Luminescence Studies of the Intercalation of Cu(TMpyP4) into DNA

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Abstract: Different types of DNA have been used to investigate binding interactions of Cu(TMpyP4), where TMpyP4 denotes the deprotonated form of *meso*-tetrakis(4-(*N*-methylpyridiniumyl))porphyrin. Physical methods employed include electronic absorption and circular dichroism as well as luminescence spectroscopy. Most of the studies have been carried out at μ = 0.2 M in a pH 7.8 Tris buffer at 25 °C. With DNA samples containing both guanine–cytosine and adenine–thymine base pairs, our results confirm that Cu(TMpyP4) can bind at external sites—most likely within one of the grooves of DNA—or by intercalation (Pasternack, R. F.; Gibbs, E. J.; Villafranca, J. J. *Biochemistry* **1983**, *22*, 2406–2414). Moreover, our results show that the distribution between the two types of sites depends on the nucleotide-to-copper ratio and that intercalation is favored at moderate ratios. A guanine–cytosine base pair in combination with any other base pair seems sufficient to define an intercalation site. Novel emission with a lifetime of about 20 ns is observed from the tripdoublet and tripquartet excited states of intercalated Cu(TMpyP4). This emission is normally quenched by a mechanism which involves coordination of the solvent at an axial position of the copper center. Deactivation is proposed to occur via a low-energy d–d state of the five-coordinate complex. Solvent-induced quenching occurs when the complex is externally bound but not when the complex is intercalated because the axial coordination sites are blocked. The results are of interest because porphyrins are important DNA-binding agents and because solvent-induced quenching is becoming recognized as an important type of exciplex phenomenon that can occur in coordinatively unsaturated complexes.

Introduction

The interactions between B-form DNA and cationic porphyrins or metalloporphyrins have been investigated by a number of groups in recent years.[1-4] Porphyrins are quite versatile systems because they can be usefully monitored by numerous physical methods and because they are capable of incorporating a variety of metal ions which in turn influence the mode of interaction with DNA. Part of the motivation for study derives from the fact that these systems are cytotoxic when illuminated; therefore, they may prove to be useful sensitizers for photodynamic therapy.[5,6] Antiviral activity has been reported as well.[7] The porphyrin *meso*-tetrakis(4-(*N*-methylpyridiniumyl))porphyrin, abbreviated below as H_2TMpyP4, has been especially well studied because it has four positively charged substituents and shows little tendency to self-associate in aqueous media.[8,9] Early on, Fiel and co-workers used optical absorption, circular dichroism (CD), and viscometry to demonstrate that H_2TMpyP4 is capable of intercalating into DNA, despite the fact that the favored orientation of substituents on the porphyrin is out of plane.[10a] A gel-mobility assay was subsequently used to confirm that H_2TMpyP4 binds by intercalation.[10b] Later, Pasternack and co-workers used absorption and CD techniques to establish that a variety of metalated forms are also capable of binding.[11,12] Numerous other techniques have since been used to study the interactions, including ^{31}P NMR spectroscopy,[13,14] linear dichroism,[15,16] viscometry,[17,18] fluorescence spectroscopy,[19] EPR spectroscopy,[20] and resonance Raman spectroscopy.[21,22]

Metalloporphyrins bind to DNA in at least three ways.[1-3] One is outside, or groove, binding which is favored by metalloporphyrins containing Fe(III), Co(III), Mn(III), Zn(II), or any other metal center which binds axial ligands. There is also an external mode of binding in which near-neighbor porphyrins, possibly stacked together along the surface of the DNA molecule, interact to produce a conservative CD spectrum.[23,24] Finally, the free H_2TMpyP4 ligand, as well as derivatives containing Pt(II), Ni(II), Cu(II), or other metal centers which do not require an axial ligand, is capable of intercalating between base pairs.[1-3] Although the preferred geometry is apparently square planar for Cu(TMpyP4), five-coordinate forms are expected to be thermally accessible. This likelihood intrigued us because previous work has shown that axial ligands can have a strong influence on the excited-state lifetimes of copper(II) porphyrins.[25,26]

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Our interest in Cu(TMpyP4) is an outgrowth of our experience with bis(phenanthroline)copper(I) systems.[27-29] In the case of the phenanthroline systems, the lowest energy excited state is a metal-to-ligand charge-transfer state in which the ability of the metal center to act as a Lewis acid is enhanced due to the increase in formal oxidation state. As a result, in donor media the emissive state of $Cu(bcp)_2^+$, where bcp denotes 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline, forms a transient five-coordinate solvate. However, the adduct is short lived because the five-coordinate form efficiently converts to the ground-state energy surface by a nonradiative relaxation pathway. One of the consequences of this process is quenching of the charge-transfer emission. Since adduct formation with DNA or RNA influences the accessibility toward solvent, we have found that luminescence methods can be used to probe the binding interactions of the bcp complex with the biopolymers.[30-32] In this report we extend our luminescence studies to the Cu(TMpyP4) system.

Experimental Section

Materials. The tosylate salt of the tetracation H_2TMPyP4 was purchased from Aldrich Chemical Co. and used to prepare the chloride salt of Cu(TMpyP4) by the procedure of Pasternack and co-workers.[11] Trisma base and salmon testes (ST) DNA, as well as DNA from *Micrococcus luteus* (*M. luteus*) and *Clostridium perfringens* (*Cl. perfringens*), were purchased from Sigma Chemical Co. The duplexes poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC) as well as single-stranded poly(dA) were purchased from Pharmacia and were used as received. $[Ru(bpy)_3]Cl_2·6H_2O$ was purchased from the GFS Chemical Co. Coumarin 440 was purchased from the Exciton Chemical Co., and dichlorodimethylsilane was purchased from Aldrich.

Methods. Prior to the spectral studies, DNA samples from *M. luteus* and *Cl. perfringens* were incubated overnight at 37 °C with 5% RNase (a gift from M. Bina of Purdue University) and extracted with an equal portion of 4/1 (v/v) phenol/chloroform. The DNA was then precipitated, washed with 70% methanol twice, and finally dried in air. The ST DNA was simply purified by precipitation with ethanol. Purified DNA samples were then dissolved in 0.025 M (pH 7.8) Tris buffer containing 0.175 M NaCl. In order to cut the naturally occurring forms of DNA into relatively short, rodlike fragments, the DNA-containing solutions were sonicated by means of a 3-mm horn. For example, a 1 mg/mL solution of ST DNA in Tris buffer was sonicated for 5 min while the flask was immersed in an ice bath. The power level of the sonifier was set at 25 W, and the output control was set at a reading of 55. Subsequent viscometric measurements established an average chain length of 800 base pairs in the case of ST DNA.[33] All viscometry measurements were carried out at 23 °C with the aid of a water bath. Prior to mixing, the stock solutions were filtered through a 0.65-μm filter, and the final DNA concentration was 0.1 mg/mL. Flow times were measured with a stopwatch.

For the spectral work, unless otherwise noted, the concentration of the porphyrin was 1.3 μM as determined from the extinction coefficient at 424 nm (2.31×10^5 M^{-1} cm^{-1}).[34] Standard volumetric techniques were used when stock solutions of DNA were combined with a buffered solution of Cu(TMpyP4) that had been filtered through a 0.45-μm filter. The total nucleotide concentration, i.e., the purine plus pyrimidine base concentration, was determined from the appropriate molar absorptivity at 260 nm: 6600 M^{-1} cm^{-1} for ST DNA;[35] 6800 M^{-1} cm^{-1} for poly(dA-dT)·poly(dA-dT);[36] 9100 M^{-1} cm^{-1} for poly(dA);[37] 6965 M^{-1} cm^{-1} for *M. luteus* DNA;[36] and 7367 M^{-1} cm^{-1} for *Cl. perfringens* DNA.[37] The extinction coefficient for poly(dG-dC)·poly(dG-dC) is 8400 M^{-1} cm^{-1} at 254 nm.[38] Prior to the spectral studies, the glassware was treated with 5% (v/v) dichlorodimethylsilane in chloroform to minimize the adsorption of the porphyrin to the glass surfaces.[39] For emission spectra, the source slit was set at 20 nm while the detector slit was set at 5 nm. A 520-nm long-wave-pass filter was placed between the sample and the emission monochromator. Emission spectra were typically recorded with 438-nm excitation or at the appropriate nearby isosbestic point. Emission quantum yields were determined from corrected spectra by the method of Parker and Rees with $Ru(bpy)_3^{2+}$ as the standard.[40,41] Luminescence lifetimes were measured with a nitrogen-pumped dye laser as described previously.[42] Excitation at ca. 438 nm was achieved by means of a 3 mM solution of Coumarin 440 in ethanol.

Instrumentation. Electronic absorption spectra were recorded with a Perkin-Elmer Lambda 4C spectrophotometer. CD spectra were recorded with a JASCO J-600 spectropolarimeter. Emission spectra were recorded with an SLM/Aminco SPF-500C spectrofluorometer. Viscometric measurements were made using a Cannon-Fenske routine viscometer (Model 25). The Vibracell VC50 sonifier was obtained from Sonics and Materials, Danbury, CT.

Table I. Physical Data for Cu(TMpyP4) in the Presence of DNA in pH 7.8 Phosphate Buffer at μ = 0.2 M and T = 25 °C

type of DNA	%GC	λ_{max}^{abs} [a] (nm)	fwhm[a,b] (cm^{-1})	$10^4 \times \Phi$[c,d]	τ[c,d] (ns)
none (control)		425	1570		
poly(dG-dC)·poly(dG-dC)	100	439	1630	1.1	22
M. luteus	73	439	1630	0.80	21
salmon testes	41	428	1380	0.34	18
Cl. perfringens	27	428	1600	0.26	19
poly(dA-dT)·poly(dA-dT)	0	428	1200		

[a] DNA-P/Cu = 50. [b] Full width at half-maximum. [c] R = 100. [d] Estimated uncertainty ±10%.

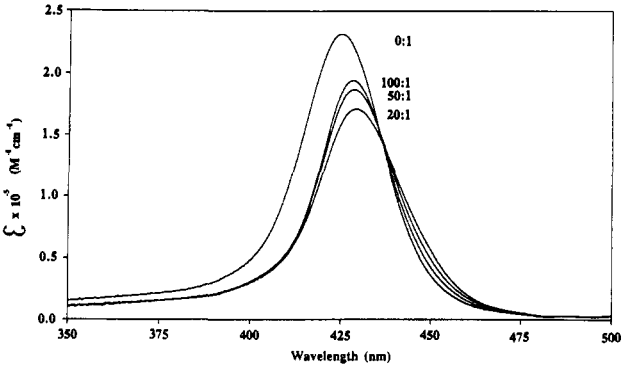

Figure 1. Absorption spectrum of Cu(TMpyP4) at various nucleotide-to-copper ratios in the presence of ST DNA. The spectra were recorded in 0.025 M (pH 7.8) Tris buffer at μ = 0.2 M and 25 °C.

Results

Absorption Spectra. The visible absorption spectrum of Cu(TMpyP4) depended on the ratio of nucleotide to porphyrin in solution. (Hereafter this ratio will be denoted as the DNA-P/Cu ratio as the phosphate concentration is equal to nucleotide concentration.) The simplest systems exhibited one of two different types of limiting spectra at high DNA-P/Cu values, i.e., in the presence of excess DNA. With excess poly(dG-dC)·poly(dG-dC) or *M. luteus* DNA in solution, the Soret band exhibited pronounced hypochromism and a significant bathochromic shift. In particular, the absorption maximum shifted from 425 to about 439 nm, and the apparent molar absorptivity decreased by about 40% at the absorption maximum. Moreover, for all values of DNA-P/Cu a fairly good isosbestic point was maintained at 437 nm. In energy units, the full width at half-maximum (fwhm) of the Soret band was 230 cm^{-1} broader at DNA-P/Cu = 10 than at DNA-P/Cu = 0 in the presence of poly(dG-dC)·poly(dG-dC).

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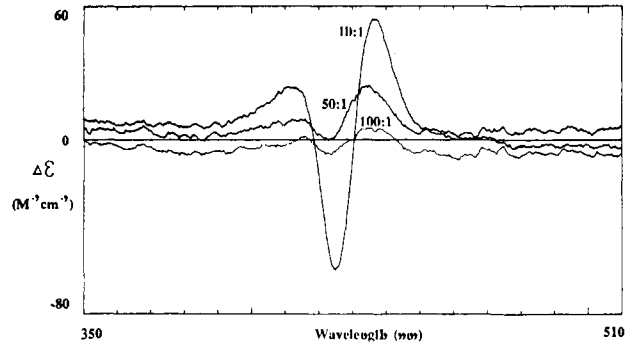

Figure 2. Circular dichroism spectrum of Cu(TMpyP4) at various nucleotide-to-copper ratios in the presence of poly(dA–dT)·poly(dA–dT). Conditions are the same as in Figure 1.

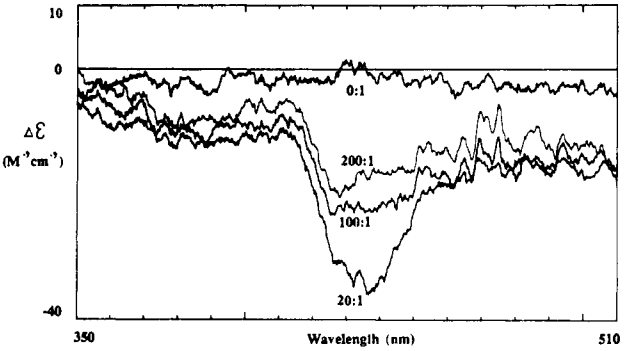

Figure 3. Circular dichroism spectrum of Cu(TMpyP4) at various nucleotide-to-copper ratios in the presence of ST DNA. Conditions are the same as in Figure 1.

However, some narrowing occurred at higher ratios, such that the net broadening was about 60 cm^{-1} at DNA-P/Cu = 50 (Table I). A different type of limiting spectrum was induced by poly(dA–dT)·poly(dA–dT) or poly(dA). With these systems the intensity of the Soret band changed little in the presence of the DNA, and the maximum shifted only about 3 nm to longer wavelength while the isosbestic point occurred at 433 nm. Also, in contrast to what was seen with poly(dG–dC)·poly(dG–dC), the fwhm of the Soret band narrowed by 345 cm^{-1} (ca. 22%) in the presence of poly(dA–dT)·poly(dA–dT) at DNA-P/Cu = 10 and by another 25 cm^{-1} at DNA-P/Cu = 50.

In the case of ST DNA, more complex behavior was observed. As the DNA was added to the Cu(TMpyP4) solution, the absorbance in the region of the Soret band initially decreased, and the absorption maximum shifted to longer wavelength. However, at higher DNA-P/Cu values the absorption intensity increased once again (Figure 1). Throughout the titration an approximate isosbestic point was maintained at 438 nm. Here the bandwidth was also a complicated function of the DNA-P/Cu ratio. By around DNA-P/Cu = 10, the fwhm had increased by 95 cm^{-1} in comparison with that of free Cu(TMpyP4), but at DNA-P/Cu = 50 the fwhm had *decreased* by 265 cm^{-1} in comparison with the control. Similar behavior was observed with DNA from *Cl. perfringens* except that the quality of the data was poorer, possibly because of scattering artifacts.

CD Spectra. The simplest results for analogous titrations monitored by CD spectroscopy were obtained with *M. luteus* DNA. In this case, at all DNA-P/Cu values a single negative band was observed in the Soret region with an extreme at 435 nm, and the limiting spectrum was apparent by DNA-P/Cu = 50. This type of spectrum is the signature of intercalated porphyrin.[2] A similar spectrum was obtained with poly(dG–dC)·poly(dG–dC); however, the wavelength at which the minimum occurred varied with the DNA-P/Cu ratio. At low DNA-P/Cu values the minimum occurred at 430 nm, but it shifted toward 435 nm at higher DNA-P/Cu values. In addition, a weak positive band at 360 nm was observed with poly(dG–dC)·poly(dG–dC).

In contrast, a conservative, derivative-like spectrum was observed with poly(dA) at DNA-P/Cu < 30 with the crossover point at about 428 nm. At higher DNA-P/Cu values, the spectrum evolved into a broad negative band with a maximum at about 420 nm. A similar derivative-like feature was apparent at low DNA-P/Cu values when Cu(TMpyP4) was combined with poly(dA–dT)·poly(dA–dT) in solution. In this case, however, the derivative signal appeared to be superimposed on another signal because there was a second positive extremum in the spectrum at about 413 nm. At higher DNA-P/Cu values the negative component was less evident, and the spectrum appeared to be evolving into a broad positive band (Figure 2), which is consistent with groove binding, i.e., binding at external sites.[2]

With ST DNA a negative band, much like that seen with poly(dG–dC)·poly(dG–dC), was observed at low DNA-P/Cu values and was most pronounced at about DNA-P/Cu = 20. At higher DNA-P/Cu values the minimum was less intense, and there was an indication of a derivative-like signal with an apparent midpoint between 420 and 425 nm superimposed on the original signal (Figure 3). In the studies with ST DNA, we also varied the salt concentration. At DNA-P/Cu = 200, an increase in the salt concentration had little effect, but at DNA-P/Cu = 20 the CD signal intensity was much reduced at μ = 1.0 M.

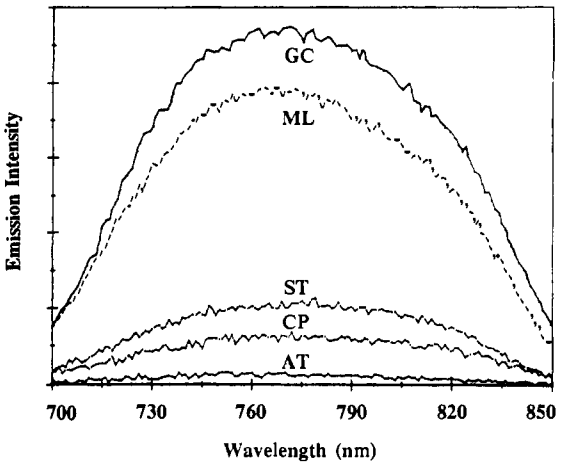

Figure 4. Uncorrected emission spectrum of Cu(TMpyP4) in the presence of different types of DNA. The conditions are similar to those in Figure 1 except DNA-P/Cu = 100 in all cases. DNA abbreviations: GC, poly(dG–dC)·poly(dG–dC); ML, *M. luteus*; ST, salmon testes; CP, *Cl. perfringens*; AT, poly(dA–dT)·poly(dA–dT).

We also made CD measurements on *Cl. perfringens* samples, but the spectral quality was rather poor because the base line shifted in the negative direction as the DNA was added, perhaps because of scattering artifacts.[43] At low DNA-P/Cu values a derivative signal, similar to the one evident in the ST samples at high DNA-P/Cu values, was observed with its midpoint at about 422 nm. This signal was also evident at higher DNA-P/Cu values but with diminished intensity.

Viscometry. Our viscometry results were similar to those of Strickland et al.[17] At low salt concentrations, the addition of Cu(TMpyP4) resulted in a decrease in the specific viscosity contributed by ST DNA. In contrast, the viscosity increased at low loading of the complex at μ = 0.2 M. More specifically, in the range of DNA-P/Cu = 30–50 the specific viscosity increased by about 30%.

Emission Data. No hint of emission could be detected from solutions of Cu(TMpyP4) in the absence of DNA. However, a broad emission band, centered at about 770 nm, was generally observed in the presence of DNA (Figure 4). The maximum in the excitation spectrum occurred at about 440 nm. Regardless of the type of DNA used, the emission lifetime was about 20 ns in the presence of excess DNA (Table I). To calculate lifetimes, the emission decay curves were fit as single exponentials, but the residual plots showed small systematic deviations, which could

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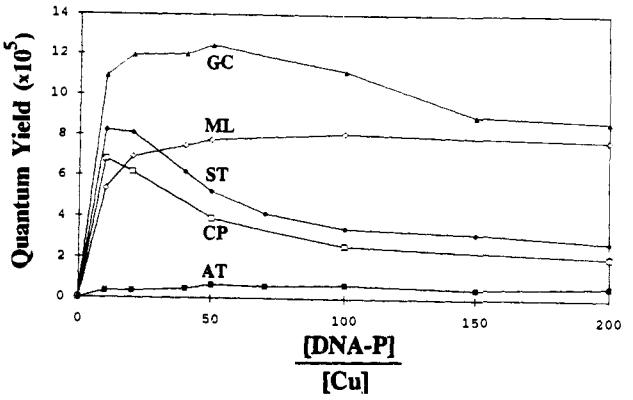

Figure 5. Emission intensity from Cu(TMpyP4) as a function of the DNA-P/Cu ratio. Conditions are the same as in Figure 1.

be indicative of multiple components. The emission varied with the guanine–cytosine (GC) content of the DNA, was strongest in the presence of poly(dG–dC)·poly(dG–dC), and was barely detectable in the case of poly(dA–dT)·poly(dA–dT). Quantum yields and lifetimes are quoted in Table I. As can be seen from the data plotted in Figure 5, the luminescence showed that at least two phases of binding occurred with ST DNA and *Cl. perfringens* DNA. In both cases, the emission intensity peaked at about DNA-P/Cu = 10 and then dropped to a limiting intensity at higher DNA-P/Cu values. Two distinct phases of binding were also observed with poly(dG–dC)·poly(dG–dC), but the fall off in intensity was more gradual and less dramatic by comparison with the results with ST or *Cl. perfringens* DNA.

Experiments with ST DNA showed that the emission intensity decreased at higher salt concentrations. For example, at DNA-P/Cu = 20 there was approximately a 10-fold decrease in emission intensity when the ionic strength was increased from 0.2 to 1.0 M. In contrast, at DNA-P/Cu = 200 the intensity decreased by only about 25% over the same range.

Discussion

Emission from Metalloporphyrins. In general, when emission is observed from transition metal complexes, it occurs from the lowest energy excited state and/or other close-lying, thermally accessible states. Because of the extensive conjugation, a $\pi\pi^*$ excited state is often the lowest energy excited state in a metalloporphyrin, and in the case of the free ligand or the zinc(II) porphyrin, the emission occurs from a state with singlet multiplicity, i.e., a $^1\pi\pi^*$ state.[44] However, when a diamagnetic heavy metal ion, such as Pt(II) or Cd(II), is bound, the rate of intersystem crossing to the $^3\pi\pi^*$ manifold is enhanced, such that the fluorescence intensity is reduced and, at low temperatures, the phosphorescence intensity is increased.[44-46] When a paramagnetic ion, such as Cr(III), is bound, intersystem crossing is also facilitated, and novel temperature-dependent emission can often be observed from a series of multiplet levels that are derived from the lowest energy $^3\pi\pi^*$ state.[47,48]

Some systems, such as Ag(II) porphyrins, are nonemissive because a charge-transfer excited state lies below the $\pi\pi^*$ states and mediates nonradiative decay to the ground state.[49] In Fe(II) and Ni(II) porphyrins the emission from the $\pi\pi^*$ states is also quenched, but the killer states are low-energy d–d, i.e., ligand field, states.[50,51] For Cu(II) porphyrins emission may be observed, but this depends upon the conditions. For example, in a nonpolar solvent like toluene, the copper complex of H_2TPP, where H_2TPP denotes 5,10,15,20-tetraphenylporphyrin, exhibits a temperature-dependent emission signal which is connected with the lowest energy $^3\pi\pi^*$ excitation of the porphyrin ring.[52] Due to the unpaired spin in the d shell of the copper center, there are actually two closely spaced emissive states, the so-called tripdoublet and tripquartet states, and their relative contributions to the total emission signal are temperature dependent.[44,52] The fact that the emission is strongly quenched in the presence of pyridine has been ascribed to formation of a five-coordinate species.[25,49]

Influence of the Axial Ligand. In an attempt to explain these results, Gouterman and co-workers have carried out iterative extended Hückel calculations which suggest that in copper(II) porphyrins a metal-to-ligand charge-transfer excited state occurs at low energy.[49] They therefore reasoned that addition of a fifth ligand could further stabilize the charge-transfer excited state such that it falls below the lowest tripquartet state and depopulates the emitting state. There are, however, problems with this model. One is the empirical evidence that copper(III) systems tend to exhibit a low-spin d^8 configuration and a preference for a planar coordination geometry.[53] Moreover, the $X\alpha$ calculations of Case and Karplus indicate that the metal-to-ligand charge-transfer excited states fall at much higher energies.[54] The $X\alpha$ calculations suggest that there is a low-energy ligand-to-metal charge-transfer excited state. More recently, Shelnutt et al. have also concluded from iterated extended Hückel calculations that a low-energy ligand-to-metal charge-transfer excited state occurs when a geometric relaxation is allowed to occur in the excited state.[55] Kim et al. have assigned this as the quenching state.[25] Because porphyrin complexes are so large and contain many electrons, at this point in time it may be asking too much of theory to predict the energies of the different excited states with such precision.

An alternative approach is to ask what type of excited state *would* be stabilized by the addition of a fifth ligand. The obvious answer is a d–d excited state, specifically the one involving $d_{z^2} \rightarrow d_{x^2-y^2}$ excitation, since it is well established that the addition of a fifth ligand destabilizes the d_{z^2} orbital and induces a red shift in the d–d spectrum.[56] Molecular orbital calculations also show that the addition of an axial ligand strongly stabilizes the $d_{z^2} \rightarrow d_{x^2-y^2}$ excited state.[55] Therefore, we suggest that it is a d–d state that quenches the emission from the tripdoublet and tripquartet states of five-coordinate copper(II) porphyrins. Regardless of the orbital parentage the deactivating state may have, the influence on the excited state is quite profound. Thus, the lifetime of the emission from Cu(TPP) is 29 ns in toluene at room temperature,[52] while the lifetime is <40 ps in pyridine.[25]

Emission Studies from DNA Adducts of Cu(TMpyP4). Although we have been unable to detect emission from Cu(TMpyP4) in aqueous solution, emission is observed in the presence of DNA, and the observed emission line shape is in keeping with emission from an aryl-substituted porphyrin. In contrast to the structured emission that has been observed from alkyl-substituted porphyrin complexes such as Cu(OEP), where OEP is the doubly deprotonated form of 2,3,7,8,12,13,17,18-octaethylporphyrin, aryl-substituted porphyrins such as Cu(TPP) exhibit a broad emission band with no resolved vibronic structure.[52,57] In either case, the excitation is to the LUMO, which is a degenerate π^* level which serves as the basis for the e representation of the D_{4h} point group. The difference is that the excitation is supposed to come from the filled a_{1u} orbital in the case of Cu(OEP) and the filled a_{2u} orbital in the case of Cu(TPP) because substituents influence the relative energies of these ligand orbitals. Kobayashi and co-workers have

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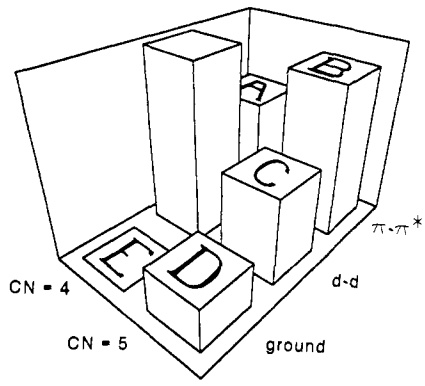

Figure 6. Schematic energy level scheme. The heights of the bars depict relative energies. The states B, C, and D of the five-coordinate porphyrin represent a $\pi\pi^*$ state, a d–d state, and the ground state, respectively. The corresponding states of the four-coordinate porphyrin are depicted to the left. An effective path for relaxation from state A, the lowest energy $\pi\pi^*$ state of the four-coordinate porphyrin, is A → B → C → D → E.

argued that the emission from porphyrins with aryl substituents in the meso positions is characteristically broad because the a_{2u} orbital is sensitive to environmental perturbations.[52] It can also be noted that, unlike the a_{1u} orbital, the a_{2u} orbital has non-zero coefficients at the $p\pi$ atomic orbitals of the meso carbons.[44] Since the a_{2u} energy is likely to be influenced by the orientation of the aryl substituents, the existence of a thermal distribution of torsion angles could be a factor determining the breadth of the emission profile.[58]

The data in Table I show that the intensity of the emission signal depends on the GC content, i.e., the percentage of guanine and cytosine in the DNA. This suggests that emission is observed from the intercalated form of Cu(TMpyP4) because of the large body of data in the literature which indicates that Cu(TMpyP4) tends to intercalate in GC-rich sequences of DNA.[1-3] Thus, the viscosity enhancement[15] and the hypochromism[11] induced by H_2TMpyP4 or Cu(TMpyP4) both increase with the GC content of the DNA used. Another indication that the emission stems from intercalated Cu(TMpyP4) is that in the case of ST DNA the excitation maximum is red shifted from the absorption maximum of the solution and agrees with the absorption maximum that is observed in the presence of poly(dG–dC)·poly(dG–dC) or *M. luteus* DNA. Thus, only a fraction of the total amount of Cu(TMpyP4) bound to ST DNA is emissive, namely, the fraction which is bound by intercalation. The observed variation of emission intensity with ionic strength is also consistent with this view, since previous work has shown that the intercalated form of binding is disfavored at high salt concentrations.[18,37]

According to the structure that has been suggested for the intercalated form of the porphyrin,[59] the axial positions should be protected from attack by external agents. This would prevent formation of a solvent-coordinated adduct which would otherwise result in the quenching of the emission. On the other hand, groove-binding modes, such as the one suggested by Marzilli,[3] are not expected to prevent axial ligation, so that the emission is quenched in spite of the DNA-binding interaction. Quenching via excimer formation is an alternative to solvent-induced quenching since porphyrins show some tendency to self-associate in aqueous solution. However, this seems unlikely to be an important process in our studies in view of the short excited-state lifetime and the low porphyrin concentrations in solution.

The energetics involved in the solvent-induced quenching mechanism can be crudely summarized in terms of the diagram presented in Figure 6. The figure shows that in the ground state the five-coordinate form of the complex is a high-energy form. Accordingly, the four-coordinate form is the dominant species in solution and is therefore the form that is excited. Excitation is followed by rapid relaxation to the multiplet levels associated with the lowest energy $^3\pi\pi^*$ state. This state is then subject to nucleophilic attack by a solvent molecule, which promotes relaxation back to the ground state. As in the ground state, addition of an axial ligand to the thermally equilibrated excited state is likely to be a thermally activated process. Nevertheless, it is still the key to an important relaxation pathway, because once the adduct is formed, the system can cascade back to the ground state via a succession of intermediate states associated with the five-coordinate form.

In essence, the quenching mechanism we are proposing is a form of exciplex quenching. By comparison with the field of organic chemistry, the incidence of exciplex quenching is rare in coordination chemistry, but a related process has been observed with copper phenanthrolines where luminescence spectroscopy has also been used as a primary means of investigation.[27-29] The crucial point is that in both cases quencher molecules are able to access the metal center through empty coordination sites.

Distribution among Binding Sites. Several lines of investigation have shown that the free ligand H_2TMpyP4 intercalates preferentially in GC-rich regions of DNA.[11,59,60] Under some conditions, Marzilli and co-workers have found that the ligand intercalation occurs specifically in a 5′CG3′ sequence of oligonucleotides,[13] and theoretical treatments have confirmed that this is a favorable intercalation site.[59,61] However, the intercalation of metalloporphyrins such as Cu(TMpyP4) does not appear to be nearly so sequence specific.[3] Our results confirm this view because the emission intensity from Cu(TMpyP4) is, to a good approximation, a strictly linear function of the %GC character. Therefore, a GC base pair in combination with any other base pair can define an intercalation site. If consecutive GC base pairs were required, a quadratic dependence on the GC content would be observed.

The CD results and the luminescence data in Figure 5 reveal that the nature of the binding interaction can vary in a complicated way with the nucleotide-to-porphyrin ratio. Thus, conservative CD spectra are observed with poly(dA–dT)·poly(dA–dT) and with single-stranded poly(dA) at low DNA-P/Cu ratios. A conservative CD spectrum typically arises when excitonic interactions occur between or among chromophores bound in a chiral arrangement.[2] However, at higher DNA-P/Cu ratios, when the porphyrin is better dispersed, this type of binding becomes less important. Marzilli and co-workers have reported similar complications in their study of the binding of Ni(TMpyP4) to poly(dA–dT)·poly(dA–dT).[62]

The GC-rich types of DNA exhibit different behavior. The simplest results are observed with *M. luteus* DNA where the CD and emission data are consistent with one dominant type of binding interaction, namely, intercalation, and saturation at high DNA-P/Cu ratios. Some degree of groove binding may also occur, but it is clear that the vast majority of the copper complex is intercalated. More complex behavior is observed with poly(dG–dC)·poly(dG–dC). Here the emission rapidly builds to a maximum and essentially levels off between DNA-P/Cu = 20 and DNA-P/Cu = 60, but by about DNA-P/Cu = 150 the emission decreases to another plateau. The transition that occurs between DNA-P/Cu = 50 and DNA-P/Cu = 150 appears to have a sigmoidal shape and may signal some type of cooperative structural transition. In the absence of porphyrin, or at high DNA-P/Cu ratios, poly(dG–dC)·poly(dG–dC) exhibits a "wrinkled" B-form structure.[63] The structure departs from standard (canonical) B-form DNA because the alternating sequences in the chains provide a regular repeat unit which is two base pairs long. One possible interpretation of the curve in Figure 5 is that poly(dG–dC)·poly(dG–dC) shifts out of the wrinkled structure as the

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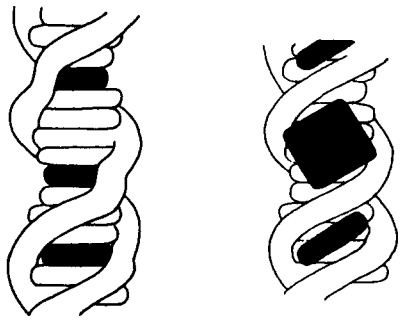

Figure 7. Scheme of binding. In the diagram on the left the solid discs represent intercalated Cu(TMpyP4), which is emissive because the axial coordination sites are blocked. In the diagram on the right the groove-bound form is depicted in the edge-on and face-on modes. The edge-bound porphyrin could be located in the major groove or the minor groove.

proportion of porphyrin to base pairs increases and the long-range repeat motif within the structure is compromised. If the rigidity of the structure and/or the disposition of the copper porphyrin within the duplex change as a result of a conformational transition, the process could easily be reflected in the emission intensity. Whatever the nature of the transition that occurs, the CD results and the absorption data rule out any significant shift away from the intercalative binding mode in poly(dG–dC)·poly(dG–dC).

It should also be noted that there are two distinct types of intercalation sites in poly(dG–dC)·poly(dG–dC), namely, 5′GC3′ and 5′CG3′ sites. Thus, the reduction in the emission intensity at higher DNA-P/Cu ratios could reflect a shift from one type of intercalation site to the other. We originally rejected this explanation because a similar intensity reduction is not observed with *M. luteus* DNA. However, a referee has pointed out that there are several possible intercalation sites in the case of *M. luteus* DNA, including the 5′GG3′, 5′CC3′, 5′TG3′, 5′GT3′ sites, etc. Since it is likely that each site would give use to a different emission yield than the next, the net effect of a redistribution among sites could amount to little or no change in the emission intensity. Furthermore, differences in the sites could be responsible for the small deviations from single-exponential decay which have been observed in the lifetime studies. Perhaps the most interesting effect in Figure 5 is observed with ST DNA. In this case the emission intensity reaches a maximum near DNA-P/Cu = 10, i.e., at a moderate ratio.

As the DNA-P/Cu ratio is then increased further, the emission rapidly drops off to a plateau at lower intensity. At the same time, a negative band grows in the CD spectrum at low DNA-P/Cu ratios, becomes most pronounced at DNA-P/Cu = 20, and then fades in intensity at higher DNA-P/Cu values where the emission intensity also drops off. Similarly complex changes also occur in the absorption spectrum, in that a strong red shift and significant hypochromism develop at lower DNA-P/Cu ratios while at higher ratios the absorption band shifts back toward higher energies (Figure 1). Independent of the DNA-P/Cu ratio, however, the emission excitation maximum occurs at ca. 438 nm. Taken together, these data indicate that the intercalative mode of binding is most favorable at moderate DNA-P/Cu ratios and that some shift to external sites occurs at higher DNA-P/Cu ratios. Steric factors probably play an important role in determining how Cu(TMpyP4) distributes among sites. Some excess nucleotide is required for intercalative binding because intercalators can occupy at most every other lattice site in B-form DNA.[64] Groove binding is likely to be an even more extensive mode of interaction. If, for example, the porphyrin binds face on within the groove, as envisioned by Marzilli,[3] several base pairs would obviously be covered by each molecule of Cu(TMpyP4). If, instead, the porphyrin binds by docking one of its edges within the groove, just as many base pairs could be eclipsed due to the edge length of the macrocycle (Figure 7). A third suggestion has been that the local DNA structure melts and the helix spontaneously reorganizes in order to accommodate the porphyrin within the groove.[4] A deformation of this type could easily entail a run of several base pairs as well.

With these ideas in mind, a simple explanation for the titration curve of ST DNA in Figure 5 can be tendered on the basis of competing steric demands: at moderate DNA-P/Cu ratios, when DNA is the limiting reagent, intercalative binding is strongly favored over groove binding. However, when excess DNA is added, so that steric clashes between neighboring porphyrins are avoided, the porphyrin binds competitively at groove sites, and the emission intensity decreases because a smaller percentage of the total amount of bound Cu(TMpyP4) is intercalated.

Previous workers have also discussed conditions which affect the distribution of H_2TMpyP4 or its metalated forms among DNA binding sites. Thus, there seems to be general agreement that H_2TMpyP4 binds to calf thymus DNA, predominantly by interaction in the presence of excess DNA, but that external binding becomes important at high loadings of porphyrin or at higher ionic strengths.[18,60,65] However, Carvlin and Fiel found a greater degree of hypochromism at a DNA-P/porphyrin ratio of 5 than they did at a ratio of 20, and they also found that the most intense negative CD signal occurred in the Soret region at a DNA-P/porphyrin ratio of about 6.[60] These data appear to be consistent with the idea that intercalative binding is most evident at moderate DNA-P/porphyrin ratios. In another study, Feng and Pilbrow considered the effect the DNA-to-porphyrin ratio has on the binding of Cu(TMpyP4) to calf thymus DNA.[66] According to their analysis, the fraction of Cu(TMpyP4) which is intercalated increases with the DNA-to-porphyrin ratio until a constant value is achieved, much like what we have proposed occurs with *M. luteus* DNA. On the basis of our results for ST DNA, a shift toward groove sites at higher DNA-P/Cu ratios would be expected because calf thymus DNA and ST DNA have similar compositions. However, it is difficult to interpret the luminescence data on a quantitative basis because the intrinsic emission yield of the bound porphyrin depends on the rigidity and the energy-transfer efficiency within the system, which are, in turn, functions of the loading.

Conclusions

We have found a rare instance of emission from a copper(II) porphyrin in room-temperature aqueous solution. The emission is from the tripdoublet and tripquartet levels of Cu(TMpyP4) bound to B-form DNA, and the emission lifetime is about 20 ns. Virtually all of the emission comes from the intercalated form of the complex, and a GC base pair in combination with any other base pair within the duplex appears to suffice to define an intercalation site. The complex also binds at external sites, but there is no significant emission from this form. The emission results and the absorption data show that the distribution of Cu(TMpyP4) among the different binding sites depends on the nucleotide-to-copper ratio and that intercalation is favored over external binding at moderate ratios, when DNA is the limiting reagent. The basis of this effect may be stereochemical because a groove-binding site probably has a larger footprint (involves more base pairs) than an intercalation site. The emissions from the free complex and the externally bound complex are quenched by the solvent due to formation of a five-coordinate solvent complex, but intercalation blocks access to the axial coordination sites about the copper. The tripdoublet and tripquartet states of the five-coordinate complex are thought to decay via a short-lived d–d excited state.

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