ M^{-2}$.[14] The similarity in behavior between CRA_3 and A–CRN–CDE indicates that the B ring does not contribute significantly to the stability of the dimer.

In region II the readings at 440 nm vary in a haphazard way as the Mg^{2+} concentration increases and there are no isosbestic points in the UV–vis spectra. Like CRA_3, the degradation product A–CRN–CDE appears to aggregate when excess Mg^{2+} is added. However, on the basis of the CRA_3 results and a CD titration performed with A–CRN–CDE,[38] we believe that region II involves the conversion of the 2:1 complex to the 1:1 complex in the presence of a large excess of Mg^{2+}.

BA–CRN–CD. The degradation product BA–CRN–CD displays behavior intermediate between CRA_3 and CRN. There are two transitions in the UV titration of the drug. As with CRA_3, the first transition is complete upon the addition of 0.50 mol equiv of metal. Isosbestic points observed in this region

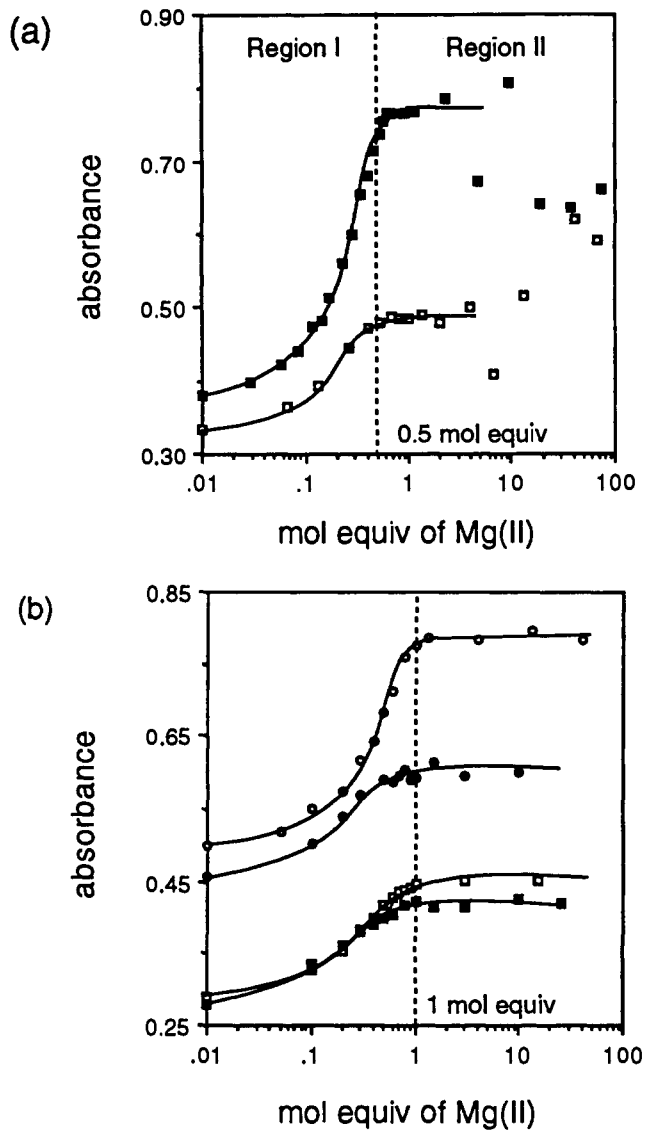

Figure 6. (a) Absorbance at 440 nm monitored for the Mg^{2+} titration of CRA_3 (■) and A–CRN–CDE (□) in methanol. Region I corresponds to Mg^{2+} concentrations from 0 to 1 mol equiv. Region II corresponds to Mg^{2+} concentrations from 1 to 50 mol equiv. (b) Absorbance at 430 nm monitored for the Mg^{2+} titration of BA–CRN–CD (■), A–CRN–C (□), A–CRN (●), and CRN (○) in methanol.

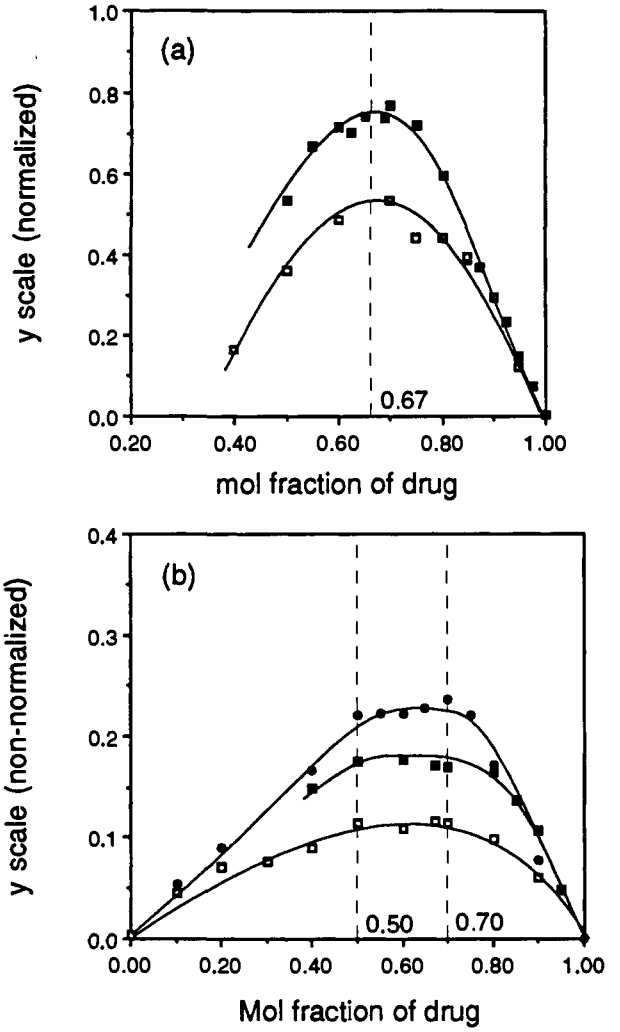

Figure 7. (a) Normalized Job plots for Mg^{2+}/CRA_3 (■) and Mg^{2+}/A–CRN–CDE (□) systems in methanol at 25 °C. For both systems the maximum of the plots occured at $x_{drug} = 0.68 \pm 0.02$, indicating the formation of a 2:1 drug–Mg^{2+} complex. (b) Normalized Job plot for Mg^{2+}/BA–CRN–CD (■), Mg^{2+}/A–CRN–C (□), and Mg^{2+}/A–CRN (●) systems in methanol at 25 °C. For the three drugs plateaus were obtained from $x_{drug} = 0.70$ to 0.50, suggesting that 2:1 and 1:1 complexes are formed. The plots also show that the 1:1 complex predominates in solution once 1.0 mol equiv of each metal is added. (The curve for the Mg^{2+}/CRN system is not shown for clarity reasons,[13] but is similar to the ones for these three degradation products.)

indicate a two-state transition in solution. These facts suggest that this first transition represents the conversion of BA–CRN–CD to its 2:1 drug–Mg^{2+} complex. Unlike with CRA_3, however, the second transition is complete upon the addition of only 1 mol equiv of metal. New isosbestic points are identified for this transition, indicating a two-state transition from the dimer to some other species. Further addition of Mg^{2+} has no effect in the UV spectrum of the drug.

In order to identify the stoichiometry of the complexes formed in solution, a Job titration for Mg^{2+} was performed at 440 nm and 25 °C. The resulting plot (Figure 7) presents a flat portion between $x_{drug} = 0.50$ and 0.70, indicating formation of more than one complex. The Job titration is consistent with the initial formation of the 2:1 drug–Mg^{2+} complex in the presence of less than 0.50 mol equiv of Mg^{2+}. Further addition of Mg^{2+} converts the 2:1 complex rapidly to the 1:1 complex. As with CRN, the conversion is complete after the addition of 1 mol equiv of metal. Therefore, removing the E ring reduces the stability of the dimer dramatically relative to the monomer. However, an analysis of the isosbestic points shows that the 2:1 complex formed by BA–CRN–CD is still slightly stabilized relative to the 1:1 complex since the latter is formed only after the addition of 0.50 mol equiv of metal. In CRN, there are no isosbestic points below 0.50 mol equiv, indicating the presence of multiple species in solution.

A–CRN–C and A–CRN. Both A–CRN–C and A–CRN behave like CRN, presenting one single transition in the UV titration, as shown in Figure 6. The transition is complete after the addition of 1 mol equiv of metal. No isosbestic points are conserved throughout the titration, suggesting that the transition is not a two-state process from the drug monomers to single metal complexes. Job titrations were performed at 430 nm and 25 °C (Figure 7). Instead of a distinct maximum, there is a plateau in both Job plots from $x_{drug} = 0.50$ to 0.70.

The data on A–CRN–C and A–CRN are consistent with the model proposed for CRN, where the drugs are converted to both 1:1 and 2:1 complexes upon addition of 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 (Scheme I). Therefore, the studies on these degradation products show that the removal of

Scheme I. Degradation Products Used in Binding Studies

form stable 2:1 drug-Mg^{2+} complex		form unstable 2:1 drug-Mg^{2+} complex
BA-CRN-CDE (CRA_3)		A-CRN-C
A-CRN-CDE	BA-CRN-CD	A-CRN
		CRN

the D and C sugars destabilizes the 2:1 complex with respect to the 1:1 complex.

Discussion

Studies in the 1960's showed that the sugars of chromomycin A_3 are essential for DNA binding,[9] although for many years there was no structural information on their role. In 1989 Gao and Patel reported an NMR study showing that CRA_3 binds as a 2:1 drug–Mg^{2+} complex in the minor groove of DNA.[10a] The β-keto phenolate of CRA_3 acts as a bidentate ligand for Mg^{2+}. A crystal structure of a drug–Mg^{2+}–DNA complex shows that Mg^{2+} occupies an octahedral site, coordinated to two CRA_3 molecules and two waters.[11] These exciting results on the structure of the drug–DNA complex stimulated new research aimed at understanding the binding process. Since six particles must come together in solution to form the drug–DNA complex (a DNA duplex, two CRA_3 molecules, a Mg^{2+} ion and two water molecules), binding involves a complicated set of equilibria.

With use of footprinting analysis, attempts have been made to determine whether the 2:1 CRA_3–Mg^{2+} complex is formed prior to DNA binding or not.[7e] We took a different approach to studying CRA_3. Rather than studying the formation of the drug–Mg^{2+}–DNA complex, we have tried to simplify the problem by studying the Mg^{2+}–drug interaction in solution. The first goal was to determine whether CRA_3 can form a stable dimeric complex with Mg^{2+} in solution. A positive answer would mean that the DNA duplex is not required for dimer formation and would suggest that CRA_3 binds as the preformed dimer complex to DNA.

It is worth pointing out that CRA_3 bears a structural resemblance to a class of well-studied acac ligands.[39a] In the presence of divalent metals such as Mg^{2+} these ligands can form either 2:1 or 1:1 ligand–metal chelates, the relative stabilities of which depend on the structure of the ligand.[39b,c] In general, bulky ligands tend to form 1:1 complexes because the 2:1 complexes are sterically hindered.[39d] Thus, one might expect that CRA_3, a bulky acac ligand, would not form a stable 2:1 complex in solution (and that the DNA duplex pays the cost of stabilizing the dimer that binds).

We found that the aglycon of CRA_3, chromomycinone (CRN), forms a 1:1 complex in the presence of 1 mol equiv of Mg^{2+} (as expected for a bulky acac ligand). However, CRA_3 forms a very stable 2:1 complex in methanol in the presence of 1 mol equiv of Mg^{2+}. These results imply that the carbohydrates on CRA_3 somehow stabilize the 2:1 complex. We have therefore concluded that it is the chromomycin sugars, not the DNA, which facilitate association of two CRA_3 molecules to form a 2:1 complex with Mg^{2+}.[15]

In this paper we have studied the role of the oligosaccharides of CRA_3 in dimer formation. In principle there are eight possible octahedral 2:1 complexes with the formula $Mg(CRA_3)_2(H_2O)_2$. A conformational analysis of the possible complexes shows that six of these are too hindered to form. Of the two remaining isomers, dynamic NMR shows that both exist in solution although one is much more stable than the other. We have characterized the structures of the major and minor 2:1 complexes and have found that in both complexes the CDE trisaccharide on one CRA_3 molecule is in close proximity to the chromophore of the other CRA_3 molecule. This interaction is apparently favorable and stabilizes both 2:1 complexes relative to the 1:1 complex. Furthermore, the major complex is essentially identical with the 2:1 complex that binds to DNA.[10]

Studies on degradation products of CRA_3 show that the entire CDE trisaccharide is necessary to stabilize the dimer (Scheme I). Removing even the terminal E sugar of the trisaccharide decreases the stability of the 2:1 complex significantly. In contrast, removing the B sugar of the AB disaccharide has no significant effect on the stability of the dimer. Unfortunately, we were unable to obtain a degradation product in which the AB disaccharide is absent but the CDE trisaccharide is intact, so we do not yet know whether the A sugar plays a role in dimer stability.[40] (This question can in principle be answered by synthesizing the appropriate analogue of CRA_3 and evaluating dimer stability.) We were also unable to remove the side chain selectively. However, it does not seem likely that the side chain plays a role in dimer formation because of its distance from the metal center. Moreover, the side chain does not present unusual chemical shifts or ROEs upon dimer formation, indicating that its environment does not change appreciably.

On the basis of the above analysis, we have concluded that the minimal structural requirements for *dimer formation* are the tricyclic chromophore with the β-keto phenolate functionality plus the CDE trisaccharide (and possibly the A sugar). It remains to be seen whether an analogue incorporating these features contains sufficient information for *DNA binding*.[41] In any event, the results described above provide a starting point for designing some simple analogues of CRA_3.[11b-e] Studies on these analogues will shed further light on the minimal structural requirements for dimer formation, DNA binding, and perhaps specificity.

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(38) A–CRN–CDE has a relatively flat CD in methanol. Addition of less than 1 mol equiv of Mg^{2+} to A–CRN–CDE leads to the formation of an intense exciton coupling-type CD band with a negative Cotton effect centered around 280 nm. Comparison between CD and UV titrations indicates that the exciton coupling-type CD band is associated with the formation of the 2:1 drug–Mg^{2+} complex in solution. In the presence of excess Mg^{2+} (more than 1 mol equiv) the CD band slowly decreases. On the basis of results obtained for CRA_3 (which has identical behavior to this degradation product), the disappearance of the CD band is attributed to the conversion of the dimer (2:1 complex) to the monomer complex (1:1 complex). This conversion is consistent with the mass-action principle.

(39) (a) Siedle, A. R. In *Comprehensive Coordination Chemistry*; Wilkinson, G., Ed.; Pergamon Press: Oxford, 1987; Vol. 2, p 365. (b) Fenton, D. E. In *Comprehensive Coordination Chemistry*; Wilkinson, G., Ed.; Pergamon Press: Oxford, 1987; Vol. 3, p 25. (c) Guter, G. A.; Hammond, G. S. *J. Am. Chem. Soc.* **1959**, *81*, 4686. (d) Irving, H.; Rossoti, H. S. *J. Chem. Soc.* **1954**, 2910.

(40) In the major isomer there are ROEs between the A ring of one CRA_3 and the C and D rings of the other CRA_3. It is therefore possible that the A ring plays a role in dimer stabilization.

(41) Indirect evidence suggests that in the CRA_3 case dimer formation correlates to DNA binding. We found that divalent cations with octahedral radius less than 0.90 Å (such as Ni^{2+}, Mg^{2+}, and Co^{2+}), which promote CRA_3–DNA binding,[9] also form stable 2:1 CRA_3–metal complexes in methanol.[14,24] Large cations (like Ca^{2+} and Ba^{2+}) neither promote DNA–drug binding nor form stable 2:1 complexes. Furthermore, the order of decreasing DNA binding for the degradation products[12] is CRA_3 > A–CRN–CDE > BA–CRN–CD > A–CRN–C > A–CRN > CRN, which is identical with the order of dimer stability determined in this paper. Taken together, these observations suggest that stable dimer formation is an important (although not necessarily exclusive) requirement for DNA binding.