

Ethidium and Proflavine Binding to a 2',5'-Linked RNA Duplex

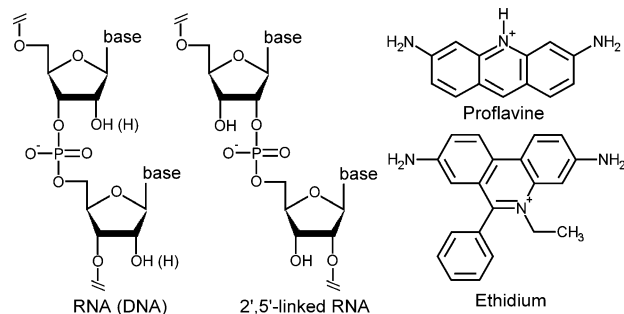
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Small-molecule intercalation of DNA and RNA has been the subject of biophysical and biochemical investigations for over 40 years.¹ Despite these studies, and the recognized importance of nucleic acid intercalation in medicine and molecular biology, fundamental questions persist regarding the energetics of intercalation.² The dramatic unwinding of DNA and RNA duplexes upon intercalation immediately suggests that the nucleic acid backbone should play a significant role in dictating the free energy of intercalation. However, the free energy contribution of the backbone is difficult to appreciate given the intertwined energetics associated with intercalation (e.g., π - π stacking and solvent effects).² Furthermore, previous intercalation studies are almost exclusively limited to natural DNA and RNA, making it difficult to predict how changes in backbone structure will promote or hinder intercalation. Here, we present binding studies of known intercalators to a duplex of 2',5'-linked RNA, a nucleic acid that is arguably the closest chemical analogue of standard RNA, but with distinct backbone atom connectivity (Scheme 1). The results presented here demonstrate that the nucleic acid backbone is a significant contributor to the free energy of small-molecule binding, even in the case of molecules known to bind nucleic acids primarily through base-stacking interactions.

Scheme 1



Proflavine and ethidium (Scheme 1) were chosen for this initial study of 2',5'-linked RNA binding because their respective interactions with natural DNA and RNA have been studied in detail. Additionally, proflavine intercalates nucleic acids with very limited contacts beyond base stacking, whereas the pendant ring of ethidium protrudes into the minor groove.³

The fluorescence intensity of proflavine and ethidium change upon intercalation, which can be used to determine binding constants.⁴ Our fluorescence titration studies reveal that proflavine binds a 2',5'-linked RNA duplex with an association constant of $K = 3.9 \times 10^5 \text{ M}^{-1}$. This is more favorable than that exhibited by DNA and RNA duplexes of the same length and sequence (Figure 1). In contrast, ethidium binds the same 2',5'-linked RNA duplex with $K = 0.4 \times 10^5 \text{ M}^{-1}$, which is less favorable than that exhibited by DNA and RNA (Figure 1). K values measured for RNA under identical conditions reveal that changing the RNA backbone linkage from

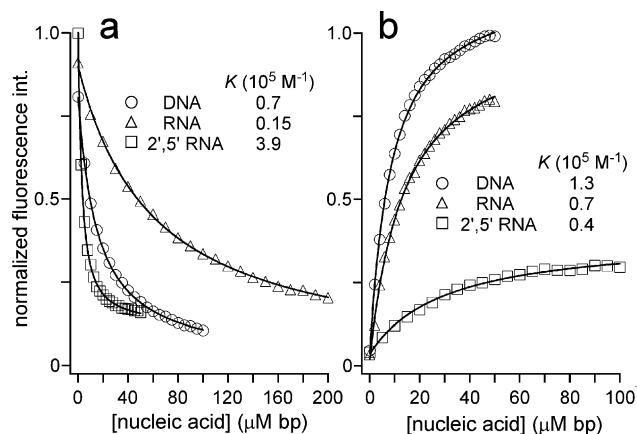


Figure 1. (a) Fluorescence intensity measurements for 1 μM solutions of proflavine in the presence of increasing concentrations of DNA, RNA, and 2',5'-linked RNA duplexes with the nucleotide sequence 5'-CCGGC-CCGGCGC and its complement. (b) Same as panel a, except for 1 μM solutions of ethidium. K values were derived from the curve fits shown. Samples were 25 $^\circ\text{C}$, 100 mM NaCl, 1 \times BPE, pH 7. Additional experimental details are provided in SI.

3',5' to 2',5' results in a 25-fold increase in the K for proflavine binding, corresponding to a $\Delta\Delta G$ of -2 kcal/mol at 25 $^\circ\text{C}$. This is an appreciable increase, considering that the ΔG for proflavine binding to RNA is ca. -8 kcal/mol at 25 $^\circ\text{C}$.⁵ The same backbone change results in a 2-fold decrease in the K for ethidium binding, corresponding to a $\Delta\Delta G$ of $+0.4 \text{ kcal/mol}$ at 25 $^\circ\text{C}$. These observed differences in the free energy of proflavine and ethidium binding are essentially independent of ionic strength (Supporting Information).

Along with changes in fluorescence, detecting an increase in duplex thermal stability is a widely used method to verify small-molecule binding to a nucleic acid. For the 2',5'-linked RNA dodecamer duplex of this study with an initial T_m of 41 $^\circ\text{C}$, proflavine and ethidium produce a ΔT_m of $+13 \text{ }^\circ\text{C}$ and $+6 \text{ }^\circ\text{C}$, respectively (SI).

Since proflavine and ethidium are well-documented intercalators of DNA and RNA, one would expect these molecules to bind duplex 2',5'-linked RNA in the same manner. However, there is the possibility of alternative binding modes (i.e., groove binding and outside stacking). In the absence of high-resolution structures that verify 2',5'-linked RNA intercalation, we present data from experiments that are sensitive to binding mode and compare this data to that acquired for DNA and RNA. First, a red-shift and a hypochromic effect of the longest-wavelength absorption band of a small molecule indicates, in most cases, binding by intercalation.⁶ The 444-nm band of free proflavine exhibits a 22-nm red-shift and 8% hypochromism upon binding to 2',5'-linked RNA, changes that are comparable to those exhibited by proflavine upon binding to DNA (15 nm, 17%) and RNA (13 nm, 18%) (SI). The 479-nm band of ethidium exhibits a 15-nm red-shift upon binding to 2',5'-linked RNA (compared to 38 and 31 nm for DNA and RNA, respectively) and 17% hypochromism

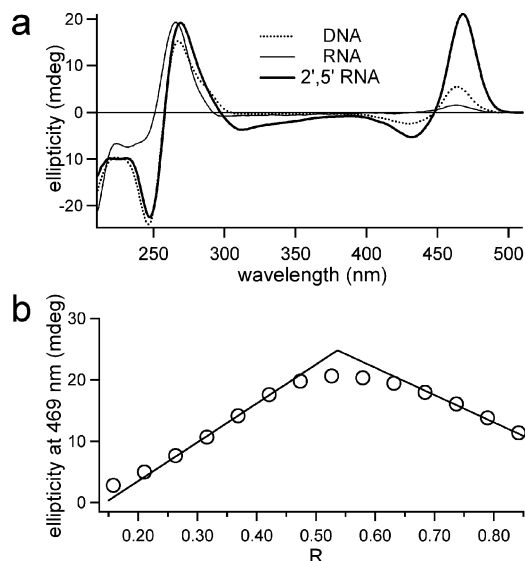


Figure 2. (a) CD spectra of nucleic acid duplexes with proflavine. All samples were 80 μ M in bp, 40 μ M in proflavine. (b) Job plot analysis of the binding of proflavine to duplex 2',5' RNA. $R = [\text{proflavine}]/([\text{proflavine}] + [\text{bp}]/2)$. Dodecamer sequence and sample conditions are given in Figure 1. Additional experimental details are provided in SI.

(compared to 21%, and 24%, respectively). Thus, our UV-vis spectroscopy measurements are consistent with intercalative binding of 2',5' RNA.

CD spectra of small molecules in the presence of nucleic acids have also been used as a means to characterize binding modes.⁷ The long wavelength CD bands of proflavine in the presence of 2',5' RNA are of the same sign and similar shape as those observed in the presence of RNA and DNA (Figure 2a). Similar CD band shapes can indicate a similar mode of binding.⁷ However, the greater intensity of proflavine CD bands with 2',5' RNA could also be indicative of nonintercalative binding, which has been reported for proflavine binding to DNA under certain conditions.⁸ Bound ethidium does not produce a significant induced CD band for any of the three duplexes (data not shown).

Another common feature of nucleic acid intercalation by small molecules is adherence to the nearest-neighbor-exclusion principle.⁹ That is, intercalators are known to bind up to a maximum of one molecule per two base pairs. We have used the CD band of proflavine at 469 nm to determine the stoichiometry of proflavine binding to 2',5' RNA by Job plot analysis.¹⁰ The inflection in the Job plot shown in Figure 2b at $R = 0.5$ indicates the binding of one proflavine molecule per two base pairs. Thus, proflavine binding to 2',5' RNA also obeys the nearest-neighbor-exclusion principle.

The free energy difference measured for proflavine binding to 2',5' RNA versus standard RNA could result from differences in the duplex structures before or after intercalation, or both. For example, base stacking in a 2',5' RNA duplex has been suggested to be less favorable than in 3',5' RNA,¹¹ which would make it easier for a 2',5' RNA duplex to create an intercalation site. However, an NMR structure of 2',5' RNA revealed an A-form helix similar to that of 3',5' RNA (albeit with 2'-endo sugar pucker),¹² which suggests that the observed increase in K could reflect a difference in the final intercalated states. The observation that ethidium binds 2',5' RNA less favorably than standard RNA, whereas the opposite is true for proflavine, suggests that the pendant ring of ethidium may make less favorable contacts in the minor groove of a locally unwound 2',5' RNA duplex than when bound to DNA or RNA. Duplex-specific solvent effects and backbone flexibility could also contribute to this difference.¹³ Clearly, explaining the origins of

our observed differences in free energies of proflavine- and ethidium binding and verification of the intercalative binding mode require additional investigations.

To the best of our knowledge, this is the first demonstration of known intercalators binding to a non-3',5'-linked nucleic acid. Armitage and co-workers have previously reported that heteroduplexes formed by DNA and RNA with locked nucleic acids (LNA) bind intercalators.¹⁴ However, LNAs possess the same backbone atom connectivity as natural DNA, but with an extra intra-sugar linkage that "locks" sugar conformation.

The results presented here demonstrate the pronounced role of RNA backbone structure in determining the thermodynamics of small-molecule binding, and suggest that more significant changes that still maintain the general structure of RNA (e.g., pyranosyl-RNA¹⁵) could result in even greater binding affinities for intercalators. This ability to enhance small-molecule binding with changes in backbone structure could be used to stabilize hybridization of antigene or antisense oligonucleotides¹⁶ and to stabilize intercalator-dependent molecular assemblies.¹⁷

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Supporting Information Available: Sample preparation and data analysis, $\ln(K)$ vs $\ln[\text{NaCl}]$ plots, UV-vis spectra, and T_m measurements. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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