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Influence of DNA Structure on Adjacent Site Cooperative Binding

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Abstract

Previous NMR studies of Hoechst 33258 with the d(CTTTTGCAAAG)₂ sequence have shown very strong ($K_2 \gg K_1$) cooperativity between two adjacent binding sites (Searle, M. S.; Embrey, K. J. *Nucleic Acids Res.* 1990, 18 (13), 3753–3762). In contrast, surface plasmon resonance (SPR) results with the hairpin analog of the same sequence show significantly reduced cooperativity. In an effort to explain the difference, two-dimensional (2-D) NMR experiments were done on both duplex and hairpin. Hoechst 33258 and an amidine analog, DB183, show very strong cooperativity with the duplex DNA but much weaker cooperativity with the hairpin. The significantly lower thermal melting temperature (T_m) of the duplex (34.8 °C) in comparison to its hairpin analog (62.3 °C) supports the idea of a dynamic difference between the two DNA structures that can influence cooperativity in binding. These results confirm the role of conformational entropy in positive cooperativity in some DNA interactions.

Introduction

Small molecules that bind cooperatively to closely spaced but not directly adjacent DNA sequences are attractive for several applications; examples include highly specific recognition of A/T-rich regions of kinetoplast DNA¹ or GC regions in promoters.² Such compounds are quite rare but would be especially useful, for example, for highly selective therapeutic targeting of similar repeated binding sites of DNA. Closely spaced AT sequences, for example, are well-characterized in mitochondrial DNA of parasitic microorganisms and have unique properties.³ The mitochondrial kinetoplast DNA of the eukaryotic parasite trypanosomes and leishmania have phased AT sequences that are targeted by dicationic drugs.^{4–7} Such sequences could be much more selectively targeted if compounds that bind cooperatively to the array of AT sequences could be designed. In another example the genomic DNA of the malaria parasite is 80% AT⁸ and has many extensive regions of closely spaced AT sequences. Such sequences could be targeted with very high selectivity by compounds with cooperative binding at adjacent sites. The utility of targeting "AT island" sequences in cancer treatment has also been described,^{9,10} and targeting such sequence tracts could clearly be improved by agents that bind cooperatively to them. Although the studies in this work are focused on parasite repeated AT sequences, the search for synthetic compounds that bind to specific sequences in a cooperative manner matches the goal of many laboratories to design agents that can selectively regulate gene expression.^{11,12}

Compounds that stack on themselves or other compounds to form a cooperative dimer and bind to a single site are relatively well-known and are very useful DNA binding agents.^{13,14}

They do not, however, meet the goal of cooperative binding to closely spaced but not directly adjacent sites. Polyamides,^{15–20} cyanine dyes,^{21,22} and some unfused heterocyclic^{14,23–25} systems form stacked dimers that are extremely useful, but that does not solve the problem of targeting multiple closely spaced sites. Some of the dimer complexes, such as with cyanine dyes, can cooperatively bind to extended AT sequences in an induced fit recognition mode.^{26,27} It seems likely that the first dimer to bind in such complexes widens the groove and converts it into a structure that is better optimized for binding additional dimers.²⁸ Single molecules of DAPI may bind to extended AT sequences with similar induced conformational changes.^{29,30} The first molecules to bind in a region would convert the local sequence to a more favorable binding conformation for binding of additional DAPI monomers.²⁹ The antibiotic echinomycin has two aromatic rings that can intercalate into DNA and are connected to a large cyclic peptide unit that binds into the DNA minor groove.^{31,32} Echinomycin binds strongly to CG sequences with the intercalated rings clamped around the CG step. The peptide forms H-bonds with the G bases of the CG base pairs that contribute to a very high binding equilibrium constant and selectivity for the compound. Interestingly, binding, NMR and footprinting experiments all indicate that echinomycin can bind to certain DNA sequences with strong positive cooperativity.³²

Searle and co-workers have found that the minor groove binding agent Hoechst 33258 binds to the two AT sites in the duplex d(CTTTTGCAAAG)₂ with very high positive cooperativity.^{33,34} The NMR studies showed that, on titration of Hoechst 33258 into the DNA, the compound bound in a 2:1 complex and there was no detectable amount of a 1:1 species.³⁴ All titrations show only the initial unbound DNA and the 2:1 complex as predicted for highly cooperative binding. On the basis of peak volumes of the NMR signals, the authors estimated that the cooperativity factor, the ratio of the equilibrium constants for binding the second molecule to the first molecule, must be greater than 1000.³³ For reasons that are not yet completely clear reversing the sequence to d(GAAAACGTTTC)₂ resulted in loss of the strong cooperativity. Molecular dynamics calculations on this system indicate that the free DNA is quite flexible, and these calculations suggest a reason for the positive binding cooperativity. In this model, binding of the first Hoechst 33258 molecule at one of the AAAA/TTTT sites reduces the dynamics of the entire DNA duplex and holds the second site in a more favorable dynamic state for binding of the next Hoechst 33258 molecule.³⁵ This is an example of positive cooperativity through a favorable addition to the binding entropy that is brought about by binding-induced changes in the dynamics of the DNA duplex. In comparison to the first molecule, binding of the second molecule of Hoechst 33258 requires a smaller entropy cost in terms of DNA conformational dynamics reduction. This model is distinct from an allosteric mechanism where cooperativity is due to the first bound molecule(s) converting the biopolymer directly to a more favorable conformation for binding additional molecules.

Given the limited information available on cooperativity for minor groove binding in closely spaced sites, we have investigated the d(CTTTTGCAAAG)₂ sequence of Searle and Laughton for development of a screening assay for compounds that can bind cooperatively to such sites. We converted the self-cDNA duplex into a similar hairpin sequence (Figure 1) for use with biosensor–surface plasmon resonance methods. Since the biosensor experiment requires constant sample flow, it cannot be used with short duplexes which gradually lose strands in a flowing solution. We have tested this sequence with Hoechst 33258 as well as a similar compound with the cyclic cation modified to an amidine (Figure 1). Interestingly, the binding cooperativity was significantly reduced in the biosensor–SPR experiments with the hairpin DNA in comparison to the duplex. We have conducted NMR and thermal melting (T_m) experiments with both the duplex and hairpin samples to help understand the differences between our observations and those published for Hoechst 33258 in the duplex.

Materials and Methods

Materials

The DNA hairpin, 5'-CTTTTGCAAAAGTCTCCTTTTGCAAAAG-3', was purchased from Midland Certified Reagent Company with HPLC purification and mass spectrometry characterization. The DNA duplex d(CTTTTGCAAAAG)₂ was purchased from Integrated DNA Technologies, Inc. Hoechst 33258 was purchased from Sigma-Aldrich, and DB183 was synthesized as previously described.³⁶ MES buffer contained 0.01 M [2-(*N*-morpholino)ethanesulfonic acid] (MES) and 0.001 M EDTA. MES 05 and MES 10 buffers contained the above components plus 0.05 and 0.1 M NaCl, respectively, all with a pH of 6.25 adjusted with 1 M NaOH solution.

Surface Plasmon Resonance

Surface plasmon resonance (SPR) experiments were conducted with a BIAcore 3000 instrument as previously described.³⁷ The DNA hairpins were immobilized on a four-cell BIAcore CM5 sensor chip after immobilization with streptavidin. The CM5 sensor chip was first activated with a fresh mixture of 50 mM NHS and 200 mM EDS at a rate of 5 μ L/min. A solution of 400 μ g/mL of streptavidin in 10 mM acetate buffer was then injected until around 3000 resonance units (RU) of streptavidin were obtained. The excess and noncovalently bound esters were removed by injection of 1 M ethanolamine hydrochloride, followed by flowing running buffer. The 5'-biotin-labeled DNA hairpins were then captured on the sensor chip via noncovalent biotin-streptavidin coupling. After treatment with 1 M NaCl and 50 mM NaOH, followed by washing with buffer, DNA at a concentration of 25 nM was manually injected at a flow rate of 1 μ L/min to the flow cell until 300–400 RU was immobilized. Three flow cells were immobilized with DNA hairpins, and one flow cell was left blank as a reference. The compounds in different diluted concentrations in degassed and filtered MES 10 buffer containing 0.005% BIAcore certified surfactant P-20 were injected over the immobilized DNA surface. Glycine (10 mM, pH 2.5) solution was used as a regeneration buffer. The RU values in steady-state regions at each concentration were averaged over a 60 s time zone and converted to r (moles of compound bound per mole of DNA hairpin) as previously described.^{37,38} The binding affinities were determined by the best fitting plot of r versus free compound concentration with a single-site binding model ($K_2 = 0$) or a two-site (K_1 K_2) binding model:

$$r = (K_1 C_{\text{free}} + 2K_1 K_2 C_{\text{free}}^2) / (1 + K_1 C_{\text{free}} + K_1 K_2 C_{\text{free}}^2)$$

where K_1 and K_2 are the macroscopic equilibrium binding constants; C_{free} is the free compound concentration at equilibrium and is actually the compound concentration in the flow solution herein.^{37,38}

Thermal Melting

Thermal melting experiments were performed using a Cary 300 BIO UV-vis spectrophotometer. The melting experiments of DNA in the absence and in the presence of a different ratio of DB183 compound in MES 05 buffer were carried out at a wavelength of 260 nm as a function of temperature. The concentration of DNA was determined by measuring the absorbance at 260 nm and then calculated using the nearest neighbor extinction coefficient.³⁹ The temperature was monitored by a temperature probe inserted into a reference cuvette, and a rate of 0.5 $^{\circ}$ C/min was used. T_m was determined by plotting the first derivative of absorbance with respect to temperature (dA_{260}/dT) versus temperature; the T_m was taken as maximum in the plot. The melting temperature increase (ΔT_m) of the

complex was obtained by subtracting the melting temperature of the free DNA from that of the complex.

NMR

The concentration of DNA was measured using a Varian 300 BIO UV-vis spectrometer at 260 nm wavelength. The final DNA concentration for NMR experiments was set to 0.4 mM. Samples were dissolved in 0.5 mL of phosphate buffer (10 mM NaH₂PO₄, 100 mM NaCl, and 0.01 mM EDTA at pH 7.0). They were dried in D₂O three times with N₂ gas and finally dissolved in 99.96% D₂O from Cambridge Isotope Laboratories, Inc. After each titration with compounds the samples were dried and again dissolved in 0.5 mL of D₂O without any further correction for pH. NMR spectra were collected using a 600 MHz Varian Unity-Inova spectrometer. Phase-sensitive two-dimensional (2-D) NOESY (nuclear Overhauser enhancement spectroscopy) and TOCSY (total correlation spectroscopy) pulse sequences were used for collecting the data. The relaxation time was set to 1 s with 300 ms mixing time for NOESY and 80 ms for TOCSY experiments. A spectral width of 6000 Hz was used with 1025 points in t₁ and 512 points in t₂ prior to Fourier transform; 24 scans were taken. A PRESAT pulse sequence was used for water suppression. VNMR software was used for 1-D and 2-D NMR processing. Data were prepared for publication using Adobe Photoshop version 7.

Results

SPR Analysis of 2:1 Binding of Hoechst 33258 to DNA

For a ligand–DNA complex with more than one ligand binding site, the interaction is noncooperative if initial binding of a compound does not affect the binding energetics of subsequent compounds. A quantitative definition of noncooperative binding for a two-identical-site complex is that the ratio of macroscopic equilibrium constants, K_2/K_1 , is equal to 0.25,⁴⁰ the purely statistical value. The interaction occurs with positive cooperativity if the ratio of K_2/K_1 is significantly larger than the statistical value⁴¹ and with negative cooperativity if the ratio is less than 0.25. Accurate determination of binding constants to obtain reliable ratios is thus critical for all investigations of cooperativity. The BIAcore biosensor–SPR method provides an excellent approach to study binding constants for multiple-site ligand–DNA complex formation for several compounds with different properties. The method responds directly to mass bound and is not dependent on spectral properties or labels, a significant advantage when working with a diverse array of molecules. Each bound compound generates essentially the same signal, and the maximum signal can be directly converted into the stoichiometry of binding. The technique is very promising for high-throughput screening for cooperative binding compounds.

To develop an SPR-based cooperativity screening method, a 5'-biotin-labeled DNA hairpin with two TTTT/AAAA binding sites (Figure 1) was captured on the surface of one flow cell of a CM5 sensor chip after immobilization of streptavidin. Hoechst 33258 samples of different concentration were injected over the surface of the DNA and blank flow cells to provide a set of sensorgrams (Figure 2). The binding rates were rapid enough that at even the lowest concentrations, a steady-state plateau was reached in about 10 min after injection. The RU values in the plateau zone for each concentration were averaged over 60 s to improve the data quality. The average is plotted versus the free Hoechst 33258 concentration (flow solution concentration) in Figure 3. The maximum RU value obtained in the titration is consistent with two Hoechst 33258 binding per duplex, as expected. Binding constants were determined by fitting the results with a two-site binding model, and the macroscopic binding constants for Hoechst 33258 with DNA hairpin are summarized in Table 1.

As can be seen, the binding of the second Hoechst 33258 molecule ($K_2 = 2.1 \times 10^7 \text{ M}^{-1}$) is weaker than that of the first molecule, which binds to free DNA ($K_1 = 6.4 \times 10^7 \text{ M}^{-1}$), and the cooperativity index, $K_{\text{coop}} = K_2/K_1$, is 0.33 at 15 °C. As discussed above, K_{coop} should be significantly above 0.25 to indicate convincing positive cooperativity. The SPR binding analysis thus shows clearly that there is only weak positive cooperativity for the 2:1 binding of Hoechst 33258 to CTTTGTGCAAAAGTCTCCTTTGTGCAAAAG DNA hairpin and that the hairpin results are completely different from those observed for the CTTTGTGCAAAAG DNA duplex.^{33,34}

Thermal Melting Analysis of 2:1 Binding of Hoechst 33285 to DNA

To qualitatively investigate the binding differences with the TTTT/AAAA DNA duplex and hairpin sequences (Figure 1), thermal melting studies with Hoechst 33258 complexes were conducted. The T_m method has been shown to be useful for indicating binding cooperativity with other minor groove binding compounds.²³ For the free DNA duplex (Figure 4 top), there is a single transition peak with a melting temperature of 34.8 °C. As the ratio of Hoechst/DNA is increased, the peak for free DNA decreases while a new high-temperature peak appears and increases as the fraction of DNA bound with Hoechst increases. At a 1:1 ratio, a biphasic melting curve was observed with a low-temperature transition phase for free DNA and a high-temperature transition for the Hoechst–DNA complex. These two melting transition peaks are not equal because Hoechst 33258 also contributes to the absorbance at 260 nm, which increases the peak intensity for the complex. At a 2:1 ratio, the low T_m peak is gone and only the high T_m peak remains. These T_m results suggest that only thermal melting transitions for free DNA and the 2:1 complex are observed under these conditions. The T_m findings qualitatively suggest that Hoechst 33258 binds to the CT4GCA4 DNA duplex with a positive cooperativity, in agreement with the published NMR results.³⁴ The hairpin DNA without added Hoechst 33258 also has a single melting transition that is at 62.3 °C, a higher temperature than for the duplex, as expected. At a 1:1 ratio of Hoechst 33258 to hairpin duplex, a new high-melting transition was observed, but the transitions at different ratios appear broadened without a clear overlap temperature as observed with the duplex (Figure 4). This result suggests that variable mixtures of the DNA, 1:1, and 2:1 complexes exist for binding with the hairpin. Thus, there is no T_m evidence for highly cooperative binding of Hoechst 33285 to the CT4GCA4 DNA hairpin, in agreement with SPR results.

NMR Experiments

NMR experiments were done with both Hoechst 33258 and a closely related amidine derivative, DB183, as part of our search for new compounds that bind to DNA with positive cooperativity. To find the conditions for the best signal-to-noise ratio, a temperature study was conducted on a 1:1 molar ratio of DB183 to duplex. In the temperature range from 15 to 55 °C, the best signal-to-noise ratio was obtained at 35 °C. NMR spectra were collected for free DNA, 1:1, and 2:1 molar ratio samples of DB183 to duplex. A TOCSY experiment shows the methyl-H6 region (Figure 5) where red peaks belong to free DNA and blue peaks are for a 2:1 ratio. Only the peaks from the free DNA and 2:1 ratios are present in the 1:1 molar ratio sample. The 7.3–7.6 ppm region is seen more clearly in a NOESY experiment. Similar results are observed in all regions of NOESY and TOCSY spectra at the 0:1, 1:1, and 2:1 ratios. These spectra are in agreement with those on the same duplex with Hoechst 33258 and show the cooperativity of binding of two molecules of DB183 to the duplex (Figure 6).

Spectra were also collected with the duplex replaced by the 28-mer hairpin as used in SPR and T_m experiments (Figure 1). The temperature for Hoechst experiments with the hairpin is 35 °C. With the hairpin, as compared to the symmetrical duplex, spectral overlap in all the

regions was a larger problem, especially after titrating with Hoechst. Figure 7 shows the methyl-H6 regions of TOCSY spectra at 0:1, 1:1, and 2:1 ratios of Hoechst to hairpin at 35 °C. The 1:1 ratio spectrum has three characteristic peaks shown in red which do not appear in the 0:1 and 2:1 complexes. These results indicate that the 1:1 ratio shows peaks that are not found in the 0:1 and 2:1 spectra. Thus, both 1:1 and 2:1 complexes are formed. A similar experiment for DB183 again shows peaks (Figure 8, red) in the 1:1 spectrum that are not seen in the 0:1 and 2:1 ratios. These results show that at a 1:1 ratio of duplex DNA–drug there is no evidence of a 1:1 complex which is in agreement with highly cooperative binding. But at a 1:1 ratio of hairpin DNA–drug there is a mixture of free DNA, 1:1, and 2:1 species, which suggests a loss of cooperativity in agreement with T_m and SPR results.

Conclusion

SPR, T_m , and NMR experiments all established that binding of DB183 and Hoechst 33258 has very strong positive cooperativity with duplex DNA ($K_2 \gg K_1$), whereas they bind to a hairpin conformation with greatly reduced cooperativity. It should be noted that the two A4T4 sites in the hairpin could have slightly different binding properties since one is closer to the hairpin loop. The observations that these two sites bind with little cooperativity suggests that the hairpin loop does not have a strong influence on the binding constant for the adjacent sites. Molecular dynamics studies conducted by Harris et al.³⁵ have shown that binding of two molecules of Hoechst 33258 to the two adjacent A/T binding sites in a cooperative manner is an entropy-driven process. Binding of the first molecule reduces the flexibility of the second binding site in a way that makes it more favorable for the binding of the second molecule. This entropy is different from the favorable entropy term which is related to water displacement from the minor groove and which accounts for a major part of the Gibbs binding free energy of many small molecules to the DNA minor groove.^{42,43}

The entropy of short DNA sequences decrease when replacing a duplex with a hairpin.⁴⁴ In the case of the hairpin, binding of the first compound does not fix the second binding site as a favorable dynamic state for increased binding affinity of the second molecule. The dynamics differences are supported by the T_m differences for the duplex and hairpin analogs (34.8 °C for duplex vs 62.3 °C for hairpin). These results show that the degree of rigidity or flexibility of biological molecules can change the binding interaction. In the SPR method it is generally necessary to use hairpin DNA to avoid losing one of the strands due to dissociation. On the basis of the data from experiments, short hairpin DNA has different dynamics than duplex so it is not able to easily introduce dynamic cooperativity in adjacent binding sites. Hairpin DNAs may actually reproduce the dynamic state of larger DNA sequences better than short duplexes, and the hairpin loop effect seems to be primarily on DNA conformational dynamics and not on binding energetics.

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Hoechst 33258

DB183

CT4GCA4G Hairpin

Figure 1.
Structures of compounds and sequences of DNAs.

Figure 2.
SPR sensorgrams for binding of Hoechst 33258 to the CT4GCA4G DNA hairpin at 25 °C in MES 10 buffer. The concentration ranges are 0 (bottom curve) to 80 nM (upper curve).

Figure 3. Binding curve for the interactions between Hoechst 33258 and CT4GCA4G DNA hairpin at 25 °C in MES 10 buffer. The binding constants were evaluated by fitting the data with a two-site binding model (see Materials and Methods).

Figure 4.

Derivative plots of the absorbance at 260 nm with respect to temperature vs temperature for CT4GCA4G DNA duplex (top) and CT4GCA4G DNA hairpin (bottom) in the presence of a series of ratios of Hoechst 33258 in MES 05 buffer.

Figure 5.

TOCSY experiments of 0:1, 1:1, and 2:1 of DB183/DNA duplex complex in the methyl-H6 region at a temperature of 35 °C with an 80 ms mixing time. The peaks which belong to free DNA are shown in red, whereas those in blue belong to the 2:1 ratio. The inset is a portion of the NOESY experiment at a 1:1 ratio to show three cross peaks more clearly.

Figure 6. TOCSY experiments of 0, 1, and 2 molar ratio of Hoechst 33258/DNA duplex in the methyl-H6 region. The temperature is 35 °C. Red peaks are free DNA, and blue peaks are 2:1 ratio. The mixing time is 80 ms.

Figure 7.

Methyl-H6 portion of TOCSY experiments of Hoechst 33258/DNA hairpin complexes in 0:1, 1:1, and 2:1 ratios at 35 °C with an 80 ms mixing time. Three peaks in the 1:1 complex are not present in 0 and 2 molar ratio. These peaks are shown in red and are seen at (F1, F2), 1.51, 7.32; 1.54, 7.46; 1.79, 7.39 ppm.

Figure 8.

Same region of the TOCSY experiments as in Figure 7 for DB183/DNA hairpin in 0:1, 1:1, and 2:1 ratios. The temperature is 35 °C with 80 ms mixing time. There are two cross peaks that belong to the 1:1 species and are not present in free DNA and the 2:1 complex. They are in red.

TABLE 1Equilibrium Constants for Binding of Hoechst 33258 to the CT4GCA4G Hairpin^a

temp (°C)	K_1 (M ⁻¹)	K_2 (M ⁻¹)	K_{coop}^b
15	6.4×10^7	2.1×10^7	0.33
25	5.3×10^7	2.5×10^7	0.47

^a All BIAcore SPR binding experiments were conducted in MES 10 buffer.^b Cooperativity index, $K_{\text{coop}} = K_2/K_1$, is 0.25 for a completely noncooperative interaction.