Studies of the Base-Dependent Binding of Cu(T4) to DNA Hairpins (H_2T4 = *meso*-Tetrakis(4-(*N*-methylpyridiumyl))porphyrin)

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Water-soluble, cationic metalloporphyrins that bind to DNA show promise as artificial nucleases and as sensitizers for photodynamic therapy, but fundamental questions remain about the binding motifs and sequence specificities. To address these issues, we have studied the interactions of Cu(T4) with a series of oligonucleotides that form hairpin structures (H_2T4 = *meso*-tetrakis(4-(*N*-methylpyridiniumyl))porphyrin). Each oligonucleotide is a 16-mer with a central run of four thymine (T) bases and complementary ends that can combine to form a specific sequence of six adenine−thymine (A═T) and guanine−cytosine (G≡C) base pairs. The techniques employed include thermal melting as well as circular dichroism (CD), absorbance, and emission spectroscopies. The number of G≡C base pairs in the stem is the most important factor that determines the melting temperature of the hairpin, and in every case investigated, the uptake of Cu(T4) stabilizes the hairpin. Depending on the nature of the adduct that forms, $\Delta\epsilon$ varies from −22 to +17 M^{-1} cm^{-1} in the Soret region of the CD spectrum, and the emission intensity from Cu(T4) changes by an order of magnitude. The results yield several useful insights regarding the binding interactions. One is that robust hydrogen bonding within a B-form duplex promotes intercalative binding of Cu(T4). Thus, if the composition is at least 50% G≡C base pairs, intercalation will occur even in the absense of a G≡C step. On the other hand, a run of four A═T base pairs defines a groove-binding site with an affinity comparable to that for intercalation at a G≡C step. Finally, at least in solutions containing excess oligonucleotide, there is no sign that either loop binding or hemiintercalation is a prevalent mode of interaction between Cu(T4) and hairpin hosts.

Introduction

Water-soluble, cationic porphyrin systems are receiving attention because of their affinity for DNA and the potential they have for applications in photodynamic therapy,[1] but many questions remain concerning the DNA-binding interactions. In what was at the time a surprising development, Fiel and co-workers first showed that readily available tetracations, like *meso*-tetrakis(4-(*N*-methylpyridiniumyl))porphyrin (H_2T4) or the copper(II) derivative Cu(T4), are capable of intercalating into B-form DNA.[2,3] This was a remarkable observation because intramolecular steric forces prevent the pyridyl substituents from residing in the plane of the porphyrin.[2,3] Subsequent work has shown that various metalated derivatives of H_2T4 form multiple types of adducts with DNA.[4−7] (In this report the term "adduct" is a generic expression for any type of associative interaction, covalent or noncovalent.) Footprinting studies have demonstrated that derivatives bearing axial ligands, like solvated Zn(T4), protect regions of DNA that are rich in adenine−thymine (A═T) base pairs,[8] especially those containing a TpA step.[9] This is one mark of an external, or groove, binding agent, and transient Raman studies by Nakamoto and co-workers have shown that Cu(T4) can undergo groove binding when the host contains a run of as few as four consecutive A═T base pairs.[10] In contrast, to a pure groove binder such as Zn(T4), footprinting studies have established that Ni(T4) and H_2T4 also protect sites containing a CpG step when they intercalate into DNA.[9] In accordance with this observation, Marzilli and co-workers reported NMR studies with oligonucleotides that indicate that H_2T4 intercalates highly specifically within a CpG step.[11] However, Gibbs et al. later found that H_2T4 intercalates into poly(dA-dC)·poly(dG-dT), which obviously has no CpG step.[12] In studies of the binding of Cu(T4) by EPR methods, Dougherty and Pasternack concluded that intercalation occurs preferentially between adjacent G≡C base pairs but that the particular sequence is not crucial.[13] However, we used luminescence techniques to study the binding of Cu(T4), and the results indicated that a single G≡C base pair is enough to support intercalative binding.[14] A new issue came to the fore when Williams and co-workers published the crystal and molecular structure of an adduct of Cu(T4) with a duplex derived from the 6-mer d(CGATCG).[15] Although the structure shows that the Cu(T4) moiety basically lodges within the CpG steps, this occurs with the expulsion of the 5′C groups from the original duplex. The authors attributed the structural reorganization to steric forces and proposed that a similar type of "hemiintercalation" with a flipped-out base will occur in solution as well.

To bring new information to bear on these issues, we have employed DNA hairpins, i.e., stem−loop structures, to investigate the base dependence of the interactions involving Cu(T4). Any oligonucleotide that contains two appropriately spaced and oriented, mutually complementary runs of bases is capable of forming a hairpin structure. Structural elements of this type occur in RNA[16] as well as DNA where they may play a role in protein-assisted regulation.[17,18] Previous investigations have utilized hairpins in conjunction with artificial nucleases to explore the effects of conformation on DNA cleavage reactions.[19,20] For the present investigation, hairpin-forming oligonucleotides are simply convenient sources of short seg-

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ments of B-form DNA that are stable at micromolar concentrations. The oligos employed are all 16-mers containing a center run of four thymines because melting studies have shown that the TTTT motif forms a stable loop.[18] In addition, NMR work has shown that a 16-mer with a central run of four thymines forms a stable hairpin when there are as few as two G≡C base pairs in the stem.[21]

Experimental Section

Materials and Methods. The Macromolecular Structure Facility of Purdue University supplied all oligonucleotides as custom syntheses. The vendor for the tetrachloride salt of H_2-T4 porphyrin was Midcentury Chemical Company (Posen, IL). All other materials were reagent grade from standard suppliers. In particular, Fisher Scientific (Chicago, IL) supplied the copper salt $CuCl_2 \cdot H_2O$, and Sigma Chemical Company (St. Louis, MO) provided the dichlorodimethylsilane.

The method of Pasternack and co-workers yielded the chloride salt of Cu(T4) from H_2T4 and copper(II) chloride.[22] For all physical studies the buffer was a $\mu = 0.1$ M, pH 6.8 solution made from appropriate potassium phosphate salts and NaCl with a total phosphate concentration of 50 mM. For the spectral studies the total copper concentration was usually 1.7 μM as determined from the extinction coefficient of Cu(T4) at 424 nm ($\epsilon = 2.31 \times 10^5\ M^{-1}\ cm^{-1}$).[23] In the case of the DNA, the absorbance at 260 nm provided a means of estimating the oligonucleotide concentration. The absorbance of a denatured sample at 80 °C along with the molar absorptivities of the component mononucleotides provided an estimate of the relevant ϵ_{260} value.[24] Important precautionary measures taken before the physical measurements included siliconizing all cuvettes and sample containers[25] and filtering each stock through a membrane with a 0.2 μm pore size. The hypochromism, expressed as %H, was the percent change that occurred in absorbance at the Soret maximum as a result of the addition of excess DNA, typically at a hairpin-to-copper ratio of 5. Spectrally monitored titrations showed that this ratio was sufficient to ensure complete complexation of Cu(T4). During the luminescence studies the excitation wavelength was generally 434 nm. The filter in the excitation beam was a 452 nm notch filter, and the emission filter was a 510 nm long-wave-pass filter. The band-pass at both slits was 20 nm. For background subtraction, the reference was a solution of the appropriate hairpin that did not contain Cu(T4). The instrumental correction factors were unreliable at the wavelengths of interest, but the emission quantum yields appeared to be of the order of 10^{-4}. Fortunately, the band shape and the emission maximum were essentially constant from sample to sample, so the intensity at the emission maximum served as a measure of the relative quantum yield (Φ_{rel}). For intensity comparisons, each sample had the same absorbance at the exciting wavelength, and the hairpin-to-copper ratio was 5. Locally written software yielded the melting temperature, T_m, from the absorbance-versus-temperature data taken at 260 nm for each hairpin.[26]

Instrumentation. The room-temperature absorbance data came from a Perkin-Elmer Lambda 4C spectrophotometer, while the emission spectrometer was an SLM-Aminco SPF-500 C model. A Jasco J-600 spectropolarimeter yielded the CD data at room temperature. For the thermal denaturation studies, the spectrophotometer was a Perkin-Elmer Lambda 3A spectrophotometer fitted with an insulated cell compartment and linked to a programmable, digital temperature controller. The pH meter was a Radiometer PHM 64 unit.

Results

The melting profiles of the different oligonucleotides were consistent with hairpin formation, and there was no indication of significant dimer formation. Thus, for those systems studied at two oligonucleotide concentrations (5 and 50 μM), the melting temperature (T_m) varied by no more than 1−2 °C. In accord with the existence of a double-helical stem, the T_m increased with the number of possible G≡C base pairs. The AT-rich systems contained only two potential G≡C base pairs, usually at either end of the stem region to facilitate closure. Of these, the systems with a C residue on the 5′ side of the loop had T_m = 52−56 °C, while those with a G in this position melted at lower temperatures. The T_m ranged from 58 to 65 °C for hairpins with 3 G≡C base pairs in the stem. When there were four G≡C base pairs, the range of T_m values was 67−75 °C. By design, most of the base variations occur in the middle of the stem structure. This explains the hairpin-naming scheme, the key to which appears in Table 1.

The melting curves of several representative substrates showed an apparent increase in T_m due to the binding of Cu-(T4). It is convenient to monitor these processes in the vicinity of 420 nm, but there are some complicating factors. One is that the absorbance of Cu(T4) itself is temperature-dependent in solution and decreases at higher temperatures. The decrease is more or less continuous above 30 °C; however, interaction with a hairpin delays the onset of the absorbance decrease. Another effect to keep in mind is that the porphyrin may also bind to the denatured form of the oligonucleotide. These effects not withstanding, the trend toward stabilization seems evident. Absorbance changes at 420 nm revealed that the binding of Cu-(T4) enhanced the T_m's of the TT and AT hairpins by 15 °C. The GG system exhibited a similar shift, while the CA hairpin showed a smaller increase, about 7 °C. For the GG and CA systems, there was an increase in the absorbance at the monitoring wavelength (424 nm).

An analysis of the spectral data shows that the AT-rich hairpins almost exclusively supported groove binding of Cu-(T4). Thus, with the exception of the TA′ hairpin, complexation with the AT-rich DNA induced only weak emission intensity from Cu(T4). Within this group, though, only the TA, TT, and TA* hosts induced the archetypical strong positive CD signal in the Soret region that is characteristic of the groove-bound porphyrin (Figures 1 and 2).[14,22] The same systems also each produced a small bathochromic shift in the Soret maximum and weak hyperchromism (Table 1). As with $[poly(dA\text{-}dT)]_2$,[14] the bandwith also narrowed; hence, there was not necessarily an increase in the integrated absorption intensity. Unlike the absorption maxima, the CD maxima shifted to shorter wavelengths. For the purposes of the discussion below, this set of hosts represents the class 1 groove binders. The adducts in question and all the other host−guest complexes investigated exhibited absorption and excitation maxima at the same wavelengths. Three other AT-rich hairpins (AT, AT′, and AT‡) formed another group, the class 2 groove binders. Interactions with these systems also produced weak emission intensities ($\Phi_{rel} \leq 0.2$) and positive, but comparitively weak, CD signals consistent with external binding. However, for this set of substrates the interaction with the DNA induced modest hypochromism. Another difference was that the induced CD signals occurred at shorter wavelengths, and sometimes there was a hint of a bisignate band shape. However, none of the class 2 adducts gave an unambiguous negative branch in the CD spectrum. The AT* hairpin showed borderline behavior. Thus, the interaction with Cu(T4) produced a hypochromic

TABLE 1: Physical Data for DNA Hairpins and Adducts with Cu(T4)

			ABS		CD		
hairpin	label	T_m, °C	λ_{max}, nm	%H[a,b]	λ_{max}, nm	$\Delta\epsilon$,[c] M^{-1} cm^{-1}	Φ_{rel}[d]
			AT-Rich Systems				
GATAAC$^{T}_{T}$T CTATTG$_{T}$T	TA	54	427	-2 ± 2	421	(17)	0.1
GATTAC$^{T}_{T}$T CTAATG$_{T}$T	TT	55	427	-2 ± 2	421	(17)	0.1
CATAAG$^{T}_{T}$T GTATTC$_{T}$T	TA*	41	429	-5 ± 2	421	(9)	0.2
GAATAC$^{T}_{T}$T CTTATG$_{T}$T	AT	50	428	5 ± 2	415 435?	(5 ± 1)	0.2
AGATAC$^{T}_{T}$T TCTATG$_{T}$T	AT′	56	429	10	413 440?	(5±1)	0.2
CAATAC$^{T}_{T}$T GTTATG$_{T}$T	AT[‡]	52	428	10	413	(5 ± 1)	0.1
CAATAG$^{T}_{T}$T GTTATC$_{T}$T	AT*	47	429	10	422	(7)	0.2
TATACC$^{T}_{T}$T ATATGG$_{T}$T	TA′	56	431	15	410? 438	(-5 ± 1)	0.4
			GC-Rich Systems				
GAGCAC$^{T}_{T}$T CTCGTG$_{T}$T	GC	75	432	20	433	(−22)	1.0
GACGAC$^{T}_{T}$T CTGCTG$_{T}$T	CG	74	434	25	434	(−27)	0.9
GAGGAC$^{T}_{T}$T CTCCTG$_{T}$T	GG	66	434	30	434	(−22)	0.8
GAGTAC$^{T}_{T}$T CTCATG$_{T}$T	GT	58	432	20	435	(−17)	0.8
GAGAAC$^{T}_{T}$T CTCTTG$_{T}$T	GA	60	432	20	435	(−22)	0.7
GAAGAC$^{T}_{T}$T CTTCTG$_{T}$T	AG	61	433	25	434	(−17)	0.7
GACAAC$^{T}_{T}$T CTGTTG$_{T}$T	CA	65	432	20	434	(−17)	0.5

[a] Measured at a hairpin-to-copper ratio of 5. [b] The estimated error is ±5% unless otherwise indicated. [c] Error of ±2 unless otherwise indicated. [d] Error of ± 0.1.

response in the Soret region, but the induced CD signal was more typical of a class 1 adduct. The TA′ system was the one AT-rich hairpin that clearly supported intercalative binding. Thus, the interaction with Cu(T4) produced a larger red shift in the Soret band, a negative CD signal in the same region, and a significant enhancement in the emission intensity (Table 1). This was undoubtedly a consequence of the CpC step in the stem, vide infra.

The properties of the complexes obtained with the seven GC-rich hairpins, which all have either three or four G≡C base pairs in the stem, are entirely consistent with intercalative binding of Cu(T4).[14,22] More specifically, each adduct showed strong hypochromism in the Soret region, a corresponding negative CD signal, and a significantly enhanced emission intensity (Table 1, Figures 1 and 2). As a rule, each of these adducts had its absorption maximum and CD signal at the same wavelength, although the position varied somewhat from hairpin to hairpin. Within this group, the hairpins with four G≡C base pairs in the stem (GG, GC, CG) exhibited slightly stronger CD signals and generally greater emission intensities.

Discussion

Binding of a Monomeric Porphyrin. With the benefit of a +4 charge, Cu(T4) binds well to DNA, even though the porphyrin is neither flat, like a classic intercalator, nor crescent-

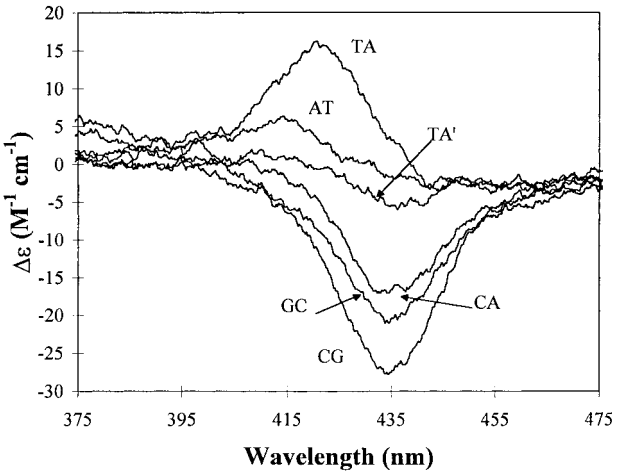

Figure 1. Representative CD spectra of adducts of Cu(T4) in pH 6.8 phosphate at 23 °C. The hairpin-to-copper ratio is 5.

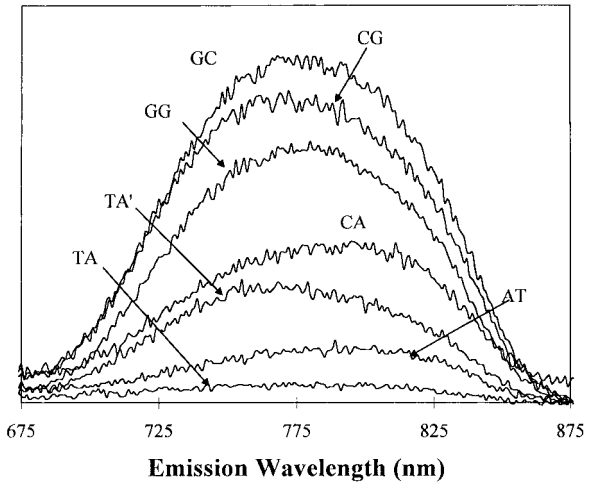

Figure 2. Emission spectra of representative hairpin adducts of Cu(T4) in pH 6.8 phosphate at 23 °C and at a hairpin-to-copper ratio of 5.

shaped like a typical groove binder. Nevertheless, as already noted, Cu(T4) is capable of intercalation as well as external groove binding. With AT-rich sequences as in $[poly(dA\text{-}dT)]_2$, the copper complex binds externally, but the interaction is intimate enough to induce a positive CD signal in the Soret region.[22] Flow-dichroism experiments indicate that the plane of a typical externally bound porphyrin lies canted, but not perpendicular, to the helix axis.[27] In addition, the absorption spectrum shows weak hyperchromism in the Soret region, and the quenching of the porphyrin emission remains almost complete.[14] In light of the significant spectral perturbations and the results of footprinting experiments,[8] the conventional wisdom is that the interaction is a form of groove binding. It may be worth noting in this regard that the narrow width of the minor groove amidst a run of A═T base pairs can enhance groove binding for some ligands.[28] (In the case of Cu(T4), the same effect may disfavor intercalative binding because the groove has to accommodate a pair of pyridyl substituents.[15]) On the other hand, for systems, like the TATA binding protein, the ease with which local structural reorganization occurs has a significant impact on the sequence specificity.[29] For metalloporphryins, the deformability of the A═T base pairs is also a potentially important factor because the binding interaction may entail a melting out of the local DNA structure and creation of a binding pocket.[30] This corresponds to an "induced-fit" mode of binding, and the porphyrin "footprint" may encompass several base pairs.[5,10,14,31] There is as yet no consensus about the locus of groove binding. DNA-cutting experiments with redox-active metalloporphyrins are consistent with edge-on binding within the minor groove.[30,32] However, the results of transient Raman studies have led to a model that supposes that Cu(T4) binds face-on in the major groove.[33]

A completely different mode of binding occurs within GC-rich sequences as in $[poly(dG\text{-}dC)]_2$. With this host the spectral perturbations include a large red shift of the Soret maximum and marked hypochromism.[14,22] Both results reveal a significant coupling between the charge clouds of the porphyrin and base residues within the double helix and are consistent with intercalative binding. Microviscosity measurements support this interpretation.[34] The observation of photoluminescence from the same type of adduct is a further indication of the way in which the DNA envelops the complex and blocks access to the axial coordination sites of the copper center.[14] There are several reasons why the presence of G≡C base pairs may foster intercalative binding. The list includes the intrinsic polarity and polarizability of the guanine moiety,[35,36] as well as interactions involving exocyclic groups.[37,38] Another potentially important factor is the robustness of the B-form structure due to the fact that a G≡C base pair has one more hydrogen bond than an A═T base pair.[39] Intercalation requires some unwinding of the double helix but generally leaves the hydrogen bonds intact. Interestingly, the structure reported by Williams and co-workers suggests that the uptake of Cu(T4) causes a disruption of the DNA structure and induces a 5′ base to flip out of the double helix.[15] It remains to be seen if the same type of binding occurs in solution, since this would entail the loss of at least three hydrogen bonds as well as some stacking energy. (In the solid-state structure, an exogenous base from an adjacent helix replaces the flipped-out base and completes the hydrogen-bonding scheme.[15]) According to theory, ordinary base pairing is compatible with the intercalation of Cu(T4) into an isolated host.[31]

For a hairpin structure, the loop region represents another potential binding site.[40] Although competitive loop binding of the porphyrin may occur, under our conditions stem binding is clearly dominant because the uptake of Cu(T4) is exquisitely sensitive to the base composition in the stem. Moreover, the CD signals, the changes in the absorption spectra, and the emission results that we have observed correlate very well with previous results, findings involving ordinary B-form DNA.[4−6,14]

Binding to AT-Rich Hairpins. The results with the AT-rich hairpins show that these structures can support groove binding and that the base sequence as well as the base composition influence the binding. The data from the three class 1 groove binders, TA, TT, and TA*, establish that a run of four A═T base pairs suffices to define a groove site for the binding of Cu(T4). The AT and the AT‡ hairpins have similar AT-rich stretches and also support external binding. However, the adducts formed by the latter systems are distinct in that they exhibit hypochromism and weaker CD signals at higher energies. Competitive formation of two types of adducts with opposing CD signals could account for the reduced intensity, but this is doubtful in view of the agreement between the excitation spectrum and the absorption spectrum. Outside stacking is apparently not a factor either because neither type of adduct exhibits a truly conservative CD signal.[22,41,42] The most likely explanation for the disparate spectral properties is that different sequences support different types of external binding. This would be entirely consistent with the induced-fit hypothesis. The difference in the two types of adducts could be that one

involves binding at a major-groove site and the other binding at a minor-groove site. However, we cannot rigorously exclude the possibility that the second type of external binding occurs in the loop region.

The spectral data obtained do not provide specific structural details but do present constraints that any viable model must accommodate. One outstanding issue is the reason for the difference in wavelength between the CD and absorption maxima of externally bound Cu(T4). In principle, this could be due to a reduction in symmetry because the Soret transition of the free porphyrin involves an orbitally degenerate E_u state.[43] However, there is no indication of band splitting in the absorption spectrum, and the line width of the Soret band is narrower for the groove-bound form. Another important facet of the groove-binding interaction is that it moderates the reactivity at the axial coordination sites of the copper center. This is evident because the adduct exhibits an emission signal, albeit a weak one. In contrast, the free Cu(T4) system is completely nonemissive in aqueous media owing to an exciplex quenching mechanism that involves the axial coordination of a water molecule.[14] An axial interaction with a water molecule or a basic moiety from the DNA molecule itself also occurs with the groove-bound form of the excited state; the rate of quenching is just slower. In fact, time-resolved resonance Raman methods have provided evidence for a five-coordinate form of the copper porphyrin,[44] in which Kruglik et al. have proposed that Cu(T4) binds face-on within the major groove to a thymine oxygen.[33] Although the Raman data pertain specifically to a covalently bound form that only has a nanosecond lifetime, our CD results indicate that the four-coordinate ground state also interacts with a similar run of A═T base pairs.

Intercalation. The emission and CD data in Table 1 also provide insights into the intercalative binding of Cu(T4). In particular, intercalation occurs readily in stretches of B-form DNA that contain at least 50% G≡C base pairs. Among such systems, the data for the GT, GA, AG, and CA systems show that intercalation occurs in the absence of a step of G≡C base pairs. These findings accord with the observation that Cu(T4) intercalates into poly(dA-dC)·poly(dG-dT), which contains exactly 50% G≡C base pairs and no G≡C steps.[12] On the other hand, the results with the TA′ system, shown below,

$$\begin{array}{l} \mathrm{TATACC^{T}T} \\ \mathrm{ATATGG_{T}T} \end{array}$$

suggest that the existence of a G≡C step can induce intercalation, even at a lower percentage composition of G≡C basepairs. Groove binding is, however, apparently a competitive process in view of the low emission yield and the weak negative CD spectrum of the TA′ adduct. Indeed, Raman studies of Cu(T4) interacting with oligonucleotides such as [d(GCGCGCATATGCGCGC)]$_2$ have shown that some degree of groove binding occurs within a run of four A═T base pairs despite the presence of multiple-flanking G≡C steps. In combination, these findings imply that the binding constants for groove binding of Cu(T4) at a triple step of A═T base pairs and intercalation at a G≡C step have comparable magnitudes. It is worth noting in this regard that both types of adduct formation induce a comparable shift in the T_m of a hairpin. The fact that the AT′ system supports groove binding implies additional qualifications. One is that the occurrence of four successive A═T base pairs is not an absolute requirement for groove binding of Cu(T4). Second, intercalation next to a single G≡C base pair is only favorable within sequences that are rich in G≡C base pairs.

Some structural implications are also evident. Although the spectral data provide no direct information about the site of intercalation, the results are consistent with binding next to a G≡C base pair or between consecutive G≡C base pairs. Thus, the presence of a step of G≡C base pairs generally enhances the hypochromism as well as the luminescence intensity, and the luminescence also maximizes at a slightly shorter wavelength. Such a preference for G≡C sites is not uncommon for DNA intercalators.[38,45,46] Although the systems studied here are not directly comparable to the oligonucleotide studied by Williams and co-workers,[15] the results suggest that hemiintercalation is unlikely to be a prevalent mode of internalizing Cu(T4) in solution. Consider the data for the AT′ and AT systems. Of all the hairpins studied, the AT′ system would appear to offer the best opportunity for hemiintercalation. This system has a limited run of A═T base pairs within the stem, and it would be possible to flip out the 5′-terminal base and retain a G≡C base pair within the putative binding site. Yet, the AT system forms a similar adduct, and in this case hemiintercalation would require the extrusion of a 5′ G. This is unlikely because the stacking interaction with a G residue is surely one of the key stabilizing factors involved in any type of intercalative binding. We cannot exclude the possibility of hemiintercalation because it is possibile that the modest hypochromism of the AT′ adduct will turn out to be the signature of this mode of binding. Be that as it may, the GT, GA, AG, and CA hairpins obviously form a very different type of adduct. The same is true of the other GC-rich hairpins.

Conclusions

Hairpin-forming hexadecamers are useful substrates for DNA-binding studies of Cu(T4) at micromolar concentrations. In general, the uptake of Cu(T4) stabilizes the system against denaturation. The results provide a number of other useful insights. (1) One is that robust hydrogen bonding within a B-form DNA duplex definitely promotes intercalative binding of Cu(T4). (2) Moreover, no one step type defines an intercalation site, and the presence of contiguous G≡C base pairs is not a requirement as long as the local sequence contains at least 50% G≡C base pairs. (3) For groove binding of Cu(T4), a run of four A═T base pairs provides an excellent site with an equilibrium constant comparable to that for intercalation at a G≡C step. (4) External binding is, however, also possible in runs of fewer than four A═T base pairs, and the spectral properties of the adduct vary with the base sequence. (5) Neither loop binding nor hemiintercalation of Cu(T4) is a prevalent mode of interaction for the hairpin hosts in solution. (6) Finally, the results lay a foundation for the design of structurally oriented studies of hairpin adducts by ^{1}H NMR techniques.

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