A Cationic Cyclophane That Forms a Base-Pair Open Complex with RNA Duplexes

M. Fernandez-Saiz,[†] H.-J. Schneider,*[,‡] J. Sartorius,[‡] and W. David Wilson*[,†]

Contribution from the Department of Chemistry and Center for Biotechnology and Drug Design, Georgia State University, Atlanta, Georgia, 30303, FR Organische Chemie, Universität des Saarlandes, D 66041 Saarbrücken, Germany

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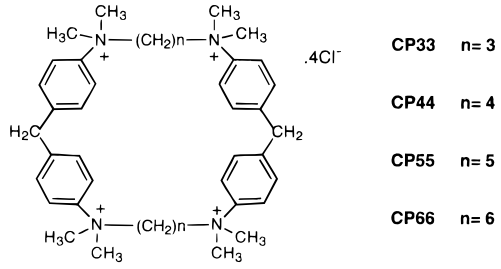
Abstract: It is now well-established that synthetic organic cations can interact with the helical conformations of DNA and RNA and can stabilize these structures. Such interactions can also perturb the function of nucleic acids, generally through modification of the interaction of nucleic acids with proteins and, thus, can be of significant therapeutic benefit against selected cells or organisms. We have investigated by T_m and viscosity studies and by CD and ^{1}H NMR spectra the interactions of tetracationic azoniacyclophanes, CPnn, where *nn* is the number of methylene groups (from 3 to 6) in the linking chains, with DNA and RNA polymers of the same sequence. All the compounds stabilize the DNA polymers, but, in a surprising result, the compounds either stabilize RNA duplexes or alternatively cause base-pair opening in RNA duplexes depending on the size of the cyclophane and the solution conditions. With RNA polymers containing A−U base pairs, the largest cyclophane, CP66, specifically binds the adenine bases into its cavity and can cause complete denaturation of the RNA at high concentrations. The NMR shift changes observed both for CP66 and the adenine base in the polymer predict an inclusion complex with the base in the cavity of CP66. These shift values can be related to those measured earlier with complexes between the same macrocycle and several adenine derivatives (Schneider, H.-J.; Blatter, T.; Palm, B.; Pfingstag, U.; Rüdiger, V.; Theis, I. *J. Am. Chem. Soc.* **1992**, *114*, 7704−7708) and reflect the NMR anisotropy effects of the aromatic units both in host and guest. The different effects of the compounds on DNA and RNA are caused by significant differences in their interactions with the duplex and single-stranded states of the nucleic acids.

Introduction

The possibility of design of highly selective drugs against viruses with RNA genomes has lead to an interest in defining the RNA recognition principles of organic cations.[1−3] To selectively target RNA it is also essential to develop an understanding of the differences between RNA and DNA interactions, and to develop a library of molecular structures that can selectively recognize specific RNA features. One of the goals is to create compounds that interact with nucleic acids through unique and specific complexes similar to those exhibited by enzymes and antibodies. As part of a search to identify compounds that have such specific interactions with nucleic acid bases, we have investigated the complexes of azoniacyclophanes, CP*nn*, with DNA, RNA and hybrid polymer duplexes.

The syntheses of CP*nn*,[4−6] where *nn* is the number of methylene groups (from 3 to 6) in the linking chains and their



complexation properties with calf thymus DNA, nucleotides, and nucleosides have been reported.[6,7] The diphenylmethane units in the CPnn derivatives shown above define cavity shapes that can form excellent interactions with flat aromatic guests. The distance between the two nitrogens in one diphenylmethane unit is ≈8 Å. To generate cavity binding sites, two or more spacers are connected by bridges having adjustable chain length. The length of the bridges between the spacers allows the cavity to be tailored to fit guests of specific size. Water solubility of the receptors is achieved by introducing four quaternary ammonium centers. CP66, for example, forms a strong and specific inclusion complex with purine nucleotides and nucleosides in aqueous solution.[7] NMR shifts demonstrate intracavity inclusion for the aromatic base with the sugar ring outside the CP66 cavity. The affinity for adenine derivatives is larger than for guanine derivatives. Pyrimidines can also be complexed by CP66, but their binding constants are very low compared with those for purine derivatives. CP44 in the protonated form has been reported to form inclusion complexes with naphthalene derivatives in aqueous solution as well as with benzene derivatives.[4]

* Address correspondence to Dr. W. David Wilson, Department of Chemistry, Georgia State University, Atlanta, GA, 30303. Telephone: 404-651-3903. FAX: 404−651−1416. e-mail address: chewdw@gsusgi1.gsu.edu.

[†] Georgia State University.

[‡] Universität des Saarlandes.

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All of the cyclophanes stabilized calf thymus DNA[8] against thermal denaturation, which indicates strong binding to the double helical conformation. Molecular modeling has shown that CP44 optimizes interactions in the large groove of the DNA double helix, thereby permitting efficient ion-pair contact between the positive $^{+}$N−CH groups and the anionic phosphate oxygen atoms.[8] Cyclophanes with $n = 3$, 5, and 6 are not as easily accommodated into the large groove of DNA, as they cannot form close van der Waals contacts and electrostatic interactions that are as favorable as with CP44.

The results with RNA polymer complexes of CPnn reported here demonstrate a different and surprising behavior: the compounds can stabilize the base paired RNA duplex or alternatively destabilize the RNA duplex through base-pair open complexes. The result obtained depends on the size of the cyclophane and the solution conditions. We have evidence that the largest cyclophane, CP66, can complex selectively with adenine bases of RNA polymers containing A−U base pairs in different sequence contexts. Such adenine complexes require breaking of base pair interactions and rotation of one or more bases out of the helical stack for formation of an extrahelical complex. DNA base pairs must open to form complexes with certain types of enzymes, such as some methyltransferases, glycosylases, or photolyases that have recently been shown to cause a flipped-out conformation of a target base.[9] The exposed base is bound into a cavity of the enzyme to allow the catalytic activity. We believe that the cyclophanes represent the first examples of small organic compounds that selectively complex with a base through a cavity complex, as with the base-flipping enzymes, to cause melting of RNA duplexes.

Results

Thermal Melting Curves. Melting curves[10] for polyd(A−T)$_2$ and polyr(A−U)$_2$ with all of the cyclophanes are compared in Figure 1A,B. All compounds cause significant and similar stabilization with the DNA polymer that is largest for CP44 in agreement with results for complexes with CT DNA.[8] The cyclophanes seem to prefer the nonalternating DNA polymers with larger ΔT_m value (T_m complex − T_m nucleic acid under the same conditions) for polydA·polydT (Table 1). With both DNA polymers, CP44 forms the most stable complex and CP66 the least stable. The difference in their ΔT_m values in both cases is 8−9 °C. CP33 and CP55 have intermediate ΔT_m values that are 5−7 °C lower than the ΔT_m values for CP44.

With the RNA polymers, polyr(A−U)$_2$ and polyrA·polyrU, strong stabilization is obtained with CP33 as with DNA. With CP44, CP55, and CP66, however, a distinct decrease in the RNA ΔT_m value relative to the DNA ΔT_ms is observed, and, with CP66, the ΔT_m value is actually negative. Qualitatively similar behavior is obtained with the hybrid duplex polyrA·polydT and with polymers that have different bases, such as polyrI·polyrC (Table 1), although the magnitudes of the ΔT_m values are sequence dependent. As with DNA, a stabilization preference for nonalternating sequences is observed with CP33, CP44, and CP55, although the magnitude of stabilization decreases significantly as the size of the cyclophane increases.

T_m values are plotted as a function of ratio for the nonalternating RNA in Figure 2 for CP33, CP55, and CP66 complexes.

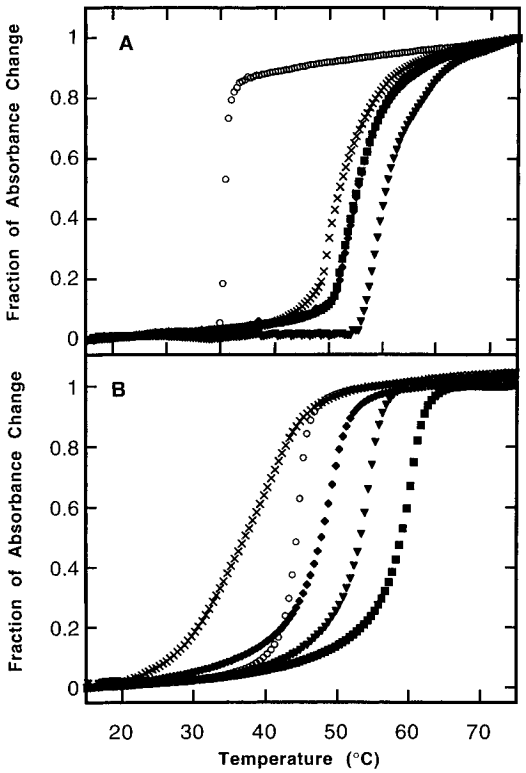

Figure 1. T_m curves in MES buffer (0.01 M 2-(*N*-morpholino)-ethanesulfonic acid, 0.001 M EDTA, and pH 6.25, [Na^+] = 0.007 M) for (A) polyd(A−T)$_2$ (○) and (B) polyr(A−U)$_2$ (○) with CP33 (■), CP44 (▼), CP55 (◆), and CP66 (×) at a ratio of 0.2 mols CP*nn* per nucleic acid phosphate.

The expected increase with increasing ratio up to saturation of the duplex is observed with CP33. With CP66, two regions can be defined in the curve: below 0.1 mol of CP66 per nucleic acid phosphate (region I) and above 0.1 (region II). In region I the T_m of the duplex RNA increases slightly (1−2 °C) as the ratio of CP66 to RNA increases, while in region II large decreases of T_m are observed with increasing ratio (Figure 2). CP55 adopts an intermediate position. Its curve reaches a maximum T_m at a ratio of 0.1 mol per RNA-P and after that begins a smooth downward slope. Above a ratio of 0.3, aggregates begin to form. Similar behavior has been observed for some metal cations[11,12] that can either stabilize nucleic acids through phosphate interactions or destabilize the duplex conformations through binding to the bases. Comparison of the cyclophanes and metal ion results suggests that CP66 has a complex interaction with RNA that involves ionic interactions with RNA phosphates as well as significant interactions with RNA bases.

Salts Concentration Effects on T_m. The importance of ionic interactions for the CP33−RNA complex is supported by the effects of salt concentration on ΔT_m (Table 2). The ΔT_m values are largest at the lowest salt concentration and continuously decrease as the salt concentration is increased. The results obtained with polyrA·polyrU and with polyrA·polydT follow the same pattern, although the ΔT_m values of polyrA·polydT are smaller in magnitude. The ΔT_m of the CP33 complex with polyrA·polyrU decreases from 27.2 to 17.8 °C on addition of 0.02 M NaCl to the buffer, and this supports the weakening of

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(10) Thermal melting experiments were conducted as previously described in the following: (a) Kibler-Herzog, L.; Kell, B.; Zon, G.; Shinozuke, K.; Mizan, S.; Wilson, W. D. *Nucleic Acis Res.* **1990**, *18*, 3545−3555. (b) Wilson, W. D.; Tanious, F. A.; Fernandez-Saiz, M.; Rigl, C. T. Evaluation of Drug/Nucleic Acid Interactions by Thermal Melting Curves. In *Methods in Molecular Medicine;* 1996 (in press).

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Table 1. Effect of Cyclophanes on DNA and RNA T_m Values[a]

cyclophane	n	ΔT_m[b] polydA·polydT	ΔT_m polyrA·polyrU	ΔT_m polyrA·polydT	ΔT_m polyr(A−U)$_2$	ΔT_m polyd(A−T)$_2$	ΔT_m polyrI·polyrC
CP33	3	29.8	27.2	10.9	15.9	23.9	17.2
CP44	4	35.9	14.1	3.6	9.5	28.4	3.7
CP55	5	28.6	6.3	0.9	4.5	23.5	2.2
CP66	6	27.4	-5.8	−6.3	−6.7	19.9	−3.2

[a] Experiments were conducted in MES buffer (0.01 M 2-(*N*-morpholino)ethanesulfonic acid, 0.001 M EDTA and pH 6.25) at a ratio of 0.2 mol of CP*nn* per mol of nucleic acid phosphate. [b] $\Delta T_m = T_m$ complex $- T_m$ nucleic acid. The ΔT_m values are obtained from first-derivative plots.

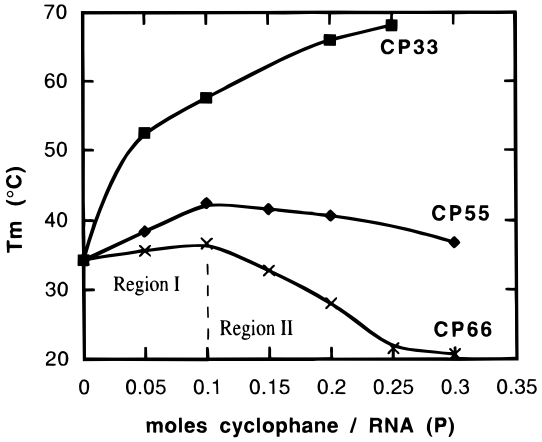

Figure 2. Variations of T_m of polyrA·polyrU as a function of cyclophane to RNA-P ratio in MES buffer for CP33 (■), CP55 (◆) and CP66 (×). For CP66 two regions can be defined.

Table 2. Salt Effects on the Binding of CP33 and CP66 with RNA Polymers

	polyrA·polyrU		polyrA·polydT	
added NaCl[a]	CP33 ΔT_m	CP66 ΔT_m	CP33 ΔT_m	CP66 ΔT_m
none	27.2	−4.5	10.9	−6.3
0.02 M	17.8	−10.0	5.0	−9.1
0.05 M	10.6	−5.5	2.3	−3.2
0.10 M	4.7	−0.8	0.0	−0.5

[a] The experiments were conducted in MES buffers (0.01 M 2-(*N*-morpholino)ethanesulfonic acid, 0.001 M EDTA and pH 6.25, [Na^+] = 0.007 M) and with addition of 0.02, 0.05, or 0.1 M NaCl, at a ratio of 0.2 mols of CPnn per mol of nucleic acid phosphate.

the ionic interactions stabilizing the complex. With CP66 the negative ΔT_m increases from −4.5 to −10 °C on addition of 0.02 M NaCl. At 0.05 M NaCl the CP33 ΔT_m is 10.9 °C, while that for CP66 is reduced to −5.5 °C, indicating that ionic interactions also play a significant role in the CP66-RNA complex. The results suggest that ionic interactions are dominant in region I, while base interactions assume increasing importance in region II. The combination of ionic and base interactions is apparently optimized near 0.02 M NaCl for the CP66 base-pair open complex.

Reversibility of Melting. Reversibility in the melting behavior of CP33 and CP66 with polyrA·polyrU is compared in Figure 3A,B for a salt concentration of 0.02 M NaCl. Figure 3A shows the melting behavior of CP33 and CP66 in region I (0.05 mol of cyclophane per nucleic acid phosphate), in which both complexes are able to renature into a duplex state on lowering the temperature from the denaturation region. However, in region II (0.2 mol of cyclophane per nucleic acid phosphate, Figure 3B), the CP33 complex can be renatured, while CP66 shows irreversible behavior suggestive of stable complex formation with RNA bases. The cooling curve of CP66 maintains a large hyperchromicity relative to the initial state (Figure 3B), indicating that the rewinding process is not occurring in this time period. Even after 24 h the absorbance of the CP66 complex has not returned to the original value.

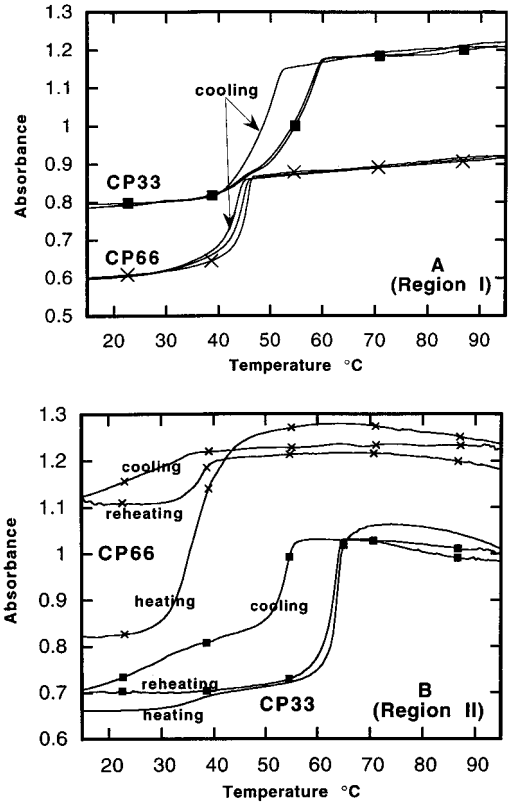

Figure 3. Melting behavior of CP33 (■) and CP66 (×) with polyrA·polyrU solutions in MES buffer with 0.02 M of NaCl adjusted to pH 6.25. The solutions contained 8×10^{-5} M RNA-p. (A) region I: for a ratio of 0.05 CP*nn* per nucleic acid phosphate. (B) region II: for a ratio of 0.2 CP*nn* per nucleic acid phosphate.

The salt concentration seems to play an important role in the process of renaturing of the complex with CP33. When no salt is added, the rewinding process is relatively slow for CP33, but with 0.02 M of NaCl the complex can renature completely within 1 min at low temperatures. CP66 shows irreversible melting behavior in region II even at higher salt concentrations. These results confirm the very different interactions that CP33 and CP66 have with RNA.

Viscometric Analysis. Titration[13] of polyrA·polyrU with CP33, 44, and 55 causes only small changes in RNA viscosity up to the highest level (Figure 4). With CP66, we can again distinguish region I below 0.1 mol of CP66 per nucleic acid phosphate and region II above that ratio. In region I CP66 behaves in a similar manner to the other cyclophanes, and relatively small decreases in the complex viscosity are detected. In region II a larger decrease in the viscosity is obtained with a 70−80% reduction in relative reduced specific viscosity at a ratio of 0.2. Above 0.25 mols of compound per RNA-p aggregates start to form with most of the compounds. The viscosity results with CP33−CP55 and CP66 in region I indicate

(13) Viscometric titrations were conducted as previously described: Jones, R. L.; Davidson, M. W.; Wilson, W. D. *Biochim. Biophys. Acta* **1979**, *561*, 77−84.

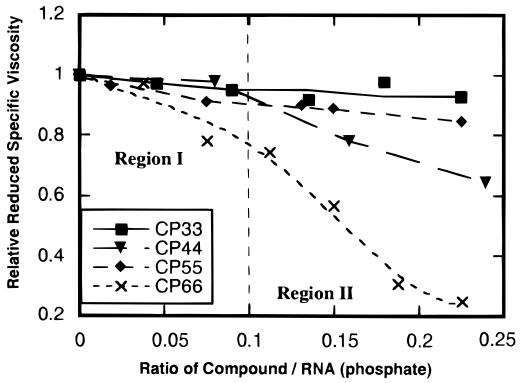

Figure 4. Viscometric titrations of polyrA·polyrU with CP33 (■), CP44 (▼), CP55 (◆) and CP66 (×) in MES buffer at 25 °C.

predominantly ionic interactions that do not cause any significantly conformational change in the RNA duplex. The viscosity results for the CP66−RNA complex in region II, however, support a model that involves stabilization of a base-pair open complex as predicted for the ΔT_m results.

Circular Dichroism Spectra. CD spectra[14] for polyrA·polyrU at 20 °C change very little on titration with CP33−55 up to ratios of 0.3 mols of compound per nucleic acid phosphate. Above that ratio the CD spectra show signs of aggregation. On the other hand, titration with CP66 causes only small changes in the intensity of the 260 nm RNA band (Figure 5) at a ratio of 0.1 mol of CP66 per nucleic acid (region I), but when the ratio is increased above 0.1 pronounced decreases in the intensity of the RNA band are produced. At a ratio of 0.3 the complex spectra approach that for denatured RNA (Figure 5).[15] This result again supports different predominant binding modes of CP66 in regions I and II. Over the same titration range, only small changes are observed in the CD spectrum of polydA·polydT for all of the cyclophanes. All the compounds behave similarly with the DNA polymers; they slightly decrease the intensity of the 260 nm band and slightly increase the 290 nm band of the DNA.

NMR Analysis of Complexes. NMR spectra as a function of the temperature were obtained for the complexes of CP66 with CT DNA and with polyrA·polyrU and chemical shift changes are shown in Figure 6. Large changes in the $\Delta\Delta\delta$ (absolute value of the chemical shift in the complex minus the value in the free compound) of CP66 were found in its complex with polyrA·polyrU. The proton signals of the alkyl chains of CP66 were the most affected: the H-γ proton signal shifted upfield over 0.6 ppm, and the H-β signal shifted in the same direction 0.5 ppm at 48 °C. The chemical shift change for the aromatic, and the Me−N proton signals were smaller but always upfield. At temperatures above 50 °C the $\Delta\Delta\delta$ values of the CP66−polyrA·polyrU complex start to decrease (Figure 6). The small temperature changes above melting, in spite of correction of temperature effects of CP66 alone without nucleic acid, can be the result of several factors: [i] part of the temperature effects are due to solvation changes, which should be different between the fully hydrated polyammonium compound and the compound partially desolvated by encapsulation of a nucleobase; [ii] the temperature change is also expected to affect the amount of CP66 complexed with CP66; and [iii] some combination of these effects.

UV melting curves for the RNA−CP66 complex were obtained in the buffer used in NMR experiments, and the UV changes parallel quite closely the chemical shift changes obtained in the NMR experiment (supporting information, Figure S1). These results clearly show that base pairs must open, and bases must move out of the helical stack to obtain the complex observed with CP66.

$\Delta\Delta\delta$ values for CP33 with polyrA·polyrU are compared in Figure 6 for the protons H-β and H-m. Both protons exhibit small changes as the temperature increases, in comparison to those for the same protons in the complex of CP66 and polyrA·polyrU.

Similar experiments for CP66 and CT DNA revealed lower $\Delta\Delta\delta$ values, around 0.2 ppm for the proton signals of the alkyl chains, and smaller chemical shift changes for the other signals than with the RNA complex. NMR experiments with CP66 and polydA·polydT were also carried out as a function of temperature, and the $\Delta\Delta\delta$ values obtained were small and very similar to those obtained with CP66 and CT−DNA.

Figure 7 shows NMR titration studies of polyrA·polyrU at 57 °C (unfolded state) with CP66. Only the chemical shifts of the adenine base peaks change on the addition of increased amounts of CP66. For instance, the adenine proton signals shift (ppm) +0.02 (H-8), −0.05 (H-2), and −0.05 (H-1′) on addition of CP66 at a ratio of 30 (polymer-p/CP66). As the amount of CP66 is increased, the adenine peaks continue to shift and broaden: at a ratio of 15 (polymer-p/CP66), the signal of H-8 shifts +0.03 ppm, the signal of H-2 shifts −0.06 ppm, and the sugar H-1′ signal shifts +0.07 ppm. It is interesting that the protons of adenine H-2 and H-1′ shift in the same direction and opposite to that for H-8. At higher ratios of CP66 versus RNA, the complex with polyA precipitated and the polyU strand was left in solution.

Discussion

The array of melting, spectroscopic, and hydrodynamic methods used in this report to analyze the CP*nn*−RNA complexes indicate that both ionic interactions of the CP*nn* cationic groups with phosphates in the RNA double helix as well as base-pair opening and base insertion in the cyclophane cavity can play important roles in complex stability. The importance of the two interaction modes change dramatically as the cavity size is increased with increasing *n*: ionic interactions are dominant with CP33, whereas base interactions, especially at ratios greater than 0.1, are significant with the CP66 complex. Surprisingly, the behavior with DNA is completely different with all cyclophanes showing only strong binding, primarily through ionic interactions, to the DNA double helix.

The dramatic differences between DNA and RNA and among cyclophanes is clearly illustrated by the thermal melting curves shown in Figure 1. All cyclophanes cause significant and similar stabilization of the DNA duplex, while RNA duplex stabilization decreases significantly as the cavity size increases. With CP66 the RNA is actually destabilized under the conditions of Figure 1. With CP33 the T_m increases monotonically with ratio and approaches a limiting T_m increase at the higher ratios (Figure 2). With CP66−RNA complexes the effects on RNA T_m change significantly with ratio. There is an initial small increase in RNA T_m on adding CP66 up to a ratio of approximately 0.1, which we have termed region I, and a second region above 0.1 where the T_m decreases with increasing ratio. All of our results suggest that the interactions of CP66 in region I are dominated by ionic interactions while base-pair opening and insertion into the CP66 cavity are dominant in region II. CP33 shows no stabilization of the base-pair open state, and ionic interactions with the duplex state define its complexes with RNA and DNA under all conditions.

(14) CD experiments were performed at 20 °C as previously described: Zuo, E. T.; Tanious, F. A.; Wilson, W. D.; Zon, G.; Tan, G.-S.; Wartell, R. M. *Biochemistry* **1990**, *29*, 8452−8461.

(15) Johnson, W. C., Jr. *Methods of Biochemical Analysis* **1985**, *31*, 62−163.

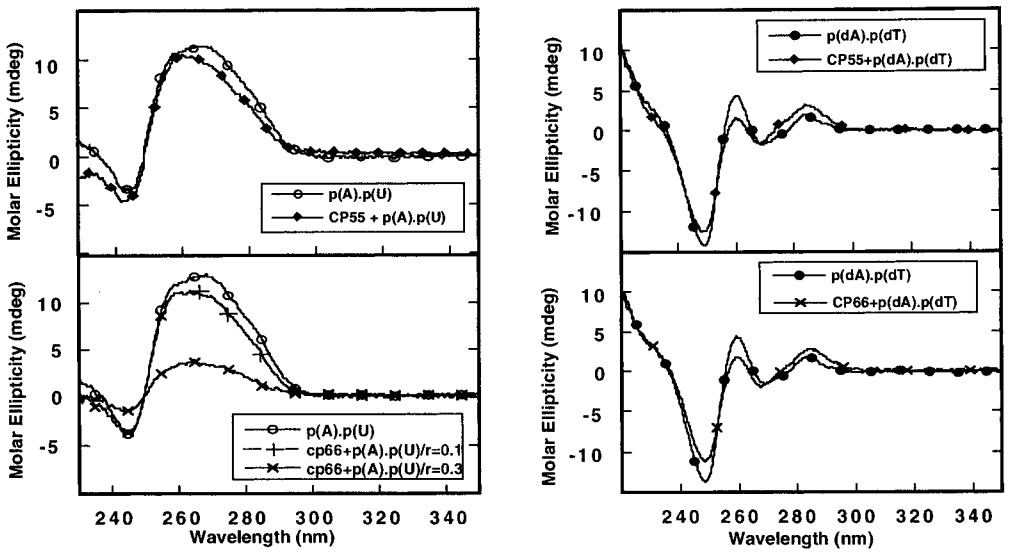

Figure 5. CD spectra obtained in MES buffer at 20 °C, for polydA·polydT (●) with CP55 (◆) and CP66 (×) at a ratio of 0.3 mols of CP*nn* per nucleic acid phosphate, and for polyrA·polyrU (○) with CP55 (◆) at ratio 0.3, and with CP66 at ratios 0.1 (+) and 0.3 (×) mols of cyclophane per nucleic acid phosphate. Spectra for the CP33 and CP44 complexes are very similar to those for CP55.

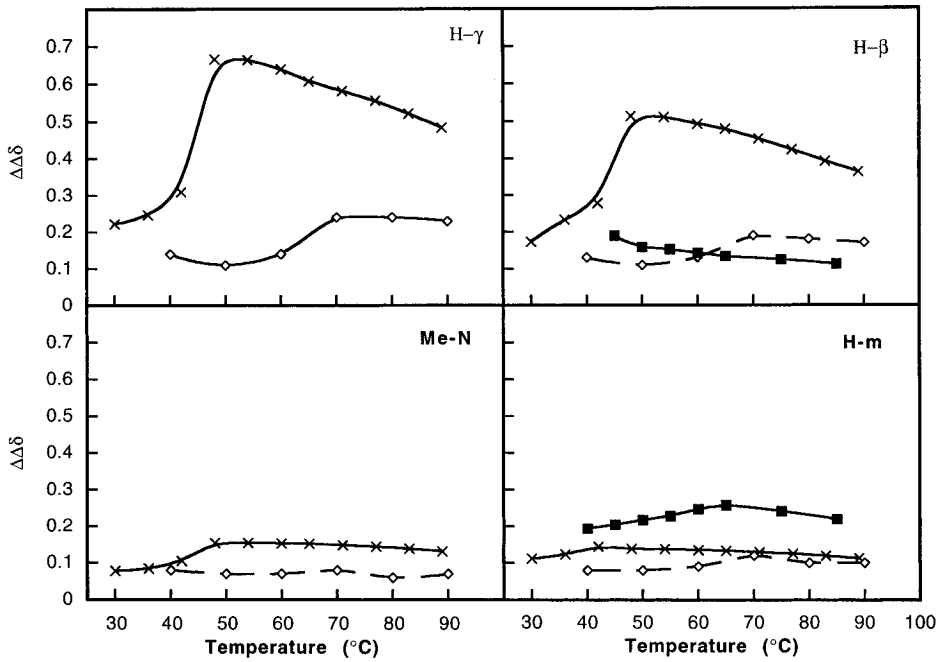

Figure 6. $\Delta\Delta\delta$ values (absolute value obtained from substraction of the chemical shift in the complex minus the value in the free compound) at several temperatures through the melting region, for protons of CP66: γ and β of the alkyl chain, *N*-methyl and aromatic meta with CT DNA (◇) or polyrA·polyrU (×); and for protons β and aromatic meta of CP33 with polyrA·polyrU (■). NMR measurements were performed in D_2O with phosphate buffer (0.02 M phosphate, 1×10^{-5} EDTA, pH = 7) using TSP ($Me_3SiCD_2CD_2COONa$) as internal reference. The ratio nucleic acid-p/CP*nn* was 20/1 in all complexes, and the concentracions of the nucleic acids were: 3×10^{-3} M-P in the complex of CP66 with polyrA·polyrU, and 6×10^{-3} M-P in the complex of CT DNA with CP66 and polyrA·polyrU with CP33.

The model with two regions that have different dominant interaction modes for CP66−RNA complexes is supported by results from renaturation, viscosity, and CD studies. The model described above predicts that CP66 complexes should behave more similarly to those for CP33 in region I where ionic interactions dominate but should exhibit significantly different behavior in region II where base interactions define the complex. An excellent example of these differences is provided by the renaturation experiments in Figure 3. It is possible to renature the CP33−RNA complexes at all ratios; however, the CP66 complexes renature in region I but not in region II as predicted by the model. The difference in RNA stabilization by CP66 and CP33 at ratios below 0.1, however, clearly demonstrates that CP66 base interactions are also occurring in region I, particularly as the temperature approaches the complex T_m.

Viscometric titrations of RNA with CP33, 44, and 55 show very little change as the ratio is increased as predicted for an external ionic interaction model. With CP66, however, the presence of two interaction regions is again supported. There are small changes in viscosity in region I, but there are significant viscosity decreases as the ratio is increased in region II. A base-pair open complex would be expected to cause a local distortion or kink in the RNA double helix with resulting significant decreases in viscosity. This is exactly what is observed with CP66 in region II in support of a base interaction model in that region.

CD spectra of CP*nn*−nucleic acid complexes provide very useful information for defining complex structures and regions in which they exist. As illustrated in Figure 5, very little change in CD is observed on addition of any of the cyclophanes to DNA. In the same manner little change in CD is observed on addition of CP33, 44, or 55 to RNA. With CP66, however, little change in CD occurs in region I, but very significant changes in CD occur as the ratio is increased in region II. At ratios of 0.3 and above, the RNA CD spectra are characteristic of a melted state suggesting that the duplex has been completely disrupted under these conditions. To summarize, melting, viscosity, and CD results indicate that there are two different types of complexes for CP66 as a function of ratio. In region I the dominant complex involves ionic interactions of CP66 with the anionic duplex, and complex formation causes very little structural change in the double helix. This is the primary complex for CP33−55 with RNA at all ratios. The viscosity and CD results strongly suggest that CP66−RNA complexes in region II are dominated by base-pair opening where one or more bases are removed from the helical state to form a cavity-inclusion complex with CP66. At high ratios this can lead to complete melting of the RNA duplex.

In order to provide additional information on the bases involved and on the structure of the CP66−complex, we conducted NMR experiments as a function of ratio and temperature. NMR experiments as a function of temperature with DNA and RNA complexes provide important insight into the interaction processes[16] (Figure 6). With polyrA·polyrU in the presence of CP66, for example, as the temperature is increased, large upfield shifts are obtained for the methylene linker protons of CP66. Such shifts suggest a complex similar to those previously observed for insertion of simple nucleotides into CP66[7] and strongly support a model involving insertion of nucleobases from melted regions of RNA into the CP66 cavity. In such a complex the methylene protons of CP66 are stacked over the aromatic base, and aromatic ring current effects cause the dramatic upfield shifts observed for the methylene proton signals. These shifts are smaller than the maximum shifts observed with adenine nucleotides,[7] and we attribute this smaller effect in the polymer to steric restriction of complete insertion of RNA bases. There may also be slight differences in insertion geometry for the polymer complex relative to less sterically constricted nucleotides.

The titration experiments of polyrA·polyrU with CP66 (Figure 7) clearly show that the base-insertion reaction of CP66 is very specific for adenine bases in this polymer, as the peaks for the uracil base do not show any significant change in chemical shift or line width. The peaks of the adenine protons, however, shift in different directions and broaden: the H-8 peak shifts downfield, while the H-2 peak shifts upfield, and the sugar H-1′ signal shifts downfield. These shifts suggest a specific orientation in the complex between the adenine bases in RNA and CP66. The H-2 proton would be inserted deep into the cavity of CP66 as it has the largest upfield shifts, and it is the proton that is sterically most accessible to the cyclophane cavity. On the other hand, H-8 is close to the sugar-phosphate backbone which hinders insertion into the cavity. Its $\Delta\Delta\delta$ value is almost zero and could be an average between the shielding and deshielding effects of the aromatic rings of CP66. We can conclude that the H-8 proton should be near the border of the cavity. H1′ experiences large deshielding and is clearly out of the cavity as would be expected from steric considerations. Surprisingly, the chemical shift values in the complex of CP66

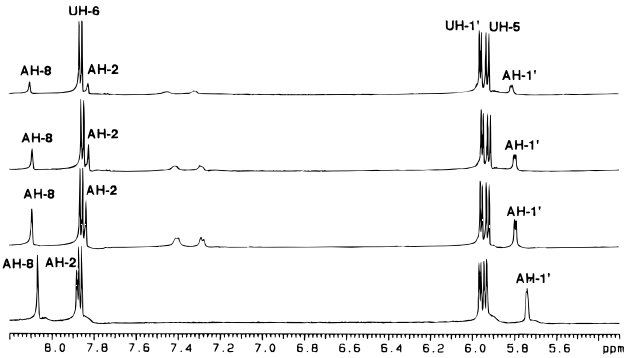

Figure 7. ^{1}H-NMR spectra of the aromatic region for the titration of polyrA·polyrU with CP66 at 57 °C. The concentration of the nucleic acid was 1×10^{-2} M (phosphate) and the buffer and conditions are as in Figure 6. From the bottom to the top: the first spectrum is polyrA·polyrU in absence of CP66 and the remainder have CP66 at ratios 30, 20, and 15 (polyrA·polyrU/CP66). The small peaks that appear between 7.2 and 7.5 ppm, correspond to the aromatic protons of CP66.

with polydA·polydT in similar titration experiments show that H-8, H-2, and H-1′ shift downfield, and this result excludes an insertion complex of the type observed with RNA. Downfield shifts were also obtained for the same protons of the CP33 complex with polyrA·polyrU, again demonstrating the unique specificity of CP66 toward adenine bases in the RNA polymer.

Other organic compounds have been reported to cause a decrease in the melting temperature of DNA and RNA, but none of those compounds show the selectivity for RNA bases exhibited by CP66. For example, the amides of L-phenylalanine, L-tryptophan, and L-tyrosine decrease the melting temperatures of polymer RNAs at low concentrations, whereas at high concentrations they increase the T_m.[17] The binding of metal complexes and ions, such us Cu(II), Pb(II),[18] Cd(II), Mn(II), and Zn(II)[11,12] can stabilize nucleic acids through phosphate interactions, but they can also destabilize the duplex through binding to the bases. Comparisons of melting results for cyclophane complexes with the metal ion results provided the basis for our initial interaction model: CP33 interacts preferentially with duplex RNA at all ratios, while, in the case of CP66, its cavity size is large enough to accommodate a purine base, as seen with nucleotides,[7] and it causes base-pair opening in region II. CP66 binds more strongly with the duplex at lower ratios (region I), but it can also interact with bases that are in open regions of the duplex, and at higher ratios (region II) the duplex completely melts. The initial interactions of CP66 are primarily ionic in nature,[8] while the base-opening process involves van der Waals and hydrophobic forces as well as ionic interactions. As the RNA polymer unfolds completely, it is possible that other types of cyclophane complexes could form.

An important question raised by these results is why base-pair opening and melting occur for the CP66 complex with RNA, while the DNA duplex is strongly stabilized by that cyclophane. Our results suggest that the dramatic differences between CP66 complexes with RNA and DNA (Table 1) are the result of two effects. First, the cyclophanes bind very tightly with duplex DNA, probably through a complex in the major groove.[7] The grooves in RNA, however, are quite different in shape from those of DNA and it seems probable that CP66 cannot form as favorable a set of interactions in a complex with an A-form RNA duplex. Second, our NMR results with single-stranded RNA or DNA clearly demonstrate a strong interaction of CP66 with an adenine base in an RNA strand compared to

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a corresponding DNA strand. Thus, the dramatic difference between CP66 complexes with RNA and DNA is the result of two opposing interactions: stronger ionic binding of CP66 with the DNA duplex than with RNA and stronger CP66 cyclophane cavity binding of RNA purine bases in a polymer strand than with bases in a similar DNA strand.

In conclusion, the results presented above clearly demonstrate the unique and surprising interaction of the azoniacyclophane CP66 with RNA bases. Only the cavity of CP66 is large enough to allow effective complexation of a purine base. This striking finding could have a parallel biological mechanism in RNA chemistry.[9] Specific reactions of RNA bases, such as modifications observed in t-RNA bases that include methylations, thiolations, and reduction of uracil to dihydrouracil,[19] may require base flipping in a similar manner to the DNA enzymes. We also note that analogous of these compounds could be used in biotechnology to selectively modify bases in RNA.

The base-flipping activity of DNA-enzymes came as somewhat of a surprise[9] but is now well-established. To find a specific base-pair open complex with a small organic molecule that is selective for RNA is particularly surprising and offers interesting new possibilities for manipulation of nucleic acids. Our results, for example, with CP66-RNA complexes suggest that it should be possible to design relatively simple organic compounds that can cause base-flipping as observed with enzymes.

Experimental Section

Materials. PolyrA·polyrU was purchased from Sigma. PolydA·polydT, poly(rA−rU)$_2$, polyrI·polyrC, poly(dA−dT)$_2$, and polyrA·polydT were purchased from Pharmacia. CP*nn* were prepared as described earlier.[5]

Thermal Melting Studies. Thermal melting experiments were conducted with a Cary 3 spectrophotometer interfaced to a Dell/486 microcomputer by following the absorption change at 260 nm (262 nm for poly(rA-rU)$_2$) as a function of temperature. The temperature was controlled by a Cary temperature controller that was programmed to raise the temperature at a rate of 0.5 °C/min. A thermistor fixed into a reference cuvette was used to monitor the temperature. Denaturation experiments were conducted in MES buffers (0.01 M 2-(*N*-morpholino)ethanesulfonic acid, 0.001 M EDTA and pH 6.25, [Na^+] = 0.007 M) without addition of NaCl, and with the addition of 0.02, 0.05, and 0.1 M NaCl, and phosphate buffer (0.02 M phosphate, 1×10^{-5} EDTA, pH = 7) with 1.0×10^{-4} M RNA or DNA phosphate, unless other concentration is specified. T_m values were determined from first-derivative plots. Compounds are compared by the increase in T_m of the nucleic acid in the presence of the cyclophane ($\Delta T_m = T_m$ of the complex $- T_m$ of the free nucleic acid) at saturating amounts of the compound. T_m values were obtained at several ratios (from 0.1 to 0.3 of compound to nucleic acid phosphate) for CP33 and CP66 cyclophanes.

Viscometric Titrations. Viscometric titrations were conducted in Ubbelohde semimicro dilution viscometers (for the Cannon series no. 75 viscometers). One milliliter of polymer solution (approximately 1.0×10^{-4} M polyrA·polyrU or polydA·polydT phosphate in MES buffer, [Na^+] = 0.007 M) was titrated with a stock solution of each cyclophane at 25 °C. The additions were made directly into the polyrA·polyrU solution by using a Hamilton syringe modified to fit into the viscometer mixing chamber.

Circular Dichroism. CD spectra were obtained with a Jasco J-710 spectrophotometer interfaced to an Dell microcomputer. All CD experiments were performed at 20 °C in 1-cm path length cuvettes with 4.5×10^{-4} M RNA or DNA phosphate. The spectra were obtained at ratio 0.3 of compound to nucleic acid phosphate in MES buffer, [Na^+] = 0.007 M. Curves presented are the average of three scans.

NMR. ^{1}HNMR measurements were obtained on Varian Unity Plus 500 or 600 MHz spectrometers in 5 mm tubes. The experiments were performed in D_2O with phosphate buffer (0.02 M phosphate, 1×10^{-5} EDTA, pH = 7) using TSP (Me3SiCD2CD2COONa) as internal reference.

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Supporting Information Available: Comparison of the melting behavior of the CP66−RNA complex in phosphate buffer by NMR and UV methods (2 pages). Ordering information is given on any current masthead page.

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