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Thermodynamic Investigation of the Association of Ethidium, Propidium and Bis-ethidium to DNA Hairpins

Dionisios Rentzeperis,† Miriam Medero and Luis A. Marky*
Department of Chemistry, New York University, New York, NY 10003, U.S.A.

Abstract—We have used a combination of calorimetric and spectroscopic techniques to investigate the association of the bis-intercalator ethidium homodimer (bis-ethidium) to short DNA hairpins with sequences: d(GCGCT$_5$GCGC) and d(CGCGT$_5$CGCG). The helix–coil transition of each hairpin, investigated by UV and calorimetric melting protocol, takes place in monomolecular two-state transitions with characteristic enthalpies of ~37 kcal mol^{-1} for disrupting the four dG·dC base pairs of the hairpin stems. Deconvolution of the bis-ethidium-hairpin calorimetric titration curves indicate that each hairpin contains two distinct binding sites for the ligand: a high affinity site in the stem (K_b ~10^7) that accommodates one bis-ethidium molecule and a lower affinity site (K_b ~10^6) located probably at the loop that accommodates two bis-ethidium molecules. The overall stoichiometries of three ligands per hairpin are in agreement with those obtained in continuous variation experiments using visible spectroscopy. The interaction of bis-ethidium for each type of sites results in enthalpy driven reactions, with average binding enthalpies, ΔH_b, of –13.1 and –12.1 kcal mol^{-1} for the stem and loop sites, respectively. Comparison to the thermodynamic profiles of ethidium and propidium binding reveals that the bis-ethidium binding to the stem site of each hairpin has a more favorable free energy term of –1.4 kcal mol^{-1} and more favorable enthalpy of –4.2 kcal mol^{-1}. These suggest that only one phenanthridine ring of bis-ethidium intercalates in the stem, while the second planar ring is exposed to solvent or weakly associated to the surface of DNA. The K_bs for bis-ethidium binding to the loop sites are about two orders of magnitude larger than the mono-intercalators and correspond to a more favorable free energy of –2.0 kcal mol^{-1}. The enthalpies of binding are also more favorable for bis-ethidium by –2 to –4 kcal mol^{-1}. Overall, the magnitude of K_b for each ligand, and for each site, is in qualitative agreement with the electrostatic contribution from the actual number of positive charges of the ligand. The increased favorable enthalpic contributions of bis-ethidium are consistent with larger hydrophobic contributions, while the increase in unfavorable entropy contributions are consistent with the higher ordering of bis-ethidium in the stem and loop sites.

Introduction

Hairpins are a common feature of RNA molecules,[1,2] and they have been found to exist in DNA in regions with palindromic sequences that are involved in gene regulation.[3–6] So far, there is not enough evidence to confirm the existence of DNA hairpins *in vivo*. However, it is thought that they may act as potential binding sites for proteins[7] especially proteins whose binding to helical DNA results in DNA supercoiling which can be relaxed by the formation of hairpins in regions of DNA with palindromic sequences.[8] Therefore, these hairpin structures have been proposed to exist in regulatory sites for replication,[9] transcription and RNA processing.[5] Short self-complementary DNA oligonucleotides with proper sequences can form hairpin structures.[10–18] These hairpins are useful model systems to conduct thermodynamic experiments because their stable formations tend to melt in monomolecular transitions at convenient temperatures.[18–23]

One type of binding mode in which a ligand can interact with nucleic acids is intercalation, whereby a planar aromatic molecule is inserted between two adjacent base pairs of the double helix. This intercalative binding mode was first suggested by Oster in 1951,[24] through studying the fluorescence quenching of an acridine dye by nucleic acids, and later established by Lerman in 1961.[25] Since then, a wide range of experimental techniques have been used to obtain a detailed understanding of the molecular basis and energetics of the specificity of intercalative ligands to DNA.[26–29] These studies have provided insight into the rational design of ligands as therapeutic agents. Earlier investigations have shown that ethidium, a well known intercalator that is widely used as a nucleic acid stain, binds to DNA with a 5'-Pyr-Pur-3' sequence preference and neighbor exclusion parameter of at least two base pairs.[30–32] This prompted our group to design short DNA hairpin molecules with a GCGC/GCGC helical stem, containing single binding sites, and loops of three to seven thymine residues and to investigate their interaction with ethidium.[33] The thermodynamic results clearly showed that each hairpin is able to accommodate one ethidium molecule in the stem. It was also shown that the thymine loops interacted with two ethidium molecules.

In this work, we have extended our thermodynamic investigations to include propidium (see Fig. 1), which contains a propyl charged side chain and intercalates to

†Present address: Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Av., Boston, MA 02130, U.S.A.

DNA with similar binding characteristics of ethidium, and bis-ethidium with two phenathridine groups joined by a cationic linker with four positive charges (Fig. 1). We also include an additional hairpin molecule with sequence d(CGCGT$_5$CG-CG), whose helical stem contains additional binding sites for monointercalators. We used a combination of spectroscopy and calorimetry techniques to obtain ligand–DNA stoichiometries and standard thermodynamic profiles for the interaction of all three ligands with each hairpin molecule. Our results indicate overall stoichiometries of 3:1 (in ligands per hairpin molecule) for the binding of each ligand to the d(GCGCT$_5$GCGC) hairpin (one ligand in the stem and two ligands in the loop); 4:1 (two in the stem and two in the loop) for ethidium and propidium with the d(CGCGT$_5$CGCG) hairpin stoichiometries, and 3:1 for bis-ethidium (one in the stem and two in the loop).

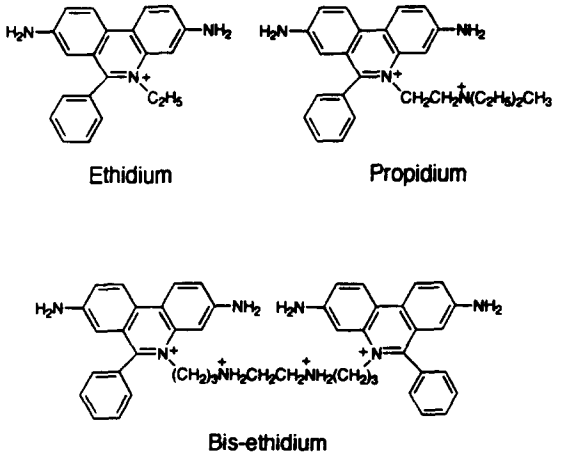

Figure 1. Structure of the ligands, ethidium, propidium, and bis-ethidium.

The interaction of bis-ethidium for each type of site results in enthalpy driven reactions, with average binding enthalpies, ΔH_b, of –13.1 and –12.1 kcal mol^{-1} for the stem and loop sites, respectively. Relative to the thermodynamic binding profiles of the monointercalators, bis-ethidium binding to the stem site of each hairpin has a more favorable free energy term of –1.4 kcal mol^{-1} and more favorable enthalpy of –4.2 kcal mol^{-1}, while binding to the loop sites corresponds to a more favorable free energy of –2.0 kcal mol^{-1} and more favorable enthalpies of –2 to –4 kcal mol^{-1}. These thermodynamic results, together with the overall stoichiometries of the complexes, are consistent with the intercalation of only one of the phenanthrydium rings in the stem, and also with the association of only one ring, from each of the two bis-ethidium molecules, to the loop sites.

Experimental

Chemicals

The oligomer sequences d(CGCGT$_5$CGCG), CG-hairpin, and d(GCGCT$_5$GCGC), GC-hairpin, were synthesized on an ABI 391 automated synthesizer, using standard phosphoramidite chemistry,[34] purified by HPLC, and desalted on a Sephadex G-10 exclusion chromatography column. The following extinction coefficients of the oligomers at 260 nm and 95 °C, in M^{-1} cm^{-1}, were determined in water by a procedure described earlier:[35,36] GC-hairpin, $\varepsilon = 1.08 \times 10^5$; and CG-hairpin, $\varepsilon = 1.104 \times 10^5$. Ethidium bromide and propidium iodide were obtained from Sigma, and bis-ethidium from Molecular Probes. All three ligands were used without further purification and their concentration determined with the following molar extinction coefficients: ethidium, 5850 M^{-1} cm^{-1} at 480 nm; propidium, 5950 M^{-1} cm^{-1} at 493 nm; and bis-ethidium, 12,120 M^{-1} cm^{-1} at 490 nm.[37] All other chemicals were reagent grade. The buffer solutions consisted of 10 mM sodium phosphate (NaP_i), 0.1 mM Na_2EDTA at pH 7.0 and adjusted to the desired ionic strength with NaCl.

Temperature-dependent UV spectroscopy

Absorbance versus temperature profiles (melting curves) of the free oligomers and of the ligand–oligomer complexes were obtained by using a thermoelectrically controlled Perkin Elmer spectrophotometer, interfaced to a PC-XT computer for the acquisition and analysis of experimental data. The instrument was set at the most convenient wavelength of 275 nm, because of larger hyperchromicity values, to monitor the melting of dG·dC base pairs. The temperature was scanned at a heating rate of 1 °C min^{-1}. Analysis of the free oligomer melting curves yields the transition temperatures, T_m, van't Hoff enthalpies, ΔH_{vH}, and the release of counterions, Δn_{Na^+}. All these parameters were determined using procedures described previously.[38]

Differential scanning calorimetry

Excess heat capacity as a function of temperature (DSC melts) for the helix–coil transition of each oligomer were measured with a Microcal MC-2 differential scanning calorimeter (Northampton, MA). Two cells, the sample cell containing 1.6 mL of oligomer solution and the reference cell filled with the same volume of buffer solution, were heated from 10 to 100 °C at a heating rate of 0.75 °C min^{-1}. Analysis of the resulting thermograms, using procedures described previously,[38] yields standard thermodynamic profiles (ΔH_{cal}, ΔS_{cal}, and $\Delta G°_{cal}$) and model-dependent enthalpies, ΔH_{vH}, for the transition of each hairpin.

High sensitivity isothermal titration calorimetry

The heat of interaction of all three ligands with each hairpin molecule was measured by using the Omega titration calorimeter from Microcal Inc.[39] In a typical titration, 1.4 mL of an oligomer solution with a 20 μM concentration in strands is titrated with a ligand solution of ~1 mM concentration using a 100-μL syringe by ~20 injections of 5–6 μL each. This syringe is also used to mix the reactants completely in the sample cell

by rotating it at 400 rpm. The reference cell of the calorimeter was filled with water and the instrument calibrated by means of a known standard electrical pulse. For each injection, the area under the resulting peak is proportional to the heat of interaction, Q. Once this heat is corrected for the titrant dilution and normalized by the titrant solution concentration it becomes equal to the binding enthalpy, ΔH_b, under unsaturating conditions. The precision of the heat of each injection is about 0.5 μcal. Therefore, one way of determining ΔH_b is to average several intermediate peaks from the different sites. In addition to the binding enthalpies, one can obtain binding affinities, and overall stoichiometries of the complexes. The method is based on a thermodynamic model, in which a ligand binds to a DNA hairpin containing two types of independent binding sites: the intercalation sites at the stem and the additional sites of the thymine loops. Each type of site is characterized by an equilibrium constant, K_b, binding enthalpy, ΔH_b, and apparent number of ligands per binding site, n. The experimental calorimetric binding isotherm is the dependence of the total heat, Q_T, (or dQ_T/dX_T) on the total concentration of ligand added, X_T. The above three parameters for each type of site are determined iteratively using the Marquardt algorithms as described previously.[40] The initial fitting procedure lets all three parameters float or fixes either the enthalpy, determined independently by averaging the heats of the intermediate peaks of a given site, or n parameters or both until the lowest standard deviation of the fit is obtained; all approaches result in similar values.

Stoichiometry of the ligand–hairpin complexes

The stoichiometry of the ligand–hairpin complexes was determined primarily by titration calorimetry experiments as mentioned in the previous section. Alternatively, these stoichiometries were measured spectroscopically using continuous variation experiments in which the hairpin was titrated with ligand at constant total concentration of ligand plus hairpin.[41] For example, first, we measured the spectra of bis-ethidium and bis-ethidium–hairpin solutions from 350 to 625 nm to determine the best wavelength of measurement, i.e. the wavelengths of maximum absorption for the free and bound ligand. For bis-ethidium, these wavelengths were determined at 490 and 527 nm. Second, the absorbance at these two wavelengths is measured as a function of the mole fraction of DNA, χ_h. The intersection of the resulting lines in these plots corresponds to mole fraction values that yield the stoichiometry of the complexes.

Results

Thermodynamic characterization of the helix–coil transition of oligomers

For the exclusive formation of monomolecular complexes, it is expected the T_m to be independent of strand concentration, which would mean that the oligomer forms intramolecular hairpins at low temperatures rather than forming a duplex with an internal loop. Therefore, UV and calorimetric melting curves were performed in the strand concentration range of 5–800 μM. For each hairpin, typical UV melting curves are shown in Figure 2a; the melting of these molecules occurs in broad monophasic transitions with hyperchromicities of 20% at 275 nm. Typical excess heat capacity versus temperature profiles are shown in Figure 2b; these curves also show monophasic transitions with no changes in heat capacities within the initial and final states. The total heat needed to dissociate the hairpins into single strands was obtained from the area under the DSC curves and normalized for the total number of moles of strands to yield molar enthalpies, ΔH_{cal}. The T_m, ΔH_{cal} and ΔH_{vH} values obtained from these curves are listed in Table 1. In this concentration range and in low salt concentration, both optical and calorimetric observables show a T_m of 71 °C for the GC-hairpin and 63.3 °C for the CG-hairpin; these T_ms increased with the increase in the salt concentration (see Table 1). The constant T_m values over a two orders of magnitude increase in the strand concentration confirms the formation of single stranded hairpins at low temperatures. Comparison of the model-dependent ΔH_{vH}s calculated from calorimetric or optical experiments and ΔH_{cal} for a given molecule allows us to draw conclusions about the nature of the transitions.[38] We obtained $\Delta H_{vH}/\Delta H_{cal}$ ratios of 0.97–1.02 for these hairpins, therefore both hairpins melt in two-state transitions.

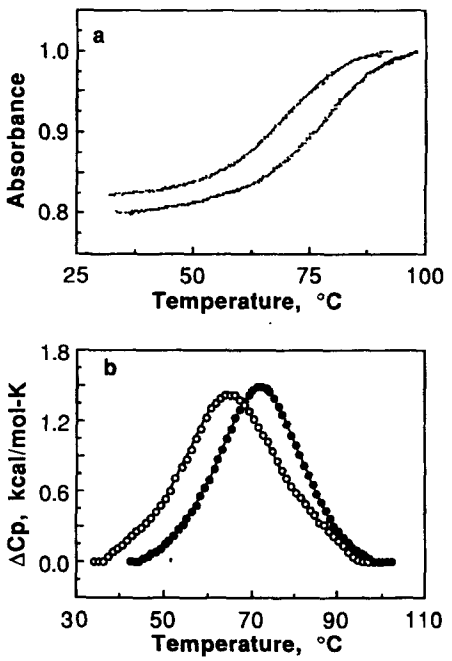

Figure 2. (a) Normalized UV melting profiles for the hairpins: d(CGCGT$_5$CGCG) (left curve) and d(GCGCT$_5$GCGC) (right curve), in 10 mM NaP_i, 0.1 mM Na_2EDTA, 0.1 M NaCl at pH 7.0 and hairpin concentrations of ~7 μM. (b) Typical excess molar heat capacity curves of the hairpins relative to buffer, consisting of 10 mM NaP_i buffer, 0.1 mM Na_2EDTA, at pH 7; d(CGCGT$_5$CGCG) (open circles) with strand concentration of 0.3 mM and d(GCGCT$_5$GCGC) (closed circles) with strand concentration of 0.8 mM.

Table 1. Spectroscopic and calorimetric melting results for the formation of hairpins at 20 °C[a]

	UV		Calorimetry					
[NaCl] M	T_m (°C)	ΔH_{vH} (kcal/mol)	T_m (°C)	ΔH_{vH} (kcal/mol)	ΔH_{cal} (kcal/mol)	$\Delta G°$ (kcal/mol)	$T\Delta S$ (kcal/mol)	Δn_{Na^+} mol Na^+ mol hairpin
				d(GCGCT5GCGC)				
0	71.0	-38	69.2	-38	-39.2	-7.4	-31.8	0.570
0.1	75.7	-39	73.9	-39	-37.2	-7.4	-29.8	0.525
1.0	76.4	-35	75.3	-34	-34.9	-7.0	-27.9	0.489
				d(CGCGT5CGCG)				
0	63.3	-39	62.6	-37	-38.4	-6.6	-31.8	0.554
0.1	66.8	-36	67.7	-35	-37.8	-7.0	-30.8	0.529
1.0	69.5	-36	69.4	-36	-35.5	-6.7	-28.8	0.492

[a]All thermodynamic parameters refer to the formation of hairpins in 10 mM sodium phosphate buffer, 0.1 mM Na_2EDTA at pH 7.0 and 20 °C. The T_ms (±0.5 °C) correspond to strand concentrations of 5–50 μM for the UV melts, and 300 μM (CG) and 800 μM (GC), for the DSC melts. The values given in parentheses are the absolute uncertainties of: ΔH_{vH} (±7%); ΔH_{cal} (±3%); $\Delta G°$(±5%); $T\Delta S$ (±8%); and Δn_{Na^+} (±5.0%).

Thermodynamic uptake of counterions

To determine the thermodynamic release of counterions, Δn_{Na^+}, we use the equation:[42] $dT_m/d\ln [Na^+] = -0.9\ (RT_m^2/\Delta H_{cal})\ \Delta n_{Na^+}$; where the $dT_m/d\ln [Na^+]$ values are determined in UV melting curves at both constant strand concentration and several salt concentrations, the term in parentheses is obtained directly from DSC melting curves, R is the universal gas constant and 0.9 is a proportionality constant of converting ionic activities into concentrations. We obtained positive slopes of 5 °C from the T_m versus dlog [Na^+] plots (see Fig. 3), yielding low Δn_{Na^+} values of 0.53 mol Na^+ per mol of hairpin that are consistent with the formation of four G·C base pairs in the stem of these hairpins.

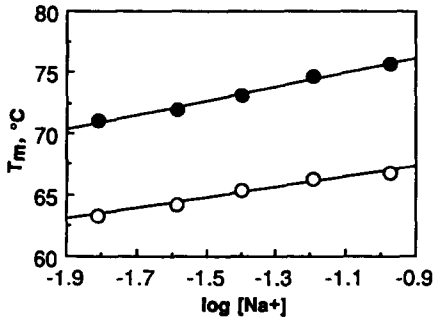

Figure 3. Salt dependence of the T_m for the hairpins, d(CGCGT$_5$CGCG) (open circles) and d(GCGCT$_5$GCGC) (closed circles), in 10 mM NaP_i buffer, 0.1 mM Na_2EDTA at pH 7 adjusted to desired NaCl concentration, and at constant strand concentration of ~5 μM.

Thermodynamic profiles for the helix–coil transition of the hairpins

The entropy, ΔS_{cal}, and the free energy, $\Delta G°_{cal}$, values were calculated from the following equations: $\Delta S_{cal} = \int(\Delta C_p/T)dT$ and $\Delta G°_{cal} = \Delta H_{cal} - 293.15\ \Delta S_{cal}$, respectively. In the Gibbs equation, both the enthalpy and entropy are assumed to be independent of temperature. Table 1 lists the thermodynamic profiles obtained from DSC melts at 20 °C, the temperature at which both molecules are ordered and exist as hairpins, and at several salt concentrations. For the overall formation of these hairpins, the average favorable $\Delta G°_{cal}$ values of –7.0 kcal mol^{-1}, resulted from the characteristic partial compensation of a favorable enthalpy of ~37 kcal mol^{-1} and unfavorable entropy terms of ~30 kcal mol^{-1}. The favorable and identical ΔH_{cal} values for the two hairpins results from the formation of both hydrogen bonds and base pair stacks, whereas the unfavorable entropy values indicate an ordering of the systems due to the uptake of sodium ions and water molecules. For each hairpin we observed that increasing the NaCl concentration from 16 mM to 1 M results in the characteristic increase of T_m, but the actual ΔG values decrease due to less favorable enthalpy contributions. However, in this salt range the formation of the GC-hairpin was more favorable by –0.5 kcal mol^{-1}, due to a less favorable entropy contribution. Since the average uptake of counterions is similar for both hairpins, this result indicates differences in the overall hydration state of the hairpins and/or differences in base stacking interactions at the stem-loop junctions, i.e. GT versus CT base steps.

Ligand binding to DNA hairpins

UV spectra and overall stoichiometry of ligand–hairpin complexes. In the visible spectra of the free bis-ethidium and that of the bis-ethidium–GC-hairpin complex (data not shown), we obtained the characteristic red shift of the bound ligand, ~40 nm, and the presence of two isosbestic points at 390 and 510 nm, as has been reported previously for the

intercalation of ethidium to DNA.[43] At the wavelength of 490 nm, corresponding to the absorbance maxima of the free bis-ethidium, we have followed the changes in absorbance as a function of the mole fraction of hairpin, χ_h. The resulting plot for this ligand is shown in Figure 4. The intersection of the two lines in each titration curve at the more sensitive wavelength of 490 nm occurs at χ_h values of 0.25; for the complexes of the GC-hairpin with each ligand, this yields stoichiometries of three ligands per hairpin. However, for the complexes of the CG-hairpin with bis-ethidium, we obtained a 3:1 stoichiometry and 4:1 with ethidium and propidium.

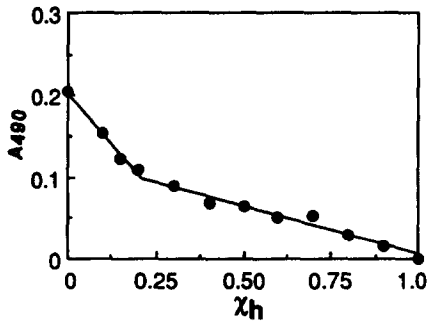

Figure 4. Absorbance versus mole fraction plot of bis-ethidium with the d(CGCGT$_5$CGCG) hairpin in 10 mM NaP_i buffer, 0.1 mM Na_2EDTA, 0.1 M NaCl at pH 7 and 20 °C. Total concentration of ligand and hairpin in all solutions was ~11 μM. Absorbances were monitored at 490 nm using a 1 cm pathlength cell.

Titration calorimetry. The integral heat as a function of total added ligand for all ligand–hairpin systems and the resulting fitted curves are shown in Figure 5. Molar binding enthalpies for the high affinity sites in the stem of these molecules were calculated either by averaging the heats of the first few points of these isotherms, which correspond to complete binding of ligand, or from the fits of these calorimetric titrations. These values are listed under the ΔH_b columns in Table 2. Inspection of this column shows that binding of ligands to each hairpin molecules is accompanied by average exothermic enthalpies of –9.1 kcal mol^{-1} for ethidium, –8.7 kcal mol^{-1} for propidium and –13.1 kcal mol^{-1} for bis-ethidium. Thus, the latter values and higher exothermicities for bis-ethidium correspond to increased van der Waals interactions.

The analysis of the calorimetric binding isotherms shows average binding affinities for the stem sites of 1.5 x 10^6 (for ethidium), 6 x 10^6 (for propidium), and 4 x 10^7 (for bis-ethidium). These binding affinities are in excellent agreement with the values obtained from a second method described earlier that uses the increase in thermal stability of the complexes relative to the unligated hairpins.[33,44] For instance, the 1:1 bis-ethidium–hairpin complexes melted in the lower salt buffer with an increase in thermal stabilities of 13.1 and 7 °C for the CG- and GC-hairpins, respectively, yielding the corresponding binding affinities of 6.4 x 10^7 and 2.9 x 10^7 M^{-1}. The magnitude and the similarity of the binding affinities[27] for a given ligand to each hairpin strongly indicates that the stem site remains unperturbed and is consistent with the intercalative binding of ligands to the CG/CG base pair stack in the stem of these hairpins. However, the values of the binding affinities increase from ethidium to bis-ethidium as expected for ligands with higher number of charges. The main observation is that the K_b for bis-ethidium is not the square of the K_b of ethidium or propidium binding, a strong indication that only one aromatic ring of bis-ethidium intercalates.

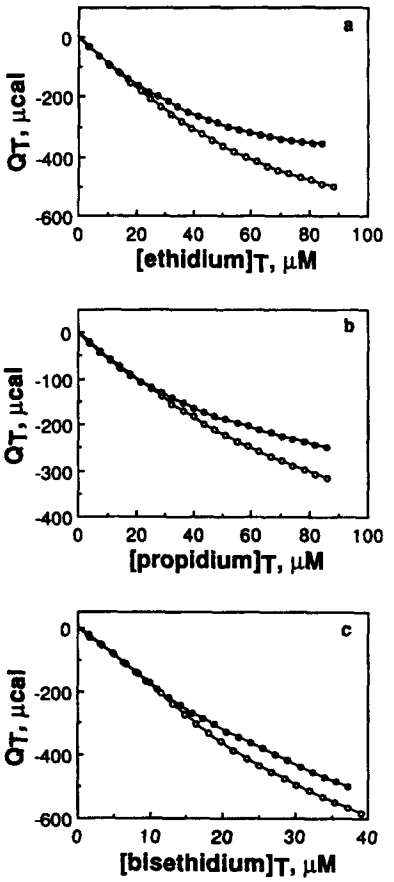

Figure 5. Calorimetric binding isotherm curves for the titration of each ligand to hairpins: d(CGCGT$_5$CGCG) (open circles) and d(GCGCT$_5$GCGC) (closed circles), in 10 mM sodium phosphate, 0.1 mM Na_2EDTA, 0.1 M NaCl at pH 7.0 and 20 °C. (a) 1.4 mL of a 20 μM DNA hairpin solution was titrated with 5.6 μL injections of a 0.9 mM ethidium solution. (b) 1.4 mL of a 20 μM DNA hairpin solution was titrated with 5.6 μL injections of a 0.9 mM propidium solution. (c) 1.4 mL of a 11.7 μM DNA hairpin solution was titrated with 6 μL injections of a 0.37 mM bis-ethidium solution.

Furthermore, in these calorimetric titrations, as in the case of ethidium,[33] we also detected secondary weaker sites for propidium and bis-ethidium in the hairpins (see Table 2) located at the thymine loops of the hairpins. The loop of each hairpin is able to accommodate two ligand molecules. These sites are characterized (see Table 3) for the binding of ethidium and propidium with K_bs of ~5 x 10^4 M^{-1} and ΔH_bs ranging from –6.9 kcal mol^{-1} (CG-hairpin) to –8.4 kcal mol^{-1} (GC-hairpin), while the binding of bis-ethidium is characterized with higher K_bs of 10^6 M^{-1} and higher exothermic ΔH_bs of –11.1 kcal mol^{-1}.

Table 2. Fitting analysis of calorimetric binding isotherms for the interaction of monofunctional and bifunctional intercalators to hairpins[a]

	Stem			Loop		
Ligand	n^b	ΔH_b (kcal/mol)	K_b (M^{-1})	n^b	ΔH_b (kcal/mol)	K_b (M^{-1})
			d(GCGCT5GCGC)			
Ethidium	0.9	-9.8	1.7×10^6	1.8	-8.9	6.5×10^4
Propidium	1	-8.3	4.0×10^6	2.0	-7.9	4.1×10^4
Bis-ethidium	1	-13.5	2.0×10^7	2	-10.2	1.4×10^6
			d(CGCGT5CGCG)			
Ethidium	2	-8.4	1.3×10^6	2	-7.3	5.1×10^4
Propidium	2	-9.0	8.0×10^6	2	-6.6	4.7×10^4
Bis-ethidium	1	-12.7	6.0×10^7	2	-12.0	2.0×10^6

[a]All values in 10 mM sodium phosphate buffer, 0.1 mM Na_2EDTA at pH 7.0 and 20 °C. The standard deviation of the non-linear fits was better than 4% in all cases.
[b]*n* is the total number of bound ligands per site.

Table 3. Thermodynamic profiles for the interaction of monofunctional and bifunctional intercalators to DNA hairpins[a]

Hairpin	Site	$\Delta G°_b$ (kcal/mol)	ΔH_b (kcal/mol)	$T\Delta S_b$ (kcal/mol)
		d(GCGCT5GCGC)		
Ethidium	stem	-8.3	-9.8	-1.5
	loop	-6.5	-8.9	-2.4
Propidium	stem	-8.9	-8.3	+0.6
	loop	-6.2	-7.9	-1.7
Bis-ethidium	stem	-9.8	-13.5	-3.7
	loop	-8.2	-10.2	-2.0
		d(CGCGT5CGCG)		
Ethidium	stem	-8.2	-7.3	+0.9
	loop	-6.3	-7.3	-1.0
Propidium	stem	-9.3	-9.0	+0.3
	loop	-6.3	-6.6	-0.3
Bis-ethidium	stem	-10.4	-12.7	-2.3
	loop	-8.4	-12.0	-3.6

[a]Values taken in 10 mM sodium phosphate buffer, 0.1 mM Na_2EDTA at pH 7.0 and 20 °C. The $\Delta G°_b$ values are within (±5%), ΔH_b (±3%), and $T\Delta S_b$ (±8%).

Standard thermodynamic profiles for ethidium–homodimer binding. Values of the thermodynamic parameters for the binding of ligands to each hairpin are listed in Table 4. Standard thermodynamic profiles such as $\Delta G°_b$, ΔH_b and $T\Delta S_b$ were obtained as follows: the $\Delta G°_b$ from the values of K_b according to the equation: $\Delta G°_b = -RT \ln K_b$. The entropy changes, $T\Delta S_b$, from the Gibbs equation: $\Delta G = \Delta H - T\Delta S$. These thermodynamic profiles indicate the following: (i) each hairpin contains two distinct binding sites for each of the ligands: a higher affinity site in the stem that accommodates one or two ethidium (or propidium) molecules depending on the stem sequence and only one bis-ethidium, and a lower affinity site (K_b ~10^6) located at the loop that accommodates two ligand molecules for all three ligands; (ii) the favorable binding of each ligand for each site takes place in enthalpy driven association reactions.

Discussion

Formation of single-stranded hairpins at low temperatures

The hairpin sequences d(GCGCT$_5$GCGC) and d(CGCGT$_5$CGCG) favor the exclusive formation of intramolecular duplexes because the formation of

intermolecular duplexes would involve four G·C base pairs interspaced by internal loops of 10 thymine residues each, which would render them unstable. This is confirmed by the higher experimental T_ms of each hairpin relative to the estimated T_m of the bimolecular duplexes of ~46 °C. Further evidence includes: (i) the T_m for the helix–coil transition for each hairpin molecule, from UV and DSC melting experiments, are similar despite a 150-fold increase in strand concentration, a result that strongly suggests that all transitions are monomolecular; (ii) all the transition enthalpies, from UV melts and DSC melts, were identical and characteristic of the two-state melting behavior of short DNA stems in double helical conformation; the ΔH_{cal} obtained from DSC for both molecules is ~35.0 kcal mol^{-1} in 1 M NaCl, which is close to the value predicted for the melting of three dG·dC base-pair stacks (~34.0 kcal);[45] (iii) the transition temperature of both hairpins has a slight dependence on Na^+ concentration with an overall thermodynamic counterion uptake of 0.52 mol Na^+ per mol of hairpin, indicative of their low charge density.

Comparison of the thermal stability of the two hairpins indicates that the GC-hairpin is more stable by 7 °C. This stabilization is entropic in origin, $\Delta(T\Delta S)$ = 1.1 kcal mol^{-1}. This small entropic stabilization is probably due to differences in their hydration state resulting from differences in loop structures, since the counterion uptake is similar for these hairpins.

Interaction of bis-ethidium to the hairpins

From our calorimetric and continuous variation experiments, we obtained overall stoichiometries of three bis-ethidium per DNA hairpin. From deconvolution of the calorimetric isotherms, we were able to distinguish two types of sites. A high affinity site (K_b ~10^7) that accommodates one ligand, and a lower affinity site (K_b ~10^6) which accommodates two ligands. We believe that the high affinity site is located in the stem of these molecules and the lower affinity site in the loops; however, one should be cautious on this assignment because none of the methods used can, with certainty, identify where the ligands are binding. Binding of bis-ethidium to each hairpin yields similar overall thermodynamic profiles for the stem and loop sites, the actual differences corresponded to a slightly favorable binding towards the CG-hairpin.

Intercalation of ligands to the stem site

The stoichiometries of 1:1 and 2:1 for ethidium and propidium binding to the stem of these hairpins together with the similarity of the thermodynamic profiles for a given ligand to each hairpin strongly indicate that each ligand insert their phenanthridinium ring in the center CG/CG base pair stack of their helical stem according to the 5'-Pyr-Pur-3' sequence specificity determined previously,[41] i.e. one ligand in the GCGC/GCGC stem and two ligands in the CGCG/CGCG stem. However, there are some slight preferences; for instance, propidium and bis-ethidium bind better (by 0.5 kcal mol^{-1}) to the CG-hairpin while ethidium shows no preference for either hairpin. Comparison of the thermodynamic profiles for propidium binding relative to those of ethidium reveals that propidium binds more favorably than ethidium by –0.6 kcal mol^{-1} (GC-hairpin) and –0.9 kcal mol^{-1} (CG-hairpin). The more favorable binding to the GC-hairpin results from a favorable differential entropy in agreement to similar differential binding profiles with poly[d(IC)]·poly[d(IC)][27] and poly[d(AT)]·poly[d(AT)],[26] while the more favorable binding to the CG-hairpin results from a favorable differential enthalpy contribution in excellent agreement with the corresponding differential binding enthalpy of binding to poly [d(GC)]·poly[d(GC)].[27] These small differences can be explained in terms of the extra positive charge on the longer alkyl chain of propidium that may form upon binding additional molecular interactions, such as removal of counterions and the uptake or removal of water molecules.

Turning to the interaction of bis-ethidium to the stem of these hairpins and relative to the monointercalators, we find that the stems of each hairpin bind only one bis-ethidium molecule; in the case of the CG-hairpin, this ligand excludes four base pairs, preventing binding of a second bifunctional ligand. At low ionic strength, the binding affinity is the lowest for ethidium and the highest for bis-ethidium, see Table 2. The binding constant obtained for the association of bis-ethidium is only an order of magnitude higher than the corresponding values for propidium and ethidium by nearly –1 to –2 kcal mol^{-1} in free energy, and corresponds to a higher negative compensation of the differential enthalpy with the differential entropy. This can be explained as an electrostatic contribution of the extra positive charges present in the ligands (ethidium has an overall charge of +1, propidium +2, and bisethidium +4); the electrostatic contribution from the nearly spherical hairpins is constant and much lower than a linear polyelectrolyte, i.e. the Δn_{Na^+} values of 0.09 or 0.07 mol Na^+ per phosphate (taking into account 6 or 8 helical phosphates in the stem) are much less than the 0.17 value for a DNA polymer. To quantify this contribution, the electrostatic free energy (ΔG_{el}) can be estimated for a polyelectrolyte by $\Delta G_{el} = z\psi RT \ln [M^+]$, where z is the charge of the ligand, ψ is the effective charge density, and $[M^+]$ is the counterion concentration.[42] In 17 mM Na^+, 20 °C and using for the stem of these hairpins a value of ψ of approximately 52% of that of a linear polyelectrolyte, the electrostatic contributions are –1.0 kcal (ethidium), –2 kcal (propidium) and –4 kcal (bis-ethidium). These values are only a fraction of the reported free energy terms and are in good agreement with the experimental differential binding free energies of bis-ethidium relative to that of monointercalators.

For the binding enthalpies, we observe an increase of ~50% in the exothermicity of the reaction relative to the value obtained for the monofunctional intercalators and due to an increase in van der Waals interactions.

These results indicate that bis-ethidium has an exclusion nearest-neighbor parameter of four, and that it behaves as a mono-intercalator. In other words, only one of the phenanthridinium rings is intercalated while the other ring behaves mostly like an outside (or surface) binder. This surface binding makes the free energy and enthalpy of interaction for bisethidium more favorable than for a mono-intercalator. If it had behaved as a bis-intercalator in its association to the CG-hairpin, we would have predicted larger thermodynamic binding functions by a factor of two than those obtained for ethidium and propidium. In addition, an intercalative–surface binding model can explain the higher neighbor-exclusion parameter of bis-ethidium, in agreement with previous viscosity and spectroscopy investigations on bifunctional intercalators that have shown their behavior as monointercalators.[46] The main reason for the high neighbor-exclusion parameter of bis-ethidium is the rigidity of its alkylamine linker that is not permitting optimal geometry for bis-intercalation. Increasing the length of the alkyl linker with proper phasing should allow bis-intercalation. We are currently in the process of investigating other bis-intercalators with longer alkylamine linkers or with more flexible functional groups.

Interaction of ligands to the thymine loops

The structure of the thymines located in the loops of DNA hairpins provide additional weaker binding sites for each ligand as detected by the calorimetric titration curves and spectroscopic Job plots. The binding of bis-ethidium to the loop is also more favorable than for ethidium and propidium, and the arguments for this observation are similar to those made for the binding of the intercalators to the stem of the hairpins, i.e. increases in electrostatic and van der Waals interactions. In addition, since we obtained overall similar stoichiometries for the loop sites with each of the intercalators, we can conclude that only one of the planar rings of bis-intercalator is being stacked in the loop while the presence of the second ring around a very crowded hairpin would contribute to the larger observed enthalpy and entropy changes.

Conclusions

We have investigated the interaction of ethidium, propidium and bis-ethidium to short DNA hairpins. First, we used melting protocol to study the helix–coil transition of the unligated oligomers and demonstrated the exclusive formation of single-stranded hairpins at ambient temperatures. Second, in the interaction of these ligands to these hairpin molecules, we show that each hairpin contains two types of binding sites, stem and loop, with different thermodynamic binding profiles. For both types, the thermodynamic driving force is enthalpic. The stem contains the stronger site, which is characterized by thermodynamic profiles typical of intercalation sites. The loops of these molecules contain weaker binding sites for all three intercalators. The binding of bis-ethidium showed that the additional charge caused an increase in the binding affinity for double-helical DNA relative to ethidium and propidium, but only one of the phenanthridinium rings is intercalated, leaving the other exposed to solvent or only weakly associated on the DNA surface. The reason for this is that the molecule contains a rigid linker with four positive charges.

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